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Transitions between reproductive systems in *Daphnia*

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Abstract

Transitions between reproductive systems are very frequent in eukaryotes. Getting a comprehensive view of the actual evolutive advantages and costs of the different reproductive systems requires the understanding of the selective forces shaping such transitions. Over the last decades, empirical studies on the ecology and genetics of reproductive systems focused on long-term consequences and were conducted on natural populations. My PhD thesis aims at showing how early steps during transitions between reproductive systems are a key component to understand their evolution. To this end, I used the water flea; *Daphnia spp.* as a model system and study the genetic consequences of new reproductive systems. First, I investigated in the literature of asexual animals, whether the traditional view of asexuality as clonality (producing identical offspring) is realistic. This project showed that asexuals retain many features associated with sexuality from which they evolved so that strict clonality is not preeminent. While secondary evolution seems to favor clonality-like reproduction, the first steps of asexual evolution were certainly not clonal, particularly due to recombination. Second, I performed sex-asex crosses in a *Daphnia* species where obligate asexuals lineages producing "rare males" co-occur with sexuals. I studied the recombination rate of these asexual males and found that asexual males recombine as much as sexual ones, while asexual females recombine much less than sexual females. These results showed that the evolution of suppression of recombination is female-specific in this species and that meiosis modifications are also probably female-specific. Together, the two projects showed that recombination is not exclusive to sexuals. Third, because males transmit asexuality genes via sex-asex crosses (a process called contagious asexuality), I also studied the reproductive modes and fitness of lab-generated asexuals compared to natural lineages. Interestingly, whereas natural asexuals are clonal, I found that new asexuals are in majority not clonal and less fit than natural ones. These results suggested that asexual lineages evolve relatively quickly to acquire the characteristics of the asexual lineages observed *in natura*. Fourth, using another *Daphnia* species, I investigated the gene expression levels of individuals with an incipient sex chromosome compared to closely related lineages whose sex is environmentally determined. I found that the evolution of genetically determined females that lost the ability to produce males is not determined by a "loss-of-function" mutation but rather by a more complex molecular mechanism. This work illustrates the relevance of using species with polymorphic reproductive systems to investigate the early evolutionary transitions between reproductive systems found in nature.

Keywords: *Daphnia* | recombination | gene expression | contagious asexuality | crosses | genotypic sex determination | sex chromosomes | clonality

Résumé

Chez les eucaryotes, les transitions entre les systèmes de reproduction sont très fréquentes. Afin d'évaluer les avantages et les coûts évolutifs des différents systèmes de reproduction, il est nécessaire de comprendre les forces sélectives qui conditionnent ces transitions. Au cours des dernières décennies, les études empiriques sur l'écologie et la génétique des systèmes de reproduction se sont principalement concentrées sur les conséquences à long terme en populations naturelles. Ma thèse a pour but de montrer comment les premières étapes de transitions entre les systèmes de reproduction sont des éléments clés pour comprendre leur évolution. Dans ce but, j'ai utilisé *Daphnia spp*. communément appelée « puce d'eau » comme système modèle afin d'étudier les conséquences génétiques de nouveaux systèmes de reproduction. Tout d'abord, à travers une synthèse bibliographique, j'ai testé si la vision traditionnelle de l'asexualité équivalente à clonalité (la production de descendants génétiquement identiques) est réaliste chez les animaux. Cette synthèse montre que les asexués conservent de nombreuses caractéristiques de la sexualité à partir de laquelle ils ont évolué, et donc que la clonalité stricte n'est pas prééminente. Bien que l'évolution secondaire de l'asexualité semble favoriser la reproduction clonale, les premières formes asexuées n'ont certainement pas été clonales, en particulier du fait de la recombinaison. Dans un deuxième temps, j'ai effectué des croisements entre lignées sexués et asexués chez *Daphnia pulex* où des lignées se reproduisant par asexualité obligatoire et produisant des « mâles rares », coexistent avec des lignées sexuées. J'ai ainsi étudié le taux de recombinaison de ces mâles asexués et j'ai montré que les mâles asexués et sexués ont le même taux de recombinaison, alors que les femelles asexuées ne recombinent pas par rapport aux femelles sexuées. Ces résultats montrent que chez cette espèce l'évolution de la suppression de la recombinaison est spécifique aux femelles ainsi que probablement les modifications de méiose à l'origine de l'asexualité. Ces deux projets montrent que la recombinaison n'est pas exclusive à la reproduction sexuée. Troisièmement, comme les mâles transmettent les gènes d'asexualité via les croisements sexués-asexués (un processus appelé asexualité contagieuse), j'ai également étudié les modes de reproduction et la valeur sélective des asexués générés en laboratoire par rapport aux lignées asexuées naturelles. Les nouveaux asexués générés sont en majorité non clonaux et ont une moins bonne valeur sélective que les lignées naturelles. Ces résultats suggèrent que les lignées asexuées évoluent relativement rapidement pour acquérir les caractéristiques des lignées asexuées observées dans la nature. Enfin, en utilisant une autre espèce, *D. magna*, nous avons étudié les différents niveaux d'expression de gènes entre des lignées composées exclusivement de femelles porteuses d'un proto chromosome sexuel et des lignées proches dont le sexe des individus est déterminé par l'environnement. Cette étude a montré que l'évolution des femelles dont le sexe est déterminé génétiquement et qui ne peuvent plus produire de mâles n'est pas déterminée par une mutation impliquant une perte de fonction mais plutôt par une base génétique plus complexe. Ce travail illustre l'intérêt d'utiliser des espèces présentant un polymorphisme dans les systèmes de reproduction afin d'étudier les premières étapes évolutives de transitions entre les systèmes de reproduction présents dans la nature.

Mots-clés : *Daphnia* | recombinaison | expression de gènes | asexualité contagieuse | croisements | déterminisme génétique du sexe | chromosome sexuel | clonalité

Introduction

Why are reproductive systems important to study?

Reproduction is the process of producing new organisms from one or several individuals. Reproductive systems (also called reproductive strategy, breeding strategy or mating system) is a term that covers all general aspects of the reproductive biology. The mode of reproduction (*i.e.,* sexual or asexual modes of reproduction) mainly determines the reproductive system, but this also includes all factors regulating investment in reproduction (reproductive effort). Reproductive systems thus reflect how one individual or population partitions its reproductive effort and associated energy costs throughout the life cycle (Williams 1966; Angelini and Ghiara 1984; Neal and Anderson 2005).

Understanding how reproductive systems evolve is of fundamental importance because they affect major evolutionary and ecological processes. Indeed, genetic and genomic evolution are shaped by patterns of inheritance as they determine levels of genetic diversity, and levels of inbreeding (Charlesworth and Charlesworth 1995; Otto and Gerstein 2006; Otto 2009). Factors linked to the ecology and reproductive biology of species, such as resource availability, partners availability, sex ratios, mating success, and colonization of new habitats influence the evolution of reproductive systems (Lehmann and Perrin 2003; West *et al.* 2005; Morris *et al.* 2010; Pannell 2015; Fouqueau and Roze 2021). Studying the reproductive systems is thus a central topic for evolutionary biologists.

Sexual reproduction is the core of eukaryotic reproductive systems

The most fundamental distinction of reproduction is between sexual and asexual modes of reproduction. Whereas prokaryotes reproduce asexually by cell division, most eukaryotes have sexual life cycles (Otto 2009; Schurko *et al.* 2009). Sexual reproduction in most organisms, is characterized by the fusion of gametes from two individuals to constitute a new individual. Sexual reproduction is associated with meiosis and recombination. Meiosis is a process reducing ploidy, allowing segregation (random separation of non-homologous chromosomes of each pair) and producing recombinant gametes. Segregation results in new combination of alleles within genes when gametes fuse. During meiosis, homologous chromosomes pair due to double-strand breaks that are repaired via chiasmata by the recombination machinery (Renkawitz *et al.* 2013). Recombination thus allows an exchange of DNA material and can create new combination of alleles between genes. Sex is by far the most used reproductive mode in eukaryotes. While the basic structure of meiosis is well preserved, the mechanisms controlling for reproductive systems including a sexual mode of reproduction are highly diverse (Neal and Anderson 2005; Aanen *et al.* 2016).

During sexual reproduction, gametes originate from independent meiotic events. Gametes may be produced by different sexes, carried by distinct individuals or by different sexual organs of the same individual (hermaphrodites). The sexual identity (*i.e.,* male, female or hermaphrodite), together with the frequency and timing of sexual reproduction (seasonality, facultative sex), are crucial factors responsible for the diversity of reproductive systems.

Sexual reproduction is often linked to the presence of separate sexes with male and female (dioecy or gonochory). However, sexual reproduction can be carried out by one individual that has both sex functions (called hermaphroditic or co-sexuals). Whether the fusion of gametes comes from the same or different genotypes has drastic genetic consequences. In the latter case, called outcrossing, this increases the genetic diversity of populations. The majority of animals are dioecious (Leonard 2010). Still, hermaphroditism is quite common in plants and also in animals, corresponding to up to one-third when excluding insects (Jarne and Charlesworth 1993; Jarne and Auld 2006; Weeks *et al.* 2014). Sexual reproduction can also occur between one sex and hermaphroditic individuals (gynodioecy or androdioecy depending on whether hermaphrodites co-occur with females or males respectively). Although rather rare compared to angiosperms (Charlesworth 2006), androdioecy and gynodioecy are also found in animals (Weeks *et al.* 2006). Eventually, sexual identity can either be determined by

genetic, environment or others factors such as haplodiploidy or paternal genome loss (Bull 1983; Charlesworth 2006; Bachtrog *et al.* 2014; Beukeboom and Perrin 2014). Sex determination -the process through which the sexual identity is established during development- and sexual reproduction are thus often, but not always, linked. The huge diversity of sex determination systems is evident both in plants and animals (Bell 1982; Barrett 2002; Bachtrog *et al.* 2014; Picard *et al.* 2021). Sex switching during the life of a given individual allows for other variations of reproductive systems (sequential hermaphroditism).

Another factor leading to alternative reproductive systems is the frequency of sexual reproduction. Sexual reproduction can be obligate, facultative or never take place (Bell 1982; Decaestecker *et al.* 2009; Schön *et al.* 2009; Stelzer and Lehtonen 2016). Indeed, there are two main modes of reproduction; sexuality and asexuality. Asexuality is defined as the production of a new individual without fusion of meiotic products from two different meioses. In this thesis, I focused on a particular type of asexuality; parthenogenesis (*i.e.,* asexual reproduction through the germline). I will use the term "asexuality" as a synonym of parthenogenesis. If sex is facultative, there is a combination of both sexual and asexual reproductive modes in a same species (Suomalainen 1950; Cáceres *et al.* 2001). Cyclical sexuality occurs when an individual alternates from asexual to sexual modes of reproduction due to seasonal signals or other environmental changes (Stelzer and Lehtonen 2016). When sexual reproduction is completely lost, the mode of reproduction is obligate asexuality.

Today, the understanding of the extent and importance of the different reproductive systems is much more developed in the plant kingdom compared to animals (Jarne and Charlesworth 1993). Overall, the multiple factors, ecological or genetic, that simultaneously influence the evolution of reproductive systems and the difficulties in matching theoretical explanations to empirical evidence leave us largely ignorant of the selective forces that maintain the high diversity of reproductive systems, especially in animals (Kondrashov 1993a; De Visser and Elena 2007; Hadany and Comeron 2008; Otto 2009; Lively 2010; Hartfield and Keightley 2012).

The multiple costs of sex

To understand the wide diversity of different and derived reproductive systems, evolutionary biologists tried to compare their benefits and costs relative to sexual reproduction. To explain the prevalence of sexual reproduction in eukaryotes, sexuality is thought to allow major benefits via DNA repairs (although few evidence exist), the purging of deleterious mutations (Muller's ratchet hypothesis) and the generation of combinations of beneficial mutations (Fisher-Muller hypothesis) through recombination and segregation (Otto and Lenormand 2002; Agrawal 2006a). This can be especially advantageous in fluctuating or stressful environments (Otto and Lenormand 2002; Agrawal 2006b; Hadany and Comeron 2008; Otto 2009; Lever *et al.* 2021). Despite the genetical and other ecological advantages of sexual reproduction, many costs of sex have also been identified (Kondrashov 1993b; Weeks 1996; Otto and Lenormand 2002; Otto 2009; Lehtonen *et al.* 2012).

First, there are costs directly associated with meiosis. Meiosis is one of the most complex molecular mechanism, taking more time and energy than mitotic cell divisions (Otto 2009; Levitis *et al.* 2017). Recombination can also have a cost (the "recombination load") by breaking the association of loci favored by selection (Charlesworth and Barton 1996; Barton and Charlesworth 1998; Otto and Lenormand 2002; Otto 2009; Roze 2009). Second, sexual reproduction is generally thought to occur between separate sexes of different genotypes (outcrossing). This implies energy cost in finding and courting a mate and represents a higher risk of predation or contracting sexually transmitted diseases (Otto and Lenormand 2002; Lehtonen *et al.* 2012). Another consequence is that the dependence to a mate reduces the reproductive assurance, which is the assurance of reproductive success ("Baker's law", Pannell 2002; Wolf and Takebayashi 2004), and which can reduce colonization of new habitat (Hörandl

2009; Pannell 2015). Lastly, with separate sexes, sexual reproduction requires allocation of resources to the production of males which usually contribute very little (only with their genes) to reproduction. This is the famous two-fold cost associated with males (Maynard Smith 1971, 1978). With a 1:1 sex ratio, sexual females have a twofold disadvantage compared to asexual females because of the male production (Lewis 1987; Jokela *et al.* 1997; Schön *et al.* 2008). Finally, if sexual reproduction occurs between gametes from similar genotypes (inbreeding) it can be costly. The most extreme case arises with a self-fertilizing hermaphroditic individual or with male and female from the same clonal lineage. In these cases, inbreeding results in loss of genetic variation (Charlesworth and Charlesworth 1995).

Because sex seems very costly in evolutionary terms, the prevalence of sexual reproductive mode is considered to be a paradox and one of the most intriguing puzzles in evolutionary biology (Maynard Smith 1971, 1978; Bell 1982; Kondrashov 1993a; Barton and Charlesworth 1998; Otto 2009). Intraspecific variation of reproductive system offers an ideal tool for studying the evolution and maintenance of sex. In particular, two main topics receive much interest in explaining the evolution of reproductive systems.

Transitions to asexual mode of reproduction and genetic sex determination

In eukaryotes, two major topics of reproductive systems have intrigued evolutionary biologists for the past decades: the evolution of asexual mode of reproduction and the evolution of genetic sex determination (GSD) and sex chromosomes.

a. Asexuality vs. sexuality and GSD vs. ESD

When seeking for the costs of sex relative to asexuality or to the evolution of sexual chromosomes, we generally assume very definite and derived categories: obligate sexuality is opposed to obligate asexuality (generally considered as clonal), and sex chromosomes are opposed to autosomes. However, both plants and animals uncover a variety of reproductive systems which can also vary among populations of the same species and thus complicate the calculation of costs/benefits relative to the ancestral state (Barrett 2002; Leonard 2018). Therefore, when the categories are more ambiguous, the transitions from sexuality to asexuality and from an environmental sex determination (ESD) to GDS are less clear than the theories predict. Thus, the study of transitions and intermediate systems possess crucial information about both questions: the evolution of asexuality and the evolution of genetic sex determination.

b. Transition from sexual to asexual modes of reproduction

Sexual reproduction has been lost across the eukaryotes independently multiple times (in fungi, Billiard *et al.* 2012; in animals, Simon *et al.* 2003; and in plants, Whitton *et al.* 2008). Many plants that are able to reproduce asexually are in fact facultative sexuals where a single individual can produce seeds both sexually and asexually (Cáceres *et al.* 2001; Aliyu *et al.* 2010). In the animal kingdom, sex-asex polymorphism occurs within species among populations, with some populations reproducing exclusively asexually and other reproducing exclusively sexually (obligate asexuality vs. obligate sexuality). In the case of cyclical or facultative asexuality, the polymorphism is among generations where a sexual generation alternates with generations of asexual reproduction. Cyclical or facultative asexuality is common in several major animal taxa including insects, trematodes, rotifers, aphids, and crustaceans (Bell 1982; Taylor *et al.* 1999; Schön *et al.* 2000; Simon *et al.* 2002; Kobayashi *et al.* 2013). These systems can be used to investigate the selective pressures for differential investment in sexual vs. asexual reproduction and explain the prevalence of sexual vs. asexual modes of reproduction. For instance, because many cyclical asexual animals are short-lived, and inhabit ephemeral ponds, reproductive assurance is, quite conceivably, an important factor shaping their life history. Obligate asexual reproduction could be seen as an extreme case with no investment in sexual reproduction.

However, if sex is facultative, the timing of sex may be such that the costs relative to asexual reproduction are minimal, which affects the whole calculation of the cost of sex (Burt 2000; Innes and Singleton 2000; Peck and Waxman 2000; Hojsgaard and Hörandl 2015).

c. Transition from environmental to genetic sex determination

Transitions to genetic sex determination (GSD) is an evolutionarily derived state widespread in most taxonomic groups, having evolved either from hermaphroditism or from ESD (Bachtrog *et al.* 2014; Blackmon *et al.* 2017). The most familiar form of GSD is through sexual chromosomes (Blackmon *et al.* 2017; Palmer *et al.* 2019), which have received the most attention (Charlesworth 1996; Bachtrog *et al.* 2014; Beukeboom and Perrin 2014). Although the evolutionary transition to GSD from hermaphroditism has received much attention (Ashman 2002; Dorken and Barrett 2004), the transition from ESD is much less documented. Indeed, the evolution of GSD from hermaphroditism evolved independently several times, in plants (Barrett 2010) and in animals, (Jarne and Auld 2006; Weeks *et al.* 2009). These transitions are likely to occur through gynodioecy or androdioecy with gynodioecy being a more common intermediate in flowering plants (Charlesworth and Charlesworth 1978b; Charlesworth 1984; Pannell 2002). Such transitions with intermediate stages have been especially studied in plants, as they can be evolutionary stable, although the complete process toward GSD is likely to occur in two steps requiring at least two closely linked sex-determining genes; the so-called "two-gene model" (Charlesworth and Charlesworth 1978b; Charlesworth 1996). Therefore, GSD may evolve from ESD also via an intermediate state (called partial GSD) in which ESD and GSD individuals coexist in the same population. Genetically determined female would carry a dominant female-determining mutation on an autosome. When hermaphroditism is the ancestral state, inbreeding depression is generally recognized as one of the main selective forces (Charlesworth and Charlesworth 1987, 1995). Indeed, sex chromosomes could have evolved to favor outcrossing vs. inbreeding (Charlesworth and Charlesworth 1978a; Charlesworth 1984; Jarne and Charlesworth 1993; Barrett 2002; Leonard 2010; Weeks 2012; Benvenuto and Weeks 2020). In the case of partial GSD, if the GSD females increase in frequency, for instance due to some fitness advantage such as obligate outcrossing, they could exert sex ratio selection for increased male function in the remaining ESD individuals, which could lead *in fine* to a full GSD system. One other particular aspect to the evolution from ESD, is that environmental unpredictability may be an important factor favoring the establishment of the initial sex-determining mutation (Werren and Beukeboom 1998; Leonard 2010), especially when specific environments are more beneficial to one sex as expected in ESD. Yet, many details, especially regarding the early stages of sex chromosome evolution, are still unknown or controversial and intermediate systems for the ESD to GSD transition, are very rare and understudied.

d. Studying the early evolutionary steps of sex-asex and ESD-GSD transitions

Many studies already addressed the long-term evolution of derived reproductive systems. We are now beginning to understand the long-term costs associated to obligate asexuality, notably caused by slower rates of adaptation, the accumulation of deleterious mutations and loss of diversity due to background selection (Muller 1964; Charlesworth *et al.* 1993; Kondrashov 1993a; Normark and Moran 2000; Neiman *et al.* 2014; Hollister *et al.* 2015) or the long-term genetic consequences of the evolution of sexual chromosomes (*e.g.* evolution of gene expression with dosage compensation, degeneration of sex chromosomes, Bachtrog 2006; Disteche 2012; Gu and Walters 2017; Muyle *et al.* 2017; Lenormand *et al.* 2020; Charlesworth 2021). However, few studies empirically investigated the initial steps of evolution of the transient reproductive modes or sex determination systems. Indeed, short term success is more difficult to study when the transition happened a long time ago, but most importantly, empirical studies comparing derived reproductive systems can be inaccurate because the selective pressures acting

on the intermediate steps may differ strongly from those acting in final reproductive systems (Simon *et al.* 2002; Engelstädter 2008; Archetti 2010; Neiman and Schwander 2011).

To address the issue of realism (how theories fit the data) and better understand how selection acts on the evolution of transient reproductive systems, we investigated species exhibiting polymorphism in reproductive systems which should give more direct insight into the early steps. In this thesis, we studied transitions from sexuality to obligate asexuality and from ESD to GSD in an animal that possesses the two types of polymorphism: *Daphnia.* I will first present the life cycle of these species.

Daphnia life cycle

Daphnia spp. are freshwater crustaceans that constitute an ideal model to study the transitions to asexual reproduction, and to genetic sex determination. Within species, both polymorphisms are found among lineages. Genomic resources are available for the two studied species such as a reference genome (*D. pulex*: Colbourne *et al.* 2011; Ye *et al.* 2017, *D. magna*: Routtu *et al.* 2014 and a genetic map (*D. pulex*: Xu *et al.* 2015, *D. magna*: Dukić *et al.* 2016).

Daphnia spp. generally reproduces via cyclical parthenogenesis (CP) characterized by a partly asexual and a partly sexual life cycle (Hebert 1978; Ebert 2005, **Figure 1**). In the asexual part of the life cycle, females produce daughters or sons that develop in the brood pouch (liveborn or subitaneous egg) via an aborted meiosis leading to clonality (Hiruta *et al.* 2010). As a result, individuals from this parthenogenetic cycle are genetically identical and constitute a clonal lineage (hereafter called "clone" in *Daphnia*). The same females can switch to sexual reproduction that is induced by environmental cues (ESD) (Kleiven *et al.* 1992; Innes and Dunbrack 1993; Ebert 2005; Fitzsimmons and Innes 2006). More precisely, male development is induced by a juvenile hormone emitted by the mother in response to specific conditions, such as shortened photoperiod and/or increased population density (Olmstead and Leblanc 2002; Roulin *et al.* 2013). These males can also be experimentally induced by adding a hormone analog to the culture medium at a precise moment of the ovarian cycle (Olmstead and Leblanc 2002). Sexual reproduction in CP is tightly linked to male production; it leads to the production of haploid diapause eggs, which has to be fertilized by males (Hebert 1978). After fertilization, the diapause eggs (now embryos) are deposited in an envelope made of maternal tissue, the "ephippium". They can withstand harsh conditions, remain viable for long periods and will give rise to female hatchlings of another genotype after diapause, as diapause embryos are the result of sexual reproduction (Cáceres 1998; Ebert 2005, **Figure 1**).

CP constitutes the ancestral reproductive mode in *Daphnia* as shown by the prevalence of CP among other Cladocera crustaceans (Taylor *et al.* 1999). However, another phenotype, which is the first focus of this thesis, is also found in the *D. pulex* and the *D. carinata* complexes (Colbourne *et al.* 2006). Some individuals reproduce exclusively via parthenogenesis: they are obligate parthenogens (OP) (**Figure 1**). Females still produce diapause eggs but they do not need to be fertilized and give rise to hatchlings of the same genotype as the mother (Omilian *et al.* 2006). The main differences between CP and OP reproductive strategies in *Daphnia spp*. is thus the ephippia production (ephippial phase), the primary dispersal stage which allows for the maintenance of the populations over years. Yet, obligately parthenogenetic lineages rarely still produce males ("rare males"), some of which are capable of haploid sperm production (Xu *et al.* 2015a), allowing them to mate with sexual females and transmit the parthenogenesis genes in a "contagious" fashion (*i.e.,* transformation of sexuals to parthenogens, Innes and Hebert 1988). Both OP and CP genotypes can coexist in the same pond, although this is apparently not common (Crease *et al.* 1989; Hebert *et al.* 1989).

Independently of the CP or OP reproductive systems, in the same population, some clones are constituted exclusively of genetically determined females that never produce males. Within these clones, which are called NMP for "non-male producer", sex is genetically determined (GSD). Thus, NMP have

a new proto sex chromosome. The ancestral state is thus an environmental sex determination (ESD), where females can produce males (MP, "male producer") via environmental cues (see above, **Figure 1**).

Assuming that male production and sexual events are regulating sexual reproduction, the different levels of investment in sexual reproduction could be classified according to the different phenotypes: CP MP clones are the ones that invest the most in sexual reproduction (they make males and reproduce sexually), then CP NMP do not produce males but still reproduce sexually, OP MP invest in sexual reproduction only through the males they still produce, and finally OP NMP neither invest in sexual mode of reproduction nor in males.

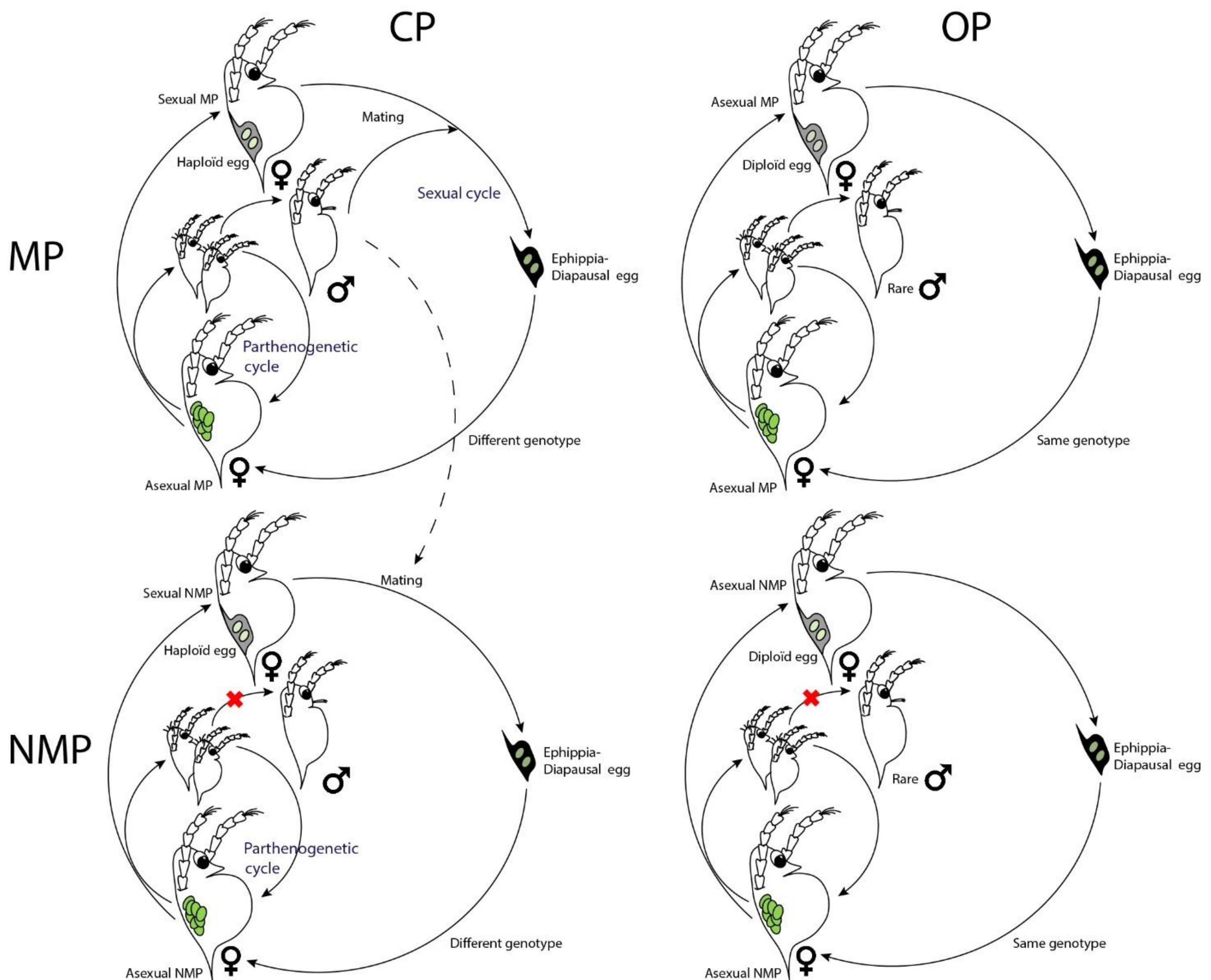

Figure 1: *Daphnia* life cycle. Cyclical parthenogenesis (CP) is in the first column and obligate parthenogenesis (OP) is in the second column. Male producing (MP) are in the first line; males are environmentally produced and are genetically identical to the mother. Non-male producing (NMP) genotypes are in the second line. These genetically determined females do not produce males anymore (represented by a red cross). Note that in CP, if the ephippia are not fertilized by males (dashed arrow), they remain empty.

Clonality and obligate parthenogenesis in *Daphnia*

a. Clonality in animals

Parthenogenesis is very rare in animals (about ~0.1%) (White 1978) when considering exclusively obligate parthenogens (OP). Parthenogenesis leading to offspring identical to the mother; clonality, is thought to be the most common type in animals (Suomalainen 1950). However, parthenogenesis is thought to have evolved from sexual relatives and thus meiosis (Ramesh *et al.* 2005). Thus, many mechanisms of parthenogenetic reproduction are in fact meiosis modifications; either a suppression of one meiotic division (meiotic apomixis), a fusion of nuclei produced by a complete meiosis (automixis), a duplication before (premeiotic doubling) or after (gamete duplication) meiosis (Archetti 2010). For all these modes of parthenogenesis, we can distinguish as major genetic consequences: intermediate loss of heterozygosity rates, a complete loss of heterozygosity or a complete retention of heterozygosity. In the latter case, the production of offspring identical to the mother generating a true "clone" is equivalent to what would be obtained via mitosis (mitotic apomixis). In the literature, parthenogenesis is often confused with clonality. In the **first chapter** of this thesis, I investigated the actual prevalence of clonality in parthenogenetic animals at a broad phylogenetic scale in collaboration with a PhD student colleague. In this survey, we investigated OP but also cyclical and other types of facultative parthenogens to better understand the actual distribution of asexual modes of reproduction nowadays, but, by inference, also when they initially arose. Looking for traces of the initial evolutionary steps from meiosis to parthenogenesis is important to better understand how asexuality emerged. Whether the majority of asexuals is truly clonal or whether most have deviations from clonality, impacts the realism of theories on benefits and costs of their reproductive modes. Indeed, "evolution of sex " theories were most often developed with the simplification that sexual reproduction is associated with meiosis while asexual reproduction is associated with mitosis (Simon *et al.* 2002; Engelstädter 2008; Archetti 2010; Neiman and Schwander 2011). The two following chapters investigate more specifically the genomic consequences of OP in *Daphnia.*

b. Obligate parthenogenesis in *Daphnia pulex*

The genetical consequences of the majority of the different asexual modes of reproduction highly depend on recombination (Archetti 2010). In OP where meiosis is not complete or even absent, meiosis-specific genes or genes involved in meiotic recombination are thought to be altered as they are supposed to be central to sexual reproduction (Smith and Nicolas 1998; Villeneuve and Hillers 2001; Tsubouchi and Roeder 2003). However, this hypothesis lacks clear empirical evidence. A case study, where this has been intensely investigated is *Daphnia pulex* where genes that suppress meiosis have been suspected (Hebert 1981; Innes and Hebert 1988). In this species, the genetic basis of OP has been investigated for decades. The first study suggested that OP in *D. pulex* is determined by a single, dominant chromosomal region (Innes and Hebert 1988) based on few crosses. However, several loci on several chromosomes have been identified by association studies, but we do not know if they cause OP, or are simply associated to secondary evolution of some traits within OP lineages (Lynch *et al.* 2008; Xu *et al.* 2011, 2015b; Eads *et al.* 2012; Tucker *et al.* 2013). A more recent study has also highlighted that modification of *Rec8* function (a gene involved in separation of sister chromatids and homologous chromosomes during meiosis) is possibly responsible for converting meiotically reproducing lineages into OP (Eads *et al.* 2012). Indeed, the estimation of OP female recombination is close to zero, but we still do not know whether the absence of recombination in females is female-specific. In the **second chapter**, we investigated the meiotic recombination ability during the ephippial production of a CP female, a CP male and an OP male to test whether the evolution of recombination rate during the CP-OP transition is sex-specific.

In addition to the meiosis and recombination features associated with sex, sexual reproduction also involves the production of males. However, in many asexual species, males are still produced by OP mothers as in the case in *Daphnia pulex* (**Figure 1**). The evolutionary significance of these OP males has been contentious. In some cases, they were supposed to be reproductive errors and evolutionary irrelevant (and therefore representing a fitness cost for the lineage producing them), while in others, they have been viewed as vectors for genetic exchange between asexuals and their sexual relatives. In particular, these OP males are thought to be able to transmit asexuality in crosses with sexual relatives, a process termed "contagious asexuality", generating new asexual lineages. Indeed, in several cases, they were found to transmit the asexual genes by mating with sexual (CP) females (Innes and Hebert 1988; Lynch *et al.* 2008; Xu *et al.* 2015b). As long as these contagion events are frequent, and pivotal for the long-term persistence of OP lineages, these rare males may therefore play a strong role in the origin and maintenance of asexuality. Moreover, contagious asexuality conferred by OP males in *Daphnia pulex*, is thought to explain the evolutionary persistence and the polyphyletic origin of OP clones (Crease *et al.* 1989; Paland *et al.* 2005). Determining how efficient contagious asexuality is in generating new asexuals will determine how beneficial OP males are compared to their presumed cost. In the **third chapter** we produced a CP x OP cross (**Figure 2**), and studied the mode of reproduction and fitness of the new asexuals generated by contagion and compared them to natural asexuals.

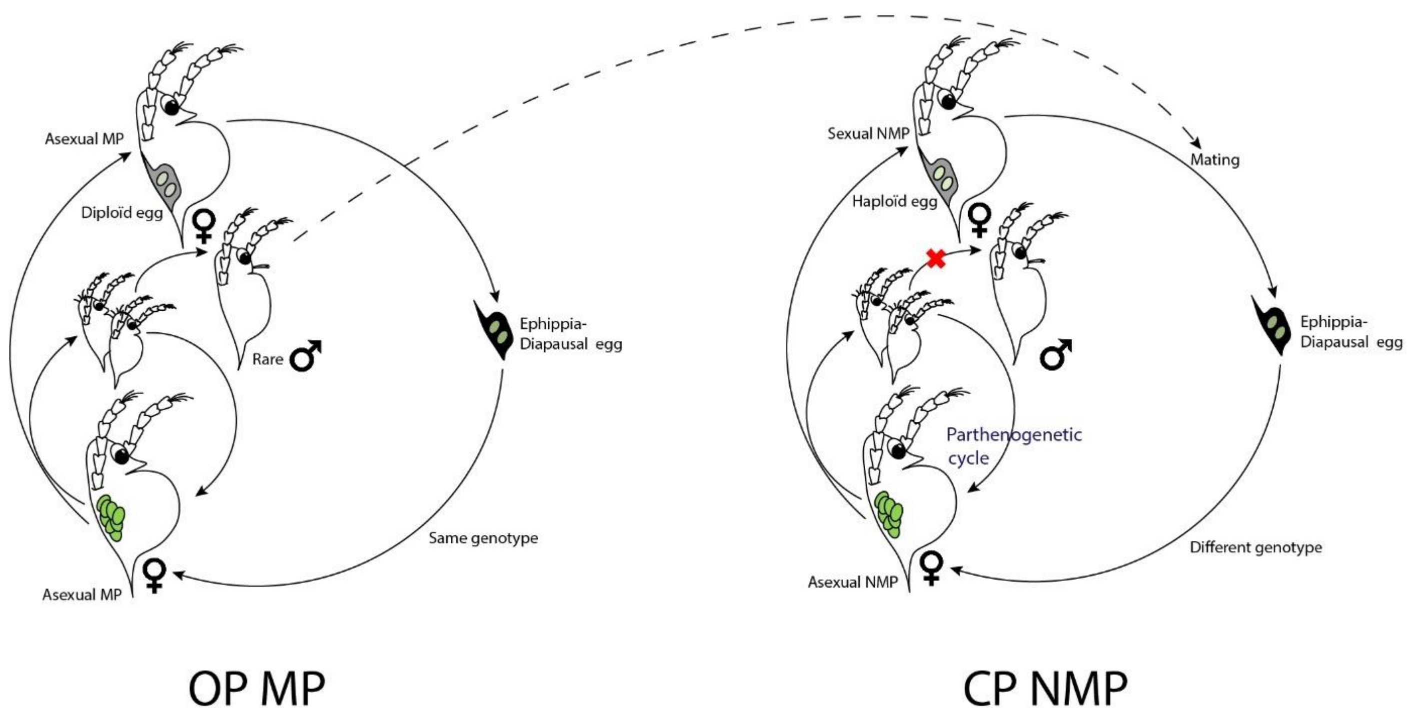

Figure 2: Experimental design showing the sex-asex (CP x OP) cross. Rare asexual males were induced from OP lineages and were manually added to aquaria each containing a CP NMP clonal lineage. Ephippial eggs are thus obligatory produced via a sexual event between CP females and OP males.

