Effects of stream degradation and recovery on parasite communities: a multiple stressors approach

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Table of contents

Summary (German) 5

Summary (English) 8

Cumulative thesis and extent of contributions 11

Introduction 13

Chapter I 19

Chapter II 40

Chapter III 54

Chapter IV 61

General Discussion 93

References 100

Curriculum vitae 110

Declarations 111

Summary (German)

Süßwasser-Ökosysteme gehören zu den am meisten belasteten Ökosystemen der Welt, da diese besonders anfällig für menschliche Aktivitäten und Umweltveränderungen sind. Stressoren wie Verschmutzung, Veränderung des Abflusses, Zerstörung oder Verschlechterung von Lebensräumen und die Freisetzung invasiver Arten führen meist zu einem Verlust der biologischen Vielfalt. Diese Tendenz wird durch den Klimawandel noch verstärkt. Trotz der Bemühungen, den Verlust der biologischen Vielfalt durch die Renaturierung von Ökosystemen rückgängig zu machen, wird der Erfolg solcher Initiativen häufig durch die komplexen Interaktionen zwischen lebenden Organismen und deren Reaktionen auf anthropogene Stressfaktoren beeinträchtigt. In diesem Kontext ist das Verständnis wenig erforschter Aspekte, wie z. B. Wirt-Parasit-Interaktionen, besonders wichtig.

Alle Parasiten sind von ihren Wirten abhängig, zumindest während bestimmter Phasen ihrer Ontogenese. Einige Parasiten sind Generalisten und nutzen ein breites Spektrum von Wirten, während andere auf eine oder wenige Wirtsarten spezialisiert sind. Heteroxene Parasiten benötigen mehrere Wirte, während monoxene Parasiten nur eine Wirtsart benötigen, um ihren Lebenszyklus zu vollenden. Daher könnte eine Verringerung der Wirtsvielfalt und -abundanz, die durch die Verschlechterung des Lebensraums verursacht wird, zum Aussterben heteroxener spezialisierter Parasiten führen. Andererseits ist es wahrscheinlicher, dass monoxene, generalistische Parasiten aufgrund ihrer einfachen Lebenszyklen und ihrer Wirtsplastizität in degradierten Umgebungen überleben können bzw. die ersten sein werden, die renaturierte Fließgewässer wiederbesiedeln. Ihre Fähigkeit zur Besiedlung und Ausbreitung kann jedoch von Art zu Art variieren und kann stark von der Gemeinschaft freilebender Arten, auch invasiver Arten, beeinflusst werden. Ebenso kann die Ausbreitung freilebender Arten durch Parasiten beeinflusst werden. Daher können ubiquitäre Parasiten mit einfachen oder komplexen Lebenszyklen und verschiedenen Wirtsspezialisierungsgraden die Geschichte der Degradierung und Erholung von Ökosystemen reflektieren. Die Ableitung der Auswirkungen multipler Stressoren auf Wirt-Parasit-Interaktionen wird jedoch häufig durch das begrenzte Wissen über Parasiten erschwert.

In vier Kapiteln werden in dieser Arbeit die Auswirkungen der Degradierung und Renaturierung von Fließgewässern auf Parasiten mit unterschiedlichen Lebenszyklen und verschiedenen Graden der Wirtsspezialisierung in Zusammenhang mit der Ausbreitungsdynamik von Wirt und Parasit und den Auswirkungen neozoischer Arten untersucht. Amphipoden, die als

Schlüsselarten in einer Vielzahl von Lebensräumen vorkommen, waren die wesentlichen Wirte in diesen Untersuchungen. Aufgrund der geringen Prävalenz von Makroparasiten liegt der Schwerpunkt der Arbeit auf Mikrosporidien, einer vielfältigen, ubiquitären und häufigen, aber wenig untersuchten Gruppe von Mikroparasiten.

In Kapitel I wurde die regionale und kontinentweite Wirtsspezialisierung von Mikrosporidien, die Amphipoden befallen, in Bezug auf degradierte und renaturierte Lebensräume in den Einzugsgebieten von Boye und Kinzig untersucht. Die Ergebnisse dieses Kapitels erweitern das derzeitige Wissen um 17 neue Wirt-Parasit Interaktionen und deuten darauf hin, dass die Mikrosporidiengemeinschaften von Amphipoden in den beiden Einzugsgebieten hauptsächlich aus generalistischen Parasiten bestehen. Wirtsdiversität und Umweltparameter hatten keinen Einfluss auf die Persistenz und Ausbreitung von generalistischen Mikrosporidien in Umgebungen, die anthropogenen Störungen ausgesetzt waren. Dies spricht dafür, dass die Persistenz und Ausbreitung von generalistischen Mikrosporidien von komplexeren Mechanismen wie der Produktion resistenter Sporen, dem Wirtswechsel und der Ausbreitung von Wirten, die individuell oder in Kombination wirken, abhängen könnte.

In Kapitel II wurde die Rolle von Mikrosporidien bei der Modulation der flussabwärts gerichteten Ausbreitung von *Gammarus pulex* durch Drift im Rotbach-Einzugsgebiet untersucht. Drei 72-Stunden-Driftversuche wurden im Oktober 2021, April und Juli 2022 durchgeführt. Die Ergebnisse zeigten, dass das Vorkommen und die Zusammensetzung von zehn Mikrosporidien saisonal, tageszeitlich und zwischen driftenden und stationären Exemplaren von *G. pulex* variierten. Bei zwei Mikrosporidien war die Prävalenz in Driftproben tagsüber am höchsten, was auf Veränderungen in der Phototaxis des Wirts hindeutet, die wahrscheinlich mit der Übertragungsweise des Parasiten und dem Infektionsort zusammenhängen. Veränderungen im Driftverhalten können daher wichtige Auswirkungen auf die Populationsdynamik von *G. pulex* und die Ausbreitung der Mikrosporidien haben.

In Kapitel III wurde die Etablierung nicht einheimischer Mikrosporidien in degradierten Ökosystemen durch die Freisetzung exotischer Wirte explorativ untersucht, indem eine verwilderte Garnelenpopulation von *N. davidi* in einem Bach (Finkelbach, Deutschland) untersucht wurde, den sie mit einheimischen Amphipoden teilt. Der molekulare Nachweis von *Ecytonucleospora hepatopenaei*, einem ökologisch und wirtschaftlich bedeutsamen Parasiten der ursprünglich aus tropischen Meer- und Brackwassersystemen stammt, sowie eines unbekannten Mikrosporidien-Isolats war der erste Bericht über Mikrosporidien in diesem Wirt. Das Vorhandensein nicht einheimischer Parasiten warf die Frage über deren Pathogenität und ihr Übertragungspotenzial auf einheimische Krebsarten auf. In Anbetracht der laufenden Ausbreitung von *N. davidi* von thermisch belasteten hin zu kälteren Gewässern sind weitere Untersuchungen erforderlich, um diese Ergebnisse und ihre Auswirkungen zu untermauern.

In Kapitel IV wurden die Ausbreitung von verwilderten *N. davidi* und ein möglicher Austausch von Mikrosporidien zwischen einheimischen (Amphipoden, Asseln) und invasiven (*N. davidi* und *Procambarus clarkii*) Krebsarten europaweit in Gebieten mit unterschiedlichem Grad an thermischer Beeinflussung und/oder Salzbelastung sowie mittels eines Infektionsversuchs untersucht. Es wurden drei neu etablierte *N. davidi* Populationen aus thermisch belasteten Gewässern in Mitteleuropa (Deutschland, Ungarn und Slowakei) gefunden. Das Vorkommen von *E. hepatopenaei* in wilden *N.davidi* Populationen in ganz Europa wurde molekular und histologisch bestätigt, und die Liste der Mikrosporidien, die diesen Wirt infizieren, wurde von zwei auf vier erweitert. Die Übertragung des generalistischen Parasiten *E. hepatopenaei* von *N. davidi* auf einheimische Amphipoden und Asseln fand unter den gewählten experimentellen Bedingungen nicht statt. Der erste Nachweis einer Übertragung eines Mikrosporidiums wurde jedoch im Feld zwischen *N. davidi* und dem invasiven Flusskrebs *P. clarkii* beobachtet. Diese Ergebnisse deuten darauf hin, dass eingeschleppte generalistische Parasiten wahrscheinlich neue, degradierte Lebensräume besiedeln, was in Verbindung mit der fortschreitenden Ausbreitung von *N. davidi* die Auswirkungen auf die einheimische Flora und Fauna verschlimmern könnte.

Die in dieser Dissertation vorgestellten Kapitel tragen zum allgemeinen Verständnis der Auswirkungen von Flussdegradation und Renaturierung auf die Wirt-Parasit-Dynamik bei. Der Nachweis einer großen Anzahl von Parasiten mit scheinbar einfachen Lebenszyklen zeigt, dass die untersuchten Fließgewässer, inklusive derer, die sich in einem Erholungsprozess befinden, immer noch unter dem Einfluss multipler Stressoren stehen. Die Ergebnisse zeigen, wie wichtig die Berücksichtigung von Parasitenmerkmalen wie Wirtsspezialisierung, Komplexität des Lebenszyklus, Pathogenität und Umweltresistenz bei der Untersuchung der Auswirkungen von multiplen Stressoren sind.

Summary (English)

Freshwater ecosystems are among the most impacted ecosystems in the world, as they are particularly vulnerable to human activity and environmental changes. Impacts such as pollution, flow alteration, habitat destruction or degradation, and release of invasive species commonly result in biodiversity loss. A loss that is further exacerbated by climate change. Despite increased efforts to reverse biodiversity losses through ecosystem restoration, the success of such initiatives is often hampered by complex interactions between living organisms and their responses to anthropogenic stressors. In this context, understanding less investigated aspects, such as host-parasite interactions, is particularly relevant.

All parasites are dependent on their hosts, at least during selected phases of their ontogeny. Some parasites are generalists and exploit a broad range of hosts, while others are specialized for one or a few hosts. Heteroxenous parasites require multiple hosts to complete their life cycle, while monoxenous parasites need just one. Consequently, a reduction of host diversity and abundance caused by habitat degradation might trigger heteroxenous specialist parasite extinctions. On the other hand, monoxenous generalist parasites are more likely to persist in degraded environments due to simple life cycles and host plasticity and, hereafter, are more likely to be the first to colonize restored streams. However, their ability to colonize and disperse may vary between species and could be strongly influenced by the community of free-living species, including invasive ones. Likewise, the dispersal of free-living species may be modulated by parasites. Hence, ubiquitous parasites with either simple or complex life cycles and various host specialization degrees may reflect ecosystem degradation and recovery histories. However, inferring the impact of multiple stressors on host-parasite interactions is often hampered by the limited knowledge on parasites.

Within four chapters, this thesis aims to investigate the effects of stream degradation and restoration on parasites with different life cycles and various degrees of host specialization, host-parasite dispersal dynamics, and the impact of novel invasive species. Amphipods, being keystone species and abundant in a wide range of environments, were the primary focal hosts in the present investigations. Due to a low prevalence of macroparasites, the thesis focuses on microsporidians, a diverse, ubiquitous, and abundant but understudied group of microparasites.

In Chapter I, regional and continental-wide host specialization in microsporidians infecting amphipods was evaluated in relation to degraded and recovering habitats across the Boye and Kinzig catchments. The results of this chapter expand the current knowledge to 17 novel host-

parasite interactions, suggesting that the microsporidian communities of amphipods in the two catchments consist mainly of generalist parasites. Host diversity and environmental parameters did not influence the persistence and dispersal of generalist microsporidians in environments that experienced anthropogenic disturbance. This finding indicates that the persistence and dispersal of generalist microsporidians might depend on more complex mechanisms such as the production of resistant spores, host switching, and host dispersal acting individually or conjointly.

In Chapter II, the role of microsporidians in modulating the downstream dispersal via drift of *Gammarus pulex*, was investigated in the Rotbach catchment. Three 72-hour drift experiments were conducted in October 2021, April, and July 2022. The results indicated that the prevalence and composition of ten microsporidians varied seasonally, diurnally, and between drifting and stationary specimens of *G. pulex*. For two microsporidians, the prevalence in drift samples was highest during daytime, suggesting changes in host phototaxis likely related to the parasite's mode of transmission and organs or tissues infected. Hence, alterations in drifting behavior may have important implications for *G. pulex* population dynamics and microsporidians' dispersal.

In Chapter III, the establishment of non-native microsporidians in degraded environments through releases of exotic hosts is exploratively assessed by screening a feral shrimp population of *N. davidi* inhabiting a stream (Finkelbach, Germany) shared with native amphipods. The molecular detection of *Ecytonucleospora hepatopenaei*, an ecologically and economically relevant parasite of tropical marine and brackish water origins, and an unknown microsporidian isolate constituted the first report of microsporidians in this host. The presence of non-native parasites raised concern about their pathogenicity and transmission potential to native crustacean species. Considering the ongoing range expansion of *N. davidi* from thermally polluted to colder water, further investigations were needed to confirm these findings and their implications.

In Chapter IV, the range expansion of feral *N. davidi* and possible spillover of host generalist microsporidians between native (amphipods, isopods) and invasive (*N. davidi* and *Procambarus clarkii*) crustaceans was assessed Europe-wide in areas impacted by different degrees of thermal and/or saline pollution and via an infection experiment. Three newly established *N. davidi* populations from thermally polluted waters in central Europe (Germany, Hungary, and Slovakia) were reported. The presence of the microsporidian parasite *E. hepatopenaei* in feral *N.davidi* populations across Europe was molecularly and histologically confirmed, and the list of microsporidians infecting this host was expanded from two to four. The transmission of the generalist parasite *E. hepatopenaei* from *N. davidi* to native amphipods and isopods did not occur under the tested experimental conditions. However, the first evidence of parasite spillover involving another microsporidian was observed in the field between *N. davidi* and the invasive crayfish *P. clarkii*. These findings suggest that introduced generalist parasites are likely to colonize novel degraded environments, which, coupled with an ongoing range expansion of *N. davidi*, may exacerbate the impact on native biota.

The chapters presented in this dissertation contribute to the general understanding of the impact of stream degradation and recovery on host-parasite dynamics. The detection of a vast number of host generalist parasites with seemingly simple life cycles indicates that the investigated environments, including those undergoing recovery, are still under the influence of multiple stressors. The obtained results highlight the importance of considering parasite traits such as host specialization, life cycle complexity, pathogenicity, and environmental resilience when investigating the impact of multiple stressors.

Cumulative thesis and extent of contributions

Cumulative thesis of Mr. Sebastian Prati

Chapter I

Prati, S., Grabner, D. S., Pfeifer, S. M., Lorenz, A. W., & Sures, B. (2022). Generalist parasites persist in degraded environments: A lesson learned from microsporidian diversity in amphipods. *Parasitology*, *149*(7), 973–982. https://doi.org/10.1017/S0031182022000452

Personal contributions: Conception (70%), fieldwork (0%), laboratory work (80%), data analysis (100%), drafting (70%), submission (100%), and revision (90%).

Chapter II

Prati, S., Enß, J., Grabner, D. S., Huesken, A., Feld, C. K., Doliwa, A., & Sures, B. (2023). Possible seasonal and diurnal modulation of *Gammarus pulex* (Crustacea, Amphipoda) drift by microsporidian parasites. *Scientific Reports*, *13*(1),), 9474. https://doi.org/10.1038/s41598-023-36630-2

Personal contributions: Conception (40%), fieldwork (40%), laboratory work (70%), data analysis (100%), drafting (70%), submission (100%), and revision (90%).

Chapter III

Schneider, R., Prati, S., Grabner, D. S., & Sures, B. (2022). First report of microsporidians in the non-native shrimp *Neocaridina davidi* from a temperate European stream. *Diseases of Aquatic Organisms*, *150*, 125–130. https://doi.org/10.3354/dao03681

Personal contributions: Conception (70%), fieldwork (50%), laboratory work (20%), data analysis (100%), drafting (40%), submission (100%), and revision (40%).

Chapter IV

Prati, S., Grabner, D. S., Hupało, K., Weiperth, A., Maciaszek, R., Lipták, B., Bojko, J., Bérces, F., & Sures, B. (2023). Invisible invaders: range expansion of feral *Neocaridina davidi* provides novel opportunities for generalist intracellular parasites. [Manuscript submitted for publication]

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Signature of the Doctoral Candidate

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Introduction

Freshwater ecosystems are particularly vulnerable to human activity and environmental changes and are among the most impacted ecosystems in the world (Dudgeon et al., 2006). Multiple stressors such as pollution, flow alteration, habitat destruction or degradation, and the release of invasive species commonly result in biodiversity loss (Birk et al., 2020; Dudgeon et al., 2006), a loss further exacerbated by climate change (Bellard et al., 2012). Areas of particular interest are those impacted by stream channelization, wastewaters, saline, and thermal pollution, where ecosystem restoration efforts to reverse biodiversity loss have soared in recent years (Fischer et al., 2021). However, despite mounting efforts to reverse biodiversity loss through ecosystem restorations, the success of such initiatives is often hampered by complex interactions within living organisms and their responses to anthropogenic stressors (Birk et al., 2020; Lorenz et al., 2018; Vos et al., 2023). In this context, understanding less investigated aspects, such as host-parasite interactions, is particularly relevant.

Parasites are dependent on their hosts, at least during certain phases of their ontogeny. Some parasites are generalists and exploit a broad range of hosts, while others are specialized in one or a few hosts (Dobson et al., 2008; Lafferty, 2012). Heteroxenous parasites require multiple hosts to complete their life cycle, while monoxenous parasites need just one. Consequently, a reduction of host diversity and abundance caused by habitat degradation might trigger heteroxenous specialist parasite extinctions (Dobson et al., 2008; Moir et al., 2010; Sures et al., 2023). On the other hand, monoxenous generalist parasites are more likely to persist in degraded environments due to simple life cycles and host plasticity (Lafferty, 2012; Poulin & Morand, 2004; Sures, 2008; Sures et al., 2023). Consequently, these parasites are more likely to be the first to colonize restored streams. However, their ability to colonize and disperse may vary between species and could be strongly influenced by the community of free-living species, including invasive ones (Sures et al., 2023). Likewise, parasites can modulate ecosystem recovery trajectories by directly or indirectly influencing free-living communities' composition and structure.

Parasites may, via their pathological effects, reduce the functional importance of hosts that are key structuring forces in the community or indirectly increase it by inducing phenotypic alterations (Poulin, 1999). Acanthocephalan and microsporidian parasites infecting freshwater crustaceans are excellent examples of such alterations. Acanthocephalans are known to induce changes in swimming activity, habitat selection, geotaxis, and phototaxis in amphipods (Giari et al., 2020). On the other hand, microsporidians can cause sex ratio distortion, influence host motility, activity level, mortality, intraguild predation between native and invasive species, and aggressive behavior (Bojko & Ovcharenko, 2019; Giari et al., 2020). Such alterations may directly or indirectly affect individual performances of amphipods and thus influence their ability and proneness to disperse. Moreover, parasite burdens may vary seasonally, either impairing host locomotory activity, as seen for the microsporidians *Pleistophora mulleri* and *Cucumispora ornata*, or enhancing it, as for *C. dikerogammari* (Bacela-Spychalska et al., 2014; Bojko & Ovcharenko, 2019; Fielding et al., 2005). Consequently, dispersal processes, including colonizing restored areas, might be impacted with possibly far-reaching consequences for the entire ecosystem.

The framework provided by the Collaborative Research Center 1439 RESIST offers an ideal opportunity to study such host-parasite dynamics in habitats with different degradation histories. The focal streams investigated in the framework belong to three German catchments: the Boye, the Rotbach, and the Kinzig. The first two are located in a lowland metropolitan area, while the third is in a more mountainous and rural area. The Boye is a tributary of the Emscher, which flows into the Rhine. The lower part of the Boye has previously been used as an open sewer and has been subject to restoration efforts in recent decades. In contrast, albeit with moderate land use, the upper part retains mostly its near-natural conditions (Winking et al., 2016). The Rotbach is a direct tributary of the Rhine. Its upper part, located near the headwater of the Boye, with minimal anthropogenic disturbance, remains in near-natural conditions (LANUV, 1999). On the other hand, the Kinzig, which is also a tributary of the Rhine, has suffered from moderate hydromorphological degradation, land use, and wastewater overflow. In all these catchments, amphipods are among the most ubiquitous and abundant macroinvertebrates and, therefore, chosen as the focal hosts for the present study.

Amphipods are keystone species in freshwater ecosystems and often represent the dominant component among macroinvertebrates (Giari et al., 2020). They are an essential component of aquatic food webs, acting as secondary producers, consumers, and food sources for a wide range of predators (Giari et al., 2020; Macneil et al., 1997). Their shredding activity contributes substantially to the diversity and functioning of microorganisms and invertebrate communities, accounting for up to 75% of the overall leaf-litter breakdown in some ecosystems (Giari et al., 2020). Amphipods thrive in a wide range of environments thanks to their adaptability and resilience to changes in abiotic conditions (Macneil et al., 1997). Hence, they are likely to be among the early colonizers of restored areas, becoming pivotal in ensuring the development and sustainment of a healthy ecosystem. Nonetheless, their dispersal into restored areas may be influenced by parasites.

Amphipods host a wide array of heteroxenous and monoxenous parasites (Bojko & Ovcharenko, 2019). These can influence almost every aspect of their life and, in the case of heteroxenous parasites, also that of the upstream hosts. Nevertheless, only a handful of these parasites have been studied more thoroughly (Bojko & Ovcharenko, 2019). Among the best-studied parasite groups of amphipods are acanthocephalans and microsporidians. While knowledge of amphipods infecting macroparasites is steadily bridging gaps, that of microsporidians is still lagging behind. Recent studies suggest that amphipods host highly diverse microsporidian communities (Bojko & Ovcharenko, 2019; Grabner et al., 2015; Ironside & Wilkinson, 2018; Park et al., 2020). Furthermore, studies conducted in German streams, comprising the Boye and nearby watercourses, show that in amphipods, microsporidians are more diverse and abundant than macroparasites (Grabner, 2017; Grabner et al., 2015). However, more profound knowledge of diversity, host specificity, seasonality, and environmental tolerances of microsporidians remains scarce. Therefore, although screening for both macroparasites and microsporidians in the current investigations, more emphasis will be given to the latter as they might be more useful to unravel multiple stressors impacts in the study areas.

Microsporidians are a ubiquitous, diverse, and successful group of eukaryotic obligate intracellular parasites known to infect a wide range of hosts spanning from protists to humans (Dunn & Smith, 2001; Stentiford et al., 2013; Wadi & Reinke, 2020). Microsporidian life cycles and transmission routes vary greatly in their complexity, with species being either monoxenous or heteroxenous and exploiting horizontal, vertical, and mixed-mode transmissions (Dunn & Smith, 2001). Horizontal transmission occurs between related or unrelated hosts via spore ingestion, venereally, or by direct invasion, and it is often associated with high host mortality (Wittner & Weiss, 1999). In contrast, vertical transmission occurs when spores are passed intergenerationally via transovarial transmission and is generally associated with low or no virulence or even increased host fitness (Fielding et al., 2005; Galbreath Slothouber et al., 2004).

In the context of multiple stressors, microsporidians' host specificity and environmental tolerances are particularly relevant. In environments with a rapidly declining diversity and availability of hosts, like those heavily affected by anthropogenic stressors, microsporidians may employ survival strategies such as releasing environmentally resistant spores and/or host-switching (Dunn & Smith, 2001). Several microsporidians are host generalists infecting hosts spanning different taxonomical groups. Hence, like other parasites, they may persist in degraded environments by switching from a rapidly declining host to an alternative, more common host, even if that entails reduced fitness benefits (Dunn et al., 2009; Moir et al., 2010). In this regard, invasive species with higher environmental tolerance than their native counterpart may potentially act as alternative hosts for native host generalist microsporidians. However, invasive species might also bring novel parasites that can severely affect native species or enhance the host's invasive capabilities (Galbreath Slothouber et al., 2004; Prenter et al., 2004). A recent study involving a nonhost-targeted screening of microsporidians across marine, freshwater, and terrestrial biomes revealed discrepancies between phylogeny and sampling environment, suggesting that transitions between different hosts and biomes might occur more frequently than initially suspected (Williams et al., 2018). Such transitions are especially relevant in the face of multiple stressors and mounting biological invasions. Therefore, exploratory studies of novel organisms and environments have the potential to shed more light on how microsporidian communities cope with biotic and abiotic disturbances.

Freshwater habitats experiencing degradation are particularly prone to biological invasions (Casatti et al., 2006; Gherardi, 2007). In these environments, the introduction of non-native species is one of the leading causes of biodiversity loss (Dudgeon et al., 2006). Introducing non-native species may negatively impact the recipient community by directly or indirectly affecting ecosystem functioning and biodiversity, even without establishment, e.g., via parasite transmission (Bojko et al., 2021; Foster et al., 2021). Non-native parasites introduced with invasive species might also enhance the host's invasive capabilities (Galbreath Slothouber et al., 2004; Prenter et al., 2004). The ongoing onslaught of European native crayfish caused by the introduction of their North American counterpart infected with *Aphanomyces astaci*, the crayfish plague's causative agent, is one such example (Svoboda et al., 2017). However, in some cases, introduced parasites may indirectly benefit native biota by acting as a controlling agent for non-native species (Bojko et al., 2015). Likewise, transmission of parasites from native to non-native species might lessen the impact of invaders (Dunn et al., 2009).

Nowadays, the ornamental pet trade is a primary pathway for introducing non-native species and associated parasites (Duggan, 2010; Lipták & Vitázková, 2015; Patoka et al., 2016). Facilitated by rising temperatures, exotic species with high environmental plasticity and fecundity, such as the ornamental shrimp *Neocardina davidi,* can rapidly adapt to local conditions becoming invasive (Blackburn et al., 2011; Klotz et al., 2013). *Neocardina davidi* can have direct and indirect impacts on the recipient community by out-competing native crustaceans relying on the same resources, altering meiofaunal assemblages and leaf-litter breakdown in invaded areas (Schoolmann & Arndt, 2017; Weber & Traunspurger, 2016). Moreover, *N. davidi* hosts a wide range of commensals and parasites, some of which have been co-introduced outside their native range and might impact native crustaceans (Maciaszek et al., 2021, 2023; Niwa & Ohtaka, 2006). However, no screening of microsporidians in this host has been carried out to date.

Neocaridina davidi has been recently introduced in the Erft, a tributary of the river Rhine with a long history of thermal pollution. It was first reported in the Gillbach in 2013, a thermally polluted tributary of the river Erft, and later from the lower and upper Erft and the Rhine (Bierbach et al., 2022; Klotz et al., 2013; Schoolmann & Arndt, 2018). While thermal pollution remains rampant in some areas of the Erft catchments, it has decreased in other areas as polluting industries have reduced or ceased their activities. As a result, decreasing water temperatures in these areas have allowed for the return of native biota (Bierbach et al., 2022). Given the environmental adaptability of *N. davidi* and an ecological niche similar to that of native amphipods, spillover of microsporidians and other parasites might occur in areas where both crustaceans co-occur. In such areas, *N. davidi* and associated parasites may, if present, affect ecosystem recovery.

This thesis aims to provide novel insight into microsporidian-host interactions in degraded and restored streams within four chapters. More specifically, in Chapter I, regional and continental-wide host specialization in microsporidians infecting amphipods is evaluated in relation to degraded and recovering habitats across two German catchments, the Boye and the Kinzig. The main hypothesis is that microsporidians in degraded environments have lower diversity and host specificity than those in near-natural sites. Secondly, for generalists microsporidians, environmental conditions and host diversity play a minor role in their persistence in degraded environments and establishment in restored areas. In Chapter II, microsporidians’ and amphipods’ reciprocal dispersal modulation is investigated by conducting drift experiments in a near-natural stream, the Rotbach (Germany). Studying such dynamics in a near-natural stream provides a baseline that can be used to shed more light on colonization dynamics in restored areas. The main hypothesis is that natural changes in amphipods’ size distribution occurring within aging host populations modulate seasonal variations in microsporidian composition and prevalence. The secondary main hypothesis is that microsporidians can modulate host drift. In Chapter III, the establishment of non-native microsporidians in degraded environments through releases of exotic hosts is exploratively assessed by screening feral *N. davidi* inhabiting a stream shared with native amphipods, the Finkelbach (Germany). Lastly, in Chapter IV possible spillover of host generalist microsporidians between native (amphipods, isopods) and invasive (*N. davidi* and *Procambarus clarkii*) crustaceans is assessed Europe-wide in areas impacted by different degrees of thermal and/or saline pollution and via an infection experiment. The main underlying hypothesis for these two chapters is that *N. davidi* carries non-native microsporidians that may spill over to co-occurring native crustaceans, potentially affecting their fitness and recovery in restored ecosystems.

Chapter I

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Abstract

The present study provides new insight into suitable microsporidian-host associations. It relates regional and continental-wide host specialization in microsporidians infecting amphipods to degraded and recovering habitats across 2 German river catchments. It provides a unique opportunity to infer the persistence of parasites following anthropogenic disturbance and their establishment in restored rivers. Amphipods were collected in 31 sampling sites with differing degradation and restoration gradients. Specimens were morphologically (hosts) and molecularly identified (host and parasites). Amphipod diversity and abundance, microsporidian diversity, host phylogenetic specificity and continental-wide β-specificity were investigated and related to each other and/or environmental variables.

Fourteen microsporidian molecular operational taxonomic units (MOTUs), mainly generalist parasites, infecting 6 amphipod MOTUs were detected, expanding the current knowledge on the host range by 17 interactions. There was no difference in microsporidian diversity and host specificity among restored and near-natural streams (Boye) or between those located in urban and rural areas (Kinzig). Similarly, microsporidian diversity was generally not influenced by water parameters. In the Boye catchment, host densities did not influence microsporidian MOTU richness across restored and near-natural sites. High host turnover across the geographical range suggests that neither environmental conditions nor host diversity plays a significant role in the establishment into restored areas. Host diversity and environmental parameters do not indicate the persistence and dispersal of phylogenetic host generalist microsporidians in environments that experienced anthropogenic disturbance. Instead, these might depend on more complex mechanisms such as the production of resistant spores, host switching and host dispersal acting individually or conjointly.

