Insights into the Maintenance and Consequences of Asexual Reproduction in Rotifera

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1 Summary

Despite much theoretical work, the evolution and maintenance of sexual reproduction in light of its apparent costs is not understood [1]. For instance, asexual reproduction possesses a large short-term advantage because only one individual is required for reproduction, whereas sexual reproduction requires two distinct sexes (typically in different individuals), of which only the female sires offspring. Consequently, sexual individuals need to produce twice as many offspring as do asexual ones to achieve the same per capita reproductive output [2]. In addition to these two-fold costs, recombination during sex (via meiosis and gametic fusion) potentially disrupts gene combinations that had proven to be fit in the current environment insofar as these parental individuals were able to reproduce [3]. In addition, the need to find a mate, the inherent energetic costs [4] and the risks of mating (e.g., higher predation risk [5] or sexually transmitted diseases [6]) increases the relative cost of sex. Yet, despite these costs, sexual reproduction is predominant among metazoans, whereas asexual reproduction is scattered among these sexual species, typically in younger, species-poor clades [7]. The implication from this pattern is that asexual reproduction has re-evolved independently on multiple occasions, with the resulting asexual species being of generally recent origin, having short evolutionary lifespans, and not giving rise to successful, species-rich clades. Thus, the lack of segregation and recombination associated with asexual reproduction might have, despite the short-term advantages of this strategy, serious long-term disadvantages, including accumulated mutations (which are often deleterious by chance) [8,9] or difficulty in adapting to changing conditions [10,11].

Generally lacking in attempts to answer the question of why sex has flourished are empirical data to test the many theoretical hypotheses that have been proposed [12] and, to turn things around slightly, why asexual reproduction so comparatively limited in its frequency and distribution is. In this context, Rotifera, a clade of small (semi-) aquatic metazoans, are a group ideally suited for empirical investigations in this area. They represent a phylum of small, fast reproducing animals that display a range of evolutionarily stable reproductive strategies that range from obligate sexuality in Seisonidea (only 3 species) to facultative asexuality in Monogononta to obligate asexuality in Bdelloidea [13]. The latter clade is the best-known exception to the rule that asexual species tend to be short-lived and do not give rise to species-rich clades [14,15,16,17,18] and, as such, have attracted much research attention.

This dissertation includes empirical experiments that try to elucidate the limits of asexual reproduction, primarily in the asexual bdelloid rotifers, but also in the facultative sexual monogonont rotifers. In particular, in manuscripts 1 and 2, I question whether bdelloids are truly asexual or if they instead possibly employ an alternative way to exchange genes, one that might help to explain their anomalous long-term survival and species richness. Nevertheless, because all evidence for gene exchange in bdelloids indicates it to be limited, either in occurrence-frequency and/or in the amount of exchange, purging deleterious mutations could still represent a major threat for these animals and in manuscript 3 I examined how bdelloids cope with UVB-irradiation that can potentially induce such mutations. Finally, in manuscript 4 I tested which of a suite of

environmental conditions, including environmental change itself, induce sexual reproduction in the facultative asexual monogononts to show when sex might be favoured as a reproductive strategy. In the following, I summarize the major finding in brief.

A possible alternative to sexual reproduction for exchanging genetic information is horizontal gene transfer as, for example, employed by bacteria [19]. Strong indications for the presence of this route in bdelloids are given by recent evidence of genes from “alien” (i.e., non-rotifer) species in their genomes [20,21,22]. Because these studies used genetic sequence data as evidence supporting horizontal gene transfer, only alien genes could be detected because of their high degree of divergence from genes in the host genome. However, although the methods used would be unable to detect it, these results do not preclude that horizontal gene transfer might also occur among more closely related bdelloid species. Instead, indirect evidence for the latter could be gained by elucidating if DNA is taken up at all and then from which donor species and subsequently by monitoring if, as expected by theory, there are heritable fitness effects after the incorporation of the foreign DNA into the host genome. In manuscript 1, I indeed show that radiolabelled DNA was taken up actively by the bdelloid *Philodina roseola* but not by the facultative sexual *Brachionus rubens*. Additionally, long-term retention of the DNA by *P. roseola* correlated roughly with the phylogenetic relatedness of the donor species, with monogonont DNA being purged after only a few hours. Crucially, in combination with a desiccation event, this DNA increased the variance of the reproductive output of the F1 (as expected by sexual theory) and also ameliorated the negative effects of DNA-lesions caused by UVB-irradiation that the animals were otherwise unable to repair (see manuscript 3). In other words, DNA is not being taken up accidentally as was assumed and, in combination with a desiccation event, has heritable fitness effects that resemble segregation and introgression of new genome parts as found in sexual reproduction. Finally, the same effects were also observed when *P. roseola* individuals were desiccated in groups without the addition of any DNA, which indicates that they not only take up environmental DNA but may also act as donors of it, a crucial perquisite for a regular horizontal transfer of conspecific genes. Thus, incorporation of DNA into the bdelloid genome appears to be connected to the important physiological changes in these (semi-) aquatic bdelloids to survive desiccation [23]. Specifically, via the double strand DNA breaks that occur at this time (and are subsequently repaired), it appears that foreign DNA present in the animals can also be incorporated.

Independent of this result, it was also proposed previously that the rebuilding of the genome after desiccation generally acts as an important check-up to prevent its degradation (visualized as a constant decrease in fitness) that occurs in constantly hydrated bdelloids [24,25]. Because bdelloids were desiccated in groups in these two studies, I tested the hypothesis whether desiccation alone is sufficient to explain this regeneration effect or whether it instead derives from instances of horizontal gene transfer that I propose occur at this time. My results in manuscript 2 show that desiccating *P. roseola* individuals in isolation had a negative effect on their fitness, reducing it to a consistent, possibly baseline level. By contrast, desiccation in the presence of other individuals had a variable, sometimes positive effect on their fitness, indicating that gene-fragments are incorporated not only in the germ-line cells (as required for heritable changes; see

manuscript 1), but apparently also in the somatic cells. Interestingly, the degradation of the fitness of the constantly hydrated individuals expected from the results in [24,25] was not observed, indicating that might not apply to *P. roseola* and/or can be prevented by rigorously selecting the fittest individuals each week, something that is likely to occur in nature as well.

In manuscript 3, I tested how mutations (DNA lesions) induced through UVB-irradiation [26] are handled by the asexual *P. roseola*, especially in comparison to the facultative sexual *B. rubens*. I found that although *P. roseola* was better shielded against UVB-irradiation, the DNA damage that occurred at high UVB-intensities could not be repaired and resulted in reduced reproductive output before and especially after desiccation. Given that desiccation is otherwise the time when bdelloids are thought to repair any damage to the genome [24,25], this latter result was unexpected. These results were in strong contrast to those obtained from *B. rubens*, which lacked effective protection against irradiation, but repaired the resulting DNA lesions quickly and without any apparent negative effects. Consequently, *P. roseola* does not appear to be suited to open water were UVB-intensities are high, but is better adapted to other, more weakly exposed habitats (e.g. in mosses or deeper in the water column because water effectively attenuates UVB-irradiation) to be protected from any damage. In addition, shielding against UVB-irradiation rather than repairing it subsequently might be the most effective strategy to prevent UV-damage to the genome during desiccation, a time when it might be needed the most and with no opportunity to repair it during the physiological preparations needed for anhydrobiosis.

Finally, in manuscript 4, I examined the combination of environmental variables (stressors) that induce sexual in a facultative sexual rotifer (*B. rubens*). In all treatments, most, but not all, of the offspring was asexual, and an increase in temperature, decrease in food quality and the combination of both did not increase the investment into sexual reproduction. Based in part on the observation that challenging low food quality conditions yielded the fewest sexual offspring in this species, I suggest that the physiological condition of facultative sexual plays an important, but as yet unappreciated role in the shift to sex. This constraint in facultative sexual rotifers at least reflects some of the increased costs of sexual reproduction, which by being connected to a resting stage has higher energetic costs (e.g., provisioning of resting eggs) as well as reduces short-term fitness compared to continuing asexual reproduction.

In summary, the long-term existence of bdelloid rotifers without sexual reproduction [27] appears to be explained in part by cryptic genetic exchange via the horizontal transfer of genes from closely related species and conspecific individuals, with the same mechanisms occasionally resulting in accidental (?) transfer from alien species [16,20,21,22]. Together with their ability to survive desiccation via anhydrobiosis, this mechanism might be a necessary adaption to the semi-aquatic (i.e. periodically dry) habitats often inhabited by bdelloids [24] and many other species of rotifer as well. Indeed, the bdelloid strategy might represent an extreme solution to the difficulty for rotifers to predict the optimal time point for reproducing sexually (i.e., shortly before conditions deteriorate to such a degree that asexual reproduction becomes a literal dead end). As shown by facultative sexual monogonont rotifers like *B. rubens*, a bet-hedging strategy is employed where there is a continuous production of some sexual offspring that is accompanied by considerable asexual reproduction. However, the potential for obligate asexuality to arise has been observed in several

monogonont species if conditions remain constant over long times and the sexual resting stage is not needed [28,29]. By contrast, the asexual phase can also be shortened considerably if the duration of favourable conditions is very short [30] or can even be lost entirely if the species are parasitic (host-parasite coevolution [31]) and restricted to small hosts like in Seisonidea [32].

Consequently, this PhD dissertation provides new insights as to when and how rotifers reproduce asexually versus sexually and underscores that rotifers are ideally suited to empirical work on the prevalence and evolution of sexual reproduction. Contrary to previous assumptions, I indicate that bdelloids might be not obligate asexuals, but require (cryptic) recombination from time to time, something that has been shown for many other species assumed to be purely asexual (see [33,34]). However, in this case, sexual reproduction is not employed, but a slightly different mechanism that resembles the horizontal gene transfer in bacteria, which itself is thought to be an important driver of adaptive diversity in these organisms [19]. Altogether, my findings reinforce that some form of genetic recombination appears to be necessary for most (all?) species to survive because of the long-term disadvantages of asexual reproduction (e.g., accumulation of deleterious mutations or reduced ability to adapt to new conditions over the long term). Nevertheless, the short-term advantages of asexual reproduction make it well suited to rapidly increase the number of individuals (including some selection among these) and reinforce the puzzle as to why it is not more common in the animal kingdom, even if only in concert with a facultative sexual lifestyle.

1.1 Zusammenfassung

Trotz intensiver (vielfach theoretischer) Forschung ist die Evolution und der Erhalt von Sexualität vor dem Hintergrund der damit verbunden Kosten ein ungelöstes Rätsel der Evolutionsbiologie [1]. Asexuelle Reproduktion birgt kurzfristig einen großen Vorteil, da nur ein einzelnes Tier erforderlich ist um erfolgreich Nachkommen hervorzubringen, während bei sexueller Reproduktion zwei zueinander passende Geschlechter (meist in unterschiedlichen Individuen) nötig sind. Da nur das Weibchen eines sexuellen Paares Nachkommen hat, müsste es doppelt so viele Nachkommen hervorbringen wie ein asexuelles Individuum um die gleiche Nachkommenzahl pro Kopf zu erreichen [2]. Zusätzlich zu diesen zweifachen Kosten kann Rekombination (teilweise) diejenigen Genkombinationen zerstören die dem Elterntier eine erfolgreiche Reproduktion ermöglichten [3]. Die Suche nach einem geeigneten Partner birgt ebenfalls Kosten [4] und Risiken, u.a. die erhöhte Chance auf die Erbeutung durch Räuber [5] oder die (sexuelle) Übertragung von Krankheiten [6]. Trotz dieser Kosten ist sexuelle Reproduktion bei Metazoen weiter verbreitet als asexuelle Reproduktion, die sich auf dazwischen verstreute, zumeist evolutionär jüngere und artenarme Gruppen beschränkt. Dies impliziert die mehrfache, voneinander unabhängige Entstehung asexueller Reproduktion, die jeweils nicht weit zurückliegt und somit wenig erfolgreiche und artenarme Gruppen hervorbringt. Das Fehlen von Segregation und Rekombination bei asexueller Reproduktion hat somit neben den kurzfristigen Vorteilen der schnellen und unkomplizierten Reproduktion scheinbar einige langfristige Nachteile, welches die Schwierigkeiten mit der Handhabung von Mutationen (oftmals nachteilig) [8,9] oder die Adaptation an sich ändernde Bedingungen [10,11] einschließt.

Zum Verständnis warum Sex so weit verbreitet ist fehlen oftmals empirische Untersuchungen um die vielen theoretischen Überlegungen zu verifizieren, bzw. um zu erklären warum asexuelle Reproduktion trotz der (zumindest kurzfristigen) Vorteile so selten ist. Das (semi-) aquatische Metazoen-Taxon Rotifera erscheint geeignet um eben jene durchzuführen, da diese kleinen, sich schnell reproduzierenden Tiere verschiedene Reproduktionsstrategien aufweisen. Diese reichen von rein sexueller Reproduktion der Seisonidae (nur 3 Arten), über die fakultative asexuelle Reproduktion der Monogononta bis hin zur rein asexuellen Vermehrung der Bdelloidea. Letztere sind die am besten untersuchte Ausnahme von der Regel, dass asexuelle Taxa nur kurze evolutive Zeitspannen überdauern und artenarm sind [14,15,16,17,18].

Diese Doktorarbeit beinhaltet Experimente die dazu dienen die Grenzen asexueller Reproduktion, vor allem innerhalb der obligat asexuellen Bdelloiden aber auch der fakultativ asexuellen Monogononten, empirisch zu testen. In Manuskript 1 & 2 habe ich untersucht ob Bdelloiden wirklich asexuell sind oder auf alternativem Wege Gene austauschen, was das lange Überleben dieser artenreichen Gruppe erklären könnte. Wenn sich die Asexualtität bestätigt oder dieser Genaustausch nur gering ist (selten und/oder Austausch von wenig Material), dann sollten Mutationen ein großes Problem für Bdelloide darstellen. In Manuskript 3 habe ich untersucht wie die Bdelloiden mit potenziell mutationsauslösender UVB-Strahlung fertig werden. Darüber hinaus testete ich in Manuskript 4 ob Umweltveränderungen sexuelle Reproduktion bei den

fakultativ asexuellen Monogononten auslösen, um herauszufinden wann sexuelle Reproduktion gegenüber asexueller Reproduktion bevorzugt wird. Im Folgenden sind die Ergebnisse kurz zusammengefasst.

Eine mögliche Alternative zum Genaustausch während der sexuellen Vermehrung stellt der horizontale Gentransfer dar, welcher z.B. von Bakterien genutzt wird [19]. Mögliche Hinweise auf diese Alternative könnten die aktuellen Nachweise fremder Gene im Genom von Bdelloiden sein [20,21,22]. Da diese Studien Sequenzdaten nutzten, konnten nur Gene von entfernt verwandten Arten als nicht zum Genom gehörig erkannt werden, was nicht ausschließt, dass auch Gene von nah verwandten Arten aufgenommen wurden. Indirekte Hinweise auf die Aufnahme von Genen nah verwandter Arten können gewonnen werden, wenn man sich anschaut ob und von welchen Arten DNA von Bdelloiden aufgenommen wird und ob dies, wie nach theoretischen Überlegungen zu erwarten, zu messbaren erblichen Veränderungen führt. Im ersten Manuskript konnte ich zeigen, dass in der Umgebung vorhandene DNA von dem Bdelloid *Philodina roseola* (im Gegensatz zum fakultativ sexuellen Art *Brachionus rubens*) aktiv aufgenommen wurde, wobei nur nahverwandte DNA langfristig gespeichert und die DNA von Monogonten zum Teil schnell wieder abgeben wurde. Die aufgenommene DNA erhöhte nach Austrocknung in der folgenden Generation die Varianz in der Nachkommenzahl oder milderte die negativen Effekte von DNA-Läsionen die durch UVB-Strahlung erzeugt wurden (vergleiche Manuskript 3) und hatte somit erbliche Folgen. Somit wird die DNA nicht zufällig aufgenommen und hat nach einer überstanden Austrocknung Auswirkungen auf die nachfolgende Generation, die den Auswirkungen von sexueller Reproduktion gleichen. Gleiches trat auf, wenn *P. roseola* in Gruppen (ohne weitere DNA) gehältert wurde, was zeigt, dass sie nicht nur DNA aufnehmen sondern auch abgeben können. Dies ist eine wichtige Voraussetzung für einen gerichteten horizontalen Transfer von arteigenen Genen. Der Einbau von der DNA erfolgte nur in Zusammenhang mit Austrocknung, welche bdelloide Rotiferen in Anhydrobiosis zu jeder Zeit überleben können [23]. Austrocknung ist in dem Zusammenhang von Bedeutung da hierdurch Doppelstrangbrüche erzeugt werden, welche während ihrer Reparatur den Einbau der aufgenommen DNA ermöglichen.

Obwohl diese Austrocknung sicherlich zu Doppelstrangbrüchen im Genom führt, wurde angenommen, dass sie eine wichtige Kontrollfunktion erfüllt, da sie den Rückgang der Fitness verhinderte, der bei kontinuierlich in Wasser gehälterten Tieren auftrat [24,25]. Da die Austrocknung in diesen Versuchen in Gruppen erfolgte, habe ich im 2. Manuskript überprüft ob die Austrocknung eines Einzeltiers oder der Austausch mit anderen Tieren via horizontalen Gentransfer dafür benötigt wird (vgl. Manuskript 1). Austrocknung allein hatte einen negativen Effekt, so dass in allen Versuchen nach Austrocknung ein zwar konstanter aber niedriger Reproduktionserfolg gemessen wurde. Die Anwesenheit anderer Individuen beeinflusste dieses Ergebnis (oftmals positiv) und war mit einer gewissen Varianz zwischen den Versuchen verbunden, was nahelegt das aufgenommene Genfragmente nicht nur in die Zellen der Keimbahn (wie die erblichen Veränderungen in Manuskript 1 zeigen) sondern auch in Körperzellen eingebaut werden. Interessanterweise kam es nicht wie erwartet [24,25] zu einer Reduktion der Fitness in den konstant im Wasser gehälterten Tieren, was entweder an der anderen untersuchten Art lag und/oder vermutlich durch die wöchentliche Selektion der fittesten Tiere erreicht wurde, wobei

letzteres wahrscheinlich der Situation in natürlichen Populationen nahe kommt.

In Manuskript 3 habe ich getestet wie die asexuelen *P. roseola* mit Mutationen (DNA-Läsionen) umgeht, die mit Hilfe von UVB-Strahlung erzeugt wurden [26] und habe diese Ergebnisse mit den fakultativ sexuellen *B. rubens* verglichen. Obwohl *P. roseola* teilweise gegen Schäden durch UVB geschützt war, traten bei hoher Strahlungsintensität einige Schäden auf, welche nicht repariert wurden. Diese Schäden verschwanden zwar nach Austrocknung, führten aber davor und vor allem danach zu einer Reduktion der Nachkommenzahl. Dies erstaunt vor dem Hintergrund der Annahme, dass Bdelloiden von der Austrocknung und der damit verbunden Reparatur profitieren sollten [24,25]. Im Gegensatz dazu fehlte bei *B. rubens* zwar ein vergleichbarer Schutz, aber die entstandenen Schäden konnten schnell und ohne Auswirkungen auf die Nachkommenzahl repariert werden. Diese Ergebnisse legen nahe, dass *P. roseola* für eine oberflächennahe Lebensweise ungeeignet ist, aber in geschützteren Bereichen (UVB-Strahlung nimmt mit der Tiefe das Wassers stark ab oder wird durch z.B. durch Moos vorher abgeschirmt) gar keine Schäden bekommt und somit keine reparieren können muss. Weiterhin ist eine aktive Reparatur während die Tiere ausgetrocknet sind nicht möglich, so dass zu diesem wichtigen Zeitpunkt nur die Abschirmung von UVB-Strahlung möglich ist.

Abschließend untersuchte ich in Manuskript 4 wie neue Temperatur-, Futterverhältnisse und die Kombination von beidem die sexuelle Reproduktion der fakultativ asexuellen *B. rubens* Individuen beeinflussen. Der größte Anteil des Nachwuchses blieb in allen Versuchen asexuell, wobei die Versuche mit schlechterer Futterqualität den wenigsten sexuellen Nachwuchs hervorbrachten. Darauf aufbauend denke ich, dass die Physiologie der fakultativen sexuellen Tiere eine wichtige Rolle bei der Induktion von sexueller Reproduktion spielt. Dieser Zusammenhang spiegelt die Kosten sexueller Reproduktion wieder, welche durch das damit verbundene Überdauerungsstadium und damit auch dem Verlust kurzfristiger Fitness gegenüber durchgehender asexueller Reproduktion erhöht sind.

Zusammenfassend scheint das langfristige Überleben der bdelloiden Rotiferen ohne sexuelle Reproduktion [27] teilweise durch den horizontalen Transfer von Genen nah verwandter (und unabsichtlich? entfernt verwandter) Arten ermöglicht zu werden [16,20,21,22]. Zusammen mit der Fähigkeit austrocknen zu können, mag dies eine notwendige Anpassung an periodisch austrocknende Habitate sein, welche oftmals von Bdelloiden bewohnt werden [24]. Auch andere Rotiferen leben dort oder in anderweitig periodisch unbewohnbaren Habitaten. Die Lebensweise der Bdelloiden ist eine extreme Strategie in Bezug auf die Schwierigkeit für die Tiere den optimalen Zeitpunkt für (pseudo)sexuelle Reproduktion vorauszusehen (kurz bevor die asexuelle Reproduktion zusammenbrechen würde und somit eine Sackgasse wäre). Fakultativ sexuelle Monogononte wie *B. rubens* nutzen dafür eine bet-hedging Strategie, welche einen (möglichst) großen Anteil asexuelle Reproduktion mit einer fortwährenden Produktion sexuellen Nachwuchs verbindet. Falls aber die Bedingungen lange unverändert bleiben, verringert die Produktion des Überdauerungsstadiums das Populationswachstum und langfristig können sich Klone durchsetzen die sich rein asexuell vermehren [28,29]. Wenn aber die Bedingungen nur kurzzeitig gut sind, kann die Phase der asexuellen Vermehrung stark verkürzt werden [30]. Ganz fehlt sie bei den Seisonidea [32], die einerseits parasitisch sind (Koevolution mit dem Wirt [31])

und nur wenig Raum zur schnellen Ausbreitung haben, welche ihnen asexuelle Reproduktion ermöglichen würde.

Insgesamt gibt die vorliegende Doktorarbeit neue Einblicke wann und wie sich Rotiferen asexuell bzw. sexuell vermehren und unterstreicht das Rotiferen interessante Modelorganismen zur Klärung der weiten Verbreitung von sexueller Fortpflanzung sind. Im Gegensatz zu der bisherigen Meinung, dass bdelloide Rotiferen rein asexuell sind, konnte ich zeigen, dass auch diese ab und an Rekombination brauchen (etwas was auch für andere scheinbar obligat asexuelle Arten zutrifft [33,34]). Dabei erfolgt der Genaustausch nicht durch sexuelle Reproduktion, sondern ist mit dem horizontalen Gentransfer in den Bakterien vergleichbar, wo er scheinbar ein wichtiger Faktor für die Diversifizierung dieser Organismen ist [19]. Insgesamt unterstreichen meine Ergebnisse, dass ein gewisser genetischer Austausch für fast alle Arten notwendig ist, um die Nachteile asexueller Reproduktion auszugleichen (wie die Anhäufung von Mutationen oder die Schwierigkeiten sich ändernden Umweltbedingungen anzupassen). Allerdings ist die asexuelle Reproduktion gut geeignet, um schnell eine große Anzahl von Individuen zu erzeugen die (inklusive möglicher Selektion zwischen diesen), was die Frage aufwirft warum sie nicht weiter verbreitet ist, zumindest im Zusammenspiel mit fakultativ sexueller Fortpflanzung.

2 Introduction

Among Metazoa and within most major groups within it, sexual reproduction involving meiosis, recombination, and the combination of gametes is the norm [35]. At the other extreme, purely asexual reproduction is extremely rare, with those lineages in which it occurs tending to be scattered among Metazoa, species poor, and short-lived [7]. An intermediate strategy that alternates between asexual and sexual reproduction ("heterogony") is more common (e.g., in some cladocerans, hemipterans, hymenopterans, nematodes, and rotifers), but is still comparatively rare [33,36,37].

Both the evolution of sex and its predominance among Metazoa has long confounded evolutionary biologists in light of its apparent costs compared to asexual reproduction ([2,3] and see below). An interesting exception to the predominance of sexual reproduction is Rotifera [38], a phylum of microscopic aquatic or semi-aquatic invertebrates encompassing about 2000 known species [39,40]. A comprehensive general introduction to this group can be found in Wallace *et al.* [13] and is summarized here. The majority of species inhabit freshwater, but some also occur in brackish water or in saltwater [41]. Body size typically ranges from 100 to 1000 µm, although the largest species can surpass 2000 µm and so are just visible with the naked eye. Size is constrained ultimately by eutely with individuals in each species possessing roughly 1000 cells [42] and also by the jaw apparatus, which is fully formed within the egg and does not grow further following hatching [43]. Their fast reproduction makes them well suited for experiments in the laboratory. First offspring in some species can be produced within a day after hatching and population densities can double within 9 h [44]. Thus, even experiments covering many generations are easily tractable.

In rotifers, obligate sexual reproduction is only found within a single family (Seisonidae) comprising three species, with the two major clades instead displaying purely asexual reproduction (Bdelloidea) or heterogony (Monogononta) [13,38]. In researching asexual reproduction generally, a great deal of attention has been focused on bdelloid rotifers. During more than 300 years of investigation, there has been no evidence of sex, meiosis, males or hermaphrodites [23,27]. Yet, the clade is conservatively estimated 35 million years old [45] and more than 450 extant (morpho-)species are described in this group [46]. Given that reproductive maturity occurs on average at five days of age [47], this asexual clade survived an impressive number of generations. How bdelloids could successfully escape the long-term disadvantages that are thought to be associated with asexual reproduction (e.g., the accumulation of deleterious mutations over time or "Muller's ratchet" [48] or adapting to environmental changes [10,11]) for such an extended timeframe remains unanswered. The ancient status of bdelloids and the species richness of this monophyletic clade, underlined by various studies in favour of their possessing true asexuality, makes bdelloids ideal candidates to understand how asexual reproduction could endure over long timescales [7,34,49] and how asexual species even diversify [46,50] or to indicate that they might also represent the proverbial exception that proves the rule.

However, this focus on bdelloids tends to disguise the fact that the prevalence of asexual

reproduction among rotifers is through heterogony much higher than in any other metazoan group, justifying an expanded approach across the group. In this dissertation, I examine what the consequences of asexual reproduction are within the group as a whole. Although my focus often lies with bdelloid rotifers given their extreme expression of this trait, I include comparisons with the heterogonic monogonont rotifers, which reproduce primarily clonally via ameiotic parthenogenesis with intermittent phases of sexual reproduction. In this, I hope to provide a more comprehensive and comparative basis on the potential limits of asexual reproduction, which in turn would help to understand the importance and prevalence of sexual reproduction.

2.1 Advantages of asexual reproduction

Compared to sexual reproduction, asexual reproduction possesses a large short-term advantage given that only one individual is required for reproduction, whereas sexual reproduction requires two sexes that are usually distributed in different individuals. Moreover, because only the female of a sexual couple sires offspring, sexually reproducing individuals need to produce twice as many offspring as an asexual individual to achieve the same per capita reproductive output [2]. In addition to these two-fold costs, there are additional costs to sexual reproduction associated with the obligate need for two sexes. The most general cost is due to genetic recombination potentially disrupting gene combinations that had proven fit in the current environment insofar as its bearers were able to reproduce [3]. Following recombination, the new genotypes are on average less fit, a phenomenon often referred to as “genetic slippage” [51,52]. However, although commonly evoked, there is no simple two-fold cost of meiosis (e.g., from the fact that only half of the maternal genome is represented in the filial generation and is mixed with another half originating the paternal genome). Instead, only those genes encoding for an asexual mode of reproduction (instead of sexual) would evoke a direct cost for sex [53,54] because formerly masked asexual genes could turn some of the offspring to asexuals and thus reduce the number of offspring that continues to reproduce sexually (for a detailed review of this problem refer to [1]).

Finally, there are the numerous additional non-genetic costs not faced by asexually reproducing individuals, ranging from those involved in finding a mate (including the risk of increased predation in so doing [5]) to the energetic costs of mating itself [4] to the risk of sexually transmitted diseases [6] and to the possibility of sexual conflict in species with separate sexes or mating types [55]. All these missing costs and missing requirements for asexual reproduction result in the obvious advantage of rapid reproduction that can successfully proceed in a newly colonized habitat from just one individual.

2.2 Asexual reproduction as a long-term strategy?

Despite this obvious advantage, the phylogenetic distribution of asexual animal species is "twiggy", comprising relatively short branches and/or species-poor lineages scattered among the much more predominant sexual species [7,56]. The implication here is that asexual reproduction has re-evolved independently on multiple occasions, but that the resulting asexual lineages are generally of recent origin, with short evolutionary lifespans, and do not give rise to successful, species-rich clades [56].