An incipient W sex chromosome in *Daphnia*

Daphnia spp. is also a good model to investigate the investment in males and sex-chromosomes. Genetic variation for diapausing egg and male production frequency, two characteristics generally associated with sexual reproduction, occurs within the CP (Larsson 1991; Innes and Dunbrack 1993; Innes 1997). This variation can be extreme as some genotypes are no longer able to produce males (Innes and Dunbrack 1993; Innes 1997; Tessier and Cáceres 2004). In *D. pulex* and *D. magna*, some clones are exclusively made of genetically determined females (GSD) that never produce males neither in nature nor under artificial hormone exposure inducing male production and are called NMP. In a same population, such clones can coexist with the others clones called MP (male producing) that have an ESD (Galimov *et al.* 2011). As explained above, the partial GSD situation is equivalent to gynodioecy although sexes are not found in the same individual, but rather in different clones of a same population: ESD individuals are able to produce both males and females (hermaphroditic-like) and GSD clones are composed of only females (sexual females).

While mainly avoiding the costs of producing males, NMP can benefit from males of other genotypes, reducing the cost associated with intra-clonal mating (inbreeding avoidance). Indeed, within a clone, although males and females are independent individuals, the situation is the same as in hermaphroditism, where a sexual reproduction event is equivalent to self-fertilization (males and females are genetically identical). Thus, the evolution of separated sexes through a gynodioecy-like system could have evolved to reduce costs associated with inbreeding depression and male production although CP NMP females may have high fitness costs associated with reproductive insurance (they are dependent on MP males to produce viable ephippia).

Previous studies already found that the NMP phenotype is determined by a locus that segregates as a single dominant locus (Galimov *et al.* 2011; Reisser *et al.* 2017; Ye *et al.* 2019). The system is analogous to a WZ/ZZ systems in which the "NMP chromosome region" is on an incipient W chromosome. Heterozygous individuals (genotype WZ) are the NMP, whereas homozygotes (ZZ) are MP, and crosses between NMP females and MP males result in 50 % NMP and 50 % MP offspring (Galimov *et al.* 2011; Reisser *et al.* 2017). In *D. pulex*, using genomic data of NMP and MP clones from five different populations, one unannotated gene (gene 8960) has been identified located within a 1.1-Mb nonrecombining region on linkage group (LG) 1 (Ye *et al.* 2019). The NMP phenotype is thought to be caused by expression change of this gene, downstream of the male-inducing signaling pathway (Ye *et al.* 2019). Although I am a co-author of this article, it is not included in the thesis as it is the result of a collaboration before the start of my thesis. In *D. magna*, the MP/NMP polymorphism is determined by a large (~2Mb), non-recombining chromosomic region on linkage group 3 (LG 3) (Reisser *et al.* 2017). However, we still do not know which of the over 600 genes in the NMP chromosome region are causing the NMP phenotype, nor do we know the identity of downstream genes and molecular networks involved in the difference between the MP and NMP phenotypes. In **the fourth chapter**, we investigated the evolution of gene expression pattern during a transition from ESD to GSD in *D. magna*. While this article was started during my Master 2, it was finalized at the beginning of my thesis.

Outline of the thesis

Sexual reproduction is often equated with meiosis, recombination and male production whereas asexuality is regarded as being characterized by mitosis, no recombination, and no males. In this thesis, I tested whether such simplification is realistic with an emphasis on empirical investigations on the model *Daphnia*. I relate these findings to the evolution and maintenance of sex (**chapters 1 to 3**). Thanks to a unique transient system in animal equivalent to gynodioecy, I also investigated the other main topic on evolution of sex: the evolution of a nascent sex chromosome. At the beginning of each unpublished manuscript, I indicated the current state of progress of each article. In the Discussion, I presented an

overview of the studies generated in the framework of this thesis, in relation with the literature. Across this thesis, I show that considering transient stages in the reproductive system is pivotal to understand their evolution.

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Chapter 1

Questioning the preeminence of clonality among parthenogenetic animals

Work in progress. The current state of the manuscript is not finished. We are still discussing the organization of the content with the authors.

Questioning the preeminence of clonality among parthenogenetic animals

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Abstract

Clonality is a form of asexual reproduction defined as the identity between parents and offspring, barring new mutations. Even though non-clonal forms of asexuality exist, clonality is considered the most common asexual reproductive mode. Likewise, theoretical models of the evolution of asexuality generally assume that it equates clonality. Yet, recent evidence has revealed non-clonal forms of reproduction in several species formerly believed to be clonal, and new theoretical results highlight potentially striking evolutionary differences between clonal and non-clonal asexuals. In fact, the problem may be more global and involve many more taxa. We argue that, for methodological and conceptual reasons, there has been a strong confirmation bias favoring clonality. In this paper, we present the different paths through which clonality can emerge in animals. We review the commonly used evidence for clonality and point out potential confusing factors and perception biases. We find that although many asexuals seem clonal, a large part of them is not strictly clonal. These small discrepancies nevertheless may have important impact. Selection for more clonal reproduction and the possible absence of mitotic parthenogens indicate that, over an evolutionary timescale, clonal species may have been non-clonal. Taken together, these conclusions do not support a preeminent role of clonality in the evolution of asexuality. We thus call for a broader inclusion of non-clonal reproductive modes for a more realistic view of asexuality.

Key-words: Parthenogenesis, apomixis, automixis, cytology, genetics, loss of heterozygosity

Introduction

Asexuality is an uncommon reproductive mode in eukaryotes, where offspring are produced by a single individual without reduction and fusion of gametes. Populations of asexual organisms are often thought to have little or no evolutionary potential due to a lack of genetic diversity. However, absence of genetic diversity is expected only under a specific mode of asexual reproduction: clonality. Clonality is defined by the absence of any genetic differences between parent and offspring, barring mutations. It has been known for long that in asexuals that reproduce through the germline (termed parthenogenesis in animals) non-clonal asexuality exists. Still, clonality is considered the most common Asexual Reproductive Mode (ARM) in eukaryotes in most empirical reviews (Suomalainen 1950; De Meeûs *et al.* 2007; Sköld *et al.* 2009).

As a consequence, the vast majority of theoretical models on the evolutionary maintenance of sex contrasts sexuality with clonality, excluding other ARMs. In these models, the switch from sex to asexuality is simple, as if it was caused by a unique mutation (Crow and Kimura 1965; Smith and Maynard-Smith 1978; Barton and Charlesworth 1998; Otto and Lenormand 2002; Otto 2009; Levitis *et al.* 2017). More generally, asexuals are also considered clonal in most models examining the consequences of asexuality on population genetics (Crow and Kimura 1965; Smith 1968; West *et al.*

1999; Hartfield and Keightley 2012), phylogeny (Birky 1996; Janko 2014), and genetic diversity (Kondrashov 1993; Mark Welch and Meselson 2000). Asexuality is also simplified as clonality when studying the link between asexuality and polyploidy (Saura *et al.* 1993), why asexuals tend to have a wider geographic distribution than sexuals (geographic parthenogenesis, Haag and Ebert 2004; Hörandl 2009; Vrijenhoek and Parker 2009) and what role asexuality plays in the success of agronomic pests (Hoffmann *et al.* 2008).

However, since the emergence of sexual reproduction is tightly linked with the evolution of eukaryotes (Hawes 1963; Otto and Lenormand 2002; Speijer *et al.* 2015; Lenormand *et al.* 2016), every parthenogenetic eukaryote species necessarily evolved from sexual reproduction. To summarize, there are four broad categories of transitions from sex to asexuality based on genetic consequences: First, several modifications of meiosis produce unreduced daughter cells, thus resulting in asexuality. These meiosis modifications can cause loss of heterozygosity (LOH), in which case they are non-clonal. 1) Some of these modifications, such as gamete duplication, terminal fusion or suppression of the second meiotic division, generally lead to high LOH and thus cannot be clonal. Others cause intermediary LOH, and can lead to clonality if the mechanism responsible for LOH is suppressed. This is the case for 2) central fusion or suppression of the first meiotic division, where LOH is caused by recombination and 3) premeiotic doubling, where LOH is caused by non-sister pairing (see **Box 1**). Lastly, asexuality could evolve through a switch from meiosis to 4) mitosis, which is always clonal because it does not cause LOH. Although many asexuals are considered mitotic, this last possibility does not appear to be the most parsimonious route of evolution to asexuality (reviewed in Lenormand *et al.*, 2016).

Hence, most routes toward the evolution of asexuality are through diverse modifications of meiosis, of which only few can lead to clonality. Still, numerous model species are considered clonal based on interpretations of early studies which are rarely questioned (Suomalainen 1950; Bell 1982). These initial sources can however be limited technically or conceptually, leading to errors in ARM identification. When clonality was questioned, it sometimes led to debates (*e.g.,* in diploid Artemia: Nougué *et al.*, 2015, aphids and *Daphnia pulex*: Blackman 1979). Yet, clonality has recently been disproved in several famously clonal species (Bdelloids: Simion *et al.* 2021; Tran Van *et al.* 2021, Timema: Schwander 2021), highlighting the fact that both methods and concepts used in identification remain unclear. This, together with the tendency of considering clonality as the "default" ARM, suggests that the perception of asexuality is biased toward clonality.

Considering the diversity of ARMs is important and has major implications. Indeed, the few existing theoretical models incorporating non-clonal asexuality found striking differences in consequences and expectations compared to clonality. Because LOH reveals deleterious alleles, it leads to genetic load. Thus, ARMs with low LOH rates are expected to be more frequent than those with high LOH rates. This can be due to selection for lineages with low LOH or to gradual evolution toward lower LOH, perhaps down to clonality (Engelstädter 2008; Archetti 2010). Therefore, as the asexuals found in nature are the most successful, we can wonder how prevalent clonality has been over time.

In this review, we assessed among parthenogenetic animals whether clonality is as prevalent as reported, how strong the bias toward clonality is, and thus whether the evolution of asexuality is mainly shaped by clonality. It was not our intention to review systematically the vast existing body of literature on asexuals, but we wished to provide the reader with examples that cover a wide diversity of taxa and reflect the different methods used for the identification of ARMs. To this end, we collected parthenogenetic species based on several reviews on asexuality in animals (Suomalainen 1950; Bell 1982; Rabeling and Kronauer 2013; Vershinina and Kuznetsova 2016; van der Kooi *et al.* 2017; Gokhman and Kuznetsova 2018). Among those, we selected species in which an ARM was identified based on any piece of evidence, including both obligate and facultative asexuals. We specifically investigated whether the species was identified as clonal in the literature, what evidence was used and

whether changes or debates over this ARM occurred. We analyzed hundreds of papers from 1940 to this day.

We structured our review by the different ways clonality can arise (see above). We further clarified erroneous methods and misconceptions that have been used as proof of clonality. On this basis, we discuss whether it is still legitimate to presume that the great majority of asexuals are clonal, and consider the implications with respect to the importance of clonality in the evolution.

Asexual reproductive modes with high LOH

First, we focus on two main mechanisms of parthenogenesis via modified meiosis that generate high LOH. Gamete duplication restores ploidy by duplication of a reduced set of chromosomes after the second meiotic division, thus resulting in complete LOH from parent to offspring. Second, "terminal fusion-like" parthenogenesis (refusion of products of the second meiotic division or suppression of the second meiotic division, see **Box 1**) leads to complete LOH except in the recombinant parts of chromosomes.

LOH is associated with high potential genetic load. Because these ARMs lead to high LOH, they are expected to be rare (Archetti 2010). Indeed, they are mainly found in tychoparthenogenetic species, which are otherwise sexual species with exceptional events of asexual reproduction. Tychoparthenogenesis with high LOH is found in certain reptiles (*e.g., Thamnophis sp.*, *Varanus sp.* and *Ophiophagus sp.:* Lenk *et al.*, 2005; Watts *et al.*, 2006; Reynolds *et al.*, 2012; Card *et al.*, 2021) and sharks (Chapman *et al.* 2007; Dudgeon *et al.* 2017). Nevertheless, ARMs with high LOH are in fact also found in species where asexuality represents a significant part of the life cycle, such as in *Meloidogyne sp.* (Triantaphyllou 1966; Van Der Beek *et al.* 1998), oribatid mites (terminal fusion-like parthenogenesis: Palmer and Norton, 1992), tardigrades (terminal fusion-like parthenogenesis: Ammermann, 1967; Bertolani, 1981; Rebecchi *et al.*, 2003), termites (gamete duplication and terminal fusion-like parthenogenesis: Matsuura, Fujimoto and Goka, 2004; Fournier *et al.*, 2016) and stick insects (gamete duplication: *Bacillus rossius*, Pijnacker, 1969). Thus, these ARMs are not as exceptional as expected. Still, most of these species are not obligate asexuals. Perhaps, the sexual part of their life cycles can compensate the costs of high LOH.

Parthenogenesis with high LOH has sometimes been wrongly inferred based on cytological evidence, due to inverted meiosis (where the equational division takes place before the reductional division). Taberly (1987) concluded to terminal fusion-like parthenogenesis in the oribatid mite *Platynothrus peltifer*, but a genetic study did not concur (Palmer and Norton 1992). It was later proposed that this species had inverted meiosis (Wrensch *et al.* 1994) and that its ARM was actually equivalent to the central fusion-like parthenogenesis (see following section). Central fusion with inverted meiosis was also suspected in *Archegozoetes longisetosus* (Laumann *et al.* 2008) after cytological observations contrasted with previous genetic results. Except for inverted meiosis, the interpretation of cytological observations is generally straightforward for this type of ARM because of the important meiosis modifications associated (extra doubling, refusion, suppression of the equational division). For instance, in annelids, Christensen (1960) showed that several species reproduce by suppression of the second meiotic division. Therefore, there does not seem to be a bias toward clonality when cytologically identifying ARMs with high LOH.

The observation of LOH by comparison of genetic markers in asexual females and their offspring is strong evidence against clonality (Pearcy *et al.* 2006; Engelstädter 2008). It is expected under gamete duplication (complete LOH) and terminal fusion-like (although heterozygosity can be retained in telomeric markers due to recombination). Thus, identifying such ARMs appears relatively straightforward with this method using a few genetic markers. However, in these ARMs, heterozygosity is lost over the whole genome in one or a few generations. Therefore, it should be difficult to find informative, *i.e.,* heterozygous markers, after an asexual reproduction event. Once heterozygosity is lost,

there would be generally no differences between parents and offspring, and no further LOH could be detected. Nevertheless, this method has proven efficient to identify these ARMs. For instance, using parent-offspring comparison, gamete duplication was identified in termites (Fournier *et al.* 2016; Hellemans *et al.* 2019) and terminal fusion in termites (Matsuura *et al.* 2004; Vargo *et al.* 2012; Yamamoto and Matsuura 2012; Luchetti *et al.* 2013) and reptiles (Lenk *et al.* 2005; Reynolds *et al.* 2012; Card *et al.* 2021). The reason why heterozygous markers were found in these species might be that in facultative or cyclical parthenogenesis, sex occurs at least occasionally, which is sufficient to restore heterozygosity.

Gamete duplication and terminal fusion-like parthenogenesis cause high LOH. Despite the high cost they generate, these ARMs are in fact found in numerous species. This could be explained by their life cycles which include sexual events. Non-obligate parthenogenesis also explains why LOH is often easily detected in these species. Still, these ARMs can appear close to clonality between successive asexual generations, as heterozygosity is not restored. This however cannot lead to clonality because recombination could happen, and any new mutation appearing in a generation would likely go through LOH in the next, with important fitness consequences for deleterious mutations.

Central fusion-like parthenogenesis

In modified meiosis where the first division is suppressed or the products of the first division fuse (regrouped under "central fusion-like" parthenogenesis), clonality is attained if paired homologous chromosomes do not recombine or in absence of pairing (Suomalainen *et al.* 1980, see **Box 1**). If the first division is suppressed, absence of pairing cytologically resembles mitosis (see next section). However, pairing of homologous chromosomes and recombination can occur. For example, in tardigrades, both pairing and recombination were found in three species (Bertolani and Buonagurelli 1975; Rebecchi *et al.* 2003). This can be the case also if meiosis I is partial or aborted (Bacci *et al.* 1961; Cognetti 1961, 1962; Scali *et al.* 2003). If recombination occurs, heterozygosity is lost from the location of crossing-over to the telomeres, or to the next location of crossing-over. This means that LOH is more likely to happen far from the centromere.

LOH, because it is costly, should not happen frequently in central fusion-like parthenogenesis (Engelstädter 2008; Archetti 2010). Accordingly, reduced LOH was found in several species with these ARMs (diploid *Artemia parthenogenetica*: Boyer *et al.* 2021; *Wasmannia auropunctata*: Rey *et al.* 2011; Cape honey bees: Goudie *et al.* 2012; Oldroyd *et al.* 2021), while to our knowledge there are no obligate parthenogens with this ARM and high LOH rates. LOH reduction can be due to low recombination rates, localization of crossing-overs near the telomeric region of chromosomes (as in *Oenothera sp.*, Ranganath 2008), or simply because recombinants do not survive (as in the Cape honey bee: Baudry *et al.* 2004; Goudie *et al.* 2012). Due to low LOH rates, species with these ARMs can be erroneously identified as clonal.

Deciphering whether pairing occurs can be especially challenging in older observations, as it was the case for the tardigrade *Ramazzottius oberhaeuseri* (Rebecchi and Bertolani 1988). Similarly, the first cytological study of the Amazon molly (*Poecilia formosa*) falsely concluded to clonality because pairing was undetectable (Rasch and Balsano 1974, rectified by Rasch *et al.* 1982). Meiosis in which Prophase I is elusive or asynchronous among chromosomes (Bishop 1994; Golubovskaya *et al.* 2002) can also complexify the detection of pairing. Polyploid asexual *Artemia* are usually described as clonal. However, Rode *et al.* (2021) reinterpreted previous cytological observations, where the number of chromosomes observed decreased succinctly before the division. They concluded that meiosis I is aborted and a brief pairing occurs before meiosis II, which could lead to recombination (and thus non-clonal asexuality). Additionally, detecting if recombination happens can also be difficult. For instance, recombination was not observed in a tardigrade, possibly because it had late pairing (Ammermann

1967). Therefore, certainty that pairing is absent is not guaranteed and mistakes can be made. Furthermore, in the case where pairing is reported, because recombination can be rare, potentially many observations are needed to be certain of clonality. These difficulties cause mistakes in identification that lead to a bias toward clonality.

Genetic methods can provide simpler evidence as they can easily give information on several reproductive events, but these methods rely on the occurrence of informative markers. The probability to observe parent-offspring LOH is increased in genetic markers the further they are from the centromere (Pearcy *et al.* 2006; Fougeyrollas *et al.* 2015). It is thus important to account for the chromosomal position of the markers used. Specifically, using centromeric markers for parent-offspring comparison could lead to deduce wrongly clonality. For example, in the parasitoid wasp *Trichogramma cacoeciae*, no LOH was found in one microsatellite marker in an iso-female line, which was interpreted as clonality. One alternative interpretation from the authors is that this species reproduces through central fusion-like parthenogenesis and that the marker may be located in the centromeric region, and thus be unlikely to lose heterozygosity. This could well be the case because this marker was highly heterozygous in wild populations among markers with variable rates of heterozygosity (Vavre *et al.* 2004). Thus, centromeric markers will be informative but will not show LOH. A further difficulty in obtaining informative markers, might be the development of markers in distal regions of chromosomes as they are constituted of highly repetitive elements (Blackburn 1991; Sohn and Nam 2018).

Variation in heterozygosity is an expected consequence of central fusion-like parthenogenesis with recombination. Due to the pattern of LOH along chromosomes, heterozygosity should be lower with increasing distance from the centromere (see **Box 1**). Based on this expectation, central fusion-like parthenogenesis was inferred in diploid *Artemia*, where F_{IS} was consistently variable among populations (Nougué *et al.* 2015). Similarly, both central and terminal fusion were detected in *Daphnia magna* thanks to chromosomal patterns of heterozygosity (Svendsen *et al.* 2015). Heterozygosity patterns on chromosomes are also affected by active selection against LOH at specific regions where it is particularly costly. For example, in the Cape honey bee worker line known as the Clone (although they reproduce by central fusion-like parthenogenesis), there are large regions of retained heterozygosity, notably around the sex locus, which is lethal if homozygous (Goudie *et al.* 2012). Under central fusion-like parthenogenesis, we thus expect that there are highly heterozygous and highly homozygous genomic regions. This can also explain why LOH may go undetected in central fusion-like parthenogenesis: In wild populations, markers that are the most likely to lose heterozygosity are probably already homozygous, and thus non-informative in parent-offspring comparisons. Hence, the remaining heterozygous markers are those with the lowest probability of LOH, either because they are centromeric or because they are linked to a recessive deleterious allele. For instance, in *Daphnia magna*, Dukić *et al.* (2019) did not find any LOH events in iso-female lines, although they used markers evenly distributed along the chromosomes. It is possible that markers where LOH could occur had already lost heterozygosity. In diploid *Artemia parthenogenetica*, no LOH occurred in iso-female populations after tens of generations (Nougué *et al.* 2015). LOH was later observed due to interpopulation crosses that restored heterozygosity along the chromosome, and especially in regions that would have already lost heterozygosity (Boyer *et al.* 2021). This approach could be used at a larger scale to improve detectability of recombination in central fusion-like ARM. To conclude, the pattern of LOH along chromosomes in central fusion-like parthenogenesis is recognizable. However, it can bias the identification of ARMs toward clonality, as heterozygous (thus informative) markers will be mainly found in parts of the chromosomes that behave the most clonally.

Rare or localized LOH can thus go undetected, and it is unclear how many markers and parent-offspring comparisons with absence of LOH are sufficient to infer clonality confidently. In the Cape honey bee, although central fusion-like parthenogenesis was identified cytologically (Verma and Ruttner 1983), Moritz and Haberl (1994) observed no LOH in parent-offspring comparison based on

DNA fingerprinting on 12 markers. However, subsequent studies based on 101 (Baudry *et al.* 2004) and 6 microsatellite markers (Goudie *et al.* 2012) reported several LOH events. Moreover, rare occurrences of LOH can look like, and are often interpreted as, other processes such as mutation, mitotic recombination or gene conversion (Tiedemann *et al.* 2005; Malysheva *et al.* 2007). We stress that gene conversion and recombination originate from the same molecular mechanism, so that they cannot be considered as completely distinct mechanisms (Keeney 2001). With the advancements of genomics, it is now easier to identify clonality by genotyping a large number of markers distributed over the length of chromosomes with known physical positions, including the telomeric region (Loxdale and Lushai 2003). However, these methods depend on the quality and resolution of genetic tools (map, assembly) which can be difficult to produce in asexuals. For instance, the first genome assembly for the Bdelloid rotifer *Adineta vaga*, suggested clonality (Flot *et al.* 2013), but a new assembly allowed the detection of LOH in this species (Simion *et al.* 2021).

In central fusion-like parthenogenesis, recombination, which can be observed cytologically, causes LOH. This generates a pattern of heterozygosity, which can complicate the detection of LOH using genetic markers. Regions that are the most likely to lose heterozygosity tend to be already homozygous and thus non-informative, so that the only informative regions are those that are the least likely to lose heterozygosity. When recombination is rare, detecting LOH is even more difficult and necessitates multiple observations. Genomics could provide a solution to these problems, although it may be complex in non-model species. If recombination is totally suppressed, this ARM is clonal. This can be achieved by suppression of recombination or by suppression of pairing. The latter, in the case where the first meiotic division is suppressed, will result in a modified meiosis very similar to mitosis.

Mitosis

Parthenogenesis through mitosis undoubtedly leads to clonality. Historically, it was assumed that asexuality exclusively arises by mitosis, however this has been clearly refuted. Although many asexuals are still considered mitotic (Levitis *et al.* 2017), there is actually no conclusive evidence for parthenogenesis through mitosis in any animal system (Archetti 2010). Still, parthenogenetic mechanisms with one equational division (equivalent to suppression of the first division or mitosis, **Box 1**) have been associated with mitosis even when pairing was observed, although pairing is a meiotic process (see previous section). For example, *Daphnia pulex* was described as "apomictic" (see **Box 1**), which was interpreted as mitotic reproduction, although several cytological observations described the first steps of meiosis including pairing (Ojima 1954; Bacci *et al.* 1961; Zaffagnini and Sabelli 1972). Later on, modified meiosis with pairing was again reported in this species (Hiruta *et al.* 2010), indicating central fusion-like parthenogenesis with no or very low levels of recombination. Therefore, mitotic parthenogenesis is still thought to be common due to conceptual or vocabulary biases, although now there is no clear indication that mitotic parthenogenesis exists in animals.

Some animals indeed reproduce through one equational division with no pairing (several species of *Meloidogyne*: Marais *et al.* 1991; Van Der Beek *et al.* 1998; Janssen *et al.* 2017; tardigrades: Bertolani 1971, 1973; Bertolani *et al.* 1987; Rebecchi and Bertolani 1988, 1999; Rebecchi 1991; Guidetti *et al.* 2019); gastropodes: Mattox 1937; Dougherty 1989; Hemiptera: Nokkala *et al.* 2008, 2017); one species of Psocoptera: Nokkala and Golub 2006). In these cases, mitotic and central fusion-like parthenogenesis are undistinguishable based on cytological observations. However, numerous cytological remnants can be observed, indicating the underlying cellular process is meiotic rather than mitotic. For instance, in *Dendrobaena octaedra*, no pairing is generally reported (Omodeo 1955; Hongell and Terhivuo 1989) but Casellato and Rodighiero (1972) observed some pairing in one sample. The extruding of a polar body (Acarida: Heinemann and Hughes 1969; *Diploscapter pachys*: Fradin *et al.* 2017, *Daphnia*: Zaffagnini 1987), the observation of lampbrush chromosomes, and the elaboration of nucleolar

ribonucleoproteins (*Poecilia formosa*: Monaco *et al.* 1984) are other cytological features normally found in meiotic oogenesis. Similarly, the activity of genes specific to meiosis, as was reported in a nematode (Fradin *et al.* 2017), provides indication on the meiotic mechanism even if some meiotic genes are missing. These traces of meiotic processes constitute evidence against mitosis. Moreover, in gynogenetic animals, oogenesis is triggered by sperm, whose function is to resume arrested meiosis (which always occurs during meiotic divisions specifically to prevent any mitotic cleavage, Lenormand *et al.* 2016). This means that gynogenetic parthenogenesis is, too, unlikely to be mitotic. Indications and clues on the underlying reproductive mechanism in possibly mitotic species, when found, all indicate modified meiosis rather than mitosis.

Once believed to be the primary ARM, mitosis is still considered a very common parthenogenetic mode. However, it can be confused cytologically with modified meiosis where the first division is suppressed (central fusion-like parthenogenesis). In these cases, small remnants of the original mechanism detected cytologically or with molecular biology are often found, and they always indicate meiosis. Whether mitotic parthenogenesis actually exists in animals is therefore still an open question.

Premeiotic doubling

In premeiotic doubling, chromosomes go through an extra replication, generally before meiosis I. During meiosis I, pairing occurs either between chromosomes originating from the same replication (sister pairing) or homologous chromosomes (non-sister pairing). Non-sister pairing can lead to LOH because it allows sister chromosomes to segregate together (with a probability of 50 %). Recombination between homologous chromosomes reverses the effect by canceling or causing LOH in the recombinant part (Archetti 2010). Sister pairing results in complete retention of heterozygosity, regardless of segregation and recombination (Uzzell 1970). Therefore, premeiotic doubling with exclusive sister pairing is clonal (see **Box 1**).

A general assumption is that sister chromosomes always pair, because they are more similar (Macgregor and Uzzell 1964; Uzzell and Goldblatt 1967) or, since this ARM often emerges from hybridization, because pairing of homeologous chromosomes (from the two parental species) is impossible (see following section). That is why this ARM is often associated with clonality, without necessarily confirming that sister pairing actually occurs. However, even though sister pairing is expected to be more frequent due to the costs of LOH, exclusive sister pairing could cause mechanistic problems regarding DSB repair (Archetti 2010). Thus, the tendency to consider species with premeiotic doubling as clonal may be erroneous.

Many species with premeiotic doubling were identified as clonal with no evidence, because deciphering sister vs. non-sister pairing was originally near impossible. An exception might be the grasshopper *Warramaba virgo*, in which one chromosome pair was structurally heterozygous, making it possible to decipher which type of pairing occurred. As there was consistent sister pairing of the chromosome, this species was identified as clonal (White *et al.* 1963). More recently developed cytological tools such as genomic and fluorescence in situ hybridization (GISH and FISH) allow to directly decipher between sister and non-sister pairing by marking each set of sister chromosomes. Using this method, non-sister pairing was detected in the hybrid salamander *Ambystoma laterale X A. jeffersonianum* (Bi and Bogart 2006; Lutes *et al.* 2010). However, it cannot always apply to every chromosome of a species. For instance, sister-pairing was found in several chromosomes and interpreted as clonality in the lizard *Apidoscelis* (Lutes *et al.* 2010; Newton *et al.* 2016) and the fish *Misgurnus anguillicaudatus* (Kuroda *et al.* 2018). Still, it is unclear whether all chromosomes should behave the same, and hence whether the type of pairing should be identified for each chromosome to conclude on the clonality of a species. The existence of random pairing was proposed (Archetti 2010) and it was suggested that both sister and non-sister pairing occurred in *A. laterale X A. jeffersonianum* (Bogart

2003). Furthermore, non-sister pairing perhaps happens rarely in species for which only sister-pairing has been found, which would not result in strict clonality. To conclude, in premeiotic doubling, clonality is often inferred with no cytological evidence for sister-pairing or based on few observations of some chromosomes. These conclusions might not be correct, and could constitute an important bias toward clonality.

Chromosome-specific or rare events of non-sister pairing can be more easily detected by parent-offspring genotype comparisons over several regions of the genome. If both sister and non-sister pairing occur, marker positions impact the detectability of LOH, which is maximal if markers are distributed in each pair of chromosomes. Few studies have performed parent-offspring genetic comparisons in species with premeiotic doubling. No parent-offspring differences were detected with DNA fingerprints and microsatellites in *Misgurnus anguillicaudatus,* although marker positions were unknown (Momotani *et al.* 2002; Itono *et al.* 2006). More often, because asexuals with this ARM tend to be hybrids, genotyping of specific markers that should be heterozygous based on parental species genotypes are used (Heppich *et al.* 1982; Dawley *et al.* 1987; Bogart and Klemens 1997; Tiedemann *et al.* 2005). However, this method is less reliable as for instance homozygosity at these alleles could be due to either mutation or recombination (Honeycutt and Wilkinson 1989). Although they could provide helpful evidence, genetic methods have only scarcely been employed in the study of this ARM, perhaps stemming from the fact that the possibility of non-clonal premeiotic doubling is not well known. Therefore, the prevalence of clonality in this ARM is still unclear.

Lastly, while it is often little discussed, the exact timing of doubling relative to the process of meiosis I can have dramatically different genetic consequences. Indeed, if doubling is not premeiotic and occurs after chromosome pairing, recombination will cause LOH as in central fusion-like parthenogenesis (see corresponding section). Such a mechanism is rare, but was observed cytologically in stick insects (Scali *et al.* 1995; Marescalchi and Scali 2001).

In conclusion, due to conceptual bias, most parthenogens reproducing through premeiotic doubling are considered clonal, based on limited evidence. Theoretical and empirical evidence indicate that premeiotic doubling might not be clonal in many cases, although we lack sufficient information for most species with this ARM.

Erroneous methods and misconceptions

Expectations under strict clonality

Phylogenetics and population genetics expectations have been used extensively as evidence for clonality. Under strict and obligate clonality, because other mechanisms never or very rarely intervene, mutation accumulation and genetic drift should generate heterozygosity at all polymorphic loci (Balloux *et al.* 2003; De Meeûs and Balloux 2005; De Meeûs *et al.* 2007). Hence, high heterozygosity is often used as evidence for clonality. In *Campeloma decisum*, Johnson (1992) suggested that populations with high heterozygosity rates should reproduce clonally whereas the ones showing fixed homozygosity should reproduce by a non-clonal ARM. An extension of this expectation is the Meselson effect, which causes entire homologous regions to diverge completely in the long term. This specifically tests whether the mutation rate is higher than the LOH rate (Mark Welch and Meselson 2000). Thus, by comparing intra- and inter-population haplotypes divergence, it has been used as evidence for clonality. Another expectation under strict clonality is complete linkage between the mitochondrial and nuclear genomes shortly after the emergence of clonality. This means that the mitochondrial and nuclear genomes evolve at the same pace (*i.e.,* diversity is produced by mutations only). This should lead to the congruence of mitochondrial and nuclear phylogenies; which was used as evidence for clonality in *Heterocypris incongruens* populations (Chaplin and Hebert 1997).

Under clonality, high heterozygosity is a long-term expectation because it is generated by mutations. It is thus particularly sensitive to confounding effects and restriction by other rare mechanisms. Several confounding factors may cause the expected high heterozygosity and even the Meselson effect, including in non-clonal parthenogens. These are hybridization, polyploidy and gene duplication (Ceplitis 2003; Simon *et al.* 2003; Mark Welch *et al.* 2009; Hollister *et al.* 2019). For instance, the Meselson effect was incorrectly inferred in the Bdelloid rotifers in which Mark Welch and Meselson (2000) detected high allelic divergence. In fact, Mark Welch *et al.* (2008) later discovered this taxon is paleotetraploid, so that the high divergence measured was actually between anciently but not presently homozygous chromosomes. Furthermore, even under clonal reproduction, biological processes such as gene conversion and mitotic recombination, if they happen more frequently than mutations, may prevent sufficient mutation accumulation from generating high heterozygosity. Such a high rate of gene conversion and recombination relative to the rate of mutation accumulation was reported in the obligate asexual *Daphnia pulex* (Tucker *et al.* 2013). In putatively clonal species, low heterozygosity was found in several species and explained by gene conversion (*Darwinula stevensoni*; Tran Van *et al.* 2021) or due to rapid sexual-asexual transition and population expansion (*Heterocypris incongruens*; Rossi *et al.* 2006). This can counter the Meselson effect (Hartfield 2016), even in fissiparous (thus truly mitotic) Nemerta (Ament-Velásquez *et al.* 2016). Similarly, mito-nuclear incongruence was found in several asexuals reported as clonal, which was explained by accumulation of mutations in a recently derived mitochondrial haplotype (Lorenzo-Carballa *et al.* 2012) or rare sex events (Schön *et al.* 2000). Finally, both heterozygosity and mito-nuclear congruence are extreme expectations that are not reliable to assess clonality.

The above predictions under clonality still constitute interesting tools to investigate the consequences of clonality in the long term. A promising direction is to study how ARMs affect different parts of the genome. For instance, specific patterns of F_{IS} along the chromosome are expected under central fusion-like parthenogenesis (see corresponding section). Heterogeneous patterns can also be indicative of a more or less clonal history within the genome: for instance, in one oribatid mite, the Meselson effect was found, but with various intensities relative to certain regions of the genome, that may reflect different histories of LOH rates (Brandt *et al.* 2021). Thus, using whole genome sequencing, it is possible to find specific regions that have been clonal for a long time in ancient asexuals, although this does not mean that the species has been reproducing clonally for a long time.