Parasitology

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Generalist parasites persist in degraded environments: a lesson learned from microsporidian diversity in amphipods

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Abstract

The present study provides new insight into suitable microsporidian–host associations. It relates regional and continental-wide host specialization in microsporidians infecting amphipods to degraded and recovering habitats across 2 German river catchments. It provides a unique opportunity to infer the persistence of parasites following anthropogenic disturbance and their establishment in restored rivers. Amphipods were collected in 31 sampling sites with differing degradation and restoration gradients. Specimens were morphologically (hosts) and molecularly identified (host and parasites). Amphipod diversity and abundance, microsporidian diversity, host phylogenetic specificity and continental-wide β-specificity were investigated and related to each other and/or environmental variables. Fourteen microsporidian molecular operational taxonomic units (MOTUs), mainly generalist parasites, infecting 6 amphipod MOTUs were detected, expanding the current knowledge on the host range by 17 interactions. There was no difference in microsporidian diversity and host specificity among restored and near-natural streams (Boye) or between those located in urban and rural areas (Kinzig). Similarly, microsporidian diversity was generally not influenced by water parameters. In the Boye catchment, host densities did not influence microsporidian MOTU richness across restored and near-natural sites. High host turnover across the geographical range suggests that neither environmental conditions nor host diversity plays a significant role in the establishment into restored areas. Host diversity and environmental parameters do not indicate the persistence and dispersal of phylogenetic host generalist microsporidians in environments that experienced anthropogenic disturbance. Instead, these might depend on more complex mechanisms such as the production of resistant spores, host switching and host dispersal acting individually or conjointly.

Introduction

Freshwater ecosystems being particularly vulnerable to human activity and environmental changes are among the most impacted ecosystems in the world (Dudgeon *et al.*, 2006). Impacts such as pollution, flow alteration and habitat destruction or degradation commonly result in biodiversity loss (Dudgeon *et al.*, 2006; Birk *et al.*, 2020). Fortunately, ecosystem restoration efforts to reverse biodiversity losses have soared in recent years (Fischer *et al.*, 2021). However, the success of such initiatives is often hampered by complex interactions within living organisms and their responses to anthropogenic stressors (Lorenz *et al.*, 2018; Birk *et al.*, 2020). In this context, understanding less investigated aspects such as host–parasite interactions is of particular relevance.

Most parasites are dependent on their hosts. Some parasites are generalist and exploit a broad range of hosts, while others are specialized on one or a few hosts (Dobson *et al.*, 2008; Lafferty, 2012). Heteroxenous parasites require multiple hosts to complete their life cycle, while monoxenous parasites need just one. Therefore, anthropogenic stressors can affect both hosts and parasites (Dobson *et al.*, 2008; Moir *et al.*, 2010). For instance, a reduction of hosts diversity and abundance caused by habitat degradation might trigger heteroxenous specialist parasite extinctions (Dobson *et al.*, 2008; Moir *et al.*, 2010). On the other hand, monoxenous generalist parasites are more likely to persist in degraded environments due to simple life cycles and host plasticity (Poulin and Morand, 2004; Lafferty, 2012). Hence, ubiquitous parasites with either simple or complex life cycles and various host specialization degrees such as microsporidians may reflect ecosystem degradation and recovery histories.

Microsporidians are a diverse and successful group of eukaryotic obligate intracellular parasites that exploit horizontal, vertical and mixed-mode transmissions (Dunn and Smith, 2001; Stentiford *et al.*, 2013; Wadi and Reinke, 2020). Horizontal transmission occurs between related or unrelated hosts *via* spore ingestion, venereally or by direct invasion, and it is often associated with high host mortality (Wittner and Weiss, 1999). In contrast, vertical transmission occurs when spores are passed intergenerationally *via* transovarial transmission and is generally less virulent (Dunn and Smith, 2001). Despite being ubiquitous, microsporidians have received little attention, particularly those infecting aquatic organisms with little or no

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commercial value but high ecological importance, such as amphipods (Quiles *et al.*, 2021). Microsporidian diversity in amphipods is high as evidenced by several studies (Grabner *et al.*, 2015; Ironside and Wilkinson, 2018; Bojko and Ovcharenko, 2019; Park *et al.*, 2020). Still, only a few common species have been characterized and described such as the horizontally transmitted *Cucumispora* spp. (Bojko *et al.*, 2015, 2017) and vertically transmitted *Nosema granulosis* (Terry *et al.*, 1999) and *Dictyocoela* spp. (Bacela-Spychalska *et al.*, 2018). However, given the fragmentary nature of available information, knowledge of host specificity in microsporidians infecting amphipods and persistence following environmental disturbance remains scarce.

The present study provides new insight into microsporidian–host associations. It relates regional and continental-wide host specialization in microsporidians infecting amphipods to a gradient of degraded and recovering habitats across 2 German river catchments offering a unique opportunity to infer the persistence of parasites following anthropogenic disturbance and their establishment in restored rivers. One catchment, the Boye, has partly been used as an open sewer since the beginning of the last century and, starting from the 1990s, it was gradually restored (Winking *et al.*, 2014, 2016). In contrast, the Kinzig catchment has suffered from moderate hydromorphological degradation, land use and wastewater overflow.

The first hypothesis is that microsporidian parasites in streams used as open sewers in the Boye catchment and those flowing in urbanized areas in the Kinzig catchment would have lower diversity and host specificity compared to sites in a near-natural state. Secondly, that to persist in such environments, microsporidian parasites should be able to use available amphipod hosts independently from their phylogenetic diversity. Thus, their presence is likely to depend on available host densities. Finally, if microsporidians have a wide geographical range and higher turnover of host species across their geographical distribution, both environmental conditions and host diversity play minor roles in their establishment in restored areas.

Materials and methods

Sampling

The sample consists of 519 amphipods collected in 31 sampling sites spread across the Boye ($n = 13$) and Kinzig ($n = 18$) catchments (Fig. 1). The source and upstream tributaries of the Boye are located in agricultural or forested areas and have never been used as open sewers, thus retaining near-natural conditions (Table 1). In contrast, the downstream section and tributaries which flow in urbanized areas were transformed into concrete channels at the beginning of the last century to transport domestic wastewater (Winking *et al.*, 2014, 2016). These open sewers were lifeless except for rare occurrences of Oligochaeta. Still, starting from the 1990s, they have undergone partial restoration and subsequent colonization by pioneer invertebrate assemblages originating from upstream sections and neighbouring catchments (Winking *et al.*, 2014, 2016). Tributaries of the river Kinzig, located in a rural landscape in the low mountain range of Hessen county, Germany, on the other hand, are in a near-natural state (Table 1). Samples were collected in March 2021 and immediately fixed in 96% ethanol. A standardized multi-habitat-sampling (Meier *et al.*, 2006) was employed to estimate benthic invertebrate densities, while additional amphipod specimens were collected separately for parasitological analyses. Amphipods were morphologically identified to the lowest taxonomical level, dissected and screened for parasites under the microscope. Intestines were removed to avoid contamination between muscular tissues and the intestinal content. A small sample of muscular tissue was then used for molecular identification of both host and parasites.

DNA isolation and sequencing

DNA was isolated from muscular tissue following a modified salt precipitation protocol described by Grabner *et al.* (2015). Molecular identification of amphipods was obtained with the universal eukaryotic primers LCO1490 and HCO2198, which amplify the CO1 region, while that of microsporidians with the universal microsporidian primers V1 and Micuni3R, which amplify the SSU rDNA region (Weigand *et al.*, 2016). If clear bands were visible, polymerase chain reaction products were sent to Microsynth Seqlab (Germany) for sequencing using LCO1490 and V1 primers, respectively.

Sequence editing and alignment

CO1 and SSU rDNA sequences were edited in Unipro UGENE version 40.0 (Okonechnikov *et al.*, 2012). Only sequences with a

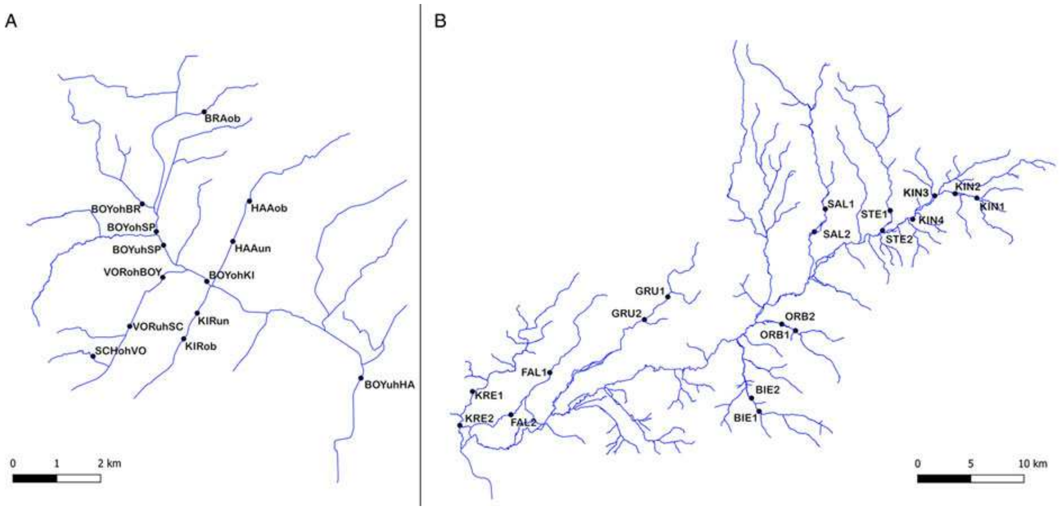

Fig. 1. Map showing sampling sites located in the (A) Boye and (B) Kinzig catchments (map created with QGIS v3.16.9).

Table 1. Sampling locations in the Boye and Kinzig catchments, with coordinates, habitat description (Boye: near-natural, <5, 6–11 and >11 years since renaturation and Kinzig: urban or rural), host MOTUs, parasite MOTUs (in bracket the number of microsporidian MOTUs including those reported in other studies conducted in the same location), number of infected hosts (infected/total host individuals in the sample) and water parameters

Catchment	Location	Coordinates	Habitat	Host MOTUs	Parasite MOTUs	Infected host	pH	EC (μs cm^{-1})	O$_2$ (mg L^{-1})	*T* (°C)
Boye	BOYohBR	51.569135; 6.927698	R > 11	1	2 (4)	2/17	7.53	593	9.5	8.7
	BOYohKI	51.553107; 6.948059	R < 5	2	1	2/10	7.80	754	11.1	7.6
	BOYohSP	51.564297; 6.930277	R > 11	1	2	4/21	7.77	700	9.9	11.4
	BOYuhHA	51.534515; 6.99774	R < 5	1	1	1/10	8.09	926	12	9.3
	BOYuhSP	51.561336; 6.932617	R > 11	2	3	9/20	7.58	706	10.2	10
	BRAob	51.588333; 6.944944	NN	1	3 (3)	6/25	7.54	873	9.1	11.1
	HAAob	51.570306; 6.960891	R6–11	2	2	2/13	7.93	829	9.1	12.7
	HAAun	51.562638; 6.955581	R6–11	1	1	1/10	7.37	1030	11.3	10.1
	KIRob	51.542223; 6.939196	R > 11	1	1	7/10	8.05	1092	9.2	9.9
	KIRun	51.547455; 6.943704	R > 11	1	2	7/15	7.65	979	10	8.2
	SCHohVO	51.539420; 6.908287	NN	1	2	3/11	7.65	584	10.3	7.5
	VORohBOY	51.554753; 6.932435	R6–11	1	4	7/10	7.37	974	11.1	9
	VORuhSC	51.544809; 6.921403	R6–11	1	1 (2)	1/10	7.37	935	9.1	9
Kinzig	BIE1	50.163394; 9.294485	Urban	1	1	3/16	7.75	182	10.8	12.3
	BIE2	50.174435; 9.284504	Rural	1	2	2/17	8.15	160	11.1	10.2
	FAL1	50.196262; 9.019494	Rural	3	3	6/13	8.15	805	12.0	9.6
	FAL2	50.160807; 8.968476	Urban	2	4	11/34	8.29	771	11.5	8.6
	GRU1	50.259856; 9.174480	Rural	1	3	6/15	7.72	146	12.2	8.2
	GRU2	50.240705; 9.144059	Urban	3	3	5/21	7.19	166	11.3	8.4
	KIN1	50.342661; 9.580566	Rural	2	4	5/26	9.04	357	12.1	8.8
	KIN2	50.346310; 9.552487	Urban	2	3	5/24	8.51	431	13.3	6.9
	KIN3	50.344639; 9.525316	Urban	2	2	4/22	8.83	402	12.1	8.4
	KIN4	50.325129; 9.496401	Rural	2	3	4/23	8.50	450	13.6	6.4
	KRE1	50.180280; 8.918023	Urban	2	2	5/16	8.17	841	15.3	12.9
	KRE2	50.151738; 8.901119	Rural	1	1	3/22	8.21	852	13.9	12.3
	ORB1	50.231196; 9.342359	Urban	2	1	8/29	7.21	111	11.6	9.3
	ORB2	50.236741; 9.324385	Rural	1	3	7/13	7.03	132	11.3	10.7
	SAL1	50.333618; 9.381166	Rural	2	2	5/6	9.54	153	13.2	10.1
	SAL2	50.314300; 9.366880	Urban	2	2	3/14	9.45	169	14.2	9.4
	STE1	50.332253; 9.466509	Rural	3	3	12/23	8.73	241	13.7	8.1
	STE2	50.315518; 9.456635	Urban	1	2	3/3	9.10	248	12.1	8.7

minimum length of 200 bp were used for the analyses. Host and parasite sequences were separately aligned using the MAFFT 7 algorithm with a standard setting (Katoh *et al*., 2019). To identify hosts and their microsporidian parasites, sequences were blasted against records contained in GenBank. Haplotypes of amphipods and parasites were grouped in molecular operational taxonomic units (MOTUs) when the Kimura-2-parameter (K2p) corrected pairwise distances were below 2% (Supplementary file 1: Table S1). A threshold of 2% was chosen to account for potential intragenomic variation present in some microsporidians while remaining below commonly observed values of intraspecific variability in amphipods (Costa *et al*., 2009; Ironside, 2013; Grabner *et al*., 2015). Obtained MOTU sequences were then compared with highly similar sequences (minimum 98% identity match) retrieved from GenBank using K2p as described above to assess if microsporidians use a wider array of hosts than what was observed locally in the present study. When host and parasite sequences from the same individual were not available, we used the closest host sequences available (e.g. from the same host population or sequences available from different areas) to build a phylogenetic tree (Supplementary file 2: Dataset S1). Thus, resulting host specificity might be conservative as splitting these hosts in genetic clades within the same species was not feasible. Maximum likelihood phylogenetic trees with bootstrap support values (1000 replicates) for both amphipods and microsporidians were produced in IQ-Tree 2.0 (Minh *et al*., 2020). Based on Bayesian information criterion scores the TIM + F + R4 substitution model was selected for amphipods and TIM3 + F + G4 for microsporidians. Sequences of the amphipod *Crangonyx islandicus* (GenBank accession number HM015162) and the amphipod-infecting microsporidia *Dictyocoela cavimanum* (GenBank accession number KY073301) were used as outgroups. The naming of undescribed Microsporidium isolates with the exception of 2 *Cucumispora* isolates (sp01 and sp02) followed

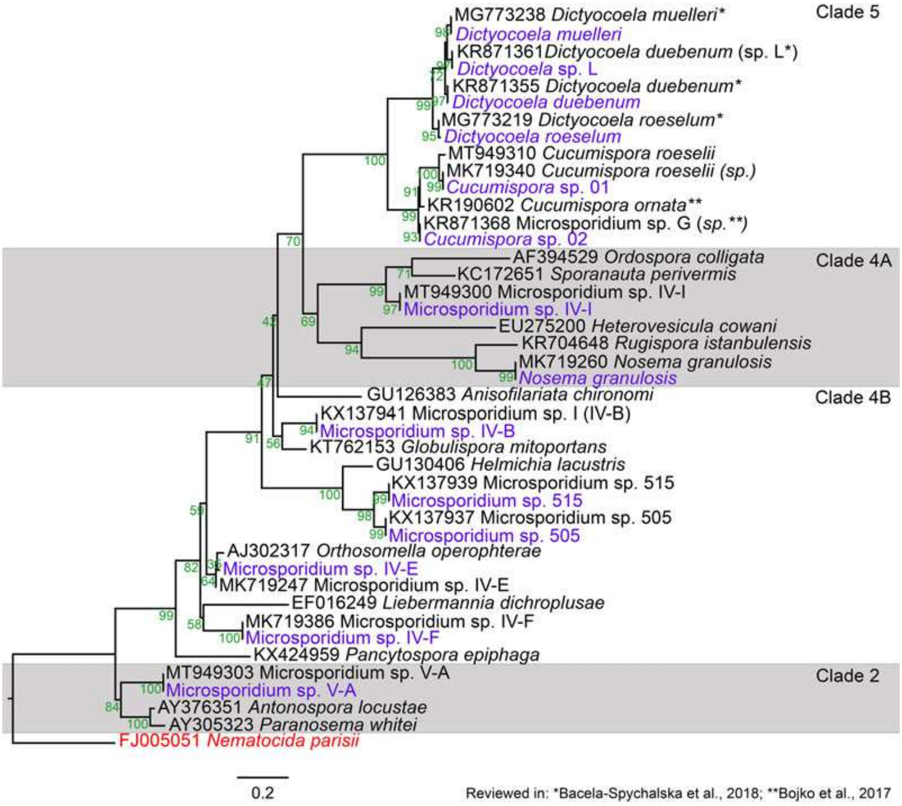

Fig. 2. Maximum likelihood phylogenetic tree obtained with IQ-Tree 2.0 (Minh *et al.*, 2020) using a TIM3 + F + G4 substitution model and based on partial small ribosomal subunit rDNA data (Supplementary file 2: Dataset S1). Labels with accession number are parasite sequences retrieved from GenBank. The name of described species and reviewed sequences are marked with asterisks. Bootstrap values (1000 replicates) are indicated in green. Outgroup is indicated in purple.

the classification used by previous studies (Bojko *et al.*, 2015, 2017; Grabner, 2017; Bacela-Spychalska *et al.*, 2018; Quiles *et al.*, 2019, 2021).

Phylogenetic and geographic specificity analyses

Analyses of phylogenetic host specificity and subsequent statistical analyses were performed with the open-source software Rstudio (version 2021.09.0, Rstudio Inc.) based on R (version 4.1.1, R Core Team). The *ape* package (Paradis *et al.*, 2004) was used to load and transform the phylogenetic tree in Newick format, while the *picante* package (Kembel *et al.*, 2010) was used to calculate Faith's PD phylogenetic diversity index (Faith, 1992) as a measure of phylogenetic host specificity. Phylogenetic host specificity represents the total length of branches linking the host species to a parasite along the phylogenetic tree (Poulin *et al.*, 2011). Thus, the higher the values, the less the parasite is species-specific. To compute β-diversity as a measure of geographic host specificity or β-specificity (Poulin *et al.*, 2011), we used the extension of the Jaccard dissimilarity index for multiple-site using the *betapart* package (Baselga and Orme, 2012). β-Specificity ranges from zero for parasites that exploit the same host across all localities to one for parasites that use completely different hosts from one locality to another.

Statistical analyses

To assess if the number of microsporidian MOTUs was correlated with the number of amphipod MOTUs and water parameters across our sampling sites, the Spearman correlation coefficient was used, while differences in microsporidian MOTU richness across habitat type (Boye: near-natural, <5, 6–11 and >11 years since renaturation and Kinzig: urban or rural) were assessed with the Kruskal–Wallis rank-sum test for the Boye catchment and the Wilcoxon rank-sum test for the Kinzig catchment. General linear models followed by analysis of variance (ANOVA) were employed to assess if microsporidian host phylogenetic specificity and β-specificity are influenced by habitat type and host MOTU richness, using phylogenetic host specificity and β-specificity, respectively, as response variables and host MOTU richness and habitat type as predictors. Furthermore, the role of host densities (individuals/m^2) in microsporidian MOTU richness and phylogenetic specificity among restored and near-natural sites in the Boye catchment was investigated using MOTU richness and phylogenetic specificity as response variables and host densities (pooled for each sampling site) and habitat type as predictors. Differences between continental- and regional-scale β-specificity were compared with the *t*-test.

Results

The sample comprised of 14 microsporidian MOTUs, infecting a total of 6 amphipods MOTUs (Fig. 2; Supplementary file 3: Fig. S1; Table 2), 3 in the Boye catchment (*Gammarus pulex* clade C, *G. pulex* clade E and *Gammarus fossarum* clade 2) and 5 in the Kinzig catchment (*G. pulex* clade D, *G. pulex* clade E, *G. fossarum* clade 1, *G. fossarum* clade 2 and *Gammarus roeselii* clade 2). Among microsporidian MOTUs 9 (*Dictyocoela duebenum*, *D.* sp.

Table 2. Microsporidian MOTU associations with amphipods host MOTUs

	Cucumispora sp. 01	*Cucumispora* sp. 02	*Dictyocoela duebenum*	*Dictyocoela muelleri*	*Dictyocoela roeselum*	*Dictyocoela* sp. L	Microsporidium sp. 505	Microsporidium sp. 515	Microsporidium sp. IV-B	Microsporidium sp. IV-E	Microsporidium sp. IV-F	Microsporidium sp. IV-I	Microsporidium sp. V-A	*Nosema granulosis*
Acanthogammarus lappaceus			a	c										
Acanthogammarus victorii			a											
Brandtia latissima			j											
Dikerogammarus haemobaphes						a/e								
Dikerogammarus villosus			a		s									s
Dorogostaiskia parasitica			a											
Echinogammarus berilloni			a/f/h											
Echinogammarus marinus			a/q											
Eulimnogammarus cyaneus			a											
Eulimnogammarus verrucosus														o
Eulimnogammarus vittatus			j											
Gammarus balcanicus C1									n					
G. balcanicus C2			m											
G. balcanicus C3				m			n	n				n	n	
G. balcanicus C4			m											
G. balcanicus C5			m	m							n			
G. balcanicus C6			m											
G. balcanicus C7			m	m										
Gammarus chevreuxi		b/r												
Gammarus duebeni			a/k				k	k						k/i
Gammarus fossarum C1			**					**						**
G. fossarum C2		**	a/f		**		e	e		**	f		**	**
Gammarus lacustris			d	j										
Gammarus locusta			a/h											
Gammarus pseudolimnaeus			a/p											
Gammarus pulex CC						a/e	f	f	f		f	**	f	
G. pulex CD					**		**	**	**					**
G. pulex CE		b/f	a/f			**	f	f	f	**	f		f	
Gammarus roeselii C1														l
G. roeselii C2	e/l	l	a/f	a/l/r	a/l	a/e	f	e/n	e/l	l	l	**	f/l	l
G. roeselii C3														l
Gammarus tigrinus			a/h											
Gammarus varsoviensis				a										
Gmelinoides fasciatus			a/j					u						

(*Continued*)

Table 2. (*Continued.*)

	Cucumispora sp. 01	*Cucumispora* sp. 02	*Dictyocoela duebenum*	*Dictyocoela muelleri*	*Dictyocoela roeselum*	*Dictyocoela* sp. L	Microsporidium sp. 505	Microsporidium sp. 515	Microsporidium sp. IV-B	Microsporidium sp. IV-E	Microsporidium sp. IV-F	Microsporidium sp. IV-I	Microsporidium sp. V-A	*Nosema granulosis*
Linevichella vortex								u						
Niphagus aquilex			g											
Niphargus schellenbergi			g						t					
Pallasea cancellus			a											
Pontogammarus robustoides						a								

All parasite–host associations are indicated by either asterisks or letter corresponding to a literature reference. New associations are indicated by double asterisks and orange colour, while associations found in our sample that were previously reported by other study are indicated with blue colour. Associations without colour marking are only reported from literature.

**New host.

(a) Bacela-Spychalska *et al.* (2018); (b) Bojko *et al.* (2017); (c) Dimova *et al.* (2018); (d) Drozdova *et al.* (2020); (e) Grabner (2017); (f) Grabner *et al.* (2015); (g) Grabner *et al.* (2020); (h) Hogg *et al.* (2002); (i) Ironside (2013); (j) Ironside and Wilkinson (2018); (k) Krebes *et al.* (2010); (l) Quiles *et al.* (2019); (m) Quiles *et al.* (2020); (n) Quiles *et al.* (2021); (o) Madyarova *et al.* (2015); (p) Ryan and Kohler (2010); (q) Short *et al.* (2012); (r) Terry *et al.* (2004); (s) Wattier *et al.* (2007); (t) Weigand *et al.* (2016); (u) unpublished GenBank sequences.

L., Microsporidium sp. 505, Microsporidium sp. 515, Microsporidium sp. IV-B, Microsporidium sp. IV-E, Microsporidium sp. IV-F, Microsporidium sp. IV-I and Microsporidium sp. V-A) were found in the Boye catchment and 11 (*Cucumispora* sp. 01, *C.* sp. 02, *D. duebenum*, *Dictyocoela muelleri*, *Dictyocoela roeselum*, Microsporidium sp. 505, Microsporidium sp. 515, Microsporidium sp. IV-B, Microsporidium sp. IV-F, Microsporidium sp. IV-I and *N. granulosis*) in the Kinzig catchment (Fig. 3). The most common microsporidians in the Boye catchment were Microsporidium sp. 505 and Microsporidium sp. IV-B with their presence being recorded in 6 sampling sites. In the Kinzig catchment, *D. duebenum* and Microsporidium sp. 515 were the most common with 10 and 9 observations, respectively (Fig. 3). Overall, 11 microsporidian MOTUs have been observed in new host MOTUs, further expanding the current knowledge on host range by 17 additional interactions (Table 2).

In both Boye and Kinzig catchments, microsporidian MOTU richness was neither correlated with host MOTU richness (the Spearman correlation coefficient, $rs = -0.051$, $P = 0.872$ and $rs = 0.400$, $P = 0.100$, respectively) nor significantly differed among habitat types (Kruskal–Wallis rank-sum test, $\chi^2 = 3.6813$, $df = 3$, $P = 0.298$ and the Wilcoxon rank-sum test, $W = 52.5$, $P = 0.2847$, respectively). Similarly, no strong correlations among water parameters and microsporidian MOTU richness were detected. However, in 2 instances, our analyses revealed diverging patterns between catchments with microsporidian MOTU richness being negatively affected by pH in the Boye catchment and by temperature in the Kinzig catchment with their counterparts remaining unaffected (Table 3).

Microsporidian phylogenetic host specificity at a continental scale was higher than 1 in all cases except for *C. roeselii*, indicating that the vast majority of microsporidians are host generalist. This was also reflected by high β-specificity values, which pinpointed exploitation of completely different hosts from one locality to another, hence high host turnover across their geographical range (Table 4). Moreover, differences between regional- and continental-scale values (t-test, $t_{(18)} = -2.77$, $P = 0.013$) highlighted the importance of a broader view when dealing with host-specialization measures (Table 4).

In both Boye and Kinzig catchments, microsporidian phylogenetic specificity was not influenced by habitat type (ANOVA, $F_{(3,23)} = 0.40$, $P = 0.754$ and $F_{(1,41)} = 0.12$, $P = 0.728$, respectively) or host MOTU richness (ANOVA, $F_{(1,23)} = 0.44$, $P = 0.515$ and $F_{(1,41)} = 0.15$, $P = 0.703$ respectively). Similarly, microsporidian β-specificity was not influenced by habitat type (ANOVA, $F_{(3,23)} = 0.55$, $P = 0.\ 650$ and $F_{(1,41)} = 0.86$, $P = 0.358$ respectively) or host MOTU richness (ANOVA, $F_{(1,23)} = 3.19$, $P = 0.09$ and $F_{(1,41)} = 0.18$, $P = 0.674$ respectively). Furthermore, in the Boye catchment, no noticeable effect on microsporidian MOTU richness across restored and near-natural sites was observed when considering differences in host densities (ANOVA, $F_{(1,23)} = 0.38$, $P = 0.546$) and habitat type (ANOVA, $F_{(3,23)} = 2.01$, $P = 0.141$). Similarly, in the same catchment, neither host densities (ANOVA, $F_{(1,25)} = 2.20$, $P = 0.150$) nor host MOTU richness (ANOVA, $F_{(1,25)} = 0.01$, $P = 0.996$) affected microsporidian phylogenetic specificity.

Discussion

Most microsporidian species infect more hosts than previously reported, underlining the importance of parasitological studies using aquatic keystone species with little or no commercial value. Furthermore, the generalist nature of the observed microsporidians in terms of host uses highlights the importance of host specificity in parasite persistence and dispersal following anthropogenic disturbance.

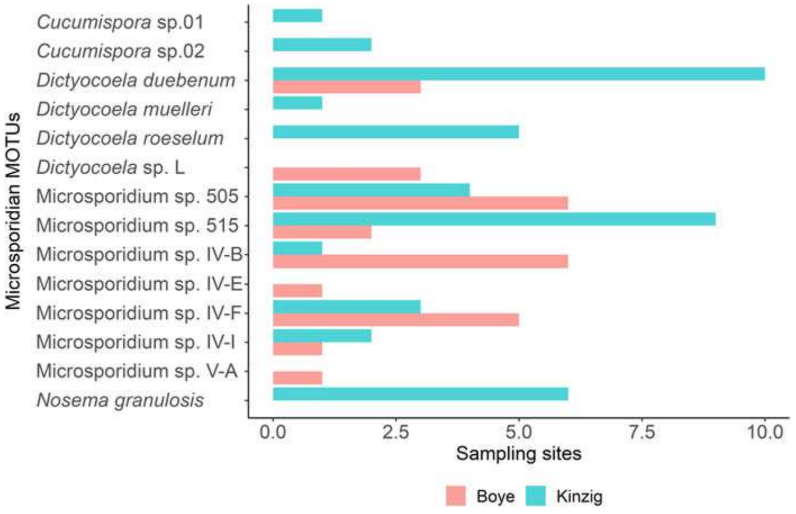

Fig. 3. Number of observed microsporidian MOTUs across sampling sites in the Boye and Kinzig catchments.

Existing and novel associations between amphipods and microsporidian parasites

More than 30 microsporidian species from 12 genera and more than 150 undescribed isolates have been reported from amphipods globally (Bojko and Ovcharenko, 2019), with a still increasing trend. Among our samples, all microsporidian MOTUs found in *G. fossarum* clade 1 and *G. pulex* clade D account for 8 novel associations. New associations with microsporidians in these 2 amphipod clades are expected as they have been investigated less intensively than *G. fossarum* clade 2 (type B in literature) or *G. pulex* clades C and E (Grabner *et al.*, 2015; Grabner, 2017; Bacela-Spychalska *et al.*, 2018). However, we also report new parasite host associations in more commonly investigated amphipods. *Gammarus fossarum* clade 2 was infected with 9 microsporidian MOTUs of which 5 are new. Similarly, *G. pulex* clades C and E were infected with 6 and 5 microsporidian MOTUs, respectively, including 3 new associations. One new association was found even in *G. roeselii* clade 2 (group C in literature), which previously underwent a massive parasitological investigation (Grabner *et al.*, 2015; Bojko *et al.*, 2017; Grabner, 2017; Bacela-Spychalska *et al.*, 2018; Quiles *et al.*, 2019, 2021). The present finding highlights the importance of further parasitological studies in amphipods.