Possible explanations for this observed pattern are two serious long-term disadvantages entailed by strict asexual reproduction. Without sexual recombination followed by selection, deleterious mutations cannot be removed from the gene pool of an asexual species and will progressively accumulate in and burden the genome of most individuals (known as Muller's ratchet [9,48]). This process should lead ultimately to the more rapid extinction of asexual species, thereby continuously limiting their number in nature. Nonetheless, because this is a long-term effect, it does not limit the number of asexual species over the short term and is only truly problematic if deleterious mutations occur above a certain threshold level. Otherwise the ratchet works either too slowly or not at all [48,57]. However, because the rate of deleterious mutations seems to differ among species, there is no simple threshold to determine the limits of sexual versus asexual reproduction across all Metazoa or even within the major groups within it [12,58,59].

Associated with and extending this long-term disadvantage is the strict tie between individual mutations with their entire genetic background because the lack of segregation and recombination cannot move these mutations to other backgrounds as is the case with sexual reproduction. This problem results in a reduced ability to adapt to changing environments [10,11], something that again is thought to limit the number of existing asexual species over time. In addition, not only are changes in the direction of selection hard to track for asexual species, these changes could also accelerate the pace of Muller's ratchet [60].

In contrast to these classical explanations for the comparative rarity of asexual reproduction among metazoans, other explanations exist that do not require high extinction rates for asexual species. For example, Schwander and Crespi [56] showed using a neutral model that a twiggy phylogenetic distribution of asexuality might result if sexual reproduction is the ancestral state (see below) and if stable asexuality is not reached often and/or speciation rates within asexual species is low. An alternative explanation also based on a neutral process might be through equilibrium clonal turnover between neutral losses (e.g., ones not caused by Muller's ratchet) and new origins of asexual clones [61]. Interestingly, this process might explain why old asexual lineages tend to be spatially separated from the sites of origin of new asexual clones or, in other words, from their sexual ancestors. However, it must be pointed out that these old lineages might also be affected in part by clonal decay and also deviate from neutral processes [62].

Another alternative reason reviewed by [56] might be that asexual species are favoured in regions with higher extinction probabilities. For instance, locally co-adapted gene complexes might be

preserved better by asexual clones in habitats where species show low productivity. By contrast, these complexes would be disrupted too often by recombination and segregation, especially if there is input from habitats with higher productivity where other genes are favored [63]. Asexuality and its associated higher productivity might thus promote a higher colonization ability of lower-quality habitats because fitness does not have to go through the bottleneck experienced by sexual species due to inbreeding [64]. Associated with this process is the fact that asexual species are often of hybrid origin [65] that might arise at the edges of the distribution of a sexual species. Thus, the resulting hybrids, potentially in combination with a polyploid origin, might represent new well adapted lineages for the intermediate habitats that are less suitable for their sexual parental species. These hybrid lineages (and their adaptive gene complexes in particular) are then stabilized by asexuality [56] and, in turn, might be less well adapted to the main habitats of the parental species resulting in their comparably small distributions [66,67]. As such, these lineages also face a greater risk of extinction should conditions change, thereby rendering the stabilized gene combinations as unsuitable again.

Consequently, it is not easily inferred from phylogenetic and geographic distributions if long-term asexuality is strongly disadvantageous or not. Thus, investigating existing old lineages that are thought to be asexual, like bdelloid rotifers, can help to understand if asexuality is indeed possible over the long term and, if so, which features are necessary to overcome the long-term disadvantages of it [7]. However, the claim of asexual reproduction is necessarily based on the verification of the absence of sex for which only negative evidence can be gathered [34]. This implies the continuous danger that the asexual status of a species merely reflects our failure to have observed sex in the past, possibly as a result of insufficient sampling of sexual individuals ([33] but compare [68]). This possibility is enhanced by the fact that rare sex is especially hard to identify, but is believed to already entail many of the benefits of sex [69,70,71]. For instance, examples are known from pathogens that show that just one round of sexual reproduction can cause dramatic positive effects, including increasing their pathogenicity dramatically [72,73,74].

2.2.1 Bdelloid rotifers – successful ancient asexuals?

Bdelloids form a monophyletic clade [75] that, based on an amber fossil [45], is at least 35 to 40 million years old; molecular estimates have the clade being twice as old [50]. The continuous absence of evidence for males, male structures, hermaphrodites or meiosis strongly supports the asexuality of the clade in general [23,27]. This alone makes bdelloids appear to be a good candidate for being an ancient asexual taxon [34]. In the following section, I review some additional genomic evidence that also points to the absence of sexual reproduction in bdelloids (although some of this evidence is ambiguous in this regard) before discussing possible strategies bdelloids have employed to survive over the long term.

2.2.1.1 Degradation of the genome as signs for the absence of sex?

One central assumption that tries to explain the overall lack of long-term success of asexuals is that their genome degrades gradually (e.g., through Muller's ratchet). Thus, at some point, the asexual species cannot survive anymore given the few possibilities available to them to undo the accumulated genetic changes and mistakes (see above). The presence of these signs of degradation, which can occur at different levels, can therefore be seen as indirect evidence for the asexual status of a species [34]. By contrast, the absence of such signs in a supposedly asexual species implies the presence of either asexual countermeasures to account for this or of hidden recombination that has been overlooked so far. In this section, I introduce the different signs of genomic degradation that could be expected for asexual species and then discuss them in the light of the results reported for bdelloids to date.

2.2.1.1.1 Degradation of homologous chromosome pairs

Meiosis plays a central role in sexual reproduction and requires the pairing of homologous chromosomes. If meiosis is missing, however, and thus pairing is not required, previously homologous chromosomes could diverge to a point where they are no longer easily identified as a pair [34,76]. Based on the assumption that this form of degradation needs time [76], it was thought at first that the lack of homologous chromosomes might be an indication of ancient asexuality. However, it is now known that the process can happen relatively quickly as well [77]. In bdelloids, the production of oocytes was described to proceed without chromosome pairing or reduction in chromosome number [78,79], but these results should be verified with more modern and exacter methods. In addition, the karyotype studies of Meselson and co-workers were unable to identify homologous chromosome pairs in bdelloids [17,80], although Pagani *et al.* interpreted the karyotypic data of one species in favour of homologous pairs [81]. However, it is generally hard to decide if there are homologous chromosome pairs in bdelloids based on karyotypic studies because the small chromosomes present in these animals often provide only little information to discern or pair them [80,81]. But even if homologous pairs are indeed absent, this situation does not exclude full pairing given that even non-homologous sex chromosomes can pair (at least in some parts) during meiosis [82,83].

2.2.1.1.2 Divergence of alleles from one gene locus

In an extension of the previous point, signs of degradation can be searched for at a finer scale by investigating if the paired alleles at diverse gene loci show a higher sequence divergence in the putative asexual species than in a comparable sexual species [34], where the sequence difference is limited by meiotic recombination. Although automixis and miotic gene conversions could also limit allelic divergence in asexuals, the absence of meiosis generally means that the alleles of a gene locus likely evolve independently from each other in these species such that

the heterozygosity between these alleles will increase [84]. This “Meselson effect” (named after its proposer [34]) is expected to be especially pronounced at neutral sites or sites. In bdelloids, highly divergent copies of two genes investigated, namely *hsp82* and *tpb*, were indeed found in four different bdelloid species [15], with the implication that these divergent genes might be important for diversification within bdelloids [85].

However, in interpreting these data, gene duplications have to be ruled out as an alternative cause for divergent (paralogous) alleles, except in cases where the alleles lie on different chromosomes [86]. This point is especially relevant for bdelloids given the mounting evidence that they might be degenerated tetraploids [16,18,87,88]. Consequently, part of the divergence found in bdelloids could be explained as arising from the divergence of duplicated chromosome pairs. Moreover, as a consequence of the apparent degenerated tetraploidy, the bdelloid genome is now thought to be organized in collinear chromosome pairs [27,89]. Whereas some genes are present on four chromosomes in the same orientation and order, intermediary gene-rich segments also exist that are present for only one of the pairs, but again also displaying conserved gene content, order and orientation.

Data in this latter regard are restricted to the regions around *hsp82* [88] and a histone gene cluster [87] from two lab cultures of *Adineta vaga* and *P. roseola*. Nonetheless, this evidence raises the question as to whether or not these collinear chromosomes indeed represent meiotic homologues. Although rare sex cannot be absolutely excluded for bdelloids and some otherwise highly divergent bdelloid species share virtually identical alleles [90], another explanation for these apparent homologues is that they are highly conserved regions due to their function in the repair of double strand genome breaks that result from the more or less common desiccation events [91].

2.2.1.1.3 Accumulation of deleterious mutations

In contrast to sexual species, which have more options to eliminate deleterious mutations (see below), asexual species are restricted to and reliant upon on back mutations to counteract the accumulation of deleterious mutations [9,48]. Thus, asexuals should possess a higher ratio of mutations leading to amino-acid changes compared to silent changes that do not alter the encoded amino acid. Older studies investigating the accumulation of mutations in bdelloids failed to verify the expected increase in nonsynonymous mutations [92,93], whereas two recent studies could only demonstrate the possibility of an increase using a more elaborate and extensive sampling method [94,95]. However, the latest of these two latter studies also indicated the possible confounding effects of different habitats in that no difference between sexual and asexual species was found when individuals were sampled from the same habitat [95]. A further general difficulty here is that bdelloids as a group diverged a long time ago from the most closely related sexual species that could be used for a meaningful comparison. Thus, further studies using more genes and controlling more carefully for potentially confounding effects (including phylogenetic divergence) are needed to answer the question if bdelloids are indeed accumulating mutations faster as a result of their supposed asexual lifestyle.

2.2.1.1.4 Transposable elements

A final, but slightly different aspect of genetic degradation lies with the presence of transposable DNA elements that are prone to moving horizontally within the genome by either pasting a copy of themselves elsewhere or by cutting themselves out and placing themselves elsewhere. Both forms of “transposition” can cause mutations due to insertions, deletions, and translocations [96,97]. Although transposable elements do spread and persist in sexual species, meiotic recombination is thought to be the only way to limit and remove deleterious transposable elements [98]. Consequently, it is thought that transposable elements might proliferate in an uncontrolled fashion in asexuals, thereby driving them to extinction in the long run [98,99] if they have no alternative way to handle them [34]. It is thus interesting that transposable elements were initially assumed to be totally absent in bdelloids [14]. Although some were found subsequently [100,101,102], they seem to be concentrated in subtelomeric regions leaving gene-rich regions largely unaffected [103]. It is thought that large population sizes might have helped bdelloids to become relatively free of transposons [104]. In addition, purging of these elements might also result from the repair of the double strand breaks in the genome following a desiccation event [89,91], where there is a limit to the number of transposable elements that can be accommodated between the collinear chromosomes [89].

2.2.1.2 Countermeasures of bdelloids to balance the disadvantages of asexual reproduction?

From the evidence above, it is clear that the expected degree of genomic degradation as required for possible indirect evidence for asexuality does not appear to be present in bdelloids. If bdelloids are indeed obligate asexuals and this lifestyle indeed possesses long-term disadvantages, additional mechanisms in bdelloids must exist to explain their long-term survival. This is the topic of the current section.

2.2.1.2.1 Surviving unfavourable circumstances in anhydrobiosis

The ability of most species of this aquatic clade to withstand desiccation via anhydrobiosis is considered to represent one of the most important ecological features underlying their success as asexual species [105,106]. Although the survival rates differ, anhydrobiosis can be entered into and survived at any life stage (e.g. as an egg, juvenile or adult stage) [107]. Survival rates are influenced both by the time the individuals have to adopt the necessary changes and the duration of anhydrobiosis. They decrease massively if the preparation time is short [108] and if the dry period continues for more than several weeks [109]. In addition to merely surviving the hostile conditions, anhydrobiotic individuals can also be dispersed by the wind at this time to potentially found new populations upon awakening. Thus, individuals can, through chance, escape unfavourable circumstances in space and time [110] where their genomes and attendant genetic load might have driven them to extinction otherwise. This escape mechanism was indeed

demonstrated in part with respect to parasitic fungi that are otherwise deadly for infected hydrated bdelloids [111,112]. Experiments showed that uninfected bdelloids in anhydrobiosis could escape if blown by chance to parasite-free sites by artificial winds (escape in space) or if they endured desiccation longer than the fungi, which themselves can also survive dryness for some time without losing any pathogenicity (escape in time).

However, desiccation could also seriously threaten the integrity of the bdelloid genome given that it is shattered to pieces at this time and numerous double-strand breaks need to be repaired. The latter represent an important potential source of harmful mutations that could speed up Muller's ratchet [113]. In this light, it is remarkable that the time spent in desiccation does not appear to count against both the total and reproductive lifespans of bdelloids (the "Sleeping Beauty hypothesis"; [106]) and, even more astonishingly, might be required to maintain fitness, with Ricci et al. [24] indicating a progressive decline in the fitness level of clonal lines of *Adineta ricciae* and *Macrotrachela quadricornifera* that were kept constantly hydrated and that could be restored to a constant level if the individuals were desiccated at least once. In addition, the rapid timescale at which the fitness loss occurred (less than one year), suggests that bdelloids might generally be restricted to desiccation prone habitats [106]. Thus, the well-developed ability of bdelloids to repair double-strand breaks following a desiccation event [89,91], aided by their unique genome structure [87,88], is thought to act as a kind of check-up to maintain genomic integrity [24] (cf., purging of transposable elements above), with the only cost seeming to be that some individuals do not awake from slumber [24,25,47].

2.2.1.2.2 Divergence of genes increasing variation?

The Meselson effect introduced earlier is often seen as a form of genome degradation resulting from obligate asexual reproduction. However, in causing the divergence of ancestral copies of genes, it might also offer the opportunity for functionally divergent copies of genes to evolve. This, in turn, could represent a mechanism to increase variation within the genome of an asexual species, especially in connection with the degenerate tetraploid genome of bdelloids, with the numerous gene copies helping ensure that the original function of the genome remains intact [86,114]. Potentially countering this tendency for alleles and individuals in an asexual population to evolve relatively independently (especially compared to the larger evolving entity facilitated by genetic exchange in sexual species) is the fact that adaptation to niches might restrict the difference between asexual reproducing individuals in a way that lets them also behave as evolving entities [46,92,115].

2.2.1.2.3 Horizontal gene transfer instead of sex?

Based on the presence of ancient alien genes located primarily in the telomeric regions of the bdelloid genome, it was recently hypothesized that bdelloids have undergone horizontal gene transfer at some time in the past [20,21,22]. Moreover, it was assumed that the horizontal gene

transfer took place during or following a desiccation event when the gut membranes are potentially disrupted [22], such that foreign DNA that had entered the organism by accident was incorporated into the genome during the repair of the latter. Although astonishingly high numbers of alien genes are seemingly functional in the bdelloid species *A. ricciae* and consequently might be a helpful genetic resource for them to increase their genetic diversity [20,21], it remains that many negative effects are often associated with incorporating highly divergent DNA (e.g., interference with the needed repair of the double-stranded breaks inflicted by desiccation) [87,88,91,116,117]. But, such negative effects might be outweighed by high individual numbers as each individual is part of the effective population size (being able to reproduce by itself), while severe (i.e. not only slightly deleterious) mutations are especially easy to select against [118].

2.2.1.3 Open questions on bdelloids regarding their asexual lifestyle

Although it is widely assumed to be true, it remains questionable if the extraordinary ability of bdelloids to withstand desiccation in and of itself is really sufficient to have allowed bdelloids to have survived without sexual reproduction for such an extended time span. Specifically, it remains to be explored if desiccation tolerance might instead in combination with degenerate tetraploidy and its greater number of gene copies, the (rare) horizontal gene transfer of alien genes, and the typically large population numbers explain how bdelloids prevent the degradation of their genomes and adapt to their current environment. Additionally, it needs to be investigated if these same factors also explain the high number of bdelloid species (see [46,50]), which contradicts the assumption of lower variation and evolvability of asexual species as outlined above. Altogether, this viewpoint raises three main questions:

1) Is horizontal gene transfer in bdelloids truly a rare, accidental event (potentially implied otherwise by the results in [20]) that typically does not include conspecific genes (as potentially implied in [16,38,90])? If not, could it form the basis for a mechanism of genetic exchange that resembles some aspects of the genetic exchange during sexual reproduction?

2) Is the repair of the broken genome after desiccation really that perfect (i.e., does not itself introducing mutations) and is this process by itself responsible for correcting any mistakes?

3) How do bdelloids prevent or handle the accumulation of deleterious mutations?

2.3 Sexual reproduction in addition to (or instead of) asexual reproduction?

The prevalence of sexual reproduction in metazoans [2,3] together with the potential limits of asexual reproduction outlined above raises the question as to which advantages can be gained by sexual reproduction, especially in light of the costs of the latter. These advantages can be subdivided broadly into two categories. The first category involves the fate of mutations, the majority of which will be deleterious by chance. In contrast to asexual species, sexual species can reduce their mutation loads, and theoretically to the point of recreating mutation-free genomes, via recombination and segregation [8,9] rather than relying on unlikely mechanisms such as back mutation. In addition, recombination and segregation also facilitate those rarer beneficial mutations being freed from genetic backgrounds of lower fitness [119,120]. By contrast, only beneficial mutations that occur in an asexual individual with an otherwise good genetic background can come to fixation within the population.

The second category concerns the generally increased genetic variability of sexual species. Although the breakdown of genetic associations through chromosomal segregation and recombination can be detrimental (the “cost of recombination”), this process also acts to increase genetic variation within a sexual population. In turn, this increased level of standing genetic variation means that sexual species should show a better ability to adapt to changing environments through natural selection as do comparable asexual species. This hypothesis extends not only to unstable or highly variable environments in general, but also to processes such as the Red Queen hypothesis [121], which states that the more genetically uniform populations of asexual species represent an easier target for diseases [35] or parasites [122] than do the more genetically diverse populations of a sexual species.

2.3.1 The evolution of sex in light of its costs

Most research into the prevalence of sex tends to focus on extant multicellular organisms that are most likely derived from sexual ancestors [33,36]. Even so, the historical difficulty in finding a universal, simple answer to explain the predominance of sexual reproduction among metazoans, has recently led to the hypothesis of a pluralistic approach. Briefly, this hypothesis states that no single factor exists that explains the maintenance of sexual reproduction in general, but that a multitude of factors instead are needed, which, in various combinations, explain the maintenance of sex in individual cases [123,124]. This approach thus draws on the diversity of high costs associated with sexual reproduction (and are listed as the advantages of asexual reproduction above) and that the magnitude of the cost of sex is far from being easily determined [1].

One complicating factor in this regard is that, through anisogamy, where the fusing gametes during sexual reproduction are of markedly unequal size [125] (with the sex producing the smaller gametes being defined as the male [35]), males directly invest very little to the zygote. Thus, in cases where the male does not influence the reproductive success of the female (i.e., neither

hinders nor assists the female), the two sexual partners will have only half the reproductive output of two asexual individuals if everything else is equal between both types of females, thereby resulting in the classic two-fold cost of sex. However, males often reduce the costs of sex to the female by providing them with resources during the mating [126,127,128,129], helping to take care of the brood [130,131,132,133], and/or minimizing the incidence of highly variable and thus sometimes relatively unfit offspring through strong selection among males [134,135,136] or their sperm [137,138]. In addition, the different strategies often used by the sexes to optimize their reproductive output (e.g., gamete survival survival via large size versus fertilization success through large numbers; see [139]) can lead to sexual conflict [55,140,141], thereby increasing the costs of sex. Finally, the number of males produced as well as the competition over resources between both sexes can also influence the cost of sex (reviewed in [1]).

Importantly, however, one needs to differentiate between the origin of sex and its maintenance, with the latter being influenced by the former. For instance, if the costs of sex were low at its origin, this would enable it to more easily arise to become the ancestral state of many descendent species, thereby explaining its later prevalence even in the face of rising costs (see above). In addition, both the rising costs and potential adaptations to sexual reproduction (e.g., attraction of partners, guarding of mates and/or securing successfully paternity) might hinder the successful origin of asexual clones from sexual species [142,143].

For the origin of sex, it is assumed that meiosis arose only once [36,144] although its exact origin is controversial (e.g., compare [145,146] and [147,148]). Although meiosis is essential for sex, a prominent cost associated with it is that it takes 5 to 100 times longer to complete than does mitosis in unicellular organisms [149]. Although the increased genetic variation yielded through meiosis is often cited when explaining the predominance of sex, this phenomenon likely only contributed to the subsequent success of sex. By contrast, sex appears to have been used initially as a genetic stabilizing mechanism [146,150,151]. For instance, the necessity for homologous chromosomes having to pair during meiosis restricts the amount of duplications, deletions and larger aneuploid changes (changes in chromosome numbers) that can occur.

This pairing of chromosomes during meiosis also potentially reduces costs associated with recombination, which occurs mainly between neighboring areas carrying homologous genetic information, where it is more unlikely to be disadvantageous. Furthermore, the sites for recombination are often more or less strictly controlled, leading to recombination hotspots and protected functional genomic regions [152,153]. Strict controls might reduce on the one hand unnecessary costs arising from to recombination at important sites (e.g., gene promoter regions as shown in *Mus musculus* [152]) and, on the other hand, maximize the variance in sites where they are more likely advantageous. However, both the mechanisms underlying protected genomic regions and recombination hotspots as well as the effect of these on reducing the costs of sex have only just been started to be explored in detail.

Interestingly, the first sexual lineages were most likely facultative sexual [36] and consequently combined the advantages of both reproductive modes, especially as rare sex entails already many of the advantages of obligate sex [69,154]. Some facultative sexual species are still to be

found among metazoan species (see above) and are even more common among the remaining eukaryotes [36]. These species, which include monogonont rotifers (see below), offer the ability to study when and why the asexual reproduction is replaced by sexual (or asexual) reproduction in one species and thus within the same genetic background. Thus, potential noise from effects/ differences not caused by the reproductive mode *per se* is minimized, even if closely related species are investigated (as in [66,155]), where the slightly different evolutionary histories might have left a different footprint in both. More generally, there is considerable variation in mutation rates within a reproductive mode [59] and the degree of parasites and predators that different species have to cope with often differ as well [156]. Likewise, the natural variance in other parameters of a species niche [157,158,159,160,161,162] can also influence the outcome of experiments based on different species that consider reactions to variable conditions. Finally, "effective" evolutionary time (speed) between species can also differ if, for example, generation times get shorter [163].

2.3.2. Comparisons of sexual vs. asexual reproduction within facultative sexual monogonont rotifer species

Monogonont rotifers normally produce clonal oocytes via ameiotic parthenogenesis, also called amictic reproduction (reproduction of monogonont rotifers is reviewed in [13,164] and is shortly summarized here). However, after the production of some sort of mixis signal, several females will start to produce eggs from which sexually active (mictic) females emerge and themselves produce oocytes via meiosis. If these mictic females are not fertilized by males, they produce haploid male eggs. By contrast, if a mictic female is fertilized by a haploid male before she has started to produce male eggs, diploid resting eggs are formed instead (figure 1). These eggs represent the diapausing stage of monogonont rotifers and typically cannot hatch until after a certain latency period and then only if certain environmental cues (e.g. temperature and light conditions) are met [164]. In rare cases, however, they can hatch with only a minor delay of some days and without conditions changing appreciably [165,166,167].

The identity of the mixis signal is often not known and, interestingly, the signal is not always species specific [168,169,170]. Crowding or changes in food availability, photoperiod or temperature are known to be responsible for triggering its release in some species [164]. The best studied signal is crowding, under which individual animals of different species of *Brachionus* produce a protein that is released into the water and induces sexual reproduction via quorum sensing [168,171,172,173,174,175]. The mixis signals can be viewed as being associated with unfavourable or changing conditions, where it is often produced and released slightly in advance of these conditions. This is of interest in the current context given that the advantages of sex under poor conditions was hypothesized to outweigh even high costs of sex in facultative sexual species [154]. Consequently, investigations into the induction of sex via multiple stressors and especially via the interactions between these stressors will serve to test the pluralistic approach of the maintenance of sex of West *et al*. [123].

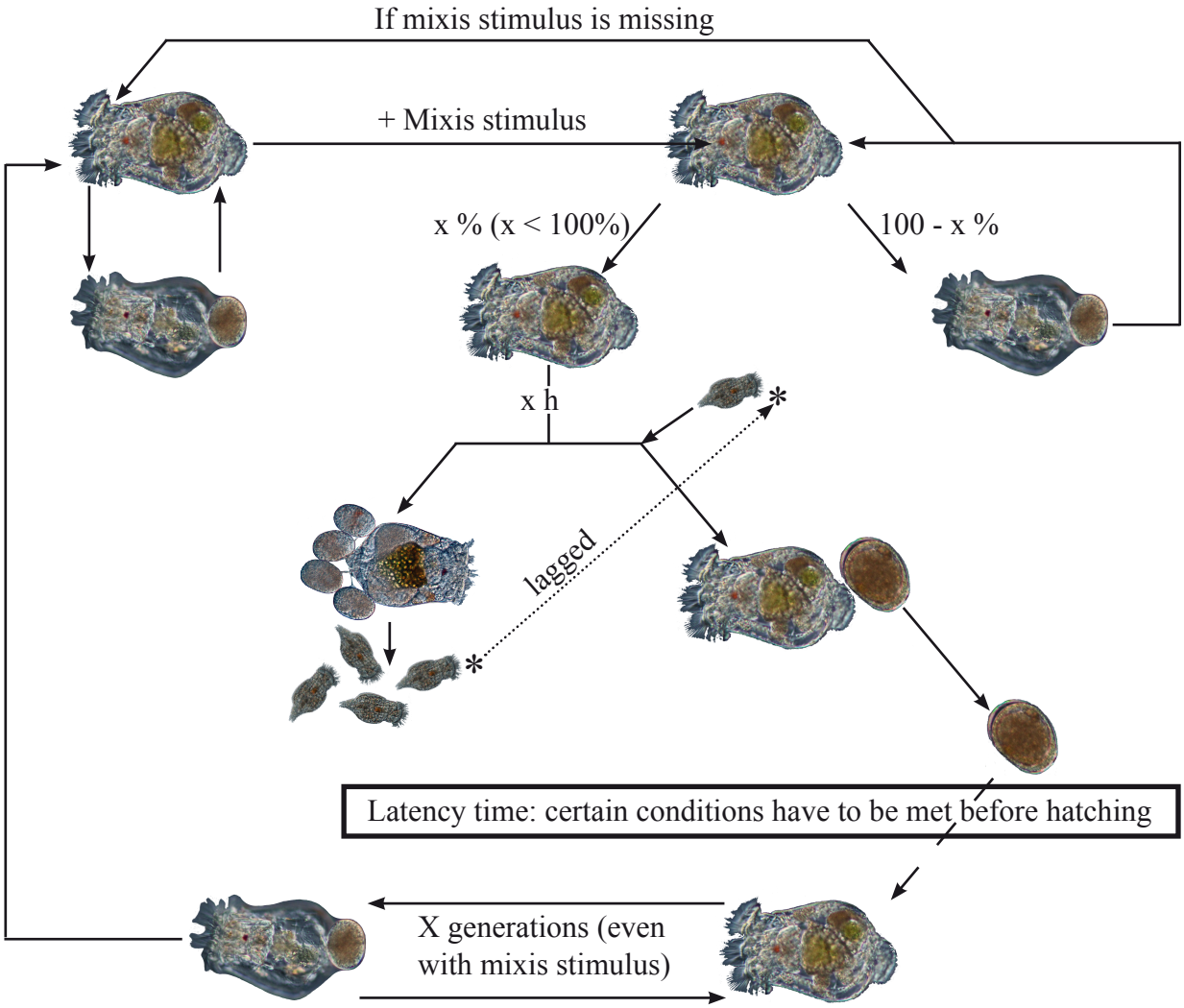

Figure 1. Life Cycle of monogonont rotifers examplified at the example of *B. rubens*. Both parthenogentic (as found in bdelloid rotifers) as well as sexual cycle is depicted

More generally, investigations of facultative sexual (or cyclical parthenogenetic) monogonont rotifers are advantageous in that they enable comparisons of sexual versus asexual reproduction within a single species and, by extension, in clones of a species [176]. Additionally, studying facultative sexual species offers the possibility to research how obligate sexual or asexual species might arise from this reproductive mode or, in other words, how stably maintained both modes of reproduction are within a given species.

Altogether, the issues involved with facultative sexuality (as present in monogonont rotifers) raise the two following questions:

1) Is sex in facultative sexual species a response to challenging and changing conditions and, as the pluralistic hypothesis predicts, is the reaction stronger if different stressors factors interact?

2) Under which circumstances is either sexual or asexual reproduction lost by facultative asexual species?

3 Focus of the dissertation

Empirical work explaining the prevalence of sex is relatively rare compared to the vast body of theoretical work (see above). In this context, rotifers are an excellent taxonomic group because of the range of reproductive strategies present within the group, including an overrepresentation of asexual reproduction [38]. In particular, verifying the ancient asexual status of the species-rich bdelloid rotifers is of vital importance to determine if and how long-term asexuality is possible. Despite the great deal of effort to prove their asexual status [27], any claim of obligate asexuality is, as mentioned, necessarily based on the verification of the absence of sex. A tantalizing possibility that remains to be investigated is that horizontal gene transfer, a documented phenomenon in bdelloids, might also occur between closely related species or conspecific individuals during desiccation and thus form the basis for a mechanism of genetic exchange (as potentially implied by [16,20,38,90]). However, even if there is no regular horizontal gene transfer by bdelloids, it remains that damage to the genome (e.g., via mutation or through the double strand breaks caused by desiccation) need to be repaired. Together, these two themes comprise the first part of my dissertation, with the following questions each treated in a separate subchapter in the form of a journal manuscript in the following chapter 4:

1) Is there evidence for the active uptake of environmental DNA by bdelloids and thus the possibility for genetic exchange based on horizontal gene transfer during desiccation (manuscripts 1 & 2)?