Other indirect methods used to test for clonality come from experimentation. The absence of response to artificial selection on an iso-female line has been interpreted as clonality (this was widely used in aphids, reviewed in Blackman 1979). The certainty of this method depends on the number of generations observed and on the genetic basis for the observed trait. Moreover, response to selection is simply a proxy for the renewal of diversity, which is not a good indicator for clonality, as other reproductive modes generate low diversity, such as selfing or sex in an inbred population. Eventually in aphids, even when variation was observed, it was interpreted as epigenetics processes (Blackman 1979). Secondly, success in grafting tissue of an asexual female on her offspring was also occasionally used to demonstrate clonality in vertebrates (Maslin 1967; Cuellar and Smart 1977; Dawley *et al.* 1987; Goddard and Dawley 1990; Cordes and Walker 2003, 2006). However, it is not reliable to detect strict clonality as we know that non-clonal grafts can be accepted. All the above-mentioned methodologies were initially developed to differentiate asexual from sexual modes of reproduction. It is clear that their designs were built under the assumption that asexuality was obligate and equivalent to clonality. However, they are not accurate enough to discriminate among all the possible ARMs, nor to be used as evidence for clonality.

Inference of clonality based on erroneous assumptions

In addition to these predictions, clonality is sometimes inferred based on certain features of the parthenogenetic species. Such features are hybridization and polyploidy, which are assumed to cause clonality. These characteristics generally co-occur with asexuality but no clear causality has been established between them so far. The "Balance hypothesis" proposes that hybridization between sexual species with a specific divergence level could directly induce (mitotic) clonality by breaking down meiosis (Moritz *et al.* 1989). However, there is no evidence of this phenomenon, and the mechanistic process that would be underlying it is unclear. Another assumption is that hybrid origin results in incorrect pairing or segregation that can only be resolved by skipping meiosis, hence reproducing clonally through mitosis (Nokkala *et al.* 2008). For the same reasons, high structural heterozygosity of homologous chromosomes and other structural anomalies are also thought to lead to clonality (aphids: Normark 1999; Darwinulid ostracods: Tétart 1978; Schön *et al.* 1998. Anisopolyploid asexuals (with uneven ploidy, *e.g.,* 3n, 5n) are, likewise, strongly assumed to only reproduce clonally (Suomalainen 1950; Bell 1982; Rasch *et al.* 1982), even though Stalker (1956) advocated that meiosis could occur in triploids. To a lesser extent, even in polyploids with an even number of chromosomes, segregation issues due to entanglement with several pairs have been demonstrated (Lloyd and Bomblies 2016). Many triploids and hybrids indeed appear clonal (*Potamopyrgus antipodarum*: Phillips and Lambert 1989; the tardigrades *Ramazzottius oberhaeuseri*: Rebecchi and Bertolani 1988; *Macrobiotus hufelandi*: Bertolani 1973; *Paramacrobiotus richtersi*: Bertolani 1971; Guidetti *et al.* 2019). However, polyploid and hybrid asexuals are not all clonal (premeiotic doubling with non-sister pairing: *Ambystoma sp.*, Bi and Bogart 2006; central fusion-like parthenogenesis with recombination: stick insects *Carassius auratus*, Zhang *et al.* 1992 and *Bacillus atticus*, Scali *et al.* 2003). Furthermore, the detection of LOH in non-clonal polyploids may be particularly difficult, because their LOH rate could be low to null (as the processes normally leading to LOH could still result in a heterozygous genotype). Thus, such species may be identified mistakenly as clonal. The same could happen in non-clonal hybrids because genetic incompatibilities, revealed by LOH events, could lead to the death of recombinants, resulting in an underestimation of LOH rate. Therefore, many more supposedly clonal polyploids and asexuals could be in fact non-clonal (as suggested for polyploid *Artemia* in Rode *et al.* 2021). Perhaps, these meiosis modifications even provide an escape to the pairing issues stemming from polyploidy and hybridization by avoiding or cancelling segregation.

Lastly, an assumption that biases the identification of asexuals toward clonality is that ARMs are thought to be shared within taxa. Thus, it is often suggested that a species' ARM is the same as that found in a related species (usually, clonality). For example, clonality was suggested for all non-marine ostracods (Butlin *et al.* 1998) and oribatid mites (Laumann *et al.* 2008), based on evidence for only a few species. Clonality was extended in the same manner from *Daphnia magna* (inferred from stability in three allozymes, (Hebert and Ward 1972) to *Daphnia pulex* (Blackman 1979). Similarly, in asexual Lepidoptera, clonality is often inferred on the basis that females in this taxon tend to be achiasmatic (Lokki *et al.* 1975). However, this was questioned by the finding of female recombination in related species (Elzinga *et al.* 2013). Perhaps the most extreme example of this "taxonomic group effect" is in vertebrates, in which premeiotic doubling is considered ubiquitous (Cuellar 1974), and premeiotic doubling is considered clonal (see previous section). This has led to infer clonality falsely in several vertebrates, which turned out to be non-clonal (*Poecilia formosa*: Rasch *et al.* 1982, *Darevskia armeniaca*: Kupriyanova *et al.* 2021, *Carassius auratus langsdorfi*: Zhang *et al.* 1992). Hence, parthenogenesis seems to arise in diverse ways within taxa, and may be a much less conserved trait than has been suggested (chapter 3 of this thesis). In fact, within a given phylum or order, several ARMs may be present. For instance, in termites (Matsuura *et al.* 2004; Fougeyrollas *et al.* 2015; Fournier *et al.* 2016), tardigrades (Ammermann 1967; Bertolani 1981; Rebecchi and Bertolani 1988; Rebecchi *et al.* 2003; Bergmann *et al.* 2018) and oribatid mites (Peacock and Weidmann 1961; Taberly 1987; Palmer and Norton 1992; Laumann *et al.* 2008), a diversity of clonal and non-clonal ARMs was identified.

ARM variation is also observed within the same genera (*Meloidogyne sp.*: Castagnone-Sereno *et al.* 2013, subspecies (*Carassius auratus gibelio and C. auratus langsdorfii*: Emelyanova O.V. 1984; Yamashita *et al.* 1993; Zhang *et al.* 2015), and species (*Meloidogyne hapla*: Triantaphyllou 1966). A single individual can reproduce through different modes with facultative parthenogenesis (*Daphnia*: Decaestecker *et al.* 2009) and rare sex (*Timema*: Schwander 2021, *Artemia*: Boyer *et al.* 2021, *Solenobia triquetrella*: Seiler 1966). Moreover, variation in ARM was reported within single genotype lineages of *Daphnia magna* (Svendsen *et al.* 2015). This suggests intra-individual variation in ARM could be found. Given the extent of diversity in asexual reproduction even at a small taxonomic scale, it is not reliable to infer ARMs based on what was found in related species or populations, as this may lead to interpretation bias. Questioning the ARM of species presumed clonal may reveal the existence of more diversity and numerous non-clonal asexuals.

Clonality has been inferred many times by testing for expectations that were either too strict and prone to confounding factors, or not accurate enough. Clonality has also been suggested for many species based on incorrect assumptions related to their characteristics or the ARMs of related species. All these means to identify clonality are unreliable and cannot be used as evidence. This means that a great part of supposedly clonal asexuals may in fact reproduce through non-clonal ARMs (**Figure 1**).

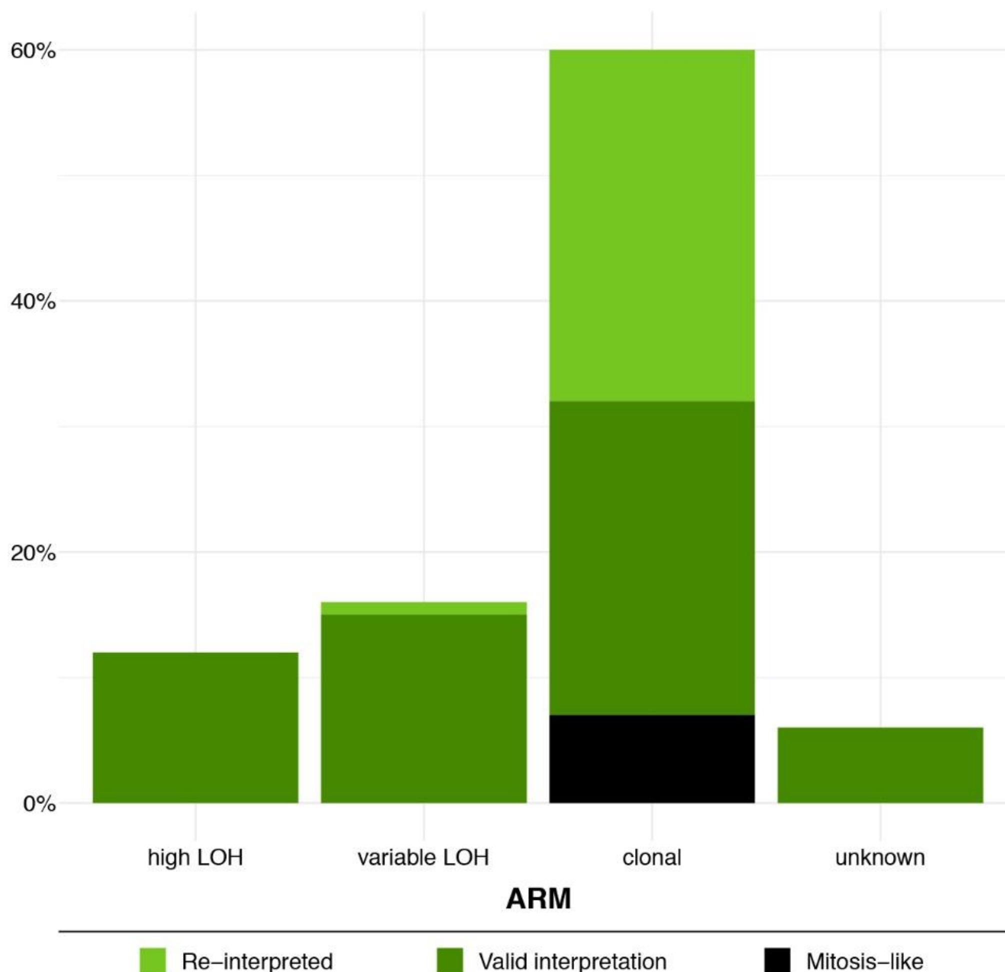

Figure 1: Distribution of the 90 species investigated according to asexual reproductive modes (high LOH ARM, variable LOH non-clonal ARM, clonal, or unknown) based on the literature. Note that four species were each subdivided into two groups in which different ARMs were identified. Light green represents species for which we re-interpreted the ARM, either because the evidence proposed was not conclusive or because the interpretation was erroneous. Dark green represents species with conclusive

evidence interpreted correctly, for the identified ARM. In black are species for which there is cytological evidence that pairing does not occur before equational division, suggesting mitosis. Note that this figure is based on a subset of investigated species (**supplementary table**), to be completed.

Prevalence of clonality

Among our sample of asexual animals investigated (90 species), the ARM of some species is still unknown according to the literature (6.4 %, **Figure 1**). Over all the other species (for which an ARM is reported), those that are presented as clonal represent about 68.2 % (**Figure 1**; "clonal"). However, as we discussed over the previous sections, it is not always possible to conclude with certainty that a species is clonal, due to inconclusive evidence or bias in interpretation. Thus, when only accounting for conclusive evidence, interpreted correctly (*i.e.,* following our indications), the proportion of clonal species falls to 54.2 %. According to predictions (Archetti 2010), we actually found that parthenogenetic animals reproduce mainly through ARMs with genetic consequences other than complete LOH, which can be equivalent or close to clonality (79.7 %, **Figure 1**; "variable LOH" + "clonal"). This still means that the number of species reproducing through gamete duplication or terminal fusion is surprisingly high considering the high LOH associated with these ARMs. As suggested in the first section, this could be explained by the fact that most of these species are not obligate asexuals. Finally, over all species included, asexuals with no evidence against reproduction through mitosis are in fact very rare (7.4 %), meaning that the great majority of asexual animals reproduce through modified meiosis.

Discussion

In this review, we investigated how common clonality is in parthenogens according to the literature, and what evidence was put forward to determine this ARM. We found many non-clonal species among those we reviewed, in spite of the belief that parthenogenesis is mainly clonal. Furthermore, we found that evidence for clonality was lacking in many species identified as such. This is partially due to technical limitations and misinterpretations leading to incorrect conclusions. Access to modern techniques, such as improved cytology marking and genomics, allows to get rid of many limitations, especially when combining cytology with genetics. Additionally, many conceptual biases, such as the misconception that polyploids and hybrids cannot go through meiosis or that premeiotic doubling is always clonal, further shift the perception of asexuals toward clonality. This shows that a common framework for identifying ARMs, accounting for the diversity in mechanisms and genetic consequences, is necessary. Because of the perception bias caused by these technical and conceptual limitations, it is possible that in addition to the known non-clonal parthenogens, an important part of so-called clonal species is in fact non-clonal too.

Consistently with the high potential costs of LOH, we found that, even though strict clonality is not as prevalent as generally thought, most non-clonal parthenogenetic animals are somewhat close to clonality: they have a low, but non-zero LOH rate. Can we thus approximate parthenogenesis as equivalent to clonality? Deviations from clonality, even if they are small, can have a great impact on the evolution of asexuals (Engelstädter 2008; Archetti 2010). This is because under clonality, it is expected that the major evolutionary force is mutation accumulation. However, recombination, even if rare, can be more frequent than mutations. If recombination is localized, it will generate clustered clonal genomic regions. Additionally, other rare events such as gene conversion and mitotic recombination may occur in otherwise strictly clonal species. Similarly, whether these events happen often enough to disturb evolutionary and genetic expectations under clonality depends on their frequency compared to the mutation rate (Engelstädter 2017). This means that, even when very rare, these events can have significant impacts on the evolution of asexuals.

Moreover, there is evidence indicating that non-clonal asexuals can evolve to become closer to clonality. For instance, fusion of meiotic products can be random (fusion of products of meiosis I or II,

Stalker 1954; Asher 1970; Svendsen *et al.* 2015). However, in many obligate asexuals, cytological mechanisms favor central fusion, which generates less LOH and is thus closer to clonality (Murdy and Carson 1959; Verma and Ruttner 1983; Suomalainen *et al.* 1987). Such mechanisms could be the result of selection, as LOH is costly. Similarly, the timing of premeiotic doubling could evolve to favor clonal-like reproduction. In cases where recombination causes LOH, the recombination rate tends to be low, and several cases of reduced recombination compared to related sexual species are known (see central fusion-like parthenogenesis section). Such reduction may result from the selection of lineages with the lowest LOH or progressive reduction of recombination within lineage. Additionally, effective LOH can be further reduced by elimination of recombinants for deleterious alleles. To conclude, evolution toward low LOH is likely to take place in non-clonal asexuals. Because parthenogenesis evolved from sexual species, mostly through meiosis modifications, mechanisms that avoid LOH may not be present at the emergence. This suggests that potentially many clonal or almost clonal species were not close to clonality in the past.

This can be extended to clonal species with an ARM suggestive of mitosis (*i.e.,* one equational division with no pairing, see mitosis section). Contrary to the general belief that many asexuals arise in one step by mitosis (Levitis *et al.* 2017), only a small part of asexual animals could concur with this origin. Moreover, it is possible that these so-called “mitotic parthenogens” in fact evolved through meiosis modifications, as traces of meiosis persist in some of them. Their ARM might be the result of a longer-term evolution toward clonality, at the beginning of which they were probably not clonal, and during which pairing was suppressed. There is so far no evidence for parthenogenesis through a complete mitotic process in animals.

Transitions from sex to asexuality happen mainly, and perhaps exclusively, through non-clonal meiosis modifications. Therefore, although clonality is frequently observed, non-clonal ARMs likely play an important part in the evolution of parthenogenesis. This means that the majority of theoretical models, which make the assumption that clonal asexuality arises spontaneously, may well be too simplistic. To tackle the challenging evolutionary questions regarding asexuality, it becomes crucial to include non-clonal ARMs, especially those that are close to clonality. In particular, models studying the emergence of asexual populations competing with sexuals need to take into account that asexuality does not likely emerge as clonal, and that there are multiple evolutionary pathways toward clonality.

Conclusion

In this review, we presented evidence that clonal asexuals do not represent a large majority. There are potentially many more non-clonal asexuals because of the strong perception bias toward clonality. Although most parthenogens are clonal or close to clonality, it might not have been the case throughout their evolutionary history. Finally, most and possibly all parthenogens evolved through meiosis modifications, and not by switching to mitosis. Therefore, the incorporation of non-clonal ARMs in theoretical models for the origin and consequences of asexuality is essential. A more accurate vision of asexuality could participate in resolving long-lasting evolutionary questions, such as the rarity of asexuality and the frequent association between asexuality, hybridization and polyploidy. The possibly intricate effects of these ARM on evolutionary, genetical, demography and ecological expectations could allow completely new theories to develop and flourish, enriching the vast field of reproductive systems evolution.

BOX 1: The vocabulary associated with ARMs is very broad and definitions have evolved over time, to the extent that publications now often need to clarify and define the terms chosen. The word "apomixis" was for a long time synonymous with clonality, whereas today one can distinguish between "mitotic apomixis" and "meiotic apomixis"; where reproduction is not necessarily clonal (Archetti 2010). Similarly, automixis was defined as involving meiosis and was therefore considered equivalent to non-clonal asexuality. However, we now know that some types of automixis can generate the same genetic consequences as clonality. Consequently, the terms originally developed for cytologically observed ARMs have evolved to accommodate the associated genetic consequences. This has led to some misunderstandings in the identification of ARMs. Indeed, suppression of the first meiotic division with absence of bivalents is clonal and may be indistinguishable from mitosis, which is why this mode of reproduction is often referred to as "mitotic division", "ameiotic parthenogenesis" or "apomixis".

To differentiate between mitosis- and meiosis-derived parthenogenesis, we propose the following terms:
-Dimeiotic: Two independent cells engage in meiosis to produce offspring. The cells originate from the same (autofecundation) or different (allofecundation) parents. In this case, they are usually of different mating types.
-Monomeiotic: A single cell engages in meiosis to produce offspring (the meiosis is necessarily modified to maintain ploidy). Different modifications exist: premeiotic doubling, central fusion like, terminal fusion like and postmeiotic doubling
-Ameiotic: No meiosis is engaged to produce offspring (*i.e.,* mitosis only).

Offspring origin	Category	Cytological mechanism	Synonym	Genetic consequence	Genetically equivalent to clonality
Monomeiotic	High LOH (loss of heterozygosity) ARM	One of the four haploid meiotic products or gametes (N) duplicate	Gamete duplication, post meiotic replication, post meiotic doubling.	Total LOH	Never
		Fusion of products of the second meiotic division	Terminal fusion-like parthenogenesis, terminal fusion, fusion of sister nuclei	Total LOH at the centromere. Recombination causes heterozygosity retention, so that it is more likely with distance from the centromere	Never
		Suppression of second meiotic division	Terminal fusion-like parthenogenesis		Never
	Central fusion-like	Fusion of products of the first division	Central fusion-like parthenogenesis, central fusion, fusion of non-sister nuclei	Total heterozygosity at the centromere. Recombination causes LOH, so that it is more likely with distance from the centromere	Only if no recombination
		Suppression of first meiotic division	Central fusion-like parthenogenesis, apomixis, meiotic apomixis		Only if no recombination
	Premeiotic doubling	Duplication of the chromosome number normally before meiosis	Premeiotic doubling, endoreduplication, endomitosis	Duplication before Prophase I: Sister-pairing leads to retention of heterozygosity, non-sister pairing leads to LOH. Duplication after Prophase I: Recombination causes LOH.	Duplication before Prophase I: Only if exclusive sister pairing. Duplication after Prophase I: Only if no recombination.
Ameiotic	Mitosis	One equational division, equivalent to the second meiotic division of meiosis	Mitotic division, apomixis, mitotic apomixis	Total retention of heterozygosity (except for mitotic recombination)	Always

Glossary

Meiotic division: Two meiotic divisions take place during meiosis: a reductional one called meiosis I and an equational one; meiosis II

Crossover: Also called recombination or chiasmata, take place during Prophase I (meiosis I). Portions of paired chromosomes are exchanged. They can be observed cytologically.

Synapsis: Pairing of chromosomes during Prophase I. Also called tetrad, bivalent or diplochromosomes.

Gynogenesis: Parthenogenesis in which the embryo contains only maternal chromosomes due to the failure of the sperm to fuse with the egg nucleus.

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Author contributions

TL, CH, CM and LB conceived the study. CM and LB conducted bibliographic research.

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Supplementary data

Supplementary table: The subset of species studied on which the figure is based. Based on the literature, each species is classed according to the asexual reproductive modes following the categories: "high LOH ARM", "variable LOH", "clonal", or "unknown". Note that four species were each subdivided into two in which different ARMs were identified. For each species we assessed whether the attributed category from the literature is conclusive (there is conclusive evidence to support the authors' interpretation, whether their conclusion is in agreement in light of the methodological or conceptual reasons developed in the review). When the class of ARM is clonal, we specified whether or not there is any evidence against mitosis as the underlying cytological process.

Species	Class of ARM	Conclusive evidence	Agreement with interpretation	No evidence against mitosis	References
Meloidogyne hapla race B	clonal	yes	yes	yes	[1], [2]
Meloidogyne africana	clonal	yes	yes	yes	[3]
Meloidogyne ardenensis	clonal	yes	yes	yes	[3]
Meloidogyne javanica	clonal	yes	yes	yes	[2]–[5]
Campeloma rufum	clonal	yes	yes	yes	[6]
Histiostoma feroniarum	clonal	yes	yes	yes	[7]
Poecilia formosa	clonal	yes	yes	yes	[8]
Epiphanes senta	clonal	yes	yes	NA	[9], [10], reviewed in [11]
Asplanchna intermedia	clonal	yes	yes	NA	[9], [12], [13], reviewed in [11]
Asplanchna amphora	clonal	yes	yes	NA	[9], [14], reviewed in [11]
Asplanchna priodonta	clonal	yes	yes	NA	[9], [15], [16], reviewed in [11]
Meloidogyne arenaria	clonal	yes	yes	NA	[5]
Meloidogyne incognita	clonal	yes	yes	NA	[5], [17]
Meloidogyne partityla	clonal	yes	yes	NA	[5]
Diplocaspter pachys	clonal	yes	yes	NA	[18]
Potamopyrgus jenkinsi = P. antipodarum	clonal	yes	no	NA	[19]–[22]
Melanoides tuberculata	clonal	yes	yes	NA	[23]
Campeloma parthenum	clonal	yes	no	NA	[24]
Campeloma decisum	clonal	no	no	NA	[25]
Dendrobaena octaedra	clonal	yes	no	NA	[26]–[30]
Ramazzottius oberhaeuseri	clonal	yes	yes	NA	[31]
Macrobiotus hufelandi	clonal	yes	yes	NA	[32]
Macrobiotus richtersi=Paramacrobiotus fairbanski	clonal	yes	yes	NA	[33]
Platynothrus peltifer	clonal	no	no	NA	[34]
Trhypochthonious tectorum	clonal	no	no	NA	[34]
Archegozs longisetosus	clonal	yes	yes	NA	[35]
AIIonothrus gigandcus	clonal	yes	yes	NA	[34]
Darwinulidae	clonal	no	NA	NA	[36]
Eucypris virens	clonal	yes	yes	NA	[37]
Daphnia pulex	clonal	yes	no	NA	[38]–[42]
Daphnia magna	clonal	yes	yes	NA	[43]
Warramaba virgo = Moraba virgo	clonal	yes	no	NA	[44], [45]
Bacillus whitei	clonal	yes	no	NA	[46], [47]
Carausius morosus	clonal	yes	yes	NA	[48]
Brevicoryne brassicae	clonal	no	no	NA	[49], [50]

Myzus persicae	clonal	yes	yes	NA	[51]–[53]
Acyrthosiphon pisum	clonal	no	no	NA	[51]
Myzus antirrhinii	clonal	yes	yes	NA	[54]
“Sitobion near fragariae”	clonal	yes	yes	NA	[52]
Sitobion avenae	clonal	yes	yes	NA	[52], [55], [56]
Amphorophora tuberculata	clonal	yes	yes	NA	[57]
Sitobion miscanthi	clonal	yes	yes	NA	[52], [58]
Aphis fabae	clonal	no	no	NA	[51]
Cathormiocerus aristatus	clonal	yes	no	NA	[59], [60]
Eusomus ovulum	clonal	no	NA	NA	[61]
Liophloeus tesselatus	clonal	no	NA	NA	[61]
Aramigus tessellatus	clonal	no	NA	NA	[62]
Otiorrhynchus ligustici	clonal	no	NA	NA	[61]
Otiorrhynchus raucus	clonal	yes	no	NA	[60], [61]
Otiorrhynchus ovatus	clonal	yes	no	NA	[61], [63]
Otiorrhynchus tristis	clonal	no	NA	NA	[61]
Polydrosus inustus	clonal	yes	no	NA	[60], [61]
Strophosoma	clonal	yes	no	NA	[59], [60]
Trophiphorus micans	clonal	no	NA	NA	[61]
Trichogramma cacoeciae	clonal	yes	no	NA	[64]
Chrosomus eos-neogaeus	clonal	no	NA	NA	[65]
Pelophylax esculentus = Rana esculenta	clonal	yes	yes	NA	[66]
Lacerta unisexualis = Darevskia unisexualis	clonal	yes	yes	NA	[67]
Apidoscelis tesselata	clonal	yes	no	NA	[68]
Bacillus lynceorum	clonal	yes	no	NA	[46], [47]
Adineta vaga	variable LOH	yes	yes	NA	[69]
Campeloma decisum	variable LOH	no	no	NA	[25]
Dactylobiotus parthenogeneticus	variable LOH	yes	yes	NA	[70]
Richtersius coronifer	variable LOH	yes	yes	NA	[71]
Artemia parthenogenetica (diploids)	variable LOH	yes	yes	NA	[72], [73]
Artemia parthenogenetica (polyploids)	variable LOH	yes	yes	NA	[74], [75]
Bacillus atticus	variable LOH	yes	yes	NA	[76]
Timema sp.	variable LOH	yes	yes	NA	[77]
Reticulitermes virginicus	variable LOH	yes	yes	NA	[78]
Reticulitermes lucifugus	variable LOH	yes	yes	NA	[79]
Embiratermes neotenicus	variable LOH	yes	yes	NA	[80]
Drosophila mangabeirai	variable LOH	yes	yes	NA	[81]
Apis mellifera capensis	variable LOH	yes	yes	NA	[82], [83]
Carassius auratus langsdorfi	variable LOH	yes	yes	NA	[84]
Ambystoma laterale x A. jeffersonianum	variable LOH	yes	yes	NA	[85]
Lacerta saxicola = Darevskia armeniaca	variable LOH	yes	yes	NA	[67]
Meloidogyne hapla race A	high LOH	yes	yes	NA	[1], [2]
Meloidogyne fallax	high LOH	yes	yes	NA	[2]
Meloidogyne floridensis	high LOH	yes	yes	NA	[86]
Dactylobiotus dispar = Macrobiotus dispar	high LOH	yes	yes	NA	[87]
Hypsibius dujardini	high LOH	yes	yes	NA	[88]
Cheyletus eruditus	high LOH	yes	yes	NA	[89]
Bacillus rossius	high LOH	yes	yes	NA	[90]
Reticulitermes speratus	high LOH	yes	yes	NA	[91]
Cavitermes tuberosus	high LOH	yes	yes	NA	[92]
Palmitermes impostor	high LOH	yes	yes	NA	[92]
Spinitermes trispinosus	high LOH	yes	yes	NA	[92]

Inquilinitermes inquilinus.	high LOH	yes	yes	NA	[92]
Meloidogyne chitwoodi	unknown	NA	NA	NA	[5]
Ramazzottius oberhaeuseri	unknown	NA	NA	NA	[93]
Brevipalpus obovatus	unknown	NA	NA	NA	[94]
Heminothrus ornatissimus	unknown	NA	NA	NA	[95]
Solenobia triquetrella =Dahlica triquetrella	unknown	NA	NA	NA	[96]
Ochthiphila polystigma	unknown	NA	NA	NA	[97]

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Chapter 2

No support for a meiosis suppressor in *Daphnia pulex*: Comparison of linkage maps reveals normal recombination in obligate parthenogenetic males

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No support for a meiosis suppressor in *Daphnia pulex*: Comparison of linkage maps reveals normal recombination in obligate parthenogenetic males

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Abstract

It is often assumed that obligate parthenogenesis (OP) evolves by a disruption of meiosis and recombination. One of the emblematic examples that appears to support this view is the crustacean *Daphnia pulex*, for which a candidate gene has been identified with a mutation that is thought to disrupt recombination in OP lineages. Yet, rare OP males, which are genetically identical to OP females and thus carry the same mutation, are able to undergo functional meiosis during spermatogenesis. Here we test whether recombination is suppressed in these meioses. Specifically, we investigate recombination of OP males but also controlled for sex-specific recombination differences (heterochiasmy) in CP. Using restriction site associated DNA sequencing (RAD-seq) approach, sex-specific linkage maps were constructed and revealed no significant variation in recombination rates and patterns between CP sexes, nor between OP male and CP males or females. Thus, recombination is not suppressed in OP males invalidating the hypothesis of a meiosis suppressor responsible for OP in *D. pulex*. As is the case in *D. pulex*, we emphasize that models where the ancestral state is a CP, by their particularity of already containing a parthenogenetic phase, then offer an alternative pathway in the evolution of the OP, without alteration of the meiosis processes. An exclusive usage or extension of the parthenogenetic phase to the formerly sexual phase, could explain CP to OP transitions.

Key words: *Daphnia*, linkage map, obligate parthenogenesis, heterochiasmy, asexuality

Introduction

The mechanisms of evolutionary transitions to obligate parthenogenesis (OP) remain poorly understood. It is now clear that these transitions more often occur through modifications of meiosis rather than through replacing meiosis by mitosis (Vanin 1985; Lynch and Conery 2000; Simon *et al.* 2003). A prominent example is the small freshwater crustacean *Daphnia pulex* for which a candidate gene has been identified with a mutation that has been hypothesized to disrupt recombination in OP lineages (Hebert *et al.* 1988, 1989; Eads *et al.* 2012). Indeed, recombination is largely or entirely absent during oogenesis of OP females (Hebert and Crease 1980, 1983). However, OP lineages do occasionally males (Hebert and Crease 1983; Lynch 1984), which are genetically identical to OP females and thus carry the same mutations. These males are nevertheless known to still be able to undergo functional (*i.e.,* reductional) meiosis during spermatogenesis (Innes and Hebert 1988; Xu *et al.* 2015a). However, whether or not recombination occurs during these meioses is unknown.

Daphnia pulex has both cyclical parthenogenetic (CP) and OP lineages, with CP being ancestral to OP. Both CP and OP share a phase of subitaneous (*i.e.,* ovoviviparous) egg production, during which females parthenogenetically produce offspring whose sex is determined by the environment. The type of parthenogenesis is an aborted meiosis, which is genetically identical to mitosis (Hiruta *et al.* 2010) except for rare cases of recombination or gene conversion leading to some loss of heterozygosity

(Omilian *et al.* 2006). Parthenogenetically produced males and females thus constitute a clonal lineage. CP and OP differ, however, in the mode of diapause egg production (here called diapause phase to distinguish it from the subitaneous phase). In CP, diapause egg production is sexual, whereas it is parthenogenetic in OP (Hebert 1978; Hebert and Crease 1980, 1983).

It has been suggested that, in species with CP, there may be an alternative route for the evolution of OP: OP may evolve by reusing the pathways for parthenogenetic reproduction that are already present in CP and extending them to the entire life cycle (Simon *et al.* 2003; van der Kooi and Schwander 2014). Considering these alternative mechanisms for the evolution of OP is important because transitions to obligate parthenogenesis are particularly common in species with CP (Hebert 1981; Kramer and Templeton 2001; Simon *et al.* 2002) and these species are often used as models to study the evolution of OP. Here, we study the recombination rate of rare OP males of *D. pulex*, with the aims to assess whether spermatogenetic meioses in these males involve normal levels of recombination (as compared to spermatogenetic meioses in CP males), as well as to elucidate different possible scenarios for the evolution of OP in this species.

Compared to CP males, levels of recombination in OP males might be reduced or absent for two main reasons. First, zero or very low rates of recombination in OP males may be due to the evolution of OP by a general recombination suppressor, affecting recombination during both male and female gametes formation. Indeed, suppression of meiosis or recombination is one of the main mechanisms invoked to explain transitions to obligate parthenogenesis, including in *D. pulex* (Simon *et al.* 2003; Schurko *et al.* 2009; Eads *et al.* 2012). Second, absent or reduced recombination in OP males may be due to a secondary reduction of recombination. Indeed, many forms of meiosis modifications that result in parthenogenesis do not necessarily involve recombination suppression (Bertolani and Buonagurelli 1975; Rebecchi *et al.* 2003; Oldroyd *et al.* 2008; Fougeyrollas *et al.* 2015). Yet, recombination may be deleterious under some forms of parthenogenesis, as it often leads to loss of heterozygosity, similar to inbreeding (Archetti 2004). As a consequence, there may be selection for reduced recombination within OP lineages (Engelstädter 2017)). This has been documented empirically in several systems (Moritz and Haberl 1994; Rey *et al.* 2011; Boyer *et al.* 2021). Even if the meiosis modification affects female gametogenesis only, as it has been suggested for OP *D. pulex* (Innes and Hebert 1988; Paland *et al.* 2005), the secondary reduction of recombination may affect OP males as well, if it is caused by recombination modifiers that are not sex-specific.

In contrast, levels of recombination in OP males might be “normal” (meaning equal to those observed in CP) for two main reasons. First, the evolution of primary or secondary recombination suppression may involve sex-limited mechanisms, *i.e.,* involve genes that affect recombination only during female gametogenesis. Second, when OP evolved from a CP ancestor, it might have re-used the subitaneous parthenogenesis pathways already present in the ancestral CP life cycle. Specifically, in OP *Daphnia*, the parthenogenesis pathways used for subitaneous oogenesis in CP may have been simply extended to diapause oogenesis. In this case, as parthenogenesis in CP is specific to oogenesis, there may be no *a priori* reason to believe that spermatogenesis should be affected as well, which goes well in hand with the observation that OP males can achieve normal, reductional meiosis (Innes and Hebert 1988; Xu *et al.* 2015a,b).

To summarize, comparing the extent to which recombination was reduced in OP females and OP males (compared to female and male CP) can inform us on the pathways that led to OP. To date, we know that recombination is very low in OP females, but recombination rates in CP females are unknown. Indeed, recombination in CP has so far only been studied through sex-average and male-specific linkage maps (Cristescu *et al.* 2006; Xu *et al.* 2015a), but never specifically in females. Furthermore, because no previous study has addressed recombination in OP males, we do not know whether OP males recombine at a normal (CP-level) or reduced rate. In this paper, we measure these missing rates to

provide a clear picture of recombination rate variation, in males and females, involved in the CP to OP transition.

As an aside, this comparison will also document the level of heterochiasmy (sex differences in recombination rates) in *D. pulex.* To date, no link between mechanisms of sex determination (genetic or environmental) and the presence of heterochiasmy has been demonstrated (Lenormand and Dutheil 2005; Stapley *et al.* 2017). However, only very few species with environmental sex determination have been studied to test any general pattern, and the data on heterochiasmy in *D. pulex* will therefore represent an interesting addition.

To compare recombination rates during diapause phase among OP males, CP males, and CP females, we performed two crosses to produce linkage maps of each of the four parents, one OP male, one CP male, and two CP females (OP females cannot be crossed and were therefore not included; their parthenogenetic recombination rate has been investigated elsewhere, though not specifically during diapause phase, Omilian *et al.* 2006; Xu *et al.* 2011; Keith *et al.* 2016; Flynn *et al.* 2017). In order to maximize the number of offspring, we used a mass mating approach with female-only clonal lines (so-called "NMP" clones for "non-male producing"). Each cross involved crossing numerous females from a CP NMP clone (a different clone in each of the two crosses) with males from another clone, either rare males from an OP clone (OP x CP cross) or males from a CP. Using Restriction-site Associated DNA sequencing (RAD-seq) we constructed highly saturated linkage maps and investigated recombination rate during gamete production in each of the four parents, according to the pseudo-testcross strategy (Grattapaglia and Sederoff 1994): SNPs that were heterozygous in both parents of a given cross ("ab × ab" SNPs) were used for the maps of both parents, while "ab × aa" and "aa × ab" SNPs (heterozygous only in the mother or only in the father) were used only for the maternal or paternal maps, respectively. For each map, meiotic recombination rates and patterns of recombination rates along the chromosomes ("recombination landscapes") were assessed by comparing genetic and physical maps (Marey maps).