Variability between catchment degradation levels and microsporidians

Contrary to our expectations, both in the Boye and Kinzig catchments, there was no apparent difference in microsporidian diversity and host specificity among restored and near-natural streams or between those located in urban and rural areas. Similarly, microsporidian diversity was generally not influenced by water parameters. The sole exception is a moderate negative effect of pH in the Boye and temperature in the Kinzig catchments. However, the pH range in the Boye catchment (7.3–8.1) varied to a lower extent compared to its counterpart (7–9.5). Hence, we argue that if pH influences microsporidian diversity, this would have been more pronounced in the Kinzig catchment. Similarly, if temperature had an important effect on microsporidian diversity, this would be observed from both catchments given a similar temperature range. Hence other processes such as transmission mechanisms, host immune system, local adaptation, dispersal constraints and competitive interactions among parasites may play a more relevant role in shaping microsporidian communities.

Table 3. Correlations among water parameters of sampling sites located in the Boye ($n = 13$) and Kinzig ($n = 18$) catchments and microsporidian MOTU richness were calculated using the Spearman correlation coefficient

	Boye		Kinzig	
Water parameters	*rs*	*P*	*rs*	*P*
pH	−0.55	0.051	0.01	0.969
Conductivity (μs cm^{-1})	−0.38	0.202	0.13	0.601
Oxygen (mg L^{-1})	−0.33	0.271	−0.05	0.853
Temperature (°C)	0.01	0.989	−0.55	0.019

Anthropogenic disturbance such as sewage discharge and reconstruction of natural watercourses, e.g. into concrete drainage channels, as in the case of the Boye catchment, may result in extinction of organisms, including parasites and their hosts. Thus, to persist, organisms should adapt to new conditions. Microsporidians may cope with similar situations by switching from a rapidly declining host to an alternative, more common host, even if that might entail reduced fitness benefits (Dunn *et al.*, 2009; Moir *et al.*, 2010). Accordingly, all microsporidians collected in the Boye catchment had low host phylogenetic specificity. In contrast, among those collected in the Kinzig catchment, *Cucumispora* sp. 01 showed the highest degree of host phylogenetic specificity, partially supporting our second hypothesis. However, phylogenetic specificity for these and other microsporidians might change over time as new hosts are discovered and it has been shown that for a single parasite species, different genotypes may be specialized on single host species or genotypes (Quiles *et al.*, 2019, 2020). Differences in host diversity between the 2 catchments might also reflect different histories of colonization and anthropogenic disturbance which could have caused host–parasite coextinction. Accordingly, *G. roeselii* is absent from the Boye catchment but present in the surrounding watercourses (Grabner *et al.*, 2015).

Unexpectedly, in the Boye catchment, host densities did not appear to influence microsporidian MOTU richness across restored and near-natural sites. Possible explanations are the release of resistant spores in the environment, which allow parasites to persist when hosts are present in low abundance or even

Table 4. (A) Regional- and (B) continental-scale phylogenetic host specificity and β-specificity calculated with Faith's PD phylogenetic diversity index (Faith, 1992) and Jaccard dissimilarity index for multiple-site (Baselga and Orme, 2012), respectively

Microsporidian MOTUs	Sampling sites	*N* host MOTUs	Phylogenetic specificity	β-Specificity
(A) Regional scale				
Cucumispora sp. 01	1	1	–	–
Cucumispora sp. 02	2	2	1.92	0.50
D. duebenum	13	4	2.90	0.88
D. muelleri	1	1	–	–
D. roeselum	5	3	2.87	0.78
Dictyocoela sp. L.	3	2	1.22	0.80
Microsporidium sp. 505	10	4	2.22	0.91
Microsporidium sp. 515	11	5	2.25	0.93
Microsporidium sp. IV-B	7	2	1.01	0.85
Microsporidium sp. IV-E	1	2	1.91	–
Microsporidium sp. IV-F	8	3	2.87	0.92
Microsporidium sp. IV-I	3	2	1.92	0.80
Microsporidium sp. V-A	1	1	–	–
N. granulosis	6	4	2.90	0.84
(B) Continental scale				
Cucumispora sp. 01	5	1	1	0
Cucumispora sp. 02	5	4	3.72	0.90
D. duebenum	41	26	17.29	0.99
D. muelleri	23	8	5.55	0.94
D. roeselum	18	5	4.07	0.86
Dictyocoela sp. L.	5	5	3.51	0.92
Microsporidium sp. 505	17	7	5.05	0.95
Microsporidium sp. 515	18	10	6.11	0.97
Microsporidium sp. IV-B	11	6	4.17	0.95
Microsporidium sp. IV-E	2	3	2.91	1
Microsporidium sp. IV-F	11	5	4.05	0.94
Microsporidium sp. IV-I	4	3	2.91	0.91
Microsporidium sp. V-A	5	5	4.05	0.96
N. granulosis	26	9	6.08	0.96

absent (Dunn and Smith, 2001), host switching (Becnel and Andreadis, 1999) and different transmission mechanisms acting as confounding factors. Microsporidians might exploit horizontal, vertical or both transmission routes (Dunn and Smith, 2001; Haag *et al.*, 2020). Among these transmission pathways, vertical and mixed transmission pathways may be relevant for parasite persistence and dispersal. For instance, a moth-infecting microsporidium, *Orthosoma operoptherae*, uses vertical transmission to over-winter in host eggs (Canning *et al.*, 1985), while *Octosporea bayeri* utilizes vertical transmission to survive diapause in *Daphnia magna* during drought (Zbinden *et al.*, 2008; Stentiford *et al.*, 2013). Among the most common microsporidians collected in the Boye catchment, horizontal transmission is believed to be the only or the predominant mode of transmission of Microsporidium sp. 505 (Grabner *et al.*, 2014), while Microsporidium sp. IV-B (also referred to as M3 or I in literature) is vertically transmitted (Terry *et al.*, 2004). However, since the biology of most microsporidians found in the current study remains understudied, their primary transmission pathway is not known, preventing us from in-depth interpretations. Furthermore, given connectivity and the relative proximity among streams in the Boye catchment, the general lack of distinct patterns may be due to underlying mechanisms related to the dispersal of hosts and parasites across sites.

The high host turnover observed in microsporidians across their geographical range suggests that neither environmental condition nor host diversity plays a significant role in their establishment in restored areas, supporting our third hypothesis. Being internal parasites, microsporidians are less exposed to changes in the external environment. Moreover, given the wide geographical distribution of Microsporidia and their occurrence in a variety of environments, it is likely that they possess a certain degree of plasticity to environmental conditions. Host diversity is less relevant in generalist microsporidians, as they may use alternative and phylogenetically distant hosts. For instance, *Enterocytozoon bieneusi* can exploit birds and mammals (Wadi and Reinke, 2020), while *Trachipleistophora hominis*, whose natural host is an insect, may infect immunocompromised humans (Watson *et al*., 2015). This might be the case also for microsporidians infecting amphipods. For instance, Microsporidium sp. 1049 has been reported to infect both *G. roeselii* and chironomid larvae (Grabner, 2017). Thus, host switching in generalist parasites might overcome environmental impediments and favour persistence and dispersal. Microsporidians are most likely infecting a wider host spectrum than reported here, as current knowledge is based only on a few host species/MOTUs. Hence our estimates may be conservative, and further investigations on host specificity targeting alternative host species are required to understand persistence and dispersal mechanisms.

Conclusion

In conclusion, host diversity and environmental parameters do not dictate the persistence and dispersal of phylogenetic host generalist microsporidians in environments that experienced anthropogenic disturbance. Instead, these might depend on more complex mechanisms such as the production of resistant spores, host switching and host dispersal acting individually or conjointly.

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Data. The dataset supporting the conclusions of this article is included within the article (and its Supplementary material). Host and parasite sequences have been uploaded to GenBank and the following accession numbers obtained: amphipod hosts (ON093813, ON093814, ON093815, ON093816, ON093817 and ON093818) and microsporidian parasites (ON113505, ON113506, ON113507, ON113508, ON113509, ON113510, ON113511, ON113512, ON113513, ON113514, ON113515, ON113516, ON113517 and ON113518).

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Author contributions. S. P. and B. S. conceived the study, and D. S. G. and B. S. supervised the project. S. M. P. and A. W. L. carried out the sampling and S. P. the data analyses. S. P. led the writing of the manuscript; D. S. G., S. M. P., A. W. L. and B. S. oversaw the analyses and writing. All authors contributed critically to the drafts and approved the final manuscript.

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Conflict of interest. The authors declare there are no conflicts of interest.

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Table S1 Parasites and hosts Kimura-2-parameters (K2p) corrected pairwise distances with Standard error estimates (in blue) obtained with bootstrapping (1000 replications). Outgroup are indicated in red

	MT949310_C.roeselii	MT949303_M.sp.V-A	MT949300_M._sp.IV-	MK719386_M.sp.IV-I	MK719340_C.roeselii	MK719260_N.granulc	MK719247_M.sp.IV-I	MG773238_D.mueller	MG773219_D.roeselu	KY073301_D.cavimar	KX137941_M.sp.I	KX137939_M.sp.515	KX137937_M.sp.505	KR871368_M.sp.G	KR871361_D.sp.L*	KR871355_D.dueben	M.sp.I_KIN3	M.sp.505_BOYohSp	KR190602_C.ornata*	M.sp.515_ORB1	M.sp.IV-E_BOYuhSp	M.sp.V-A_BRAob	N.granulosis_GRU1	D.roeselum_BIE2	D.muelleri_STE1	D.sp.L_BOYohBr	D.dubemum_SAL1	C.sp.02_FAL1	C.sp.01_KIN4	M.sp.IV-F_VORohBo	M.sp.IV-I_KRE1
MT949310_C.roeselii		0.162	0.108	0.099	0.011	0.166	0.100	0.055	0.060	0.066	0.063	0.115	0.124	0.023	0.059	0.055	0.063	0.124	0.026	0.115	0.100	0.162	0.166	0.061	0.055	0.059	0.055	0.023	0.011	0.099	0.106
MT949303_M.sp.V-A	0.892		0.081	0.063	0.148	0.150	0.066	0.107	0.108	0.112	0.098	0.080	0.081	0.158	0.110	0.109	0.098	0.081	0.177	0.080	0.066	0.000	0.150	0.098	0.107	0.110	0.109	0.158	0.148	0.063	0.081
MT949300_M._sp.IV-I	0.628	0.478		0.087	0.102	0.085	0.063	0.086	0.084	0.091	0.063	0.075	0.083	0.092	0.080	0.086	0.063	0.083	0.103	0.075	0.063	0.081	0.085	0.078	0.086	0.080	0.086	0.092	0.102	0.087	0.000
MK719386_M.sp.IV-F	0.612	0.367	0.503		0.093	0.152	0.030	0.106	0.107	0.132	0.060	0.073	0.075	0.096	0.110	0.102	0.060	0.075	0.099	0.073	0.030	0.063	0.152	0.093	0.106	0.110	0.102	0.096	0.093	0.000	0.088
MK719340_C.roeselii	0.037	0.832	0.596	0.579		0.173	0.090	0.048	0.054	0.058	0.059	0.110	0.117	0.021	0.051	0.049	0.059	0.117	0.023	0.110	0.090	0.148	0.173	0.054	0.048	0.051	0.049	0.021	0.000	0.093	0.100
MK719260_N.granulosis	0.984	0.908	0.541	0.864	1.002		0.144	0.163	0.165	0.169	0.079	0.117	0.111	0.154	0.158	0.169	0.079	0.111	0.161	0.117	0.144	0.150	0.000	0.161	0.163	0.158	0.169	0.154	0.173	0.152	0.086
MK719247_M.sp.IV-E	0.588	0.379	0.359	0.160	0.537	0.820		0.091	0.089	0.104	0.055	0.064	0.078	0.096	0.090	0.088	0.055	0.078	0.102	0.064	0.000	0.066	0.144	0.075	0.091	0.090	0.088	0.096	0.090	0.030	0.063
MG773238_D.muelleri*	0.345	0.652	0.537	0.614	0.308	0.980	0.528		0.012	0.025	0.069	0.101	0.117	0.047	0.009	0.010	0.069	0.117	0.049	0.101	0.091	0.107	0.163	0.014	0.000	0.009	0.010	0.047	0.048	0.106	0.085
MG773219_D.roeselum*	0.363	0.653	0.536	0.628	0.335	0.989	0.522	0.038		0.023	0.070	0.096	0.115	0.044	0.010	0.011	0.070	0.115	0.049	0.096	0.089	0.108	0.165	0.005	0.012	0.010	0.011	0.044	0.054	0.107	0.082
KY073301_D.cavimanum	0.423	0.669	0.551	0.723	0.361	0.977	0.585	0.127	0.106		0.074	0.118	0.128	0.050	0.024	0.026	0.074	0.128	0.054	0.118	0.104	0.112	0.169	0.021	0.025	0.024	0.026	0.050	0.058	0.132	0.089
KX137941_M.sp.I	0.394	0.538	0.368	0.351	0.369	0.501	0.305	0.430	0.439	0.456		0.061	0.057	0.057	0.070	0.069	0.000	0.057	0.059	0.061	0.055	0.098	0.079	0.067	0.069	0.070	0.069	0.057	0.059	0.060	0.062
KX137939_M.sp.515	0.644	0.452	0.440	0.432	0.620	0.707	0.371	0.583	0.568	0.646	0.346		0.021	0.106	0.095	0.101	0.061	0.021	0.109	0.000	0.064	0.080	0.117	0.093	0.101	0.095	0.101	0.106	0.110	0.073	0.075
KX137937_M.sp.505	0.685	0.471	0.489	0.439	0.656	0.656	0.444	0.661	0.655	0.687	0.318	0.088		0.113	0.115	0.117	0.057	0.000	0.116	0.021	0.078	0.081	0.111	0.114	0.117	0.115	0.117	0.113	0.117	0.075	0.084
KR871368_M.sp.G	0.117	0.851	0.547	0.589	0.105	0.926	0.555	0.285	0.269	0.325	0.342	0.593	0.628		0.047	0.046	0.057	0.113	0.011	0.106	0.096	0.158	0.154	0.046	0.047	0.047	0.046	0.000	0.021	0.096	0.090
KR871361_D.sp.L*	0.362	0.668	0.507	0.643	0.312	0.950	0.522	0.027	0.031	0.115	0.439	0.568	0.655	0.293		0.009	0.070	0.115	0.051	0.095	0.090	0.110	0.158	0.012	0.009	0.000	0.009	0.047	0.051	0.110	0.078
KR871355_D.duebenum*	0.338	0.660	0.537	0.600	0.309	1.002	0.515	0.027	0.031	0.132	0.430	0.583	0.661	0.280	0.027		0.069	0.117	0.050	0.101	0.088	0.109	0.169	0.012	0.010	0.009	0.000	0.046	0.049	0.102	0.084
M.sp.I_KIN3	0.394	0.538	0.368	0.351	0.369	0.501	0.305	0.430	0.439	0.456	0.000	0.346	0.318	0.342	0.439	0.430		0.057	0.059	0.061	0.055	0.098	0.079	0.067	0.069	0.070	0.069	0.057	0.059	0.060	0.062
M.sp.505_BOYohSp	0.685	0.471	0.489	0.439	0.656	0.656	0.444	0.661	0.655	0.687	0.318	0.088	0.000	0.628	0.655	0.661	0.318		0.116	0.021	0.078	0.081	0.111	0.114	0.117	0.115	0.117	0.113	0.117	0.075	0.084
KR190602_C.ornata**	0.135	0.903	0.591	0.604	0.122	0.955	0.576	0.308	0.297	0.343	0.361	0.609	0.636	0.036	0.316	0.315	0.361	0.636		0.109	0.102	0.177	0.161	0.050	0.049	0.051	0.050	0.011	0.023	0.099	0.101
M.sp.515_ORB1	0.644	0.452	0.440	0.432	0.620	0.707	0.371	0.583	0.568	0.646	0.346	0.000	0.088	0.593	0.568	0.583	0.346	0.088	0.609		0.064	0.080	0.117	0.093	0.101	0.095	0.101	0.106	0.110	0.073	0.075
M.sp.IV-E_BOYuhSp	0.588	0.379	0.359	0.160	0.537	0.820	0.000	0.528	0.522	0.585	0.305	0.371	0.444	0.555	0.522	0.515	0.305	0.444	0.576	0.371		0.066	0.144	0.075	0.091	0.090	0.088	0.096	0.090	0.030	0.063
M.sp.V-A_BRAob	0.892	0.000	0.478	0.367	0.832	0.908	0.379	0.652	0.653	0.669	0.538	0.452	0.471	0.851	0.668	0.660	0.538	0.471	0.903	0.452	0.379		0.150	0.098	0.107	0.110	0.109	0.158	0.148	0.063	0.081
N.granulosis_GRU1	0.984	0.908	0.541	0.864	1.002	0.000	0.820	0.980	0.989	0.977	0.501	0.707	0.656	0.926	0.950	1.002	0.501	0.656	0.955	0.707	0.820	0.908		0.161	0.163	0.158	0.169	0.154	0.173	0.152	0.086
D.roeselum_BIE2	0.353	0.581	0.464	0.506	0.322	0.912	0.402	0.048	0.007	0.088	0.388	0.515	0.603	0.264	0.039	0.035	0.388	0.603	0.288	0.515	0.402	0.581	0.912		0.014	0.012	0.012	0.046	0.054	0.093	0.076
D.muelleri_STE1	0.345	0.652	0.537	0.614	0.308	0.980	0.528	0.000	0.038	0.127	0.430	0.583	0.661	0.285	0.027	0.027	0.430	0.661	0.308	0.583	0.528	0.652	0.980	0.048		0.009	0.010	0.047	0.048	0.106	0.085
D.sp.L_BOYohBr	0.362	0.668	0.507	0.643	0.312	0.950	0.522	0.027	0.031	0.115	0.439	0.568	0.655	0.293	0.000	0.027	0.439	0.655	0.316	0.568	0.522	0.668	0.950	0.039	0.027		0.009	0.047	0.051	0.110	0.078
D.dubemum_SAL1	0.338	0.660	0.537	0.600	0.309	1.002	0.515	0.027	0.031	0.132	0.430	0.583	0.661	0.280	0.027	0.000	0.430	0.661	0.315	0.583	0.515	0.660	1.002	0.035	0.027	0.027		0.046	0.049	0.102	0.084
C.sp.02_FAL1	0.117	0.851	0.547	0.589	0.105	0.926	0.555	0.285	0.269	0.325	0.342	0.593	0.628	0.000	0.293	0.280	0.342	0.628	0.036	0.593	0.555	0.851	0.926	0.264	0.285	0.293	0.280		0.021	0.096	0.090
C.sp.01_KIN4	0.037	0.832	0.596	0.579	0.000	1.002	0.537	0.308	0.335	0.361	0.369	0.620	0.656	0.105	0.312	0.309	0.369	0.656	0.122	0.620	0.537	0.832	1.002	0.322	0.308	0.312	0.309	0.105		0.093	0.100
M.sp.IV-F_VORohBo	0.612	0.367	0.503	0.000	0.579	0.864	0.160	0.614	0.628	0.723	0.351	0.432	0.439	0.589	0.643	0.600	0.351	0.439	0.604	0.432	0.160	0.367	0.864	0.506	0.614	0.643	0.600	0.589	0.579		0.088
M.sp.IV-I_KRE1	0.618	0.481	0.000	0.506	0.586	0.544	0.361	0.528	0.528	0.542	0.360	0.443	0.493	0.537	0.499	0.528	0.360	0.493	0.581	0.443	0.361	0.481	0.544	0.456	0.528	0.499	0.528	0.537	0.586	0.506	

Parasites Kimura-2-parameters (K2p) corrected pairwise distances with Standard error estimates (in blue) obtained with bootstrapping (1000 replications). Outgroup in red
Reviewed in: *Bacela-Spychalska et al., 2018; **Bojko et al., 2017

	MN322616_Pontogammarus_robustoides	KT075268_Dikerogammarus_haemobaphe	MT800937_Pallacea_cancellus	MN148355_Linevichella_vortex	MK887783_Gammarus_lacustris	MK887755_Eulimnogammarus_vittatus	MK887733_Eulimnogammarus_cyaneus	MK887577_Eulimnogammarus_verrucosu	MK403736_Gammarus_tigrinus	MG214958_Gmelinoides_fasciatus	MF187975_Gammarus_locusta	KY706685_Niphargus_aquilex	MK160074_Echinogammarus_marinus	KU056447_Gammarus_balcanicus_c3	KU056443_Gammarus_balcanicus_c1	KU056394_Gammarus_balcanicus_c5	KU056390_Gammarus_balcanicus_c2	KU056353_Gammarus_balcanicus_c7	KU056304_Gammarus_balcanicus_c6	KT633989_Niphargus_schellenbergi	KT075266_Dikerogammarus_villosus	KT075263_Echinogammarus_berilloni	KP789691_Gammarus_roeselii_c1e	KF053264_Gammarus_balcanicus_c4	JN393823_Acanthogammarus_victorii	JN393806_Acanthogammarus_lappaceus	JF965987_Gammarus_varsoviensis	HM015165_Crangonyx_islandicus	KP789701_Gammarus_roeselii_c3g	GQ341716_Gammarus_chevreuxi	FJ756321_Dorogostaiskia_parasitica	FJ756302_Brandtia_latissima	EF582876_Gammarus_pseudolimnaeus	EF468643_Gammarus_duebeni	Gammarus_pulex_cC	Gammarus_pulex_cD	Gammarus_pulex_cE	Gammarus_roeselii_c2	Gammarus_fossarum_c2	Gammarus_fossarum_c1
MN322616_Pontogammarus_robustoides		0.039	0.047	0.042	0.044	0.050	0.048	0.045	0.041	0.050	0.060	0.049	0.611	0.051	0.052	0.045	0.052	0.051	0.054	0.049	0.031	0.046	0.054	0.055	0.053	0.057	0.048	0.060	0.053	0.048	0.056	0.063	0.245	0.049	0.046	0.045	0.047	0.054	0.050	0.052
KT075268_Dikerogammarus_haemobaphes	0.284		0.047	0.041	0.044	0.050	0.050	0.049	0.046	0.056	0.053	0.052	0.429	0.047	0.049	0.052	0.052	0.049	0.058	0.047	0.033	0.061	0.045	0.048	0.068	0.055	0.050	0.058	0.049	0.045	0.063	0.053	0.309	0.053	0.045	0.045	0.044	0.048	0.058	0.054
MT800937_Pallacea_cancellus	0.387	0.386		0.046	0.052	0.045	0.045	0.051	0.047	0.049	0.059	0.048	0.983	0.043	0.051	0.048	0.052	0.049	0.059	0.050	0.043	0.056	0.053	0.054	0.072	0.062	0.055	0.055	0.059	0.062	0.053	0.061	0.229	0.044	0.049	0.051	0.047	0.057	0.057	0.056
MN148355_Linevichella_vortex	0.341	0.329	0.370		0.040	0.042	0.040	0.043	0.045	0.036	0.052	0.043	0.601	0.035	0.037	0.035	0.040	0.047	0.038	0.041	0.041	0.050	0.044	0.040	0.047	0.046	0.034	0.043	0.045	0.044	0.048	0.044	0.283	0.049	0.036	0.035	0.037	0.050	0.040	0.040
MK887783_Gammarus_lacustris	0.341	0.329	0.411	0.319		0.040	0.041	0.046	0.048	0.048	0.051	0.047	0.387	0.037	0.039	0.040	0.040	0.046	0.045	0.040	0.049	0.040	0.038	0.045	0.051	0.053	0.038	0.051	0.043	0.047	0.046	0.053	0.265	0.035	0.039	0.038	0.039	0.040	0.043	0.045
MK887755_Eulimnogammarus_vittatus	0.407	0.399	0.343	0.328	0.322		0.034	0.028	0.042	0.045	0.040	0.046	0.501	0.045	0.035	0.037	0.040	0.039	0.041	0.043	0.051	0.051	0.042	0.044	0.042	0.042	0.041	0.051	0.043	0.048	0.045	0.043	0.205	0.037	0.042	0.043	0.040	0.043	0.048	0.044
MK887733_Eulimnogammarus_cyaneus	0.393	0.403	0.344	0.311	0.322	0.238		0.036	0.040	0.045	0.048	0.042	0.640	0.036	0.037	0.040	0.044	0.042	0.041	0.044	0.050	0.046	0.042	0.042	0.047	0.045	0.041	0.047	0.041	0.045	0.039	0.037	0.264	0.041	0.045	0.047	0.040	0.043	0.051	0.048
MK887577_Eulimnogammarus_verrucosus	0.345	0.379	0.386	0.338	0.357	0.206	0.252		0.039	0.052	0.044	0.040	0.659	0.046	0.039	0.038	0.047	0.046	0.046	0.049	0.045	0.055	0.048	0.041	0.045	0.041	0.044	0.048	0.049	0.046	0.042	0.043	0.230	0.040	0.045	0.046	0.047	0.051	0.048	0.048
MK403736_Gammarus_tigrinus	0.327	0.353	0.369	0.339	0.365	0.344	0.310	0.311		0.048	0.042	0.044	0.653	0.038	0.041	0.042	0.049	0.046	0.042	0.045	0.044	0.045	0.041	0.045	0.049	0.054	0.039	0.044	0.042	0.044	0.061	0.051	0.213	0.048	0.042	0.041	0.034	0.044	0.051	0.051
MG214958_Gmelinoides_fasciatus	0.396	0.446	0.397	0.275	0.401	0.360	0.355	0.411	0.391		0.066	0.045	0.821	0.048	0.045	0.041	0.047	0.047	0.050	0.049	0.055	0.049	0.050	0.042	0.057	0.048	0.046	0.061	0.057	0.053	0.053	0.053	0.320	0.050	0.053	0.048	0.051	0.062	0.051	0.051
MF187975_Gammarus_locusta	0.470	0.420	0.470	0.422	0.410	0.338	0.393	0.350	0.335	0.524		0.048	0.560	0.046	0.046	0.043	0.052	0.049	0.047	0.049	0.050	0.045	0.044	0.046	0.052	0.055	0.044	0.043	0.045	0.040	0.048	0.051	0.392	0.040	0.042	0.047	0.047	0.048	0.055	0.053
KY706685_Niphargus_aquilex	0.389	0.390	0.385	0.335	0.351	0.371	0.343	0.313	0.332	0.370	0.393		0.615	0.042	0.042	0.035	0.043	0.043	0.048	0.036	0.049	0.047	0.046	0.048	0.054	0.051	0.043	0.047	0.047	0.046	0.046	0.044	0.220	0.038	0.045	0.047	0.040	0.046	0.047	0.046
MK160074_Echinogammarus_marinus	2.372	2.044	2.767	2.360	1.870	2.146	2.399	2.356	2.266	2.718	2.204	2.385		0.745	1.047	0.761	0.515	0.872	0.670	0.469	0.872	0.427	0.786	0.522	0.868	0.813	0.550	0.593	0.504	0.449	0.532	1.104	10.39	0.475	0.522	0.532	0.465	0.697	0.851	1.048
KU056447_Gammarus_balcanicus_c3	0.390	0.355	0.342	0.267	0.283	0.332	0.265	0.335	0.293	0.375	0.357	0.328	2.494		0.031	0.030	0.030	0.033	0.032	0.042	0.047	0.047	0.043	0.036	0.053	0.052	0.040	0.040	0.040	0.041	0.048	0.047	0.304	0.041	0.043	0.041	0.043	0.044	0.046	0.044
KU056443_Gammarus_balcanicus_c1	0.396	0.370	0.390	0.298	0.298	0.274	0.286	0.291	0.317	0.380	0.358	0.322	2.531	0.217		0.037	0.032	0.038	0.027	0.043	0.050	0.047	0.042	0.032	0.049	0.046	0.044	0.043	0.045	0.039	0.044	0.046	0.339	0.044	0.038	0.039	0.039	0.045	0.052	0.049
KU056394_Gammarus_balcanicus_c5	0.355	0.401	0.388	0.283	0.315	0.286	0.307	0.302	0.339	0.343	0.352	0.274	2.568	0.216	0.263		0.035	0.034	0.032	0.042	0.045	0.044	0.042	0.036	0.054	0.045	0.039	0.044	0.045	0.044	0.039	0.048	0.264	0.038	0.038	0.038	0.036	0.044	0.046	0.045
KU056390_Gammarus_balcanicus_c2	0.400	0.387	0.413	0.311	0.305	0.314	0.330	0.347	0.381	0.388	0.411	0.338	2.156	0.209	0.228	0.260		0.038	0.035	0.042	0.051	0.042	0.043	0.036	0.047	0.042	0.042	0.053	0.049	0.050	0.043	0.046	0.286	0.037	0.043	0.042	0.044	0.049	0.051	0.048
KU056353_Gammarus_balcanicus_c7	0.382	0.381	0.387	0.367	0.362	0.314	0.325	0.349	0.360	0.377	0.376	0.343	2.635	0.235	0.267	0.252	0.288		0.042	0.042	0.049	0.056	0.045	0.041	0.057	0.054	0.043	0.043	0.048	0.053	0.047	0.042	0.227	0.039	0.037	0.039	0.045	0.042	0.040	0.040
KU056304_Gammarus_balcanicus_c6	0.433	0.430	0.458	0.309	0.353	0.314	0.310	0.344	0.318	0.398	0.373	0.384	2.381	0.228	0.198	0.243	0.260	0.316		0.042	0.053	0.044	0.049	0.033	0.047	0.043	0.043	0.045	0.049	0.048	0.038	0.048	0.355	0.039	0.043	0.040	0.040	0.045	0.042	0.042
KT633989_Niphargus_schellenbergi	0.378	0.364	0.420	0.327	0.301	0.352	0.357	0.391	0.353	0.384	0.404	0.279	2.063	0.346	0.341	0.330	0.329	0.340	0.327		0.042	0.044	0.042	0.045	0.055	0.049	0.045	0.047	0.045	0.047	0.045	0.051	0.306	0.043	0.036	0.037	0.043	0.046	0.048	0.050
KT075266_Dikerogammarus_villosus	0.224	0.232	0.358	0.326	0.383	0.412	0.403	0.341	0.358	0.444	0.399	0.377	2.736	0.354	0.387	0.351	0.385	0.367	0.413	0.335		0.055	0.052	0.049	0.061	0.050	0.052	0.053	0.054	0.049	0.064	0.068	0.281	0.046	0.046	0.047	0.046	0.055	0.054	0.054
KT075263_Echinogammarus_berilloni	0.360	0.472	0.439	0.391	0.299	0.416	0.376	0.429	0.351	0.391	0.352	0.380	1.981	0.368	0.373	0.354	0.351	0.452	0.338	0.348	0.446		0.049	0.046	0.055	0.049	0.039	0.052	0.057	0.038	0.045	0.054	0.308	0.037	0.049	0.050	0.046	0.055	0.050	0.052
KP789691_Gammarus_roeselii_c1e	0.445	0.363	0.419	0.358	0.295	0.329	0.337	0.396	0.318	0.402	0.352	0.350	2.549	0.330	0.335	0.332	0.354	0.360	0.383	0.333	0.406	0.384		0.042	0.050	0.044	0.037	0.050	0.016	0.045	0.051	0.045	0.226	0.040	0.045	0.051	0.043	0.018	0.050	0.045
KF053264_Gammarus_balcanicus_c4	0.433	0.356	0.422	0.305	0.346	0.338	0.320	0.330	0.352	0.333	0.363	0.373	2.109	0.261	0.246	0.274	0.273	0.319	0.241	0.348	0.396	0.364	0.329		0.052	0.044	0.043	0.045	0.044	0.042	0.047	0.051	0.486	0.044	0.040	0.042	0.042	0.052	0.054	0.052
JN393823_Acanthogammarus_victorii	0.439	0.504	0.558	0.370	0.422	0.330	0.349	0.345	0.381	0.455	0.406	0.424	2.540	0.420	0.395	0.395	0.383	0.449	0.359	0.428	0.458	0.458	0.402	0.403		0.035	0.054	0.050	0.052	0.043	0.043	0.046	0.522	0.051	0.050	0.047	0.050	0.057	0.048	0.044
JN393806_Acanthogammarus_lappaceus	0.448	0.415	0.481	0.358	0.414	0.336	0.342	0.307	0.428	0.394	0.434	0.422	2.625	0.407	0.365	0.356	0.335	0.426	0.331	0.394	0.396	0.412	0.362	0.349	0.265		0.045	0.053	0.050	0.046	0.042	0.046	0.265	0.042	0.048	0.047	0.049	0.053	0.049	0.048
JF965987_Gammarus_varsoviensis	0.383	0.384	0.417	0.265	0.280	0.322	0.322	0.356	0.303	0.354	0.362	0.327	2.234	0.309	0.341	0.309	0.334	0.343	0.338	0.360	0.410	0.297	0.285	0.334	0.428	0.359		0.046	0.038	0.042	0.044	0.043	0.264	0.040	0.038	0.039	0.040	0.040	0.041	0.042
HM015165_Crangonyx_islandicus	0.478	0.445	0.423	0.346	0.384	0.419	0.375	0.377	0.334	0.501	0.343	0.373	2.339	0.315	0.344	0.367	0.416	0.356	0.361	0.382	0.440	0.405	0.396	0.373	0.417	0.428	0.362		0.049	0.044	0.049	0.049	0.299	0.045	0.042	0.048	0.048	0.053	0.050	0.050
KP789701_Gammarus_roeselii_c3g	0.446	0.400	0.471	0.358	0.337	0.345	0.328	0.414	0.334	0.460	0.365	0.367	2.146	0.311	0.364	0.356	0.394	0.379	0.383	0.380	0.428	0.448	0.090	0.342	0.417	0.406	0.291	0.392		0.043	0.054	0.052	0.275	0.043	0.046	0.049	0.044	0.022	0.051	0.047
GQ341716_Gammarus_chevreuxi	0.376	0.364	0.471	0.343	0.356	0.375	0.363	0.353	0.339	0.415	0.313	0.345	2.039	0.312	0.295	0.336	0.383	0.384	0.366	0.370	0.380	0.304	0.351	0.342	0.351	0.361	0.312	0.355	0.349		0.055	0.046	0.513	0.047	0.043	0.043	0.046	0.051	0.052	0.055
FJ756321_Dorogostaiskia_parasitica	0.446	0.495	0.418	0.388	0.364	0.342	0.298	0.311	0.468	0.442	0.380	0.364	2.245	0.379	0.332	0.298	0.348	0.367	0.311	0.369	0.489	0.361	0.412	0.365	0.338	0.320	0.339	0.407	0.441	0.426		0.030	0.358	0.045	0.041	0.042	0.042	0.051	0.052	0.055
FJ756302_Brandtia_latissima	0.489	0.408	0.457	0.348	0.411	0.325	0.277	0.321	0.392	0.421	0.410	0.340	3.024	0.349	0.351	0.358	0.345	0.327	0.363	0.408	0.497	0.422	0.356	0.373	0.353	0.346	0.336	0.404	0.411	0.361	0.223		0.512	0.046	0.044	0.043	0.050	0.048	0.054	0.052
EF582876_Gammarus_pseudolimnaeus	1.444	1.671	1.446	1.584	1.514	1.314	1.549	1.396	1.345	1.705	1.889	1.396	15.29	1.613	1.713	1.496	1.585	1.427	1.768	1.662	1.592	1.656	1.428	2.138	2.094	1.541	1.516	1.662	1.565	2.106	1.796	2.150		0.210	0.299	0.338	0.298	0.192	0.253	0.224
EF468643_Gammarus_duebeni	0.395	0.407	0.342	0.378	0.288	0.309	0.312	0.331	0.370	0.417	0.330	0.307	2.137	0.322	0.331	0.295	0.299	0.322	0.313	0.344	0.368	0.298	0.332	0.344	0.401	0.330	0.311	0.358	0.356	0.375	0.348	0.361	1.368		0.043	0.042	0.041	0.040	0.042	0.041
Gammarus_pulex_cC	0.354	0.356	0.394	0.279	0.299	0.327	0.352	0.335	0.317	0.406	0.329	0.339	2.156	0.338	0.293	0.291	0.329	0.279	0.345	0.279	0.353	0.380	0.338	0.318	0.392	0.367	0.289	0.339	0.351	0.323	0.313	0.334	1.599	0.339		0.010	0.020	0.044	0.046	0.046
Gammarus_pulex_cD	0.343	0.346	0.403	0.270	0.290	0.335	0.357	0.348	0.308	0.378	0.373	0.358	2.165	0.317	0.297	0.289	0.322	0.293	0.319	0.285	0.358	0.389	0.378	0.327	0.367	0.360	0.302	0.383	0.375	0.323	0.326	0.330	1.700	0.330	0.037		0.020	0.046	0.042	0.044
Gammarus_pulex_cE	0.364	0.342	0.370	0.287	0.294	0.315	0.300	0.354	0.251	0.394	0.373	0.302	2.081	0.319	0.299	0.281	0.326	0.338	0.314	0.332	0.357	0.362	0.337	0.320	0.394	0.389	0.310	0.370	0.345	0.346	0.322	0.369	1.599	0.318	0.122	0.124		0.045	0.046	0.047
Gammarus_roeselii_c2	0.433	0.393	0.464	0.405	0.316	0.344	0.345	0.414	0.333	0.491	0.394	0.358	2.478	0.343	0.361	0.344	0.394	0.335	0.359	0.366	0.427	0.432	0.108	0.385	0.447	0.420	0.315	0.416	0.139	0.406	0.416	0.368	1.252	0.338	0.336	0.353	0.347		0.051	0.047
Gammarus_fossarum_c2	0.370	0.420	0.448	0.301	0.313	0.365	0.392	0.358	0.387	0.403	0.435	0.363	2.746	0.358	0.391	0.349	0.384	0.314	0.333	0.373	0.400	0.398	0.381	0.392	0.376	0.391	0.305	0.415	0.394	0.379	0.407	0.405	1.504	0.337	0.341	0.319	0.342	0.394		0.008
Gammarus_fossarum_c1	0.387	0.399	0.436	0.293	0.321	0.334	0.376	0.360	0.396	0.408	0.414	0.355	2.996	0.345	0.377	0.346	0.372	0.320	0.333	0.383	0.400	0.409	0.350	0.384	0.350	0.380	0.315	0.416	0.371	0.396	0.431	0.399	1.396	0.332	0.346	0.334	0.348	0.373	0.028	