2) How well does desiccation in and of itself rebuild the genome of bdelloids or does the potential uptake and incorporation of environmental DNA also play an important role (manuscript 2)?

3) Does the amount of lesions induced by UVB-irradiation (which can possibly also induce mutations) and their subsequent repair differ between the asexual bdelloid *P. roseola* and the facultative sexual monogonont rotifer *B. rubens*? In addition, what role, if any, does desiccation / sex, respectively, play in the repair of these lesions in these species (manuscripts 3)?

Finally, given the historical difficulties to find a simple, universal answer to the question as to why sexual and not asexual reproduction predominates among metazoans, I conducted experiments with the monogonont rotifer *B. rubens* to test the pluralistic hypothesis to this question [123,124]. Like other facultative sexual rotifer species, individuals of *B. rubens* reproduce primarily, but not exclusively asexually, switching periodically to sexual reproduction according to environmental conditions. Thus, I investigated if there are differences in the percent investment into sex under different stressors as well as in combination of them. Should the effect of multiple stressors, perhaps accelerated by their interaction, induce sex, this would underscore the idea that different factors in combination explain the maintenance of costly sex. This idea is explored in the fourth subchapter of chapter 4:

4) Is increased rates of sex a response to changing and challenging food and temperature conditions in the facultative sexual rotifer *B. rubens* and is the reaction intensified through the combination of these two stressors (manuscript 4)?

In summary, this dissertation tries to establish if bdelloids are indeed asexual using *P. roseola* (figure 2A) as a model system and, if so, how they cope with it over the long term. Supplementing this information are data as to the circumstances under which the monogonont rotifer *B. rubens* (figure 2B) abandons asexual reproduction to switch to sexual reproduction. Given that *Brachionus* sp. are among the best examined species in this regard, my experiments add considerable, comparative data to this question. In general, all experiments seek to elucidate how long and under which circumstances rotifers are able to maintain asexual reproduction, which, in turn, provides important hints toward the question of the prevalence of sex among metazoans.

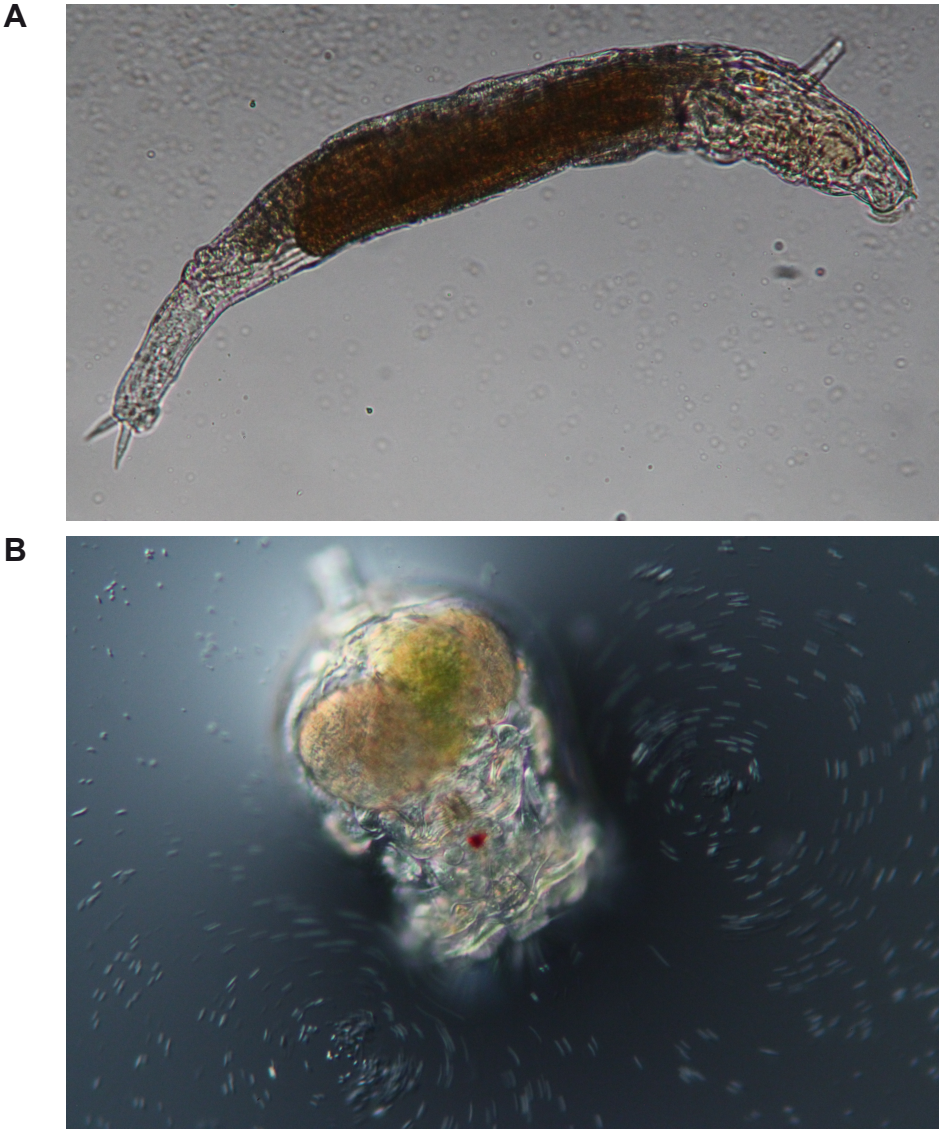

Figure 2. (**A**) *Philodina roseola* female (**B**) *Brachionus rubens* female (Photographs by: Claus Fischer).

4 Manuscripts

4.1 Explaining an evolutionary scandal? A potential process of genetic exchange in the "ancient asexual" bdelloid rotifer *Philodina roseola*

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Abstract

A long-standing evolutionary puzzle is the prevalence of sexual reproduction over the less costly asexual reproduction in multicellular organisms. Nevertheless, obligate asexual reproduction clearly entails competitive disadvantages over time: the phylogenetic distribution of this trait is highly scattered, with asexual lineages being comparatively short-lived and species-poor because they are less able to adapt to changing environments and the accumulation of deleterious mutations increases genetic load. Equally puzzling, therefore, are bdelloid rotifers, an ancient, species-rich clade of aquatic invertebrates lacking sex, males and meiosis. How these "evolutionary scandals" have thrived for so long remains a mystery, although recent studies linking ancient horizontal gene transfer to their ability to withstand desiccation to escape unfavourable conditions hint at a more general system of genetic exchange, including material from conspecific individuals.

We show that environmental DNA is taken up regularly by adults of the bdelloid *Philodina roseola* but not of the facultative asexual monogonont rotifer *Brachionus rubens*. Four additional results both exclude artifactual DNA uptake and suggest that the DNA taken up by *P. roseola* has heritable effects in combination with a desiccation event and could underlie a mechanism of genetic exchange in this species. 1) DNA uptake correlates with the phylogenetic relatedness of the donor species: DNA from more closely related species is retained longer and in greater amounts. 2) Providing undamaged DNA during desiccation to UVB-irradiated individuals partly, but significantly ameliorated the negative effects on the reproductive output of their untreated F1 generation. 3) We observed additional fitness effects in agreement with sexual theory (increased variance of the reproductive output of the untreated F1 generation) when the parental generation underwent desiccation in the presence of environmental, conspecific DNA. 4) These fitness effects also exist when the animals were desiccated in groups (and can therefore act as DNA donors), but not individually, indicating the potential for the mechanism under natural conditions.

Together, our results suggest a mechanism for regular genetic exchange in the bdelloid *P. roseola* that favours closely related taxa. Its dependency on desiccation parallels the switch to sexual reproduction during worsening environmental conditions in facultative asexual species. If similar mechanisms hold across bdelloids, it helps answer how this clade of "ancient asexuals" have flourished for over 35 million years and reinforces that obligate asexuality apparently cannot be maintained over the long term.

Keywords cryptic sexual reproduction, heterogony, horizontal gene transfer, Muller's ratchet, Rotifera, Bdelloidea, desiccation, anhydrobiosis

Introduction

Rotifera is a phylum of approximately 2000 species of microscopic aquatic invertebrates, notable in part for the diverse reproductive strategies present within the group. These strategies characterize the major lineages of rotifers, with the major clades Monogononta and Bdelloidea possessing facultative sexual and obligate asexual reproduction, respectively, whereas species of the genus *Seison*, for which the phylogenetic affinities remain unclear, are obligate sexuals.

In this regard, the asexual Bdelloidea represent a puzzle (or “scandal” in the eyes of John Maynard Smith; see [177]) that has long confounded evolutionary biologists. Although the prevalence of the more costly sexual over asexual reproduction is still not understood fully [34], it remains that strict asexual reproduction entails many disadvantages. For instance, without sexual recombination followed by selection, deleterious mutations cannot be removed by asexual species and will progressively accumulate in and burden the genome as “genetic load”. This process, known as Muller’s ratchet [48], ultimately results in the extinction of asexual species over the mid to long term, thereby continuously limiting their numbers in nature. Furthermore, the close dependency between individual mutations with their genetic backgrounds slows adaption to changing environments [10,11], thereby also limiting the numbers of asexual species over time. Yet, despite hundreds of years of investigation, there has been no evidence of sex, meiosis, males or hermaphrodites [23,27] among any of the more than 450 extant species of bdelloid rotifers [46] and, by extension, among the more than 35 million years of evolutionary history of the group as a whole [45]. With reproduction commencing on average at five days of age [47], this makes an impressive number of asexual generations and begs the question as to how bdelloids have escaped the long-term threats posed by asexuality for so long.Experimental evidence shows that a tendency to obligate asexuality might be present more widely within Rotifera, with this trait occasionally evolving in lab strains of the monogonont *Brachionus calyciflorus* held under constant conditions [29], where an asexual strategy might be more favourable [178]. However, this fact only serves to deepen the mystery over bdelloid rotifers because, through their ability to withstand desiccation (anhydrobiosis) [107], they are common and otherwise well-adapted to highly unstable environments where sexual reproduction is more advantageous. One would instead expect bdelloids to follow a classic heterogonic strategy, with asexual reproduction being used under more constant or favourable environmental conditions before switching to sexual reproduction with the onset of unfavourable or changing conditions [165].

Nevertheless, the ability of most bdelloid species to enter and withstand anhydrobiosis at any life stage [107] is traditionally considered an important feature underlying the success of the group in the face of their asexual lifestyle [105]. They can survive in this dehydrated state for several weeks [109] during which wind-dispersal enables the founding of new populations. In so doing, individuals are able to potentially escape unfavourable circumstances in space and time [110], whereby their genomes and attendant genetic load might have driven them to extinction otherwise [111,112].

More excitingly, it was recently hypothesized that bdelloids have undergone horizontal gene transfer based on the presence of ancient alien genes located primarily in the telomeric regions

of the genome [21,22], with some of these genes retaining their functionality [21]. In conjunction with the previous point, it is assumed that the horizontal gene transfer took place in bdelloids during or following desiccation when the gut membranes are disrupted [22] and the genome, both that of the oocytes as well as of the somatic cells [89], is subject to numerous double stranded breaks that need to be repaired during rehydration. Although the mechanism for the latter process remains unknown, it is believed that the scaffold provided by the degenerated tetraploid genome plays a crucial role [91] and it is conceivable that new, homologous genetic material could also be incorporated at this time by a similar process. Because only genes from distantly related species can be detected easily using sequence-based analyses, it remains a tantalizing possibility that undetected horizontal gene transfer from more closely related species might also occur to potentially form the basis for an unspecific mechanism of genetic exchange (as potentially implied by [16,90]). Importantly, even if the genetic transfer as such remains undetected, sexual theory predicts that it should result in heritable effects in the form of an increased variance in the fitness of the (untreated) F1 generation compared to the parental generation, thereby forming the basis of a testable hypothesis.

In this paper, we provide initial empirical support for potential mechanism of genetic exchange in bdelloid rotifers by showing that *Philodina roseola* is able to take up and, in combination with a desiccation event, incorporate environmental DNA. Although *P. roseola* internalizes both conspecific and “alien” DNA initially, long-term retention of radiolabelled environmental DNA correlates with its similarity to the host DNA, with conspecific DNA being preferentially retained. Importantly, this ability appears to underlie an indirect mechanism of genetic exchange given that the uptake of DNA in the parental generation in combination with desiccation has heritable fitness effects on the untreated F1 generation predicted by sexual theory (i.e., increased variance in fitness compared to the parental generation) and can prevent heritable fitness loss that we induced through UVB-irradiation of the parental generation. Altogether this general mechanism could provide a potential route of genetic exchange in bdelloid rotifers and thereby a possible explanation as to how this clade of ancient asexuals might have escaped being ground down by Muller’s ratchet for so long.

Material and Methods

Rotifer and algal cultures

Our *P. roseola* colony descends from a continuously hydrated culture derived from a single egg in 1989. Thus, although clonal in origin, accumulation of mutations over these ~1500 generations means that some genetic variation should now be present. Our colony was cultured under a 9/15h light/dark regime in ten different 90x15 mm plastic Petri dishes at 20±1°C, with weekly transfers to new Petri dishes. The algae *Cryptomonas sp*. SAG 26.80 was used as food source and was cultivated separately under continuous illumination (13W/840 Osram Daylight neon tubes) in 500ml Erlenmeyer flasks with COMBO medium [179]. A second bdelloid species, *A. ricciae*, was cultured similarly as was the facultative asexual monogonont rotifer *B.rubens*, apart from being fed the algae *Monoraphidium minutum* SAG 243-1; for the latter species only two Petri dishes were used.

Quantifying DNA uptake

The ability of *P. roseola*to uptake conspecific DNA was tested in parallel in both hydrated individuals as well as those desiccated for one week (= Protocol D of Ricci et al.[180]). Tubes were incubated for either 3 h (10 tubes each) or 15 h (30 tubes each). Twenty batches of 80 individuals of *B. rubens*, each similarly exposed to radioactive DNA for either 3 or 15 h, were used as a control. Thereafter, the differential ability of *P. roseola* to uptake alien DNA was tested in hydrated individuals using DNA from *B. rubens* (for 1.5 or 15 h) and *A. ricciae* (15 h) as well as from the additional monogonont species *E. dilatata* (15 h; individuals obtained from the Ems-Jade Channel near Mariensiel, northwest Germany, N +53.0991, E+8.1003). For each donor species, tests were run in parallel using the uptake of radioactive *P. roseola* DNA as a reference point (15 tubes each of 75 individuals).

All source DNA was obtained from batches of 70 individuals, with algae and other contaminants filtered out using a 60 µm mesh. DNA was extracted using a 300 µl digestion solution of 10% Chelex (Biorad) and 0.07 µg μl^{-1} proteinase K. Samples were incubated at for 30 min 55°C, followed by a 10-min heat-inactivation step at 95°C before being cooled for at least 30 min at 5°C. Following sedimentation of Chelex at 15000 rpm for 15 sec, the clear supernatant was transferred into sterile Eppendorf tubes and stored at -18°C until use. Twenty five nanograms of DNA was labelled in vitro using the standard assay from the High Prime DNA Labelling Kit (Roche) using [alpha-^{32}P]-dCTP (Hartmann Analytic); unincorporated dNTPs were removed with Sephadex G-50 (fine) QuickSpin columns (Roche).

For all experiments, labelled DNA with around 25000 counts min^{-1} (roughly 1 µl) was added to 2 ml test tubes (Qiagen) each containing 75 individuals of *P. roseola* in 200 µl algae-free COMBO medium; this procedure also served to reanimate the desiccated individuals. At the end of the respective incubation times, tubes were placed on ice for 10 minutes and non-internalized DNA was washed out subsequently. Following centrifugation for 6 minutes at 0 °C and 9500 rpm using a Heraeus Biofuge fresco, all but 20 µl of fluid in a test tube was removed and 400 µl fresh medium (without DNA or algae) was added. Centrifugation and subsequent washing was repeated 6 times. After the last centrifugation step, 300 µl of digestion solution instead of medium was added and DNA was extracted as described above. Radioactivity was measured in a Wallac© 1415 Scintilation counter using 20 ml PE-vials (Perkin Elmer) containing 200 µl of the supernatant from the DNA extraction mixed with 5 ml LumaSafe (Lumac LSC). For each test, two tubes containing only COMBO-medium and marked DNA were used to control for background radioactivity that could not be removed by washing.

Quantifying reproductive output (figure 3)

Using the number of eggs laid as proxy for fitness, we tested if incorporated DNA influences the variance of the reproductive output of the offspring as predicted (i.e., is inheritable). Individuals of *P. roseola* were desiccated either in groups with no additional DNA or individually with 350 ng bare *P. roseola* DNA added to the culture medium before desiccation. For both trials, offspring from continuously hydrated parents were used as reference with additional controls for the effects of DNA addition without desiccation (hydrated individuals with DNA) and for desiccation itself

(desiccated individuals without DNA). For each treatment, control and reference group, 96 eight-day old adult individuals formed the parental generation. They were maintained in 24-well flat-bottom plates (Falcon© 353935) with an algal density of around 10^6 cells ml^{-1} as necessary. Plates were desiccated for 7 days in custom-built humido-thermostatic chambers. One egg from each parental individual contributed to the tested F1 generation, where number of eggs laid was counted every 24 h and removed.

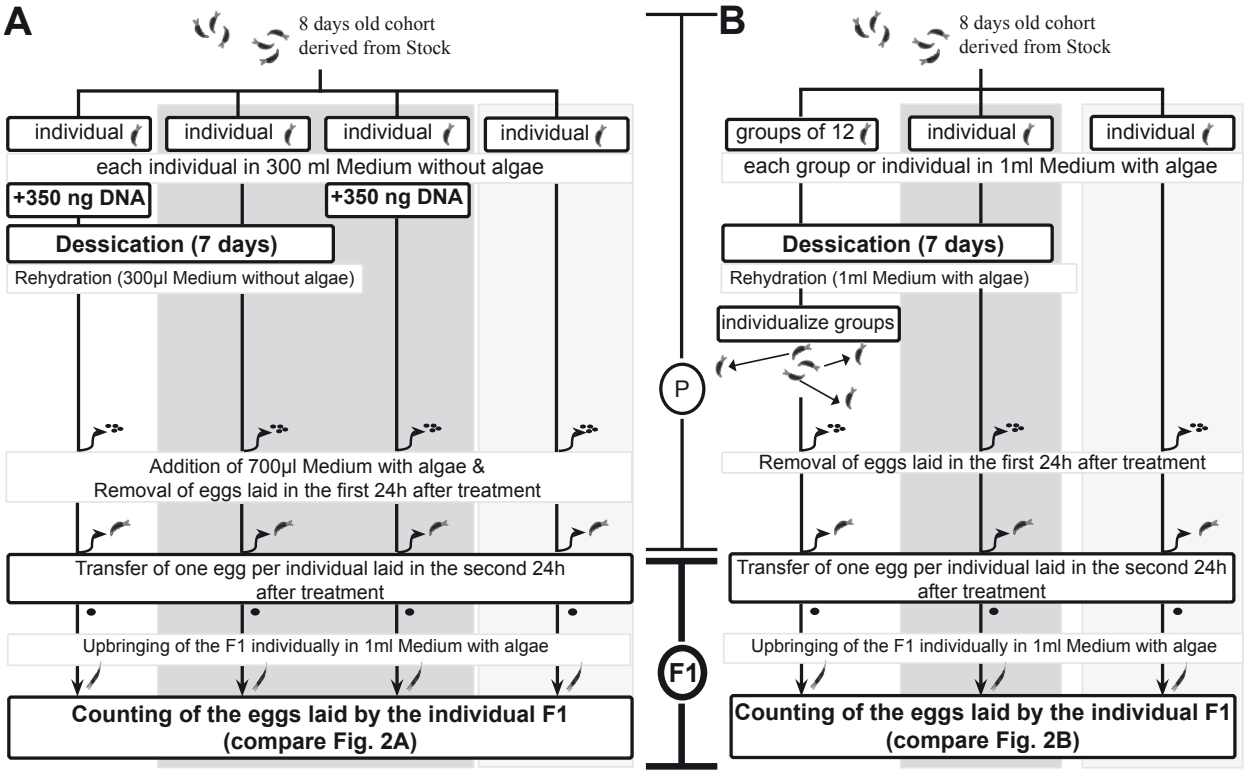

Figure 3. Experimental procedure to quantify changes in reproductive output of the F1 generation of Philodina roseola. The parental generation was either (**A**) desiccated individually with bare *P. roseola* DNA added or (**B**) desiccated in groups with no DNA added. Controls (shaded grey): constantly hydrated *P. roseola* with DNA added (A only) and individually desiccated *P. roseola*. Reference (shaded light grey): constantly hydrated *P. roseola*.

UVB-irradiation experiments

As an additional, more direct test for any heritable effects of DNA uptake, we also performed UVB-irradiation experiments that we showed elsewhere to have negative effects on the reproductive output of the untreated F1 generation of *P. roseola* [181]. In particular, we compared two groups of individuals, both of which were exposed to UVB-radiation and subsequently desiccated, but with only one group being desiccated in the presence of undamaged DNA.

We seeded four populations (two per treatment), each with 50 individuals, in Petri dishes with a diameter of 5.5 cm and a depth of 1.3 cm and 15 ml COMBO medium with algae. In addition to the normal illumination, each population was exposed over a course of 10 days to UVB-irradiation in four 15-minute time blocks per day, each interspersed by an hour of normal light. UV-irradiation was provided by a Fisher bioblock scientific VL-6.LM UV-lamp with an intensity of 3.5 W m^{-2} UVB at 312 nm as measured with a USB 2000+ U-Vis fiber-optic spectrometer (Ocean Optics).

Thereafter, each population was desiccated in groups for seven days in custom-built humido-thermostatic chambers, with either 600 ng of undamaged DNA or 15 µl Chelex-supernatant without DNA being added to each of two Petri dishes. One day after rehydration, all living adult individuals were transferred to fresh Petri dishes containing 15 ml COMBO medium with algae. After four days, 48 newborn individuals from each Petri dish were transferred individually into separate wells of a 24-well flat-bottom plate containing 1 ml COMBO medium with algae. The numbers of eggs laid within the first five reproductive days by these F1 individuals were counted every 24 h and removed.

Results and Discussion

Differential DNA uptake according to phylogenetic relatedness

Continuously hydrated individuals of *P. roseola* display a remarkable ability to take up DNA, one that is consistently shown in all trials even after comparatively short incubation times (e.g., 1.5 or 3 hours, figure 4 & table 1). This finding is in stark contrast to that for the monogonont rotifer *Brachionus rubens*, which did not internalize any DNA even after 15 hours. The ability to take up DNA is also present, albeit reduced, in *P. roseola* individuals that were previously desiccated for one week before being reanimated in the presence of environmental DNA (figure 4A & table 1).

Table 1. Pairwise comparisons of the uptake of radioactively marked DNA by *Philodina roseola*.

Comparison of hydrated *P. roseola* + *P. roseola* DNA with:	**Time [h]**	**Paired *t*-test (two-sided) of the amount of radioactive DNA taken up**	**Contingency table for uptake frequency in test / reference (each *df* = 1 in all cases)**
Desiccated *P. roseola* + *P. roseola* DNA	3	$t = 2.416$, $df = 9$, $P = 0.039$	7 / 10, $\chi^2 = 3.529$, $P = 0.060$
	15	$t = 4.352$, $df = 29$, $P < 0.001$	30 / 30, $\chi^2 = 0$, $P = 1.000$
P. roseola + *B. rubens* DNA	1.5	$t = -0.725$, $df = 14$, $P = 0.480$	14 / 15, $\chi^2 = 1.035$, $P = 0.309$
	15	$t = 3.855$, $df = 14$, $P = 0.002$	7 / 15, $\chi^2 = 10.909$, $P < 0.001$
P. roseola + *E. dilatata* DNA	15	$t = 2.333$, $df = 14$, $P = 0.035$	8 / 15, $\chi^2 = 9.130$, $P = 0.003$
P. roseola + *A. ricciae* DNA	15	$t = 0.589$, $df = 14$, $P = 0.565$	13 / 15, $\chi^2 = 2.143$, $P = 0.143$

Statistical tests compared both the number of trials in which DNA was taken up as well as the amount taken up in terms of amount of radioactivity measured (see also figure 4).

Here, DNA was taken up in fewer trials (although not significantly fewer) compared to hydrated individuals and always at significantly reduced levels. Taken together, our results for these two species show that DNA uptake appears to be occurring actively at all adult life stages in *P. roseola* and that DNA is not simply entering by chance sometime during the desiccation process.

DNA uptake appears to be unselective initially (figure 4B & table 1), with hydrated individuals of *P. roseola* internalizing conspecific (*P. roseola*) and alien (*B. rubens*) DNA equally after 1.5 hours, both in terms of frequency and amount. Thereafter, however, alien DNA appears to be preferentially removed (or conspecific DNA preferentially retained). Thus, after 15 hours of incubation, both the number of trials in which *B. rubens* DNA was detected in *P. roseola* as well as the overall amount were significantly reduced compared to values for conspecific DNA after the same time or to the initial values for *B. rubens* DNA after 1.5 hours. Similar results to those for *B. rubens* DNA were also found for donor DNA from a second monogonont species (*Euchlanis dilatata*) after 15 hours. By contrast, uptake parameters compared to conspecific DNA were not significantly reduced for donor DNA from another bdelloid species (*Adineta ricciae*).

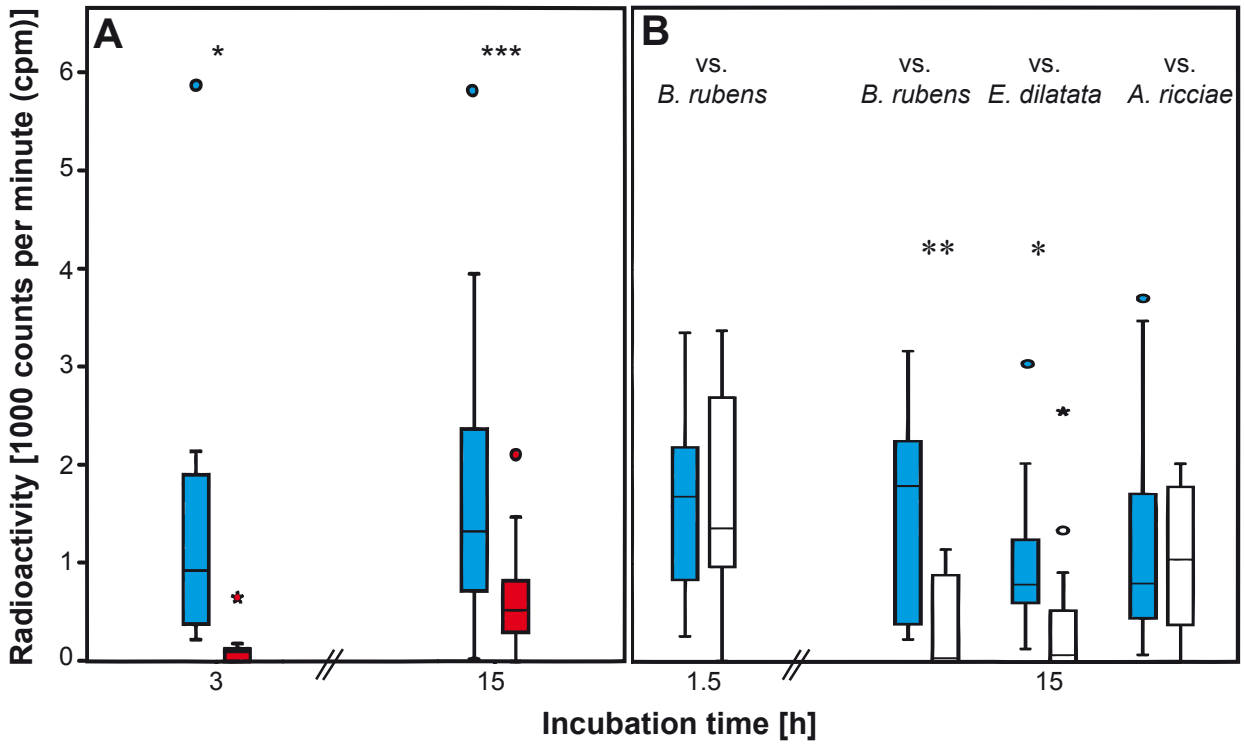

Figure 1. Pairwise comparisons of the uptake of radioactively marked DNA. (**A**) Uptake of conspecific DNA by desiccated (red) and continuously hydrated (blue) Philodina roseola. (**B**) Uptake by P. roseola of DNA from the monogonont rotifers Brachionus rubens and Euchlanis dilatata and the bdelloid rotifer Adineta ricciae (all white) compared to conspecific DNA (blue). Significant pairwise differences in radioactivity levels are indicated (see table 1).