Materials & Methods

Material

We performed two mapping crosses, using four parental clones that originated from three different North American *Daphnia pulex* populations, called LPB, STM, and TEX (**Table S1**): The first cross, "CP x CP", was carried out using males of the CP clone TEX-1 and females of the CP clone LPB-87, while the second cross, "OP x CP", was carried out using rare males of the OP clone STM-2 and females of the CP clone TEX-114. Both crosses were thus inter-population crosses, and the fact that males of TEX-1 were used in one cross and females of TEX-114 in the other, allowed comparing male and female maps between clones from the same population. Both clones used as females (LPB-87 and TEX-114) are non-male producing (NMP) clones, that is, they are unable to produce males and thus they participate in sexual reproduction only as females (Innes and Dunbrack 1993; Tessier and Cáceres 2004; Galimov *et al.* 2011; Ye *et al.* 2019). The use of NMP clones meant that mass-mating could be performed without occurrence of within-clone mating (*i.e.,* with obligate outcrossing between the two clones). To initiate a given cross, we introduced males of the father clone into a mass culture of the mother clone. Specifically, we regularly (about once every two weeks) introduced a small number of males into two 10L aquaria containing mass cultures of females (one for each of the two crosses), across a period of six (CP x CP) to eight (OP x CP) months. In total, 165 males were used for the CP x CP cross and 299 males for the OP x CP cross. Both crosses produced several thousands of ephippia, which were collected and stored at 4°C in the dark for two months or longer (necessary to break the diapause). Differences in male numbers used and in the duration of ephippia production were explained by the fact that many ephippia from the OP x CP cross were empty (*i.e.,* did not contain any viable embryos) and

because we wanted to ensure that we would be able to obtain a sufficient number of hatchlings for linkage analysis in each of the two crosses. Hatching was induced by bathing ephippia in a solution of pure water for two hours, followed by eight minutes of bleach solution and abundant rinsing with osmotic water (Retnaningdyah and Ebert 2012; Paes *et al.* 2016). The ephippia were then exposed to high light for 24h and then placed to standard laboratory conditions. The hatching vials were carefully inspected every two days for hatched juveniles, and any juvenile present was isolated individually in a new vial to initiate a clonal culture. We obtained a total of 104 clonal cultures of F1 offspring from the CP x CP cross (*i.e.,* hatchlings that survived to adulthood and established a clonal culture by parthenogenesis). However, due to low hatching success, only 44 clonal cultures of F1 offspring of the CP x OP cross were obtained. All parent and offspring clones were kept under standard conditions in the laboratory, fed with the microalgae *Tetraselmis chuii*.

DNA extraction and RAD-sequencing

One batch (offspring clones) to three batches (parent clones) of 15 to 20 individuals were collected, frozen in liquid nitrogen, and stored at -80°C. Total genomic DNA was extracted from each batch using the DNeasy® Blood & Tissue kit (Qiagen). DNA concentration and quality were examined by electrophoresis on 1 % agarose gels and with a Qubit 3.0 (high sensitivity) fluorometer. The replicate batches of the parent clones were extracted and sequenced separately to increase sequencing depth (reads from all replicates of a given parent were pooled prior to analysis). Library construction was carried out according to the RAD-sequencing protocol described by Svendsen *et al.* (2015). The libraries were sequenced on four Illumina HiSeq2500 lanes, using 100 bp single-end sequencing by the Montpellier GenomiX platform (MGX, Montpellier, France).

SNP calling and filtering

Raw sequencing data were demultiplexed with Stacks v.2.41 (Catchen *et al.* 2013) using process_radtags. Reads were aligned to the *D. pulex* reference genome V1.1 (Colbourne *et al.* 2011) using BWA (version: bwa-0.7.17-r1188), and reads with a mapping quality of 30 or less were removed using samtools v1.7 (Li *et al.* 2009). This procedure resulted in 5’217 to 4’695’427 reads per F1 of both crosses. Even though most F1 were well-covered (83 % of F1 had more than one million reads), also low-coverage F1 were kept because the downstream analyses in Lep-MAP3 (Rastas 2017), specifically take genotypes likelihoods into account, and removal of low-coverage individuals is recommended against for these analyses. Parents were all highly covered with 3’381’813 to 6’000’981 reads per parent clone (all three replicates per parent clone combined).

The Stacks module “gstacks” with default parameters was used (--model marukilow and --var-alpha 0.05) to call SNPs and to infer genotype likelihoods. SNP markers were named according to their location, that is, scaffold name and base pair position in the reference genome. SNP markers were filtered using the module “population”, with 0.25 as the maximum proportion of missing values allowed per SNP marker across all F1 of a given cross. After this filtering step, 40’975 SNP markers were retained in the CP x CP cross and 41’917 SNP markers in the OP x CP cross.

Linkage maps construction and analysis

Linkage maps

Linkage maps were constructed using Lep-MAP3 (Rastas 2017). Relationships between parents and offspring in each family were confirmed through the IBD (identity by descent) module in Lep-MAP3. The module "ParentCall2" was used to re-call missing or erroneous parental genotypes based on genotype likelihoods of the offspring, as well as to remove non-informative markers (*i.e.,* markers that were homozygous in both parents). The module “Filtering2” was used to remove strongly distorted markers (*p*-value < 0.0001, as recommended for single-family data). These filtering steps reduced the

numbers of retained markers to 25’951 and 32’654 for the CP x CP and the OP x CP cross, respectively. The stronger reduction in the number of markers in the CP x CP cross is explained by a higher proportion of distorted markers (21 %) compared to the OP x CP cross (9 %).

The initial assignment of markers to linkage groups (LGs) followed the previous linkage map of *D. pulex* (Xu *et al.* 2015a), which was based on the same reference genome. Specifically, all markers on scaffolds that were present on the previous map, were assigned to the corresponding LGs of these scaffolds in the previous map. Second, we used the module “JoinSingles2All” to add markers on unmapped scaffolds (lodLimit=18). After the subsequent ordering steps, the initial assignment of markers to LGs was re-evaluated and corrected (if needed) using Lep-Anchor (see below). To order markers within each LG and to estimate linkage map distances, we used the module “OrderMarkers2”. The analyses were conducted separately for each parent of the two crosses using a pseudo-testcross design (Grattapaglia and Sederoff 1994).

Finally, we used Lep-Anchor (Rastas 2020) to detect potential assembly errors (“chimeric scaffold”), split them, if needed, and rerun the Lep-MAP3 pipeline using the split scaffolds. We ran three rounds of Lep-Anchor + Lep-MAP3 on the maps, until no further chimeric scaffolds were detected. This procedure identified 19 cases of likely assembly errors (assignment of parts of scaffolds to two distinct LGs or to different parts of the same LG, separated by a gap of at least 20 cM, **Table S2**). The final maps were based on 15’577 SNPs for LPB-87 (female of the CP x CP cross), 13’733 SNPs for TEX-1 (male of the CP x CP cross), 16’492 SNPs for TEX-114 (female of the OP x CP cross), and 21’405 SNPs for STM-2 (male of the OP x CP cross).

Physical distances between markers

To estimate physical distances between markers, we performed a final ordering and orientation of scaffolds, using two additional rounds of Lep-Anchor + Lep-MAP3 with SNP markers from all four linkage maps combined. This resulted in a single ordering of the scaffolds containing at least one informative marker in at least one of the four maps. Using this ordering, we estimated physical distances (in bp) between markers, using a custom R script, assuming no gaps between adjacent scaffolds and forward orientation of scaffolds whose orientation could not be determined based on the information of the linkage maps.

Integrated linkage map

Based on the single physical ordering of the scaffolds, we also produced a single linkage map (“integrated linkage map”) using information of both crosses. First, a sex-averaged linkage map (using the option “sexAveraged=1” in the module “OrderMarkers2”) was produced for each of the two crosses. Second, these two sex-averaged maps were combined by averaging. Specifically, for each physical position, we estimated the cM position by linear extrapolation of the nearest markers in each sex-averaged map using a custom R script and averaged these values to obtain the integrated map.

Recombination rate

Genome-wide recombination rate (in cM/Mb) was estimated by summing cumulative genetic lengths of all LGs and dividing it by the total length of the *D. pulex* genome, 197.3 Mb (Colbourne *et al.* 2011) or, alternatively, by the sum of the physical lengths of the anchored scaffolds 148.3 Mb. An average recombination rate for each LG was estimated using the total genetic length of a given LG, divided by the sum of the physical lengths of the scaffolds anchored on that LG.

We also estimated the within-LG recombination parameter, $\bar{r}_{\text{intra}}$ (Veller *et al.* 2019), which, in addition to the number of crossover events also takes into account their locations to estimate the average amount of shuffling of genes that occurs within a chromosome per meiosis (central and widely-spaced crossovers generate more shuffling than tightly-spaced or terminal crossovers, (Veller *et al.* 2019). To

estimate $\bar{r}_{intra}$ we used the MATLAB script from Veller *et al.* (2019), considering, as measure of physical length, the total length of anchored scaffolds. Following Veller *et al.* (2019), we also estimated $\bar{r}_{inter}$, which is the probability of allele shuffling due to random assortment (*i.e.,* segregation).

Comparison of recombination rate between maps

To visualize variation in recombination rates within LGs and to compare this variation among the different parents, we used Marey maps, which plot cumulative genetic distances (cM with respect to the first marker) against cumulative physical distances (Mb with respect to the first marker) for each marker of a given LG. The Marey maps were constructed using all markers. To quantitatively compare recombination rates between the four parents, we then used a subset of the data only (the "reduced data set"), with truncated LGs in order to ensure identical terminal positions for all four maps. Specifically, the Mb position of the most interior terminal markers on any of the four maps was used (one at each LG end), and, in maps where no marker was present at that specific physical position, the cM position was estimated by linear extrapolation of the cM positions of the two nearest markers. The cM position of all markers was subsequently adjusted (by subtracting the cM position of the first marker) to ensure that the corrected cM-position of the first marker was zero. To test for differences in total genetic lengths among the four parents, we conducted an ANOVA on genetic lengths, using each LG as a unit of replication and looking for a parental map effect. We used pair-wise post-hoc tests with the adjusted Tukey HSD method to investigate pairwise differences between any pair of parents.

To investigate potential differences in the linkage map length among the four parents at smaller scales, we divided each LG into three zones of equal length, each of them being composed of five windows (again of equal length). Linkage map positions of the boundary positions of zones and windows were estimated for each map by linear extrapolation of the linkage map positions of the nearest markers. We first tested whether specific LGs showed differences in genetic length among the parents. Second, we investigated whether specific zones within LGs showed such differences. Finally, to test for differences in crossover occurrences, independently of the total map length of the LG, we normalized all four maps to the same genetic length. Using this normalized data set, we again tested for differences among the four parents restricted to specific LGs or specific zones within LGs. Due to the frequent occurrence of windows without any crossover events, the assumptions of ANOVA were not met. We thus analyzed the truncated data (both normalized and non-normalized) with pairwise, non-parametric tests: For each LG or each zone, we performed a Wilcoxon rank test (ZIW) modified for zero-inflated data (Wang *et al.* 2021). To test for effects of specific LGs, 72 (12 LGs*6 pairs) pairwise tests were performed. The effect of specific zones within LG was assessed through 216 (12 LGs*3 zones*6 pairs) pairwise tests, using windows as units of replication. The *p*-values were adjusted according to the Benjamini and Hochberg (1995) correction for multiple tests. Note that ten zones were not tested because all windows within these zones showed zero recombination in the two maps that were compared.

Data availability

Linkage maps including all anchored markers and their positions are given in File S1 (available here). RAD-seq data on parents and offspring of the two crosses would be available when submitted.

Results

Linkage maps construction and analysis

Linkage maps

The linkage maps of all four parent maps, including the OP male, where highly similar (**Figure 1, Table 1**). We therefore first present the characteristics of the integrated map only (**Figure S1, Table 2**). The corresponding results for the four individual maps are given in **Table S3**. In the second part of

the results section, we then use the reduced data set to analyze potential differences among the four individual maps.

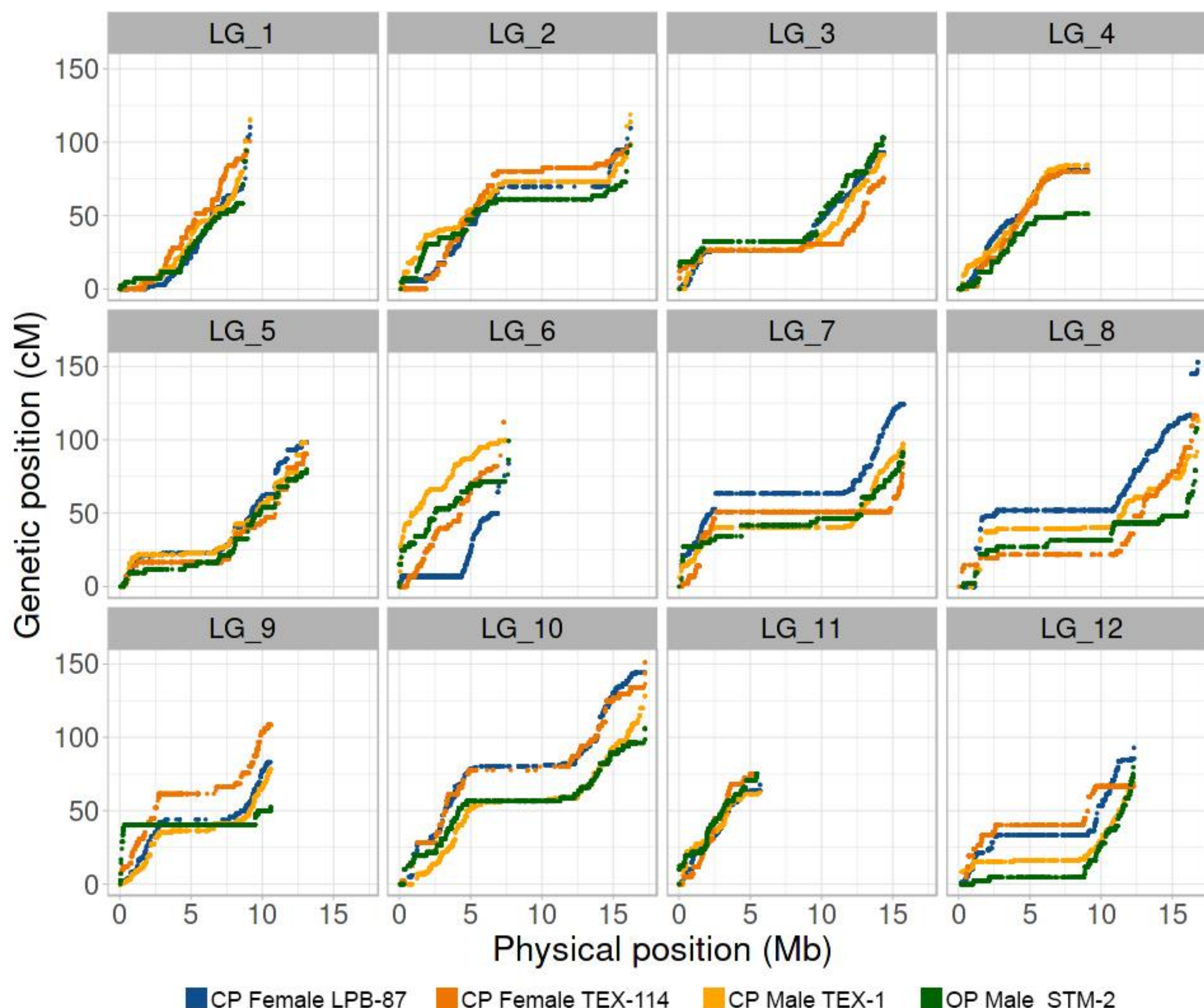

Figure 1: Marey maps, showing the genetic position (in cM) vs. the physical position (in Mb) of each SNP marker (dot) per linkage group (LG) and parents (color code: blue, CP_Female_LPB-87; orange, CP_Female_TEX-114; yellow, CP_Male_TEX-1; green, OP_Male_STM-2; total, non-reduced data set in all cases).

Table 1: Total genetic length (in cM), total physical length of all anchored scaffolds (in Mb), and recombination rate (cM/Mb) across all LGs for each of the four parents, based on the non-reduced data set.

Parent	Genetic length (cM)	Physical length (Mb)	Recombination rate (cM/Mb)
CP_female_LPB-87	1240.60	142.80	8.69
CP_male_TEX-1	1157.16	144.29	8.02
CP_female_TEX-114	1160.55	142.22	8.16
OP_male_STM-2	1037.20	145.56	7.13

Table 2: Number of markers, total genetic length (in cM), total physical length of all anchored scaffolds (in Mb), and recombination rate (cM/Mb) for each LG of the integrated *D. pulex* linkage map. The last row ("Total") refers to sums across all LGs, except for recombination rate where it refers to the average.

LG	Number of markers	Genetic length (cM)	Physical length (Mb)	Recombination rate (cM/Mb)	$\bar{r}$ intra
1	3900	104.09	9.22	11.28	9.08E-04
2	4894	117.00	16.21	7.22	2.40E-03
3	3835	89.59	14.37	6.23	1.50E-03
4	3375	69.96	9.13	7.66	8.28E-04
5	3853	89.78	13.16	6.82	1.50E-03
6	3500	99.75	7.78	12.83	5.73E-04
7	4759	104.73	15.77	6.64	1.50E-03
8	5081	125.17	16.78	7.46	2.20E-03
9	4349	82.35	10.62	7.76	7.00E-04
10	5171	129.47	17.25	7.51	3.00E-03
11	2396	72.76	5.70	12.76	2.99E-04
12	3502	89.40	12.31	7.26	9.66E-04
Total	48615	1174.04	148.31	7.92	1.64E-02

The integrated map

The integrated *D. pulex* map contains 345 of the 5191 scaffolds of the Xu *et al.* (2015a) assembly (**Table S2**). Note that the LG numbering is equivalent to the one in Xu *et al.* (2015a), but we added suffixes "_1", "_2" or "_3" for scaffolds that were split during the Lep-Anchor analysis (*i.e.,* due to evidence that these likely are chimeric scaffolds). The total length of the 345 anchored scaffolds is 148.3 Mb (**Table 2 and S2**), which represents 75.2 % of the combined length of all scaffolds of the reference genome used here (Colbourne *et al.* 2011). The total estimated physical length of each LG ranged from 5.7 Mb on LG 11 to 17.2 Mb on LG 10 (**Table 2**). The four individual maps were on average 3.1 % shorter than the integrated map, missing, on average, 50 (range 41 to 64), mostly smaller scaffolds (**Table S3, File S1**). Our integrated *D. pulex* linkage contains a total of 48'615 SNP markers (Table 2), with an average inter-marker distance of 0.02 cM (**Table 2**). The total map length is 1'174 cM with the different LGs spanning between 69.96 cM on LG 4 and 129.47 cM on LG 10 (**Table 2**). The two sex-averaged Marey maps of each cross as well as the integrated Marey map are represented in **Figure S1**.

Recombination rates

The estimated genome-wide recombination rate of the integrated map is 7.92 cM/Mb or 5.95 cM/Mb (ranging from 5.26 to 6.29 cM/Mb among the four linkage maps), depending on whether the total linkage map length was divided by the total length of anchored scaffolds or by the estimated total genome size (197.3 Mb) of *D. pulex* (**Table 2**). The genome-wide intra-chromosomal recombination parameter $\bar{r}_{\text{intra}}$ across all LGs is 0.0164, while inter-chromosomal recombination parameter $\bar{r}_{\text{inter}}$ is 0.45. Recombination rates of individual LGs varied between 6.2 cM/Mb on LG 3 and 12.8 cM/Mb on LG 6 (**Table 2, Figure S1**), and the intra-chromosomal recombination parameter $\bar{r}_{\text{intra}}$ ranged between 3x10^{-4} on LG 11 and 3x10^{-3} on LG 10 (**Table 2**). The $\bar{r}_{\text{intra}}$ was positively correlated with the total genetic length (in cM) across LGs (Pearson r = 0.83, d.f. = 10, p = 0.0007) but negatively correlated with the recombination rate (in cM/Mb) (Spearman ρ = -0.68, d.f. = 10, p = 0.01). As evident from the Marey maps (**Figure 1**), recombination rate varied extensively within LGs. In most LGs, we detected a large region with zero or almost zero recombination, putatively the peri-centromeric regions (Svendsen *et al.*

2015), although centromere locations are unknown in *D. pulex*. In contrast, recombination rates were high towards the ends of the LGs (**Figures 1 and S1**).

Comparison of recombination rate among maps

Overall genetic length

All comparisons between maps were based on the reduced data set (truncated to identical terminal positions), which was 2.3 % shorter (in terms of the number of base pairs included) than the integrated map (**Table S4**). Overall, we found a slight but significant variation in the total genetic length among the four maps (ANOVA, $F = 3.59$, $p = 0.02$, **Table 3**), with only one of the pairwise post-hoc tests being significant (OP male vs. CP female LPB-87, $p = 0.01$, **Table 3**). Regarding sex-differences, the map length of the CP male (TEX-1) was slightly (average 6 %) but non-significantly lower than the map lengths of the two CP females (**Figure 2, Table S4, Table 3**). Regarding the difference between CP and OP, the genetic length of the OP male was 11.9 % lower than that of the CP male and 15.5 % lower compared to the mean of the three CP parents (**Table S4, Figure 2**). As stated above, only one of the pairwise post-hoc tests was significant (**Table 3**).

Table 3: Post-hoc tests for differences in the overall genetic length (using LGs as replicates) in all pair-wise comparisons between parents ("Contrast"). *P*-values are adjusted according to the Holm method.

Contrast	z_value	*P_adj*
CP_Female_TEX-114 vs. CP_Female_LPB-87	-0.74	0.92
CP_Male_TEX-1 vs. CP_Female_LPB-87	-1.34	0.54
OP_Male_STM-2 vs. CP_Female_LPB-87	-3.14	0.01
CP_Male_TEX-1 vs. CP_Female_TEX-114	-0.60	0.92
OP_Male_STM-2 vs. CP_Female_TEX-114	-2.40	0.08
OP_Male_STM-2 vs. CP_Male_TEX-1	-1.80	0.29

Genetic length of specific LGs and zones within LGs

We tested whether the differences in total genetic length among the four maps were driven by just some of the LGs or even more narrowly by just some zones within LGs. None of the LGs differed significantly among maps (after correcting for multiple testing) in any of the pairwise comparisons (**Table S5**). Only LG 9 showed a tendency for being shorter (in terms of genetic length) in the OP male, compared to each of the three CP individuals (**Figures 1 and 2, Table S5**). Two zones within LGs showed significantly different genetic lengths among maps (**Table S5**): the middle zone of the LG 7 was significantly longer (p_adj < 0.003) in the OP male compared to each of the three CP individuals, and the middle zone of the LG 9 showed significant differences (p_adj < 0.003) between most pairs, being shorter in the OP male than in most CP individuals (**Table S5**).

Normalized maps

We used the normalized data set to test for differences in the localization of crossovers, independent of the total length of the maps. Again, none of the LGs showed a significant difference in any of the pairwise comparisons and the only two zones that showed significant differences were the same ones already identified when considering non-normalized maps (**Table S5**).

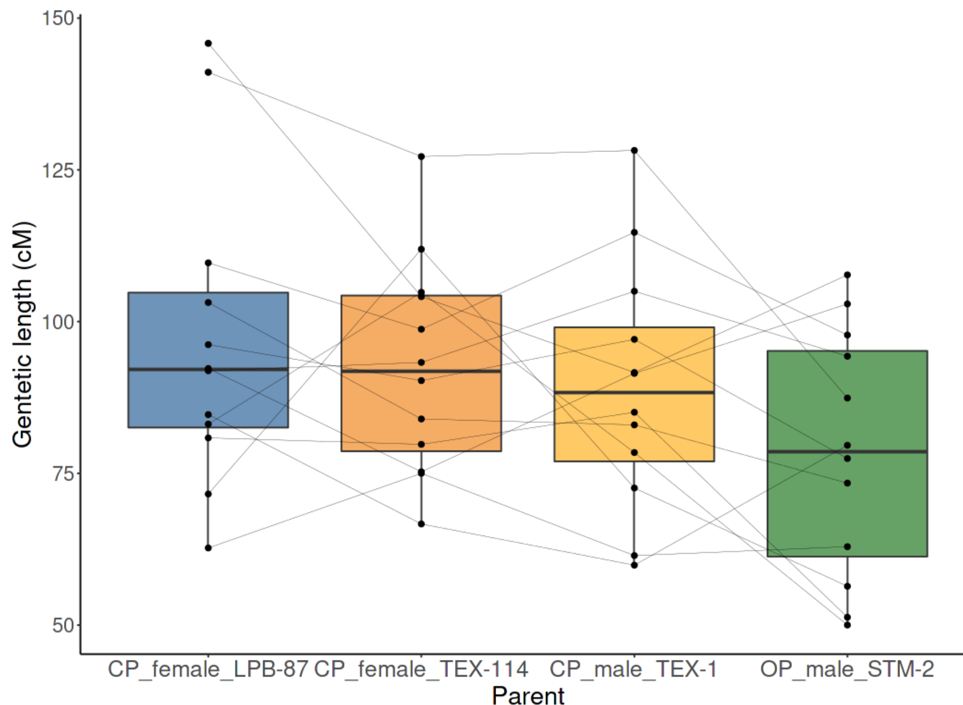

Figure 2: Genetic length of LGs in each of the four maps, based on the reduced data set. Dots represent individual LGs, and the fine lines identify the same LGs in the different maps. The thick horizontal lines represent the medians, the box the 25th and 75th percentiles, and the error bars are the 95 % confidence intervals. Color code as in Figure 1.

Discussion

No recombination differences between OP males and CP males

The main goal of this study was to examine how recombination changed in males and females in the CP to OP transition. Our results demonstrate that recombination is not absent in OP males. Rather, the OP male showed highly similar levels of recombination compared to both the CP male and the CP females. While recombination rate was slightly lower than in CP, this effect was mainly local, being largely explained by LG 9 and a few zones within other LGs. These may correspond to regions that affect asexuality itself (Lynch *et al.* 2008; Eads *et al.* 2012; Tucker *et al.* 2013; Xu *et al.* 2013). The asexuality-determining regions are highly heterozygous in OP, due to hybrid origin of these regions (Xu *et al.* 2015b). This high heterozygosity (*i.e.,* high levels of divergence between homologs) may be the cause of these local reductions in recombination, as demonstrated in other systems (Lukacsovich and Waldman 1999). Overall, our results clearly support the fact that OP males can be fully functional, producing sperm by a normal meiosis including normal recombination.

This contrasts with OP females, in which the diapause phase is clonal (or nearly clonal), based on the non-segregation of allozymes (Hebert and Crease 1980; Innes and Hebert 1988; Hebert *et al.* 1989). Similarly, recombination is absent (or extremely low) during the subitaneous phase of CP and OP females (Omilian *et al.* 2006; Xu *et al.* 2011; Keith *et al.* 2016; Flynn *et al.* 2017). Overall, the presence of recombination in OP males but not in OP females (diapause phase) shows that recombination suppression only concerned females, but not males in the CP to OP transition.

Possible mechanism underlying the evolution of OP in *D. pulex*

The meiosis suppression and the Rec8 hypothesis

Given that recombination is not suppressed in OP males, it is unlikely that OP has evolved due to a *de novo* mutation leading to general suppression of recombination. General meiosis suppression, for

instance due to pseudogenization (Li *et al.* 1981) of an essential recombination gene, has been put forward as one of the possible mechanisms of OP evolution (Simon *et al.* 2003; Schurko and Logsdon 2008). In *D. pulex*, a particular haplotype containing a frameshift mutation in one of the three genomic copies of the Rec8 gene (Rec8-B) consistently occurs (in heterozygous form) in OP but not in CP (Eads *et al.* 2012). Rec8 is involved in the cohesin complex that binds sister chromatids during meiosis and is therefore a good candidate for a gene that might lead to recombination suppression if its function is disrupted. Rec-8 is not specific to the female meiosis: all Rec-8 paralogs are expressed in both sexes of CP *D. pulex* (Schurko *et al.* 2009) and there is so far no evidence for male-biased or female-biased expression of Rec8-B.

However, our data indicates that disrupting Rec8-B does not lead to recombination suppression in OP males. The males in our experiments are genetically identical to OP females and therefore also heterozygous for the loss of function mutation in Rec8-B, while still having a functional copy of Rec8-B, just as the females. Thus, our result shows that there is no evidence for a causal involvement of Rec8-B in the evolution of OP. Rather, the Rec8-B mutation may have occurred secondarily in OP. Loss of function mutations can indeed occur secondarily in genes that are no longer under strong selection pressure (Normark *et al.* 2003).

The sex-limited meiosis suppression hypothesis

Normal recombination in OP males is consistent with a scenario where OP evolution is caused by mutation(s) affecting recombination only during oogenesis. This is the idea of a sex-limited meiosis suppression gene (Hebert *et al.* 1988, 1989). This sex-specific suppression might have occurred in CP through de novo mutations. We do not observe heterochiasmy in CP (see below), suggesting that this type of variation is not frequent, or at least that mechanisms differentially adjusting recombination in males and females do not pre-exist in CP.

Another possibility is that OP evolved by reusing the subitaneous parthenogenesis oogenesis pathways already present in CP and extending them to oogenesis during diapause egg formation. In this scenario, the sex-limited meiosis suppression is based on an already existing pathway and only requires that it becomes used in a different part of the life cycle. Because this modification is likely to be minor (*e.g.,* involve different signaling or expression patterns during diapause egg production), it may be a common route to evolve OP in *Daphnia* and other CP-OP systems. In aphids, OP has evolved though a genetic change that prevents individuals from entering the diapause phase and are typically observed in temperate regions with mild winters (Simon *et al.* 2002, 2010; Dedryver *et al.* 2013). The identified candidate region in the pea aphid (*Acyrthosiphon pisum*) contains genes involved photoperiod sensitivity (Jaquiéry *et al.* 2014). Similarly, in rotifers, the transition to OP is thought to be caused by a genetic change that prevents individuals from responding to chemical signals that induce sexual reproduction in CP (Stelzer 2008; Stelzer *et al.* 2010). In contrast to aphids, OP *Daphnia* still enter diapause phase, so that the mechanism is probably different. It cannot involve only an altered sensitivity to environmental signals. However, the general principle may be the same. Once parthenogenesis is present in a part of the life cycle, a transition to OP can simply be achieved by extending it to the entire life cycle, rather than by evolving a new, female-limited, parthenogenetic pathway.

Secondary evolution in OP male

In our experiment, we deliberately used an OP strain known to be able to undergo successful, reductional meiosis (Xu *et al.* 2015b). Indeed, other OP strains exist, in which males do produce diploid or aneuploid sperm (Xu *et al.* 2015b). Doing a mapping cross with a male from such a strain would either have been impossible (in case of unviability of the produced offspring) or technically too challenging (interpretation of segregation patterns in offspring with a potential mixture of diploid and triploid loci). We therefore do not know whether spermatogenesis in these males involves normal

recombination. Yet, it is likely that non-reductional (and potentially non-recombining) spermatogenesis in these males is explained by secondary evolution, a scenario in line with the expected secondary loss of males or male functions in OP following a relaxation of selection pressure (Innes *et al.* 2000; Wolinska and Lively 2008; van der Kooi and Schwander 2014). Indeed, the emergence of new OP lineages occurs through contagious asexuality where males transmit OP genes, which originated in a hybrid lineage, to new lineages by mating with CP females (Innes and Hebert 1988; Crease *et al.* 1989; Hebert *et al.* 1989, 1993; Taylor and Hebert 1993; Paland *et al.* 2005; Xu *et al.* 2015a). As all known OP lineages (with the exception of high arctic ones, Beaton and Hebert 1988; Dufresne and Hebert 1995) are diploid, the males transmitting OP genes to these lineages must have been able to undergo reductional meiosis, just as in our experiment.

No heterochiasmy in CP *D. pulex*

We produced both male-specific and female-specific linkage maps of *D. pulex*, which allows us to evaluate how recombination changed in males and females in the CP to OP transition. Even though the CP male recombined slightly less than the two CP females, we found no evidence for genome-wide heterochiasmy in *D. pulex*. This is the first evidence for the absence of heterochiasmy in a species with environmental sex determination and no sex chromosomes. The result is congruent with very recent finding in *D. pulicaria*, the sister species of *D. pulex*, in which also no heterochiasmy was found (Wersebe *et al.* 2022). The only other case of an ESD animal where sex-specific recombination rate was investigated, is the saltwater crocodile where there is strong heterochiasmy (Miles *et al.* 2009). Hence, our findings tend to confirm that there is no special pattern of heterochiasmy in ESD species, and no global association between mechanisms of sex determination (genetic or environmental) and the presence of heterochiasmy (Lenormand and Dutheil 2005; Stapley *et al.* 2017). We also observed that the female LPB-87 has a non-recombining region at the beginning of the LG 6 but this difference was not shared with the female TEX-114, and thus is more likely to be explained by a population difference rather than by the sex. This also highlights the fact that taking into account inter-population variability may be important when studying heterochiasmy, either by using within-sex biological replicates from different populations or males and females from the same populations (both were done here).

A new reference map for *D. pulex*

The sex-specific and integrated maps presented in the current study constitutes an important addition to existing genomic resources for *D. pulex*. The first *D. pulex* linkage was based on microsatellite data (Cristescu *et al.* 2006). Subsequently, Xu *et al.* (2015a) produced a second-generation, male-specific map, based on single sperm methodology. An additional map, which was published as an appendix of a new reference genome for the species (Ye *et al.* 2017), is likely erroneous, as it predicts, on average, over eight crossovers per chromosome and meiosis, as opposed to just a bit over two in our map and that of Xu *et al.* (2015a). We therefore compare our results, mainly to the linkage map from Xu *et al.* (2015a), which was also based on the same genome assembly as used here (Colbourne *et al.* 2011). Xu *et al.* (2015a) anchored 187 scaffolds (131.9 Mb) and have an average inter-marker distance of 0.87 cM, while our integrated map anchors 345 scaffolds (148.3 Mb) with 0.02 cM 0.02 cM between markers on average. The main improvement thus comes from the mapping of many additional, mostly smaller scaffolds. In addition, while there was a high degree of collinearity between the maps, we identified and corrected 19 likely assembly errors (chimeric scaffolds), and placed the part-scaffolds back to the linkage map. Still, about one fourth of the total assembly (197.3 Mb) remains unmapped, either due to smaller scaffolds containing no SNPs, scaffolds with SNPs only in repetitive regions (which are filtered during mapping due to a low mapping score), and perhaps also due to the presence of contaminant scaffolds (*e.g.,* DNA from microbial symbionts) in the reference genome.

Regarding the genome-wide recombination rate, the estimates from our study and that of Xu *et*

al. (2015a) are very similar (7.9 cM/Mb and 7.3 cM/Mb, respectively). These estimates are also similar to those from other *Daphnia* species (*D. pulicaria*, 7.4 cM/Mb, Wersebe Matthew 2021 and *D. magna*, 6.8 cM/Mb, Dukić *et al.* 2016), suggesting conservation of recombination rates in the genus.

Regarding the individual maps, there appears to be large variation among individuals in the ranking of the longest to the shortest LG. Inspection of the Marey maps (**Figure 1**) suggests that the differences are largely due to a small group of terminal markers per LG, while the recombination patterns were otherwise (apart from the few notable exceptions discussed above) remarkably similar among individuals. Two factors may have contributed to differences in estimated recombination rates in terminal markers among individuals. First, the observation may be entirely artefactual because the estimation of recombination rate is less reliable for terminal markers than for more central ones. Indeed, to counter the well-known fact that erroneous genotype information artificially increases recombination rate, Lep-MAP3 (Rastas 2017) uses information on several flanking markers to smoothen spikes in apparent recombination rates due to unreliable markers. Second, as most LGs exhibited higher recombination rates in more peripheral parts, the estimated total length of LGs may be rather sensitive to inclusion or not of an additional, slightly more terminal marker as well as to sampling variation among the different maps.