Hosts Kimura-2-parameters (K2p) corrected pairwise distances with Standard error estimates (in blue) obtained with bootstrapping (1000 replications). Outgroup in red.

Dataset S1 Parasites and hosts sequences used in the current study.

Location code	**Coordinates (lat.; long.)**	**Country**	**Microsporidia name**	**Microsporidia sequence**	**Genetic coverage**	**Genetic similarity**	**Host name**	**Host sequence**
BOYohBR	51.569135;6.927698	Germany	Dictyocoela sp. L				*G. pulex* clade E	
			Microsporidium sp. 505				*G. pulex* clade E	
			Microsporidium sp. IV-F	KR871373	100%	100%	*G. pulex* clade E	KT075231
			Microsporidium sp. IV-B	KR871371	100%	100%	*G. pulex* clade E	KT075231
BOYohKI	51.553107;6.948059	Germany	Microsporidium sp. 505				*G. pulex* clade C	
			Microsporidium sp. 505				*G. pulex* clade E	
BOYohSP	51.564297;6.930277	Germany	*Dictyocoela dubenum*				*G. pulex* clade E	
			Dictyocoela sp. L				*G. pulex* clade E	
BOYuhHA	51.534515;6.99774	Germany	Microsporidium sp. 505				*G. fossarum* clade 2	
BOYuhSP	51.561336;6.932617	Germany	*Dictyocoela dubenum*				*G. fossarum* clade 2	
			Microsporidium sp. 515				*G. fossarum* clade 2	
			Microsporidium sp. IV-E				*G. fossarum* clade 2	
			Microsporidium sp. IV-E				*G. pulex* clade E	
BRAob	51.588333;6.944944	Germany	*Dictyocoela dubenum*				*G. fossarum* clade 2	
			Microsporidium sp. IV-F	KR871373	100%	100%	G. fossarum clade 2	KT075259
			Microsporidium sp. V-A				*G. fossarum* clade 2	
			Dictyocoela dubenum	KR871355	100%	100%	*G. fossarum* clade 2	KT075259
HAAob	51.570306;6.960891	Germany	Microsporidium sp. 505				*G. pulex* clade E	
			Microsporidium sp. IV-F				G. pulex clade C	
HAAun	51.562638; 6.955581	Germany	Microsporidium sp. 505				*G. pulex* clade E	
KIRob	51.542223;6.939196	Germany	Microsporidia sp. IV-B				G. pulex clade C	
KIRun	51.547455;6.943704	Germany	Microsporidium sp. IV-B				G. pulex clade C	
			Microsporidium sp. IV-I				G. pulex clade C	
SCHohVO	51.53942;6.908287	Germany	Microsporidium sp. IV-B				G. pulex clade C	
			Microsporidium sp. IV-F				G. pulex clade C	
VORohBOY	51.554753;6.932435	Germany	Dictyocoela sp. L				*G. pulex* clade C	
			Microsporidium sp. 515				*G. pulex* clade C	
			Microsporidium sp. IV-B				G. pulex clade C	
			Microsporidium sp. IV-F				G. pulex clade C	
VORuhSc	51.544809;6.921403	Germany	Microsporidium sp. IV-B				G. pulex clade C	
VORuhSc			Microsporidium sp. IV-B	KR871371	100%	100%	G. pulex clade C	KT075251

VORuhSc			Microsporidium sp. 505	KR871376	100%	100%	*G. pulex* clade C	KT075251
BIE1	50.163394; 9.294485	Germany	Microsporidium sp. 515				*G. pulex* clade D	
BIE2	50.174435; 9.284504	Germany	Microsporidium sp. 515				*G. pulex* clade D	
			Dictyocoela roeselum				*G. pulex* clade D	
FAL1	50.19626231; 9.01949369	Germany	*Cucumispora sp. 02*				*G. fossarum* clade 2	
			Cucumispora sp. 02				*G. roeselii* clade 2	
			Dictyocoela dubenum				*G. fossarum* clade 1	
			Dictyocoela roeselum				*G. fossarum* clade 2	
			Dictyocoela roeselum				*G. roeselii* clade 2	
FAL2	50.16080729; 8.96847607	Germany	*Cucumispora sp. 02*				*G. roeselii* clade 2	
			Dictyocoela dubenum				*G. fossarum* clade 2	
			Microsporidium sp. IV-F				G. fossarum clade 2	
			Dictyocoela roeselum				*G. fossarum* clade 2	
			Dictyocoela roeselum				*G. roeselii* clade 2	
GRU1	50.259856; 9.174480	Germany	*Dictyocoela dubenum*				*G. fossarum* clade 2	
			Microsporidium sp. 505				*G. fossarum* clade 2	
			Nosema granulosis				*G. fossarum* clade 2	
GRU2	50.240705; 9.144059	Germany	*Dictyocoela dubenum*				*G. fossarum* clade 1	
			Nosema granulosis				*G. fossarum* clade 2	
			Nosema granulosis				*G. roeselii* clade 2	
			Dictyocoela roeselum				*G. roeselii* clade 2	
KIN1	50.342661; 9.580566	Germany	*Dictyocoela dubenum*				*G. fossarum* clade 2	
			Microsporidium sp. 505				*G. fossarum* clade 2	
			Microsporidium sp. IV-F				G. fossarum clade 2	
			Nosema granulosis				*G. roeselii* clade 2	
KIN2	50.3463103; 9.55248653	Germany	*Dictyocoela dubenum*				*G. fossarum* clade 2	
			Microsporidium sp. 515				*G. fossarum* clade 1	
			Nosema granulosis				*G. fossarum* clade 1	
KIN3	50.34463942; 9.52531583	Germany	Microsporidium sp. 515				*G. fossarum* clade 2	
			Microsporidium sp. IV-I				G. roeselii clade 2	
KIN4	50.32512937; 9.49640061	Germany	*Cucumispora sp. 01*				*G. roeselii* clade 2	
			Microsporidium sp. 515				*G. fossarum* clade 2	
			Microsporidium sp. IV-F				G. roeselii clade 2	

KRE1	50.180280; 8.918023	Germany	Microsporidium sp. 515				*G. pulex* clade E	
			Microsporidium sp. IV-I				G. roeselii clade 2	
KRE2	50.151738; 8.901119	Germany	*Dictyocoela roeselum*				*G. roeselii* clade 2	
ORB1	50.231196; 9.342359	Germany	Microsporidium sp. 515				*G. fossarum* clade 2	
			Microsporidium sp. 515				*C. pulex* clade D	
ORB2	50.236741; 9.324385	Germany	Microsporidium sp. 505				*C. pulex* clade D	
			Microsporidium sp. 515				*C. pulex* clade D	
			Microsporidium sp. IV-B				G. pulex clade D	
SAL1	50.33361787; 9.38116571	Germany	*Dictyocoela dubenum*				*G. fossarum* clade 1	
			Dictyocoela dubenum				*G. fossarum* clade 2	
			Microsporidium sp. 515				*G. fossarum* clade 2	
SAL2	50.3143, 9.36688	Germany	*Dictyocoela dubenum*				*G. fossarum* clade 2	
			Dictyocoela dubenum				*G. roeselii* clade 2	
			Nosema granulosis				*G. fossarum* clade 2	
STE1	50.33225282; 9.46650921	Germany	*Dictyocoela dubenum*				*G. fossarum* clade 2	
			Dictyocoela muelleri				*G. roeselii* clade 2	
			Nosema granulosis				*G. fossarum* clade 2	
			Nosema granulosis				*G. pulex* clade D	
			Nosema granulosis				*G. roeselii* clade 2	
STE2	50.31551791; 9.45663466	Germany	*Dictyocoela dubenum*				*G. fossarum* clade 2	
			Microsporidium sp. 505				*G. fossarum* clade 2	
AL01	41.678;19.853	Albania	*Dictyocoela dubenum*	MT932352	100%	99.43%	*G. balcanicus* clade 4	KF053264
AL02	41.584;20.032	Albania	*Dictyocoela dubenum*	MT932357	100%	99.72%	*G. balcanicus* clade 4	KF053264
AL03	41.074583;20.487933	Albania	*Nosema granulosis*	MK719238	97%	100%	*G. roeselii* clade 1 E	KP789691
			Nosema granulosis	MK719238	97%	100%	G. roeselii clade 3 G	KP789701
AT01	47.89154;13.31564	Austria	*Cucumispora sp. 02*	MK719284	100%	100%	*G. roeselii* clade 2	MT325259
AT02	48.028974;15.433093	Austria	*Nosema granulosis*	MK719277	97%	100%	*G. roeselii* clade 2	MT325399
BE01	50.4121 5.9535	Belgium	*Dictyocoela dubenum*	MN959957	100%	99.15%	Niphagus aquilex	KY706685
BE02	50.0706 5.0979	Belgium	*Dictyocoela dubenum*	MN959955	100%	99.15%	Niphagus shellenbergi	KT633989
BG01	43.184; 23.497	Bulgaria	*Dictyocoela dubenum*	MT932333	95%	99.70%	G. balcanicus clade 7	KU056254
BY01		Belarus	*Dictyocoela muelleri*	MG773238	100%	100.00%	G. varsoviensis	JF965987
DE01	51.6643410; 6.9762790	Germany	*Cucumispora sp. 02*	KR871368	100%	100%	*G. pulex* clade E	KT075230
			Dictyocoela dubenum	KR871355	100%	100%	*G. pulex* clade E	KT075231

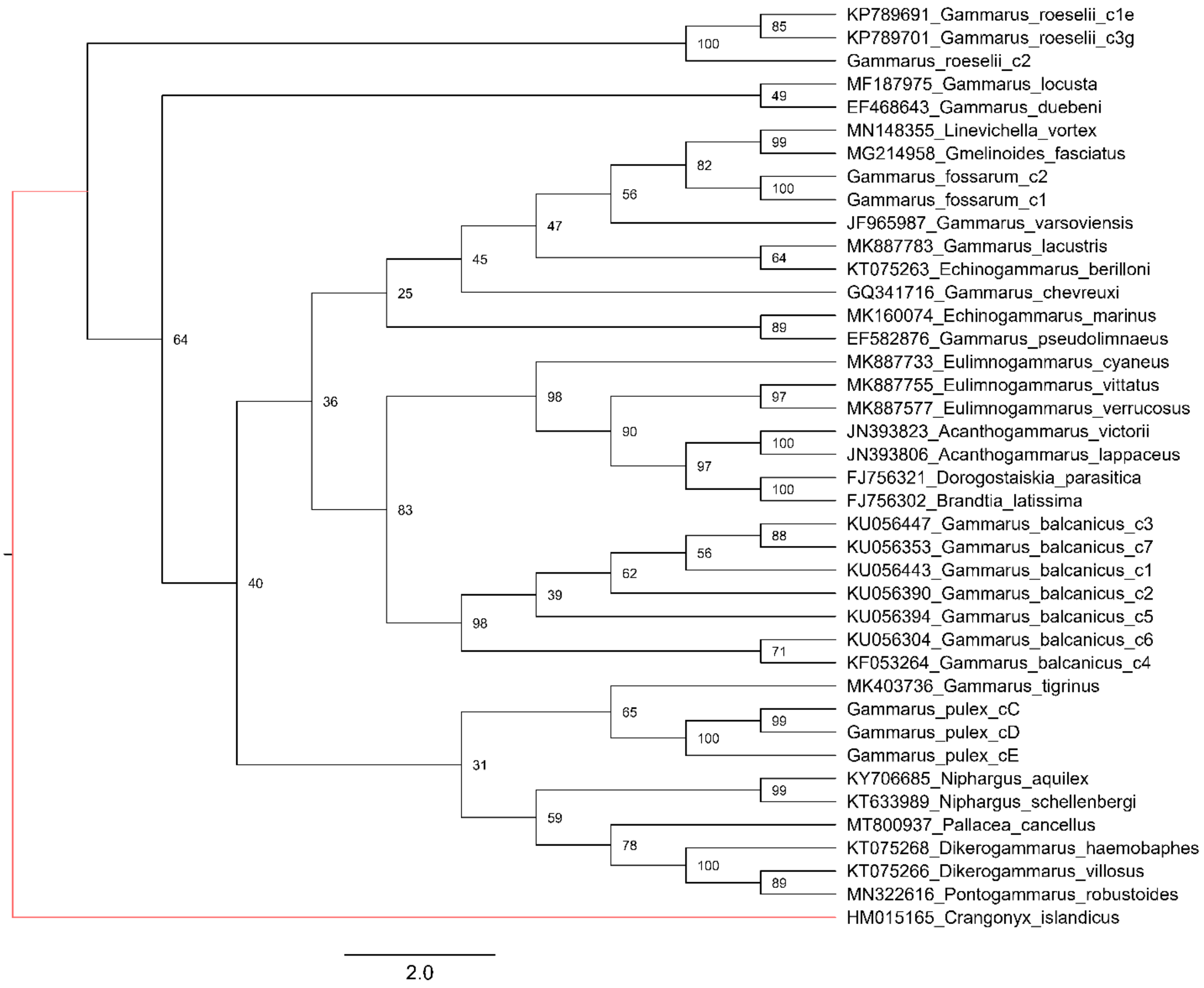
			Dictyocoela dubenum	KR871356	100%	100%	G. pulex clade E	KT075231
			Microsporidium sp. 505	KR871379	100%	98.55%	*G. roeselii* clade 2	KT075225
			Microsporidium sp. V-A	KR871372	100%	100%	G. roeselii clade 2	KT075225
DE02	51.4229100; 7.2679480	Germany	*Dictyocoela dubenum*	KR871355	100%	100%	*G. roeselii* clade 2	KT075225
			Dictyocoela dubenum	KR871356	100%	100%	*G. roeselii* clade 2	KT075225
			Dictyocoela sp. L	KR871361	100%	100%	*G. pulex* clade C	KT075246
			Dictyocoela sp. L	KR871361	100%	100%	*G. roeselii* clade 2	KT075225
			Dictyocoela sp. L	KR871362	100%	99%	D. haemobaphes	KT075268
DE03	51.2815; 7.4127	Germany	*Dictyocoela dubenum*	MN959951	100%	99.15%	*N. schellenbergi*	KY706852
DE04	52.78102;8.45392	Germany	*Dictyocoela roeselum*	MK719358	100%	99.36%	*G. roeselii* clade 2	MT325333
DE05	51.5639119; 6.9117179	Germany	Microsporidium sp. 505	KR871376	100%	100%	*G. pulex* clade C	KT075256
			Microsporidium sp. 505	KR871377	100%	100%	*G. pulex* clade E	KT075231
			Microsporidium sp. 515	KR871354	100%	100%	*G. pulex* clade E	KT075231
			Microsporidium sp. V-A	KR871372	100%	100%	G. pulex clade C	KT075256
			Microsporidium sp. V-A	KR871372	100%	100%	G. pulex clade E	KT075231
DE06	51.5983583; 6.9386889	Germany	Microsporidium sp. 505	KR871376	100%	100%	*G. pulex* clade C	KT075236
			Dictyocoela dubenum	KR871356	100%	100%	G. pulex clade E	KT075231
DE07	51.4867500; 7.5230420	Germany	Microsporidium sp. 515	KR871354	100%	100%	*G. pulex* clade C	KT075255
DE08	51.5812470; 7.3192820	Germany	Microsporidium sp. IV-F	KR871373	100%	100%	*G. pulex* clade C	KT075249
			Dictyocoela dubenum	KR871355	100%	100%	*G. fossarum* clade 2	KT075259
DE09	51.5856151; 6.9540089	Germany	Microsporidium sp. V-A	KR871372	100%	100%	G. pulex clade C	KT075256
			Microsporidium sp. 505	KR871376	100%	100%	*G. pulex* clade C	KT075256
DE10	47.95388;12.74870	Germany	*Nosema granulosis*	MK719285	97%	100%	*G. roeselii* clade 2	MT325310
DE11	47.88381;12.41757	Germany	*Nosema granulosis*	MK719287	97%	100%	*G. roeselii* clade 2	MT325282
DE12	47.65674;11.35559	Germany	*Nosema granulosis*	MK719293	97%	100%	*G. roeselii* clade 2	MT325308
			Dictyocoela muelleri	MK719301	100%	100.00%	*G. roeselii* clade 2	MT325308
DE13	48.07664;11.13394	Germany	*Nosema granulosis*	MK719304	97%	100%	*G. roeselii* clade 2	MT325298
DE14	51°21022.6″N; 7°10015.5″E	Germany	*Cucumispora sp. 01*	KX137940	100%	100%	*G. roeselii* clade 2	KT075225
			Microsporidium sp. IV-B	KX137941	100%	100%	*G. roeselii* clade 2	KT075225
			Microsporidium sp. 515	KX137939	100%	100%	*G. roeselii* clade 2	KT075225
			Microsporidium sp. 515	KX137938	100%	100%	*G. fossarum* clade 2	KT075259
			Microsporidium sp. 505	KX137937	100%	100%	*G. fossarum* clade 2	KT075259
DE15	49.9399; 10.5631	Germany	*Dictyocoela dubenum*	MN959956	100%	99.15%	*Niphagus shellenbergi*	KY706852

DE16	50.6774; 8.6460	Germany	*Dictyocoela dubenum*	MN959958	100%	99.15%	*Niphagus aquilex*	KY706685
DE17	51.4208960; 7.2966850	Germany	*Dictyocoela dubenum*	KR871357	100%	98.58%	*Echinogammarus berilloni*	KT075263
DE18	52.433415; 9.489048	Germany	*Dictyocoela muelleri*	MK719365	100%	100.00%	*G. roeselii* clade 2	MT325233
DE19	48.00710; 10.87965	Germany	*Dictyocoela muelleri*	MK719303	100%	100.00%	*G. roeselii* clade 2	MT325356
DE20	48.97343;11.35182	Germany	*Dictyocoela muelleri*	MK719308	100%	100.00%	*G. roeselii* clade 2	MT325349
FR01	47.814312;7.546001	France	*Cucumispora sp. 01*	MK719418	98%	100%	*G. roeselii* clade 2	MT325277
			Dictyocoela roeselum	MK719420	100%	99.36%	*G. roeselii* clade 2	MT325277
			Microsporidium sp. IV-B	MK719421	100%	99.68%	G. roeselii clade 2	MT325277
			Dictyocoela roeselum	MG773219	100%	99.68%	G. roeselii clade 2	MT325277
			Dictyocoela muelleri	MG773235	100%	98.78%	G. roeselii clade 2	MT325277
FR02	47.699722;2.542222	France	*Dictyocoela muelleri*	MK719433	100%	100%	*G. roeselii* clade 2	MT325236
FR03	47.148305;5.2774815	France	*Dictyocoela muelleri*	AJ438956	100%	100%	*G. roeselii* clade 2	MG779536
			Dictyocoela roeselum	MK719541	100%	99.36%	*G. roeselii* clade 2	MG779536
			Nosema granulosis	MK719479	97%	100%	*G. roeselii* clade 2	MG779536
FR04		France	*Dictyocoela roeselum*	EF095542	100%	99.68%	*D. villosus*	KT075266
FR05	48.5755556;6.10777778	France	*Dictyocoela roeselum*	MK719397	100%	99.36%	*G. roeselii* clade 2	MT325330
			Dictyocoela muelleri	MK719396	100%	100%	*G. roeselii* clade 2	MT325330
FR06	47.5272;3.5408	France	*Dictyocoela roeselum*	MK719431	100%	99.36%	*G. roeselii* clade 2	MT325357
			Dictyocoela muelleri	MK719430	100%	100%	*G. roeselii* clade 2	MT325357
FR07	46.1466667;4.09722222	France	*Dictyocoela roeselum*	MK719445	100%	99.36%	*G. roeselii* clade 2	MT325352
FR08	49.89183;3.17069	France	*Nosema granulosis*	MK719375	97%	100%	*G. roeselii* clade 2	MT325316
FR09	49.92377;2.22939	France	*Nosema granulosis*	MK719383	97%	100%	*G. roeselii* clade 2	MT325297
			Dictyocoela muelleri	MK719385	100%	100%	*G. roeselii* clade 2	MT325297
FR10	49.072827; 6.326767	France	Microsporidium sp. IV-F	MK719386	100%	100%	*G. roeselii* clade 2	MT325297
			Dictyocoela muelleri	MK719392	100%	100%	*G. roeselii* clade 2	MT325297
FR11	48.5394306; 6.13936667	France	*Dictyocoela muelleri*	MK719430	100%	100%	*G. roeselii* clade 2	MT325297
FR12			*Nosema granulosis*	EF091822	100%	99.68%	D. villosus	KT075266
GR01	41.282; 23.332	Greece	*Dictyocoela dubenum*	MT932356	100%	99.72%	*Gammarus balcanicus* clade 7	KU056307
GR02	40.228;20.971	Greece	*Dictyocoela dubenum*	MT932344	100%	99.43%	Gammarus balcanicus clade 6	KU056304
		Greece	*Nosema granulosis*	MK719374	100%	99.68%	Gammarus roeselii C 1 E	KP789691
HR01	43.977; 16.435	Croatia	*Dictyocoela muelleri*	MT932409	100%	98.78%	*Gammarus balcanicus C 7*	KU056346
HR02	43.451; 17.182	Croatia	*Dictyocoela muelleri*	MT932398	100%	98.78%	*Gammarus balcanicus C 7*	KU056353
HR03	45.149;14.560	Croatia	*Dictyocoela dubenum*	MT932354	100%	99.43%	*Gammarus balcanicus* clade 7	KU056373