These results strongly suggest that donor DNA is somehow being “filtered” according to its overall similarity with that of the host animal, with more similar DNA being preferentially retained. A potential mechanism involves homologous pairing with complementary genomic DNA, possibly in combination with the degenerate tetraploid scaffold of these animals, given that DNA from more closely related species will be more similar to that of the host genome on average. Another

possibly involves GC ratios, which differ among bdelloid families [17] as well as between *Brachionus plicatilis* and bdelloids [93], and which are known to be used by some host organisms to identify foreign DNA originating from horizontal gene transfer events [182]. Because bdelloids are eutelic [81] and do not undergo cell division as adults, it seems likely that any internalized DNA is merely being somehow stored (e.g., in the vesicles in the stomach cytoplasm [108,183]) rather than incorporated into the genome in constantly hydrated animals. Instead, recombination of any stored internalized DNA likely occurs only in association with desiccation (see below).

Importantly, this combination of results enables us to exclude them as being artefacts of an unselective DNA ingestion either in the form of ^{32}P (i.e., from degraded DNA) or indirectly through bacteria that have internalized the radiolabelled DNA. In either case, we would also expect to detect some, if not equal amounts of radioactivity within the monogonont *B. rubens*, whereas none was detected. Given that *B. rubens* is a filter feeder, there is no reason why either radiolabelled phosphorus or bacteria were not also ingested (or were equally quickly purged), even if, in the latter case, this species feeds on algae and not bacteria [184]. Similarly, the cold washing process we used to remove non-ingested DNA (see Material and Methods) should affect both species equally, given that it was far too rapid for *P. roseola* to effectively shield its ingested DNA by entering anhydrobiosis, a prolonged and complicated morphological and physiological process [21,183]. Moreover, the unselective natures of both alternative explanations, which would tend to "anonymize" the donor DNA, completely fail to explain why *P. roseola* preferentially retained DNA (or, more inexplicably, just the radiolabelled phosphorus) from more closely related donor species over the long term. We cannot exclude bacteria as a possible vector for the uptake of DNA, especially given that *P. roseola* does feed on bacteria ([185]; although our colony was maintained on algae; see Material and Methods). However, some selective post-processing of the DNA (which could include its internalization from the gut) must still be occurring to explain the apparent preference for bdelloid over monogonont donor DNA we observed in this species.

Fitness effects of DNA uptake

Importantly, the DNA taken up by *P. roseola* individuals apparently can impact the reproductive output of the F1 generation in combination with a desiccation event, suggesting that it can also be incorporated in the genome of the germ-line cells of the parental generation under these conditions and is therefore heritable. Using the number of eggs laid as a proxy for fitness, there is significantly higher variation in the number laid by the F1 generation where isolated individuals of the parental generation were desiccated with DNA than by those where the parents were either desiccated without DNA (F = 1.853, df = 46,43, P = 0.043) or continuously hydrated with (F = 1.832, df = 46,76, P = 0.016) or without DNA (F = 2.166, df = 46,84, P = 0.003); no significant differences existed among the latter three treatments (hydrated vs. hydrated with DNA: F = 0.846, df =76,84, P = 0.458; hydrated vs. desiccated: F = 0.855, df = 76,43, P = 0.545; and hydrated with DNA vs. desiccated: F = 1.012, df = 84,43, P = 0.988, figure 5A). In addition, there was no effect of the desiccation procedure (T = -0.870, df = 1,290, P = 0.385), the addition of DNA (T = 0.127, df = 1,290, P = 0.899) or their interaction (T = 0.779, df = 1,290, P = 0.437) on the mean number of eggs laid.

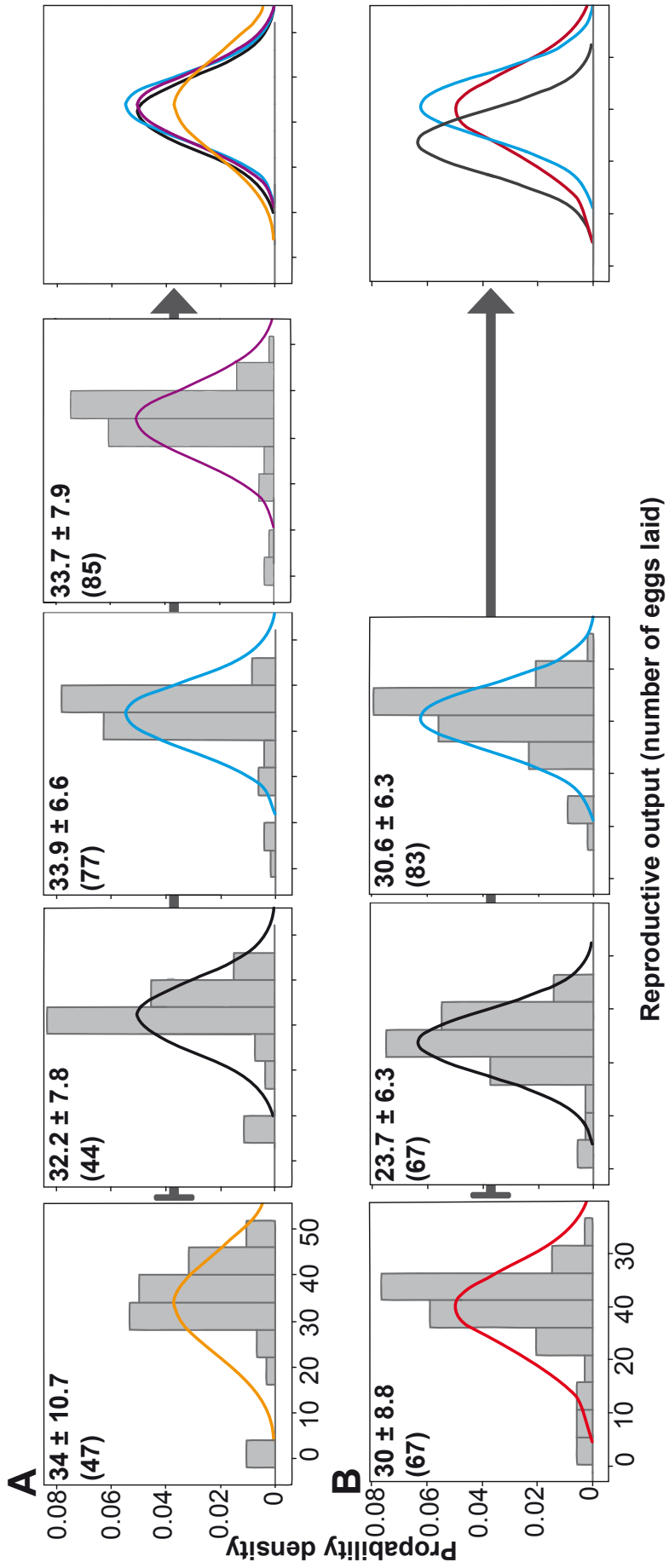

Figure 2. Quantification of changes in reproductive output of the F1 generation of treated *Philodina roseola* individuals. The parental generation was either (**A**) desiccated individually with bare *P. roseola* DNA added (orange) or (**B**) desiccated in groups with no DNA (red). Controls: constantly hydrated *P. roseola* with DNA added (purple; a only) and individually desiccated *P. roseola* (black). Reference: constantly hydrated *P. roseola* (blue). The mean number ± standard deviation of eggs laid as well as the number of individuals laying at least one egg (in brackets) are given in each histogram.

Moreover, the same fitness effects are also present under more natural conditions. When the parental generation was desiccated in groups of individuals, the variance in the number of eggs laid by their progeny was significantly higher than when the individuals were desiccated in isolation (F = 1.968, df = 66,66, P = 0.007) or were continuously hydrated (F = 1.915, df = 66,82, P = 0.005; again, no significant difference existed between the latter two groups, F = 1.028, df = 66,82, P = 0.914, figure 5B). By contrast, the mean of the reproductive output of the F1 generation of *P. roseola* desiccated individually was significantly lower than for the other treatments (F = 20.22, df = 2,213, P < 0.0001): individually desiccated vs. hydrated T = - 6.028, P < 0.0001; respectively vs. desiccated in groups T = - 4.887, P < 0.0001), with no difference between the latter two (T = -0.987, P = 0.664).

More direct evidence for heritable effects of the incorporated DNA is provided by UVB-irradiation experiments. Elsewhere, we have demonstrated the *P. roseola* is largely unable to repair the damage to its genome induced by UVB-irradiation [181], which results proximately in DNA lesions in the form cyclobutane-pyrimidine dimers (CPDs) that subsequently result in C →T mutations [26,186]. Whereas this damage negatively affects the reproductive output of both the irradiated animals and their untreated offspring, the effect is intensified after a desiccation event [181]. Here, desiccating *P. roseola* in groups previously exposed to UVB-radiation in the presence of undamaged DNA still results in a reduced reproductive output in their unexposed F1 compared to control animals (mean difference ± SE = -1.460 ± 0.552, T = 2.794, df = 94, P = 0.036), but significantly less so than if the irradiated parental generation is subsequently desiccated without additional DNA (mean difference ± SE = 1.250 ± 0.586, T = 2.132, df = 94, P = 0.0063). Again, the implication of these heritable effects is that the animals are taking up environmental DNA and incorporating it into their (germ-line) genomes during the desiccation event.

Altogether, these results appear to support an indirect analogue of sexual reproduction in *P. roseola* in that internalized, foreign DNA can also be transferred to the offspring. Here, the fitness effects that we observed agree with sexual theory, which predicts that the breakdown of existing genetic associations through the transfer of genetic material will primarily alter the variance of traits in the following generation [187], with trait means being less strongly or even slightly negatively affected [165]. For example, good alleles can potentially eliminate their association with bad genetic backgrounds (e.g., deleterious mutations accumulated in the asexual phase) through their release and uptake into another genome. At the other extreme, bad alleles can find their way into bad genetic backgrounds. Together with intermediate scenarios, the end effect is to increase the variance found in the subsequent generation, providing greater diversity for selection to act upon. Unlike in true sexual recombination, which involves mixing half of the genetic material, the limited exchange of gene fragments in the current system renders large changes in non-additive gene interactions (e.g., dominance or epistasis), and thus on mean fitness, unlikely.

Other potential explanations for our observed fitness effects can be largely ruled out through the experimental design (see Material and Methods for details). For instance, the result is unlikely to be a general effect of desiccation (e.g., due to potential errors during reconstruction of the DNA, the disruption of the hydrated, “active” life cycle, or by epigenetic changes [24]) because it would also have been present in the individually desiccated *P. roseola* used as controls. Similarly, any

density-dependent effects due to the higher density of the group treatment, albeit short in duration, would mean that the same fitness effects should not have been observed for animals desiccated individually with DNA added. Finally, if the added DNA is merely being used as a supplemental food source, any effect should be identical across all groups and it is difficult to explain why it has a heritable effect that influences the variance of the reproductive output of the F1 generation (and then only in combination with desiccation).

Finally, the group desiccation trials reinforce that, in addition to environmental DNA, bdelloid rotifers themselves can apparently act as DNA sources during the desiccation process. Although it is unclear if the donor DNA is released through the normal cell apoptosis associated with desiccation and/or with the death of individuals, it is noteworthy that genes associated with apoptosis, transportation and translation, among others, are upregulated in *A. ricciae* 24 hours after the onset of dehydration [21]. For the observed fitness effects, DNA would have to be released, taken up, and stored before desiccation was complete to enable it to be incorporated in the genome during its reconstruction in the reanimation phase. Supporting this hypothesis is the observation that reanimated individuals took up smaller amounts of environmental DNA, consistent with a reduced DNA uptake during the reanimation process when the priority is to reconstitute both the genotype and phenotype. Ecologically, this timing would also ensure proportionately larger amounts of DNA from closely related individuals in the limited amount of water present near the end of the desiccation process, something that would be more difficult to achieve under normal, favourable conditions.

The importance of desiccation

It would therefore appear that desiccation not only represents a strategy to overwinter unfavourable conditions by *P. roseola*, but is also a necessary component for the DNA to be incorporated into the genomes of its germ-line cells. In addition to disrupting the additional membrane that delimits these cells from the soma [183], desiccation would also serve to produce the double-stranded breaks (which are as common in oocytes as in somatic cells [89]) required to incorporate any stored foreign genetic material (e.g., from the vesicles in the stomach cytoplasm; see above) via the well-developed DNA repair mechanisms in bdelloids [91] in combination with the template provided by their degenerate tetraploid genome [88] (e.g., through homologous recombination of sufficiently similar DNA strands). Distribution of any internalized DNA throughout the animal might occur via the lipid droplets present not only in hydrated specimens [108], but also in larger numbers within the gut of desiccated bdelloids [183]. Interestingly, lipids are not only used in a very effective DNA-transfection lab-protocol [188], but they are also not consumed by bdelloid rotifers during anhydrobiosis [183]. Alternatively, the internalized DNA might already be globally distributed at the onset of desiccation in association with the rearrangement of nuclei already documented in desiccated bdelloids [183].

Although the connection between desiccation and incorporation of foreign DNA in bdelloid rotifers has previously been raised in the context of horizontal gene transfer (see [22]), our results suggest that this process might be more common than previously thought and might underlie an indirect form of sexual reproduction within the group. As a side effect, the end result of this process

must be that reanimated, adult bdelloids are naturally chimeric given the virtual impossibility of incorporating the same piece of DNA in all cells of these eutelic animals.

If our hypothesis is true, the general reproductive system of *P. roseola* thereby bears intriguing resemblances to the heterogonic system of monogonont rotifers, whereby the asexual reproduction that occurs in stable environments is interspersed with bouts of sexual reproduction as conditions worsen and the (future) environment becomes more unpredictable [30,165,178]. The parallels between the two systems go even further. To overwinter unfavourable conditions, monogonont rotifers produce sexual resting eggs, which can also serve as a dispersal stage; both traits are also functions of the anhydrobiotic stage of bdelloid rotifers. Thus, for both monogonont and bdelloid rotifers, an exchange of genetic material is favoured by environmental instability or worsening conditions, albeit using two different mechanisms.

However, unlike the monogonont system incorporating true sexual reproduction, the mechanism of genetic exchange employed by bdelloid rotifers is potentially better adapted to highly unstable environments in which these animals are often found. The production of true resting eggs, as in monogonont rotifers, is more time intensive than undergoing anhydrobiosis given that it requires both a change to mictic females as well as the production of males [30]. This strategy is thus less suited to environments where the duration of favourable conditions is too short and frequently interrupted.

In addition, genetic exchange in bdelloids is more unspecific than a true heterogonic system and is probably driven by the degree of similarity between the donor and host DNA. Nonetheless, this selection mechanism is probably sufficient to avoid many of the negative effects associated with incorporating highly divergent DNA (e.g., interference with the needed repair of the double-stranded breaks inflicted by desiccation) [87,88,91,116,117] and is strengthened by other, indirect mechanisms that promote exchange with potentially advantageous donors [116,189]. For example, *P. roseola* tend to stay together both while laying eggs and, more importantly, also during desiccation when they can potentially act as DNA donors. Environmental DNA from non-conspecifics can still be incorporated, presumably dependent upon having sufficient similarity with the host DNA to enable homologous recombination. This would explain the occasional presence of ancient, alien DNA in the bdelloid genome as more of an accidental instance of horizontal gene transfer. By contrast, the apparently greater preference for bdelloid DNA in general would explain the observation that otherwise divergent bdelloid species often share several virtually identical alleles [16,90].

Conclusions

Our observations of the active uptake of DNA fragments by *P. roseola*, their (passive?) retention according to the degree of relatedness of the donor species, as well as their heritable effects following a desiccation event point to a potential mechanism for regular (contra [16,90]) genetic exchange in bdelloid rotifers. The potential importance of this proposed process of genetic exchange is underlined by the fact that several species of bdelloid rotifer that are kept continuously hydrated appear to lose significant amounts of fitness over time [24]. Like with the global repairing of the genome following desiccation, we are unfortunately unable to suggest the precise cellular

mechanisms underlying this apparent process at this time.

It remains to be investigated how universal these results are across bdelloid rotifers and how they apply in particular to those few species that do not undergo anhydrobiosis [107]. If genetic exchange in bdelloids is indeed dependant upon desiccation, then these latter species would represent the only truly obligate asexuals within the group, with their limited number and scattered distribution matching that of asexual lineages in general. More importantly, if this or similar mechanisms of genetic exchange do indeed exist across the group, the status of bdelloids as "ancient asexuals" must be called into question, even if the process is not strictly equivalent to true sexual reproduction. In this regard, bdelloids would serve as yet another, but extremely high-profile, case study where the "cryptic" exchange of genetic material was found in what was widely thought to be an asexual lineage, as was recently the case for the parasitoid wasp taxon *Lysiphlebus* [190], the aphid taxon *Tramini* [191], fungi of the taxon *Candida*, of the taxon *Aspergillus* [192] and of the taxon Glomeromycota [193], *Entamoeba histolytica* [33], colpodean ciliates [194], while the asexual status of others is often not unambiguously supported [34]. Even the horizontal gene transfer found throughout the otherwise asexual bacteria can be viewed in this light and was also held recently to be an important mechanism driving adaptive diversity in these organisms [19]. Altogether, these findings reinforce the notion that some form of genetic recombination appears to be necessary for most species to survive over the long term such that evolutionary biologists remain confronted with the puzzle behind the prevalence of sexual reproduction in general.

Author's contributions

All authors designed the project for which C.F. performed the research and performed most of the analyses. C.F. and O.B.E. wrote the manuscript.

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4.2 Immediate and heritable effects of desiccation in isolation versus in groups on the life history of the bdelloid rotifer *Philodina roseola*

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Abstract

The long-term survival of the ancient asexual bdelloid rotifers, a clade of small aquatic invertebrates, is thought to have been made possible in part through their ability to enter a state of anhydrobiosis. This ability has both clear benefits (e.g., survival of desiccating conditions coupled with random wind dispersal to potentially colonize (more) suitable habitats), but considerable costs as well (e.g., subsequent repair of the genome with its many double stranded breaks). Despite the latter, recent studies have shown that the time spent dry seems to be effectively ignored with respect to life expectancy (the Sleeping Beauty hypothesis) and that the genomic repair after a desiccation event might actually be a necessary process to repair any mistakes that accumulated from the obligate asexual reproduction while the animals were hydrated. In this paper, we propose that these latter positive effects do not derive from desiccation *per se* but instead result from the genetic exchange that we hypothesize occurs between individual bdelloid rotifers when desiccated in groups (see chapter 4.1). By comparing individuals of *Philodina roseola* desiccated in groups versus in isolation, we document actual costs to desiccation in the latter treatment group that impact negatively on lifespan and reproductive output. In addition, in comparing both groups with constantly hydrated individuals, we see neither strong evidence for the Sleeping Beauty hypothesis in this species nor any decline of fitness over a period of six months for the latter group. Finally, many of the treatment effects appear to be at least partly heritable and were also found in the untreated F1 generation. In particular, both individuals desiccated in groups and their offspring could reproduce faster than the offspring of constantly hydrated individuals. As such, our results lend further support to our hypothesis of potential genetic exchange during desiccation events in *P. roseola* and highlight the importance of desiccation for the fitness and shaping of the genetic information of this species that need to be considered in future studies.

Keywords anhydrobiosis, desiccation, Sleeping Beauty, cryptic sexual reproduction, heterogony, horizontal gene transfer, Rotifera, Bdelloidea

Introduction

Bdelloid rotifers are a clade of small, aquatic invertebrates that inhabit a diverse range of water bodies including unstable, desiccation prone habitats like mosses or soil [23]. Their status as an ancient asexual taxon [14,15,16,17,18] has long puzzled evolutionary biologists because, compared to (facultative) sexually reproducing organisms, the absence of sexual recombination should make them more vulnerable to the accumulation of deleterious mutations [57] and the resulting lower level of standing genetic variation should make them less able to adapt as quickly to changing environments [10,11].

Instead, the long-term success of the group is often explained as being tied to the ability of most species to withstand desiccation through anhydrobiosis [105], where individuals at any life stage undergo morphological changes leading to a characteristic tun-shape [108] that enables survival for several weeks [109]. Apart from the obvious immediate benefit of surviving a hostile environment, desiccated individuals can also undergo wind-dispersal at this time to enable the founding of new populations. In so doing, the dispersal can act as a form of adaptation: individuals are able to potentially escape unfavorable circumstances in space and time and can randomly colonize habitats that are more suitable for their genetic makeup [110,111,112]. However, desiccation comes with a considerable cost. During this process, the genome is initially shattered to pieces and the resulting double-stranded breaks must be repaired upon rehydration, a potential source of harmful mutations and structural rearrangements that could impair gene functioning [113]. Differences in survival rates among the life stages have also been documented for this process and are highest in adult individuals [107].

Nevertheless, these costs would appear to be relatively low in that the time spent in the desiccated state does not appear to count against an individual's lifespan and is, in effect, simply ignored (the Sleeping Beauty hypothesis; [24,25,47]). Even more surprising, however, is the contention of Ricci et al. [24] that desiccation, despite its apparent costs, might instead provide important and necessary benefits for the genetic integrity of the organisms. As one explanation of their observation that a combination of fitness components in constantly hydrated, clonal lines of the bdelloid rotifers *Adineta ricciae* and *Macrotrachela quadricornifera* progressively declined with time, but could be reset to normal levels if they were desiccated at least once, they argued that the repair of double-stranded DNA breaks during rehydration acts simultaneously to somehow proofread the genome and correct any errors that might have accumulated (e.g., through mutation or epigenetic effects) [24]. This explanation is in accordance with the surprisingly good ability of bdelloid rotifers to repair double-stranded DNA breaks found later on [89,91], one that is possibly aided by their degenerate tetraploid genome structure [87,88].

An alternative explanation for these results derives from our recent proposal that the bdelloid rotifer *Philodina roseola* is apparently able to take up environmental DNA that, based on fitness effects on the F1 generation, can be incorporated into the genome during a desiccation event (compare 4.1). These effects were also present when individuals of *P. roseola* were desiccated in groups and so could act reciprocally as DNA donors. Thus, because animals were also desiccated in groups in the Ricci et al. [24] experiments, their results might instead obtain primarily from the

incorporated genetic material being responsible for repairing any mutations or epigenetic changes that had accumulated in the parallel continuously hydrated control rather than the process of desiccation in and of itself with its attendant genome rebuilding.

Our primary goal in this paper, therefore, is to re-examine the phenomenon observed by Ricci et al. [24] in the light of our hypothesis that *Philodina roseola* and perhaps other bdelloid rotifers can take up and, during a desiccation event, incorporate environmental DNA in their genomes. Specifically, we tested if individuals of *Philodina roseola* desiccated in isolation show the same positive fitness effects as those desiccated in groups. If the exchange of DNA is indeed needed to mask the cost of desiccation and perform repairs to the genome, then a positive effect should only be present (or more strongly present) in the group-desiccation treatments. If, however, desiccation itself acts as a check-up as Ricci et al. [24] proposed, then no significant difference between the two desiccation treatments is expected. Furthermore, by comparing data from the treated animals and their offspring, we hoped to discern any immediate costs from heritable ones.

In so doing, our experiments also address two additional hypotheses put forth for bdelloid rotifers. First, we will verify whether or not continuously hydrated individuals of *P. roseola* also lose fitness over very short timespans (i.e., months), such they would effectively be excluded from permanent waters where they cannot undergo desiccation and therefore repair their genomes [23,24,106]. Second, the data we recorded for both organismal and reproductive lifespan will serve to test the Sleeping Beauty hypothesis that there is no appreciable cost to anhydrobiosis beyond surviving the process in the first place.

Material and Methods

Rotifer and algal cultures

All *P. roseola* individuals under investigation descended from a small group of animals obtained at the end of 2009 that were themselves derived from a single egg in 1989. Since the end of 2009, our colony was fed a diet of the algae *Cryptomonas spec*. SAG 26.80 as opposed to that of *Escherichia coli* that they were fed before this point. Algae were cultivated under continuous illumination (13W/840 Osram Daylight neon tubes) in 500 ml Erlenmeyer flasks with COMBO medium [179]. Rotifers were cultivated under a 9/15 h light/dark regime in ten different 90x15 mm plastic Petri dishes at 20 ± 1°C. Each week, around 50-75 of the largest, healthiest looking individuals were transferred to new Petri dishes, with individuals from different Petri dishes being mixed periodically to keep the continuously hydrated stem-culture as fit as possible. Algal density was continuously maintained at roughly $1x10^6$ cells per ml.

Experimental procedure

To measure any decline in fitness in the continuously hydrated stock culture, five samples were taken from it at time intervals of one to three months, with the fifth and last trial commencing six months after the first one. Each time sample consisted of batches of equally aged *P. roseola* that were generated by transferring about 100 or 300-400 eggs (trials 1–3 and trials 4–5, respectively) laid by the stock culture on a particular day to a single new Petri dish containing fresh algae.

Individuals were raised in the Petri dish until they reached an age of eight days to ensure that all *P. roseola* were sexually mature before the experiments started.

From these populations, the largest and healthiest looking individuals (to exclude any damage incurred during the transfer of eggs) were assigned randomly to one of three treatment groups — hydrated in isolation, desiccated in isolation, or desiccated in groups of ten or twelve (trials 1–3 and trials 4–5, respectively) — and transferred to 24-well flat-bottom plates (Falcon© 353935), with each well containing 1 ml COMBO medium with algae. Desiccation, if applicable, followed protocol D of Ricci et al. [180], with a 1 cm² piece of KIMTECH science© delicate task wipe being added to each well, followed by seven days of desiccation in a humido-thermostatic chamber (trial 1-4: Snijders scientific B.V. ECP01E; trial 5: a custom-built chamber from the University of Oldenburg workshops). Thereafter, 1 ml of fresh medium with algae was added to each well to begin the rehydration process. After 24 h, the rotifers were transferred individually to new wells containing 1 ml of medium and fresh algae as were individuals in the hydrated treatment group from the start of the experiment.

Fitness in each treatment group was measured as the number of offspring laid by each individual over its lifespan. For those treatment groups involving desiccation, any eggs present in the original wells and/or on the piece of task wipe within them were also included. In the case of *P. roseola* individuals desiccated in groups, we calculated the average number of offspring for the 10 or 12 individuals. In so doing, the average includes any individuals that died during the desiccation process because it was unclear how many eggs they laid before dying and essentially represent conservative estimates of per individual fitness. Eggs were counted every 24 h and removed from the well. Trials ended once all individuals in a treatment group were deceased or, in the case of trial 4, after no eggs were laid for one week.

Finally, for trial 5 only, the effects of these treatments on the reproductive output of the untreated F1 generation were also measured. F1 individuals were obtained by transferring one egg from each parent (i.e., from each well) to a new plate, with the egg being taken either on the fourth day after the treatment for the hydrated group or on the third day after the transfer to the final well for either of the two desiccated groups. Thereafter, the total number of eggs for each individual was counted in a similar manner as above for the parental generation. All F1 individuals were continuously hydrated throughout the experiment such that any effects of the treatments would have to be inherited from the parental generation.

Statistical procedure

The comparison of the overall mean of the reproductive output across all five trials used a general linear mixed model (GLMM) to exclude random effects between the treatment groups in combination with Tukey contrasts to control for multiple comparisons. Variation between the trials was analyzed with ANOVAs for each treatment group separately. Additionally, lifespan and the duration of reproduction were each compared using another GLMM to again exclude random effects between the treatment groups; trial 4 was excluded from these analyses because of the differing circumstances under which it was terminated (i.e., after egg deposition had ceased for one week; see above). Finally, any differences between the treatments in the otherwise untreated

F1 generation (trial 5 only) were analyzed using general linear models (GLMs), again with Tukey contrasts to control for multiple comparisons. All statistical tests were computed in R 2.12.0 [195].

Results

The impact of desiccation on fitness and longevity

In contrast to the results from Ricci et al. [24], we saw no evidence for a continual decline in fitness (as measured by the mean number of eggs laid by the hydrated treatment group) within a constantly hydrated population of *P. roseola* individuals over a period of six months (table 2). Instead, a high and significant amount of variation between the five trials was observed ($F_{4,196}$ = 9.283, P < 0.0001). In addition, no significant difference in average fitness between this group and that comprising animals desiccated in groups was observed over all trials ($Z_{229,201}$ = -1.220, P = 0.441, figure 6), with the latter treatment group also displaying a high variation between trials ($F_{4,224}$ = 6.116, P < 0.001). By contrast, individuals of *P. roseola* that were desiccated in isolation showed significant differences to the other two treatment groups in that the average fitness over all trials in this treatment group was reduced significantly (versus constantly hydrated individuals: $Z_{162,201}$ = -3.741, P < 0.001; versus those desiccated in groups: $Z_{162,229}$ = -2.599, P = 0.025) and there was no significant variation in average fitness among the trials ($F_{4,157}$ = 0.268, P = 0.898, table 2).