The high prevalence of peripheral crossovers likely has also contributed to the observed low $\bar{r}_{intra}$ (within-LG recombination parameter) because terminal recombination contributes only little to effective gene shuffling. The excess of recombination in peripheral parts was mainly noted in (physically) larger LGs, a pattern also observed in many other animal and plant species (Haenel *et al.* 2018). This pattern might amplify the very well-known negative relationship between the recombination rate (cM/Mb) and the physical size of LGs, caused by the constraint of at least one crossover per LG and meiosis (Mather 1938; Hunter 2007). It might thus also contribute to the observed positive and negative correlations of $\bar{r}_{intra}$ with cM length and cM/Mb recombination rate across LGs, which are likely explained by the same factors.

Overall, we found that the inter-chromosomal recombination parameter $\bar{r}_{inter}$ was much larger than the intra-chromosomal one, $\bar{r}_{intra}$. This is not surprising given that the species has 12 different chromosome pairs of more or less similar physical size (suggesting that the probability of a random pair of genes to be on two different chromosomes is about $\frac{11}{12}$), and given that recombination within chromosomes is not free. Nonetheless, this finding illustrates that the reduction of crossover numbers or an evolution to more terminal crossover locations would have minor effects on overall shuffling. This highlights the fact that even if recombination rates were reduced in OP males, gene shuffling reduction would be efficient only if segregation was reduced at the same time.

Conclusion

We found that the CP to OP transition in *D. pulex* involves a considerable reduction in female recombination rate, that male recombination is not affected, and that recombination is not initially different between male and female CP during the diapause stage. These findings favor the hypothesis that the subitaneous parthenogenetic pathway was re-used and extended to the production of diapause egg in *D. pulex*. This may be a common way to evolve obligate parthenogenesis in species with mixed sex-asex reproductive systems.

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Author contributions

CH, CM, and TL conceived the study. CM conducted the analysis.

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Supplementary data

File S1: Excel file with five sheets, containing the integrated linkage map (sheet 1) and the four parental maps (sheets 2 to 5). In each sheet, each line corresponds to a marker ("Marker_ID"), whose name is based on the reference genome (scaffold and bp position within scaffold). For each marker, its LG and cM position are given, a well as its cumulative physical position within the LG (see materials and methods). Two additional columns indicate whether the marker is included in the "Reduced data" set, and whether it is on a "Split scaffold". Available here.

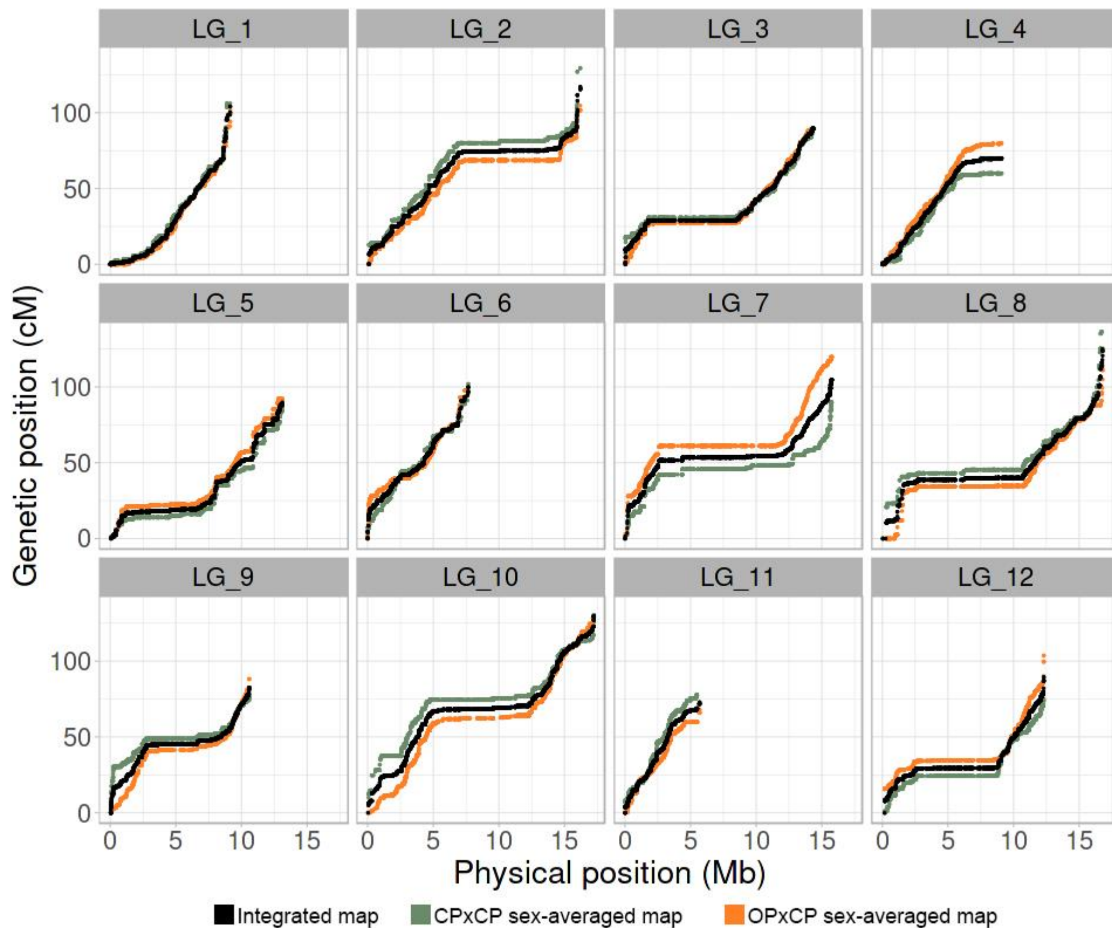

Figure S1: Marey maps, showing the genetic position (in cM) vs. the physical position (in Mb) of each SNP marker (dot) per linkage group (LG) for the integrated map and the two sex-averaged maps from each cross (color code: black, integrated map; green and orange, sex-averaged maps from the CP x CP and OP x CP cross respectively; total, non-reduced data sets in all cases).

Table S1: Names and origins of clones used in the study, as well as their use as mother or father line in each of the two crosses.

Clone name	Origin	Cross	Parental line
LPB-87	Long Point Pond B, Ontario, USA	CPxCP cross	mother line
TEX-1	Textile Road, Michigan, USA	CPxCP cross	father line
TEX-114	Textile Road, Michigan, USA	OPxCP cross	mother line
STM-2	St. Mattieu-du-Parc, Quebec, Canada	OPxCP cross	father line

Table S2: Physical locations of the 345 anchored scaffolds in the integrated map. Scaffolds are named according to Xu *et al.* (2015a), with suffixes "_1", "_2", or "_3" for scaffolds split during the analysis (due to evidence that the original scaffolds were chimeric). For each scaffold its location is indicated by the linkage group (LG) to which it is assigned, the start and end positions (in bp) of the scaffold within that LG, as well as the orientation ("up" for the same orientation as in the reference genome, "down" for the opposite, *i.e.,* highest position first). For split scaffolds, bp positions after which they were split are indicated, based on the unsplit scaffold in original (*i.e.,* "up") orientation. Available here.

Table S3: Total genetic length (in cM), total physical length of all anchored scaffolds (in Mb), and number of markers for each LG and for each of the four parents, based on the non-reduced data set. Physical lengths differ slightly among parents because of different numbers of anchored scaffolds. Totals refer to sums across LGs.

	CP Female LPB87			CP Male TEX1			CP Female TEX114			OP Male STM2		
LG	**Physical length (bp)**	**Genetic length (cM)**	**Number of markers**	**Physical length (bp)**	**Genetic length (cM)**	**Number of markers**	**Physical length (bp)**	**Genetic length (cM)**	**Number of markers**	**Physical length (bp)**	**Genetic length (cM)**	**Number of markers**
1	9224380	110.32	1448	8965511	115.69	1097	9224380	100.6	1364	9067762	94.29	1487
2	13267051	109.68	1643	16100122	118.84	1422	15976790	98.74	2006	16148797	97.79	2009
3	14363272	93.12	1242	14090285	91.46	1192	13927103	75.25	1324	13574107	102.9	1703
4	8996529	80.84	1104	9111636	85.06	1014	9111636	79.78	1129	9122884	51.29	1469
5	13128336	98.16	1285	12754333	98.04	1146	12761360	90.28	1261	12551488	79.77	1625
6	7727419	83.83	1834	7476569	99.55	727	7435323	111.92	919	7763553	99.3	1207
7	15635506	123.98	1491	15703832	97.01	1329	15473559	83.96	2111	15522597	91.05	1953
8	16233883	152.84	1450	15381548	114.64	1254	15316892	116.47	1376	16542103	107.7	2580
9	9594813	83.11	838	10617469	78.43	1151	10380930	108.5	1072	10617469	52.33	2710
10	16623951	144.19	1639	16314704	128.2	1578	15001577	151.09	1458	17157501	105.87	2451
11	5702396	67.68	672	5527719	62.42	766	5507181	75	882	5507181	75.28	960
12	12306890	92.88	927	12246553	67.83	1057	12098393	68.99	1590	11982477	79.65	1251
Total	142804426	1240.6	15573	144290281	1157.16	13733	142215124	1160.55	16492	145557919	1037.2	21405

Table S4: Physical and genetic lengths of each LG in each of the four parents in the reduced data set. Totals refer to sums across LGs. Details on the positions of the terminal markers are given in File S1.

LG	**Physical length (bp)**	**Genetic length (cM)**			
		CP Female LPB-87	**CP Male TEX-1**	**CP Female TEX-114**	**OP Male STM-2**
1	8874044	91.90	105.00	93.27	94.29
2	16108229	109.68	114.70	98.74	97.79
3	14296715	92.27	91.46	75.25	102.90
4	9009149	80.84	85.06	79.78	51.29
5	12803662	96.20	97.07	90.28	77.44
6	7244772	71.59	72.58	111.92	56.39
7	15502991	103.14	82.98	83.96	73.39
8	16385810	145.84	91.61	104.11	107.70
9	10530459	83.11	78.43	104.83	50.00
10	16732901	141.08	128.20	127.19	87.40
11	5302574	62.71	61.45	75.00	62.92
12	12081464	84.68	59.87	66.66	79.62
Total	144872770	1163.02	1068.41	1110.98	941.13

Table S5: Zero-inflated Wilcoxon rank tests (ZIW) for differences in recombination between pairs of parents ("Contrast") based on the normalized or non-normalized data set ("Data type) and either for specific LGs or specific zones within LGs ("Genome region"). *P*-values adjusted by the Benjamini & Hochberg method (Benjamini and Hochberg 1995). Available here.

Chapter 3

Asexuality is not faithfully transmitted by contagion in *Daphnia pulex*

In preparation. The current state of the manuscript is not complete. Some small analyses will be added and the discussion will be refined.

Asexuality is not faithfully transmitted by contagion in *Daphnia pulex*

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Abstract

In some taxa, new emergences of asexual lineages are possible through contagious asexuality, where rare males from obligate asexual lineages can transmit asexuality to new lineages by cross-mating with sexual females. With such "contagious asexuality" scenario, it is often assumed that asexuality can be immediately transmitted intact from the asexual to the new hybrid lineages. In this paper, we investigate in detail whether asexuality is faithfully transmitted in such crosses. We studied the reproductive modes of F1s produced by crossing sexual females to males from an obligate parthenogen lineage in *Daphnia pulex*. While the parental asexual lineage is an obligate parthenogen reproducing clonally, we find that the F1s show a wide diversity of reproductive modes. We do not find discrete classes of sexual vs. asexual F1s. Rather, some F1s appear to be able to reproduce both sexually and asexually. Moreover, when they are able to reproduce asexually (about 20 % of F1s), they do not reproduce clonally, as shown by frequent loss of heterozygosity (LOH) among their parthenogenetic offspring. Such LOH can lead to large fitness reduction by revealing recessive deleterious mutations, which may therefore largely impact the chance of establishment of contagiously-produced asexual lineages. We also found that these F1s are difficult to produce and have strongly reduced fertility rates, particularly for asexual F1s compared to natural ones, indicating that the initial fitness of these contagiously-produced asexual lineages is also often low. These findings prompted us to verify that natural asexuals were not able to also reproduce sexually, and we did find that it can occur rarely. Together, our results indicate that asexuality is not transmitted intact with "contagious" crosses. Such crosses rather result in diverse, non-binary, and non-clonal offspring, on which subsequent selection may act.

Keywords: *Daphnia*, asexual modes of reproduction, obligate parthenogenesis, mixed reproduction, new asexuals

Introduction

The rarity of obligate asexual species among eukaryotes remains one of the greatest puzzles of contemporary evolutionary biology (White 1978; Otto and Lenormand 2002; Schön *et al.* 2009; Hartfield and Keightley 2012). The puzzle is a theoretical one because sex involves very strong costs relative to asexual reproduction (Maynard Smith 1978). Yet, it is also an empirical challenge as experimental estimates of the relative fitness of asexuals versus sexuals have proven hard to obtain, despite intensive effort. Asexuals are often studied through comparisons with closely related sexuals (Barton and Charlesworth 1998; Neiman and Schwander 2011; Meirmans *et al.* 2012). However, a major empirical limitation that remains is that the asexuals sampled in nature represent a highly biased subset of the most successful lineages and these successful lineages may provide little information about the properties of average novel asexual lineages. Yet, because all extant asexuals have evolved from sexual relatives (Ramesh *et al.* 2005), the limited success of asexuals may well be determined by the properties of these novel lineages. Indeed, asexuality, more often evolves through modifying meiosis than through

replacing meiosis by mitosis (Bell 1982; Archetti 2010). Several of these meiosis modifications have genomic consequences that strongly differ from pure clonality (*e.g.,* they lead to loss of heterozygosity, Archetti 2004, 2010; Engelstädter 2008, chapter 1). Thus, the selection pressures acting on the newly arising asexuals may differ substantially from those acting on established asexuals found in nature (Simon *et al.* 2002; Engelstädter 2008; Archetti 2010; Neiman and Schwander 2011). Studying newly arising asexuals may therefore be a crucial step to understand why sex-asex transitions are so rare.

In most systems, studying newly arising asexuals is exceedingly difficult, as sampling these young lineages is almost impossible. However, some asexuals still rarely produce males, which, by mating with related sexual females, can transmit asexuality-determining genes to their offspring, thus creating new asexual lineages (Simon *et al.* 2003). This so-called "contagious asexuality" is one of several ways by which new asexual lineages arise in nature and is known to occur in systems across a wide taxonomic range (earthworm: Jaenike and Selander 1979, water flea: Innes and Hebert 1988, rotifer: Stelzer *et al.* 2010; brine shrimp: Maccari *et al.* 2013; aphid: Jaquiéry *et al.* 2014; parasitoid wasp: Sandrock and Vorburger 2011 and in a Brassicaceae plant: Mau *et al.* 2021).

Here we use the unique opportunity offered by contagious asexuality in *Daphnia pulex* to generate new asexual lineages in the laboratory and to compare them not only with closely related sexuals, but also with established asexual lineages. *Daphnia pulex* is one of the most emblematic and best documented cases of contagious asexuality (Innes and Hebert 1988; Simon *et al.* 2003; Lynch *et al.* 2008). In natural lineages of *Daphnia*, asexuality occurs through a modified meiosis, which is genetically equivalent to clonality, during the production of liveborn offspring (CP and OP), as well as during asexual production of ephippial embryos in OP (Hebert and Crease 1980, 1983; Hebert 1981; Hiruta *et al.* 2010). Several independent genomic regions (on different chromosomes) have been found to differ between CP and OP in association studies (Lynch *et al.* 2008; Tucker *et al.* 2013; Xu *et al.* 2015). It is possible that these regions together determine the asexuality phenotype of OP, such that breakup of these regions due to segregation (some of the regions are heterozygous in OP, Tucker *et al.* 2013) may lead to a non-faithful transmission of asexuality phenotype.

We set up several crosses between males from obligate parthenogenetic (OP) lineages and females of cyclical parthenogenetic (CP) lineages of the crustacean *Daphnia pulex*. Both CP and OP lineages produce liveborn offspring asexually, but resting eggs are produced sexually in CP and asexually in OP (OP are thus obligately asexual). Crosses led to the formation of diapause embryos contained in diapause capsules (called "ephippia"). To assess how easily new asexual lineages are generated, we estimated the hatching rate of these ephippial embryos, the rate at which asexuality is transmitted to offspring, and, as a proxy of asexual fitness, the hatching rate of asexually produced F2 offspring. We also investigated the mode of asexual reproduction of the F1s by comparing their genotypes, at several microsatellites loci, to that of their F2 offspring. Overall, we tested whether asexuality was faithfully transmitted upon contagion and whether the new asexual lineages produced by contagion had a high fitness, such that they could establish *in natura*.

Materials & Methods

D. pulex clones

Daphnia pulex clones come from North America (**Table S7**). We used 12 different clonal lineages (six OP clones and six CP clones) for the crosses. Each clonal lineage is constituted of a single genotype (descendant of a single female obtained from nature and maintained by clonal reproduction of liveborn offspring in the laboratory). Clones were maintained in the laboratory under standard culturing conditions (Adam medium, Klüttgen *et al.* 1994) 18°C, daily fed with freeze-dried microalgae *Tetraselmis chuii* diluted in Adam medium). Males were produced by adding the hormone methylfarnesoate in the culture medium (Toyota *et al.* 2015). The offspring of hormone-treated females

were sexed morphologically under a binocular loupe, and males were checked a second time when they reached at least pre-adult age.

Crosses

In total, we performed four control crosses CP x CP, 13 CP x OP crosses (CP females x OP males) and two OP x CP crosses (OP females x CP males; **Table S1**). In all crosses, clones used as females (LPB-87, TEX-114, KAP-87, NFL-92, DIS-47 and DIS-85) are non-male producing (NMP) clones, that is, they are unable to produce males and thus they participate in sexual reproduction only as females (Innes and Dunbrack 1993; Tessier and Cáceres 2004; Galimov *et al.* 2011; Ye *et al.* 2019). The use of NMP clones strongly simplifies the use of mass-mating as ephippia are produced by obligate outcrossing with the chosen father clones (unless they are produced asexually). Obviously, all clones used as males are male producing clones (MP).

CP x CP (control) and CP x OP crosses (sex-asex)

We performed CP x OP crosses to produce newly generated asexual clones, as well as CP x CP crosses used as control. For these crosses, we regularly (about once every two weeks) introduced a small number of males into 10L aquaria containing mass cultures of females, across a period of six (CP x CP) to eight (CP x OP) months. In total, 799 males were used for the CP x CP crosses and 1'832 males for the CP x OP crosses (**Table S1**). All crosses produced several thousands of ephippia, which were collected and stored at 4°C in the dark for at least two months (necessary to break the diapause). The mass-mating approach and the duration of the experiment were set to obtain a sufficiently large number of embryos to hatching (about 940 on average for each CP x CP and CP x OP crosses, **Table S8**).

OP x CP crosses (rare sex in OP)

Two natural NMP OP clones and two natural MP CP clones were used to test whether OP females are able to (rarely) produce diapause embryos sexually (test for rare sexual reproduction in OP). We let the OP clonal cultures grow in numbers (by clonal production of liveborn offspring) and produce ephippia for nine to 13 weeks. To each OP clone, we manually added 54 to 226 males once a week (1'904 males added in total, **Table S1**). Compared to other crosses, the extra effort put into the number of males is justified by the fact that sexual reproduction in OP clones is expected to be rare (if there is any) and we wanted to make sure that no male-limitation occurred if there was any sexually receptive female.

MP vs. NMP phenotyping of F1 offspring

The F1 offspring that hatched from the CP x OP crosses (see **Appendix A**.) were phenotyped with respect to their ability to produce males (MP vs. NMP). Phenotyping was determined using, first a hormonal treatment (Toyota *et al.* 2015) and then using a diagnostic locus (part of the gene *Dp8960*) responsible for the NMP phenotype in *D. pulex* (Ye *et al.* 2019). For details, see the protocol and primers used in **Appendix B., E. and G**.

Fill rates data:

We studied the reproductive mode of the F1 offspring phenotypically and genetically with emphasis on one particular CP x OP cross in which rare OP males were known to produce haploid sperm (Xu *et al.* 2015): the TEX-114 x STM-2 cross (the "main cross"). First, we used a phenotypic approach only for the NMP using the number of embryos found in the ephippia (called "fill rate"). Fill rate were based on 28 NMP F1s, opening between eight to 210 ephippia per F1 for the main cross and opening between 26 to 183 ephippia per F1 of the 36 NMP F1s from other crosses (**Table S2**). By definition, embryos in the ephippia of NMP clones are produced asexually, thus these clones are able to reproduce

asexually, whereas a fill rate equal to zero in absence of male is expected under exclusive sexual reproduction. However, two other reasons can lead to a zero-fill rate observation: limited sample size (false-negative) or a sterility, which we define as the inability to produce diapause embryos regardless of the presence of the males. We also evaluated the fill rates of 18 MP F1s from our main cross. For MP F1s, sterility is directly given by a zero-fill rate (**Table S2**). Then, to discriminate between exclusive sexuality and sterility, we evaluated fill rates after manually adding males from a CP clone in five NMP F1 clones with zero fill rate from our main cross. We checked whether the fill rate of F1 clones with males increased. We also added males to five NMP F1 clones able to reproduce asexually to check for the exclusive asexual mode of reproduction. In total, sterility can be assessed through 23 F1 offspring of our main cross (MP and NMP).

LOH data

We extracted DNA from adult F1 offspring following the protocol described in **Appendix B.** and from individual diapause embryos (F2) produced by the F1 offspring (see protocol in **Appendix C.**). We screened for LOH by genotyping the F2 embryos and the mother (F1) at one to four heterozygous microsatellite loci. Detailed protocols for LOH analyses are described in **Appendix E., F**. Given the low fill rate and time required for embryo dissection, it was difficult obtaining sufficient numbers of diapause embryos for many F1s. In addition, since there was low amplification success in the embryo DNA extraction protocol, even more diapause embryos were needed, explaining why this was done on a single CP x OP cross. To prevent erroneous interpretation of LOH due to allelic dropout (*i.e*., a selective allele amplification during PCR), we performed independent PCRs for each embryo and only kept data for which we were able to obtain at least three independent runs with clear microsatellite profiles from the same embryo. LOH rate for each F2 embryo was calculated as an average across loci (with 2.5 diagnostic, *i.e.,* heterozygous in the mother, microsatellite loci per F2, on average). LOH analyses were carried out on diapause embryos produced by 17 MP F1s and by five NMP F1. The number of analysed F2s ranged from two to 29 (mean = 10, variance = 35) per F1 and in total LOH was assessed for each 224 F2 progeny of the 22 F1 offspring. A total of fifteen offspring of four natural OP clones were also investigated (**Table S6**). Finally, we performed a paternity test on two F1 NMP able to reproduce asexually after placing them in contact with males (**Table S2**).

Rare sexual reproduction in natural OP clones

For each OP x CP cross, diapause embryos were pooled by groups of ten embryos, and DNA of each pool was extracted using the protocol described in **Appendix H**. In total, 14 pools were analyzed for one cross and 45 pools for the other cross. For each pool, two DNA fragments were amplified by PCR, with primers specific to the CP MP clones from which the males are originating (*i.e*., primers placed on an indel polymorphism with an insertion in the male clone relative to the OP clone). Successful amplification of the DNA fragment in a pool of embryos therefore indicates that at least one embryo of the pool was an offspring of a sexual reproduction event. Using the frequency of negative pools (*i.e.,* pools of 10 offspring that were produced without contribution of the male), we calculated the proportion of embryos produced by sex in each of the crosses. All amplifications also contained positive controls (manually assembled pools containing nine asexually produced embryos and one sexual embro produced by the CP MP clone) as well as both parent clones. To rule out contamination of the cultures, 200 females (about 50 % of all females present) of one of the crosses were screened at the end of the experiment (see **Appendix H.**).

Model design

We designed likelihood models in order to estimate (1) the proportion of the different categories of F1s (*sexual*, *mixed*, *sterile*), (2) the different proportion of sexually or asexually produced F2s for

mixed F1s, and (3) the LOH pattern of the asexually produced F2s (**Table S5**). These models were written and fitted with Mathematica 11.1 (Wolfram Research 2017). These models used three sources of information that are combined in a single likelihood function jointly estimating all parameters.

First, they use the LOH data in the offspring produced by each F1. LOH events were modelled using binomial error (number of LOH events per F2 observed among all the heterozygous loci present in the F1 parent). Here, we did not incorporate a specific effect of the different loci. For sexually produced F2s, the expected LOH rate was set to 0.5 (corresponding to within-clone mating). For asexual F2s, we considered different types of variation for the LOH rates. We compared models where the LOH rate was (1) fixed for all F1s, (2) variable among F1s, but where each F1 produces all its F2 asexual offspring with the same LOH rate, (3) variable among asexual F2s, but where the LOH rates among those F2s are drawn in the same distribution for all F1s. In models considering variable LOH rates, we described this variation with a Beta distribution. This part of the likelihood therefore provided information on the proportion of sexual and mixed F1s, as well as on the distribution of LOH rates in asexually produced F2s.

Second, the likelihood models used the ephippial fill rate data observed for NMP F1s. When an isolated NMP clone produces non-empty ephippia, it indicates that it can reproduce asexually (since no males are present). However, if it only produces empty ephippia, it indicates that it is either sterile, or sexual. However, in the last case, we may also miss the non-empty ephippia if only few of them are observed. Hence, the number of non-empty ephippia can be modelled using binomial error to provide information on the proportion of *sexual*, *sterile* and *mixed* F1s.

Third, the likelihood model used the number of sterile MP and NMP clones observed in an experiment where males are present (*i.e.,* added for NMP clones). In this experiment, a sterile clone is defined as a clone producing only empty ephippia in presence of males. Hence, the number of sterile vs. fertile F1 clones can be modelled with a binomial error to provide information on the proportion of sterile clones.

In this full likelihood models, we first evaluated whether the different parameters (proportion of *sexuals,* parameters describing the distribution of LOH rates in asexually produced F2s) differed between MP and NMP. We then compared the different type of variation of LOH rates (variable among F1s or among F2s, see above), and a set of models used to evaluate specific hypotheses (*e.g.,* assuming that all MP were sexuals). We considered many simplifications of the full model, constraining or not the different parameters (see **Table S5**). Models were compared using Akaike's information criterion and we calculated the support limits of each estimated parameters of the best models (**Table S4**).

Fitness estimation

We also investigated the fitness of the newly generated F1s from CP x OP crosses compared to CP x CP crosses and the fitness of F1s able to reproduce asexually compared to the obligate asexuals from natural clones. We used the hatching rate and the fill rate as proxy for reproductive fitness. Indeed, they give a direct estimate of the reproductive component for next year of the fitness, which is crucial in the life of a clonal lineage in *Daphnia sp.* (Pietrzak and Slusarczyk 2006).

Fill rates

Fill rate is equivalent to fertilization rate when ephippia are sexually produced (*i.e.,* the mother clone is CP). The number of manually added males was not correlated with the fill rate for all CP x CP and CP x OP crosses investigated (Spearman correlation test, *p*-value =0.09 altogether or *p*-values=0.79 and 0.35 for each CP x CP or CP x OP crosses respectively). We also compared the fill rate between F1 offspring and natural clones.

Hatching rate

The hatching rate success of the F1 offspring from CP x CP crosses vs. CP x OP crosses was evaluated using four CP x CP crosses, five CP x OP crosses. We did not investigate the hatching rates of offspring from within-clone mating in CP MP as we did not want to have confounding effect with inbreeding depression. Hatching rate of F2 offspring was evaluated using 14 F1 offspring from the main CP x OP cross investigated and compared with hatching rates of F1s from CP x OP crosses and offspring of six natural OP. For each clone, the number of ephippia to hatch was estimated by homogenizing the ephippia in their total volume and counting the number in three independent subsamples. Hatching rate was then calculated as the fraction of the number of hatched embryos over the number of expected embryos to hatch (**Table S8**).

Results

Contagious asexuality: a difficult route to generate new F1 clones

We set up a total of 13 CP x OP crosses. To facilitate the production of a large number of F1 embryos by mass-mating, all CP clones used as females in the crosses were so-called non-male producers (NMP), that is, clones that do not produce males (Galimov *et al.* 2011; Reisser *et al.* 2017; Ye *et al.* 2019) contrary to male-producers (MP) clones. Mass-mating was achieved by growing high-density cultures of clonal lineages of these females (generated by asexual reproduction via liveborn offspring, hereafter "clone"), and by adding males from OP clonal lineages to these cultures. In *Daphnia*, high density stimulates the production of diapause offspring. Because NMP clones produce no males, all produced diapause offspring were necessarily outcrossed offspring between CP females and the added OP males.

Despite the high numbers of ephippia (diapause capsules) that were produced, most crosses yielded zero or less than 10 offspring that hatched, survived to adulthood, and successfully established clonal F1 clones. Only two crosses were moderately successful, each yielding ~40 F1 clones (**Table S1**). The low success of these crosses was explained by a combination of a high number of empty ephippia and low hatching success of the embryos after diapause. At high density, *Daphnia* produce ephippia even if no diapause embryo is subsequently deposited in the ephippium. In CP clones, which produce ephippia sexually, an empty ephippia may be the result of male limitation (*e.g.,* due to an insufficient number of males added in our experiment), low fertilization success (*e.g.,* due to low sperm quality) or due to early embryo mortality (causing embryos to decay). To quantify success in depositing viable embryos into ephippia, we used the "fill rate", the average number of embryos per ephippium divided by two (each ephippium can contain up to two embryos).

The average fill rate in CP x OP crosses was only 4.7 % (**Figure 1B**). This is lower than in CP x CP control crosses (20.6 %), though not significantly so (Mann-Whitney test, *p*-value = 0.06, **Figure 1B**). A part of this low fill rate might indeed be explained by male limitation, though the number of males added to the CP x OP crosses was not smaller than those added to the CP x CP control crosses (*t*-test, *p*-value = 0.67, **Table S1**), suggesting that the low fill rate in CP x OP crosses is in part also explained by low fertilization success or early embryo mortality. When exposed to hatching stimuli, the remaining viable embryos had an average hatching rate (number of hatchlings per viable embryo) of only 0.5 %. Hatching rate in CP x CP control crosses was somewhat higher (2.9 %), though not significantly so (Mann-Whitney test *p*-value = 0.10 **Figure 1A**). The low hatching rate in both CP x OP and control crosses suggests that, in addition to possible embryo mortality or developmental failure, the chosen hatching stimuli were ideal for the studied clones. In either case, the combined effect of low fill rates and low hatching rates in CP x OP crosses explains why so few F1s could be studied and suggests low overall success of the CP x OP crosses: Indeed, the success of ephippia production (hatching rate *

fill rate) was nearly significantly lower in CP x OP crosses compared to CP x CP control crosses (Mann-Whitney test *p*-value = 0.03).

A. **B.**

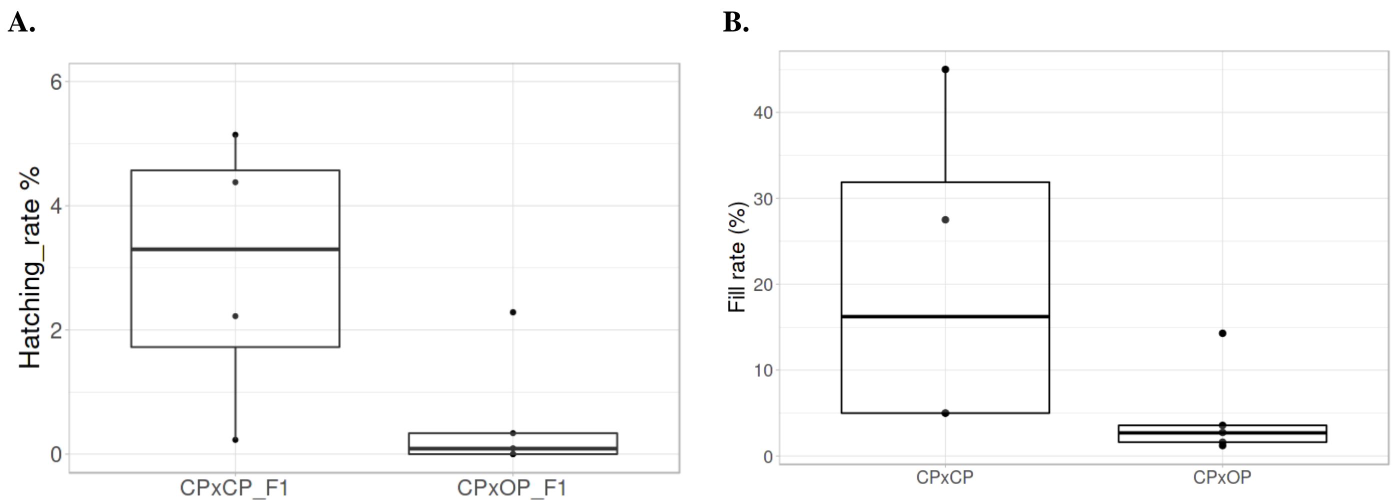

Figure 1: Hatching rates and fill rates in for the four CP x CP crosses and five CP x OP crosses. The thick horizontal line represents the median of each category, the box the 25th and 75th percentiles, and the error bars are the 95 % confidence interval. **A.** Hatching rate of the F1s produced by four inter-clonal lineages CP x CP crosses (CP x CP_F1) and five CP x OP crosses (CP x OP_F1). **B.** Ephippial fill rate in four CP x CP crosses and five CP x OP crosses.

Evidence for unfaithful transmission of asexuality

Surprising reproductive phenotypes of the F1 offspring

To assess the reproductive modes of the F1 offspring, and to test for successful contagious asexuality, we grew high-density clonal cultures of F1 offspring to stimulate ephippia production. In theory, asexual reproduction of F1 offspring (and thus successful contagious asexuality) could be demonstrated by testing for successful reproduction in the absence of males (*e.g.,* by verifying that ephippia produced in the absence of males contain diapause embryos, (Innes and Hebert 1988; Xu *et al.* 2015). In practice, however, it is difficult to assure that not a single male was present in high-density cultures (males are reliably separable from females only under a stereo-microscope). We therefore again took advantage of the NMP clones, in which no males are present in clonal cultures. More specifically, we first evaluated the reproductive mode of only those F1s that were NMP. Indeed, a genetic marker and a phenotypic test showed that 64 of the 107 F1s clones were NMP (NMP is a Mendelian trait, transmitted to 50 % of the offspring, Reisser *et al.* 2017). Of these 64 NMP clones, 20 produced at least one non-empty ephippium, demonstrating that they were capable of asexual reproduction and thus that contagious asexuality indeed occurred in our crosses. However, closer inspection of the F1 phenotypes revealed several surprises. First, the proportion of F1s that were capable of asexual reproduction differed among crosses (*p*-value = 0.019, **Table S2**): In one of the two crosses that produced more than five NMP offspring, 13 out of 28 F1s (46 %) were able to reproduce asexually, in the other one, only four out of 28 (14 %, **Table S2**). This suggests that, contrary to the current model of contagious asexuality (Paland *et al.* 2005), either the genetic basis of asexuality differs among clones or survival of offspring capable of asexual reproduction relative to those that are exclusively sexuals, differs among clones. Second, the addition of males to some of the cultures confirmed sexual reproduction: In clones that produced ephippia with zero embryos, the ephippial fill rate increased as expected (from zero to an average of 68%) after the addition of males. The few clones that still did not produce any non-empty ephippia are considered sterile for the production of diapause offspring). We could thus demonstrate the existence of potential sexual reproduction and sterility among the F1 offspring. Unexpectedly, however, in several of the clones that were capable of asexual reproduction, the ephippial fill rate equally increased after the

addition of males (from 1.67 % to 46.5 %), suggesting that at least some F1 clones were capable of both sexual and asexual production of diapause offspring. To confirm this “mixed” reproduction, we carried out paternity tests on the diapause embryos and were indeed able to find paternal alleles (*i.e.*, alleles specific to the clone of the added males) in eight out of 14 tested embryos (**Table S2 and S3**). Mixed production of diapause embryos has never been observed in *Daphnia pulex* before and represents a novel reproductive phenotype in the F1 offspring.