HU01	47.827417;19.644917	Hungary	*Dictyocoela roeselum*	MK719251	100%	99.36%	*G. roeselii* clade 2	MT325362
HU02	47.29465;18.0536	Hungary	*Dictyocoela roeselum*	MK719263	100%	99.36%	*G. roeselii* clade 2	MT325353
			Nosema granulosis	MK719261	97%	100%	*G. roeselii* clade 2	MT325353
HU03	46.677617;16.565203	Hungary	*Dictyocoela roeselum*	MK719274	100%	99.36%	*G. roeselii* clade 2	MT325360
HU04	47.9952;19.865	Hungary	*Nosema granulosis*	MK719260	100%	100%	*G. roeselii* clade 2	MT325315
HU06	47.936;18.832	Hungary	*Dictyocoela dubenum*	MT932353	100%	99.43%	*Gammarus balcanicus* clade 3	KU056310
IE01	53.23; 6.45	Ireland	Microsporidium sp. 505	FN434085	100%	99.64%	*Gammarus duebeni*	EF468643
IE02	53.27000;9.19120	Ireland	Microsporidium sp. 515	FN434086	100%	100%	*Gammarus duebeni*	EF468643
IE02		Ireland	*Nosema granulosis*	FN434087	100%	99.68%	*Gammarus duebeni*	EF468643
IS01	64.12; 22.83	Iceland	*Dictyocoela dubenum*	FN434091	100%	98.29%	*Gammarus duebeni*	EF468643
IT01	45.6557;12.2195	Italy	*Nosema granulosis*	MK719289	97%	100%	*G. roeselii* clade 2	MT325346
LU01	50.1565805; 6.0283959	Louxemburg	Microsporidium sp. IV-B	KT633992	97%	100%	*Niphargus schellenbergi*	KT633989
LU02	50.0734; 6.0467	Louxemburg	*Dictyocoela dubenum*	MN959959	100%	99.15%	*Niphargus schellenbergi*	KT633989
PL01	52.335833;14.833567	Poland	*Cucumispora sp. 01*	MK719333	100%	100%	*G. roeselii* clade 2	MT325184
			Dictyocoela roeselum	MK719329	100%	99.36%	*G. roeselii* clade 2	MT325244
PL04	52.67564;14.57197	Poland	*Dictyocoela roeselum*	MK719341	100%	99.36%	*G. roeselii* clade 2	MT325299
			Dictyocoela muelleri	MK719344	100%	100%	G. roeselii clade 2	MT325299
PL05	51.042117;17.158933	Poland	*Nosema granulosis*	MK719309	97%	100%	*G. roeselii* clade 2	MT325340
PL06	51.278956;16.364215	Poland	*Nosema granulosis*	MK719320	97%	100%	*G. roeselii* clade 2	MT325142
PL07	53.537015;18.796849	Poland	*Cucumispora sp. 01*	MK719326	98%	100%	*G. roeselii* clade 2	MT325142
			Dictyocoela muelleri	MK719327	100%	100%	*G. roeselii* clade 2	MT325142
PL08	53.225867;14.497300	Poland	*Dictyocoela muelleri*	MK719352	100%	100%	*G. roeselii* clade 2	MT325142
PL09	52.458668; 21.030227	Poland	Dictyocoela sp. L	MG773243	100%	99%	Pontogammarus robustoides	MN322616
RO01	44.942533;21.661000	Romania	Microsporidium sp. IV-E	MK719247	100%	100%	*G. roeselii* clade 2	KP789693
RO02	47.752;26.201	Romania	Microsporidium sp. IV-I	MT949300	100%	99.68%	*G. balcanicus* clade 3	KU056402
			Dictyocoela dubenum	MT932348	100%	99.43%	G. balcanicus clade 3	KU056402
RO04	46.135;24.521	Romania	Microsporidium sp. IV-F	MT949298	96%	100%	Gammarus balcanicus clade 5	KU056394
			Dictyocoela muelleri	MT932392	100%	99.69%	*Gammarus balcanicus* clade 5	KU056394
			Dictyocoela dubenum	MT932358	100%	99.72%	*Gammarus balcanicus* clade 5	KU056394
RO05	47.626;24.768	Romania	*Dictyocoela dubenum*	MT932355	100%	99.72%	*Gammarus balcanicus* clade 2	KU056390
RO06	45.751; 22.888	Romania	*Dictyocoela muelleri*	MT932391	100%	99.08%	*Gammarus balcanicus* clade 3	KU056440
RO07	46.950; 22.289		Microsporidium sp. 515	MT949289	100%	98.16%	*Gammarus balcanicus* clade 3	KU056332
RO08	47.059;22.042	Romania	Microsporidium sp. 505	MT949301	98%	99.27%	*Gammarus balcanicus* clade 3	KU056335

RU01	53.500000, 108.000000	Russia	*Dictyocoela dubenum*	MH753357	100%	99.43%	*G. lacustris*	MK887783
			Dictyocoela dubenum	FJ756212	100%	99.43%	*E. cyaneus*	MK887733
			Dictyocoela muelleri	KY458984	100%	98.78%	*Acanthogammarus lappaceus*	JN393806
			Dictyocoela muelleri	MG027875	100%	98.78%	*G. lacustris*	MK887783
			Dictyocoela dubenum	MG027888	100%	98.29%	*Eulimnogammarus vittatus*	MK887755
			Dictyocoela dubenum	MG029373	100%	98.29%	*Gmelinoides fasciatus*	MG214958
			Dictyocoela dubenum	FJ756210	100%	98.01%	*Acanthogammarus victorii*	JN393823
			Dictyocoela dubenum	MG029394	100%	98.01%	*Brandtia latissima*	FJ756302
			Dictyocoela dubenum	FJ756204	100%	98.29%	*Dorogostaiskia parasitica*	FJ756321
			Dictyocoela dubenum	FJ756201	100%	98.29%	*Acanthogammarus lappaceus*	JN393806
			Dictyocoela dubenum	FJ756198	100%	98.29%	*Pallacea cancellus*	MT800937
			Microsporidium sp. 515	FJ820191	100%	98.54%	*Gmelinoides fasciatus*	MG214958
			Microsporidium sp. 515	FJ756007	100%	98.16%	*Linevichella vortex*	MN148355
			Nosema granulosis	KM657356	100%	99.05%	*Eulimnogammarus verrucosus*	MK887577
RU02	51.9207; 105.072	Russia	*Dictyocoela dubenum*	MH753359	100%	99.72%	*Gammarus lacustris*	MK887783
SK01	47.918198; 18.645932	Slovakia	*Dictyocoela roeselum*	MK719270	100%	99.36%	*G. roeselii* clade 2	MT325301
			Nosema granulosis	MK719264	97%	100%	*G. roeselii* clade 2	MT325301
SK02	49.096; 19.145	Slovakia	*Microsporidium sp. 515*	MT949290	97%	99.24%	*G. balcanicus* clade 3	KU056441
UA02	48.084; 24.656	Ukraine	*Dictyocoela muelleri*	MT932369	100%	98.78%	*G. balcanicus* clade 3	KU056441
UA03	48.190; 24.558	Ukraine	Microsporidium sp. V-A	MT949303	100%	100%	*Gammarus balcanicus* clade 3	KU056447
UA04	44.435;34.042	Ukraine	Microsoporidium sp. IV-B	MT949291	100%	98.41%	Gammarus balcanicus clade 1	KU056443
UA05	46.257; 30.419	Ukraine	*Dictyocoela dubenum*	MG773213	100%	98.58%	*Dikerogammarus villosus*	KT075266
UK01	50.180, 3.495988	United Kingdom	*Cucumispora sp. 02*	AJ438962	100%	100%	*G. chevreuxi*	GQ341716
UK02	Isle of Man, Cumbrae	United Kingdom	*Dictyocoela dubenum*	*AF397404*	100%	98.29%	*G. duebeni*	EF468643
			Dictyocoela dubenum	*AF397404*	100%	98.29%	*G. tigrinus*	MK403736
			Dictyocoela dubenum	*AF397404*	100%	98.29%	*G. locusta*	MF187975
UK03	Inner Bay, Inverkeithing, Scotland	United Kigndom	*Dictyocoela dubenum*	JQ673483	100%	98.01%	Echinogammarus marinus	KX224026
UK04	Scotland	United Kingdom	*Nosema granulosis*	JX213743	100%	100%	*G. duebeni*	EF468643
US01	Kalamazoo County, Michigan	USA	*Dictyocoela dubenum*	HM991451	100%	98.58%	*Gammarus pseudolimnaeus*	EF582876

Figure S1 Ultrametric Phylogenetic tree of Host MOTUs used to calculate parasite phylogenetic host specificity.



Chapter II

Prati, S., Enß, J., Grabner, D. S., Huesken, A., Feld, C. K., Doliwa, A., & Sures, B. (2023).
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Abstract

In lotic freshwater ecosystems, the drift or downstream movement of animals (e.g., macroinvertebrates) constitutes a key dispersal pathway, thus shaping ecological and evolutionary patterns. There is evidence that macroinvertebrate drift may be modulated by parasites. However, most studies on parasite modulation of host drifting behavior have focused on acanthocephalans, whereas other parasites, such as microsporidians, have been largely neglected. This study provides new insight into possible seasonal and diurnal modulation of amphipod (Crustacea: Gammaridae) drift by microsporidian parasites. Three 72 h drift experiments were deployed in a German lowland stream in October 2021, April, and July 2022.

The prevalence and composition of ten microsporidian parasites in *Gammarus pulex* clade E varied seasonally, diurnally, and between drifting and stationary specimens of *G. pulex*. Prevalence was generally higher in drifting amphipods than in stationary ones, mainly due to differences in host size. However, for two parasites, the prevalence in drift samples was highest during daytime suggesting changes in host phototaxis likely related to the parasite's mode of transmission and site of infection. Alterations in drifting behavior may have important implications for *G. pulex* population dynamics and microsporidians' dispersal. The underlying mechanisms are more complex than previously thought.

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Possible seasonal and diurnal modulation of *Gammarus pulex* (Crustacea, Amphipoda) drift by microsporidian parasites

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In lotic freshwater ecosystems, the drift or downstream movement of animals (e.g., macroinvertebrates) constitutes a key dispersal pathway, thus shaping ecological and evolutionary patterns. There is evidence that macroinvertebrate drift may be modulated by parasites. However, most studies on parasite modulation of host drifting behavior have focused on acanthocephalans, whereas other parasites, such as microsporidians, have been largely neglected. This study provides new insight into possible seasonal and diurnal modulation of amphipod (Crustacea: Gammaridae) drift by microsporidian parasites. Three 72 h drift experiments were deployed in a German lowland stream in October 2021, April, and July 2022. The prevalence and composition of ten microsporidian parasites in *Gammarus pulex* clade E varied seasonally, diurnally, and between drifting and stationary specimens of *G. pulex*. Prevalence was generally higher in drifting amphipods than in stationary ones, mainly due to differences in host size. However, for two parasites, the prevalence in drift samples was highest during daytime suggesting changes in host phototaxis likely related to the parasite's mode of transmission and site of infection. Alterations in drifting behavior may have important implications for *G. pulex* population dynamics and microsporidians' dispersal. The underlying mechanisms are more complex than previously thought.

Dispersal is a pervading feature of most animals and has an important role in shaping ecological and evolutionary patterns in natural populations[1,2]. Dispersal, or the movement of individuals across space, entailing potential consequences for gene flow[2], is influenced by many factors. Among them, intra- and inter-specific competition (e.g., for food, habitat, mating partners), predation, habitat loss, lack of resources, and environmental stochasticity (e.g., flood and storm events) seem to be the prevailing mechanisms driving dispersal[3,4]. However, dispersal also depends on the individual performance of an organism[5]. Any alteration of the individual performance, including that directly or indirectly induced by parasites, may influence the ability and proneness of an organism to disperse.

Parasites can modulate animal behavior indirectly through pathogenicity, defense response induction, and directly via host manipulation[6–9]. Parasite-induced changes might, for instance, affect mobility[10], habitat selection[7], foraging[11], reproduction[12], longevity[13], and host morphology[14]. Hence, the trade-off between the costs and benefits of dispersal might be influenced by parasites. For instance, seabirds might disperse more to escape infested habitats[15]. Meanwhile, condition-dependent dispersal in large terrestrial herbivores reduces the dispersal propensity of hosts with higher parasite load[16]. Parasites might also manipulate their host to disperse more frequently, increasing the chance of contact with the next host and thereby enhancing the transmission rate[17]. In aquatic ecosystems, for example, uninfected amphipods prefer dark and shaded areas. In contrast, conspecifics infected with acanthocephalan cystacanths show reverse geotaxis or reverse phototaxis and are more likely to be found in open water[18]. Such behavioral changes in intermediate hosts may enhance the individuals' vulnerability to predation and thus lead to higher transmission rates[6,19,20]. However, host manipulation might also work in the opposite direction, e.g., by limiting drift in mermithid-infected mayfly nymphs to avoid predation by fish, which would result in the death of both host and parasite[21]. Nevertheless, not all parasite-induced alterations in

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host phenotype are part of an adaptative manipulation strategy. Some alterations may result from non-adaptive pathological side effects of infection[22].

Amphipods are keystone species in aquatic ecosystems and generally harbor a wide range of parasites, some of which might affect dispersal[18,23]. Amphipods can actively disperse upstream to avoid resource limitation and competition, often in single massive migration events during times of limited food availability and high population densities[24,25]. On the other hand, drift or downstream dispersal with the water current can be either active or passive and is comparatively more consistent over time[24,25]. Drift plays a fundamental role in the population dynamics of amphipods and enables them to escape unfavorable conditions due to competition for food or predation risk[26]. Drift occurs mainly at night, about one hour after sunset and just before dawn[6,27,28]. In temperate regions, drift is often positively correlated with temperature, being at its lowest during winter and increasing toward summer[29]. However, like free-living biota, parasite communities vary seasonally due to temporal changes in abiotic and biotic factors, thus influencing the ecological dynamics of host-parasite relationships[30,31]. The burden of parasites in amphipods may fluctuate heavily between seasons and either impair host locomotory activity, as seen for the microsporidians *Pleistophora mulleri* and *Cucumispora ornata,* or enhance it, as for *Cucumispora dikerogammari*[23,32,33]. Hence, the influence of parasites on locomotory activity and, consequently, on host drifting behavior may result in alterations of host population dynamics that can be assumed to have broad ecological implications[34].

The vast majority of studies looking at parasite modulation of host drifting behavior have focused on acanthocephalans[6,35,36], whereas the influence of other parasites, such as microsporidians, remains largely unexplored. Microsporidians are a successful group of eukaryotic obligate intracellular parasites with relatively simple life cycles that exploit horizontal, vertical, and mixed-mode transmission to infect many hosts[37,38]. Horizontal transmission occurs via spore ingestion, venereally, or by direct invasion. Their transmission is often favored by cannibalistic behaviors, which are of common occurrence in amphipods[39]. On the other hand, vertical transmission occurs when spores are passed intergenerationally via transovarial transmission[37]. Generally, horizontal transmission is linked to high virulence, while vertical transmission is associated with low or no virulence or increased host fitness[32,40]. To our knowledge, only one study addressed the role of amphipod-infecting microsporidians on the drift behavior of their host[41]. The authors found that *Gammarus duebeni celticus* that were infected with the vertically transmitted microsporidian *Pleistophora* sp. were less abundant in the drifting fraction of the population. The influence of microsporidians and the differential role of their transmission routes on the drifting behavior of amphipod hosts remain largely unknown.

The present study aims to provide new insight into possible seasonal and diurnal modulation of amphipod drift by microsporidian parasites. Therefore, three drift experiments took place in October 2021, April, and July 2022, in a German lowland stream, the Rotbach, located within a nature-protected area. Three hypotheses drove our experimental design. Firstly, seasonal variations in microsporidian composition and prevalence are driven by shifts in amphipod body size distribution resulting from natural changes in age composition structure within amphipod populations. Secondly, horizontally transmitted microsporidians will be more represented in drifting amphipods than vertically due to possibly differential pathogenic effects on the host. And thirdly, microsporidians might alter the phototaxis of amphipods, resulting in a higher portion of infected individuals drifting during the day than at night. The mode of transmission of parasites might also influence drift timing due to different pathogenicity and site of infection.

Methods

Sampling.

Amphipods were collected in three field experiments conducted in October 2021, April 2022, and July 2022. Each experiment lasted 72 h and took place in a nature-protected section of the Rotbach stream, North Rhine-Westphalia, Germany (51°34′03.4″ N, 6°51′48.8″ E). The Rotbach is a tributary of the Rhine, and its upper section, with only minimal anthropogenic disturbance, is one of the last standing natural sections of sand-bottom lowland streams in North Rhine-Westphalia and listed among the Federal State's reference streams[42]. Drifting amphipods were collected at two hours intervals using drift nets (30 × 30 cm, mesh size 500 µm). The drift nets were placed diagonally to cover the entire width of the stream and avoid reciprocity bias in catches (Fig. 1). Water velocity was measured with an anemometer (Schiltknecht MC20 with C-53084 adapter) at 20% and 80% depth in the middle of each drift net at each emptying. Velocity varied depending on net placement, time, and season (range: 2–45 cm/s, mean ± SD: 20.1 ± 11.8 cm/s). After the conclusion of drift experiments, stationary amphipods were collected during the day, through kick sampling at two sites located 50 m and 100 m upstream of the uppermost drift net, in areas with differing flow velocity (range: 0.2–24 cm/s, mean ± SD: 12.8 ± 7.75 cm/s). The kick sampling consisted of triplicates for each flow velocity classes (low: < 7.5 cm/s, medium: 7.5–15 cm/s, and high > 15 cm/s) at each site.

All amphipods were immediately fixed in 96% ethanol. Amphipods were subsequently measured (fourth coxal plate length), morphologically identified to the lowest taxonomical level, dissected, and screened for parasites under the microscope. Moreover, amphipods were divided into four size classes based on coxal plate lengths (< 1, 1–1.99, 2–2.99, and > 3 mm) to investigate possible infection patterns related to ontogeny. Amphipod's sex, however, was not initially recorded. In later samples, sex could not be assessed with certainty for most of the collected individuals, as distinctive sex characteristics are fully developed only in adults. Thus, sex was excluded from subsequent analyses. Only six individuals, one belonging to the stationary sample and five trapped in the drift nets, were infected with cystacanths of Acanthocephalans. Thus, acanthocephalans-infected amphipods were excluded from further analyses. Amphipods were dissected to investigate microsporidians, and guts removed to avoid detecting eventually enclosed spores. After gut removal, the remaining tissues were used for DNA extraction allowing the molecular identification of both host and microsporidian parasites. During a survey conducted between May and June 2021 at the same location, 40 amphipods were molecularly identified as belonging to

Figure 1. Drift nets placed diagonally in the stream to cover its width.

Gammarus pulex clade E (99.8–100% similarity to KT075231). Therefore, an additional batch of 20 randomly selected individuals for each experiment was molecularly identified to account for possible variability in host composition between experiments for a total of 100 individuals.

If present, *G. pulex* eggs of infected specimens were extracted individually to identify possible vertical transmission of parasites. Additionally, to evaluate the potential role of fish in microsporidian spore dispersal, the gastrointestinal content of ten *Barbatula barbatula* individuals, the most common fish inhabiting the investigated stretch of the Rotbach, were also subject to DNA extraction. The fish were collected in the same area used for the drift experiment in May 2022 as part of an ongoing study of parasite diversity within the collaborative research center CRC 1439 RESIST framework.

DNA isolation and sequencing. DNA was isolated from *G. pulex* tissues, individual eggs, and gastrointestinal fish content using a modified salt precipitation protocol according to Grabner et al.[43]. Molecular identification of hosts was obtained with the universal eukaryotic primers LCO1490 (5′-GGTCAACAAATCATAAAG ATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′)[44], while that of microsporidians with the universal microsporidian-targeted primers V1 (5′-CACCAGGTTGATTCTGCCTGAC-3′)[45] and mic-uni3R (5′-ATTACCGCGGMTGCTGGCAC-3′)[46]. PCR reaction volumes used for host tissues and gastrointestinal fish content were prepared following Weigand et al.[46] using AccuStart II PCR ToughMix (Quanta Bioscience). One reaction contained 10 µL of 2 × ToughMix, 0.5 µM of each primer, and 1 µL of DNA. MilliQ water was added up to a total volume of 20 µL. Whereas PCR reaction volumes used for individual eggs were adjusted to account for a lower DNA yield than whole amphipods and gastrointestinal fish content by increasing the volume of DNA to 2 µL. PCR cycle conditions were set as follows: initial denaturation for 3 min at 94 °C, followed by 35 cycles of 35 s (host tissues) or 40 cycle of 35 s (eggs), denaturation at 94 °C and 40 s annealing at 68 °C, and a final elongation of 5 min at 68 °C. PCR products of hosts and microsporidians were sent to Microsynth Seqlab (Germany) for Sanger sequencing using LCO1490 and V1 primers, respectively.

Sequences editing and alignment. Raw sequences were quality-checked and edited using Geneious v2023.0.1 (Biomatters). Only sequences with a minimum length of 200 bp were used for the analyses. Host and parasite sequences were separately aligned using the MAFFT v7.490 algorithm with standard settings[47]. Haplotypes of hosts and microsporidians were grouped in molecular operational taxonomic units (MOTUs) when the Kimura-2-parameter (K2p) corrected pairwise distances were below 2%. A threshold of 2% was chosen to account for potential intragenomic variation among microsporidians while remaining below commonly observed values of intraspecific variability in amphipods[43,48]. For microsporidians, a maximum likelihood phylogenetic tree with bootstrap support values (1000 replicates) was produced in IQ-Tree 2.2.0[49]. The TIM3 + F + G4 substitution model was selected based on Bayesian information criterion scores. To identify hosts and their microsporidian parasites, obtained MOTU sequences were compared against records contained in GenBank using megablast. The microsporidian *Metchnikovella dogieli* (MT969020) was used as an outgroup. The naming of undescribed Microsporidium isolates except for Microsporidium sp. RB 01, RB02, and RB03 followed the classification used by previous studies[50,51].

Statistical analyses. Statistical analyses were performed with the open-source software RStudio (version 2022.07.2, RStudio Inc.) based on R (version 4.2.2[52]).

Body size and flow velocity may influence drift, and parasitism, in turn, may influence both the body size and drift of amphipods[41]. However, the body size is expected to vary seasonally following the natural cycle of birth, growth, and death of *G. pulex*. Hence, the size (4th coxal plate) of pooled drifting and stationary amphipods was firstly compared between months using the Dunn post hoc test with FDR adjustment (rstatix package, version 0.7.1[53]) as data did not follow a normal distribution. Afterward, differences in *G. pulex* body size between stationary (day) and drifted samples (day and night) were compared for each part of the day and month separately, using a pairwise Wilcoxon rank sum test with FDR correction (rstatix package, version 0.7.1[53]).

A binomial generalized linear model (GLM) with parasite prevalence as the dependent variable and host body size, sample type (either stationary or drift), average flow velocity, and season as independent variables was employed to investigate the effect of body size on parasite prevalence in stationary and drifting amphipods. Since stationary amphipods were collected solely during the day, only the prevalence of amphipods drifting during the day was used in the model. The variance inflation factor (VIF; analyzed using the car package version 3.1-2 [54]) remained below two for all descriptors. Multicollinearity was not an issue, and stepwise backward regression (MASS package version 7.3-56[55]) did not reduce the number of variables; thus, we retained all the variables. Including an interaction between sample type and season did improve the model. The results were reported as odd ratios. To assess diurnal differences in prevalence, we separated *G. pulex* in drift net samples into day (first and last sampling taken entirely in daylight) and night (sampling that included sunset, night, and sunrise). Prevalence differences in relation to diurnal drift were assessed with Fisher's exact test (rstatix package, version 0.7.1[53]) for each experiment separately using pooled sample of parasites. When feasible, diurnal differences in prevalence were investigated for each parasite species separately using GLMs, and the results were reported as odd ratios. These were performed using parasite prevalence as the dependent variable, host body size, average flow velocity, and time of the day (either day or night) as the independent variable for each experiment separately.

Results

The sample comprised 1893 *G. pulex* clade E specimens (all 100 molecularly identified individuals showed 99.7–100% similarity to KT075231). Of these, 897 individuals were collected by kick sampling, and 996 were trapped in drift nets. Drift rates of *G. pulex* peaked during the night before plunging during the daytime. This pattern remained stable across seasons irrespective of flow velocity (Fig. 2).

Gammarus pulex body size differed substantially between seasons (Kruskal–Wallis test, H = 234, *df* = 2, *P* = < 0.001), with larger individuals being more common in April and smaller individuals in July. However, in all three seasons, recently hatched *G. pulex* were detected, and in April, individuals were either large or very small (Fig. 3). On the other hand, their size distribution in October was more balanced. The observed differences were congruent for each season combination following pairwise comparisons (Dunn post hoc test, all *Padj* = < 0.001). Moreover, the body size of drifted *G. pulex* was generally larger than that observed in stationary individuals (Wilcoxon rank sum test, all *P* = < 0.001, Fig. 3).

Ten microsporidian taxa belonging to four different clades sensu Bojko et al.[56] were detected in *G. pulex* clade E (Fig. 4). Half of the identified microsporidians belonged to the Enterocytozoonida clade. These included Microsporidium sp. 505 (99.6–100% similarity to KX137937), Microsporidium sp. 515 (99.4–100% similarity to KX137939), Microsporidium sp. IV-B (100% similarity to KX137941), Microsporidium sp. IV-F (99.7–100% similarity to KR871373), and Microsporidium sp. 03RB. The latter is a possible new undescribed species showing 97.2% similarity to *Helmichia lacustris* (GU130406). Among them, Microsporidium sp. 505 and Microsporidium sp. 515 are supposedly horizontally transmitted, while Microsporidium sp. IV-B may be horizontally and vertically transmitted[57].

Two microsporidians, *D. duebenum* (100% similarity to KR871355) and *Dictyocoela* sp. H9 (100% similarity to KR871363) belonged to the Glugeida clade. While *Nosema granulosis* (99.7–100% similarity to FN434088) and Microsporidium sp. 01RB (96.6–98% similarity to FJ865224 Microsporidium sp. 4-YYS-2009d, closest described species *Heterovesicula cowani* with 86% similarity) belonged to the Nosematida clade. *Nosema granulosis* and *Dictyocoela* spp. are usually vertically transmitted[50,58,59]. The remaining Microsporidium sp. 02RB belonged to the Neopereziida clade and showed 96.2% similarity to Microsporidium sp. OTU9332 (MZ405830), with the closely described species being *Jirovecia sinensis* (MN752318), with 86.3% similarity. Both Microsporidium sp. RB02 and RB03 were detected in single *G. pulex* individuals.

Among the 24 ovigerous females out of 61, a total of 126 eggs were molecularly screened for microsporidians. However, none of the eggs was infected. Molecular analyses of microsporidians in the fish gut revealed the presence of Microsporidium sp. IV-F (100% similarity to KR871373) and an unknown Microsporidium (99.3% similarity to KX137921 Microsporidium sp. 7, closest described species FN794114 *Octosporea muscaedomesticae* with 93.1% similarity) each in a single *B. barbatula* specimens.

Stationary *G. pulex* individuals were infected with all parasites except for Microsporidium sp. IV-F. Overall, the most prevalent microsporidian identified in this study was Microsporidium sp. IV-B, while Microsporidium sp. 515, and Microsporidium sp. 505 were the only microsporidians found every season and among drifted and stationary samples (Table 1). Microsporidian richness and prevalence were largely dependent on *G. pulex* size. The largest diversity of parasites was detected in the smallest specimens, while larger individuals had a higher prevalence than smaller ones (Table 2). Accordingly, the prevalence in *G. pulex* with a 4th coxal plate length above 2 mm ranged between 32.3 and 45.3%, while that in the two smaller size classes ranged between 4.18 and 9.59% (Table 2). Drifting *G. pulex* (day only) were generally larger than stationary individuals (4th coxal plate length range: 0.22–3.88 mm, mean ± SD: 1.45 ± 0.98 mm vs. range: 0.11–3.63 mm, mean ± SD: 1.04 ± 0.73 mm) and had a higher prevalence with 18% compared to 7.13%. Correspondingly larger specimens were more likely

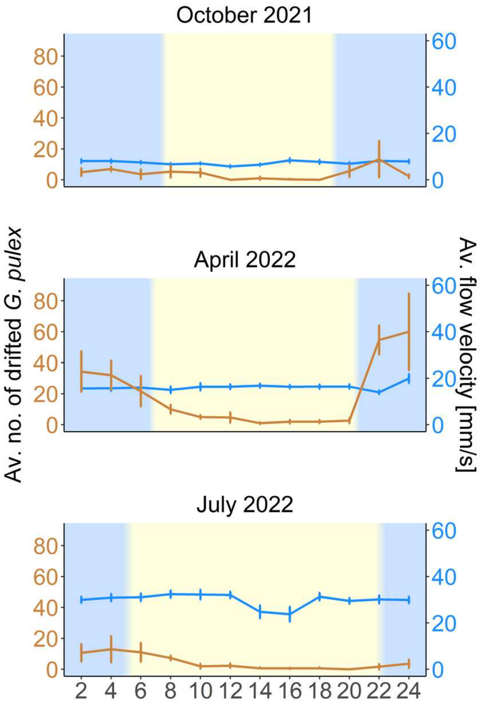

Figure 2. Line plot showing the average number of drifted *Gammarus pulex* captured in two hours intervals (in orange, each temporal point represents the average number of amphipods collected during the prior two hours) and the measured average water flow velocity in mm/s (in blue), including the standard error. The bluish background indicates night, while the yellowish background and transition areas represent day and sunrise/sunset. Please note that the two y-axes differ in scale.

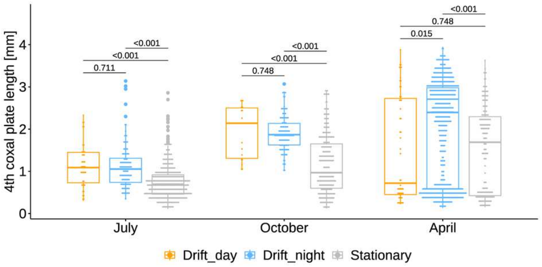

Figure 3. Size differences among drifted (day) and stationary (day and night) *Gammarus pulex* for each drift experiment. Horizontal bars within each experiment show size comparisons between drifted and stationary individuals, including *P*-values obtained pairwise Wilcoxon rank sum test with FDR correction.

to be infected than smaller ones (OR 3.51, 95% CI 2.48–5.08), while stationary individuals were less likely to be infected than drifted ones (OR 0.36, 95% CI 0.14–0.94) primarily due to the smaller size of stationary *G. pulex* collected in July (OR 5.69, 95% CI 1.29–31.73). There was no evidence of flow velocity influencing parasite prevalence (OR 0.99 95% CI 0.96–1.03).