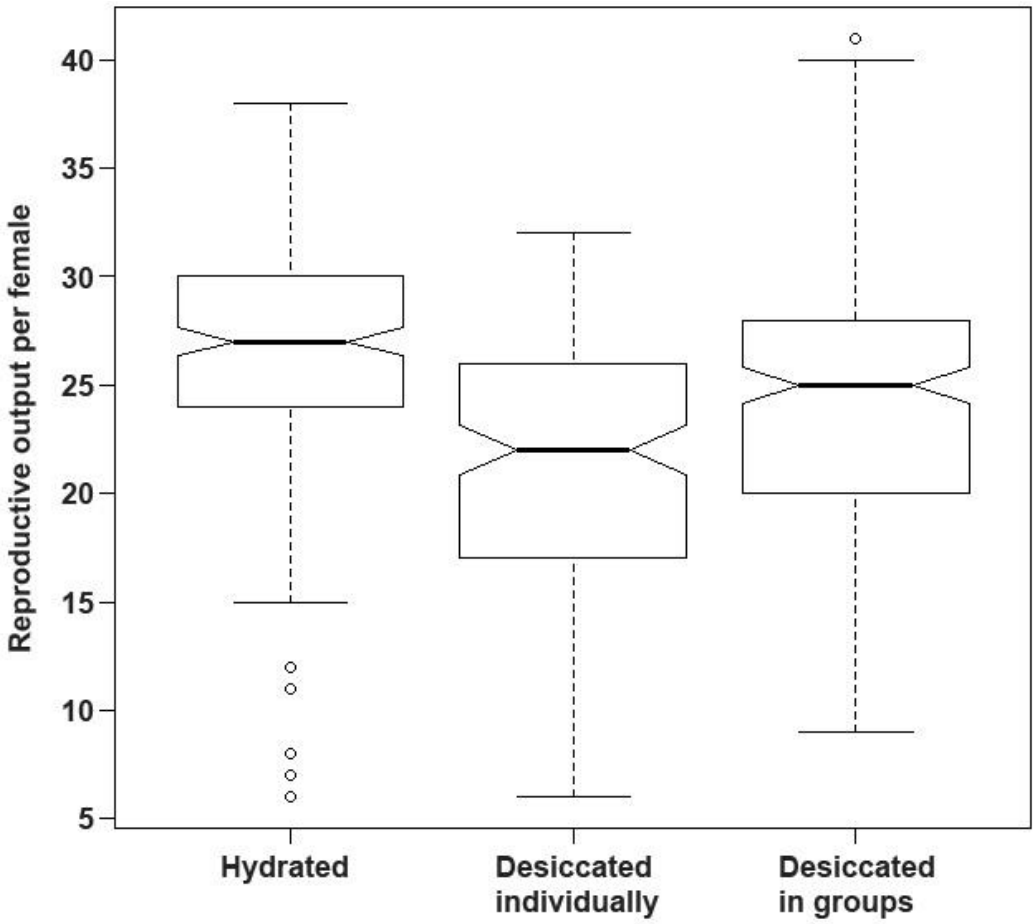

Figure 6. Mean number of eggs laid since an age of 8 days by individuals of *P. roseola* subdivided according to the treatment group.

Table 2. Reproductive output of *P. roseola* subdivided according to treatment group and measured as the mean number ± standard deviation (s.d.) of eggs laid (starting date of trials given in parentheses). For trial 5, values for both the treated parental generation and its untreated F1 generation (total reproductive output) are given. The numbers of individuals for each data point are given in parentheses and thereby exclude any individuals that died during desiccation, were lost during transfer or could not deposit eggs.

Treatment group	**Trial 1 (February 17, 2010)**	**Trial 2 (February 25, 2010)**	**Trial 3 (March 9, 2010)**	**Trial 4 (May 5, 2010)**	**Trial 5 (August 11, 2010)**	
					Parental generation	**Untreated F1 generation**
Hydrated	26.4 ± 6.3 (12)	23.6 ± 4.4 (24)	24.0 ± 6 (23)	25.4 ± 3.2 (46)	28.7 ± 5 (96)	30.6 ± 6.3 (83)
Desiccated in isolation	21.0 ± 6.1 (17)	21.5 ± 3.7 (13)	20.1 ± 5.7 (16)	21.8 ± 3.8 (31)	21.1 ± 6.2 (85)	23.7 ± 6.3 (67)
Desiccated in groups	27.7 ± 6.5 (29)	25.4 ± 3.6 (27)	22.9 ± 6.5 (20)	21.7 ± 6.4 (65)	24.2 ± 5.5 (88)	30.0 ± 8.8 (67)

Significant differences in lifespan between the treatment groups across all trials were generally absent, regardless if any time spent in desiccation was included in the comparison or not ("total" versus "active" lifespan respectively) (table 3 & 4). The only significant difference found was for active lifespan where animals desiccated in groups had significantly shorter lifespans than did those that were continuously hydrated. A similar tendency for animals desiccated individually was also observed, although it was marginally non-significant.

Table 3. Comparison of the lifespan of the treated generation with a GLMM over all trials, either using the total (above the diagonal) or the active lifespan for the desiccation treatments (= whole lifespan - seven days; under the diagonal).

Treatment	**Hydrated**	**Desiccated in isolation**	**Desiccated in groups**
Hydrated		Z = -0.683, P = 0.773	Z = -0.625, P = 0.806
Desiccated in isolation	Z = -2.328, P = 0.0520		Z = 0.082, P = 0.996
Desiccated in groups	Z = -2.346, P = 0.0497	Z = 0.033, P = 0.9994	

Table 4. Average total lifespan in days (± standard deviation (s.d.)) of *P. roseola* subdivided according to the treatment group (starting date of trials given in parentheses). The average active lifespan for the desiccated treatment groups is the total value minus seven days. Data from trial 4 are not presented because the trial was ended prematurely (see Methods).

Treatment group	**Trial 1 (February 17, 2010)**	**Trial 2 (February 25, 2010)**	**Trial 3 (March 9, 2010)**	**Trial 5 (August 11, 2010)**	
				Parental generation	**Untreated F1 generation**
Hydrated	51.1 ± 6.3	30.0 ± 11.4	42.1 ± 9.8	56.4 ± 19.2	62.7 ± 15.9
Desiccated in isolation	46.0 ± 12.5	40.2 ± 11.5	37.8 ± 9.5	48.2 ± 19.2	58.6 ± 15.1
Desiccated in groups	46.8 ± 12.8	40.2 ± 8.5	36.8 ± 11.6	48.6 ± 16.0	50.5 ± 15.9

The active reproductive lifespan (i.e., excluding any time spent desiccated when no eggs can be laid; table 5), is significantly shorter in the treatments involving desiccation (individually desiccated vs. constantly hydrated: $Z_{162,201} = -4.231$, $P < 0.0001$; desiccated in groups vs. constantly hydrated: $Z_{162,229} = -3.135$, $P = 0.0049$), but did not differ between these treatments (desiccated in groups vs. individually desiccated: $Z_{229,162} = 1.282$, $P = 0.405$). Thus, in keeping with the observation that individuals desiccated in groups tended to show a burst of reproductive activity following desiccation [196], animals in this treatment group laid their eggs faster than did those in the continually hydrated treatment, given that both groups laid equal numbers of eggs (see above).

Heritable effects of desiccation on the untreated F1

Although the untreated F1 of parents desiccated in groups laid as many eggs as those from hydrated parents ($T = -1.323$, $P = 0.384$), they needed significantly fewer days to finish reproduction ($Z = -4.504$, $P < 0.0001$) and also had a significantly shorter lifespan ($T = -4.715$, $P < 0.0001$). The reproductive output of the F1 of parents desiccated in isolation is significantly reduced (vs. constantly hydrated: $T = -5.717$, $P < 0.0001$, vs. desiccated in groups: $T = -4.175$, $P = 0.00013$). Nevertheless, the days needed to finish reproduction ($T = 0.308$, $P = 0.95$) as well as lifespan ($T = -1.598$, $P = 0.248$) is comparable to those of parents hydrated and thus both higher than of the F1 of parents desiccated in groups (reproductive days: $T = 4.522$, $P < 0.0001$, lifspan: $T = -2.915$, $P = 0.0011$).

Discussion

Table 5. Duration of active reproduction of *P. roseola* subdivided according to the treatment group, measured as the mean number ± standard deviation (s.d.) days until reproduction was finished (starting date of trials given in parentheses). Values do not include any time spent desiccated.

Treatment group	**Trial 1 (February 17, 2010)**	**Trial 2 (February 25, 2010)**	**Trial 3 (March 9, 2010)**	**Trial 4 (May 5, 2010)**	**Trial 5 (August 11, 2010)**	
					Parental generation	**Untreated F1 generation**
Hydrated	24.6 ± 6.0	21.3 ± 3.2	23.0 ± 4.0	20.7 ± 2.1	28.8 ± 3.8	35.3 ± 4.0
Desiccated in isolation	18.7 ± 4.6	18.7 ± 3.1	19.3 ± 4.2	20.1 ± 3.8	23.7 ± 5.2	35.6 ± 5.3
Desiccated in groups	21.0 ± 4.1	18.7 ± 2.9	21.0 ± 4.2	20.1 ± 3.8	24.0 ± 3.7	30.9 ± 7.4

Ricci et al. [24] explained the resetting of declining fitness levels that they observed after a desiccation event as the result of a “rescue effect” involving repairing accumulated errors in the genome (genomic repair) and/or ridding the animals of accumulated parasites that cannot withstand desiccation (ecological repair) [111,112]. Although we did not observe any progressive deterioration of fitness in our continually hydrated population (see below), we nevertheless interpret their results as potential support for our hypothesis that bdelloid rotifers possess the ability to take up DNA from the environment and to incorporate it in their genomes during a desiccation event. This process has the potential to both repair any existing genomic damage and to facilitate an indirect form of genetic exchange. Instructive here are our experiments in which individuals of *P. roseola* were desiccated in isolation, a treatment that Ricci et al. [24] did not examine. The significantly lower fitness of this treatment group compared to that of either continuously hydrated individuals or those desiccated in groups reveals that there do indeed appear to be tangible costs associated with anhydrobiosis, and presumably at the genomic level because they are passed on to the subsequent (untreated) generation in the form of similarly reduced fitness levels. The differential effects we observed between the treatment groups involving desiccation would tend to exclude the alternative explanation of ecological repair in this study (or indicate its importance to be very secondary) because desiccation should have the same effect on parasites in either group.

Thus, contra a main hypothesis of Ricci et al. [24], our results indicate that desiccation alone seems to be insufficient to correct any mutational errors in the genome that derive from extended parthenogenesis without recombination and itself imposes costs on the organisms. These costs are reflected in three ways. First, without the uptake of environmental DNA that is similar to their own (e.g., as would be the case from conspecific or closely related organisms), the animals have only their own degenerate tetraploid genome [91] to use as a reference for error correction,

which, given the decrease in fitness observed for animals desiccated in isolation, seems to be insufficient for this purpose as well as potentially introducing additional errors. Importantly, the negative effects would appear to be heritable given that decreased fitness was also present in the untreated F1 of this treatment group. Also, despite producing fewer eggs, the offspring of individuals desiccated in isolation also needed as much time to finish reproduction as the F1 of hydrated parents and even more time than the F1 of parents desiccated in groups.

Second, *P. roseola* is not behaving unequivocally according to the Sleeping Beauty model proposed for *M. quadriconifera* and *A. ricci* [24,25,47] and the tardigrade species *Milnesium tardigradum* [197] in that the time spent desiccated does indeed appear to count at least in part against lifespan. Results for organismal lifespan were somewhat mixed, with few significant differences for either active or total lifespan. Nevertheless, it was clear that total lifespans of desiccated animals were more similar to – and still slightly shorter than – those of continually hydrated individuals than were their active lifespans. It is important to note, however, the Sleeping Beauty model is not universal among organisms able to undergo anhydrobiosis, being absent in the nematode *Panagrolaimus rigidus* [198] for example. By contrast, active reproductive lifespans were clearly impacted negatively in desiccated individuals, although this did not affect the quantity of eggs produced for animals desiccated in groups because eggs were laid at a higher rate, particularly following rehydration (see also [196]). Given this latter result, we interpret the reduced fitness of animals desiccated individually to stem more from insufficient genomic repair than the observed reduction in reproductive lifespan.

A final cost to desiccation is hinted at by the striking and unique absence of any significant variation in the average number of eggs laid across the five trials by individuals that were desiccated in isolation. Thus, fitness would not only appear to be reduced by desiccation, but reduced to some minimal level rather than by some fixed cost compared to the other two treatment groups. A possible explanation for this result again revolves around insufficient genomic repair and/or errors introduced through the genome repair process, although other, at this point unknown scenarios, cannot be excluded.

These apparent costs to desiccation get ameliorated by the presumed transfer of genetic material that occurs when individuals are desiccated in groups (see 4.1), with group sizes as small as ten to twelve individuals being sufficient for this purpose. The lack of any significant difference in fitness compared to the constantly hydrated individuals is especially noteworthy in that the values for individuals desiccated in groups are likely slight underestimates because individual survival during the desiccation process could not adequately be accounted for. In addition, desiccation in groups also appears to yield some positive heritable effects in addition to an increased variance in reproductive output of the F1 (see 4.1), especially in contrast to being desiccated in isolation. For instance, in addition to producing higher egg numbers, the untreated F1 for this treatment group also finished reproduction earlier, with this combination thereby resulting in the fastest growth rate of all treatment groups. Coincident with the accelerated reproduction, however, was a reduced total lifespan, which might reflect the trade-off in these two traits that has been witnessed in other organisms (e.g. [199,200]).

Although the overall timeframe of our experiments was admittedly only half as long as that of Ricci et al. [24] (12 versus six months), we still failed to observe any decline in fitness in the constantly hydrated treatment group over time, a clear tendency that was already apparent in their results after only four months. A number of possible explanations exist for these differing results. First, Ricci et al. [24] measured fitness as a combination of six different variables of which only three were examined here and then separately: number of eggs laid, active reproductive lifespan, and (total or active) lifespan. Second, although they observed the effect in two species from different genera, it might be species specific and does not occur in *P. roseola*. Indeed, from the results of Ricci et al. [24], one can see that the effect was not so strong for *M. quadricornifera* as it was for *A. ricciae*. Finally, our use of a non-clonal population that was recently switched to a different food source and not a clonal population fed a constant food source for years would initially present a larger standing variation in the population and one that might be accentuated by the selection pressure presented by the new food source. Together with our use of only the most robust individuals for the ongoing culture as well as for our experiments, this would result in selection for higher fitness, which might have been sufficient to prevent a decrease in fitness in the timeframe of our experiment.

However, variation is undoubtedly present in natural populations as well giving the most robust individuals a likely competitive advantage. Thus, it is unclear how strongly dependent bdelloid rotifers are in general for living in desiccation-prone habitats (as suggested by Ricci et al. [24]) or how important desiccation in and of itself is for maintaining fitness. A potentially important counterargument here is that although many bdelloid species do undergo anhydrobiosis, this trait is not universal for the group [107]. Should the hypotheses of Ricci et al. [24] and us (compare 4.1) regarding the importance of desiccation for maintaining fitness and genetic variation, respectively, be correct, then it would be important to discover how these latter, exceptional species have managed to survive over the long term without any apparent negative effects. At the very least, even though the exact mechanisms remain uncertain at this point, future studies on the unusual features of bdelloid genomes are challenged to also consider their desiccation history (compare [89]), which appears to play an important role in shaping the genetic information in these species.

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4.3 How does the "ancient" asexual *Philodina roseola* (Rotifera: Bdelloidea) handle potential UVB-induced mutations?

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Abstract

Like other purely asexual species, bdelloid rotifers are expected to suffer from degradation of their genomes through processes including the accumulation of potentially deleterious mutations. However, sequence-based analyses in this regard remain inconclusive, although bdelloids are assumed to be ancient asexuals. Instead of looking for historical footprints of mutations, we directly examined the susceptibility to and/or ability to repair point mutations by the bdelloid *Philodina roseola* Ehrenberg, 1832 by inducing potential mutations (CPDs: cyclobutane-pyrimidine dimers) via UVB-exposure (light in the wavelength range of 280-320 nm). For comparison, we performed analogous experiments with the facultative asexual monogonont rotifer *Brachionus rubens* Ehrenberg, 1838. Different strategies were found for the two species. *P. roseola* appeared to shield itself from CPD induction through uncharacterized UV-absorbing compounds and, except for the genome reconstruction that occurs after desiccation, had virtually no ability to repair UVB-induced damage. By contrast, although *B. rubens* was more susceptible to UVB-irradiation, it was able to repair all induced damage in about two hours. In addition, whereas UV-irradiation had a significant negative impact on the reproductive output of *P. roseola* and especially so after desiccation, that of *B. rubens* was unaffected. Although the strategy of *P. roseola* might suffice under natural conditions where UVB-irradiation is less intense, the lack of any immediate CPD repair mechanisms in this species remains perplexing. It remains to be investigated how typical these results are for bdelloids as a group and therefore how important desiccation-dependent genome repair is for these animals to correct potential DNA damage given their obligate asexual lifestyle.

Keywords Genome degradation, asexual reproduction, cyclobutane-pyrimidine dimers, genome repair, UV-damage, bdelloid rotifer, desiccation tolerance, anhydrobiosis, deleterious mutations

Introduction

Bdelloid rotifers are a clade of small invertebrates that inhabit a diverse range of water bodies and are renowned for their paradoxical status as a successful and ancient asexual clade [23,27]. Although the widespread prevalence of sexual reproduction among metazoans is still not understood in view of its greater costs compared to asexual reproduction [1], a purely asexual lifestyle also entails distinct disadvantages such that the long-term success of a group like the bdelloidsis equally confounding [7]. For instance, the lack of segregation and recombination in the genome of purely asexual lineages should result in its progressive degradation [7,48,201] through the effects of transposable elements [98,99], the degeneration of homologous chromosomes [34,76], the independent functioning and therefore evolution of the alleles of a given gene locus (the Meselson effect [7,34]), and especially the accumulation of deleterious mutations over time [9,48].

Evidence from sequence-based analyses for the latter in bdelloids is inconclusive, however. Whereas older studies indicated that bdelloids did not accumulate slightly deleterious mutations faster than their sister group, the facultative sexual monogonont rotifers [92,93], two newer studies using more elaborate and extensive sampling strategies indicated the possible existence of this effect [94,95]. However, these studies, which examine the rate of historical accumulation of deleterious mutations, are difficult to interpret because of a number of confounding factors. For instance, the latest study showed that no increased accumulation in bdelloid rotifers compared to monogonont rotifers was observed when comparisons were made between monogonont and bdelloid rotifers from the same habitat only [95]. In addition to habitat, other potential confounding factors include both methodological aspects (e.g., sample-size effects [95] or the long divergence time separating bdelloid and monogonont rotifers [94]) as well as natural factors (e.g., mutations with severe effects that do not go to fixation [94]) or the frequency and impact of any repair/recombination events (i.e., desiccation in bdelloids, sex in monogononts). These difficulties notwithstanding, the observations above, together with the long-term existence of bdelloids as a group (estimated to be at least 40 million years [45]) would indicate that some mechanism(s) to prevent degradation of the genome must be occurring in these animals.

Evidence of ancient horizontal gene transfer in bdelloids [20,21,22] and the possible advantageous aspects of the Meselson effect [85,114], may be acting in this regard, at least to increase genetic variation. In terms of more immediate countermeasures to genomic degeneration, it has been shown that bdelloids can efficiently repair double-stranded breaks in the genome [91], especially in the context of having to reconstruct their genomes through an as yet uncharacterized repair mechanism following a desiccation event [24], which bdelloids survive via anhydrobiosis [106]. However, it is unclear if this mechanism would also be effective against single deleterious mutations *per se* nor is it clear which mechanisms are present to ward off their progressively negative effects.

In investigating the latter, a more direct and therefore potentially more profitable route would be to check the susceptibility to and/or ability to repair point mutations by inducing them directly instead of looking for their historical footprints. One possible way to do this is via UVB-irradiation, which

causes lesions in the DNA, with cyclobutane-pyrimidine dimers (CPDs; T-T and 5' T-C dimers [202]) being the most frequent form [203]. Here, adjacent pyrimidine nucleotides on the same DNA strand become linked via a four-carbon (cyclobutane) ring, thereby disrupting the base-pairing with the other strand and altering the DNA configuration. In doing so, CPDs immediately inhibit gene transcription and DNA replication by obstructing both RNA- and DNA polymerases [204,205]. In addition, the otherwise stable cytosine bases are unstable in CPDs and readily deaminate to uracil that, in turn, will give rise to C → T and CC → TT mutations (reviewed in [26]).

In this paper, we investigated how the bdelloid rotifer *Philodina roseola* Ehrenberg, 1832 handles the threat of possible mutations by inducing CPD lesions via UVB-exposure. To this end, we quantified both the damage level (i.e., number of CPDs) and the rate of its repair, both under constantly hydrated conditions and under conditions where desiccation, and therefore DNA repair (see above), was allowed to occur. As a comparison, we performed analogous experiments on the facultative sexual monogonont rotifer *Brachionus rubens* Ehrenberg, 1838. Furthermore, we ascertained any fitness effects associated with this DNA damage by recording the reproductive outputs of control and irradiated individuals of both species with and without the ability to repair the DNA via desiccation or sex. Our data represent the first of their kind for bdelloid rotifers and therefore provide another important clue towards having maintained their status as ancient asexuals in the face of the many negative aspects of this mode of reproduction.

Material and Methods

Rotifer and algal cultures

Multiple populations of *P. roseola* and *B. rubens* were each cultured under a 9/15 h light/dark regime in 90x15 mm plastic Petri dishes at 20 ± 1°C, with weekly transfers of a small number of individuals from each population to new Petri dishes. The algae used as the food source for each species (*P. roseola*: *Cryptomonas sp*. SAG 26.80; *B. rubens*: *Monoraphidium minutum* SAG 243-1) were each grown in 500 ml Erlenmeyer flasks with COMBO medium [179] under continuous illumination (13W/840 Daylight neon tubes: Osram, Munich, Germany).

Each species was then used for two series of experiments, the first focusing solely on CPD accumulation and its repair and the second focusing on its impact on the reproductive capability of the animals.

I: Quantification of CPD accumulation and repair

Rotifers were filtered using a 60 µm mesh and transferred to algae-free COMBO medium for roughly one hour before batches of 50 animals in 2 ml of medium were transferred into one of 16 even-sized chambers with a maximum capacity of 5.5 ml and uniformly arranged as pie slices on a round plate. Each rotifer species was assigned randomly to half the chambers. One chamber per species served as a control through being covered with a UV-opaque Plexiglas (233, Röhm, Darmstadt, Germany) that removed virtually all UV-irradiation (< 1.5% transmission for wavelengths < 360 nm) but did not remove the radiation needed for photosynthesis (400-700 nm). The filled vessel was mounted on a custom-built rotation system (roughly 0.5 rotations per

minute) to ensure equal exposure of each chamber and the chambers were placed in a 2 l water bath equipped with a RC6 CP thermostat (Lauda, Lauda-Königshofen, Germany) to maintain them at a constant temperature (20°C). This procedure was replicated several times to obtain the desired number of measurements, while the order of the treatments (with or without time to repair and high or low irradiation intensity) was chosen at random.

UVB exposure originated from an MSR 400-HR bulb (Phillips, Amsterdam, The Netherlands) and was passed through a UVC cut-off filter and a quartz diffusor to exclude any UVC damage and to furthermore ensure equal irradiation of each chamber. Experiments used two different UVB irradiation levels differing by a factor of about four (table 6). Actual irradiation intensity striking the chambers was measured with a USB 2000+ U-Vis fiber-optic spectrometer (Ocean Optics, Dunedin, Florida, USA).

Table 6. Irradiances (in W m^{-2}) of the different spectral ranges used in the first experimental series.

Trial	Treatment phase	UVB (280-320nm)	UVA (320-400nm)	PAR (400-700nm)
High	Damage	2,2	30	195
	Repair	< 0,1	7,5	160
	Control	< 0,1	10	188
Low	Damage	0,5	7	134
	Repair	< 0,01	2	120
	Control	< 0,01	3	127

Series I experiments consisted first of all of 20 batches of 50 animals of each species (including controls) being exposed to UVB-irradiation for four hours. An additional 20 batches of 50 animals were likewise irradiated for four hours, with the chambers subsequently being covered by UV-opaque Plexiglas for an additional one or two hours to allow light-dependent repair of any UV damage. Immediately following irradiation and any repair time, the vessels were put on ice and 2.5 ml of 99% ethanol was added to each chamber to fix the rotifers. After 15 minutes, most of the liquid in each chamber was replaced with fresh 99% ethanol and each batch of 50 rotifers from a given chamber was transferred to a 1.5 ml tube (Eppendorf, Hamburg, Germany).

Finally, *P. roseola* was tested for its ability to repair CPDs over longer time periods at low ambient light intensities. To this end, batches of 50 *P. roseola* individuals were irradiated for four hours at the high UVB-intensity (see table 6) and subsequently transferred to 24-well flat-bottom plates (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA), with each well containing either 2.0 ml or 0.5 ml of algae-free COMBO-medium. The batches of 50 individuals in each of the lower-volume wells were desiccated for 3.5 days (= Protocol D [180]) in custom-built humido-thermostatic chambers (University of Oldenburg workshops), rehydrated with 1.0 ml algae-

free COMBO-medium and maintained for an additional six hours before ethanol was added as described before. The batches in the higher-volume wells were kept continuously hydrated for four days before ethanol was added for fixation. Each of the lower- and higher-volume treatments was replicated 20 times.

II: Quantification of the impact of CPD accumulation and repair on reproductive output

To investigate the potential fitness effects of UVB induced DNA mutations and their interaction with potential, secondary DNA repair phases (i.e., desiccation or sexual reproduction), we quantified and compared the reproductive output of both *P. roseola* and *B. rubens* under four treatment conditions: 1) an untreated control group, 2) untreated animals that could undergo desiccation or sex (for secondary DNA repair; as appropriate for the species), 3) irradiated animals, and 4) irradiated animals that could undergo desiccation or sexual reproduction as appropriate.

Each treatment group was seeded with a starter population of 50 individuals that was then subjected to the following experimental cycle: 10 days of continuous hydration, seven days of the experimental treatment (i.e., continued hydration versus desiccation / sexual reproduction), 14 days of continuous hydration, seven days of a second round of the experimental treatment, and a final four days of continuous hydration. During the hydrated phases (including those corresponding to the relevant experimental treatments), each treatment population was maintained at 20 ± 1°C in separate Petri dishes (5.5 cm diameter and a depth of 1.3 cm), each with 15 ml COMBO medium with algae under a 9/15h light/dark regime (illumination via 13W/840 Daylight neon tubes, Osram); half of the medium with algae and rotifers was aspirated and renewed every second day.

Treatment populations receiving UVB irradiation were only exposed during the first two (10- or 14-day) hydrated phases and then in addition to the normal illumination. UVB-irradiation was provided in four 15-minute time blocks per day, each interspersed by an hour using a bioblock scientific VL-6.LM UV-lamp (Fisher, Illkirch, France) with an intensity of 3.5 W/m^2 UVB emitted at 312 nm as measured with a USB 2000+ U-Vis fiber-optic spectrometer (Ocean Optics). No irradiation was provided on the final day of the hydrated phase.

For the secondary DNA repair phases, treatment populations of *P. roseola* were desiccated for six days in groups in their Petri dishes following Protocol D [180] in custom-built humido-thermostatic chambers (University of Oldenburg workshops) after half the medium was aspirated. Reanimation was induced through the addition of medium with algae. The production of sexual resting eggs by *B. rubens* occurred throughout the experiment, but was accelerated on the final day of the continuously hydrated phases by increasing the culture temperature to 26°C. Thereafter, half of the medium was aspirated from the Petri dish and the remaining fluid was evaporated within a day using the custom-built humido-thermostatic chambers. The dry Petri dishes were then held in the dark at 5-8°C for five days before hatching of the resting eggs was induced by adding 15 ml COMBO medium with algae at 20°C. Irradiation, where applicable, was first applied the day following the addition of the medium.

After the experimental cycle, 48 freshly hatched individuals were randomly chosen from each treatment group and transferred individually into separate wells of either 48-well plates (Becton,

Dickinson and Company) with 0.5 ml medium with algae (*B. rubens*) or 24-well plates (Becton, Dickinson and Company) with 1 ml medium with algae (*P. roseola*). Beginning with the first offspring (or eggs in the case of *P. roseola*), each offspring produced was counted and removed from the wells for 5 days at 12 h control intervals.

DNA extraction and quantification of CPDs

DNA was extracted at most one week after the experiments. Ethanol in the samples was drawn off as much as possible, with the remainder being evaporated at 50°C. DNA was extracted for each batch of 50 rotifers using a 70 µl digestion solution of 10% Chelex (Biorad; Munich, Germany) and 0.07 µg μl^{-1} proteinase K (Carl Roth, Karlsruhe, Germany). The samples were incubated for 30 min at 55°C and heat-inactivated for 10 min at 95°C before being cooled for at least 30 min at 5°C. Following sedimentation of the Chelex at 15000 rpm for 15 sec, the clear supernatant containing the DNA was transferred into sterile Eppendorf tubes and stored at -18°C until further processing.