As a control for the new mixed reproductive phenotype found in F1 offspring, we investigated whether females from natural OP clones are also capable of sexual reproduction, contrary to what is currently thought. Investigating diapause embryos produced by cultures of two OP clones to which males of a CP clone were added, we indeed found evidence for cryptic sexual reproduction, as evidenced by was assessed by male-specific alleles present in a low proportion (see **Appendix H**. for details, 0.52 %, 95% C.I.: 0.036 % to 7.05 %) of these diapause embryos. Several lines of evidence strongly suggest that the male-specific amplificated bands in embryos are indeed due to rare sexual reproduction rather than contamination: 1) the double-phenotyping (sexing) prior to the addition of males, 2) finding rare sexual events in two independent crosses, 3) a contamination test based on genotyping of a large number of females at the end of the experiment, finding no contaminant CP female in these cultures. Our results thus demonstrate that females from natural OP clones are capable of rare sexual reproduction. Nevertheless, sexual reproduction in OP clones is much rarer than in the “mixed” F1s of the CP x OP crosses (see below), thus still suggesting that these F1s presented a phenotype that was not present in the parent.

A complementary way of investigating the reproductive mode of F1s from the CP x OP crosses is by genotyping the diapause embryos produced by the MP F1s. Indeed, for MP clones, non-empty ephippia can be due to either sexual or asexual reproduction. Empty ephippia are indicative of sterility, which we indeed also found in one MP F1. Mating within an MP clone is equivalent to self-fertilization as males and females are clones (liveborn offspring are produced clonally). We therefore expect a loss of heterozygosity (LOH) rate of 0.5 in this case. The majority of the offspring of the MP F1s indeed showed LOH rates around 0.5, as expected under sexual reproduction (**Figure 2A**). This is consistent with the finding of a high prevalence of sexual reproduction in the presence of males in NMP F1s (including both F1s incapable of asexual reproduction and “mixed” ones with a high rate of sex). An LOH rate of zero is expected under clonality, and this was observed in the offspring of one MP F1 (**Figure 2A**). Similarly, the few MP F1 clones whose offspring had LOH rates slightly below 0.5 are likely explained by mixed reproduction. However, the offspring of a few F1s had higher LOH rates than expected under sexual reproduction, suggesting other non-clonal modes of asexuality (Archetti 2010; Engelstädter 2017), which were so far unknown in *D. pulex*. To investigate this further, we analyzed the genotypes of asexually produced diapause offspring of NMP F1s in one of the crosses. These analyses confirmed that different modes of asexuality occur in different F1 clones. Of the five F1 clones that were tested in detail, only one produced identical (clonal, zero LOH) offspring. The offspring of the other four F1 clones showed varying, non-zero rates of LOH, including very high rates of LOH in offspring of three of these clones (**Figure 2B**). In contrast, no LOH was found in the asexually produced diapause offspring of four natural OP clones (**Figure 2B**). This demonstrates that the asexual phenotype is transmitted non-faithfully during contagious asexuality. Rather, contagious asexuality led to a variety of asexual modes in the F1s, including modes with non-zero recombination (needed to explain intermediate rates of LOH) and possibly a variety of cytological mechanisms.

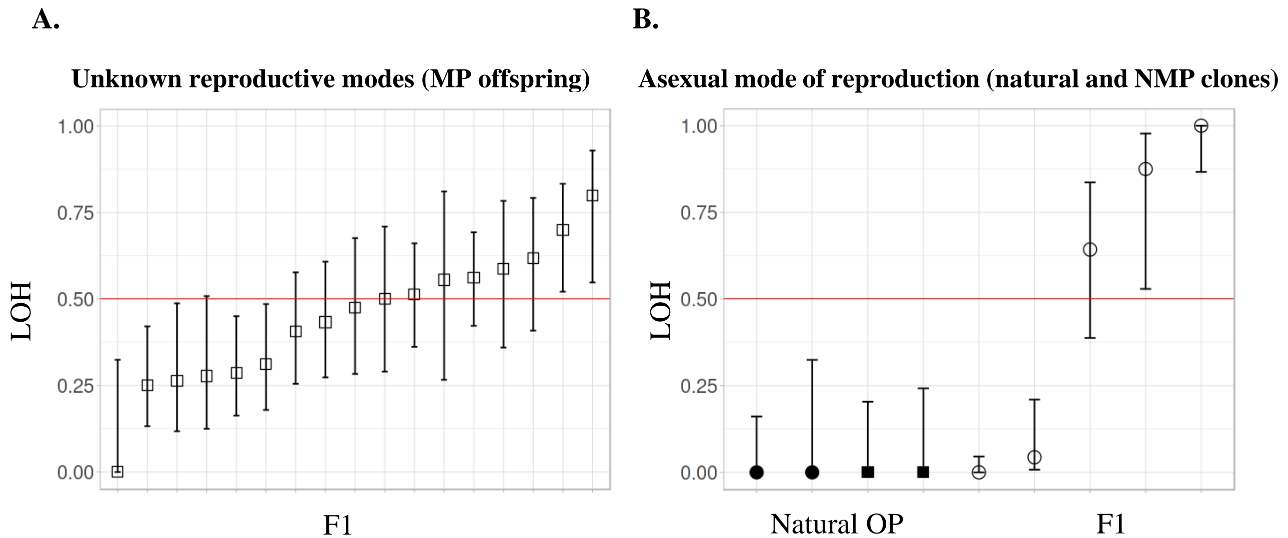

Figure 2: Loss of heterozygosity (LOH) rate in ephippial F2 embryos. Natural clones reproduce through the ephippial production either clonally (LOH=0, black filled points) or sexually (LOH = 0.5 expected under sexually produced embryos within a clone, red line). Circles (for non-male producing clones, "NMP") and squares (for male-producing clones, "MP") represent the mean LOH over all F2 embryos genotyped and across all microsatellite loci. Error bars represent the confidence interval of the binomial error of the LOH proportion (F2 embryos and loci are considered independent). **A.** LOH of the F2 embryos produced by unknown mode of reproduction by 17 MP F1s (non-filled). **B.** LOH of the asexually produced embryos from four natural OP clones (black filled) and asexually produced F2s from five NMP F1s (non-filled).

Global estimation of different reproductive modes proportions in F1 offspring

To obtain a quantitative estimate of the proportions of asexually produced F2s, mixed, and sexual F1s, as well as to test for a possible difference between MP vs. NMP offspring, we used a likelihood model, based on the data from a single cross (**Table S2**). This allowed us to combine the information from several technically difficult to obtain and therefore somewhat disparate data sets, but all from a single CP x OP cross. Indeed, F2 offspring (diapause embryos) for each F1 clone were difficult to obtain because of low fill rates which meant that we had to produce a lot of ephippia for each clone. In addition to that, the extraction of DNA was difficult to perform on embryos, and we lost many embryos this way. The models used data on fill rates in presence and absence of males sets (in NMP F1s and, for the estimation of the sterility rate also in MP F1s) and data on LOH in F2s (asexual offspring of NMP F1s and all offspring of MP F1s), while accounting for differences in sample size (*e.g.,* different numbers of ephippia checked for fill rates, different numbers of F2s genotyped, different number of variable loci). Knowing that mixed reproduction can occur, it was important to analyze our data at the F2 level which was not done before. We also jointly analyzed LOH rates during asexual reproduction (see materials and methods).

According to our best model (**Figure 3**), 10.2 % of the F1 offspring are sterile (support limits: 1.61 - 28.5 %), 32.2 % produce ephippia exclusively by sexual reproduction (support limits: 7.58 - 51.4 %), and the remaining 57.6 % are mixed (support limits: 38.3 – 82.7 %; **Figure 3A**). Note that exclusive asexuality may rarely occur (*e.g.,* only one NMP F1 whose fill rate did not increase when males were added). However, given the small number of F1 clones suggesting this possibility, there was too little data specifically to test this possibility. Our parameter for the proportion of sexual or asexual F1s were allowed to vary freely and our statistical approach could have detected whether exclusive asexuality was common. Furthermore, each mixed F1 produces an estimated 64.6 % (support limits: 46.7 - 79.5 %) of the F2 progeny by sexual reproduction (**Figure 3A**) and the models in which this proportion is constant

are better supported than models in which this proportion varies among mixed F1s (**Table S4**). Our models also confirmed that LOH rates in F2s produced by MP F1s cannot be explained by sexual reproduction only (**Table S5**). Our models do not support any difference in the distribution of LOH rates of F2s asexually produced between MP F1s and NMP F1s (**Table S5**). However, the model comparison could not conclusively discriminate whether the proportion of exclusively sexual F1 differed between MP and NMP, because this proportion was very poorly estimated in MP (**Tables S4 and S5**). There is thus no evidence for any difference in estimated parameters between MP and NMP and we therefore consider the simplest model (with no difference) as the best one.

Regarding the LOH rate of asexually produced F2s (**Figure 2B**, **Table S6**), our model comparison supports variable LOH rates among F1s, but not among F2 asexual offspring of a given F1 (the model comparison rejects that LOH rates are variable among asexual F2s of a given F1, but drawn in the same distribution for all F1s, **Table S5**). The average LOH rate across all F1s was estimated at 37 % (support limits: 15.8 – 61.2 %), but with a strongly bimodal distribution (**Figure 3B**). This analysis thus further supports the finding of high variation in LOH rates indicative of both recombination and different asexual modes of reproduction to produce the F2s, depending on the F1s. According to the models, most F2s are produced either in a manner close to the clonality (*i.e.,* with zero LOH), and thus close to the mode of reproduction of natural OP, or by an asexual mode that entails almost complete loss of heterozygosity (such as suppression of the second meiotic division without recombination or gamete doubling, (Bell 1982; Archetti 2010; Engelstädter 2017). Yet, intermediate rates of LOH, which are indicative of recombination (Archetti 2004) also occur. It should also be remembered that the models were fitted to the data from one cross only and that the fill rate of ephippia produced by NMP F1s suggested that at least the proportion of clones capable of asexual reproduction varies a mong crosses (see above).

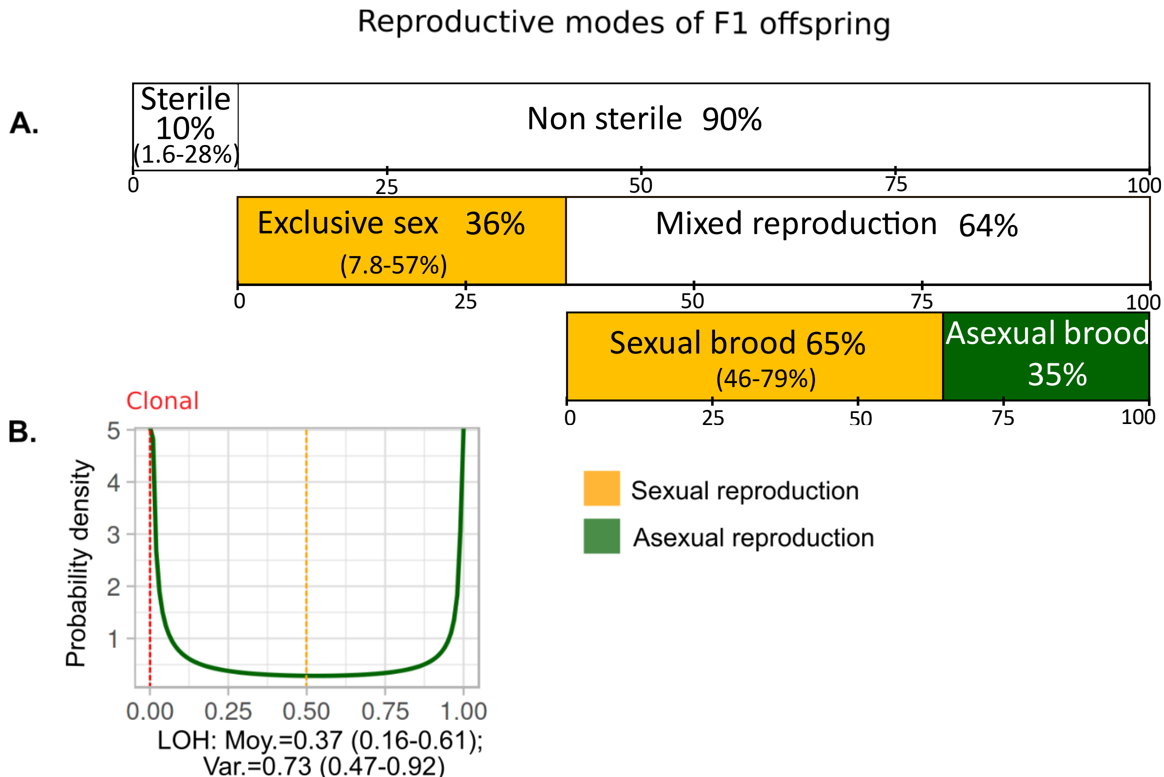

Figure 3: Reproductive modes of F1 offspring when producing ephippia, according to the best model, based on the data from one CP x OP cross. **A.** Frequency of different reproductive modes among F1 offspring. F1 offspring are either "sterile" or "non-sterile". Non-sterile F1s may reproduce either

through "exclusive sexuality" when producing ephippia, like natural CP clones, or by "mixed reproduction", where each F1 is capable of reproducing diapause offspring both sexually and asexually. The proportions of the different classes and their support limits are reported in the figure. **B.** LOH distribution of all F2 offspring produced by asexual reproduction; the red line is indicative of clonality (LOH=0), non-zero LOH is indicative of non-clonality and the yellow line is the expected LOH under sexual reproduction (LOH=0.5). Support limits of the model estimates are reported in brackets.

F1 offspring have low fitness, especially asexual ones

Hatching rates and fill rates (see above) were used as fitness proxies (both are not correlated, Spearman rank correlation, *p*-value = 0.34). Because of sterility, low fill rates and difficulties in ephippia production, only 14 F1s from the main CP x OP cross could be used to estimate hatching rate of their F2s. The mean hatching rate of the F2 offspring ranged from 0 to 18 % with an average of 2.15 % (SD = 2.29; **Figure 4A**). Hatching rate was substantial (around 18 % and 9 %, respectively) only in two batches of offspring: the first batch was produced asexually by the only NMP F1 that reproduced in a manner similar to the natural OP (zero LOH), the second batch was produced by a putatively sexual NMP F1 after the addition of males. All the other F2s (produced asexually by another NMP F1 or by unknown reproduction mode by 11 MP F1 clones) had hatching rates of close to zero (average 0.2 %). Overall, both the hatching rate of the F1 ephippia produced by the CP x OP crosses and the hatching rate of the F2 ephippia were significantly lower than the hatching rate of offspring produced by the six natural OP clones (Mann-Whitney tests, *p*-values on **Figure 4A**).

Ephippia production by the F1s of the different crosses resulted mostly, though not uniformly in low fill rates (**Figure 4B**). There was a clear difference between ephippia produced by the 20 NMP F1 clones capable of asexual reproduction, which all had low fill rates (mean = 3.9 %), and ephippia produced by 17 MP clones. The latter had strongly variable fill rates, ranging from 0.1 to 83 % (mean = 45 %; **Figure 4B**). Despite this variation, the difference is highly significant (Mann-Whitney test, *p*-value=8.10^{-6}). Note that NMP F1s were able to produce diapause embryos only asexually, whereas MP F1s were able to produce them asexually or sexually, as long as males were present. Because this was not systematically controlled for, it is possible that some of the low fill rates of the MP F1s were explained by absence of males from these cultures. Moreover, fill rate of the NMP F1s able to reproduce asexually from the CP x OP cross used in the likelihood models, was estimated at 4.2 % (support limits: 3.5 – 5 %, **Table S4**). Ephippia produced by NMP F1s had also significantly (Mann-Whitney test, *p*-value = 0.0004) lower fill rates than ephippia produced by natural OP clones (mean = 34.3 %, **Figure 4B**). The highest fill rates were achieved by ephippia produced in three natural CP clones (mean 70 %). This is significantly higher than the fill rates of ephippia produced by natural OP clones (Mann-Whitney test, *p*-value = 0.004). Overall, these results indicate that offspring of CP x OP crosses have low fitness in F1s and in F2s, particularly regarding asexual reproduction. Contagious asexuality in *D. pulex* thus seems to lead to asexuals with much lower fitness than asexuals found in nature.

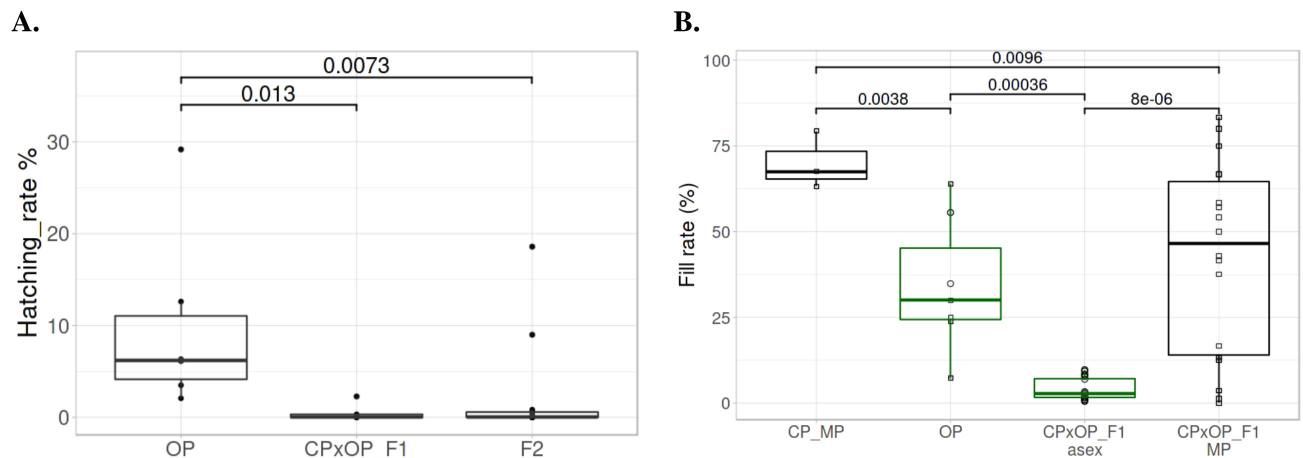

Figure 4: Hatching rate and fill rates of the natural clones and the F1s and F2s generated by crosses. The thick horizontal line represents the median of each category, the box the 25th and 75th percentiles, and the error bars are the 95 % confidence interval. *P*-values for significant Mann-Whitney tests between two categories are given (except between CP_MP and CPxOP_F1_MP where a *t*-test was used). **A.** Hatching rate of six asexual natural clones (OP) and F2s from 14 CPxOP_F1. We also added the five CPxOP_F1 data from Figure 1 for comparisons. **B.** Ephippial fill rate in three sexual male-producing natural clones (CP_MP), seven OP natural clones, F1s from CP x OP cross that reproduce asexually (20 CPxOP_F1_asex) and the other that reproduce either sexually or by mixed reproduction with non-zero fill rate expected (17 CPxOP_F1_MP). Circles represent the non-male producer (NMP) and squares the male-producer (MP) clones. Green highlights the categories where ephippia are asexually produced.

Discussion

Inheritance patterns of asexuality in F1s

In this study, we evaluated the inheritance pattern of parthenogenesis of *Daphnia pulex* using contagious asexuality. We found variable proportions (14 % and 46 %) of NMP F1s able to reproduce asexually in two CP x OP crosses. In addition, the majority of F1s able to reproduce asexually are not obligate asexual, but rather mixed F1s. We have revealed the existence of a mixed reproduction in F1s where individuals are both able to reproduce asexually and sexually through the ephippial stage. This result is highly surprising as such phenotype was never observed in *Daphnia* before. The possibility of mixed reproduction, makes the usual simple phenotypic reproductive test for OP with empty vs. non-empty ephippia in absence of males more difficult to interpret. In the light of our results, the different inheritance patterns of OP that was previously found in *Daphnia pulex,* make sense, but with a different interpretation. A first study (Innes and Hebert 1988) carried out CP x OP crosses to assess the inheritance pattern of OP. Using the fill rate methodology in absence of males, they phenotyped four F1s as asexuals and six as sexuals suggesting that OP should be transmitted as a single dominant locus. Later, associations studies found that at least four loci on four distinct chromosomes may be driving the OP phenotype (Lynch *et al.* 2008; Xu *et al.* 2015) questioning the single dominant inheritance pattern. In the 2000’s, Lynch *et al.* 2008 and Xu *et al.* 2015 performed new CP x OP crosses using different combinations of parental clones and, using the same methodology, only two out of 31 F1s and three out of the seven F1s tested, respectively were assessed as OP. All these conclusions might not be correct if the OP phenotype is actually not faithfully transmitted, as in our study (situation that cannot be revealed using filling rates in absence of males). All these proportions cannot therefore reveal the genetic basis (number and dominance of genes) of the OP phenotype. In view of our results, as a contagious event

never transmit all the genes causal for the OP phenotype, we show that the OP phenotype is not determined by a single gene, in agreement with association studies that suggest a polygenic basis.

Besides the possible variable inheritance patterns of asexuality, the majority of F1s able to reproduce asexually were non-clonal, with likely different asexual modes of reproduction and with recombination as showed with variable loss of heterozygosity (LOH) rates in asexually-produced F2s. Recombination in modified meiosis can drastically reduce fitness when it reveals deleterious mutations ("Loss of complementation hypothesis", Archetti 2004). Several genes, controlling for example the frequency of sexual versus asexual events or recombination rates, may together be required to produce the "asexual phenotype". In the context of contagion, in order for the phenotype of the OP lineage to be transmitted, all these genes would have to be directly transmitted (Hojsgaard and Schartl 2021). Variable LOH rates in asexually produced F2s also implies that genetic data are needed in order to have a realistic idea of the proportion of F1s able to reproduce clonally as the natural parthenogens. Again, this result is also very surprising because no non-clonal mode of asexuality has been identified in natural OP. The surprising variability in reproductive modes we found is present in newly-formed asexuals, but may not persist long in natural populations. This is also consistent by the fact that we find traces of a mixed mode of reproduction in OP natural clones, since they can still (rarely) reproduce sexually.

Fitness of F1 offspring

Our results suggest that OP males might have difficulties to fertilize CP females which could in part explain the low number of F1 offspring produced. In addition, these F1s had very low fill rates and when producing F2, the F2s had very low hatching rates. Altogether, this indicates that F1 offspring from a contagious event have low fitness compared to natural clones, especially the "asexual" ones. Our findings are consistent with the fact that not all rare OP males are able to produce haploid sperm (Xu *et al.* 2015), suggesting a great inefficiency of transmission of asexual genes into new clonal lineages through contagious asexuality. Our findings are also comparable to the results of Innes and Hebert (1988) and Xu *et al.* (2015) who performed the same kind of CP-OP crosses in *Daphnia pulex* and found that the majority of newly generated F1s either had difficulties in producing ephippia, or only produces empty ones even in the presence of males. As a result, in the study of Xu *et al.* (2015), only seven out of 122 F1s have been investigated with respect to their reproductive mode. Incompatibility between parental clones may explain a part of variability of F1 hatching rates. However, our results show a strong tendency for fitness proxies to be much lower for CP x OP crosses than for CP x CP crosses although both are inter-clonal lineages. This observation strongly suggests that the problems encountered in F1s are not caused by incompatibilities between clones.

Rare sexual events in OP

Our study showed that rare sexuality is found in natural OP clones of *Daphnia pulex*. First, this result echoes the new mixed phenotype found in the F1s. However, it should be noted that the proportion of sexual events is orders of magnitude lower than the estimated proportions of sexual reproduction in the mixed F1s studied. Thus, the rare sexual events may be a remnant of mixed reproduction in very derived natural OP. If LOH is found in OP clones, this could now be possibly explained by classical inbreeding from rare sex between males and females from the same OP clone (within-clone mating). Second, it is possible that rare sexual events of OP females may result in contagious asexuality: asexuality may not only be transmitted through rare OP males, but also through rare sex in "OP" females (though in this case, they would not really be "obligate" parthenogens). The observation is remarkable because it suggests that some "OP" females are still able to successfully undergo regular meiosis during oogenesis. In other asexual animal taxa as for instance, in *Artemia parthenogenetica*, rare events of sex realized by asexual females resulted in the formation of new asexuals (Boyer *et al.* 2021). In the obligate parthenogen *Cacopsylla myrtilli*, the presence of rare diploid females is also thought to be associated

with contagious asexuality (Nokkala *et al.* 2015). Thus, cryptic sexual events, if they occur in many parthenogens, call into question the prevalence of purely asexual reproduction in animals. Indeed, detecting rare sexual events in asexual females is difficult and it is possible that contagious asexuality is more frequent in other taxa, via the females, even when males are not produced anymore. Rare events of sex can drastically change the evolutionary consequences of asexuality allowing the advantages of sex while minimizing its costs (Engelstädter 2017). The absence of true OP, especially during sex-asex transitions, if common, would challenge the way the "paradox of sex" is usually presented and analyzed.

Contagious asexuality: a maintenance process of asexual taxa?

A strong assumption underlying the concept of contagious asexuality is that it should transmit the asexual phenotype and thus create new asexual clones without the need to evolve asexuality from scratch after each cross (Engelstädter *et al.* 2011). Thus, contagious asexuality may constantly re-create new asexual lineages, which may compensate for their higher rate of extinction. After each contagion, new asexual clones carry half of the genome of the sexual parent, which can "rejuvenate" the genome and decrease the genetic load. If contagion is frequent, it can create a diversity of asexual clones, upon which selection can act efficiently (Neiman and Linksvayer 2006; Janko *et al.* 2008). With many attempts, some asexuals may even turn out to be better competitors than sexuals (Vrijenhoek and Parker 2009). Overall, if contagious asexuality is frequent, it allows for high clonal turnover, and a dynamic equilibrium between extinction and recovery of clones (Janko *et al.* 2008) explaining their long-term persistence. As a result, contagious asexuality is thought to be essential for explaining the maintenance and distribution of asexual reproduction (Lynch 1984; Burt 2000; Janko *et al.* 2008; Janko 2014; Neiman *et al.* 2014). The benefit associated with contagious asexuality should also explain the maintenance of rare males in many asexual species (Joshi and Moody 1995, 1998).

In *D. pulex*, the contagious nature of asexuality is thought to produce new clonal clones explaining their multiple and recent origins (Innes and Hebert 1988; Crease *et al.* 1989; Hebert *et al.* 1989; Paland *et al.* 2005). However, assuming that OP is polygenic, contagious asexuality may actually not work so easily, especially since the genes and alleles required for the OP phenotype may not be all transmitted upon contagion. They are likely to recombine and segregate during the sexual event between the CP female and the OP male. Nevertheless, even under a four-locus model, contagious asexuality is still view as a powerful mechanism to rapidly displace CP clones (Ye *et al.* 2021).

In *Daphnia pulex*, we showed that contagious asexuality did not actually occur as hypothesized in the literature (Paland and Lynch 2006; Decaestecker *et al.* 2009). Newly-formed asexual after contagion do not share the characteristics of their asexual parent, and do not reproduce like the clonal lineages observed *in natura.* Asexual genes have been transmitted, but the actual OP phenotype was not faithfully transmitted. Instead, a huge variability of reproductive modes is revealed with the maximum variability we could expect from the known asexual modes of reproduction (Archetti 2010).This idea is consistent with the observation that empirical studies have difficulties in obtaining a first-generation clonal hybrids that resemble the parents in fitness and asexuality phenotype (Vrijenhoek 1989). For instance, in *Artemia*, Boyer *et al.* (2021), found that F1s between sexual and asexual species, and further backcrosses have higher recombination rate than the asexual parental species. In the same species, while the ability to reproduce asexually seems to be a dominant trait, the results from Boyer *et al.* (2021) suggest that other genes are involved in the global "asexual phenotype".

Conclusion

In conclusion, our study does not support contagious asexuality as a faithful transmission of asexual phenotype and fitness. Our results suggest that the "obligate parthenogenesis" phenotype may be more complex than previously thought, involving several genes and probably secondary modifications within the asexual lineages. Indeed, non-clonal asexual modes of reproduction are not

reported in natural clones of *Daphnia pulex*, suggesting that secondary modifications or very strong direct selection may act to only select a particular type of asexual reproduction. Contagious asexuality in *D. pulex* thus needs "evolutionary incubation" and therefore successful contagion events may be much rarer than previously thought. Asexual phenotype found in nature may need to be shaped, refined and improved by selection each time a new asexual arises. This process may often fail, which may explain the rarity of new transitions to asexuality (Engelstädter 2008; Hojsgaard and Schartl 2021). Our findings give empirical support for this theory and thus call for a change in the way contagious asexuality is conceptualized and modelled.

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Author contributions

CH, TL, CM conceived the study. CM, CC and HC acquired the data. CM, TL conducted the analysis

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Supplementary data

The Files are available here.

Table S1 Crosses production.
Crosses production.
Names and identification of mother or father line used for the crosses. For each cross production, we give the number of males from the father-line that were manually added and the type of crosses (asex-sex cross for the rare sex investigation "OP x CP", sex-asex cross to mimic contagious asexuality "CP x OP" and control crosses sex-sex "CP x CP"). Number of hatched embryos from each cross as well as the number of F1 that constituted a new clonal lineage used in the study are reported.

Cross names	Mother clone	Father clone	Nb added males	Mother clone x Father clone	Nb hatched embryos	Nb F1 used in the study
DIS-47xTEX-1	DIS-47	TEX-1	1268	OPxCP	NA	NA
DIS-85xKAP-65	DIS-85	KAP-65	636	OPxCP	NA	NA
KAP-87xMT-107	KAP-87	MT-107	119	CPxOP	46	42
KAP-87xSED-2	KAP-87	SED-2	170	CPxOP	1	0
KAP-87xTEX-1	KAP-87	TEX-1	177	CPxCP	NA	NA
KAP-87xTRO-3	KAP-87	TRO-3	102	CPxOP	2	2
LPB-87xKAP-65	LPB-87	KAP-65	146	CPxCP	NA	NA
LPB-87xSED-2	LPB-87	SED-2	201	CPxOP	8	8
LPB-87xSTM-2	LPB-87	STM-2	54	CPxOP	0	0
LPB-87xTRO-3	LPB-87	TRO-3	113	CPxOP	0	0
NFL-92xKAP-65	NFL-92	KAP-65	131	CPxCP	NA	NA
NFL-92xMT-107	NFL-92	MT-107	80	CPxOP	1	1
NFL-92xSED-2	NFL-92	SED-2	96	CPxOP	0	0
NFL-92xTRO-3	NFL-92	TRO-3	68	CPxOP	0	0
TEX-114xMT-107	TEX-114	MT-107	257	CPxOP	2	0
TEX-114xSED-2	TEX-114	SED-2	178	CPxOP	6	4
TEX-114xSTM-2	TEX-114	STM-2	299	CPxOP	70	50
TEX-114xTEX-1	TEX-114	TEX-1	345	CPxCP	NA	NA
TEX-114xTRO-3	TEX-114	TRO-3	95	CPxOP	0	0

Table S2 F1 offspring.
F1 offspring investigated.
For each cross, F1 offspring were names by numbers (ID) were identified as male-producing ("MP") or non-male producing ("NMP"). Some F1 offspring were investigated according to their fill rate (number of embryos found by opening ephippia), some of them have been exposed to males manually added in order to obtain a fill rate in presence of males ("Fill rate with males") or the presence of male-specific allele in the offspring ("Specific male allele in F2"). The two last columns indicate whether each F1 is included in the fill rate data or LOH data used in the likelihood models. Available here.

Table S3 Paternity tests.

Paternity tests on two F1 NMP and one F1 MP of the main cross.

Paternity tests were performed using males from one natural clonal lineage and different F1 clones (F1_cross_ ID). Each line corresponds to an individual (Male, MP or NMP mother or offspring) for which we have the genotypes of the five microsatellites investigated ("*Dp256*"; "*Dp339*"; "*Dp496*"; "*Dp502*"; "*Dpu7*"). We highlighted in blue genotypes for which we have a male-specific allele in the offspring.

Clonal lineage	Individual	Dp256		Dp339		Dp496		Dp502		Dpu7	
TEX-1	MP male	230	230	161	163	200	208	135	141	114	114
F1_TEX-114xSTM-2_30	MP mother	232	232	161	163	200	205	NA	NA	110	114
F1_TEX-114xSTM-2_30	Offspring	230	232	161	161	200	199	135	144	114	114
F1_TEX-114xSTM-2_33	NMP mother	232	232	161	163	205	208	NA	NA	110	114
F1_TEX-114xSTM-2_33	Offspring	232	232	161	163	205	208	135	144	110	114
F1_TEX-114xSTM-2_33	Offspring	232	232	161	163	205	208	135	144	110	114
F1_TEX-114xSTM-2_33	Offspring	232	232	161	163	205	208	135	144	110	110
F1_TEX-114xSTM-2_33	Offspring	232	232	161	163	205	208	135	144	110	114
F1_TEX-114xSTM-2_33	Offspring	232	232	NA	NA	205	208	135	144	110	114
F1_TEX-114xSTM-2_33	Offspring	232	232	NA	NA	205	208	135	144	110	114
F1_TEX-114xSTM-2_39	NMP mother	230	232	161	163	208	213	135	138	110	114
F1_TEX-114xSTM-2_39	Offspring	230	232	161	163	208	213	135	135	110	114
F1_TEX-114xSTM-2_39	Offspring	230	230	161	163	208	208	135	138	114	114
F1_TEX-114xSTM-2_39	Offspring	230	232	161	163	208	208	135	138	110	114
F1_TEX-114xSTM-2_39	Offspring	230	230	NA	NA	200	208	135	135	114	114
F1_TEX-114xSTM-2_39	Offspring	230	232	NA	NA	208	213	135	135	110	114
F1_TEX-114xSTM-2_39	Offspring	230	232	NA	NA	208	213	138	141	114	114
F1_TEX-114xSTM-2_39	Offspring	230	230	NA	NA	208	208	138	141	110	114
F1_TEX-114xSTM-2_39	Offspring	230	230	NA	NA	200	208	135	135	110	114
F1_TEX-114xSTM-2_39	Offspring	230	230	NA	NA	200	213	138	141	NA	NA
F1_TEX-114xSTM-2_39	Offspring	230	232	NA	NA	208	213	135	138	NA	NA
F1_TEX-114xSTM-2_39	Offspring	230	230	NA	NA	208	213	138	141	NA	NA
F1_TEX-114xSTM-2_39	Offspring	230	230	NA	NA	208	208	135	135	NA	NA
F1_TEX-114xSTM-2_39	Offspring	230	232	NA	NA	200	213	135	135	110	114
F1_TEX-114xSTM-2_39	Offspring	230	230	NA	NA	208	208	135	141	114	114

Table S4 Estimated parameters.

Estimated parameters for the two best likelihood models.

For the two best likelihood models based on all data ($\Delta AIC < 2$), we give the estimated parameters: the mean and the variance of the LOH rates of the asexually produced F2 offspring (*mean_LOH* and *var_LOH*), the proportion of the sexually produced F2 offspring for a mixed F1 offspring *psex*, the fill rate of the asexual F1 offspring *f*, the proportion of sterile *q* and the proportion of sexual F1 offspring; s for both MP and NMP F1 of *s_MP* and *s_NMP* only for MP and NMP F1 respectively. The support limits are given in brackets.

Model code	*mean pLOH*	*Variance pLOH*	*psex*	*f*	*q*	*s nmp*	*s mp*	*s*
H E D2 MP+D NMP	0.3705 (0.1582-0.6118)	0.7256 (0.4671-0.9213)	0.6459 (0.4567-0.7946)	0.0421 (0.0353-0.0496)	0.1025 (0.0161-0.2848)			0.3590 (0.0781-0.5729)
I E D2 MP+D NMP	0.3802 (0.1838-0.5953)	0.7008 (0.4493-0.9092)	0.6943 (0.5252-0.8269)	0.0423 (0.0355-0.0498)	0.0870 (0.0135-0.2324)	0.4405 (0.2204-0.6548)	2.63E-20 (0-1)	

Table S5 Likelihood models.
Likelihood models fitted to the modes of reproduction of F1 offspring and their ΔAIC.
Models were fitted using MP data only, NMP data or both based on LOH data only, or both LOH and fill rate data with sterility data. For each dataset considered, the best fitted model is in bold. Models with categories 1, 2 or 3 assume that for each F1, the F2 are produced by sexual reproduction uniquely: *psex*=1, with a constant (cste) or with a variable proportion depending on the F1 offspring and where α and β are the parameters of the Beta distribution. Models with categories A and B assumed that the asexually produced F2 have a LOH rate fixed at *pLOH*= 0.5 or constant, respectively. Models with categories C and D assume that the LOH rate of the asexually produced F2 can vary and a and b are the parameters of the Beta distribution; either the variation is among F2 progenies of a same F1 (C) or among F1 offspring but all F2 are asexually produced with a same LOH rate (D). Models with categories E and F assumed that the LOH rates of the asexually produced F2 are the same or different relative to the MP or NMP phenotype of their F1 mother, respectively. Models with G, H or I account for the proportion of *sexual* F1 offspring *s* only for MP data, both MP and NMP or independent rates for MP and NMP respectively. The parameters *f* and *q* represent the fill rate of the asexual F1 and the sterile proportion of F1 offspring. Two best models are found to fit all the data, with ΔAIC < 2.