Pooled microsporidians prevalence in drift (= 24 h) had minor variations between day and night in every season (Fisher's exact test, all $p > 0.184$). However, when looking at each parasite separately, considering host size and average flow velocity, fewer *G. pulex* but with a higher prevalence of Microsporidium sp. 505 drifted during the day compared to the night in October and April samples (Table 1, Table S1). These differences were more evident in October than in April (OR 0.11, 95% CI 0.02–0.56 vs. 0.33, 95% CI 0.07–2.40). A similar pattern was observed in Microsporidium sp. 515 during April and July, with diurnal differences in prevalence more visible in the first (OR 0.13, 95% CI 0.04–0.43 vs. 0.32 95% CI 0.03–2.96). On the contrary, Microsporidium sp. IV-B,

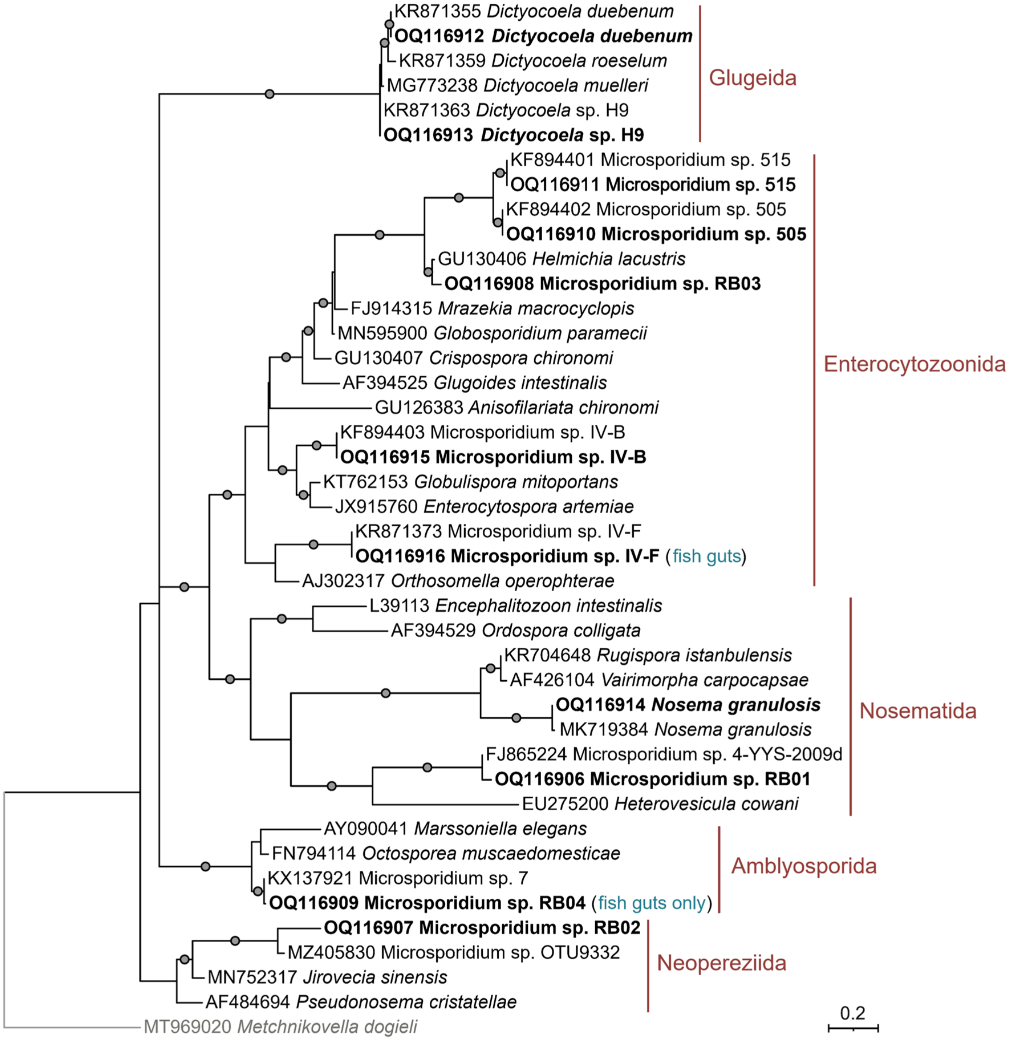

Figure 4. Maximum likelihood phylogenetic trees of microsporidians identified in amphipods and fish guts. The tree was obtained IQ-Tree 2.2.0 using the TIM3 + F + G4 substitution model. Dots represent bootstrap branch support values (1000 replicates) above 90%. The names and circumscriptions of microsporidians clades sensu Bojko et al.[56] are indicated in red. Microsporidians, which were also present in fish guts or fish guts only, are noted in blue.

which was detected in the drift only in April, had a higher prevalence during the night. This, however, was mostly due to diurnal differences in host size (Table 1, Table S1). In none of the parasite taxa, flow velocity influenced prevalence (all OR 95% CI ranged from below 1 to above 1).

Discussion

Seasonal parasite prevalence and composition variations driven by *G. pulex* size occurred in drifting and stationary samples. For two parasites, the prevalence in drifting *G. pulex* was highest during daytime, thus, suggesting changes in host phototaxis are likely related to the parasite's mode of transmission or site of infection. Such findings underline the limited understanding of infection mechanisms related to microsporidians and their role in host population ecology. A better understanding of these mechanisms is crucial as any indirect and direct influence of parasites on host population structure and dynamics in keystone species such as amphipods are likely to affect ecosystem functioning[34,60]. Furthermore, the identification of three new isolates, Microsporidium sp. RB01, RB02, and RB03 in a single host population highlight the need for further research on this group of ubiquitous but often neglected parasites.

The present study expands the current knowledge of host-parasite interaction in *G. pulex* clade E by five interactions. Within the *G. pulex* complex, *Dictyocoela* sp. H09 was previously found in individuals belonging to the clade C, while *Nosema granulosis* in those of the clade D[43,61]. Microsporidium sp. RB01 is closely related to the

Microsporidian parasites	**Microsporidian prevalence of drifted *Gammarus pulex* in %**								
	October 2021			**April 2022**			**July 2022**		
	Drifted		**Station.**	**Drifted**		**Station.**	**Drifted**		**Station.**
	Day (n = 18)	**Night (n = 127)**	**Day (n = 280)**	**Day (n = 52)**	**Night (n = 638)**	**Day (n = 187)**	**Day (n = 41)**	**Night (n = 120)**	**Day (n = 430)**
Dictyocoela duebenum	0	0.79 (1)	0	0	0.47 (3)	0.5 (1)	0	0	0.2 (1)
Dictyocoela sp. H9	0	1.57 (2)	0.4 (1)	0	0	0.5 (1)	0	0	2.1 (9)
Microsporidium sp. 01RB	5.56 (1)	1.57 (2)	0	0	0	0	0	0	0.9 (4)
Microsporidium sp. 02RB	0	0	0	0	0	0	0	0	0.2 (1)
Microsporidium sp. 03RB	0	0	0	0	0	0	0	0	0.2 (1)
Microsporidium sp. 505	22.2 (4)	3.15 (4)	0.7 (2)	3.85 (2)	1.41 (9)	0.5 (1)	0	0.83 (1)	2.1 (9)
Microsporidium sp. 515	0	7.87 (10)	1.8 (5)	9.61 (5)	2.04 (13)	1.6 (3)	4.88 (2)	1.67 (2)	1.86 (8)
Microsporidium sp. IV-B	0	0	0	9.61 (5)	23.82 (152)	6.4 (12)	0	0	0.9 (4)
Microsporidium sp. IV-F	0	0	0	0	0.78 (5)	0	2.44 (1)	0.83 (1)	0
Nosema granulosis	0	0	0.4 (1)	0	0.16 (1)	0	0	0.83 (1)	0

Table 1. Seasonal and diurnal prevalence of microsporidians in drifted and stationary (Station.) *Gammarus pulex* clade E for each drift experiment. The number of infected individuals is reported in brackets.

Microsporidian parasites	**Microsporidian prevalence in *Gammarus pulex* for each size classes in %**			
	< 1 mm (0.56 ± 0.22 mm, n = 885)	**1–1.99 mm (1.49 ± 0.29 mm, n = 417)**	**2–2.99 mm (2.49 ± 0.29 mm, n = 412)**	**> 3 mm (3.29 ± 0.22 mm, n = 412)**
Dictyocoela duebenum	0.23 (2)	0	0.24 (1)	0
Dictyocoela sp. H9	1.02 (9)	0.96 (4)	0.73 (3)	0
Microsporidium sp. 01RB	0.45 (4)	0.48 (2)	0.24 (1)	0
Microsporidium sp. 02RB	0.11 (1)	0	0	0
Microsporidium sp. 03RB	0.11 (1)	0	0	0
Microsporidium sp. 505	0.90 (8)	1.20 (5)	1.94 (8)	6.14 (11)
Microsporidium sp. 515	0.90 (8)	2.40 (10)	5.82 (24)	3.35 (6)
Microsporidium sp. IV-B	0.34 (3)	3.84 (16)	22.33 (92)	34.64 (62)
Microsporidium sp. IV-F	0.11 (1)	0.24 (1)	0.97 (4)	0.56 (1)
Nosema granulosis	0	0.48 (2)	0	0.56 (1)
Pooled samples	4.18 (37)	9.59 (40)	32.28 (133)	45.25 (81)

Table 2. Prevalence of microsporidians in *Gammarus pulex* clade E for each size class. The number of infected individuals is reported in brackets.

isolate Microsporidium sp. 4-YS-2009d found in the barklouse *Polypsocus corruptus*[62], and the closest described species is *H. cowani* which infects the adipose tissue of Mormon crickets, *Anabrus simplex*[63]. Microsporidium sp. RB02 is closely related to the isolate Microsporidium sp. OTU9332, for which no information relative to the host is available, as it was detected in lacustrine environmental samples[64]. However, the closest described species *J. sinensis* was recently described from a freshwater oligochaete, *Branchiura sowerbyi*[65]. Microsporidium sp. RB03, on the other hand, is closely related to *H. lacustris,* a microsporidian found in the midge larvae of *Chironomus plumosus*[66]. With half of the detected microsporidians representing new host-parasite interactions, the present finding highlights the need for further parasitological studies in amphipods.

Parasite composition varied between size classes, seasons, and sample types. Differences in host size mainly explained such variations. Overall, smaller individuals had lower parasite prevalence but a more variable community than the larger ones. This can happen if small differences in the exposure to parasites occurring at early stages generate large intraspecific differences, while the parasite community of larger individuals might become more homogeneous through repeated parasite exposure, as commonly observed in other aquatic organisms[67,68]. However, parasites appearing in early stages might become dominant over time, leading to the homogenization of parasite communities in larger individuals[69]. Homogenization of parasite communities in larger *G. pulex* individuals might also be favored by cannibalism and size-selective predation, the latter of which might be connected with the removal of large infected individuals (e.g., by predatory fish) and/or small infected individuals (e.g., by predatory macroinvertebrates such as dragonfly larvae) or by a combination of both[70,71]. Such mechanisms might, in turn, influence observed differences in parasite communities. Infections with several microsporidian taxa in one amphipod individual are considered rare as an initial infection may create a bottleneck for successive infections[72]. However, it was impossible to assess coinfections in the present study due to the limitation of the sequencing method used.

Like parasite composition, prevalence varied seasonally. Such differences were particularly evident in Microsporidium sp. IV-B. Interestingly, this species was not detected in October and was present in low prevalence during July but peaked in April. Seasonal differences in microsporidians prevalence were mainly explained by *G. pulex* size, with larger individuals being more infected than smaller ones. Accordingly, host size varied between seasons following natural population dynamics, with larger individuals mainly present in April, smaller ones in July, and middle-sized ones in October, supporting our first hypothesis. Such population dynamics are consistent with those observed in other *G. pulex* populations in which a cohort of juveniles was observed from spring throughout summer, while large individuals were nearly absent from July to October[73].

A positive correlation between host size and parasite prevalence is commonly observed in aquatic organisms such as fish and snails[74,75]. As hosts grow, the exposure time to the parasite's infective stages increases proportionally, enhancing the likelihood of becoming infected. Furthermore, ontogenetic dietary shifts leading to predation of conspecific and heterospecific individuals might occur, bolstering parasite infections. Size-asymmetric cannibalism is common in many taxa, including fish and aquatic invertebrates, mainly in size-structured species with overlapping generations in time and space[76]. Cannibalism is commonly observed in *G. pulex* and might potentially favor the transmission of parasites via ingestion of infected individuals[39]. In turn, microsporidian parasites might influence conspecific and heterospecific predatory interactions through enhanced aggression[77].

Host size, likewise, explained differences in microsporidians prevalence between drifting and stationary *G. pulex*, with larger individuals being more common in the drift compared to stationary samples. This suggests that either large specimens are more dispersive or higher prevalence in larger specimens drives dispersal. Generally, a larger body size implies higher energy costs and food intake but also lowers locomotion costs per unit of body mass, favoring movement to richer patches and enhancing dispersal[78,79]. Accordingly, in a microcosm experiment, the home range of *G. insensibilis* was greater in larger individuals, as these could not fully exploit patches. On the other hand, smaller individuals with lower energetic requirements tended to use a limited set of patches[80]. Nevertheless, resources in the field might not be as limited as in experimental setups, and individual habitat preferences coupled with inter and intraspecific interactions might influence home range.

Although no clear pattern between *G. pulex* size and water flow was detected in the present study, smaller individuals are likely to prefer low water velocity near the banks[81]. In these microhabitats, leaves accumulate providing hiding opportunities from predation by larger conspecifics. Such habitat preferences may influence the size of *G. pulex* going into the drift. However, confounding mechanisms such as parasite pathogenicity might have a pivotal role. Accordingly, Microsporidium sp. 505, Microsporidium sp. 515, and Microsporidium sp. IV-B, all belonging to the Enterocytozoonida clade, were more often represented in drifting specimens than stationary ones, but the opposite was true for *D. duebenum* and *Dictyocoela* sp. H9, both belonging to the Glugeida clade. Similarly, in another study *G. duebeni celticus* infected with the microsporidian *Pleistophora* sp., belonging to the Glugeida clade, were less abundant in the drifting fraction of the population[41].

Microsporidians of the Enterocytozoonida clade are generally tissue and organ-specific, mainly infecting epithelial cells of the midgut, particularly the hepatopancreas of aquatic arthropods. They can be transmitted horizontally and vertically or horizontally alone[56,82]. On the other hand, species belonging to the Glugeida clade are common in host muscle tissue and transmit horizontally, vertically, or both[56]. Correspondingly, vertical transmission is known in *D. duebenum* and other species of the same genus, which are found in the ovarian tissue and adjacent muscles[59,83]. Microsporidium sp. 505 and Microsporidium sp. 515 seem to be predominantly horizontally transmitted, while Microsporidium sp. IV-B is suspected of vertical transmission[57,59]. However, in the current study, no eggs of individuals infected with Microsporidium sp. IV-B tested positive, suggesting that horizontal transmission is the prevailing infection pathway in the studied area. The pristine status of the upper part of the Rotbach might possibly explain this discrepancy. Accordingly, in parasites with a mixed transmission mode, a switch from horizontal to vertical transmission may occur during phases of adverse environmental conditions and is an important survival strategy[84]. Alternatively, the PCR protocol used, although modified to maximize sensibility, might have failed to detect, if present, the very small number of spores contained in the eggs.

Horizontally transmitted parasites are generally linked to high virulence, which might reduce host fitness. For instance, *Enterocytozoon hepatopenaei,* a microsporidian belonging to the Enterocytozoonida clade, infects the hepatopancreas of various shrimps inducing storage consumption of lipids, downregulation of lipid metabolism and thus energy production[85]. It is, therefore, plausible that a reduction in fitness might occur and hinder the ability of *G. pulex* to withstand water flow and, thus, be more likely found in the drift. An enhanced drift of infected individuals is likely to benefit horizontally transmitted parasites by favoring spore dispersal. Although drift might result in spore dilution and limit cannibalism, many spores persist in the environment even in the absence of suitable hosts[86]. Depending on the microsporidian species, spores can be more or less resistant. Thus, from an evolutionary perspective, this might suggest that horizontally-transmitted parasites might produce more environmentally-resistant spores than vertically-transmitted parasites. If this was the case, long-lasting spores transported downstream might accumulate over time in areas of low water flow, reaching densities suitable for a successful infection. Amphipods likely use such areas as feeding grounds as leaves and other organic matter tend to accumulate. Infection may then occur when spores are inadvertently ingested during feeding activities.

Vertically-transmitted parasites are less virulent than horizontally-transmitted ones and can have either positive or neutral effects on host fitness[72]. They rely on successful host reproduction for their transmission. Hence host drifting might be counterproductive for several reasons. First, drift exposes the host to predation, possibly ending the parasite's life cycle. Second, drift might reduce the host's chances of encountering suitable partners due to a possible dilution effect. Amphipods are often aggregated and can have patchy distributions in streams[81,87]. Therefore, drifting from areas of higher to lower individual densities could impact *G. pulex* reproductive success hindering the vertical transmission of microsporidians. Differences in the site of infection, pathological stage, and host size may explain differences in the ratio of likely horizontally and vertically-transmitted microsporidians among drifting and stationary specimens.

Diurnal differences in the drift of *G. pulex* were observed only in individuals infected with Microsporidium sp. 505 and Microsporidium sp. 515 after accounting for size differences between day and night. Infected *G.*

pulex seemed to drift mainly during daytime and to a lesser degree during nighttime. Such differences were particularly evident in October for Microsporidium sp. 505 and April for Microsporidium sp. 515. Drift activity of stream invertebrates typically is greatest during the nighttime hours in running waters throughout the world, presumably to minimize predation risk by visually hunting drift-feeding fishes[88]. Thus, the shift in the drift rate of infected individuals towards daytime suggests a change in the phototaxis of infected *G. pulex*, which supports the third hypothesis of our study.

Altered phototaxis has previously been observed in a variety of aquatic and terrestrial hosts infected by acanthocephalans, cestodes, fungi, nematomorphs, and trematodes. It is suspected to enhance transmission to the final host via predation or force the host to a suitable habitat where parasite life cycle completion can occur[89,90]. Only one experiment investigated phototaxis alteration in amphipods infected with the microsporidian *Dictyocoela roeselum*. This particular microsporidian did not have any relevant effect on phototaxis[91]. However, considering the contrasting life strategy of horizontally vs. vertically transmitted parasites, the opposite might be true for Microsporidium sp. 505 and Microsporidium sp. 515. An alteration of phototaxis toward daytime might be detrimental for vertically transmitted parasites as *G. pulex* might be more likely predated by visual feeding predators, hindering reproductive success and thus transmission. The same might not hold for horizontally transmitted parasites. A shift toward daytime might enhance predation risk, which could benefit the parasite by further enhancing spore dispersal following prey digestion. Their detection in fish guts suggests that these might be suitable vectors for amphipods infecting microsporidians. Even if there is no known phase of development in the fish, microsporidian spores might pass the digestive tract and be released into the environment while still being infective[92]. Therefore, microsporidians depending on their mode of transmission might benefit from the tendency of large amphipods to drift more, which may result in increased predation risk and consequently enhanced infections via spore dispersal even without direct manipulation of the host. Further studies, including morphological data, are thus required to shed light on the dispersal strategies of microsporidian parasites and the potential role of predators feeding on their hosts.

In conclusion, our findings show that microsporidian infections and population dynamics may influence host drift behavior. However, the underlying dispersal mechanism of microsporidian parasites with differing transmission routes might be more complex than previously thought, requiring further studies.

Data availability

The raw data supporting the conclusions of this article are available in the OSF repository, https://doi.org/10.17605/osf.io/b9tvk. The nucleotide sequence data reported are available in the GenBank database under the accession numbers OQ116906-OQ116916 for microsporidians and OQ121128 for *G. pulex* clade E.

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Author contributions

S.P., J.E., C.K.F., and B.S. conceived the study. C.K.F., D.G., and B.S. supervised the project. S.P., J.E., and A.H. carried out the sampling. A.H., A.D., and S.P. performed laboratory analyses. S.P. performed the data analyses. S. P. led the writing of the manuscript, and J.E., D.G., A.H., C.K.F., A.D., and B.S oversaw the analyses and writing. All authors contributed critically to the drafts and approved the final manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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Table S1 Logistic regression output table from GLMs relative to factors (day/night, average flow velocity, and amphipod size) influencing microsporidians prevalence in *Gammarus pulex* clade E for each drift experiment and pooled experiment samples. The table includes coefficients, standard errors of coefficients, Z values, P values, Odd Ratios, Odd Ratios 95% confidence intervals, and McFadden pseudo-R^2.

Experiment	**Variable**	**Coef.**	**SE Coef.**	**Z**	**P**	**OR**	**95% CI**	**Mcfadden pseudo-R^2**
				Microsporidium sp. 505				
October 2021	Night	-2.225	0.835	-2.665	0.008	0.108	0.019-0.562	
	Size	1.288	0.818	1.574	0.115	3.625	0.782-20.478	0.165
	Av. flow	0.081	0.175	0.465	0.642	1.085	0.775-1.556	
April 2021	Night	-1.183	0.862	-1.297	0.195	0.327	0.070-2.397	
	Size	2.390	0.762	3.139	0.002	10.916	3.068-58.191	0.221
	Av. flow	0.126	0.105	1.198	0.231	1.134	0.926-1.405	
Pooled samples	Night	-1.904	0.534	-3.562	<0.001	0.149	0.054-0.453	
	Size	1.293	0.332	3.894	<0.001	3.644	2.013-7.496	0.175
	Av. flow	-0.083	0.050	-1.677	0.094	0.920	0.828-1.006	
				Microsporidium sp. 515				
April 2021	Night	-2.080	0.586	-3.549	<0.001	0.125	0.041-0.426	
	Size	0.785	0.270	2.908	0.004	2.193	1.349-3.961	0.117
	Av. flow	-0.096	0.076	-1.262	0.207	0.909	0.780-1.051	
July 2022	Night	-1.156	1.079	-1.071	0.284	0.315	0.032-2.956	
	Size	1.514	0.775	1.953	0.051	4.546	0.926-22.462	0.126
	Av. flow	0.017	0.096	0.180	0.857	1.017	0.822-1.226	
Pooled samples	Night	-1.198	0.457	-2.623	0.009	0.302	0.129-0.792	
	Size	0.567	0.195	2.900	0.004	1.762	1.216-2.628	0.058
	Av. flow	-0.035	0.029	-1.211	0.226	0.965	0.908-1.019	
				Microsporidium sp. IV-B				
April 2021	Night	0.928	0.520	1.783	0.075	2.528	0.983-7.861	
	Size	1.112	0.124	8.992	<0.001	3.041	2.414-3.927	0.178
	Av. flow	0.019	0.033	0.576	0.565	1.019	0.956-1.087	

Chapter III

Schneider, R., Prati, S., Grabner, D. S., & Sures, B. (2022).
First report of microsporidians in the non-native shrimp *Neocaridina davidi* from a temperate European stream.
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Abstract

The release of ornamental pets outside their native range can directly or indirectly impact the recipient community, e.g., via the co-introduction of associated pathogens. However, studies on parasites associated with non-native species, in particular freshwater decapods, have focused mainly on a limited set of pathogens. Here we provide data for the first time on microsporidian parasites of the non-native ornamental shrimp *Neocaridina davidi*, collected in a stream in Germany. Furthermore, we confirm an ongoing range expansion of the warm-adapted *N. davidi* from thermally polluted to colder water. In the investigated shrimps, the microsporidian parasite *Enterocytozoon hepatopenaei* and an unknown microsporidian isolate were detected, raising concerns about their transmission potential and pathogenicity on native crustacean species.

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NOTE

First report of microsporidians in the non-native shrimp *Neocaridina davidi* from a temperate European stream

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ABSTRACT: The release of ornamental pets outside their native range can directly or indirectly impact the recipient community, e.g. via the co-introduction of associated pathogens. However, studies on parasites associated with non-native species, in particular freshwater decapods, have focused mainly on a limited set of pathogens. Here we provide data for the first time on microsporidian parasites of the non-native ornamental shrimp *Neocaridina davidi*, collected in a stream in Germany. Furthermore, we confirm an ongoing range expansion of the warm-adapted *N. davidi* from thermally polluted colder water. In the investigated shrimps, the microsporidian parasite *Enterocytozoon hepatopenaei* and an unknown microsporidian isolate were detected, raising concerns about their transmission potential and pathogenicity on native crustacean species.

KEY WORDS: *Enterocytozoon hepatopenaei* · Atyidae · Invasive species · DNA barcoding · Shrimp diseases

1. INTRODUCTION

The ornamental pet trade has flourished in recent years, which has increased the risk of non-native species introductions (Patoka et al. 2018). Freshwater habitats, especially those experiencing degradation, are prone to biological invasions (Casatti et al. 2006). Thermal pollution of temperate streams or rivers, for example, may facilitate the establishment of exotic species (Klotz et al. 2013, Weiperth et al. 2019). The introduction of non-native species can have negative impacts on the recipient community by directly or indirectly affecting ecosystem functioning and biodiversity, even without establishment, e.g. via pathogen transmission (Simberloff et al. 2013).

Biological invasions are often linked to commercial success in the global pet trade (Gippet & Bertelsmeier 2021), and decapods are no exception. Warm-adapted decapods have a low probability of becoming established in temperate waters, except for in thermally polluted waterbodies (Weiperth et al. 2019). Established populations of the atyid shrimp *Neocaridina davidi*, a popular aquarium pet originating from eastern and central China, have been reported in the USA, Japan, Israel, and Europe (Klotz et al. 2013, Jabłońska et al. 2018, Levitt-Barmats et al. 2019, Weiperth et al. 2019). Until now, *N. davidi* has primarily been investigated for epibionts such as *Holtodrilus truncatus*, *Scutariella* sp., *Vorticella* sp., and *Cladogonium* sp. (Ohtaka et al. 2012, Liao et al. 2018, Bauer et al. 2021), which have been found in native and non-native populations of *N. davidi* (Ohtaka et al. 2012, Patoka et al. 2016, Liao et al. 2018, Maci-

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aszek et al. 2021). However, other potential parasites such as microsporidians, of which the genus *Triwangia* has been described from atyid shrimps (Weng et al. 2022), have received little or no attention.

Microsporidians are eukaryotic obligate intracellular parasites with simple or complex life cycles (Stentiford et al. 2013). They infect all major animal groups, have zoonotic potential, and can cause important diseases in aquatic organisms (Stentiford et al. 2013). The spread of microsporidians as pathogens may occur via invasive species that introduce these parasites into new areas. For instance, the microsporidian *Cucumispora dikerogammari*, which was initially described in the invasive amphipod *Dikerogammarus villosus*, has spread to native amphipod species, leading to high mortality levels (Cormier et al. 2021).

We sampled *N. davidi* from the Finkelbach, a cold-water tributary of the thermally polluted River Erft, North Rhine-Westphalia, Germany, for the presence of microsporidian parasites. The present study provides new insights into parasites of this non-native shrimp. We also provide further evidence of an ongoing range expansion of *N. davidi* in European temperate streams.

2. MATERIALS AND METHODS

Neocaridina davidi took were sampled on 14 December 2021 at the Finkelbach (50.9808° N, 6.5781° E), a tributary of the River Erft located 25 km upstream of the nearest reported shrimp population (Klotz et al. 2013, Schoolmann & Arndt 2018; Fig. 1). *N. davidi* was first reported from the Gillbach in 2013, another tributary of the River Erft, and later from the lower Erft and the Rhine (Klotz et al. 2013, Schoolmann & Arndt 2018). The Finkelbach receives water from a communal wastewater treatment plant via the Elsdorfer Fließ. Upstream of the estuary of the Elsdorfer Fließ, the Finkelbach is dry and serves only as a drainage during heavy rain. For several decades, the Finkelbach, like most streams in this region, has not had contact with groundwater due to coal mining activities. Until 2009, the stream also directly received water from 3 wastewater treatment plants. Additionally, a sugar factory released warm water into the Elsdorfer Fließ until the

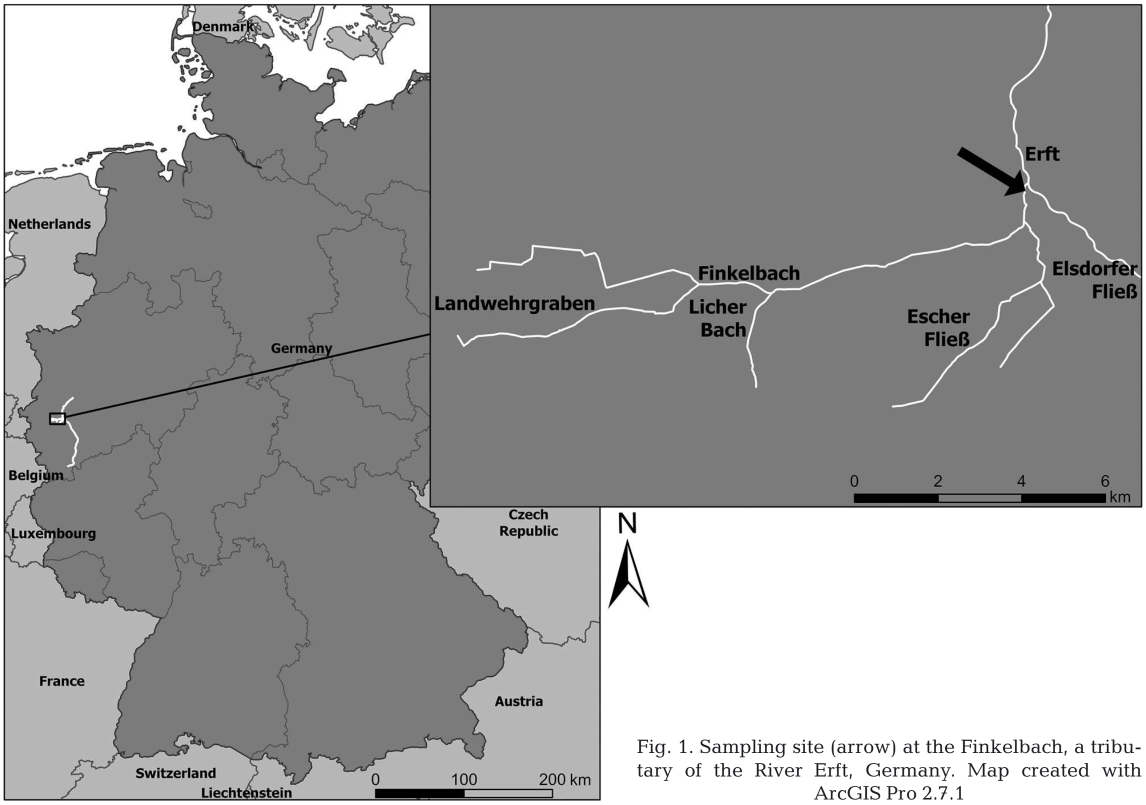

Fig. 1. Sampling site (arrow) at the Finkelbach, a tributary of the River Erft, Germany. Map created with ArcGIS Pro 2.7.1

early 2000s. Currently, there are irregular rinse-water discharges from the sugar factory and the local water company into the Elsdorfer Fließ (U. Rose pers. comm.). At the time of sampling, water temperature was 9°C, conductivity 620 ms cm^{-1}, dissolved oxygen 9.8 mg l^{-1}, and pH 7.65. Individuals of *N. davidi* were collected using dip nets and preserved in 96 % ethanol. Morphological identification, carapace length measurements, and parasitological analyses were performed in the laboratory. Additionally, a small sample of muscular tissue was retrieved for molecular identification of both host and microsporidian parasites.

DNA was isolated from muscular tissue using a modified salt precipitation protocol described by Grabner et al. (2015). Molecular identification of 3 randomly selected *N. davidi* individuals was achieved with the universal eukaryotic primers LCO1490 and HCO2198 (Folmer et al. 1994) and that of microsporidians with the universal microsporidian primers V1 and Micuni3R (Weigand et al. 2016). PCR products of infected amphipods were sent to Microsynth Seqlab (Göttingen, Germany) for sequencing using LCO1490 and V1 primers, respectively. Obtained sequences were compared against GenBank records using BLAST (blast.ncbi.nlm.nih.gov). Sequences were edited in Geneious version 2022.0.1 (Biomatters) and aligned using the MUSCLE algorithm, and maximum likelihood phylogenetic trees were produced in MEGA 11 version 11.0.8 (Tamura et al. 2021) using the K2+G substitution model for microsporidians and the T92+G substitution model for *N. davidi*.