CPDs were quantified using an immunofluorescent thymine dimer detection method [206]. For each sample, 100 ng DNA, as determined using a NanoQuant plate (Tecan, Männedorf, Switzerland) on an Infinite 200 Pro (Tecan), was denaturated for 10 min at 95°C, cooled for 30 min on ice, and vacuum-blotted in parallel on nitrocellulose-membrane (Portran BA79, 0.1 µm pore size, Whatman, Sanford, Maine, USA) using a Minifold 1 dotblot apparatus (96 well, Whatman) and a vacuum pump (KNF Neuberger, Freiburg, Germany). Two calibration series of UV-irradiated calf thymus DNA (Sigma-Aldrich, St. Louis, Missouri, USA) for which the CPD concentrations were determined beforehand (100 ng = 10-320 CPDs per 1 million base pairs) were included on each blot. After vacuum-blotting of the DNA, the membrane was washed with 200 µl phosphate-buffered saline (PBS, Carl Roth) while still in the dot-blot apparatus. DNA was then fixed to the membrane by heating it at 60°C for two hours. Nonspecific binding sites were blocked with 5% skimmed-milk powder (Sucofin, Zeven, Germany) in PBST (phosphate buffered saline and 0.1% (vol./vol. (volume/volume)) Tween 20: Sigma-Aldrich) for 30 min at room temperature. After washing with PBST (3 x for 10 min each), the samples were incubated overnight at 5°C with a monoclonal anti-thymine dimer antibody specific for CPDs (clone H3 produced in mouse, Sigma-Aldrich; 1:3000 diluted in PBST with 0.5% skimmed milk powder [wt./vol.] (weight/vol.)). Thereafter, the samples were again washed with PBST (3 x for 10 min each) before being incubated with *horseradish peroxidase* conjugated rabbit anti-mouse (Dako, Glostrup, Denmark) at room temperature for two hours (1:15000 diluted in PBST with 0.5% skimmed milk powder [wt./vol.]). Antibody labeled CPD complexes were detected by exposing the sealed membrane to photosensitive films (Amersham hyperfilm ECl, GE Healthcare, Chalfont St. Giles, Great Britan) after incubation with ECL western blotting detection reagent (GE Healthcare). Grey-scale values of the scanned films were determined in ImageJ1.46r [207] using a dot-blot analyzer plugin [208], with the final CPD concentrations being determined on the basis of the two calibration series.

Statistical procedure

All statistical analyses used SPSS Statistic 20 (IBM, Armonk, New York, USA) with a nominal alpha of 0.05. The occurrence of repair was inferred on the basis of a significant reduction in CPD levels in the short-term experiments as determined using linear regression.

The influence on reproductive output (number of offspring) of UV-irradiation with or without secondary DNA repair (i.e., desiccation or sex) was analysed separately for each species with a full factorial generalized linear model using a Poisson error structure with log as the link function. A post hoc test to disentangle the contributions of individual factors to a significant effect through their combination was done in a pairwise fashion using a Holm-Bonferroni [209] correction for multiple comparisons.

Results

Table 7. Amount of CPDs per million base pairs (mean ± SD) measured for *P. roseola* and *B. rubens* as a function of the amount of time allowed for repair after irradiation at two different intensities (see table 6). For *P. roseola*, the amount of CPDs present after four days at low ambient light intensities (with or without desiccation) following high-intensity irradiation is also given.

Species	Trial	Time for repair [h]	CPDs
B. rubens	Low	0	25.4 ± 15.2
		1	8.2 ± 10.0
		2	1.1 ± 3.0
	High	0	111.5 ± 129.2
		1	31.7 ± 29.2
		2	9.5 ± 12.5
P. roseola	Low	0	16.5 ± 15.3
		1	16.1 ± 26.6
		2	16.3 ± 17.5
	High	0	20.8 ± 12.8
		1	19.5 ± 16.4
		2	19.1 ± 14.2
		96 h	4.7 ± 4.7
		96 (with desiccation for 3.5 days)	0.7 ± 2.5

In the first series of experiments, striking differences between the two species were apparent with respect to their susceptibility to UVB-induced CPD formation and their ability to repair this damage (table 7). Although UVB-exposure generated comparable amounts of CPDs in both *B. rubens* and *P. roseola* in the low-irradiation treatment (two-sample $t = 1.825$, df (degrees of freedom) = 38, $P = 0.076$), the amount of CPDs increased significantly in the high-irradiation treatment for *B. rubens* compared to the low-irradiation treatment (two-sample $t = 2.963$, $df = 19.529$, $P = 0.008$), but not for *P. roseola* (two-sample $t = 0.962$, $df = 38$, $P = 0.342$).

CPD repair by *P. roseola* over a two-hour timeframe was negligible regardless of the irradiation level (low: $F_{1,58} = 0.001$, $P = 0.971$, adjusted $r^2 = 0.017$; high $F_{1,58} = 0.136$, $P = 0.713$, adjusted $r^2 = 0.015$). Even after four days, significant amounts of CPDs still remained in individuals of *P. roseola* that were kept continuously hydrated after a high-UVB treatment (one-sample *t*-test against zero = 4.445, $df = 19$, $P < 0.001$), although these values were significantly lower than initial values (two-sample $t = 5.269$, $df = 24.162$, $P < 0.001$). By contrast, nearly no CPDs were found in individuals that had been desiccated for four days (one-sample *t*-test against zero = 1.227, $df = 19$, $P = 0.217$). This resulted in a significant difference between the two different long-term treatments (two-sample $t = 3.347$, $df = 28.6$, $P = 0.001$).

By contrast, *B. rubens* repaired almost all induced CPDs over a period of at most two hours, with the repair rate of CPDs at either irradiation intensity following a linear regression (low: $F_{1,58} = 49.833$, $P < 0.0001$, adjusted $r^2 = 0.453$, average rate of repair ± s.e.m. = 12.1 ± 1.7 CPDs per hour starting from an initial value ± s.e.m. of 23.7 ± 2.2 CPDs; high $F_{1,58} = 16.747$, $P < 0.0001$, adjusted $r^2 = 0.211$, average rate of repair ± s.e.m. = 51.0 ± 12.5 CPDs per hour from an initial value ± s.e.m. of 101.9 ± 16.1 CPDs).

In the second series of experiments, reproductive output (figure 7) in *P. roseola* was significantly reduced by UV-irradiation (Wald $c^2 = 211.207$, $df = 1$, $P < 0.001$), desiccation (Wald $c^2 = 83.945$, $df = 1$, $P < 0.001$) and the interaction between both (Wald $c^2 = 78.495$, $df = 1$, $P < 0.001$; omnibus test of the fit of overall GLM model: Likelihood-Quotient $c^2 = 318.286$, $df = 3$, $P < 0.001$). In teasing apart these effects, the post hoc test revealed that desiccation without UV-irradiation had no significant influence on its own (mean difference ± s.e.m. to the control = -0.21 ± 0.765, $P = 0.785$). However, in combination with UV-irradiation, it increased the negative effect of the latter significantly (UV-irradiation alone: mean difference ± s.e.m. to the control = -3.23 ± 0.717, $P < 0.001$; in combination with desiccation: mean difference ± s.e.m. to the control = -9.52 ± 0.619, $P < 0.001$, figure 7A). The reproductive output of *B. rubens* was, by contrast, only slightly, but significantly reduced by sexual reproduction (Wald $c^2 = 9.517$, $df = 1$, $P = 0.002$); otherwise, UV-irradiation alone (Wald $c^2 = 0.055$, $df = 1$, $P = 0.814$) or in combination with sex (Wald $c^2 = 0.018$, $df = 1$, $P = 0.893$) had no effect (figure 7B, omnibus test of the fit of the overall GLM model: Likelihood-Quotient $c^2 = 9.610$, $df = 3$, $P < 0.022$).

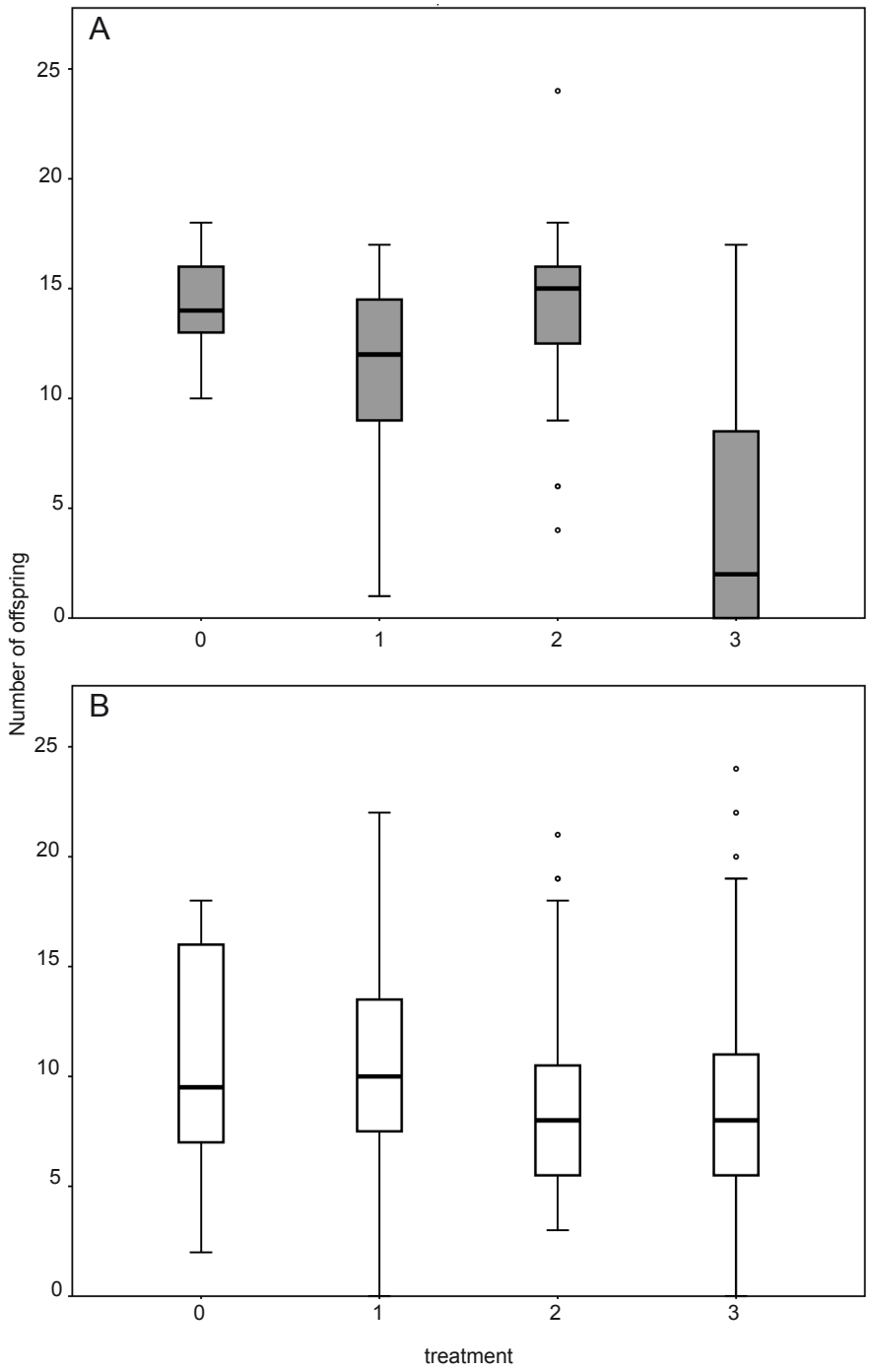

Figure 7. Reproductive output (measured as number of eggs laid during the first five reproductive days) without any treatment (0), with UV-irradiation (1), with desiccation or (as appropriate for the species) (2), and with both together (3) for both P. roseola (**A**) and B. rubens (**B**).

Discussion

In dealing with potential DNA damage and mutations induced through UVB-exposure, two main strategies are possible: prevention and/or subsequent repair. Prevention is possible both by behaviorally minimizing the exposure to UV-irradiation (not allowed here) and through chemical mechanisms such as UV-absorbing compounds (e.g., carotenoids or mycosporine-like amino acids in phytoplankton) [210]). When considering repair, several potential mechanisms exist.

These include highly efficient and rapid photoreactivation reactions mediated by specific light-dependent photolyases [186,211,212] or more versatile mechanisms such as nucleotide excision and recombinational repair [186]. An extension of the latter would arguably include the genome repair bdelloid rotifers must necessarily undergo following a desiccation event, using homologous recombination of sufficiently similar DNA strands of their degenerated tetraploid genome to rebuild it [88,91]. However, all these latter mechanisms are slower and more energetically expensive than photoreactivation [213]. Moreover, they can also introduce their own mutations, especially if the lesions are frequent and in the relative vicinity of one another [186].

Although these two strategies would not seem to be mutually exclusive, our results highlight that *P. roseola* and *B. rubens* employ either one strategy or the other to deal with DNA lesions and mutations induced by high UVB levels. Based on our experiments, *P. roseola* appears to be highly resistant to DNA damage, regardless of irradiation intensity. A potential candidate for a UV-absorbing compound in *P. roseola* (given that behavioural avoidance was excluded) might also be carotenoids given that this species is often reddish (which, interestingly, diminishes with age). Yet, *P. roseola* appears largely unable to actively repair any damage that occurs except through a desiccation event. The slight reduction in the number of CPDs after four days in this species appears to be due more to the natural deamination of cytosine to uracil (resulting in a C → T mutation) that occurs within CPDs [26]. Using an intermediate value of 50 h for the half-life of a cytosine within a CPD (from published estimates ranging from 2 to 100 h [214,215]), we would expect only five CPDs to remain after four days based on our initial measured values, which is in good agreement with our observations. The significant reduction in reproductive output observed for irradiated (but not desiccated) individuals of *P. roseola* also highlights the inefficient or missing DNA repair mechanisms in this species.

By contrast, *B. rubens* showed irradiation intensity-dependent DNA damage that was found to be largely repaired within the course of a few hours. Interestingly, the rate of repair was not constant, but seemed to scale with the level of damage such that all CPDs were removed in about two hours regardless of the initial level of damage. Although our methodology could only reveal the repair of CPDs in *B. rubens per se* and did not include the possible introduction of point mutations following repair, the lack of any significant reduction in the reproductive output of irradiated individuals would indicate the latter effect to be small at best. This fact, together with the observed high repair rate, would indicate that photolyases are likely used by this species.

Although both species were subjected to unnaturally high levels of UVB-radiation in our experiments (normally a maximum of 0.2 W m^{-2} in temperate regions [210]), our results are valuable in revealing species specific strategies. However, the reason behind their use of differing strategies is unclear. As mentioned, the two general mechanisms of prevention and repair are not mutually exclusive and would provide additional protection when combined. The apparent lack of short-term DNA repair in *P. roseola* is especially puzzling, firstly because asexual species in general are more susceptible to accumulating deleterious mutations and secondly because CPDs are exceptionally detrimental in that they obstruct gene transcription [204,205]. However, this conclusion is consistent with the “sleeping beauty” hypothesis for bdelloid rotifers [24]. Based on the observation that continually hydrated individuals show a decrease in fitness that is reset after

a desiccation event [24], it was hypothesized that individuals of these species regularly need to undergo desiccation to repair any DNA damage that they have accumulated but seem unable to repair while in the hydrated phase. However, desiccation does not appear to be a cure-all for DNA damage given our observations that irradiated animals still showed a reduction in fitness following desiccation. In other words, although CPDs were removed during this process, they nevertheless would appear to induce errors that are spread throughout the genome.

In explaining the apparent lack of immediate CPD repair in *P. roseola*, it might be that this species is not normally confronted with significant UVB levels. For instance, irradiation intensity attenuates relatively fast in water [216,217] and bdelloids often occur in habitats like mosses or soil that (partly) shield them against UV-irradiation [23]. However, although this means that our experimental set up was highly unnatural in that the water depth was at most 1 cm and behavioural avoidance of UV-irradiation by the animals was also prevented, our results still indicate what *P. roseola* is capable of in dealing with the resultant DNA damage.

By contrast, because the planktonic rotifer *B. rubens* might be exposed to higher UV-irradiation at the water surface, fast and effective DNA repair might represent a more profitable strategy than the accumulation of UV-absorbing components. Importantly, in the absence of any immediate DNA repair and insufficient protection from irradiation, any residual damage will be exposed in the haploid males at the initiation of sex [13] and thus might be disadvantageous for long-term survival if this situation prevents or severely hinders successful sexual reproduction.

That being said, increased exposure to UV-irradiation would be expected in bdelloids during the lead up to anhydrobiosis when desiccation removes any protecting water layer as well as limits any potential behavioural avoidance. At this time, the immediate repair of any lesions via photolyases (as inferred for *B. rubens*) would likely be impossible given that the metabolism is being progressively silenced and protection by UV-absorbing compounds would represent an effective alternative strategy. However, if this protection fails, as in our experiment, survival might depend critically on the built-in genomic redundancy provided by the degenerate tetraploid genome together with the frequency of desiccation. In the latter case, desiccation needs to occur frequently enough to prevent the accumulation of excessive numbers of mutations that would impact negatively on the probability that recombinational repair within the homologous parts of the genome can eliminate chance mutations via gene conversions [218,219].

Importantly, the combination of strategies in *P. roseola* might not be foolproof and, although the effect was undoubtedly magnified under our experimental conditions, can lead to drastic reductions in fitness even if desiccation has occurred. A similar effect might be expected if the period between desiccation events is too infrequent leading to the accumulation of mistakes and mutations. In either case, the reduced fitness or even survival of individuals with high mutation loads (in analogy to inbreeding that happens within homologous parts of the degenerate tetraploid genome) might help to explain the difficulties in finding robust indications for the expected faster accumulation of (slightly) deleterious mutations in bdelloids and the increased tendency to find these animals in desiccation prone habitats [106].

Finally, it is important to stress that our results hold only for the two species examined and the

trends should not be extended to all monogonont and bdelloid rotifers. For instance, the presence of UV-absorbing compounds has been documented in some monogonont rotifer species (e.g., *Polyarthra dolichoptera*, *Synchaeta grandis*, *Synchaeta pectinata* and *Keratella cochlearis*) [220]. In addition, as shown by [95] in a related context and implicit in our arguments above, habitat is a potentially important confounding factor. As such, it is important to extend investigations to include other rotifer species, with a view to comprehensive sampling in terms of both habitat and evolutionary relatedness. In addition, given that our experiments focused on a specific type of DNA damage (DNA lesions through UVB-irradiation), it would be instructive to explore further how bdelloids repair their genome in general and deal with point mutations derived from other sources, what kinds of damage can be repaired and when such repair is possible (e.g., is it necessarily restricted to when the genome is reconstructed following a desiccation event?).

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4.4 Fitness affects the reproductive strategy in a facultative sexual rotifer

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Abstract

Facultative sexual species employ a dual reproductive strategy (heterogony) comprising primarily asexual reproduction with intermittent phases of sexual reproduction. Understanding when and why sexual reproduction is favored in these species might help to unravel the paradoxical prevalence of it in across metazoans instead of the less costly asexual reproduction as well as explicitly test the hypothesis of a pluralistic explanation of sex. To this end, we examined the influence of changes in food quality, water temperature, physiological adaptation and all combinations of these factors on both the lifetime reproduction and investment into sexual offspring in a clonal population of the monogonont rotifer *Brachionus rubens*. The investment into sex, both absolutely and relative to lifetime reproduction, was most closely tied to individual fitness (total number of offspring) and not to changing or challenging conditions as was the case in other studies. Indeed, investment into sex increased after a period of physiological adaptation to the new conditions was allowed, probably because of the amelioration of short-term stress effects. This emphasizes the increased costs of sexual reproduction in these animals, both on a physiological level (e.g., through provisioning of the resting eggs) as well as with respect to its inherent time lags and slower population growth compared to asexual reproduction. This study supports the bet-hedging nature of facultative sexual reproductive and the need to balance the short-term, cheaper fitness gains from asexual proliferation with the long-term, but costlier survival ensured through the sexual resting eggs.

Key-words asexual reproduction, bet-hedging, evolution of sex, heterogony, influence of environmental changes, stress

Introduction

Although sexual reproduction entails substantial costs compared to asexual reproduction (as reviewed in [1]), it is nonetheless widespread and prevalent in nature [56], a contradiction that has puzzled evolutionary biologists for decades [2,143,221]. Facultative sexual species represent an ideal system for empirical work into the evolution of sex because they utilize both strategies, reproducing primarily asexually with intermittent switches to sexual reproduction. Understanding the conditions under which sexual reproduction becomes favored in such species (e.g., any or all of variable environmental conditions, mutations, parasites or predators) could help test the many associated hypotheses regarding the evolution and persistence of sex in general [123,124]. Such investigations could also help test the relatively recent proposal of a pluralistic explanation to sex [123,124] that, in the apparent absence of a simple, universal explanation, emphasizes that multiple factors might instead interact to reduce the actual costs sex has to overcome, especially with respect to any single factor in isolation. Extending the concept further, it might be that different sets or weighting of factors might explain sex in different species, even between closely related ones. Research in this regard, however, remains sparse.

Monogonont rotifers, a clade of aquatic, microscopic invertebrates, represent a promising group for investigations of this general topic [222], with species belonging to the genus *Brachionus* having been particularly well studied here (see below). Generally, most monogonont species employ a classic heterogonic strategy tied to their often temporally unstable aquatic environments, under which asexual reproduction is favored under optimal / stable environmental conditions to increase population sizes relatively rapidly with sexual reproduction to produce resting eggs becoming favored as deteriorating conditions no longer permit individual survival.

For species of *Brachionus* in particular, several factors are known to induce sex (e.g., starvation or temperature; see below), all of which are indirectly linked to crowding and/or habitat degradation to varying degrees. Indeed, crowding seems to be the proximal trigger for sex, as individuals release a protein into the water that, upon reaching a threshold concentration, induces sexual reproduction via quorum sensing [172,174]. Although the reaction is assumed to be proportional to the amount of crowding [174], sex is never induced in all individuals of a population, even under strong stimulus conditions [175,223]. In addition, the threshold concentration to induce sex seems not be fixed even within species [224] and can be quite low [225]. However, crowding might itself be acting as a proxy for subsequent deteriorating conditions given that it is connected with higher levels of intraspecific competition and might also indicate the evaporation of water. Indeed the propensity of sex in *Brachionus calyciflorus* was higher under experimental conditions mimicking ephemeral compared to permanent habitats [224]. Likewise, starvation, which could be a consequence of crowding / deteriorating conditions, was shown to induce sex in *Brachionus plicatilis* [226]. More generally, changing food quality conditions were recently shown to influence the propensity of sex in *B. calyciflorus*, with the frequency of sex increasing if the quality fluctuated periodically between good and bad, but remaining low if food quality was either constantly good or bad [178]. With only a single change in food quality, increased occurrence of sexual reproduction resulted only during the period of adaptation to the new condition [165]. Finally, higher temperatures have also been shown to yield a higher proportion of sexual offspring in

different studies in *Brachionus* ([165,178] vs. [175]), potentially by indicating evaporation, evoking temperature stress or by simply increasing growth rates and thus density.

Whatever the trigger, the shift to sexual reproduction in monogonont rotifers entails significant additional costs and risks that go beyond those usually associated with sex [1]. Once sexual reproduction is induced, females must first asexually produce a new egg-type from which sexually active (mictic) female clones emerge [13] in addition to the normal asexual eggs yielding further amictic female clones. Unfertilized mictic females produce haploid male eggs via meiosis, where the males, upon hatching, can fertilize mictic females before they start to produce male eggs. These fertilized females then exclusively produce diploid resting eggs that normally hatch only after a certain latency period and only if certain cues like temperatures and light conditions are met [164].

Thus, although it represents a necessary strategy to potentially overwinter unfavorable conditions [1,164], sexual reproduction in monogonont rotifers delays population growth considerably compared to a purely asexual strategy [223]. Ideally, sexual reproduction would thus be delayed for as long as possible until conditions become unsuitable for survival of the population without resting eggs (the “bang-bang strategy”; [227]) while still providing the males sufficient time to find and fertilize mictic females [228]. Even so, some form of bet hedging using intermediate mictic rates to balance between the short-term advantages of asexual reproduction and the long-term insurance provided by sex [229] might be the optimal strategy to guarantee long-term survival [230,231] when the onset of suboptimal conditions is uncertain or difficult to predict (both in its timing and if these conditions will really occur or persist) [232].

In this paper, we examine the influence of three different factors known to induce sex in *Brachionus*: (food quality, water temperature, and time for adaptation), both separately and in combination, on the lifetime reproduction and production of sexual offspring by clonal individuals of *Brachionus rubens* Ehrenberg, 1838. Our results thereby provide a test of the pluralistic theory for the evolution of sex as well as provide data on the apparent inherent trade-off between asexual and sexual reproduction in facultative sexual species, including important information as to the factors that might determine when each strategy is favored.

Materials and methods

Culture conditions and experimental setup

All experimental individuals came from a clonal, stock culture of *B. rubens* (density of ~100 individuals ml^{-1} at the start of the experiments) that was derived from a single individual five months previously. Both the stock culture and all experimental individuals were incubated at 20 ± 1 °C under a 9/15h light/dark regime and were fed the algae *Monoraphidium minutum* SAG 243-1 raised in COMBO medium [179].The stock culture was maintained in several plastic Petri dishes (90 x 15mm) and was restarted weekly with ~50 individuals and fresh algae in new Petri dishes.

Individuals were assigned randomly to one of four treatment groups: 1) food and temperature as in the stock culture (control), 2) algae raised in COMBO medium with an additional 0.5 g NaCl l^{-1} and

a nitrogen concentration of 80 µM instead of 350 µM ("low-quality food"), 3) incubated at 25 ± 1 °C ("high temperature") and 4) high temperature combined with low- quality food. Individuals were tested both with and without the chance to adapt to the treatment conditions. In the former case, an adaptation period of 12 days was used, in line with the results from a similar experiment [165] showing that differences were already apparent after only seven days. For each treatment, 200 individuals were transferred to a Petri dish with 40 ml of the corresponding algae (with 50% of the algae being changed every second day) and held under the appropriate temperature conditions.

To measure individual lifetime reproduction, 35 females per treatment were transferred individually into single wells of a 48-well plate (cellstar suspension culture plate, Greiner bio-one, Kremsmünster, Austria), each containing 400 µl of the appropriate algae. Each female was controlled every 12 h over its entire lifespan for (F1) offspring, which were similarly transferred individually to new wells filled with 400 µl of the corresponding algae after taking note of their birth-rank (e.g., rank 1 for the first offspring produced, rank 2 for the second, and so on). If more than one offspring was present during the control period, the larger one was taken to be older. Parental females that turned out to be mictic (i.e., produced male F1 offspring) were excluded from further testing so that the effective sample sizes were lower than 35 (see table 8). The F1 offspring were kept until their own first offspring (F2) was sired, with the former being scored as either sexual if a male F2 was born or asexual if a female F2 was born. If no F2 offspring were produced, no status for the F1 offspring was given. For all test individuals, 50% of the algae were renewed in each well every two or three days over the entire course of the experiment.

To our knowledge, the minimum requirement for the emergence from resting eggs from our clone is to slowly dry the eggs out and keep them dry at 8 °C for about 2-3 days before adding medium and algae again (pers. obs.). Nevertheless, as a control to determine whether or not any sexually derived offspring could have hatched during our experiments, 40 (sexual) resting eggs from the stock culture were kept under the respective experimental conditions for 12 days as were all resting eggs that were produced under the experimental populations and that were still present at the end of the experiment. In no case did any resting eggs hatch, strongly indicating that all results were obtained from clonal individuals derived from asexual reproduction only and that the stock culture was likely clonal as well.

Data Analysis

All statistical analyses were performed in SPSS Statistic 20 (IBM). The influence of temperature (temp; 20°C or 25°C), food quality (food; high or low) and adaptation time in the new conditions (time; zero or 12 days) were analyzed as fixed factors on the percentage of sexual offspring produced by the parental female generation (= percent investment in sex) using a full-factorial generalized linear model (GLzM). A negative binominal error structure with a logistic link function was used because many females showed zero investment into sexual offspring and thus the distribution of the percent sexual offspring was overdispersed at low values. A second GLzM (Poisson with logistic link, $\alpha = 0.025$) using the same factors but with the total number of offspring as the dependent variable was also performed given indications that this variable also varied considerably among the factors and might parallel the results for the number and percentage of

sexual offspring (table 8). Both variables were not analyzed together due to the different error structures that fitted to the data. Additionally, we analyzed whether or not females produced any sexual offspring at all as a categorical, yes/no variable in a GLzM with the same factors as above (without interactions) and the total number of offspring as a covariate using a binary structure and a logistic link function. Finally, a stepwise logistic regression was used to analyze how each significant factor (ordered from those having the highest to the lowest Wald c^2 values) enhances the correct prediction as to whether or not an individual is reproducing sexually.

Table 8. Investment into sex and overall offspring by the parental female generation as influenced by three factors (food, temperature, and adaptation) either alone or in combination.

Treatment	**Percent sexual offspring per female**	**Total number of offspring per female**	**Number of amictic females producing sexual offspring**
stock culture	19.17 ± 17.85	9.45 ± 4.87	20 of 29
high temperature	31.21 ± 12.99	14.03 ± 4.7	29 of 29
low quality food	4.52 ± 12.11	4.74 ± 2.03	4 of 27
low quality food + high temperature	7.53 ± 11.44	5.43 ± 2.49	11 of 30
adaptation (to stock culture)	33.93 ± 23.94	12.2 ± 5.21	26 of 30
adaptation + high temperature	23.73 ± 14.96	12.97 ± 4,38	29 of 30
adaptation + low quality food	6.60 ± 14.53	5.37 ± 2.33	8 of 30
adaptation + low quality food + high temperature	11.45 ± 12.35	6.31 ± 1.67	17 of 29

All values represent means ± SD; the overall sample size for each treatment can be found in the last column.