Data	**Model code**	**MP**	**NMP**	**MP**	**NMP**	**MP**	**MP**	**MP**	**NMP**	**NMP**	**MP&NMP**	**ΔAIC**
		pLOH	*pLOH*	*pLOH*	*pLOH*	*psex*	*psex*	*s*	*s*	*f*	*q*	
		a,b	*a,b*	cste	cste	*α,β*	cste	cste	cste	cste	cste	
MP	A1_MP	0		½		0	1	0				25.52
MP	B3_MP	0		yes		yes	0	0				29.69
MP	B2_MP	0		yes		0	yes	0				5.26
MP	C2_MP	yes		0		0	yes	0				2.34
MP	C3_MP	yes		0		yes	0	0				3.55
MP	D3_MP	yes		0		yes	0	0				1.98
MP	**D2_MP**	**yes**		**0**		**0**	**yes**	**0**				**0**
MP	G_D2_MP	yes		0		0	yes	yes				2
NMP	B_NMP		0		yes			0				125.77
NMP	C_NMP		yes		0			0				45.33
NMP	**D_NMP**		**yes**		**0**			**0**				**0**
LOH:MP+NMP	E_C3_MP+C_NMP	yes	yes	0	0	yes	0	0				48.07
LOH:MP+NMP	E_D3_MP+D_NMP	yes	yes	0	0	yes	0	0				2.05
LOH:MP+NMP	E_D2_MP+D_NMP	yes	yes	0	0	0	yes	0				0
LOH:MP+NMP	F_C3_MP+C_NMP	yes	yes	0	0	yes	0	0				52.07
LOH:MP+NMP	F_D3_MP+D_NMP	yes	yes	0	0	yes		0				5.17
LOH:MP+NMP	F_D2_MP+D_NMP	yes	yes	0	0	0	yes	0				3.19
LOH:MP+NMP	F_A1_MP+D_NMP	yes	yes	0	0	0	1	0				28.71
LOH:MP+NMP	F_A1_MP+C_NMP	yes	yes	0	0	0	1	0				74.04
LOH:MP+NMP	G_E_D3_MP+D_NMP	yes	yes	0	0	yes	0	yes				4
LOH:MP+NMP	G_E_D2_MP+D_NMP	yes	yes	0	0	0	yes	yes				2
LOH:MP+NMP/ Fill rate/Sterile	**H_E_D2_MP+D_NMP**	**yes**	**yes**	**0**	**0**	**0**	**yes**	**yes**		**yes**	**yes**	**0.88**
LOH:MP+NMP/ Fill rate/Sterile	**I_E_D2_MP+D_NMP**	**yes**	**yes**	**0**	**0**	**0**	**yes**	**yes**	**yes**	**yes**	**yes**	**0**
LOH:MP+NMP/ Fill rate/Sterile	H_E_D3_MP+D_NMP	yes	yes	0	0	yes	0	yes		yes	yes	2.88
LOH:MP+NMP/ Fill rate/Sterile	I_E_D3_MP+D_NMP	yes	yes	0	0	yes	0	yes	yes	yes	yes	2.01

Table S6 LOH data.
Loss of heterozygosity (LOH) data.
LOH rate in offspring of five natural clones and 22 F1 offspring. We give the parental clone's name, its sexual (CP) vs. asexual (OP), male-producing (MP) vs. non-male producing (NMP) status as well as the number of informative microsatellite loci investigated and the number of homozygous loci found in each offspring (corresponding to each line). The last column gives the LOH rate calculated overall loci for each offspring. Available here.

Table S7 Clonal lineages origin.
Clonal lineages origin.
Names and origins of clones used in the study, as well as their use as their sexual (CP) or obligate asexual (OP) identity and their use as mother or father line in the crosses.

Clone name	Origin	CP or OP	Parental clone
LPB-87	Long Point Pond B, Ontario, North America	CP	mother clone
TEX-1	Textile Road, Michigan, North America	CP	father clone
TEX-114	Textile Road, Michigan, North America	CP	mother clone
STM-2	St. Mattieu-du-Parc, Quebec, Canada	OP	father clone
MT-107	Maki Turn, Wisconsin, North America	OP	father clone
KAP-65	Kickapond, Illinois, North America	CP	father clone
KAP-87	Kickapond, Illinois, North America	CP	mother clone
NFL-92	North Flatley, Indiana, North America	CP	mother clone
SED-2	Sedgy, Minnesota, North America	OP	father clone
TRO-3	Troy II, Maine, North America	OP	father clone
DIS-85	Disputed Pond, Ontario, North America	OP	mother clone
DIS-47	Disputed Pond, Ontario, North America	OP	mother clone

Table S8 Fitness proxies.
Fitness proxies through hatching rates and fill rates.
This table is composed of two sheets "Hatching rates" and "Fill rates". In the "Hatching rates" sheet, hatching rates have been calculated for some F1 offspring, F2 or offspring of natural clones "natural offspring". We give the information of the parental clones; whether it is male-producing ("MP"), non-male producing ("NMP), sexual (CP) or asexual (OP) and the name of the parental clone or the same information for each parent in this order: mother-line x father -line. For each clone, we give the number of opened ephippia, the fill rate of the ephippia used for the hatching rate allowing us to estimate the number of embryos to hatch, the number of hatched embryos and the hatching rate.
In the "Fill rates" sheet, fill rates have been calculated for some crosses, F1 offspring and natural clones "natural". Again, we give the informations of MP or NMP, CP or OP and the names of clones investigated. For the crosses the informations are ordered as mother-clone x father-clone. For the F1 offspring, the name also depends on the parent cross. For each clone we give the number of opened ephippia to calculate the fill rate. For crosses, we also added the number of males from the father-clone. Available here.

Appendix

A. Hatching protocol & new clonal cultures

The collected ephippia were rinsed to remove as much algae as possible. Hatching was induced by bathing ephippia in a solution of pure water for two hours, followed by seven minutes of bleach solution and abundant rinsing with osmotic water (Retnaningdyah and Ebert 2012; Paes *et al.* 2016). The ephippia were then exposed to high light for 24 hours, then placed in standard culturing conditions. Every two days, we monitored and isolated any hatched juvenile (F1) in a new vial to initiate a clonal culture.

B. Hot shot DNA extraction protocol for adult *Daphnia*

DNA was extracted in 15 µL HotShot extraction buffer (Sigma) in a thermocycler at 95°C for 10 min and 20°C for 10 min. The extraction product was then diluted with 25 µL dilution buffer (Sigma).

C. DNA extraction for embryos

Derived from Proteinase K extraction protocol from Sen Xu

- Open ephippia under the binocular to extract the embryos. With a pipette P10 and some water (µL for the PCR=3.3µL), take the embryo and place it on a well. Grind each embryo with clean pest.
- Add 10 µL of extraction buffer (1 % Proteinase K (20mg/ml Qiagen); 0.5 % of Tween20 solution at 1 %; 0.5 % of NP40 (Nonidet P-40, a nonionic surfactant used in the isolation of membrane complexes) IGEPAL CA-630 at 1 %; 5 % of KCl (1M); 1 % of TRIS HCl (1M PH 8.3) and 92 % of ddH2O).
- Vortex and incubate at 50 °C overnight.
- In a thermocycler, denature proteinase K at 95 °C for 10 min.

D. Microsatellite protocol

The four informative loci were the *Dpu7*; *Dp339*; *Dp496*; *Dp256*. A special PCR mix for paternity test was used with the loci: *Dpu7*, *Dp502*, *Dp496* and *Dp256*. The primers were developed from Colbourne *et al.* 2004 and ordered from Eurogentec. In primer mix the concentration of each primer was 0.15 µM (except for *Dp496*: 0.25 µM).

E. PCR preparation

- **for adult *Daphnia***

We added 1 µL of DNA extract to 9 µL of PCR mix containing 5 µL of Multiplex buffer (Qiagen), 0.7 µL of forward and reverse primers [0.15 µM except for Dp596 at 0.25 µM], and 3.3 µL of sterile water. PCR amplification was conducted in a thermocycler with an initial denaturation step at 95 °C for 15 min, 35 cycles consisting of 30s at 94°C, 30 s at 55°C and 45 s at 72°C, and finally a supplementary extension step of 30 min at 60 °C.

- **for diapause embryos**

To the 2.5 µL of DNA extract (containing water) we added a PCR mix containing 5 µL of Multiplex buffer (Qiagen), 0.7 µL of forward and reverse primers [0.15 µM except for *Dp596* at 0.25 µM]. PCR amplification was conducted in a thermocycler with an initial denaturation step at 95 °C for 15 min, 45 cycles consisting of 40s at 93°C, 30 s at 55°C and 45 s at 72°C, and finally a supplementary extension step of 15 min at 60 °C.

F. Preparation for genotyping

We added 3 µL of diluted PCR product to 15 µL of HI-DI TM formamide (Applied Biosystems) and 0.2 µL of GeneScan-500 LIZ size standard. Samples were analysed on an ABI 35000XL 24 capillary sequencer DNA Analyzer (Applied Biosystems) at the LabEx CeMEB sequencing platform (Montpellier, France). Fragment analysis and scoring were carried out using GeneMapper v. 3.7 (Soft Genetics, State College, PA, USA). The DNA fragment sizes measured from the peaks were converted into discrete alleles by comparison with reference lists of allele sizes.

G. *Dp8960* (NMP gene) sequencing protocol for adult *Daphnia*

Primers were designed using Primer3 web software (Untergasser *et al.* 2012).

Name	OLIGO	Length (bp)	Tm (°C)	GC%	Sequence
Dp_8960_2F	LEFT PRIMER	20	58.71	50.00	AATGCGCAACTACCGAGATG
Dp_8960_2R	RIGHT PRIMER	21	58.22	47.62	GTGCATGCGAGTTTGAGATTC

We added 1 µL of DNA extract to 19 µL of PCR mix containing 10 µL of Multiplex buffer (Qiagen), 4 µL of forward and reverse primers [2 µM], and 4 µL of sterile water. PCR amplification was conducted in a thermocycler with an initial denaturation step at 95 °C for 15 min, 30 cycles consisting of 30s at 94°C, 30 s at 58°C and 60 s at 72°C, and finally a supplementary extension step of 10 min at 72°C. Products were sent for Sanger sequencing at Eurofins Genomics. Data were analysed using CodonCode Aligner Version 6.0.2 (CodonCodeCorporation, Dedham, MA, USA).

H. Investigation for rare sexual reproduction in OP natural clonal populations

Primers for DNA sequence present in CP populations and absent in OP populations

Name	OLIGO	Length (bp)	Tm (°C)	Sequence
D47_Sc1_2F	RIGHT PRIMER	20	59.01	CTCTCTCCGTTTTCTTCGCG
D47_Sc1_2R	LEFT PRIMER	20	58.98	ATCCGCCAATGTGAATGTCG
D85_Sc8_1F	RIGHT PRIMER	20	59.77	CAACTCGACCAGGAAAACGC
D85_Sc8_1R	LEFT PRIMER	20	59.47	CAGGTTGCCATCTTTGCTCC

The first pair of primers was used to amplify a DNA fragment present in the CP MP clonal population TEX1 (father line of the cross) and absent in the OP NMP clonal population DIS47 (mother lineage). The second pair of primers was used to amplify a DNA fragment present in the CP MP clonal population KAP-65 (father line of the cross) and absent in the OP NMP clonal population DIS-85 (mother line). DNA of the embryos contained in the ephippia of each cross was extracted by pooling ten embryos following the DNA extraction protocol for embryos described above. To the 6 µL of DNA extract (containing water) we added a PCR mix containing 10 µL of Multiplex buffer (Qiagen), 4 µL of forward and reverse primers [0.15 µM]. PCR amplification was conducted in a thermocycler with an initial denaturation step at 95 °C for 15 min, 30 cycles consisting of 30s at 94°C, 30 s at 58°C and 60 s at 72°C, and finally a supplementary extension step of 10 min at 72 °C. Electrophoresis gel were conducted using an agarose gel preparation (1.5 %), were a mix of 2 µL of loading buffer 5x and 5 µL of amplified DNA was deposited.

I. Paternity tests on NMP F1 able to reproduce asexually

Paternity tests were conducted on two F1 able to reproduce asexually after adding males to them NMP (F1_TEX-114xSTM-2_33 and F1_TEX-114xSTM-2_39, Table S2).

In one asexual NMP F1, at least eight out of 14 investigated progenies showed male-specific allele on at least one of the two informative loci. However, in the other one, no specific allele of the male lineage was found in the 18 possible genotypes of the F2 offspring where the test would have been informative (six F2 offspring investigated, two loci). This suggests that sexual reproduction should be less than 16 % in this particular NMP F1. See **Table S3**.

Results

We obtained two positive bands from one cross and one from the other cross. Using the known proportion of negative amplifications, the probability of having at least one event of sexual reproduction is 1.5 % (0.077 - 23.8) in the first cross, 0.22 % (0.006 - 8.27) in the second cross and 0.52 % (0.036 - 7.05) for both (1.4 %, 0.22 % and 0.51 % respectively under the assumption that exactly one embryo comes from sexual event in a positive pool of 10 embryos).

Control tests

Tests for DNA sequence amplification detection were performed using different frequencies of paternal lineage embryos with respect to maternal lineage embryos: 1:9 to 1:1. All tests identified an electrophoretic band signaling the presence of paternal lineage embryos. The use of embryo pools did not restrict the detection of even rare sexual reproduction events (only one in the 10 pooled embryos). A test in one of the two crosses was performed to verify that there was no contamination when adding males. To do so, DNA from 200 females (representing about 28 % of the total estimated number of adult females in the cross) was extracted and PCR were performed to amplify the insertion specific to the father line clonal population. The results were analyzed with an electrophoresis gel. We also stress that the protocol of adding males followed a double control in order to decrease a possible identification error.

References of supplementary data

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Untergasser, A., I. Cutcutache, T. Koressaar, J. Ye, B. C. Faircloth, M. Remm, and S. G. Rozen. 2012. Primer3-new capabilities and interfaces. Nucleic Acids Res. 40:e115–e115.

Chapter 4

Evolution of gene expression during a transition from environmental to genetic sex determination

Published manuscript.

Abstract

Introduction

Results

Discussion

Materials & Methods

References

Supplementary data

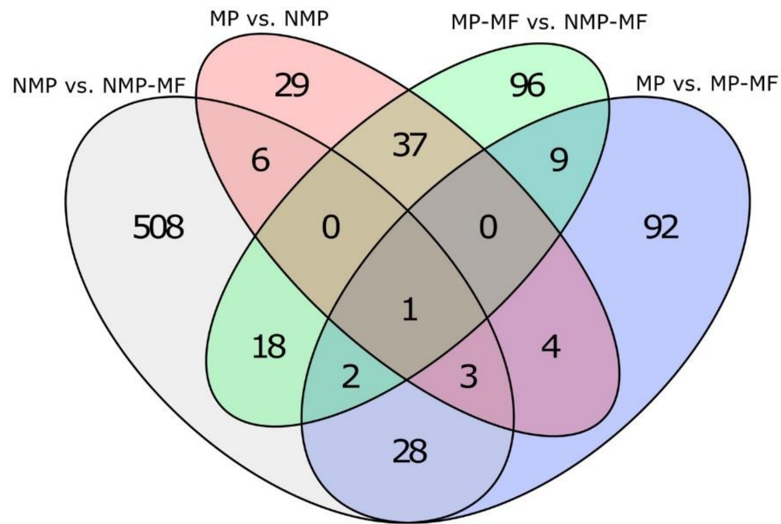

Figure S1: Venn-diagram showing the number of DE genes (p-adj < 0.05, |log2FC| > 1) in each combination of the four contrasts.

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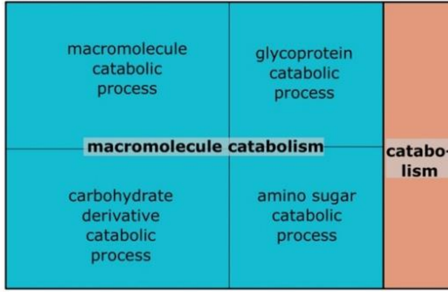

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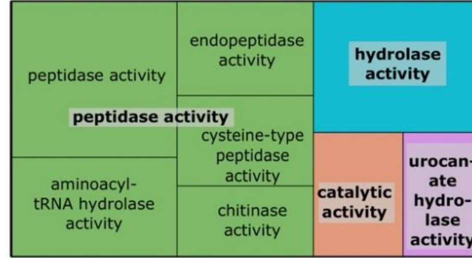

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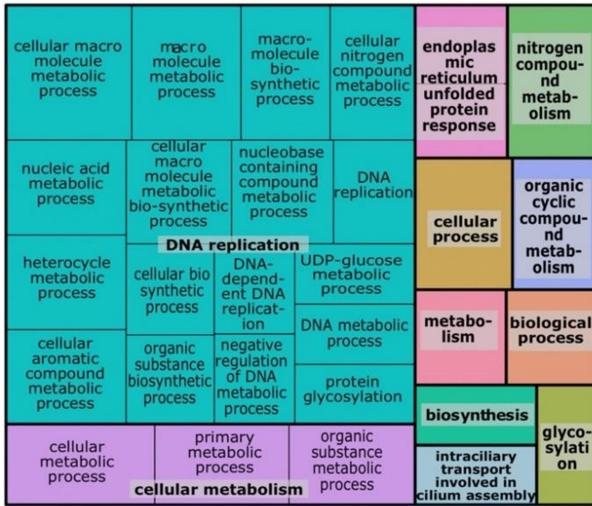

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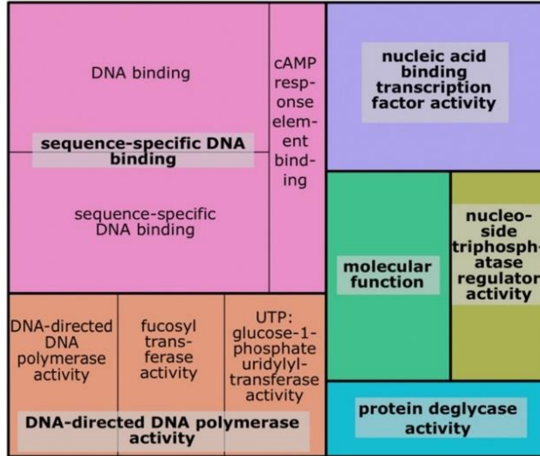

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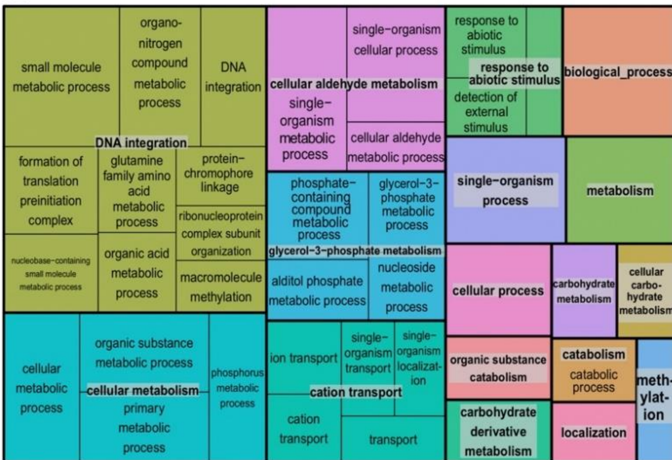

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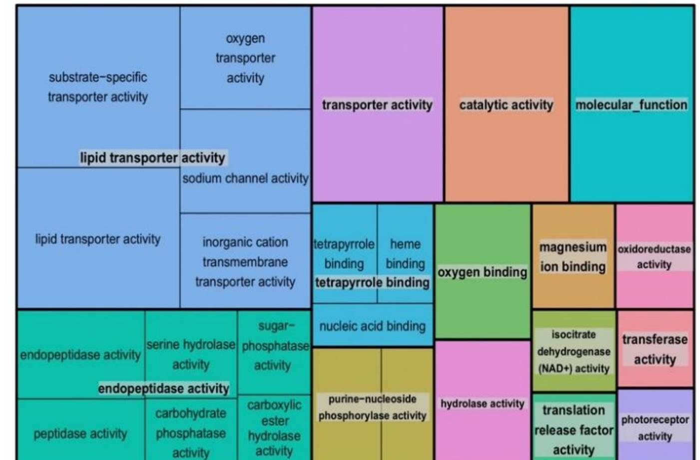

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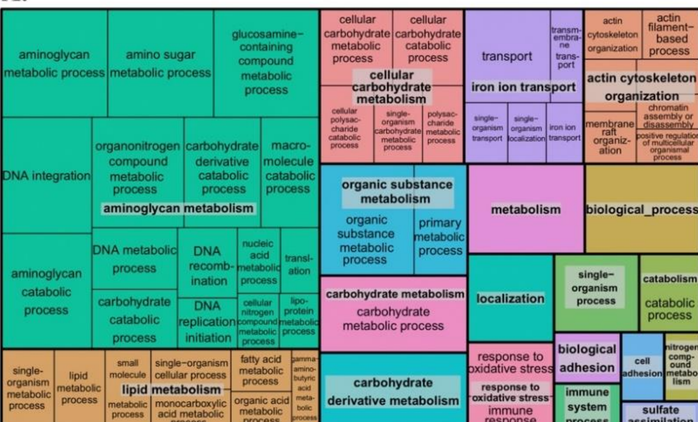

B.

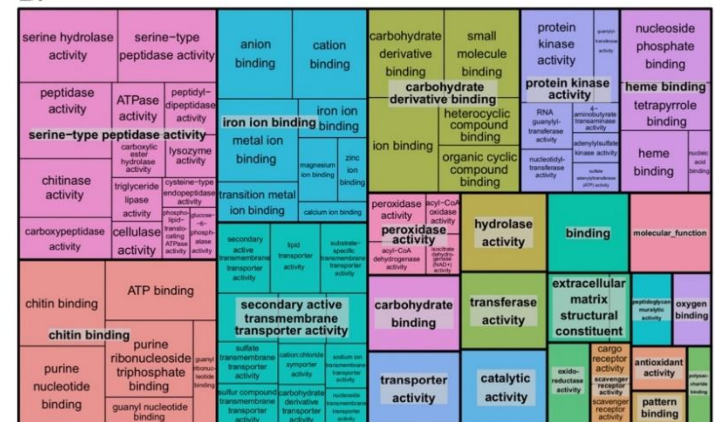

Figure S2: Over-represented functional categories with p-values < 0.001 in the GO-term enrichment analysis were used to generate a treemap colored by functional category with REVIGO (Supek *et al.* 2011) between MP vs. NMP (1.), between MP-MF vs. NMP-MF (2.), between MP vs. MP-MF (3.), between NMP vs. NMP-MF (4.). The size of each rectangle is proportional to the p-value for its category. UniprotKB was used to determine Gene Ontology Biological Process (A.) and Molecular Functions (B.) that were over-represented among genes DE between the corresponding contrast.

Table S1: Number of reads obtained by paired-end sequencing and retained after trimming and mapping.

Sample	Raw reads (in millions)	Trimmed reads (in millions, % of raw)	Mapped reads (in millions, % of raw)
MP1	76.24	75.50 (99.02%)	69.66 (92.26%)
MP2	64.59	64.00 (99.07%)	58.89 (92.01%)
MP3	68.13	67.46 (99.02%)	62.33 (92.39%)
MP4	73.42	72.75 (99.10%)	67.37 (92.61%)
NMP5	67.41	66.77 (99.05%)	61.35 (91.88%)
NMP6	74.86	74.20 (99.11%)	68.59 (92.44%)
NMP7	69.99	69.40 (99.15%)	64.39 (92.79%)
NMP8	71.27	70.62 (99.08%)	65.44 (92.67%)
MP1-MF	64.79	64.15 (99.01%)	60.21 (93.86%)
MP2-MF	62.72	62.25 (99.25%)	58.64 (94.21%)
MP3-MF	61.39	60.66 (98.81%)	56.39 (92.96%)
MP4-MF	57.7	57.16 (99.06%)	53.42 (93.46%)
NMP5-MF	82.66	81.93 (99.12%)	75.82 (92.54%)
NMP6-MF	92.48	91.68 (99.13%)	84.80 (92.50%)
NMP7-MF	74.75	73.98 (98.96%)	68.78 (92.97%)
NMP8-MF	73.14	72.47 (99.08%)	66.71 (92.06)

Table S2: List of genes that are DE in at least one of the four contrasts. Available here.

Table S3: List of genes that are strongly DE in at least one of the four contrasts and their functional annotations. Available here.

Table S4: List of DE genes in the NMP region and their functional annotations.

	MP vs. NMP		MP-MF vs. NMP-MF		NMP vs. NMP-MF		Annotation results from BLAST2GO				
Locus tag	**log2FC (MP relative to NMP)**	***p-adj***	**log2FC (MP relative to NMP)**	***p-adj***	**log2FC (Control relative to MF)**	***p-adj***	**Description**	**GO IDs**	**GO Names**	**Enzyme Codes**	**Enzyme Names**
APZ42_029238					1.15	5.01 E-04	AChain The Structure Of Endoglucanase From Nasutitermes At Ph	P:GO:0030245; F:GO:0008810; F:GO:0030247	P:cellulose catabolic process; F:cellulase activity; F:polysaccharide binding	EC:3.2.1.4	Cellulase
APZ42_026001	-1.08	3.57E-02					ecdysone 20-monooxygenase isoform X1	F:GO:0005506; F:GO:0016705; P:GO:0055114; F:GO:0004497; F:GO:0020037	F:iron ion binding; F:oxidoreductase activity. acting on paired donors, with incorporation or reduction of molecular oxygen; P:oxidation-reduction process; F:monooxygenase activity; F:heme binding		
APZ42_025994					-1.12	2.71 E-03	organic cation transporter	C:GO:0005887; F:GO:0015101; P:GO:0015695; P:GO:0055085	C:integral component of plasma membrane; F:organic cation transmembrane transporter activity; P:organic cation transport; P:transmembrane transport		
APZ42_029237			1.10	2.57E-12			probable peptidyl-tRNA hydrolase 2	F:GO:0004045	F:aminoacyl-tRNA hydrolase activity	EC:3.1.1.29; EC:3.1.1.1	Aminoacyl-tRNA hydrolase; Carboxylesterase
APZ42_025969	-1.85	4.70E-13	-1.22	1.50E-02			Rolling stone	C:GO:0016021	C:integral component of membrane		
APZ42_010828			1.75	2.27E-02			NA				
APZ42_014081	-1.40	1.43E-02	-1.50	1.06E-03			NA	F:GO:0003824; F:GO:0046872	F:catalytic activity; F:metal ion binding		
APZ42_017775	-2.6	6.92E-08	-2.56	2.04E-05			NA				
APZ42_017776	-3.07	1.25E-11	-3.52	1.03E-11			NA				
APZ42_018570					-1.31	3.19 E-08	NA	C:GO:0016021	C:integral component of membrane		
APZ42_020301			1.19	3.53E-02			NA				
APZ42_026030	-2.2	8.51E-06	-3.09	2.49E-20			NA				
APZ42_033362			-1.62	2.10E-02			NA				
APZ42_011846	1.42	3.70E-02					NA				

Table S5: List of genes known to be involved in sex determination in *Daphnia magna* or related species. Available here.

Discussion

Evolution of obligate asexuality

In eukaryotes, obligate asexuals have evolved from sexual relatives (Ramesh *et al.* 2005). It is therefore interesting to ask to what extent obligate asexuals maintain traits that characterize sex, such as meiosis, recombination or male production. This is part of the broader question: How asexuals arise and evolve in the first place? If they maintain some sexual traits, do they still have a short-term advantage over sexuals as predicted by theory based on the hypothesis that they reproduce by mitosis? In this PhD thesis, I provide some elements to answer these questions (**chapters 1 to 3**).

a. What sexual traits do obligate asexuals still possess and what does this imply?

As a consequence of the evolution of asexuality from sexuality, it is known that many sexual traits are still found in asexuals (even in obligate ones), such as meiosis, recombination and male production.

Meiosis and recombination

Meiosis and recombination are intrinsic features of sexual reproduction. Therefore meiosis or recombination genes are often hypothesized to causally underlie the evolution of obligate parthenogenesis (OP), for instance through a loss of function or pseudogenization of these genes (Li *et al.* 1981; Vanin 1985; Simon *et al.* 2003; Lehtonen *et al.* 2012). This hypothesis has led to the development and use of a meiosis genes "toolkit" to identify parthenogenesis (Normark *et al.* 2003; Schurko and Logsdon 2008). Indeed, the idea received some support from studies in plants, such as *Arabidopsis thaliana* or *Oryza sativa*, where meiosis can be turned into a mitotic-like division by mutations in three different genes (D'Erfurth *et al.* 2009; Mieulet *et al.* 2016). In animals (and certainly in plants too), the majority of OP still possess cytological mechanisms of modified or even complete meiosis (**chapter 1**). Recombination too, has been found in OP, albeit sometimes at very low rates (**chapter 1**, Bell 1982; Archetti 2010; Lenormand *et al.* 2016). More specifically, in *Daphnia pulex*, it is known that meiosis in OP is not entirely disrupted. First, cytological observations showed that OP occurs through an abortion of meiosis I (Bacci *et al.* 1961; Zaffagnini and Sabelli 1972). The precise moment of the abortion has been demonstrated in parthenogenesis of CP to occur after pairing of homologous chromosomes, thus after the moment when recombination could potentially be achieved (Hiruta *et al.* 2010). Second, rare OP males are able to produce haploid sperm (Xu *et al.* 2015). Our study has added new evidence to this literature for conserved meiotic processes in OP *D. pulex*: We found that also OP females are able to rarely produce diapause embryos by sexual reproduction (**chapter 3**). So far, we do not know if the resulting offspring are diploid or triploid, and thus whether OP females underwent reductional meiosis. However, "mixed" F1s from a sex-asex cross were able to reproduce via sexual reproduction involving reductional meiosis during oogenesis (the offspring were diploid, **chapter 3**). As rare sex in OP females may be a remnant of such "mixed" reproduction, meiosis may not even be completely suppressed in OP females. If OP females can mate with males and OP males can produce normal meiosis (with recombination and reduced gametes), the hypothesis of pseudogenization of genes essential for meiosis is not supported. Moreover, the pseudogenization of genes usually occurs over long periods of time (Lynch and Conery 2000) after gene duplication and relaxed selective pressures. In many species, the origin of OP is rather young in terms of evolutionary time, as in *D. pulex,* where it has been dated between 1'250 and 187'000 years ago (Tucker *et al.* 2013). Thus, it is unlikely that pseudogenization of meiosis genes have enough time to evolve. Moreover, many genes involved in meiosis may also be involved in other important functions, such as DNA repair, and may thus be highly constrained. This might explain why functional meiosis genes are found in many asexuals, including some "ancient asexuals" (protists: Arkhipova and Morrison 2001; Malik *et al.* 2008, animals: Rice 2015; Tvedte *et al.* 2017). As many meiotic features are still found in asexuals, pseudogenization of meiotic genes should not be considered as one of the main causes of the transition to asexuality.

The maintenance of meiotic features in asexual organisms has also important implications regarding the genetic consequences of asexuality. Indeed, whereas mitosis leads to clonality, the genetic consequences of meiosis-derived asexual modes are less straightforward, and may deviate from clonality. Clonal vs. non-clonal genetic consequences of asexuality will depend on the cytological mechanisms of asexual reproductive modes as well as on recombination. Recombination may generate variability among asexually produced offspring, suggesting that they may benefit to some degree from the advantages if recombination, for instance improved adaptation and purging (reviewed in De Visser and Elena 2007; Neiman and Schwander 2011). On the other hand, they may also suffer from costs of recombination, which in asexuals are likely to be larger than in sexuals (Archetti 2004; Engelstädter 2008). Indeed, in asexual modes of reproduction where heterozygosity is maintained, recombination leads to loss of heterozygosity, which results in loss of complementation, akin to inbreeding. Due to these costs, recombination may be suppressed or reduced by secondary evolution, as suspected in some parthenogens (Engelstädter 2017; Haag *et al.* 2017). In *Daphnia pulex*, CP females recombine whereas OP females do not or only at very low rates (**chapter 2)**. Furthermore, asexual reproduction of F1s from a sex-asex cross often involves recombination (**chapter 3**). This indicates that suppression of recombination is not causally responsible for the evolution of OP and suggests that suppression of recombination in OP females is due to secondary evolution. If recombination is secondarily suppressed also in other asexual species, this may lead to an underestimation of the importance of non-clonal asexuality in nature, especially during their early evolution (**chapter 1**). It also indicates that the evolution towards asexuality may be costly. This cost may depend on the initial recombination rate in sexual females (Rauwolf *et al.* 2011; Haag *et al.* 2017). Therefore, if selection against recombination is necessary, it calls into question the classical advantage of asexual reproduction over sexuals which considers a direct short-term advantage of asexuality (without costs due to meiosis or recombination). To conclude, part of the costs and/or benefits of meiotic recombination are also applicable to asexuals.

Males

Males, although generally ascribed to sexual reproduction, are also present in OP. What maintains non-zero male production in OP? Do these asexual males confer an advantage through contagious asexuality? Theory predicts that rare asexual males might be beneficial because by transmitting asexuality during crosses with sexuals, they generate new, variable asexual lineages (Joshi and Moody 1998; Engelstädter *et al.* 2011). These genetically diverse assemblage of asexual lineages may enable increased levels of evolutionary potential. Yet, models assume faithful and efficient transmission of asexuality. In addition, the supposed advantage of males only takes place when sexuals and asexuals coexist in the same habitat. In *Daphnia pulex*, many OP lineages are known to produce rare males (Hebert *et al.* 1989). We and others (Jaenike and Selander 1979; Innes and Hebert 1988; Stelzer *et al.* 2010; Sandrock and Vorburger 2011; Maccari *et al.* 2013; Jaquiéry *et al.* 2014) have shown that males indeed transmit asexuality genes (**chapter 3**) and we showed that OP males recombine contrary to OP females (**chapter 2**). Thus, males could be beneficial despite their costs. Besides, in *Daphnia*, it has been shown that even if male production is found in OP, this male production is reduced compared to CP in the field as well as under laboratory conditions (Innes and Singleton 2000). In addition, OP have also inherited variation in male allocation as in CP: Hebert *et al.* (1989) found that rare clonal lineages were more likely to produce males than common ones. The authors suggested that the success of the common lineages may be partially due to avoidance of the cost of males; lineages with little or no allocation to males' production being favored. Indeed, the cost of males has been investigated in CP lineages; the more males the clones produce, the less fit the clones are (Innes *et al.* 2000), and between CP, MP OP and NMP OP lineages; by comparing the number of ephippia, females or males produced, lower fitness in CP was demonstrated to be due to the cost of males (Wolinska and Lively 2008). However, when both are in competition, CP outcompete OP lineages, suggesting sexuals could have a

reproductive or survival advantage in competition (Wolinska and Lively 2008). More interestingly, there was no evidence for a cost of males in OP; MP OP have better fitness than NMP OP, raised separately or in competition. This result could explain why male production is maintained in OP. Compared to sexuals, males can be particularly costly, especially if they produce non-functional sperm, as they cannot provide any benefit (Xu *et al.* 2015). Our study adds to this knowledge by showing that, even when OP males produce viable sperm, transmitting asexuality genes is linked to additional costs. In **chapter 3**, we found that only 20 % of F1s from a contagion event are able to reproduce asexually in *Daphnia pulex*. Moreover, the more genes that determine the asexuality phenotype (not only the ability to reproduce asexually, but the whole phenotype) and the less dominant they are, the higher the cost associated with males. Indeed, we have shown that the OP phenotype (the fact that asexuality is obligate and clonal) is not faithfully transmitted. Furthermore, in our study new asexuals seem to have a very low fitness; their hatching rates is lower than natural clones and they produce fewer diapause embryos (**chapter 3**). These are the first fitness comparisons between newly produced asexuals and those found in the wild, although earlier trials of sex-asex crosses also suggest that F1s are difficult to hatch and produce few or no ephippia (Innes and Hebert 1988; Xu *et al.* 2015). Taken together, the production of OP males, even considering their potential benefits via contagious asexuality, seem very costly.