3. RESULTS

A total of 75 adult shrimps (31 females and 44 males) were collected, including 1 ovigerous female. Morphological identification of *Neocaridina davidi* was confirmed by molecular identification. All 3 sequenced shrimp individuals belonged to the same haplotype (GenBank accession nos. OM468121, OM468122, OM468123) and showed 99.68 % similarity to *N. davidi* collected in Taiwan (MG734300). Altogether, PCR revealed that 4 shrimps (5.3 % prevalence) were infected with microsporidians. Three of those individuals (4 % prevalence), were infected with a microsporidian that was 99.56 % similar to *Enterocytozoon hepatopenaei* (EHP) isolates found in India, China, and Vietnam (MH260592, KX981865, and KP759285, respectively). However, only 2 (OM467903, OM467904) out of the 3 EHP sequences were of suitable quality to be used in our phylogenetic analyses and were consequently uploaded to GenBank. One of the 4 microsporidian-positive individuals (1.3 % prevalence) was infected with an unknown microsporidian isolate, referred to here as Microsporidium sp. EFB01 (OM467902) showing 85.24 % similarity to Microsporidium sp. BIVIC3 from lake Baikal (FJ756174) and 84.54 % similarity to the *Vittaforma*-like Microsporidium sp. Q2-DKR-D (MF374895) found in Taiwanese drinking water (Fig. 2). The most closely related and taxonomically characterized species is *V. corneae*, with 84.06 % similarity (MH351756). Reference sequences of the genera *En-*

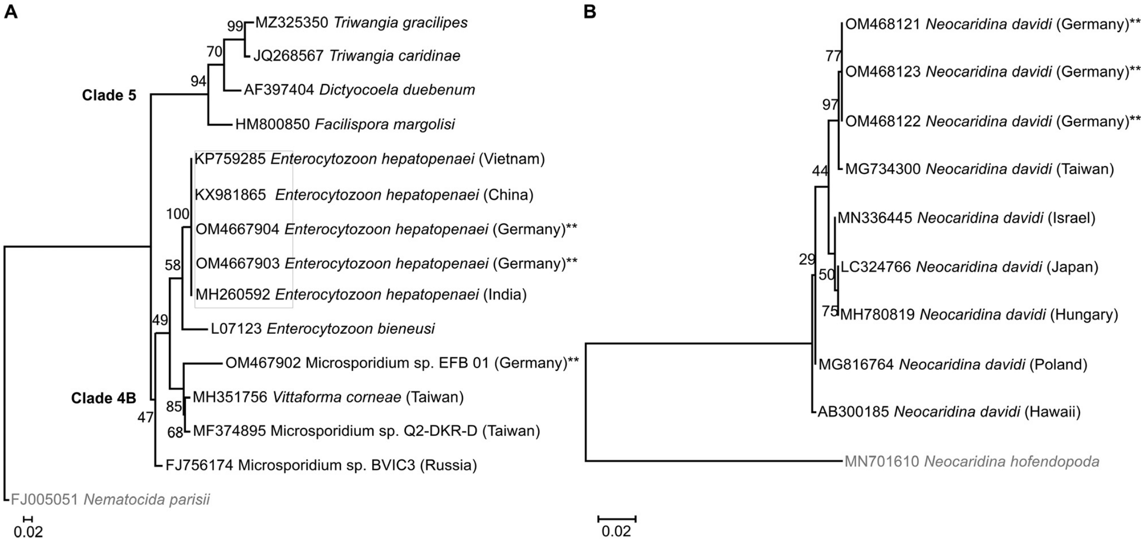

Fig. 2. Maximum likelihood phylogenetic tree with bootstrap values (999 replicates) of (A) microsporidian isolates (K2+G substitution model) and (B) *Neocaridina davidi* (T92+G substitution model). Outgroup sequences are indicated in grey font. **Specimens from this study

terocytozoon and *Vittaforma* all belong to the microsporidian Clade 4B (Park & Poulin 2021) (Fig. 2).

With an average carapace length of 4.04 ± 0.19 mm (SD), *N. davidi* individuals infected with EHP were smaller than uninfected individuals (5.47 ± 0.86 mm, Table 1). No epibionts or other parasites were found in or on *N. davidi*.

4. DISCUSSION

The release of ornamental pets and their associated parasites into novel environments can seriously threaten native biodiversity (Svoboda et al. 2017, Patoka et al. 2018). Despite that, there is a lack of knowledge on 'hitchhiking' pathogens, in particular concealed ones like microsporidians. The present study provides the first report on the occurrence of microsporidian parasites in non-native *Neocaridina davidi* from a German stream flowing into a thermally polluted river. To our knowledge, this is the first record of microsporidians in *N. davidi* to date.

The discovery of EHP in *N. davidi* is surprising, as this microsporidian is mainly known from brackish and marine water, where it has become a critical threat to the shrimp farming industry, particularly in Southeast Asia (Kim et al. 2022). However, EHP has been reported from waters with low to high salinity (2–40 ppt) (Aranguren Caro et al. 2021). Moreover, EHP is not limited to warm tropical and subtropical waters, as evidenced by its recent discovery in grow-out ponds located in the Korean province of Chungcheongnam-do, an area with climatic conditions similar to central Europe (Kim et al. 2022). Therefore, the survival of this parasite under German climatic conditions is plausible.

The occurrence of EHP and *Mirosporidium* sp. EFB01 in the Finkelbach is likely the result of co-introduction of *N. davidi* by aquarium owners in the Erft catchment. *N. davidi* is farmed intensively in ponds, mainly in southern Taiwan, in close proximity to the coast, an area known for intensive farming of penaeid shrimps such as *Litopenaeus vannamei* and *Penaeus monodon*. Penaeid shrimps such as *L. vannamei*, a euryhaline shrimp species, are common hosts for EHP (Aranguren Caro et al. 2021, Kim et al. 2022). Thus, it cannot be excluded that EHP might have inadvertently entered *N. davidi* ponds and infected shrimps destinated for aquarium trade export. Although EHP sequences from penaeid shrimp farmed in the same area are not available, the similarity with sequences found across Asia seems to support this transmission route. On the other hand, the origin of Microsporidium sp. EFB01 remains uncertain due to a low similarity to the best matching isolates currently hosted on GenBank.

The exclusive occurrence of EHP in small-sized *N. davidi* might be an indication of reduced growth of infected shrimps. Observations from brackish and marine shrimp farms indicate reduced feeding and retarded growth in infected shrimps (Kim et al. 2022). However, due to the small number of infected *N. davidi* individuals, this observation should be treated with caution. The prevalence of EHP in cultured shrimps such as *L. vannamei* typically ranges between 11 and 93 % (Shen et al. 2019). Therefore, it is likely that the low infection level observed in *N. davidi* is related to reduced water salinity, which might hinder EHP infectivity, as observed by Aranguren Caro et al. (2021). Alternatively, *N. davidi* might not constitute an ideal host for this parasite, constraining its reproduction and transmission rate. An analogy could be the case of *Cucumispora dikerogammari*, a microsporidian that typically infects the amphipod *Dikerogammarus villosus* but is also able to infect other crustacean species in which it can be found at low prevalences (Bacela-Spychalska et al. 2012).

Ectoparasites and epibionts are commonly reported from *N. davidi*, including non-native populations (Ohtaka et al. 2012, Patoka et al. 2016, Liao et al. 2018, Maciaszek et al. 2021). For instance, the prevalence of *Scutariella japonica* (Platyhelminthes: Temnocephalida) in native *N. davidi* populations was between 25.2 and 100 % (Ohtaka et al. 2012), while that of *Vorticella* sp., a peritrich ciliate, in non-native populations reached up to 82.5 % (Maciaszek et al. 2021). Their absence in our sample might be due to a very low prevalence in the study area or their inability to adapt to the local water parameters. Similarly, a previous study in the same catchment also did not detect epibionts (Klotz et al. 2013).

Table 1. Infection, sex, and carapace length of *Neocaridina davidi*

	n (prevalence [%])	Sex	Carapace length (mm) Mean ± SD	Range
Enterocytozoon hepatopenaei	3 (4.00)	1F/2M	4.04 ± 0.19	3.88–4.30
Microsporidium sp. EFB01	1 (1.33)	1F	4.29	4.29
Uninfected	71	29F/42M	5.47 ± 0.86	3.67–7.99
Total	75	31F/44M	5.40 ± 0.89	3.67–7.99

The presence of *N. davidi* in the cold water of the Finkelbach suggests a range expansion towards colder water. Despite the relatively cold temperature of 9°C, an ovigerous female was present among the collected shrimps, indicating that local reproduction even at low temperature seems possible. In a previous study, non-native *N. davidi* individuals were observed sporadically during winter at a water temperature of 6°C in a thermally polluted stream (Weiperth et al. 2019). However, no ovigerous females were found in that study. As *N. davidi* has been present in the Erft catchment for over a decade, it is likely that, over time, the gradual insurgence of cold-adapted individuals among the shrimp population allowed for an expansion towards temperate water, as predicted by Klotz et al. (2013). Thus, a further spread into nearby watercourses can be expected.

The range expansion of *N. davidi*, coupled with the presence of microsporidian parasites, is cause for concern, as the transmission potential of EHP and its pathogenicity for the native crustacean fauna are unknown. Given the scarcity of information, further investigations, including screening of the native fauna to assess the transmission potential of these pathogens, are recommended.

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Chapter IV

Prati, S., Grabner, D. S., Hupało, K., Weiperth, A., Maciaszek, R., Lipták, B., Bojko, J., Bérces, F., & Sures, B. (2023)

Invisible invaders: range expansion of feral *Neocaridina davidi* provides novel opportunities for generalist intracellular parasites.

[Manuscript submitted for publication]

Invisible invaders: range expansion of feral *Neocaridina davidi* offers new opportunities for generalist intracellular parasites

Prati, S., Grabner, D. S., Hupało, K., Weiperth, A., Maciaszek, R., Lipták, B., Bojko, J., Bérces, F., & Sures, B.

Abstract

The release of ornamental pets and associated pathogens outside their native range might directly or indirectly impact the recipient community. In temperate regions like central Europe, pet-traded freshwater species of tropical and sub-tropical origins are mainly constrained to thermally polluted waters and thermal springs. However, species with high environmental plasticity and reproduction rates, such as the shrimp *Neocaridina davidi*, may adapt to colder water regimes over time. Such thermal niche widening can eventually overcome thermal barriers, resulting in further range expansion that may also provide new transmission opportunities for host generalist parasites.

The present study assesses the range expansion of *N. davidi* and associated parasites in Europe. We report three newly established *N. davidi* populations from thermally polluted waters in central Europe (Germany, Hungary, and Slovakia) and provide further evidence of its range expansion into colder environments. We confirm the presence of the microsporidian parasite *Ecytonucleospora hepatopenaei* in feral *N. davidi* populations across Europe and expand the list of microsporidians found in this host from two to four. Furthermore, we provide the first evidence of parasite spillover from/to the invasive crayfish *Procambarus clarkii*. This discovery highlights the possibility of parasite exchange, which, coupled with an ongoing range expansion of *N. davidi*, may exacerbate the impact on native biota.

Keywords: Atyidae, DNA barcoding, *Ecytonucleospora hepatopenaei*, Invasive species, Ornamental trade, Shrimp diseases

Introduction

Aquatic species (e.g., fish, mollusks, and crustaceans) are commonly introduced into lakes and rivers worldwide, causing irreversible ecological damage (Gozlan and Newton 2009; Essl et al. 2020; Bernery et al. 2022). Introducing non-native species may negatively impact the recipient community by directly or indirectly affecting ecosystem functioning and biodiversity, even without establishment, e.g., via pathogen transmission (Roy et al. 2017; Foster et al. 2021; Bojko et al. 2021). Freshwater habitats are particularly prone to biological invasion, especially those experiencing degradation (Casatti et al. 2006; Gherardi 2007).

The introduction of a broad spectrum of non-native organisms in freshwater habitats is commonly associated with stocking, ballast water of ships, aquaculture, recreational angling, and ornamental pet trade (Cambray 2003; Savini et al. 2010; Capinha et al. 2013; Patoka et al. 2018; Bernery et al. 2022). The ornamental pet trade is a primary pathway for the introduction of non-native species and their associated symbionts (Padilla and Williams 2004; Duggan 2010; Lipták and Vitázková 2015; Patoka et al. 2016). Atyid shrimps (*Neocaridina* spp. and *Caridina* spp.) are among the most common organisms involved in the ornamental pet trade worldwide (Bláha et al. 2022). Thanks to their vibrant colors (Maciaszek et al. 2018) and their availability in the pet trade, their trade is constantly increasing, at the same time providing opportunities for illicit releases. Once released, low winter temperatures commonly prevent their survival and range expansion. However, thermally polluted waterbodies can provide ideal conditions for the permanent establishment of these invaders (Veselý et al. 2015; Weiperth et al. 2019).

Species with high environmental plasticity and fecundity, including the ornamental shrimp *Neocardina davidi,* are known to rapidly adapt to local conditions and become invasive (Blackburn et al. 2011). Water temperatures in the native habitat of *N. davidi* in Southeast Asia can range from 6°C in winter to 30°C in summer (Klotz et al. 2013). Those temperatures are higher than winter temperatures in central Europe; however, a warming climate might facilitate the establishment of this thermophilic species (Klotz et al. 2013; Jabłońska et al. 2018).

Established feral populations of *N. davidi* have been reported worldwide, including records from the USA, Japan, Israel, Germany, Poland, Hungary, and the French overseas territory of la Reunion (Klotz et al. 2013; PM 2017; Jabłońska et al. 2018; Levitt-Barmats et al. 2019; Weiperth et al. 2019). The effects of its introduction are known to be multi-dimensional. Reported ecological impacts of *N. davidi* are, for instance, the replacement of native shrimps with similar ecological niches (Onuki and Fuke 2022), alteration of meiofaunal assemblages (Weber and Traunspurger 2016), and changes in leaf-litter breakdown in invaded areas (Schoolmann and Arndt 2017). The high feeding rate of *N. davidi*, estimated to be over half of its body weight per day, implies that other less efficient native crustaceans relying on the same resources may be outcompeted (Schoolmann and Arndt 2017). However, the ecological impact of *N. davidi* on European native species has not yet been thoroughly investigated. Likewise, the risk posed by co-introducing symbionts associated with *N. davidi* remains unclear.

Neocaridina davidi hosts a wide range of commensals and parasites (Ohtaka et al. 2012; Liao et al. 2018; Bauer et al. 2021; Maciaszek et al. 2023), some of which have been co-introduced with *N. davidi* outside their native range (Niwa and Ohtaka 2006; Maciaszek et al. 2021b; Kakui and Komai 2022). Among them, *Ecytonucleospora (=Enterocytozoon) hepatopenaei* (EHP) (Wang et al. 2023), has been detected using molecular tools in a German population of feral *N. davidi* (Schneider et al. 2022). EHP is known also to infect cultured Penaeid shrimps (Chaijarasphong et al. 2021) but also infects freshwater, brackish, and marine invertebrates (Karthikeyan and Sudhakaran 2020; Krishnan et al. 2021; Munkongwongsiri et al. 2022; Wan Sajiri et al. 2023). Most recently, EHP has been reported in dragonflies with persisting infections in their terrestrial imago stage (Dewangan et al. 2023). Therefore, it is imperative to confirm the presence and extent of EHP and other microsporidian infections in feral *N. davidi* populations as they might pose a risk to native biota.

Thus, the main aims of this study are to assess the range expansion of *N. davidi* and its associated parasites in Europe, confirming the presence of EHP in the feral populations and testing experimentally the possibility of pathogen transmission between alien and native biota.

Materials & methods

Sampling sites

The sampling of *N. davidi* took place between September 2021 and May 2023 in multiple locations across Germany, Hungary, Slovakia, and Poland (Table 1, Fig. 1). Two catchments have been investigated in Germany: the Erft in North Rhine-Westphalia and the Blies in Saarland. The Erft is a tributary of the river Rhine with a long history of thermal pollution (Schoolmann and Arndt 2018). *Neocaridina davidi* was first reported from the Gillbach in 2013, a thermally polluted tributary of the river Erft, and later from the lower and upper Erft and the Rhine (Klotz et al. 2013; Schoolmann and Arndt 2018; Bierbach et al. 2022; Schneider et al. 2022). Two citizen scientists also reported sightings of *N. davidi* in the upper part of the Erft via the iNaturalist app (aymac 2021; drmichaelbraun 2021). The heated waters of the Gillbach, which are fed entirely by the cooling water of the nearby lignite-fired powerplant of Niederaußem, hosts a community of organisms commonly found in the pet trade (Klotz et al. 2013; Lukas et al. 2017b).

The second German catchment, the Blies, is a tributary of the River Saar, subject to post-mining water discharges (Franzaring 2022). Warm and saline waters from a former coal mine now used as a geothermal plant are released in one of its tributaries, the Sinnerbach. The heated water is discharged into a so-called 'water garden' for cooling, which flows into a nearby creek, the Klinkenbach, before converging into the Sinnerbach. The water garden is an artificial recreational area characterized by cloudy, saline waters (pH 7.5-7.9, DO 3.6-9 mg/l, 1850-1900 µs) and a temperature constantly above 26°C. The garden is home to a thriving community of ornamental pets, which includes Poecilidae, cichlids, and *Procambarus clarkii* (Lukas et al. 2017a). *Neocaridina davidi* was first filmed on-site in 2020 by a YouTuber (Krabbelkeller, 2020). From the outflow of the water garden into the Klinkenbach, the water temperature still remains above 20°C until the upper part to the Sinnerbach.

Two localities have been investigated in Hungary: the thermal water of Miskolctapolca and those of the Városliget Park in Budapest. In Miskolctapolca, the thermal waters of the Békás pond and the outflowing Hejő Creek, a tributary of the River Tisza, host a wide variety of ornamental fish, shrimp, crayfish, amphibians, and aquatic reptiles which have been extensively described in recent publications (Weiperth et al. 2019; Maciaszek et al. 2021a; Bláha et al. 2022). Here, *N. davidi* was first recorded in the autumn of 2017 and has maintained a stable population since then. Occasionally, shrimps have been observed to venture into the colder downstream water of Hejő Creek (<6°C) between late autumn and early spring (Weiperth et al. 2019). The second location, the thermal water of Városliget Park, comprises large and small thermal spring ponds. The warmest of the large ponds hosts pet-traded crayfish such as *P. clarkii*, *P. virginalis*, and five *Cherax* species (Weiperth et al. 2020). All ponds are interconnected and drain via a subsurface channel into the main arm of the Danube River. Among these is also Szent István spring pond, a small artificial pond at the base of a fountain fed by thermal water (28.9°C, pH 8.37, DO 3.1 mg/l, 796 µs).

In Slovakia, *N. davidi* was first observed in 2021 by Martin Dobrota, a citizen scientist in the upper part of the Čepčínsky Creek, a tributary of the river Turiec (Váh River Basin). The creek originates from a thermal spring and has a length of seven km. Its upper section hosts a population of *P. clarkii*, which likely escaped from one of Europe's largest aquaculture facilities focused on breeding exotic fish species, mollusks, and crustaceans for the aquarium pet trade (Lipták et al. 2023).

Neocaridina davidi has been present in Poland at least since 2003 in the Ciepły canal, a thermally polluted artificial channel in the lower section of the River Oder, near Gryfino (Jabłońska et al. 2018). The 3.5 km long channel receives heated water from the Dolna Odra Power Station cooling system. In its proximity, the annual mean temperature is 20.9°C (Maciaszek et al. 2021b). Besides *N. davidi,* several alien species, including pet trade-related ones, are well-established on-site (Jabłońska et al. 2018; Maciaszek et al. 2021b; Ondračková et al. 2023). However, in the summer of 2022, fish and mollusks living in the River Oder, including the artificial canal where *N. davidi* was previously collected, experienced unprecedented mass mortality caused by the toxic golden alga *Prymnesium parvum*, an alga associated with saline waters (Marchowski and Ławicki 2023).

Sampling collection and processing

Neocaridina davidi were collected using hand nets and immediately preserved in 96% ethanol. Additionally, the carapax, including a section of the hepatopancreas, of 50 freshly dissected individuals collected from an EHP-infected population (Kleine Erft, Germany) were fixed in Davidson's freshwater solution for 24h and later transferred to 70% ethanol for histological examination. The rest of the body was preserved in 96% ethanol. A total of 50 pet-traded *N. davidi* were later bought from different retailers and private keepers. These were euthanized by ice submersion until the absence of eye stalk responses. After death, they were cut in half lengthwise, one side preserved in 96% ethanol for molecular screening of parasites and the other homogenized for a subsequent infection experiment with isopods and amphipods.

Since other native and invasive crustaceans inhabiting the same environment as *N. davidi* may potentially share pathogens, we also collected local amphipods and isopods. All hosts were morphologically and molecularly identified, measured, and dissected in the laboratory. The hosts were screened for internal parasites, and the guts were removed to avoid microsporidian contamination in downstream molecular analyses. Additionally, small portions of preserved muscles from five *P.clarkii* individuals collected during another study (Lipták et al. 2023) were also molecularly screened for microsporidians. Epibionts were not analyzed as their conservation in ethanol-preserved specimens is generally poor.

Molecular analyses

DNA was isolated from hosts using either a modified salt precipitation protocol described by Grabner et al. (2015) or using a 10% solution of Chelex 100 resin (Bio-Rad Laboratories, Hercules, CA, USA) following the protocol described in Hupało et al. (2023). Host molecular identification was performed using the universal eukaryotic primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al. 1994) or LCO1490-JJ (5'-CHACWAAYCATAAAGATATYGG-3′) and HCO2198-JJ (5'-AWACTTCVGGRTGVCCAAARAATCA-3′) (Astrin and Stüben 2008). When possible, ten *N. davidi* individuals for each sampling point and each retailer were randomly chosen for molecular identification. Other crustaceans were identified following the same procedure.

Microsporidians presence in the examined hosts was initially screened with the universal microsporidian primers V1F (5′-CACCAGGTTGATTCTGCCTGAC-3′) (Zhu et al. 1993) and Micuni3R (5′-ATTACCGCGGMTGCTGGCAC-3′) (Weigand et al. 2016) targeting the small subunit ribosomal RNA (SSU rRNA) gene. Subsequently, infected individuals (i.e., bands clearly visible in the gel) were screened for EHP using a single PCR with the species-specific primers ENF779 (5′-CAGCAGGCGAAAATTGTCCA-3′) and ENR779 (5′-AAGAGATATTGTATTGCGCTTGCTG-3′) targeting the SSU rRNA gene (Tangprasittipap et al. 2013) and a nested PCR with the SWP1F (5′-TTGCAGAGTGTTGTTAAGGGTTT-3′), SWP1R (5′-CACGATGTGTCTTTGCAATTTTC-3′), SWP2F (5′-TTGGCGGCACAATTCTCAAACA-3′), and SWP2R (5′-GCTGTTTGTCTCCAACTGTATTTGA-3′) primers targeting the spore wall protein of EHP (Jaroenlak et al. 2016). All PCR reactions excluding those used for the LCO1490-JJ and HCO2198-JJ primer pair consisted of 20 µL composed of 10 µL of 2x AccuStart II PCR ToughMix (Quantabio), 1 µL of each primer (0.5 µM), 0.35 µL of 50x GelTrack Loading Dye (Quantabio), 6.65 µL MilliQ water and 1 µL of DNA template. For the LCO1490-JJ and HCO2198-JJ primer pair, the PCR reaction consisted of 20 µL assay with 10 µL of Dream-TaqTM Hot Start Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), 1.6 µL (5 uM) of each primer, 4.8 µL of nuclease-free water and 2 uL of DNA template per reaction. PCR settings for DNA fragment amplification varied between different primer sets and are described in detail in the Supplementary file (S1). PCR products were sent either unpurified to Microsynth Seqlab (Germany) or purified (LCO1490-JJ and HCO2198-JJ) to Eurofins Genomics (Cologne, Germany) for Sanger sequencing. Enzymatical purification was obtained with 10 U of Exonuclease I (ExoI) and 1 U of thermosensitive alkaline phosphatase (FastAP) (both Thermo Fisher Scientific) for 5 µL of PCR product with an incubation step at 37 °C for 25 min followed by an inactivation step at 85 °C for 15 min.

Raw sequences were quality-checked and edited using Geneious v2023.0.1 (Biomatters, Ltd., New Zealand). Only sequences with a minimum length of 200 bp were retained for the analyses. Obtained sequences were compared against GenBank records using BLASTN (blast.ncbi.nlm.nih.gov). Host and parasite sequences were separately aligned using the MAFFT v7.490 algorithm with standard settings (Katoh et al. 2019). Maximum likelihood phylogenetic trees with bootstrap support values (1000 replicates) were produced in IQ-Tree 2.2.0 (Minh et al. 2020) using the TIM3+F+G4 substitution model for microsporidians and the HKY+F+G4 substitution model for *N. davidi*. A haplotype network was inferred using the Minimum Spanning Network method in PopArt v1.7 (Leigh and Bryant 2015), whereas the visualization of *N. davidi* haplotypes for each area investigated was obtained with the R package mapmixture (Jenkins 2023). All the sequences generated in this study were submitted to NCBi GenBank (accession numbers OR610860-71 and OR613110-12 for hosts and OR616363-71 for microsporidians).

Histological analyses

Whole animal preparations were processed for histological analyses via initial fixation in Davidson's freshwater fixative (~48 hours) before being transferred into 70% ethanol. Only infected individuals (confirmed via molecular analyses) were used. These were infiltrated with paraffin wax in an automated tissue processor (ethanol-xylene substitute wax). Wax-infiltrated tissues were then embedded into wax blocks and sectioned at 4 µm to attain a single section per specimen through the center of the animal. Each section was then mounted upon a glass slide and stained with hematoxylin and eosin, following a standard de-wax and rehydration protocol. The slides were screened for pathogens using a Leica DM500 light microscope and photographed with the integrated camera system.

Infection experiment

Potential horizontal transmission of EHP from *N. davidi* to the native macroinvertebrates *Gammarus pulex* and *Asellus aquaticus* (co-habitants at the invasion site) was experimentally assessed. Three groups of ten *G. pulex* and ten *A. aquaticus* were transferred from the respective rearing tanks into the experimental tanks (acclimatization: 24 hours). These were lab-reared, microsporidian-free individuals (F2 of individuals collected in the Boye catchment, NRW, Germany). After that, homogenized tissues of five EHP-infected *N. davidi* (EHP positive using nested PCR and preserved at 4°C <24 hours) were provided as feed (inoculant) over three days to the treatment groups (two groups of ten individuals per species). In parallel, homogenized tissues of five EHP-free *N. davidi* individuals were fed to the control groups (one group of ten individuals per species). Each homogenized shrimp was diluted in 4 ml of water and evenly distributed among tanks on days one and three to minimize ammonia production. Both *G. pulex* and *A. aquaticus* were observed to feed on the material provided. After 20 days, all *G. pulex* and *A. aquaticus* were collected and molecularly screened for EHP following the procedure described in section 2.3.

All *G. pulex* and *A. aquaticus* were held in 3-litre containers illuminated daily for eight hours with 6500K LED bars. The container setup consisted of a thin layer of gravel barely covering the bottom, moss (*Vesicularia dubyana*), three alder leaves, and water from the respective rearing tank (17.1 °C, pH 7.92, 426 µs). No aeration or heating was used. The experimental tanks were left for four weeks before the experiment to reduce potential ammonia spikes when feeding homogenized *N. davidi* and avoid water changes that could reduce the number of infective spores in the tank.

Statistical analyses

Descriptive and statistical analyses were performed with the open-source software R (version 4.2.3) via the RStudio GUI (version 2023.06.0, RStudio Inc.). As females *N. davidi* generally grow larger than males, gender was accounted for when investigating size differences between sampling sites. For this, sites with less than four sexed individuals were excluded. These were Erft near Neuss, Gillbach at the power plant outflow, the lower and middle part of the water garden, and the garden outflow in the Klinkenbach. Afterward, a linear model followed by ANOVA with size as the dependent variable and sex and pooled locations as interacting independent variables was employed. Temperature-dependent sex ratio was investigated using a pairwise chi-square test based on temperature categories (<15, 15-19.99, 20-24.99, and >25°C) and using sexed individuals from all sampling sites. EHP infections in all feral *N. davidi* were investigated using a binomial generalized linear model (GLM) with infected/uninfected as the dependent variable and carapax size, sex (female, male, and unsexed), and temperature categories as independent variables. The results were reported as odd ratios. The model did not include water conductivity, a proxy for salinity, as this has not been consistently measured (Table 1). Body size and prevalence relationships among pet-traded *N. davidi* were not investigated as pet-traded individuals are sex and size-biased. Furthermore, confinement in restricted spaces (aquariums) might result in an unnatural high exposure to infective spores.

Results

A total of 658 shrimps were collected, all of which were *N. davidi*. Morphological identification of feral *N. davidi* was corroborated by molecular identification with five haplotypes detected, here named 'Ndh1', 'Ndh2', 'Ndh3', 'Ndh4', and 'Ndh5' (Fig. 2, Fig. 3). Ndh1 showed 100% similarity to feral *N. davidi* previously collected in Germany (OM468123). 'Ndh2' was 100% similar to feral and pet-traded individuals previously found in Poland (MG816764 and MG816765) and wild individuals from Taiwan (MG816764 and MG734262), while 'Ndh3' and 'Ndh5' had 100% similarity to wild Taiwanese samples (MG734280 and MG734258). 'Ndh4' was 100% similar to feral individuals previously collected in Hungary (OM214466).

Neocaridina davidi was present at all sites, including the Gillbach, where other pet-traded species disappeared, and the Ciepły canal, which was affected by the mass mortality event in the Oder during the summer of 2022. In the water garden (Blies catchment), *N. davidi* cooccurred with pet-traded species, including previously unreported fish species (*Ancistrus* sp., *Hemichromis* spp., *P. sphenops,* and *Xiphophorus maculatus*) as well as the crustacean *Procambarus virginalis*. In the Szent István spring pond *N. davidi* cooccurred with pet-traded fish (e.g., Poecilidae) and the invasive crayfish *P. virginalis*.