Potential differential investment into sex over time (i.e., whether a tendency existed for the F1 offspring of a given rank to be sexual or asexual) was analyzed as in [175]. Briefly, for each individual treatment group (including the control), the fate of any given offspring was modeled using a cumulative binomial function (i.e., reflecting the two possible outcomes: sexual or asexual) with the probability of it being sexual being taken from the overall proportion of sexual offspring across all ranks for that treatment group. For a given rank, the observed proportion of sexual offspring was then compared to the expected value obtained from the model, with confidence intervals on the latter being derived based on the number of reproductive females still present at that rank. Deviations from the expected value were held to be significant at $\alpha = 0.05$ if the

observed proportion of sexual offspring fell outside the 95% confidence intervals constructed for that rank (for more details, see [175]).

Results

Factors influencing sexual reproduction

Both the percent of sexual offspring as well as the total number of offspring responded nearly identically to all three factors (and their combinations) examined in this study (tables 8 & 9). Both showed strong, significant reductions in response to low quality food, whereas higher temperatures and having a period of adaptation induced slight, but still significant increases. By contrast, none of the interactions of these factors had a significant influence on either variable. Additionally, the two variables also interact with one another, in that there is a higher relative investment into sexual offspring with higher total numbers of offspring (figure 8A). In absolute terms, females producing more than seven offspring are more likely to also produce sexual offspring than those

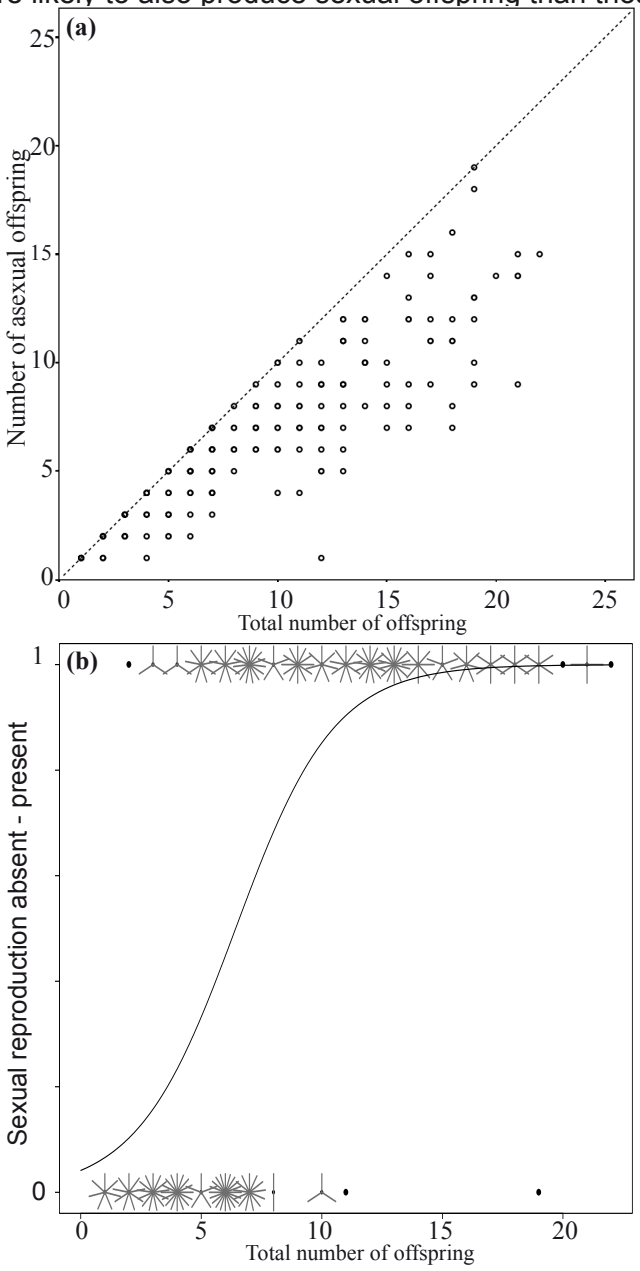

Figure 8. Relationship between the number of asexual offspring (**A**) and the production of any sexual offspring (**B**) with the lifetime reproduction (overall total number of offspring produced) of all females. The thin broken line in (A) indicates total investment into asexual offspring. In (B) points represent one individual with the total number of offspring and the according state (state 1 with sexual offspring, state 0 only with asexual offspring), while attached dashes count the cases if there are more than one. Data in (B) is fitted ignoring the minor influence of food quality and temperature (see results).

producing less than this (figure 8B).

Similar tendencies were noted when examining the number of individuals investing into sex at all (table 8). Again, the propensity for any sexual reproduction increased significantly with the

Table 9. Influence of all factors and factor combinations on the percent offspring and total number of offspring per female (each test in the first seven rows with *df* = 1).

Factors	**Influence on percent sexual offspring per female**	**Influence on total number of offspring per female**	**Direction of influence**
low quality food	Wald χ^2 = 91.343, *P* < 0.001	Wald χ^2 = 273.071, *P* < 0.001	negative
high temperature	Wald χ^2 = 4.736, *P* = 0.03	Wald χ^2 = 15.391, *P* < 0.001	positive
low quality food * high temperature	Wald χ^2 = 2.899, *P* = 0.089	Wald χ^2 = 0.677, *P* = 0.411	none
adaptation	Wald χ^2 = 3.996, *P* = 0.046	Wald χ^2 = 5.471, *P* = 0.019	positive
adaptation * high temperature	Wald χ^2 = 2.162, *P* = 0.141	Wald χ^2 = 2.580, *P* = 0.108	none
adaptation * low quality food	Wald χ^2 = 0.835, *P* = 0.361	Wald χ^2 = 0.255, *p* = 0.614	none
adaptation * low quality food * high temperature	Wald χ^2 = 2.608, *P* = 0.106	Wald χ^2 = 3.507, *P* = 0.061	none
Goodness of fit	Pearson χ^2 = 416.66, *df* = 226	Pearson χ^2 = 360.37, *df* = 226	n/a
Omnibus test of the overall fit of the GLzM versus a constant model	Likelihood-Quotient χ^2 = 99.461, *df* = 7, *P* < 0.001	Likelihood-Quotient χ^2 = 339.432, *df* = 7, *P* < 0.001	n/a

number of total offspring the female produced (Wald $c^2 = 27.805$, $df = 1$, $P < 0.001$) as well as with exposure to higher temperatures (Wald $c^2 = 10.722$, $df = 1$, $P = 0.001$), but decreased significantly with low food quality (Wald $c^2 = 13.331$, $df = 1$, $P < 0.001$). In contrast to previous results, however, the presence of a period of adaptation had no significant effect (Wald $c^2 = 2.266$, $df = 1$, $P = 0.132$; goodness of fit of the overall model Pearson $c^2 = 755.001$, $df = 86$, Omnibus test: Likelihood-Quotient $c^2 = 143.830$, $df = 8$, $P < 0.001$). The strongest effect on whether or not sexual offspring were produced derives from the total number of offspring ($c^2 = 120.204$, Nagelkerkes $r^2 = 0.546$). The addition of food and then temperature as factors improves the fit of the model only slightly, but still significantly (food: additional increase of ca. 2% in fit and 9.775 for c^2, $df = 1$, $P = 0.002$, new Nagelkerkes $r^2 = 0.579$; temperature: additional increase of ca. 0.4% in fit and 11.553 for c^2, $df = 1$, $P = 0.001$, new Nagelkerkes $r^2 = 0.616$).

The timing of sexual reproduction

The results into potential differential timing of sexual reproduction showed no clear pattern (figure 9), in part because investment into sex was very low for the treatments involving low food quality. Animals in both the control and temperature treatments (with or without adaptation) show a significant tendency to increased sex at the early intermediate ranks, with animals in the control group also showing significantly reduced sex at the earliest ranks. Otherwise, no clear trends were apparent and the values appear to vary relatively randomly within the 95% confidence intervals. As mentioned, rates of sex were extremely low in treatments involving low food quality. Under these conditions, a maximum of 10 offspring were produced (compared to 21 to 22 for the other treatments) and no sexual offspring were produced past rank 8 (and usually sooner). By contrast, sexual offspring were produced for longer in the remaining treatments (generally until at least rank 17), with animals from the high temperature treatment producing sexual offspring for longer compared to the corresponding control treatment. No clear effect for the period of adaptation was observed.

Discussion

Our results clearly indicate the total number of offspring produced by female *B. rubens*, and thus their general (physiological) condition, to be a key, proximal determinant of sexual reproduction in these animals. Although the environmental factors investigated also influenced the propensity for sexual reproduction (and food quality in particular), they appear to be working indirectly via their effects on the physiological condition of the animals. Thus, overall fitness would instead appear to be determining if individual females are fit enough for sex and how much they can invest. (The positive influence of the higher temperature might reflect that 25°C is closer to the optimal temperature of the species and/or of the food source.) Support for this hypothesis derives from theoretical demographic models for *B. plicatilis* [230] and heterogonic organisms in general [231], which predict intermediate investment of the total reproductive output into sex, with the optimal level being dependent on how good the conditions are and increasing with better conditions [230]. Additional support is provided by the adaptation treatments. Although the period of physiological adaptation was relatively short at 12 days (which together with the apparent absence of any offspring from sexual resting eggs excludes any evolutionary adaptation), both total number of

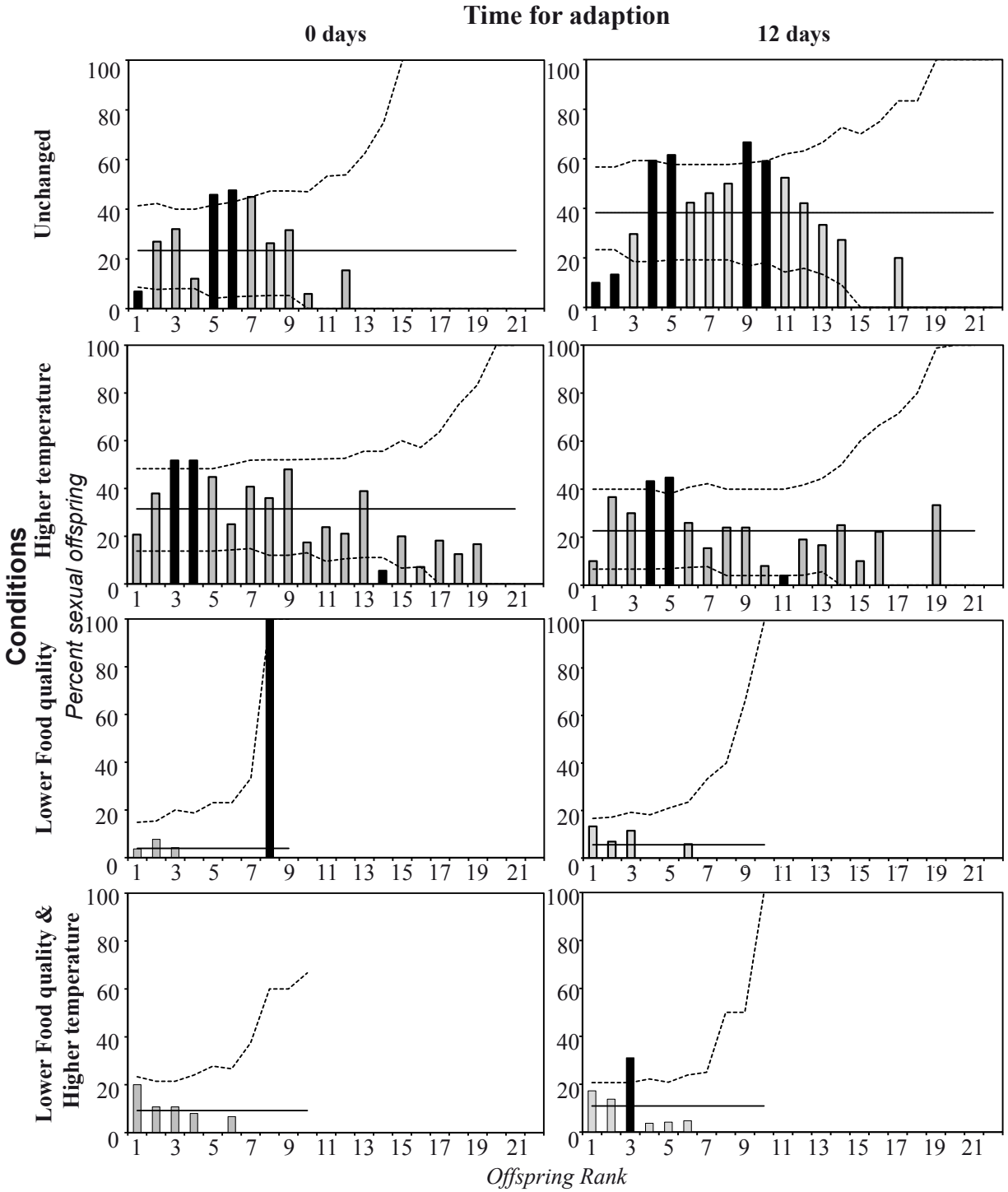

Figure 9. Percentage of sexual offspring as a function of their birth-rank for three different treatments (high temperature, low-quality food, physiological adaptation) and all combinations thereof. Bars in black represent significant deviations from the expected percentage of sexual offspring across a given treatment (continuous line) by falling outside the 95% confidence intervals of this value for that rank (broken line; lower limit is zero if not visible).

offspring and investment into sex were nevertheless increased significantly in all these treatment groups. Thus, adaptation would appear to improve the physical condition of the animals by ameliorating any short-term stress reactions to the changing environment.

An important caveat here is that all our experiments were conducted under conditions of more or less constant density of ca. 2-3 females ml^{-1} and therefore excludes any density-dependent effects. For instance, it has been shown theoretically that the amount invested into sex by *B. plicatilis* [230] is dependent on how close a given population is to its carrying capacity. In addition, the release of the mixis stimulus that occurs under increased densities is known to directly stimulate sexual reproduction [172,174]. That being said, our results still highlight that sex does occur in the apparent absence of the mixis stimulus and that the degree of investment in sex can also change independently of the mixis stimulus. It is also noteworthy that our results are comparable to those of [175] in which investment into sex was counted in a comparable way but where the experiments were conducted at higher densities (30 females ml^{-1}). An unanswered question at this point is the relationship between the mixis stimulus and fitness in inducing sex in so far to what degree individual females can ignore the mixis stimulus should they be too unfit to increase their investment into sex. The possibility for this scenario certainly exists given that it is known that variation exists within a species with respect to the threshold level of the signal that will induce sex [224] and that a flexible response in general might be advantageous, in part because the mixis signal is not always species specific [168,169] and so might always be present in the background, even under otherwise favorable / non-crowded conditions for the species.

Thus, *B. rubens* would appear to be continually following a mixed, bet-hedging strategy that balances the short-term gains of the less costly asexual reproduction against the long-term insurance of the more costly sexual reproduction. The balance, however, is clearly shifted toward asexual reproduction, which yielded at least 70% of all offspring regardless of the treatment group. This observation together with the apparent primacy of overall fitness as a determinant of sex emphasizes the many, diverse costs of the latter in addition to the baseline “two-fold” cost of sex. First, sexual reproduction entails several inherent time lags that effectively decrease the net birth rate, including the need to first generate males, the delay until the first offspring hatch from the resting eggs, and the delay until these offspring can react to a mixis stimulus [30,233]. Thus, the shift to sexual reproduction reduces population sizes through decreased net birth rates (see [165,178,223]), with simulation studies showing that the delays are large enough to effectively halt population growth entirely at high levels of sexual reproduction (e.g., individuals of *B. plicatilis* producing 75% or more sexual offspring) [230]. Second, recombination and segregation means that some proportion of the sexual offspring will be less well adapted / fit than the parent or its cloned offspring [187]. Likewise, many males also will not fertilize any females and so contribute nothing to the fitness of their mother [230]. Finally, sexual reproduction entails substantial energetic resources for sufficient provisioning of the resting eggs and offspring therefrom [234]. This latter, physiological cost could also explain the tendency towards the slightly decreased investment into sex at later offspring ranks, with the increased time between successive offspring also implicating aging and a decrease in the efficiency to gather and process the resources needed for reproduction at all [175]. However, when individual reproduction is high, these costs

might become proportionately cheaper because sufficient asexual offspring will be produced to ensure short-term fitness. In addition, the relative cost of sex might also be context dependent in that simulation studies have shown that individuals of *B. plicatilis* should invest more into sex when the population is closer to its carrying capacity [230]. Under these conditions, which are analogous to crowding, the increased competition that would be present among the asexual offspring apparently lowers the relative costs associated with producing time-delayed sexual offspring.

Nevertheless, the costs associated with sex effectively reduce the short-term fitness for any given individual investing in it and thus provides a short-term competitive advantage to those that manage to stop or reduce sexual reproduction (especially in more permanent environments) [223]. Indeed, individual variation in this trait is known [235] and was also witnessed here, with the presence of females with low reproductive output that nevertheless produced sexual offspring as well as, albeit much less frequently, the converse situation. Together, these facts help explain observations of a gradual decrease of the investment into sex within populations over time [165,178,224] as well as of the tendency for obligate asexuals to arise in *Brachionus* sp. in constant environments [28,29,236,237]. It also cannot be excluded that latter tendency might be more widespread among monogonont rotifers; however, data in this regard are missing entirely.

In addition, the costs associated with sex are apparently severe enough that sex is expected to be largely absent in the severely degraded or challenging environments (as in our low quality food treatment), when conventional wisdom would otherwise lead us to expect the highest levels of sex to ensure long-term transmission of the genetic information of an individual. Thus, although the production of asexual offspring potentially represents a dead-end strategy should the environment continue to deteriorate, it might also represent the only one available to these animals at this time and using a suboptimal strategy is better than no reproduction at all. Importantly, the bet-hedging strategy employed in this species, with its continual investment into sex and increased investment under better conditions, means that long-term genetic transmission has already been assured, even for those individuals that find themselves in a dying population, by their clones from previous generations.

It remains unclear how well *B. rubens* can track and react to a changing environment [238]. Some evidence for this ability is present in *B. calyciflorous*, which responds to altered environmental conditions (food quality) with increased rates of sexual reproduction (which temporarily decreases population sizes), a tendency that disappears if the environment remains constant thereafter [165,178]. However, the inherent delay in having to first generate males obviously limits how rapid any reaction could be. As noted above, an analogous tendency is missing from our results and the degree and investment into sexual reproductive seems tied more directly to the physical condition of a given individual. Another potential mechanism to track a changing environment involves not so much the amount of investment into sex, but rather the timing of this investment by each individual. A reasonable expectation here might be for the animals to shift to sexual reproduction earlier in an altered environment to ensure long-term survival of the genes before switching back to asexual reproduction for the later offspring. Importantly, such a switch that postpones asexual reproduction, in combination with the time lags inherent to sexual reproduction

could help explain the initial decreases in population size as well as the relative higher proportion of sexual reproducing individuals in the population observed by Becks and Agrawal [165] in their study. However, our own timing data are inconclusive on this point and show no clear trends. At best, a tendency for the earliest offspring to be asexual exists (and significantly so for individuals held under control conditions), with an increased investment into sex only at early intermediate ranks. Again, this hints at a bet-hedging strategy that here seeks to insure and maximize short-term fitness rather than any direct tracing of the environment.

Our results reinforce that that evolution of sex and the drivers behind it are complex. Although sex in monogonont rotifers, like many other animals with a heterogonic reproductive strategy, is closely associated with an overwintering stage, this connection is not absolute in that asexual resting eggs are known within the group [239]. How widespread this ability is or how frequently such resting eggs are produced remains unknown. However, the long-term disadvantages of a purely asexual strategy [10,11,48], would argue against their high prevalence and the necessity of at least occasional sexual reproduction [69,71,240]. Finally, the apparent primacy of individual fitness for determining the propensity of and investment into sex in *B. rubens* does not necessarily contradict the pluralistic explanation of sex in general. In the first instance, many different factors, both biotic and abiotic, can affect physical condition and variation among individuals was also present in our results. More importantly, our results apply strictly speaking only to *B. rubens* and other studies show different tendencies in closely related *Brachionus* species, including the influence of and ability to react to a changing environment (see above) and whether or not the proportion of mictic females produced is age dependent [164]. It is to be expected, therefore, that other drivers and/or combinations thereof will likely apply throughout Rotifera and beyond. To what extent these drivers might be shaped by evolution and therefore reflect phylogenetic relatedness remains an open question, however.

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5 Summary of main results and general discussion

5.1 Summary of main results

Although the empirical portion of my dissertation focussed on only two different rotifer species (the bdelloid *P. roseola* and the monogonont *B. rubens*), the results nevertheless provide important new insights into the question of the missing prevalence of sex in rotifers. Both species mirror rotifers in general by favouring the faster asexual reproduction, with *P. roseola* obviously being the more extreme of the two. Consequently, results for both species together help to explain what might be required to abandon sexual reproduction altogether or at least reduce it to a minimum and those circumstances under which reproduction with sex or some alternative, analogous form of introgression of new genetic material is required.

The absence of sex and the associated recombination and segregation is thought to lead to long-term difficulties with respect to the handling of mutations (mostly deleterious by chance [48]) and changing environments (adaption should be slower in asexuals [10,11]). Thus, the complete absence of sexual reproduction in the ancient and successful clade of bdelloid rotifers has long puzzled evolutionary biologists [14,15,16,17,18] (see above). However, my findings in the first and second manuscripts indicate that there might be still a relatively frequent exchange of genetic material in *P. roseola* (and possibly other bdelloid species). However, instead of occurring vertically, as in sexual recombination, the exchange seems to be done horizontally [19] and thus indeed without males, meiosis or other classical attributes of sexual reproduction. In so doing, I built on recent studies linking evidence of supposedly rare, ancient horizontal gene transfer to the ability of the “ancient asexual” bdelloids to withstand desiccation via anhydrobiosis [20,21,22], where foreign DNA that had entered through the disrupted gut membranes of dried animals was subsequently incorporated into the genome during the repair of double-strand breaks caused by desiccation [20,21,22,91]. That this process can be extended to one that mimics sexual reproduction through a backdoor process has been implied previously [16,90], but was never demonstrated. For this process to function, it simply needs to be assumed that bdelloids do not take up and store foreign DNA by accident but actively, and somehow prefer DNA from more closely related species. The documented uptake of alien DNA in the genome of some species [20,21,22] thus represents more of a mistake and/or might only be used by some specialized species given that incorporating genes from alien sources are more likely to cause problems [116,117,189] (see also 2.2.1.2.3).

In accordance with this hypothesis, I showed that DNA is indeed taken up actively by the bdelloid *P. roseola* (in contrast to the facultative sexual *B. rubens*) and retained roughly according to phylogenetic relatedness. Furthermore, adding DNA to the medium before desiccation (thus making it available to the individuals for subsequent uptake and incorporation) increased the

variance of the reproductive output of the untreated F1 as expected by theory if fragments of DNA, be they beneficial or deleterious for fitness, enter a new genetic background [187]. Other explanations for the results were ruled out using a series of controls where the parental generation was kept hydrated, kept hydrated with added DNA, and desiccated individually without DNA. The first control excludes that possibility that the increased variation was caused by the transfer of a single randomly chosen F1 per female (i.e., excludes random effects). The second control shows that although the uptake of environmental DNA does occur in hydrated individuals, it can only be incorporated into the genome in association with a desiccation event. Finally, the third control excludes the possibility that desiccation alone is sufficient to cause the observed increase in variation in the F1 generation.

A subsequent trial comparing the reproductive output of the untreated F1 of individuals desiccated in groups with those of individuals desiccated in isolation and without DNA added yielded similar results, thereby indicating the *P. roseola* individuals can themselves act as DNA donors and that this mechanism of genetic exchange should also function under more natural conditions. The incorporation of foreign DNA in the genome is additionally underscored by the fact that the addition of undamaged DNA to irradiated individuals of *P. roseola* before desiccation ameliorated the effect of applied UVB-irradiation. By contrast, desiccation in groups without any added DNA, such that only UVB-damaged DNA could be exchanged between the individuals, did not.

A direct proof of this hypothesis of the horizontal transfer of DNA in association with desiccation was not possible so far. Sequence-based analyses, as have been used previously in this context [20], cannot easily detect the horizontal transfer of conspecific genes because only genes from (highly) distantly related species are easy to identify as having been acquired horizontally. Importantly, the trials I performed demonstrate that the long-term retention of foreign DNA, and thus the changes of its horizontal transfer, increases with its similarity to the host DNA. Confounding a direct proof of horizontal transfer further is that it is unknown if all the added DNA in the experiments is incorporated during desiccation (which seems unlikely) and how similar the foreign DNA must be to ensure reasonable chances of its incorporation while still being able to detect it afterwards. The situation is even more complicated if incorporation is restricted to certain DNA fragment sizes and/or if there are some protected genome sites or sites where incorporation is fatal. Altogether, these problems mean that the chances to detect the successful incorporation of foreign DNA in a single individual are low.

These results expand upon the widely acknowledged importance of bdelloids being able to survive desiccation at any life stage is traditionally considered an important ecological feature of the bdelloids [107]. Of the many advantages anhydrobiosis provides (see sections 2.2.1.2.1 and 2.2.1.2.3), the most important in the context of this dissertation is the suggestion that the necessary repair of the genome following a desiccation event also functions as a check-up to repair mistakes that seem to accumulate in constantly hydrated bdelloids [24]. However, an alternative explanation of these results is that it is the hypothesized horizontal gene transfer that also occurs at this time that is the primary cause for the restored fitness observed.

To this end, the results in my second manuscript showed that fitness (measured as numbers

of eggs laid) varies over time in constantly hydrated populations of *P. roseola*, but need not necessarily decline over time if only some individuals (in my experiments the fittest) are "selected" and exchanged among subpopulations while the remaining individuals are discarded at weekly intervals. Indeed, this situation might mimic natural circumstances better than would a continuous culture of a single, large population as used in [24]. Desiccation of single individuals originating from the same populations (and measured at the same time points) resulted in an astonishingly invariant reproductive outcome that was significantly below that of the hydrated populations. Consequently, although desiccation in and of itself does have an effect on the fitness, it does not appear to be the rescue effect proposed in [24]. Instead, the repair of the double-strand breaks with the help of the degenerated tetraploid genome could possibly reduce heterozygosity, epigenetic changes and/or mutations recognized as such by epigenetic signatures. In the context of this experiment, these "mistakes" might be initial adaptions towards the recently changed food source I used for this population. In any case, the exact mechanism behind this genome repair remain unknown, but should still be explored in the future where it might point to other yet unknown scenarios. Importantly, desiccating individuals in groups alters the outcome found for single individuals by inducing some variability in fitness and yielding a significantly higher reproductive output, presumably due to the horizontal transfer of DNA during the desiccation process. Importantly, these results indicate that DNA fragments are not only incorporated into and have an effect on the untreated F1 via the germline cells of the parental generation, but also into the somatic cells of the desiccated individuals themselves.

To look directly at the susceptibility of asexual species to the threat of accumulated mutations, the effects of applied UVB-irradiation on both *P. roseola* and *B. rubens* was examined in the third manuscript. Although UVB-irradiation causes cyclobutane-pyrimidine dimers (CPDs) and not mutations per se, it can lead to the latter because cytosine bases are unstable in CPDs and deaminate to a uracil, thereby leading to C → T and CC → TT mutations at the sites of uracil-containing CPDs produced by deamination [26]. This effect, however, is not expected if the repair of CPDs is relatively rapid, as is the case with photoreactivation via specific light dependent photolyases [186,211,212]. By contrast, it could be accentuated by alternative, slower [213], but more versatile repair mechanisms such as nucleotide excision repair and recombinational repair that can even introduce additional mutations [186].

My results in the third manuscript showed that *P. roseola* and *B. rubens* use different strategies to protect their genomes against UVB-induced damage. Whereas *P. roseola* was shielded against UVB-irradiation to some degree, it could not repair any damage to the genome that occurred at high irradiation levels. By contrast, *B. rubens* was not shielded against UVB-irradiation, but could repair the damage quickly and seemingly error-free. In addition, this lack of repair had a negative influence on the reproductive output of *P. roseola*. This was especially true if the animals were desiccated after irradiation at high UVB-intensities, which was unexpected given that desiccation is generally seen as the time to repair the genome in bdelloids. One explanation for this is that the initial damage might be especially problematic if the double strand breaks in the DNA get repaired by recombinational repair [26]. More importantly, these results underscore that desiccation itself is not a cure-all for DNA damage in bdelloids, even given the possibility of horizontal transfer

(albeit of similarly damaged DNA in this case). However, if instead undamaged DNA is available before desiccation (see manuscript 1) the incorporation of this DNA can ameliorate the effects of the previous UVB-irradiation.

The different strategies used by the both species might derive from the fact that the fast-acting photolyases would not be effective during the physiological changes occurring during a desiccation event, which might be the time when UV-damage is most likely to occur. Protective compounds, by contrast, would still work at this time. Although *P. roseola* likely does not suffer from UV-damage under natural circumstances (unlike *B. rubens*), it is important to note that there might be no effective DNA repair in this species when hydrated, although the UV-lesions obstruct gene transcription and may give rise to mutations. An analogous lack of effective DNA repair might also characterize bdelloids generally given studies reporting a decline in fitness in hydrated bdelloids [24,47]. Thus, bdelloids might be restricted to desiccation prone habitats for their long-term survival, where repair and recombination events occur often enough to overcome any accumulated errors. This fact does not exclude their presence in other habitats, but in such cases they might suffer from the disadvantages of pure asexual reproduction to a greater degree. In turn, *B. rubens* is more suited to open water habitats, where it appears to be more costly to shield themselves from UV-damage than to efficiently repair the lesions with photolyases before the lesions potentially turn into harmful mutations.