Finally, in CP *Daphnia*, fertilization of ephippia is constraint by the presence of males. If the timing or the ratio of number of males vs. number of ephippia to be fertilized is not well adjusted, then the females would produce empty ephippia which are extremely costly to produce (Lynch 1983). Thus, it was assumed that OP should not suffer from the cost of producing empty ephippia, as ephippia embryos can be produced without males. However, in our lab, we noticed that natural OP lineages seem to produce lots of empty ephippia, which has also been noticed in a previous study (Innes and Hebert 1988; Wolinska and Lively 2008).

b. How do obligate parthenogens evolve at first?

In the review (**chapter 1**), we have seen that all investigated parthenogens seem to originate from meiosis or at least there is no evidence contradicting an origin though modification of meiosis. That parthenogens evolve from sexual ancestors is well known in the literature, but our review reveals the extent to which evidence for any truly mitotic apomixis is missing. In the initial phases of their evolution, the majority of parthenogenetic species may have been non-clonal (**chapter 1**). Moreover, even in cases where asexuality nowadays is close to clonality, such as in OP *Daphnia pulex*, newly formed asexuals by contagion are non-clonal (**chapter 3**). This illustrates the possible biases that can occur by studying extant, natural asexuals because asexuals found in nature are only a selected subset of lineages (**chapters 1** and **3**). For instance, OP *D. pulex* are thought to originate from an ancient hybridization event with introgression of its sister species (*D. pulicaria*) alleles into a *D. pulex* genomic background (Xu *et al.* 2013). Indeed, hybrids between *D. pulex* and *D. pulicaria* found in nature are always OP (Hebert and Finston 2001). However, in line with what we found with contagious asexuality in *D. pulex*, F1s fom *D. pulicaria* and *D. pulex* produced in laboratory are always CP (Heier and Dudycha 2009). Thus, it could mean that, asexuals need secondary modifications to become what is observed in natural populations. A similar case occurs in *Poecilia formosa*, where crosses intended to mimic the initial hybridization event that lead to asexual reproduction involving suppression of meiosis I, instead lead to hybrids reproducing by random gamete fusion (Lampert *et al.* 2007). Moreover, the authors concluded that the majority of all offspring were sterile and suggested that hybrids suffer from lower fertility than their parental species. In conclusion, transitions to parthenogenesis have certainly been more complex than if they arose by mitosis and might even be costly to evolve. Our results, in line with findings on other parthenogens, imply secondary changes in the evolution of asexuality: suppression of recombination, evolution towards a specific asexual mode of reproduction, and/or a very high selection of newly produced asexuals.

From an evolutionary point of view, this is important because it is thought that sexuality does not need to confer major benefits if new asexual lineages have lower fitness than competing sexuals or are rarely or never produced (Burt 2000; Engelstädter 2008). Previous studies found that fitness of OP was reduced compared to CP for fecundity, hatching success and offspring survival in *D. pulex* (Lynch 1984; Lynch *et al.* 1989). The combination of these traits measured the OP fecundity, but did not took into account the possible avoidance of the cost of males. This cost could offset OP reproductive output explaining why OP and CP coexist. In **chapter 3**, we showed for the first time that newly produced asexuals have reduced fecundity compared to OP found in the wild, although earlier trials of sex-asex crosses also suggest that F1s are difficult to hatch and produce few or no ephippia (Innes and Hebert 1988; Wolinska and Lively 2008; Xu *et al.* 2015a). This suggests that arising asexuals should have very low fitness compared to competing sexuals. Asexuals are thought to experience a long-term cost, as often predicted by theoretical models about the long-term evolution of asexuals. Our results suggest that these costs of asexuality may also include short-term costs.

Because OP should suffer from a genetic load of deleterious mutations compared to CP, OP should age faster than CP (Paland *et al.* 2005; Paland and Lynch 2006). However, in *D. pulex*, no differences in lifespan have been found between OP and CP, with even a tendency for sexuals to age quicker (Dudycha and Hassel 2013). This may be the result of a high selection for the fittest clones in OP. Studies that compare related sexual and asexual species should take into account that they do not compare sexuals with a random sample of newly evolved asexual lineages.

A final indication that asexuals are difficult to produce is that even if there are highly genetically diverse OP clones, there are very few different clones that have invaded the whole world (Mergeay *et al.* 2006; So *et al.* 2015; Ye *et al.* 2021b). It seems that the invasion by few "superclones" can reflect the fact that very few OP have actually a high fitness. In conclusion, the obstacles in the evolution of asexuality that we have highlighted may explain the rarity of asexuals. In any case, this hypothesis proposes a different view of the initial evolution of asexuality.

c. Perspectives

We found that F1s *D. pulex* from a sex-asex cross were able to reproduce asexually (though not obligatory and not clonally). This indicates that the asexuality genes are at least partially dominant. In this thesis, we did not identify the underlying responsible genes for OP because of the surprising diversity of reproductive phenotypes in F1s. Indeed, the initial purpose of the CP x OP crosses was to map the asexuality genes. Using parent and offspring genotypes from one cross, we performed a preliminary QTL analysis (*i.e.*, Quantitative Trait Loci analysis that links phenotypic and genotypic data) using the discrete classes of NMP F1s: able or not to reproduce asexually, but also looked for any QTL associated with the fill rate in NMP F1s as a continuous trait. Both led to a nearly significant region on LG 7 and a second region on LG 9. Finding a region potentially related to our "asexual" phenotype on LG 7 is rather surprising as this chromosome is not part of the four LGs (LGs 5, 8, 9 and 10) found in earlier studies to be associated with the OP phenotype (Lynch *et al.* 2008; Eads *et al.* 2012; Tucker *et al.* 2013; Xu *et al.* 2015b). In associations studies besides the causal regions, it is possible that some regions are only correlated to the OP phenotype, which are the result of secondary modifications. The difficulties we had in phenotyping the F1s compromise the chance to identify the causal regions of the OP phenotypes, but we still expected to find some of the same associated regions. Our data are too limited to draw any conclusion, but it would still be interesting to do this analysis again, adding few but other data (some RAD-seq data from NMP F1s of other crosses have been produced).

Another possibility for the transition from sexuality to parthenogenesis is through epigenetic modifications. In this case, mutations may be found in other parts of the DNA (such as promoters) that might silence one or more genes. By modifying gene expression, it might be easier to obtain a sex-limited effect (female-only suppressor). Under this scenario, it may be possible to reconcile the idea that

meiosis genes are modified for the OP phenotype with the idea that these lineages retain the ability to produce functional rare males. In *D. pulex*, a recent study evaluated the allele-specific expression of the OP and CP lineages (Ye *et al.* 2021a). A major question in that study was to assess whether the OP was due to gene expression unbalanced due to a hybrid origin (whereas no such unbalanced was present in CP). The authors found that the OP lineages have more genes with differential allele-specific expression than the CP lineages, and suggested that some of these genes are directly involved in meiosis-related processes (Ye *et al.* 2021a). To better understand the epigenetic basis of parthenogenesis, one possible approach could be based on the differential expression of genes between the sexes of an OP lineage. In *D. pulex*, about 50 % of the genes show sex-biased expression during parthenogenetic reproduction in CP (Eads *et al.* 2007). It might be interesting to examine also the differentially expressed genes between OP males (which can perform normal meiosis) and OP females (which cannot or only very rarely), controlling for sex-specific gene expression in CP.

Evolution of GSD from ESD

The control of sexual reproduction associated with the production of ephippia or males is generally considered to be environmental in *Daphnia*, but different genotypes also allow for different intensities of energy allocated to sexual reproduction. Deng (1996) found a gene x environment interaction in the production of ephippia and the number of males produced in *D. pulicaria*. But the most extreme case is that of genetically determined females via the NMP locus (Innes 1997; Galimov *et al.* 2011; Ye *et al.* 2019). This new genotype with a GSD has evolved from ESD independently in *D. magna* and *D. pulex* (Innes and Dunbrack 1993; Galimov *et al.* 2011). In animals, the evolution of GSD from ESD via a transient state equivalent to gynodioecy is only found in *Daphnia*. In *Daphnia magna,* NMP evolution does not seem to be a simple loss of function but rather an active and tightly controlled suppression of male production (**chapter 4**). Even if the evolutionary costs and benefits of NMP in *Daphnia* still have to be fully elucidated, our study suggests that NMP phenotypes may have a selective advantage under certain circumstances. In addition, a preliminary analysis of a master's internship carried out this year, which I co-supervised, suggested that, in addition to the NMP phenotype having evolved independently in two species of *Daphnia*, it may also have evolved independently at least twice within the species *D. magna*. These results, based on a Pool-sequencing analysis of several geographically distant populations with the MP/NMP polymorphism, suggest that one population appears to have a different genetic basis of the trait.

One of the main potential selective advantages of NMP is the avoidance of inbreeding depression due to obligate outcrossing with other (MP) clones. Indeed, to optimize the benefit of sexual reproduction, we have explained in the introduction that sexual reproduction is more advantageous if occurring between different genotypes in order to avoid inbreeding depression. However, inbreeding depression alone is not expected to drive the evolution of separated sexes (Charlesworth and Charlesworth 1978, 1987). Moreover, NMP clones have also evolved in aphids, even if the genetic control remains to be demonstrated (Rispe *et al.* 1999; Helden and Dixon 2002). In some species of aphids however, males have wings and inbreeding depression is not expected to be high. Remarkably, aphids are also cyclical parthenogens, suggesting that such gynodioecy-like system may be advantageous especially in CP. Finally, if the cost of males is high enough, it could lead to the evolution of lower male production, although this would also reduce general sex investment in CP. Indeed, we have seen above that male production is associated with sexual resting eggs which are costly to produce, but essential to survive recurring adverse periods in CP (Simon *et al.* 2002). In *Daphnia*, as in other CP species, males are produced only at a specific moment of the cycle, reducing their cost, and thus ESD is generally thought to be adaptive in such systems (Bull 1983; Janzen and Phillips 2006). Thus, if environmental conditions are continuously favorable, a reduction or total lack of investment in sexual

reproduction is less risky. For instance, in aphids, some clones have become "OP" by totally abandoning male and resting egg production, staying forever in the parthenogenetic part of the CP life cycle (Rispe *et al.* 1998). Similarly, in rotifers, low sex investment is selected in short term (when environmental conditions are goods) but selected against by between-year-cycle selection (Carmona *et al.* 2009). To conclude, we still lack a sufficient understanding of the evolutionary advantage of GSD and more empirical studies are needed in *Daphnia*. For example, investigating whether specific ecological factors correlate with the occurrence of NMP or comparing inbreeding depression rates between sets of populations with and without NMP would be important data that would help explaining the evolution of this phenotype. Perhaps, surveys of other CP species, where similar traits can be found, would also be a promising avenue.

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Conclusion

Reproductive systems are much more labile than generally admitted. Indeed, in animals, the sexual vs. asexual reproductive modes can behave as plastic traits. For instance, asexuality can be reversed to sexuality (Domes *et al.* 2007; Christiansen and Reyer 2009). Likewise, sex determination is also known to be highly plastic (Barske and Capel 2008; Quinn *et al.* 2011). For instance, several genetic sex determination systems are found in a cichlid species (Moore *et al.* 2021) and a possible coexistence of ESD and GSD has been suggested in another fish *Odontesthes bonariensis* (Yamamoto *et al.* 2014). To echo what Barrett (2002) said about the reproductive systems in flowering plants, I would like to emphasize that animals too show a remarkable ecological and evolutionary lability in their reproductive systems. Today, the study of transient systems, to which my doctoral thesis contributed, and the search for even more cryptic reproductive strategies are increasingly being considered because of their importance in providing important insights into the costs and benefits relative to sexual reproduction.

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Extended abstract in french/ Résumé étendu en français

Transition entre les systèmes de reproduction chez *Daphnia*

La reproduction sexuée est le mode de reproduction le plus fréquent chez tous les eucaryotes. Pourtant, on observe une multitude de systèmes de reproduction qui incluent ou non un mode de reproduction sexué (Otto 2009 ; Schurko *et al.* 2009). De nombreuses transitions entre systèmes de reproduction se retrouvent à différentes échelles, même au sein d'une espèce (Barrett 2002 ; Leonard 2018). Du fait que l'évolution des systèmes de reproduction est soumise simultanément à de multiples facteurs, écologiques ou génétiques et des difficultés à concilier les explications théoriques aux preuves empiriques, les forces sélectives qui maintiennent la grande diversité des systèmes de reproduction restent aujourd'hui encore à élucider, en particulier chez les animaux (Kondrashov 1993 ; De Visser and Elena 2007 ; Hadany and Comeron 2008 ; Otto 2009 ; Lively 2010 ; Hartfield and Keightley 2012). Lors de ma thèse, je me suis intéressée en particulier aux conséquences génomiques de l'évolution de la sexualité vers l'asexualité, ainsi qu'à l'évolution d'un déterminisme sexuel environnemental (ESD) vers un déterminisme sexuel génétique (GSD). Les études théoriques et empiriques qui traitent ces sujets se fondent sur de nombreuses simplifications qui reflètent les caractéristiques des systèmes de reproduction souvent très dérivés trouvées dans la nature. Plus précisément, peu d'études empiriques ont examiné les étapes initiales dans l'évolution des transitions de la sexualité vers l'asexualité ou vers un nouveau chromosome sexuel à partir d'un déterminisme environnemental. En effet, les avantages à court termes sont plus difficiles à étudier lorsque la transition s'est produite il y a longtemps. Mais surtout, les études empiriques comparant les systèmes de reproduction dérivés peuvent être erronées car les pressions de sélection qui agissent sur les étapes initiales ou intermédiaires peuvent différer fortement de celles agissant sur les systèmes de reproduction finaux (Simon *et al.* 2002 ; Engelstädter 2008 ; Archetti 2010 ; Neiman and Schwander 2011). Ainsi, les étapes de transitions sont essentielles afin de mieux comparer les coûts et les avantages de ces nouveaux systèmes de reproduction par rapport à leur état ancestral et afin d'avoir une compréhension plus juste et complète des forces évolutives sous-jacentes.

Dans cette thèse, j'ai étudié les transitions de la sexualité vers l'asexualité obligatoire et de l'ESD à la GSD chez un animal qui possède les deux types de polymorphisme : *Daphnia spp*, un petit crustacé d'eau douce. La reproduction sexuée est généralement associée aux caractéristiques telles que la méiose, la recombinaison et la production de mâles, tandis que l'asexualité est considérée comme étant caractérisée par la mitose, l'absence de recombinaison et l'absence de mâles. J'ai testé si de telles simplifications, largement utilisées dans la littérature, sont réalistes. Grâce à un système transitoire équivalent à la gynodioécie, unique chez un animal, j'ai également étudié l'évolution des premières étapes d'un chromosome sexuel naissant. Dans la Discussion, j'ai présenté un aperçu des études générées dans le cadre de cette thèse, en relation avec la littérature. A travers cette thèse, je montre que tenir compte des étapes transitoires entre les systèmes de reproduction est essentielle pour comprendre leur évolution. Afin de traiter mon sujet, j'ai combiné les approches d'expérimentations en laboratoire, un travail bibliographique et l'utilisation de marqueurs génétiques et génomiques.

Evolution de l'asexualité obligatoire

La première partie de cette thèse est consacrée à la transition entre la reproduction sexuée et la reproduction asexuée. Chez les eucaryotes, la majorité des organismes se reproduisent de manière sexuée. Pourtant, certains organismes sont capables de parthénogénèse, une forme d'asexualité où la production des descendants par lignée germinale se réalise sans fertilisation. Même s'il existe des formes non clonales d'asexualité (Bell 1982 ; Archetti 2010), la clonalité (la production de descendants génétiquement identiques à leur mère, hormis les nouvelles mutations) est considérée comme le mode de reproduction asexuée le plus courant (Suomalainen 1950). De même, les modèles théoriques de l'évolution de l'asexualité supposent généralement une équivalence à la clonalité. Dans un premier chapitre, j'ai évalué si cette simplification était réaliste à travers les modes de reproduction parthénogénétiques chez certains animaux. En effet, la parthénogénèse ayant évolué à partir de la reproduction sexuée et donc de la méiose, de nombreux mécanismes parthénogénétiques sont en fait des modifications de méiose (Ramesh *et al.* 2005). Cela peut impliquer des conséquences génétiques très différentes de celles de la clonalité, bien que certaines forment de méioses modifiées peuvent également mener à la clonalité. De plus, de récentes preuves ont révélé des formes non clonales de reproduction chez plusieurs espèces autrefois considérées comme clonales, et de nouveaux résultats théoriques mettent en évidence des différences évolutives potentiellement importantes entre les asexués clonaux et non clonaux. En réalité, le problème pourrait être plus global et concerner beaucoup plus de taxons. Dans le chapitre 1 de cette thèse, nous avons ainsi pu mettre en avant les raisons méthodologiques et conceptuelles menant à un biais de perception fort dans lequel l'asexualité est assimilée à la clonalité. Nous avons présenté les différentes voies par lesquelles la clonalité peut émerger chez les animaux. Nous avons également cherché les preuves de clonalité couramment utilisées dans la littérature en soulignant les facteurs de confusion et les biais de perception potentiels. Les résultats de cette synthèse montrent que bien que de nombreux asexués semblent clonaux, une grande partie d'entre eux n'est pas strictement clonale. Ces petites divergences peuvent néanmoins avoir un impact important dans l'évolution de la sexualité. L'absence possible d'asexués se reproduisant par mitose ainsi qu'une sélection préférentielle des modes de reproduction qui miment la clonalité, indiquent que, sur une échelle de temps évolutive, les espèces clonales aujourd'hui ne l'ont peut-être pas toujours été. Cela suggère que la transition vers un mode parthénogénétique ne devrait pas être associée à l'apparition directe de la clonalité. Par contre il est possible que secondairement, les modes de reproduction asexués aient évolué vers la clonalité qui est certainement moins coûteuse d'un point de vue génétique dans de nombreux cas. Ces conclusions ne soutiennent pas un rôle prééminent de la clonalité dans l'évolution de l'asexualité.

Dans les chapitres 2 et 3, j'ai étudié plus particulièrement les conséquences génomiques de la transition vers la parthénogénèse obligatoire chez mon organisme modèle : *Daphnia pulex.* En effet, au sein de cette espèce, deux types de systèmes de reproduction sont reportés et peuvent coexister (Crease *et al.* 1989 ; Hebert *et al.* 1989). Le cycle de vie ancestral est une parthénogénèse cyclique (CP) ; les individus de ces lignées alternent entre plusieurs évènements de reproduction asexuée lorsque les conditions environnementales sont clémentes et un mode de reproduction sexué lorsque ces conditions se dégradent (Hebert 1978 ; Ebert 2005). En effet, la reproduction sexuée est liée à la production d'œufs de diapause encapsulés dans un ephippium afin de résister aux mauvaises conditions environnementales (Hebert 1978 ; Ebert 2005). Certaines lignées se reproduisent par parthénogénèse obligatoire, les œufs de diapause sont donc également produits par parthénogénèse (Omilian *et al.* 2006). La parthénogénèse, que ce soit lors de la formation d'un ephippium ou non, se réalise par une modification de méiose qui a pour conséquence génétique celle de la clonalité (c'est-à-dire une transmission du génome maternel sans recombinaison ni ségrégation, (Zaffagnini and Sabelli 1972 ; Hiruta *et al.* 2010). De plus, les lignées parthénogénétiques peuvent produire des mâles rares capables de méiose fonctionnelle pendant la

spermatogénèse (Xu *et al.* 2015). Ces mâles peuvent donc se reproduire avec des femelles sexuées, et transmettre les gènes d'asexualité obligatoire (Innes and Hebert 1988). Lors de ma thèse, j'ai réalisé de tels croisements entre lignées sexuées (parthénogénèse cyclique) et asexuées (parthénogénèse obligatoire) ainsi que des croisements contrôles entre lignées sexuées (c'est-à-dire les lignées CP). Les descendants issus de ces croisements, ou F1, ont été maintenus en laboratoire, chaque descendant constituant une nouvelle lignée clonale. En utilisant l'approche de séquençage d'ADN associé à un site de restriction (RADseq), nous avons séquencés les parents et les descendants d'un croisement contrôle («sex-sex») et d'un croisement entre femelles sexuées et mâles («sex-asex»).

Dans la littérature, on suppose souvent que la parthénogenèse obligatoire (OP) évolue par une perturbation de la méiose et de la recombinaison. *Daphnia pulex*, est justement l'un des exemples emblématiques qui semble soutenir ce point de vue (Hebert 1981 ; Innes and Hebert 1988). En effet, un gène candidat a été identifié avec une mutation qui est censée perturber la recombinaison dans les lignées OP (Eads *et al.* 2012). Pourtant, de rares mâles OP, qui sont génétiquement identiques aux femelles OP et donc porteurs de la même mutation, sont capables de réaliser une méiose fonctionnelle pendant la spermatogenèse.

Dans le chapitre 2, nous avons examiné si la recombinaison est supprimée dans ces méioses. Plus précisément, nous avons étudié le taux et les patrons de recombinaison des mâles OP et nous avons également contrôlé s'il existe des différences de recombinaison spécifiques au sexe (appelé aussi hétérochiasmie) chez les CP. En utilisant les données génomiques des croisements, nous avons pu construire des cartes génétiques spécifiques à chacun des sexes. Les résultats de ce chapitre montrent qu'aucune différence majeure n'a été révélée entre les taux et les patrons de recombinaison entre les sexes de CP, ni entre les lignées de mâles OP et CP. Ainsi, la recombinaison n'est pas supprimée chez les mâles OP, invalidant l'hypothèse d'un suppresseur de méiose responsable de l'OP chez *D. pulex*. Nous avons également discuté des hypothèses alternatives quant à l'évolution de l'OP à partir d'une CP. Plus particulièrement, dans les cas où l'état ancestral du système de reproduction est une parthénogénèse cyclique. Ainsi, nous avons souligné une voie alternative possible : comme les CP contiennent déjà une phase parthénogénétique, les transitions de CP vers OP pourraient avoir évoluées sans altération des processus de méiose, mais simplement en étendant ou en utilisant de manière exclusive cette phase parthénogénétique vers la phase antérieurement sexuée.

Dans le chapitre 3, j'ai plus particulièrement étudié les nouvelles lignées générées grâce à l'asexualité contagieuse. Dans certains taxons, de nouvelles émergences de lignées asexuées sont possibles grâce à l'asexualité contagieuse : grâce aux mâles rares produits par les lignées asexuées obligatoires, ces derniers peuvent transmettre l'asexualité à de nouvelles lignées en se reproduisant avec des femelles sexuées (Simon *et al.* 2003). Avec un tel scénario, on suppose souvent que l'asexualité peut être transmise intacte aux nouvelles lignées hybrides (Joshi and Moody 1995 ; Engelstädter *et al.* 2011). Nous avons étudié en détail si l'asexualité est en effet transmise de manière fidèle dans de tels croisements. Plus précisément, nous avons déterminé les modes de reproduction des F1 produits par le croisement « sex-asex ». Alors que la lignée parentale asexuée se reproduit par parthénogénèse obligatoire et de manière clonale, nous avons été surpris par la grande diversité des modes de reproduction des F1. Nous n'avons pas trouvé de classes discrètes de F1 c'est-à-dire les classes sexuées ou asexuées. Au contraire, certains F1 semblent être capables de se reproduire à la fois de manière sexuée et asexuée. De plus, lorsque ces F1 sont capables de se reproduire de manière asexuée (environ 20 % des F1), ils ne se reproduisent pas de manière clonale, comme le montre la perte fréquente d'hétérozygotie parmi leurs descendants parthénogénétiques. Nous avons également constaté que ces F1 sont difficiles à produire et ont des taux de fertilité fortement réduits, en particulier pour les F1 asexués par rapport aux lignées naturelles. Cela indique que la valeur sélective initiale de ces lignées asexuées produites par contagion peut être faible. Ces résultats nous ont incités à vérifier si les asexués

de la nature ne pouvaient pas également se reproduire de manière sexuée. Nous avons mis en évidence que ces asexués qui ont été définis comme des parthénogénétiques « obligatoires » peuvent néanmoins rarement se reproduire de manière sexuée. L'ensemble des résultats de ce chapitre indiquent que l'asexualité n'est pas transmise intacte par des croisements « contagieux » comme supposé dans la littérature (Crease *et al.* 1989 ; Paland *et al.* 2005). A l'inverse, nos résultats montrent que ces croisements produisent des nouvelles lignées dont les modes de reproduction sont très divers, non binaires et non clonaux, et sur lesquelles une sélection ultérieure peut agir.

L'ensemble des résultats des chapitres 1 et 3 démontrent l'importance d'étudier les premières étapes dans l'évolution de l'asexualité car les asexués échantillonnés dans la nature ne représentent qu'un sous-ensemble sélectionné des meilleures lignées et ne peuvent donc pas contenir toute l'information sur les propriétés des nouveaux asexués. Notamment, la recombinaison peut être encore présente chez les nouveaux asexués (chapitres 1, 2 et 3) et peut entrainer un fardeau génique fort en révélant les mutations délétères (Archetti 2004). D'ailleurs, nous avons mis en évidence une valeur sélective bien réduite des nouveaux asexués générés par « contagion » (chapitre 3). L'évolution de l'asexualité pourrait alors être coûteuse, si seuls certains types de reproduction asexuée sont sélectionnés du fait de leur fardeau génétique moindre, comme la clonalité (Archetti 2004 ; Engelstädter 2008, 2017). Il est alors essentiel d'inclure dans les modèles théoriques, des modes de reproduction non-clonaux, afin d'avoir une vision plus réaliste de l'évolution de l'asexualité.

Evolution d'un nouveau chromosome sexuel

Lors d'un évènement de reproduction sexuée, il y a fécondation entre gamètes produits généralement par deux sexes. Le sexe des individus peut être déterminé soit par l'environnement (ESD), soit par leurs gènes (GSD). Le déterminisme génétique sexuel (GSD) peut évoluer à partir de l'ESD via un état intermédiaire dans lequel l'ESD et le GSD coexistent dans la même population. De telles populations mixtes ESD/GSD sont trouvées chez plusieurs espèces de daphnies dont *Daphnia magna*. L'état ancestral est une ESD où les individus peuvent être mâles ou femelles ; les individus avec ESD sont donc capables de produire des mâles (MP) en réponse à des signaux environnementaux (Kleiven *et al.* 1992 ; Innes and Dunbrack 1993 ; Ebert 2005 ; Fitzsimmons and Innes 2006). En laboratoire, la production de mâles peut également être artificiellement induite en exposant les mères à une hormone juvénile (Olmstead and Leblanc 2002). Les individus ayant une GSD sont des femelles génétiquement déterminées, appelées « NMP » et ne produisent pas de mâles (Innes and Dunbrack 1993 ; Innes 1997 ; Tessier and Cáceres 2004). Cela fait de *Daphnia magna* un des rares modèles animal pour l'étude des transitions évolutives de l'ESD vers la GSD. Le polymorphisme est causé par une grande région non recombinante (la « région NMP »), héritée à la manière d'un chromosome sexuel W (Galimov *et al.* 2011 ; Reisser *et al.* 2017). Bien que cette région ait été localisée, les gènes et les mécanismes moléculaires impliqués dans l'évolution de la GSD à partir de l'ESD restent inconnus. Afin de mieux comprendre comment le phénotype NMP est régulé, nous avons effectué une analyse transcriptomique chez des femelles MP et NMP dans des conditions de contrôle (lorsque les femelles produisent des femelles) et sous traitement hormonal (induisant la production de mâles chez les MP, ou pas, chez les NMP). Cette étude a montré que les phénotypes MP et NMP présentent un nombre substantiel de gènes différentiellement exprimés même en l'absence de traitement hormonal (environ 100 gènes), notamment dans la région NMP. De plus, lorsque les femelles sont soumises au traitement hormonal, beaucoup plus de gènes ont changé leur expression chez les femelles NMP (600) par rapport aux femelles MP (100), avec une surexpression de ces gènes presque systématique chez les NMP. Ces observations suggèrent que le phénotype NMP n'est pas déterminé par une simple mutation de « perte de fonction ». Au contraire, l'homéostasie de la production de descendants femelles lorsque les mères sont soumises au

traitement hormonal, semble être un état actif, étroitement régulé par des mécanismes complexes impliquant de nombreux gènes. De plus, la nature complexe des patrons d'expression génétique qui permettent le maintien de la production de femelles suggère que l'évolution du phénotype NMP n'est pas un événement très récent et que la région du nouveau chromosome sexuel (la région du chromosome NMP) n'est pas très jeune et qu'elle a probablement subi des changements secondaires, y compris des mutations éventuellement antagonistes du sexe et une suppression de la recombinaison. D'un point de vue plus général, cela illustre que ce système de transition vers l'évolution de la GSD, bien que potentiellement initiée par une seule mutation, conduit probablement déjà à une régulation secondaire impliquant de nombreux gènes et mécanismes moléculaires.

Conclusion

Les systèmes de reproduction sont beaucoup plus labiles qu'on ne l'admet généralement. En effet, chez les animaux, les modes de reproduction sexués ou asexués peuvent se comporter comme des traits plastiques. Par exemple, l'asexualité peut ré-évoluer vers la sexualité (Domes *et al.* 2007 ; Christiansen et Reyer 2009). De même, la détermination du sexe est également connue pour être très plastique (Barske et Capel 2008 ; Quinn *et al.* 2011). Par exemple, chez les poissons, plusieurs systèmes de détermination génétique du sexe sont présents chez une même espèce de cichlidés (Moore *et al.* 2021). Pour rejoindre les propos que Barrett (2002) a tenu sur les systèmes de reproduction des plantes à fleurs, cette thèse souligne que les animaux aussi présentent une remarquable variabilité tant dans l'évolution de leurs systèmes de reproduction. A l'heure actuelle, les études sur les transitions des systèmes de reproduction à laquelle ma thèse de doctorat a contribué, sont de plus en plus considérées car elles permettent de fournir des éléments essentiels sur leur évolution.

Curriculum vitae

Cécile MOLINIER - PhD student in Evolutionary biology

Education

2018-21 PhD student, Centre d'Ecologie Fonctionnelle et Evolutive (CEFE), Montpellier, supervisors: Haag Christoph & Lenormand Thomas - *Transitions between reproductive systems in Daphnia.*

2017 Master degree 2, Ecology, Evolution, Genomics (M2 EEG), Lyon (France)- Rank: $\frac{2}{16}$

2016 Master degree 1, Biology Ecology and Evolution (M1 BEE Darwin), Montpellier (France)- Ranks: $\frac{10}{14}$; $\frac{8}{14}$

2013-15 Bachelor degree, Ecology and Biology of Organisms (L3 EBO), Montpellier (France)- Ranks: $\frac{4}{194}$; $\frac{11}{198}$; $\frac{1}{90}$; $\frac{16}{92}$

2013 1st year of CPGE in Biology – Joffre High school (France)

2012 Scientific Baccalaureate with highest honor- Jean Vilar High school (France)

Research experiences

2018 (2,5 mos) Collaboration at the Arizona State University (ASU) in Dr. Lynch's group - *Association mapping on a population of Daphnia pulex in which individuals with environmental or genetic sex determination occur.*

2017 (3 mos) Fixed-term contract for the M2 internship prolongation at the CEFE, supervisor: Christoph Haag.

2017 (5,2 mos) M2 internship at the CEFE of Montpellier, supervisor: Christoph Haag - *Evolution of gene expression during a transition from environmental to genetic sex determination in Daphnia magna.*

2017 (3,5 mos) M1 internship at the Institut de Recherche pour le Développement (IRD), Montpellier, supervisor: Valérie Poncet - *Detection of selection signals in wild populations of Coffea canephora in tropical Africa*.

2015 (2 mos) Internship at the Centre de Biologie et de Gestion des Populations (CBGP-INRA), Montpellier- supervisor: Réjane Streiff *Implementation and monitoring of survival and development (time, weight) in reciprocal transplantation of Ostrinia nubilalis and Ostrinia scapulalis larvae on corn and artemisia.*

International peer-review publications

Boyer, L.*, **Molinier, C.***, Lenormand, T. & Haag, C. R. **[*in prep.*]** Questioning the preeminence of clonality among parthenogenetic animals. ***Authors contributed equally to this work***

Molinier, C., Haag, C. R., & Lenormand, T. **[*in prep.*]** Asexuality is not faithfully transmitted by contagion in *Daphnia pulex*.

Molinier, C., Haag, C. R., & Lenormand, T. **[*ready to submit*]** No support for a meiosis suppressor in *Daphnia pulex*: Comparison of linkage maps reveals normal recombination in obligate parthenogenetic males.

Molinier, C.*, Boyer, L.*, & Villamil, N.*. (2021). Digest: Evolution and maintenance of androdioecy in a haplodiploid insect. Evolution. ***Authors contributed equally to this work***

Ye, Z., **Molinier, C.**, Zhao, C., Haag, C. R., & Lynch, M. (2019). Genetic control of male production in *Daphnia pulex*. Proceedings of the National Academy of Sciences, 116(31), 15602-15609.

Molinier, C.*, Reisser, C. M.*, Fields, P. D., Ségard, A., Galimov, Y., & Haag, C. R. (2019). Evolution of gene expression during a transition from environmental to genetic sex determination. Molecular biology and evolution, 36(7), 1551-1564. ***Authors contributed equally to this work***

Molinier, C.*, Reisser, C. M.*, Fields, P., Ségard, A., Galimov, Y., & Haag, C. R. (2018). Identification of general patterns of sex-biased expression in *Daphnia*, a genus with environmental sex determination. G3: Genes, Genomes, Genetics, 8(5), 1523-1533. ***Authors contributed equally to this work***

Scientific communications

Molinier C., Haag C. R., Lenormand T. (2021). *Contagious asexuality in Daphnia pulex reveals a surprising diversity of reproductive modes.* **Talk** at the satellite symposium of the ESEB Congress "Genomic signatures and consequences of sex and asexuality".

Molinier C., Haag C. R., Reisser C., Fields P., Ségard A., Galimov Y. (2018). *Evolution of gene expression during a transition from environmental to genetic sex determination in Daphnia magna*. **Talk** at the 25th EMPSEB (European Meeting of PhD Students in Evolutionary Biology), 26 May -1 June 2019 in Pedrógão Pequeno, Portugal.

Molinier C., Haag C. R., Reisser C., Fields P., Ségard A., Galimov Y. (2018). *Evolution of gene expression during a transition from environmental to genetic sex determination in Daphnia magna*. **Poster** at the CNRS, Jacques Monod Conference "Sex uncovered: the evolutionary biology of reproductive systems", 23-27 April 2018 in Roscoff, France.

Reviewer and outreach contributions

2020 Reviewed for Freshwater Biology (1)
2019 Reviewed for BMC Genomics (1)
2019 Cover image for MBE (Molecular Biology and Evolution) journal
2018 Organizing committee of the non-permanent scientific day at the CEFE

Teaching and supervision experiences

2018-19 48h of graduate teaching (Bachelor 1 and 3). Fundamental concepts in evolutive ecology, Animals and plants development, Mendelian genetics
2021 (5,5 mos) Supervision of Cassandra Clément (1st year master's student)
2021 (3 mos) Supervision of Victor Lezaud (1st year master's student)
2020 (1 mo) Supervision of Héloïse Calzan (3rd year bachelor's student)
2020 (4 mos) Supervision of Pauline Guérin (2nd year master's student)

Fellowships

Funding of mobility (3000 €) from the Mediterranean Centre for Environment and Biodiversity Laboratory of Excellence (CeMEB LabEx), France

Scientific skills

Data-acquisition:
- Experimental design conception,
- Field and lab experimentations: husbandry and handling *Daphnia sp*., cross-mating, hormonal protocol
- Molecular biology: primer design, DNA extraction, PCR, genotyping using microsatellites, libraries construction

Computing:
- Linux exploitation system
- Programming software: R, bash
- High sequencing data handling: RAD-Seq, RNA-Seq, Pool-Seq

Analyses:
Differential gene expression analysis (DEseq2, WGCNA), genetic map construction (LepMap3, LepAnchor), QTL mapping (RQtl), Population genetic structure (Bayesian classification methods, GWAS), Model constructions (GLMM, Mathematica)