Although monogonont rotifers also inhabit desiccation prone habitats, their ability to survive the adverse conditions is often only possible through a sexually formed resting egg (figure 1). One implication of this strategy is that monogonont rotifers can proliferate through asexual reproduction, but their long-term survival depends on the resting eggs from which a new generation hatches. Despite this advantage, the latency time associated with the hatching of the resting eggs removes (at least temporarily) all energy invested into them and thus into reproduction into the ongoing population [236]. Consequently, individuals must effectively “choose” between the short-term advantages of asexual reproduction (investing into the current population) and the long-term insurance provided by sex (possibly surviving if the current population collapses).

In the fourth manuscript, I tested if and how environmental changes (food quality and/or temperature) influence how much is invested into sex by *B. rubens* under the assumption that more is invested into sex if conditions become more unfavourable (see section 2.3). However, my results indicate that the investment into sexual offspring per individual increased neither absolutely nor relatively to the total number of offspring under challenging conditions and indeed declined severely in the poor-quality food treatment. Instead, there seemed to be a relatively close relationship between the total number of offspring with the amount of sexual offspring, as might be expected if investing too little into short-term asexual reproduction reduces the chances of future sexual reproduction. Crucially, individuals of *B. rubens* invested more into sex under good conditions (as indicated by when they produced the greatest number of total offspring) where they are better able afford this more expensive investment to likely produce more high quality sexual offspring and not in poor conditions where it was previously assumed that the long-term advantages of sex should outweigh its high costs in theoretical models [154].

Nevertheless, a continuous investment into sex was always observed, presumably given the difficulties in predicting the exact timing of an optimal shift to sexual reproduction as required by the bang-bang strategy. Although other studies indicate that the occurrence of sex might be shifted towards earlier offspring ranks if conditions change, with this shift again relaxing after some adaption to these new conditions [165,178], I did not find a similar result here. If true, however, a consequence of this strategy would be that asexual reproduction gets postponed upon encountering uncertain future conditions because the gain from it cannot be assured. Consequently, the relative frequency of sex within the population increases, even if the investment into sex is depending principally on the overall condition of the individuals.

5.2 General discussion

5.2.1 Rotifers as an ideal model system for empirical work on the prevalence of sex

Rotifers present many ideal qualities necessary for empirical research into the limits and advantages of asexual reproduction as well as the triggers for when to start reproducing sexually. First, the short generation times [38,44] means that experiments can be conducted in manageable amounts of time. Second, facultative sexual species present many particular benefits. For instance, it is possible to monitor (semi-) naturally the balance between sexual and asexual reproduction in such species as well as to determine experimentally if and how long the tested individuals/populations have been reproduced asexually. Moreover, it can also be influenced if and when sexual reproduction occurs or at least the hatching of sexual offspring can often be prevented. Furthermore, sexual and asexual reproduction can easily be compared without differences in the genetic background that would arise from comparing different species and the results can be linked directly to potential differences in the sexual mode, including the tendency of *B. calyciflorus* to produce obligate asexual clones [29,237]. Third, as a group, rotifers present a diversity of reproductive modes ranging from obligate asexual (Bdelloidea) to facultative sexual (Monogononta) to obligate sexual (Seisonidae).

Importantly, empirical research into sexual versus asexual reproduction in rotifers is limited to only a handful of species (mostly from *Brachionus*; [38,164]), meaning that there are still ample opportunities for comparisons with other rotifer species, thereby providing a more detailed picture on the prevalence and drivers of sex in this group. For instance, it could be investigated in more detail if and for how long some rotifer species have given up sexual reproduction completely and to what degree they suffer from or are able to prevent the long-term disadvantages associated with obligate asexuality. Likely candidates here are certainly within bdelloids, particularly those species that do not cope well with desiccation [107], a process that is seemingly required for the hypothesized horizontal gene transfer (see manuscripts 1 and 2) and to repair any deleterious mutations accumulated by the genome through extended asexual reproduction [24,47]. However, it cannot be excluded that there also might be some obligate asexual monogononts among the more rare and/or bottom dwelling species (where sexual partners are harder to find), those species that live in highly stable habitats (where variation generated by sexual reproduction as well as the resting stage are not needed, as demonstrated by certain lab clones of *B. calyciflorus* [28,29]) or those species that can produce the resting stage asexually. It is noteworthy in this context that males of many monogonont rotifer species are not known yet [13,241].

By contrast, it would be equally interesting if very unstable habitats yield purely or predominantly sexually reproducing monogonont species. For instance, some but not all of the females hatching from resting eggs in small temporary ponds in Chihuahuan Desert were sexual, meaning that a part of the population skipped asexual reproduction entirely, with the remainder still reproduced as classic facultative sexuals [30]. A strong selection towards obligate sex could also be present

in parasitic species, given that host-parasite coevolution is often thought to be strongly favouring the maintenance of sex (the Red Queen hypothesis) [242]. The only known examples of obligate sex within rotifers, namely all species of Seisonidae, fit into this context [32]. Here, the parasitism/commensalism of these rotifer species is associated with limited space on the host crustacean, which, in turn, severely reduces the advantage of rapid reproduction that is provided by asexual reproduction. Although the third and last described species of Seisonidae was found without a host, this situation might derive from untargeted sampling [243] or because this species descended from the two “parasitic” species and lacks a host. If the latter case is indeed true, it would provide a unique opportunity to research if this species has remained an obligate sexual or if has managed to resume asexual reproduction, at least as part of a facultative sexual strategy. A final phenomenon interesting in this regard are those species where amphoteric females can produce combinations of either haploid males and diploid females, females and resting eggs, or males and resting eggs, thus combining sexual and asexual properties that are normally restricted to different females (and generations – compare figure 1) [13].

Another worthwhile line of investigation would be to examine the potential for different bdelloid species to include alien genes via horizontal gene transfer. It might be that some species try to exclude such genes during the reconstruction of the genome after desiccation ([22]; see also manuscript 1), whereas others have acquired astonishingly high proportions of alien genes [20], many of which are even functional. Furthermore, the mechanisms of underlying the horizontal gene transfer in bdelloids could be compared to those in other groups engaging in this process, including bacteria [182,244]. Apart from the implications of the hypothesized horizontal gene transfer regarding segregation and recombination in bdelloids, the successful incorporation of DNA fragments as well as the effective repair of large numbers of double-strand breaks are in itself important areas for future research.

Finally, the evolution of the different reproductive strategies could be reconstructed on a phylogenetic basis as soon as sufficient data become available, both genetically and with respect to the reproductive strategies used. Doing so would elucidate how reproductive modes have evolved and how they have influenced the evolution of the whole genome (e.g., under frequent horizontal gene transfer). These studies would also include an investigation of the different male types found in rotifers, which range from dwarf males without a digestive system in some species [13] to fully developed males that are not smaller than females in other species [245] to a series of intermediate states [246].

Altogether, rotifers represent an ideal group to study the prevalence of sex empirically given the clear tendency to produce asexually as much as possible. Although this is generally done quite successfully within the group, it appears that genetic exchange is ultimately needed at some time point for most species because of the short generation times of rotifers and the fact that they sometimes have to withstand rapid environmental changes (e.g., habitats that are only shortly filled with water) and/or changes they are unable to cope with. Examples of the latter might be extensive seasonal changes in temperature or food availability, where long-term survival is only possible via the resting stage formed by sexual reproduction in monogonont rotifers. The analogue for bdelloid rotifers, albeit on a comparably shorter timescale that can be endured,

would be anhydrobiosis or starvation, both of which involve a comparable state of metabolic stasis [247]. However, genetic exchange would only seem to be possible through anhydrobiosis given that the reconstitution of the double strand breaks seems to be a perquisite for bdelloids to incorporate any retained foreign DNA.

5.2.2 Are bdelloids asexual or not?

It might be argued that the experiment in manuscript 1 failed to demonstrate directly that foreign DNA was incorporated into the genome of *P. roseola* and that the indirect evidence presented does not exclude alternative explanations. However, it was already demonstrated for some bdelloids, including *P. roseola* [22], that DNA is found within the genome that is likely of alien origin. As such, some form of horizontal gene transfer has occurred within the group and if alien DNA can be incorporated in the genome (which should be risky because it often will not fit well to the remaining genome [116,117,189]), why should DNA from closely related species not be incorporated as well? Indeed, my results make the latter seem likely given that DNA was indiscriminately taken up in the experiments reported in manuscript 1, but with the DNA from more closely related species being preferably retained after some hours. In addition, both the transfer of alien and more similar DNA utilize the same process (i.e., incorporation into the genome during the DNA repair that occurs following desiccation). Together, these results and inferences suggest that the transfer of alien genes might be accidental and occurs either when there is insufficient time to discard it before a desiccation event or it could not be discriminated as alien. Consequently, one might consider those studies that report the presence of horizontally transferred alien genes as an indication that genetic exchange with more closely related species might be occurring in these same species as well. This would include *P. roseola* [22], *A. vaga* [22] and *A. ricciae*, with the amount of retained alien genes in the latter species being remarkably high [20,21]. Likewise, the evidence of shared alleles by otherwise highly divergent bdelloid species, e.g. *M. quadricornifera* and *P. roseola*, also points to the possibility of horizontal gene transfer among bdelloids [16].

Problematic in this regard is the widespread acceptance of bdelloids as a successful clade of asexual reproducing species, with several studies seeming to underline this hypothesis [23,27]. Even given that it is hard to prove that sexual reproduction in any group is permanently absent (with rare sex being especially hard to exclude [89]), the idea that the well-studied bdelloids [34], are *ancient* asexuals already implies that bdelloids must have some mechanisms to overcome the degradation of the genome expected for asexual species (see 2.5) to have survived and flourished for as long as they have. Instead, the fact that bdelloids are unusually (morpho) species-rich for an asexual clade is attributed to their specialization into distinct niches [46], resulting in even higher diversification rates for them compared to their facultative sexual sister taxon the Monogononta [50]. Additionally, the widespread acceptance of desiccation with its diverse advantages as one of the important “tricks” of bdelloids might deflect attention away from any additional properties of it.

Nevertheless, hints in the literature are present. For instance, the alternative explanation of horizontal gene transfer as for bacteria is mentioned by Fontaneto et al. [46] in the first paragraphs of their introduction. However, the authors did not discuss if the data presented for the bdelloids

might also fit to this hypothesis, although they even point out that bacteria were falsely used as an example for clonal speciation. In addition, despite evidence of ancient and massive horizontal gene transfer, this mechanism was likewise not suggested as a possible explanation in Fontaneto et al. [50] for the higher diversification rates of *CO1* observed in the asexual bdelloids compared to (facultative) sexual monogont rotifers. However, the tendency for horizontal gene transfer to happen more frequently between closely related species (as it is more likely to be successful here) [248,249] fits both to the fact that the possible exchange underlying the shared alleles between *M. quadricornifera* and *P. roseola* happened long ago [16] and to the observation that higher diversification rates of *CO1* tends to be shifted towards the root of the tree [50].

At last, the nature of the proposed horizontal gene transfer between closely related bdelloid species has important differences to normal sexual reproduction. Consequently, some indications suggesting the absence of sexual reproduction (e.g., lack of males or meiosis) might still be present although there is segregation and recombination via horizontal gene transfer (see above). In addition, whereas one half of a genome recombines with another half from a partner in normal sexual reproduction, at best only some fragments of DNA that had been taken up are incorporated into the genome via horizontal gene transfer, either because the amount of DNA taken up is limited and/or because some parts of the genome do not or only rarely recombine (e.g., by chance or because they are somehow protected). Also, because the proposed mechanism appears to depend on desiccation, no recognisable genetic exchange occurs as long as there is no desiccation (as in permanent water habitats or possibly in lab cultures) or if genetically deviant individuals are absent. An additional confounding factor could be the effects of the intragenomic repair within the tetraploid genome that also occur after desiccation (see manuscript 2).

Thus, it remains unclear if bdelloids really represent ancient asexuals or not. Classical sexual reproduction, even in the form of rare sex, has likely been missing for a long time, but there are several signs that point to the use of horizontal gene transfer in combination with desiccation as a replacement for the advantageous effects of sexual reproduction, especially given that bdelloids occur quite often in desiccation-prone habitats [106]. The possibility that this horizontal gene transfer might more routinely include genes from closely related species or conspecific individuals needs to be explored in more detail. Should evidence accumulate that it does indeed happen relatively frequently (according to the frequency of desiccation), this would further underscore that some kind of gene transfer is essential for the long-term survival of even bdelloid rotifers.

5.2.3 When and why sex in rotifers

If one views the indirect evidence presented here for *P. roseola* in favour of semi-regular (based on the frequency of desiccation) horizontal gene transfer, preferably with closely related species, then there is an interesting parallel to the facultative sexuality present in *B. rubens*. Both species use asexual reproduction primarily for normal and rapid proliferation, with genetic exchange in the form of either horizontal gene transfer or sex, respectively, being associated with a latency time (anhydrobiosis or resting eggs, respectively) that precedes the newly awakened individuals potentially being confronted with new environmental conditions. Thus, in both species, extended

phases of asexual reproduction get interrupted, with the next phase of asexual reproduction being started by a genetically variable set of individuals formed by segregation and recombination. However, despite these coarse similarities, certain differences between bdelloids and monogononts remain, with both of the respective reproductive systems being in stark contrast to the obligate sexual reproduction used within Seisonidae.

5.2.3.1 Bdelloids

Bdelloids like *P. roseola* need to invest neither energy nor time into sexual reproduction, which might be important in desiccation-prone habitats where sexual reproduction might be too slow. However, without desiccation any DNA that is taken up apparently cannot be incorporated into the genome. In addition, the amount of DNA that is segregated and recombined during the hypothesized horizontal gene transfer is presumably (much) lower than what occurs in normal sexual reproduction where half of the genome is recombined. In addition, the somewhat unspecific nature of the mechanism means that DNA from other sources can be included (as was the case for alien genes), which might be advantageous in some cases and potentially speed up diversification in bdelloids (see above).

Consequently, bdelloids seem to be critically dependent on desiccation to gain the potential advantages of genetic recombination, with the frequency of recombination being determined by the habitat the given species is found in. Thus, in habitats with permanent water or highly infrequent desiccation events, bdelloids are expected to suffer more greatly from the disadvantages of purely asexual reproduction. This fact, in turn, influences how long bdelloids can survive in such habitats and/or how often they must be recolonized. Altogether, this fact might explain why bdelloids tend to preferentially be found more often in unstable aquatic habitats [106].

5.2.3.1 Monogononts

Monogononts like *B. rubens* use true sexual reproduction in addition to extended periods of asexual reproduction. As such, they are at a certain disadvantage if sexual reproduction is required very often [30], in part because the need to produce males means that two generations are needed for it to be completed (figure 1), thus diverting resources from the growth of the current population. Nevertheless, the timing and the amount of sexual reproduction can be influenced greatly with respect to at which population density monogononts preferentially engage in sex (partners still need to be found) [224], how long sexual reproduction is not possible after individuals have hatched from resting eggs [30,167], and how long resting eggs indeed stay at rest [164]. Here, there is still much to be learned how these factors act together and are influenced in different species. Indeed, many of the factors that promote sex likely remain to be discovered, with the distinct possibility that they may differ between species, even in the same genus!

Fitting this latter idea, is that my reported results for *B. rubens* seem to differ substantially from published results for *B. calyciflorus* [165,178] or *B. plicatilis* [174], which differ in and among

themselves (e.g. only in the formost species was crowding seemingly no perquisite for sexual reproduction). For instance, in contrast to *B. calyciflorus*, changing/challenging conditions was not the primary driver of sexual reproduction in *B. rubens*, with overall condition instead appearing to fulfill this role. The fact that investment into sex occurs preferentially under good conditions in *B. rubens* might reflect the various costs of sex, including the two-fold cost of a pair of individuals being needed for reproduction [2], the cost of recombination [3], the energetic costs [4], and risks of mating [5,6]. By contrast, the results for *B. calyciflorus* agree with the results of a theoretical study showing that sex under poor conditions might outweigh the large costs of sex in facultative sexual species [154]. One reason for this difference in the two species might be that the resting eggs of *B. calyciflorus* hatch without an appreciable latency time or special conditions, whereas dry and cold conditions are required for the resting eggs of *B. rubens* to hatch, conditions that would wipe out the current population. Another difference might lie with the different experimental approaches of both studies. Whereas my study of *B. rubens* looked at individual life histories, the study with *B. calyciflorus* used mainly the dynamics of entire populations. Thus, it might be possible that the amount that can be invested into sex still depends on the individual condition in both cases, whereas the timing of sexual reproduction could influence the dynamics observable over the population. For instance, if asexual reproduction is postponed in favour of earlier sexual reproduction upon changing/challenging conditions, population growth will slow down and the relative proportion of sexual offspring will increase.

In summary, it would be instructive to investigate if and what kind of phylogenetic (vs. ecological) components those factors inducing sex possess: do closely related species react more similarly or are ecological factors more important? An example in favour of the latter might be the differences between *B. calyciflorus* and *B. rubens* mentioned above, where the resting eggs in the former are not needed for long-term survival, which enables the species to track ecological changes directly. However, it should be noted that if environmental conditions prevent current survival of the population, this strategy would be a dead end. Such research on monogonont rotifers might also permit exploring those circumstances under which facultative sexual reproduction is stable and when it might be tipped to either obligate sexual or asexual reproduction (see below).

5.2.3.1 Seisonidae

The species-poor, Seisonidae represent the only rotifers that reproduce obligate sexually. The parasitic lifestyle of these species means both that a resting stage is not needed and that the advantages associated with fast asexual proliferation might be negated by the restricted space on the host. Indeed, parasitism might be actively driving obligate sexual reproduction in this case, given that the latter is a prominent factor that is thought to (partly) explain sex via the Red Queen hypothesis [31] (see 2.3.1).

5.2.4 Why are facultative sexual species not more common?

My results seem to underscore the general importance of some form of genetic recombination in the long run, although it need not necessarily occur via true sexual reproduction (see also [249,250]). Secondarily, my results also highlight the high potential of asexual reproduction to rapidly increase population numbers to ensure having enough individuals that can engage in sex. These latter individuals, in turn, ensure that at least some offspring are produced that might be able to cope with potential environmental changes, prevent the accumulation of deleterious mutations and increase the possibility of retaining any favourable mutations in the population. Although the phase of asexual reproduction can be extended to some degree, it needs to be balanced against the time point at which the long-term disadvantages of asexuality will catch up with a population that is not reproducing sexually early enough. (That being said, it might be difficult to reduce the mutation levels to bearable levels in facultative sexual species after prolonged asexual phases even with recombination and segregation.)

However, the question also exists as to why facultative sexual reproduction, which can draw on the advantages of both sexual and asexual reproduction [69,154,251], is not more common. This might be a problem of perspective to some degree, which tends to be focused on metazoans, with facultative sexuality being more common in other groups [36,69]. In addition, the difficulty in detecting rare sex [33] means that some species that are thought to be asexual might turn out to be facultative sexuals.

More interestingly, although true sexual reproduction includes segregation and recombination, proliferation in this context depends on multiple mitotic cell divisions of germline cells before meiosis occurs. In other words, each gamete is a product of a asexual/sexual lifecycle and thus profits from the advantages of both [252]. As a consequence, asexual reproduction (and selection on it) also happens, albeit invisibly, within the germline cells of obligate sexual species. In multicellular species, this facilitates rapid asexual proliferation to happen to the extent potentially needed to enable natural selection to occur on these clones before sexual reproduction occurs (e.g., during sperm competition [137,138]). By contrast, if this step would be missing too many offspring with low fitness might arise during the recombination of random gametes.

Regardless of the system, an interesting effect of facultative sexuality is that the relation of asexual and sexual reproduction can be kept under a strict control that provides the optimum advantages from both. In existing facultative species, this might/can be balanced by external factors as outlined above for monogonont rotifers. However, this trade-off also includes the risk that facultative sexual reproduction might be unstable in the long term [69], leading to obligate asexuality or sexuality.

Indeed, obligate asexual clones can potentially originate from normally cyclical parthenogenetic monogonont rotifers, as is the case for recently derived obligate asexual *B. calyciflorus* clones being investigated by C.P. Stelzer and colleagues [29,223,236]. Although these clones still produce the mixis signal that induces sexual reproduction, they do not react to it [237]. The

transition to obligate asexuality appears to be easier than is usually assumed and, for the *B. calyciflorus* clones seems to involve the simple Mendelian inheritance of a recessive allele, which in combination with linked genes or via pleiotropic effects additionally causes a reduction in body size to approximately half the size of the normal cyclical parthenogenetic clones [29]. Accordingly, this effect is most pronounced when the recessive allele occurs in the homozygous state [253]. By contrast, the minimal effect in the heterozygous state means that clones carrying the allele can invade the cyclical parthenogenetic clones without their transition being recognized. Following invasion, any newly derived homozygous asexuals can potentially outcompete cyclic parthenogenetic clones unless the latter are investing into sex on a very low level only [236]. Importantly, both this and other transitions to obligate asexuality in various species of *Brachionus* were achieved under constant laboratory conditions [28] where asexuality is favoured. It remains to be investigated how often obligate asexual monogonont clones actually arise in nature and how successful these clones would be in less constant habitats. Under such scenarios, although individuals investing entirely in sex have zero fitness initially and will be removed from the current population (for possible exceptions see [165,166,167]), there genes will likely be the only ones to survive over the long term if conditions do not permit survival of asexual clones or even sexual offspring in between. Thus, the costs for asexuality versus sexuality and which reproductive mode is favoured are very dependent on the circumstances and timeframe [178,254,255].

Although a shift to obligate sexual reproduction has not been documented for facultative sexual rotifer species, it would nevertheless be worthwhile to investigate those monogonont species that experience circumstances comparable to those of Seisonidae (e.g., close association with a host and limited potential for rapid proliferation). In this regard, it would also be interesting to verify if *Seison africanus* indeed has a host [243] and, if not, if it is able to reproduce asexually as well. More generally, it would be interesting to investigate if frequent environmental disturbances that otherwise require formation of the sexually formed resting stage for long-term survival can lead to obligate sex (see [30]), if constant but traceable environmental changes also have this potential (see [165,178]), or if there is always some (low) level of asexual reproduction to ensure rapid reproduction and clone selection (see above).

Thus, rotifers again provide an ideal model system that could provide interesting insights into the evolutionary stability of facultative sexual species, either by investigating various different monogonont rotifer species, exploring the evolutionary history of the three reproductive modes found within rotifers, and/or by modelling populations using the various factors that might have an influence on sexual versus asexual reproduction.

6 Own contributions to the publications

Manuscript I: Explaining an evolutionary scandal: evidence for a mechanism of genetic exchange in the "ancient asexual" bdelloid rotifer *Philodina roseola*?

I designed the project with input from O.B.E. and W.H.A. for which I performed the research and performed most of the analyses. (The analysis of the uptake of radioactive DNA was done with O.B.E.). All authors discussed the results. I wrote the initial draft of the manuscript with input from the other authors. The manuscript was finalized by O.B.E. and myself.

Manuscript II: Immediate and heritable effects of desiccation in isolation versus in groups on the life history of the bdelloid rotifer *Philodina roseola*

I designed the project with input from O.B.E. and W.H.A. for which I performed all the research and analyses. All authors discussed results. I wrote the initial draft of the manuscript with input from the other authors. The manuscript was finalized by O.B.E. and myself.

Manuscript III: How does the "ancient" asexual *Philodina roseola* (Rotifera: Bdelloidea) handle potential UVB-induced mutations?

I designed the project with input from O.B.E. and W.H.A., with W.v.P. and A.B. teaching me the methods for the detection of CPDs. All research was performed by me as were all analyses with input from W.v.P. All authors discussed results. I wrote the initial draft of the manuscript with input from the other authors. The manuscript was finalized by O.B.E., A.B., and myself.

Manuscript IV: Fitness affects the reproductive strategy in a facultative sexual rotifer

I designed the project with input from O.B.E. and W.H.A. for which I performed all the research and analyses. All authors discussed results. I wrote the initial draft of the manuscript with input from the other authors. The manuscript was finalized by O.B.E. and myself.

The description of the candidate's contribution to the scientific publications contained in this dissertation is accurate.

Olaf R.P. Bininda-Emonds

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8 Curriculum vitae

Personal data

Date of birth:	19.12.1979
Place of birth:	Oldenburg
Nationality:	German

Education

11/2009-heute	PhD at the Carl von Ossietzky University Oldenburg - Title: „ Insights into the Maintenance and Consequences of Asexual Reproduction in Rotifera” - Funding: DFG (BI 825/6-1)
10/2000-11/2007	Diploma landscape ecology at the Carl von Ossietzky University Oldenburg. - Title: „Biotic and abiotic parameters affecting the coexistence of the Asexual *Poecilia formosa* and its host *P. latipinna*: populations, community, predators, extreme temperatures and landuse”
10/2000-11/2006	Diploma biology at the Carl von Ossietzky University Oldenburg. - Title: „Zur Morphologie, Phylogenie und Evolution der *Cephalodella* und die Erstbeschreibung einer neuen Rotifera-Art“
06/1999	Abitur at the Gymnasium Eversten Oldenburg

Grants

11/2009-12/2012	DFG (BI 825/6-1) „Properties and costs of sex in rotifers”
08/2009	Kongress-Stipendienprogramm der Oldenburger Bankleiter for attending Rotifera XII in Berlin (16.-28. August 2009)
11/2006-02/2007	DAAD scholarship for the preparation of a thesis abroad (USA)

Further activities

06/2007-today	Freelancing (part time): Acquisition of fish distribution data (electrofishing) with Dr. Oliver D. Finch – Tierökologische Fachbeiträge
09/2008-09/2010	Freelancing (part time): Maintenance of aquaria for AquaDeko, Rastede
12/2007-10/2009	Preparation and drafting of a DFG grant

Publications

Fischer C., Ahlrichs W.H., Buma, A.G.J., van de Poll, W.H. and O.R.P. Bininda-Emonds. Submitted to Journal of Experimental Biology. How does the “ancient” asexual *Philodina roseola* (Rotifera: Bdelloidea) handle potential UVB-induced mutations?

Fischer C., Ahlrichs W.H. and O.R.P. Bininda-Emonds. Submitted to Functional Ecology. Fitness affects the reproductive strategy in a facultative sexual rotifer.

Fischer C., Ahlrichs W.H. and O.R.P. Bininda-Emonds. In preparation. Explaining an evolutionary scandal: evidence for a mechanism of genetic exchange in the “ancient asexual” bdelloid rotifer *Philodina roseola*?

Fischer C.,Ahlrichs W.H. and O.R.P. Bininda-Emonds. In preparation. Immediate and heritable effects of desiccation in isolation versus in groups on the life history of the bdelloid rotifer *Philodina roseola*.

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Poster

Fischer C. and W.H. Ahlrichs.2009. Revisiting the *Cephalodella*trophi types.Rotifera XII Berlin 2009.

Fischer C. and W.H. Ahlrichs. 2006. *Cephalodella*n.sp (Notommatidae, Monogononta), a new rotifer species from North-West Germany. Rotifera XI Mexiko 2006.

Talks

Fischer C., Ahlrichs W.H. and O.R.P. Bininda-Emonds. 2013. Explaining an evolutionary

scandal: evidence for a mechanism of genetic exchange in the “ancient asexual” bdelloid rotifer *Philodina roseola*? DZG Meeting Evolutionary Biology in Göttingen, Germany.

Fischer C., Ahlrichs W.H. and O.R.P. Bininda-Emonds. 2012. Evidence for a mechanism of genetic exchange in an ancient asexual bdelloid rotifer. Annual meeting of the German Zoological Society in Konstanz, Germany.

Fischer C. 2012. Essen auf Rädern – Rädertiere als Aufzuchtfutter. First ‚Klimahaus-Kongress Süßwasser-Aquaristik‘ in Bremerhaven, Germany.

Popular science

Hinz. C., K. Rohmeyer and C. Fischer. 2012. Ein „falscher“ Zwergflusskrebs - Beobachtungen an *Orconectes nana*. Caridina 1/12.

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Fischer C. and C. Hinz. 2010. Zucht und Pflege von *Epiplatys infrafasciatus rathkei*. Aquaristik-Fachmagazin (Nr. 211; Februar/März), 36-39.

9 Erklärungen gemäß § 10 der Promotionsordnung

Hiermit erkläre ich gemäß § 10 der Promotionsordnung, dass ich mit dieser Dissertation den Titel Dr. rer. Nat. (Doktor) anstrebe.

Hiermit erkläre ich gemäß § 10 der Promotionsordnung, dass ich die Arbeit selbstständig verfasst und nur die angegebenen Hilfsmittel benutzt habe.

Hiermit erkläre ich gemäß § 10 der Promotionsordnung, dass ich meine Dissertation weder in ihrer Gesamtheit noch in Teilen einer anderen wissenschaftlichen Hochschule zu Begutachtung in einem Promotionsverfahren vorgelegt habe.

Oldenburg, den

Unterschrift

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