Among the *N. davidi* collected, 316 were females (119 of whom were ovigerous), 237 males, and 105 individuals of undetermined gender. With an average carapace length of 5.09±1.00 SD (range 3.06-7.99) mm, females were larger (ANOVA, $F(1, 504) = 164.59$, $p < 0.001$) than males 4.38±0.75 SD (3.01-6.38) mm. Overall, the size of sexed *N. davidi* varied greatly between sampling sites (ANOVA, $F(19, 504) = 15.36$, $p < 0.001$). Such differences were, however, influenced by differences in sex ratios across sampling sites (ANOVA, $F(17, 501) = 2.01$, $p = 0.001$). As the proportion of females and males was relatively similar across temperature categories (Chi-square test, $\chi^2(3, 553) = 6.88$, $p = 0.076$), there was no clear evidence of a temperature-dependent sex ratio. Populations of *N. davidi* living at temperatures between 15 and 20°C showed the highest proportion of ovigerous females (61.54%) and those living above 25°C the lowest (14.63%).

Among the investigated feral *N. davidi*, 5.62% were infected with microsporidians, mostly EHP (5.02%, Table 2). However, neither carapace length, sex (female, male, unsexed), nor temperature predicted EHP infections (all OR 95% CI ranged from below 1 to above 1). Half of the investigated feral population were infected with EHP, and the highest prevalence was found in the Erft catchment (9.30%). EHP was the only microsporidian found in pet-traded *N. davidi*, reaching 14% prevalence. EHP was not found in other macroinvertebrates collected in the wild, and no infected specimen was found in the infection experiment of *G. pulex* and *A. aquaticus* with EHP. The detection of EHP relied on molecular analyses. SSU rRNA sequences showed 99.9-100% similarity with those obtained from the prawn *Penaeus vannamei* (India, China, South Korea, and Vietnam) and polychaete worms *Marphysa* spp. collected in India. All infected *N. davidi* shared the same EHP haplotype (100% similarity). Likewise, EHP spore wall protein sequences belonged to a single haplotype, which showed 98.64% similarity to sequences obtained from *P. vannamei* collected in India, Thailand, and Malaysia. Histology of EHP-positive *N. davidi* revealed infected hepatopancreatic tubules, presenting eosinophilic cytoplasmic inclusions of early spore development as well as later mature spore stages (Fig. 4).

Besides EHP, three other microsporidian isolates, two of which were new to science, were detected in *N. davidi* (Table 2, Fig. 5). Those in Čepčínsky Creek were infected with an undescribed microsporidian isolate, here named Microsporidium sp. SCC01. The closest described species was *Enterocytospora artemiae* (JX915760; 83.23% identity, 99% coverage, e-value = 6e-80). The same microsporidian was also detected in two *P. clarkii* collected on site. One *N. davidi* collected from the Békás pond was infected with an undescribed isolate, here named Microsporidium sp. HMI01. The closest described species was *Jirovecia branchilis* (AF484694; 83.97% identity, 81% coverage, e-value = 1e-69). One individual from Finkelbach was infected with Microsporidium sp. EFB01 (OM467902; 99.50% identity, 69% coverage, e-value = 1e-94), a microsporidian isolate previously observed in *N. davidi* by Schneider et al. (2022).

Six other microsporidians were detected exclusively in amphipods or isopods (Table 2, Fig. 5). Among these were *Nosema* sp. clade B *sensu* Bacela-Spychalska et al. (2023) (OR165989; 99.60% identity, 100% coverage, e-value = 9e-174), *Dictyocoela roeselum* (MG773219; 100% identity, 100% coverage, e-value = 0.0), and Microsporidum sp.V-B (MK719363; 99.06% identity, 100% coverage, e-value = 5e-157) found in *G. roeselii,* Microsporidium sp. 505 (FN434085; 99.14% identity, 100% coverage, e-value = 5e-171) found in *G. pulex* and two undescribed microsporidians. The first of which was found in *G. roeselii* and here named Microsporidium sp. HMI02. The closest described species was *H. lacustris* (GU130406; 90.46% identity, 84% coverage, e-value = 6e-86). The second, here named Microsporidium EFB02, was found in *A.*

aquaticus. The closest described species was *Euplotespora binucleata* (DQ675604; 84.23% identity, 96% coverage, e-value = 2e-74). None of the other hosts were infected (Table 2).

Discussion

The release of ornamental pets and associated parasites into novel environments might pose severe risks to native biota (Sures 2011; Svoboda et al. 2017; Patoka et al. 2018). Deepening the knowledge of invading hosts and parasites is paramount, especially for commercially successful ornamental pets. Commercially successful ornamental pets, like *N. davidi*, are widely available, easy to breed, and often tolerant to a wide range of environmental variables. This makes them more likely to arrive and establish in new locations (Lipták and Vitázková 2015; Bláha et al. 2022). We present the discovery of three newly established *N. davidi* populations across Europe and mounting evidence of an ongoing range expansion into colder water. Furthermore, we confirm the presence of EHP infections in feral *N. davidi* as well as other microsporidian parasites, including one shared with the highly invasive crayfish *P. clarkii*, highlighting a possible parasite spillover.

The presence of feral *N. davidi* in central Europe dates back to around 2003, with the establishment of a population in Poland (Jabłońska et al. 2018). Afterward, more populations were discovered in Germany and Hungary (Klotz et al. 2013; Weiperth et al. 2019). The current study increased this list to three thermally polluted sites in Germany, Hungary, and Slovakia. The latter is the first report of this species in the country. The geographic distribution of feral *N. davidi*, as evidenced by their haplotypes, primarily reflects what is available in the pet trade of the respective countries. The haplotype 'Ndh2' was dominant in Poland, Slovakia, and the Hungarian population of Városliget Park, Budapest. The dominance of this haplotype in the eastern part of central Europe is unsurprising, as the pet market in these countries often shares similar suppliers. Accordingly, this haplotype has previously been reported from the Polish pet trade (Jabłońska et al. 2018), and the Slovakian *N. davidi* population may have escaped from the nearby aquaculture farm that supplies the Slovak, Hungarian, and Polish markets. On the other hand, 'Ndh1' and 'Ndh3' were only found in Germany, where these haplotypes are traded.

All investigated sites are characterized by different degrees of thermal and salt pollution. Interestingly, *N. davidi* seems to be relatively unaffected by perturbations in the thermal regime of cooling water systems. As seen for the Gillbach, where most ornamental species, except for *N. davidi*, perished following a temperature decrease due to the partial decommissioning of the powerplant in 2020 and 2021. Likewise, most ornamental species hosted in the water garden were also present in the lower warm part of the Klinkenbach until its confluence with the Sinnerbach, where cold and warm water mixed, with only *N. davidi* occurring in the colder downstream waters. Similarly resilient was the *N. davidi* population in the Ciepły Canal. This population survived oxygen deprivation associated with the algal explosion event that infested the saline waters of the Oder River in the summer of 2022 (Marchowski and Ławicki 2023).

Temperature did not strongly influence *N. davidi* sex ratios. This contradicts previous experimental findings in which the sex ratio appears to be heavily influenced by temperature (Serezli et al. 2017). However, such experimental studies do not take predation into account. Females are larger and often more colorful than males, hence more visible to predators, which might result in a more balanced sex ratio. This might hold particularly true for water nearing 20°C, in which 80% of the population is expected to be composed of females (Serezli et al. 2017). Ideal breeding conditions seem to be found between 15 and 20°C, as shown by the large proportion of ovigerous females. However, ovigerous females were also observed at temperatures below 10°C, suggesting that reproduction in these waters is not limited to the summer season. This finding indicates that species' range expansion to colder waters might be possible in the upcoming years. A range expansion of *N. davidi* into colder waters is unsurprising; previous studies have postulated such a prediction (Klotz et al. 2013; Schneider et al. 2022). Although considered thermophilic, *N. davidi* is not strictly a tropical species. In truth, its native habitat includes waters ranging from 6 °C in winter to 30 °C in the summer (Klotz et al. 2013). Winter temperatures are 2-4°C higher than those registered in central European water bodies, unaffected by thermal pollution, but similar to those reported from thermally polluted rivers such as the River Rhine, Europe's seventh-longest and busiest waterway (Wetzel 2001; van Vliet et al. 2011), known for its enormous proportion of invasive species (Sures et al. 2019). Nevertheless, a rise in water temperatures, an increase in the frequency and duration of heat waves, and a decrease in recurring cold spells during winter facilitate the acclimatization of thermophilic species (van Vliet et al. 2013; Jabłońska et al. 2018; Woolway et al. 2021). The critical thermal minima and the lower incipient lethal temperature in thermophilic freshwater decapods depend on acclimation time and temperature (Díaz Herrera et al. 1998; Kır and Kumlu 2008). Since *N. davidi* has been present in Europe for two decades, it is likely that, over time, a gradual insurgence of cold-adapted individuals among the established populations may result in a thermal niche expansion. Such adaptations are not unexpected, as shown by *Neocaridina denticulata* inhabiting a Korean stream where temperatures drop below 5°C in winter (Oh et al. 2003). Furthermore, hybrids between *N. denticulata*, *N. davidi,* and *N. palmata* are found in nature (Onuki and Fuke 2022) and in the pet trade, as suggested by the relatively high degree of genetic variability in feral *N. davidi* across the globe. If one of the parent populations was genetically better adapted to the cold, it could be assumed that there is still some adaptation in the gene pool of the *N. davidi* traded today, which allows for an expansion of the thermal niche.

An expansion of *N. davidi* into colder water might also result in the spread of associated pathogens. *Neocaridina davidi* is a known host for a wide range of commensals and parasites (Ohtaka et al. 2012; Liao et al. 2018; Bauer et al. 2021; Maciaszek et al. 2023), some of which have been co-introduced outside their native range (Niwa and Ohtaka 2006; Maciaszek et al. 2021b; Kakui and Komai 2022; Schneider et al. 2022). Among these, the microsporidian EHP, previously detected in a German population of feral *N. davidi* (Schneider et al. 2022), can be included. This parasite has been reported from a variety of invertebrate hosts and can be considered host generalist (Karthikeyan and Sudhakaran 2020; Krishnan et al. 2021; Munkongwongsiri et al. 2022; Wan Sajiri et al. 2023). The presence of EHP-infected feral *N. davidi* in sites with different temperatures and salinity indicates that this parasite is resilient to various environmental conditions. Accordingly, EHP is tolerant to salinity between 2 and 40 ppt, albeit its transmission at low salinity is reduced (Aranguren Caro et al. 2021), and its distribution range spans from tropical to temperate areas (Kim et al. 2022). Considering water salinization across Europe, bolstered by industrial discharges and droughts, EHP's prevalence might increase in the near future.

The spread of EHP into European water can be attributed to pet trade dynamics as the vast majority of *N. davidi* sold on the global market are imported from Taiwan due to cheaper production costs (Maciaszek et al. 2018). Taiwanese farms are located in the southern part of the island in close proximity to the coast, an area also known for the intensive farming of *L. vannamei*, the primary host for EHP (Aranguren Caro et al. 2021; Kim et al. 2022). This area heavily relies on groundwater for aquaculture, a water that is subject to salinization due to seawater intrusion (Dibaj et al. 2020). Water salinization coupled with intensive farming enhances the infectivity of EHP (Aranguren Caro et al. 2021); consequently, a higher load of spores is available in the environment. Thus, EHP spores might spread across aquaculture facilities via water movement and flying insects reaching *N. davidi* farms. Accordingly, dragonflies, often abundant near shrimp farms, are a suitable host for EHP (Dewangan et al. 2023). Infected *N. davidi* may then end up unnoticed in the global market, favored by loose regulations and a lack of relevant information.

EHP was not detected in amphipods and isopods collected from the field nor in *G. pulex* and *A. aquaticus* subjected to experimental infection. However, it is not excluded that EHP, as seen for parasites found in other invasive crustaceans (Bacela-Spychalska et al. 2012; Svoboda et al. 2017), may spread to native biota under favorable transmission conditions. The ongoing rise in temperature and salinization of freshwater ecosystems is likely to bolster the spread of *N. davidi* and the transmissibility of EHP, enhancing the availability of propagules that might infect native biota. These might become infected via predation of EHP-infected *N. davidi* or by inadvertently ingesting spores while feeding on organic matter. On the other hand, parasite spillover seems to have occurred between *N. davidi* and the invasive crayfish *P. clarkii*, as they shared a common microsporidian. Given the distant relationship between the two hosts, we can assume that this parasite is of a generalist nature. The other two microsporidians found in *N. davidi* were not detected in other biotas. Nevertheless, the number of microsporidians found in this host doubled compared to a previous study (Schneider et al. 2022), and it is likely to grow further with the current range expansion of feral *N. davidi.* Moreover, it is also possible that other parasites like trematodes, such as Plagiorchidae, which are common in central Europe, might find in *N. davidi* a suitable host, as seen for *N. denticulata* in its native range (Okabe and Shibue 1952).

To conclude, the range expansion of *N. davidi* in Europe is ongoing and is likely to continue in the future, supported by warming temperatures. The increasing presence of feral *N. davidi* and ongoing water salinization presents unique opportunities for the spread of EHP and potentially other host generalist parasites to native biota, leading to unpredictable outcomes. Therefore, it is imperative to closely monitor the expansion of feral *N. davidi* and undertake comprehensive experimental infection studies on a broader range of potential native hosts using various environmental conditions, spore concentrations, and exposures.

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Tables

Table 1 Sampling areas and sampling sites in which *N. davidi* were collected, including temperature and conductivity measured at the date of collection.

Sampling area	Sampling sites	Coordinates	Date	Temperature [°C]	Conductivity [µs/cm]
Erft catchment (DE)	Erft in Bergheim	50.966607, 6.613078	26/10/2021	21.6	750
	Erft near Neuss	51.168308, 6.704281	26/10/2021	17.6	856
	Kleine Erft near industrial outflow	50.942189, 6.659522	24/02/2022	22.1	-
	Kleine Erft near industrial outflow	50.942189, 6.659522	24/11/2022	18.4	1068
	Finkelbach	50.980763, 6.578115	14/12/2021	9.0	620
	Finkelbach	50.980763, 6.578115	24/02/2022	9.7	-
	Gillbach 200 m below powerplant outflow	50.997865, 6.661693	26/10/2021	21.2	1470
	Gillbach 3 km below powerplant outflow	51.014611, 6.684583	26/10/2021	19.8	1470
	Gillbach 11 km below powerplant outflow	51.089833, 6.688056	26/10/2021	16.7	1542
Blies catchment (DE)	Water garden near geothermal plant outflow	49.351179, 7.114634	16/09/2021	26.5	1850
	Water garden 300 m below geothermal outflow	49.352081, 7.119069	16/09/2021	28.8	1900
	Water garden 500 m below geothermal outflow	49.352328, 7.121528	16/09/2021	28.2	1870
	Klinkenbach 750 m below geothermal outflow	49.353775, 7.123450	16/09/2021	28.6	2000
	Klinkenbach 750 m below geothermal outflow	49.353775, 7.123450	03/02/2022	29.3	2274
	Klinkenbach 1 km below geothermal outflow	49.354401, 7.126394	03/02/2022	27.7	2134
	Sinnerbach 2.8 km below geothermal outflow	49.352848, 7.146605	03/02/2022	21	1775
	Sinnerbach 3.7 km below geothermal outflow	49.349784, 7.158135	04/03/2023	20.5	1852
	Blies 5 km below geothermal plant outflow	49.350809, 7.170278	04/03/2023	13.2	1076
Miskolctapolca (HU)	Békás thermal pond	48.062361, 20.747833	29/11/2022	24.6	551
	Hejő Creek near thermal pond outflow	48.062444, 20.748389	29/11/2022	14.8	536
	Hejő Creek 100 m below thermal pond outflow	48.063361, 20.749611	29/11/2022	13.1	551
	Hejő Creek 900 m below thermal pond outflow	48.068194, 20.756861	29/11/2022	11.1	554
Városliget Park (HU)	Szent István thermal spring pond	47.518364, 19.084177	21/05/2023	28.9	796
Čepčínsky Creek (SK)	200 m below thermal spring	48.861667, 18.838556	08/05/2023	20.2	-
	1 km below thermal spring	48.867944, 18.835306	08/05/2023	15.7	-
Dolna Odra (PL)	Ciepły canal 600 m below powerplant outflow	53.211742, 14.467465	27/05/2023	27.4	735
	Ciepły canal 1 km below powerplant outflow	53.215342, 14.470639	27/05/2023	24.0	740

Table 2 Prevalence of microsporidians in different crustacean species, including pet traded decapods. The Genbank accession number of each host is indicated in the bracket after the host name.

Host (Genbank accession number)	**Pooled sample**	**N**	**Prevalence %**	**Microsporidian**
Asellus aquaticus (OR613110)	Blies catchment (DE)	4	0	
	Erft catchment (DE)	35	2.86 (1/35)	Microsporidium sp. EFB02
	Miskolctapolca (HU)	2	0	
	Dolna Odra (PL)	4	0	
Crangonyx pseudogracilis (OR610871)	Erft catchment (DE)	31	0	
Dikerogammarus haemobaphes (OR610868)	Dolna Odra (PL)	13	0	
Dikerogammarus villosus (OR610869)	Erft catchment (DE)	30	0	
Gammarus fossarum (OR610870)	Blies catchment (DE)	4	0	
Gammarus pulex (OR610867)	Blies catchment (DE)	18	27.78 (5/18)	Microsporidium sp. 505
Gammarus roeselii (OR613111)	Erft catchment (DE)	93	1.08 (1/93)	Microsporidium sp. V-B
			33.33 (31/93)	*Nosema* sp. clade B
	Miskolctapolca (HU)	101	2.97 (3/101)	*Dictyocoela roeselum*
			4.95 (5/101)	Microspordium sp. HMI02
			0.99 (1/101)	*Nosema* sp. clade B
Gammarus tigrinus (OR610864)	Dolna Odra (PL)	5	0	
Neocaridina davidi (OR610860-65)	Blies catchment (DE)	140	5.00 (7/140)	*Ecytonucleospora hepatopenaei*
	Čepčínsky Creek (SK)	110	1.82 (2/110)	Microsporidium sp. SCC01
	Erft catchment (DE)	193	9.30 (18/193)	*Ecytonucleospora hepatopenaei*
			0.52 (1/193)	Micorsporidium sp. EFB01
	Dolna Odra (PL)	36	0	
	Miskolctapolca (HU)	168	4.76 (8/168)	*Ecytonucleospora hepatopenaei*
			0.59 (1/168)	Microspordium sp. HMI01
	Városliget Park (HU)	11	0	
	Pet trade (DE)	50	14.00 (7/50)	*Ecytonucleospora hepatopenaei*
Pontogammarus robustoides (OR610866)	Dolna Odra (PL)	8	0	
Procambarus clarkii (OR613112)	Čepčínsky Creek (SK)	5	40.00 (2/5)	Microsporidium sp. SCC01

Figure Captions

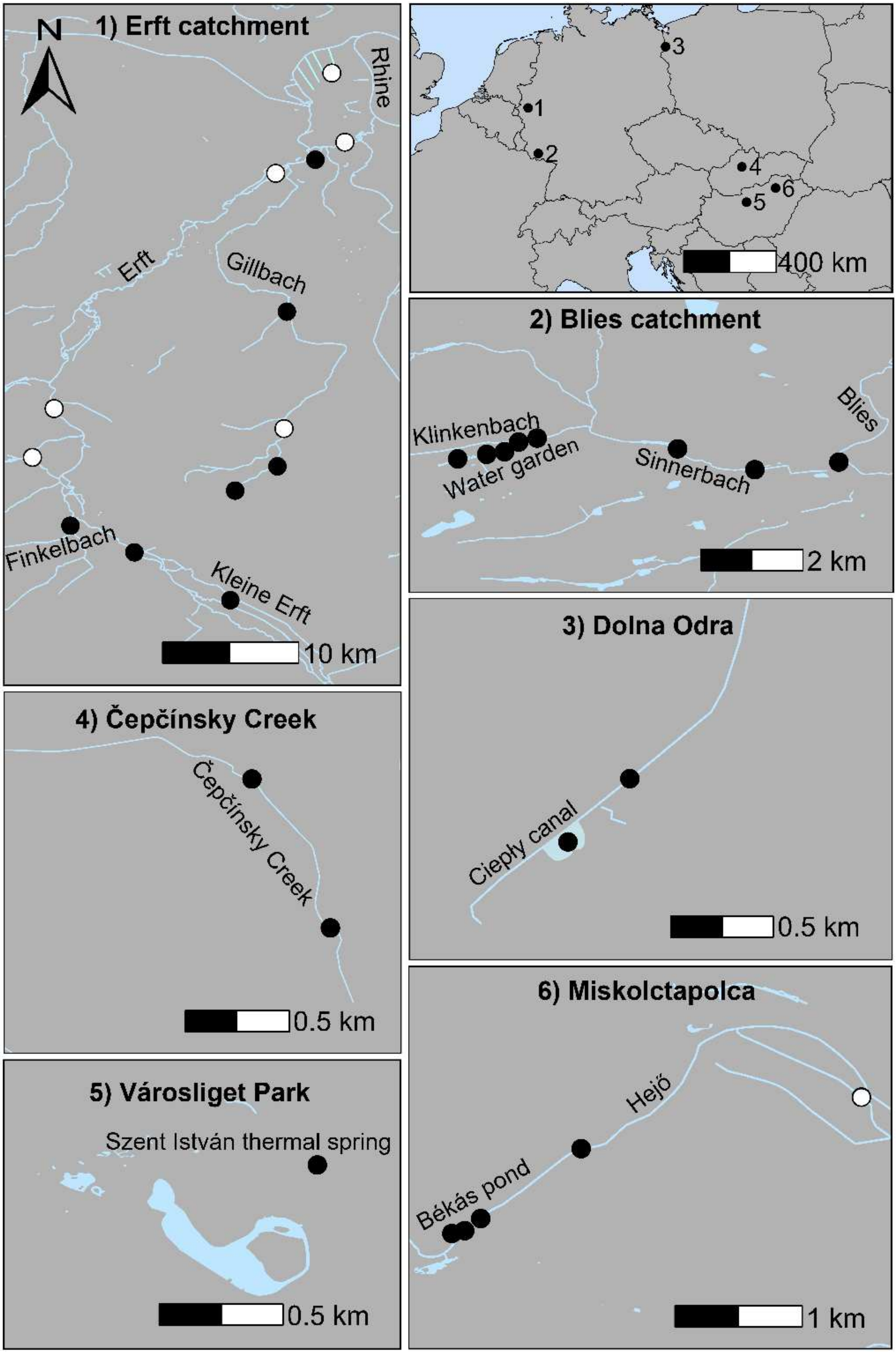
Fig. 1 Map of sampling areas where *Neocaridina davidi* individuals were collected. Black dots indicate sites visited in the present study. White dots indicate additional areas in which *N. davidi* has been observed in previous studies or reported by citizen scientists via the iNaturalist app. The maps were generated using ArcGIS with data extracted from OpenStreetMap.

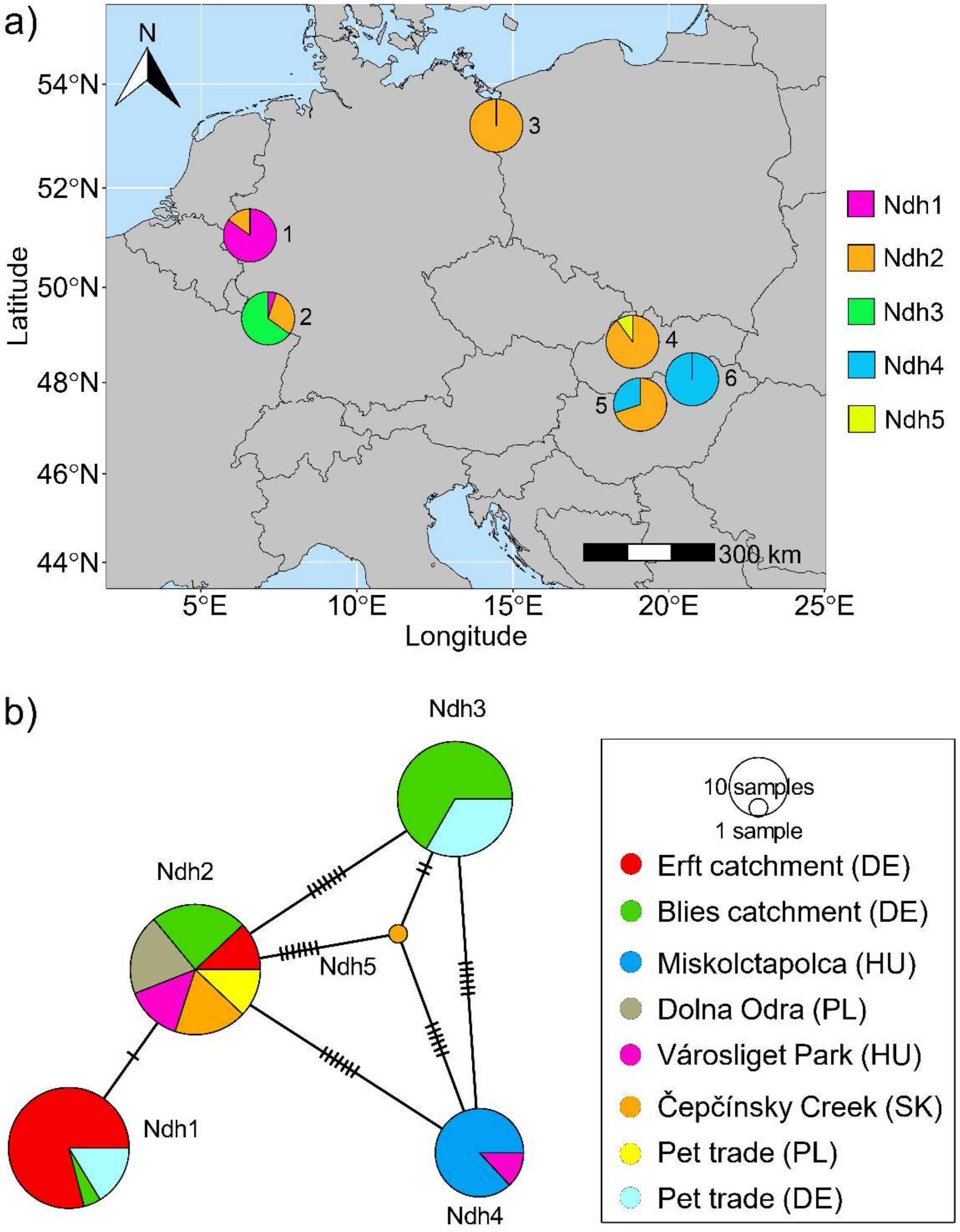
Fig. 2 a) Map of feral *Neocaridina davidi* haplotypes ('Ndh1' to 'Ndh5') across Europe and b) Minimum Spanning haplotype network including pet traded individuals. 1) Erft catchment, 2) Blies catchment, 3) Dolna Odra, 4) Čepčínsky Creek, 5) Városliget Park, and 6) Miskolctapolca.

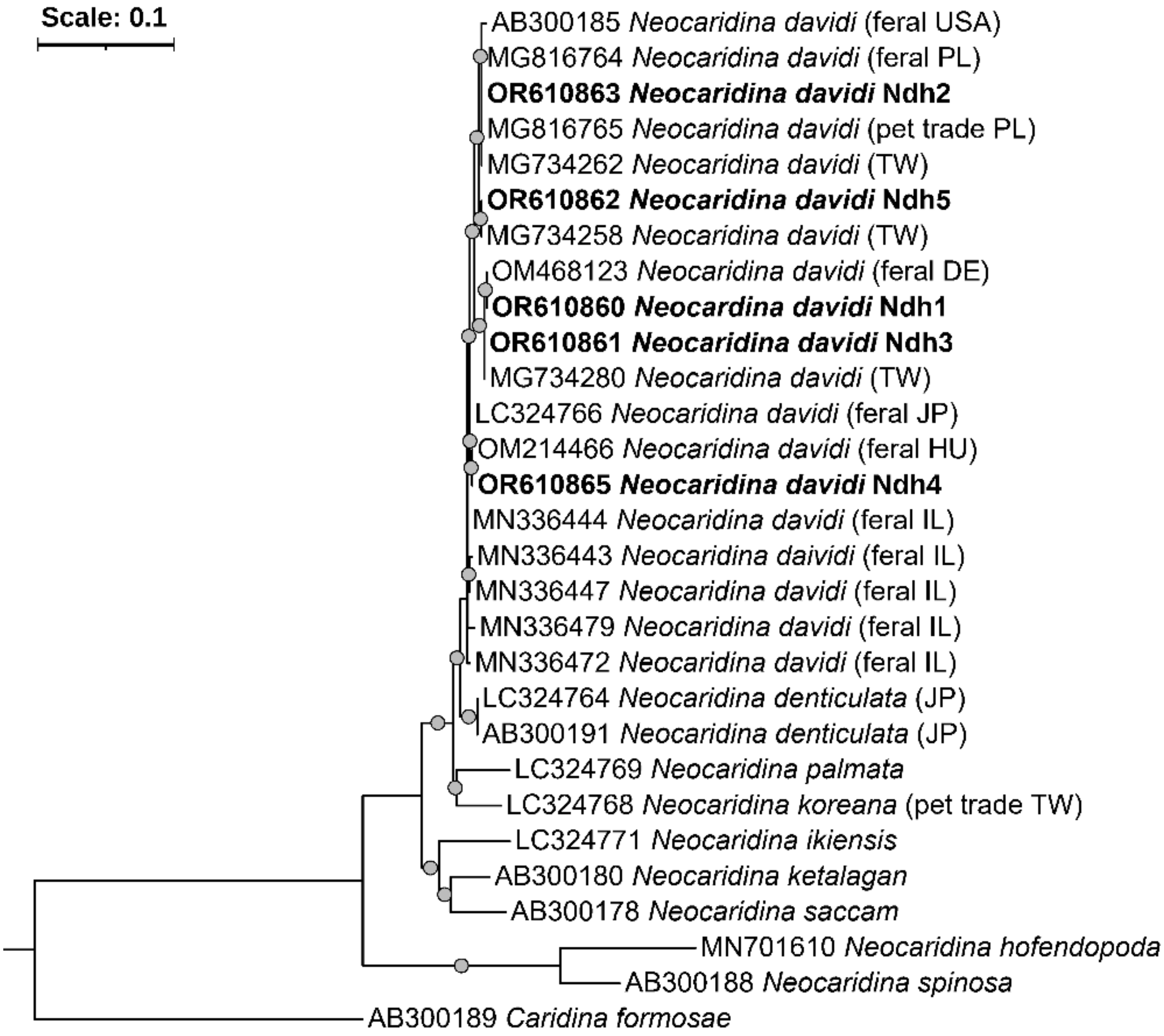
Fig. 3 Maximum likelihood phylogenetic tree of feral *Neocaridina davidi*. Grey dots represent bootstrap support values above 90%. Sequences obtained in this study are indicated in bold, and the outgroup sequence is highlighted in grey.

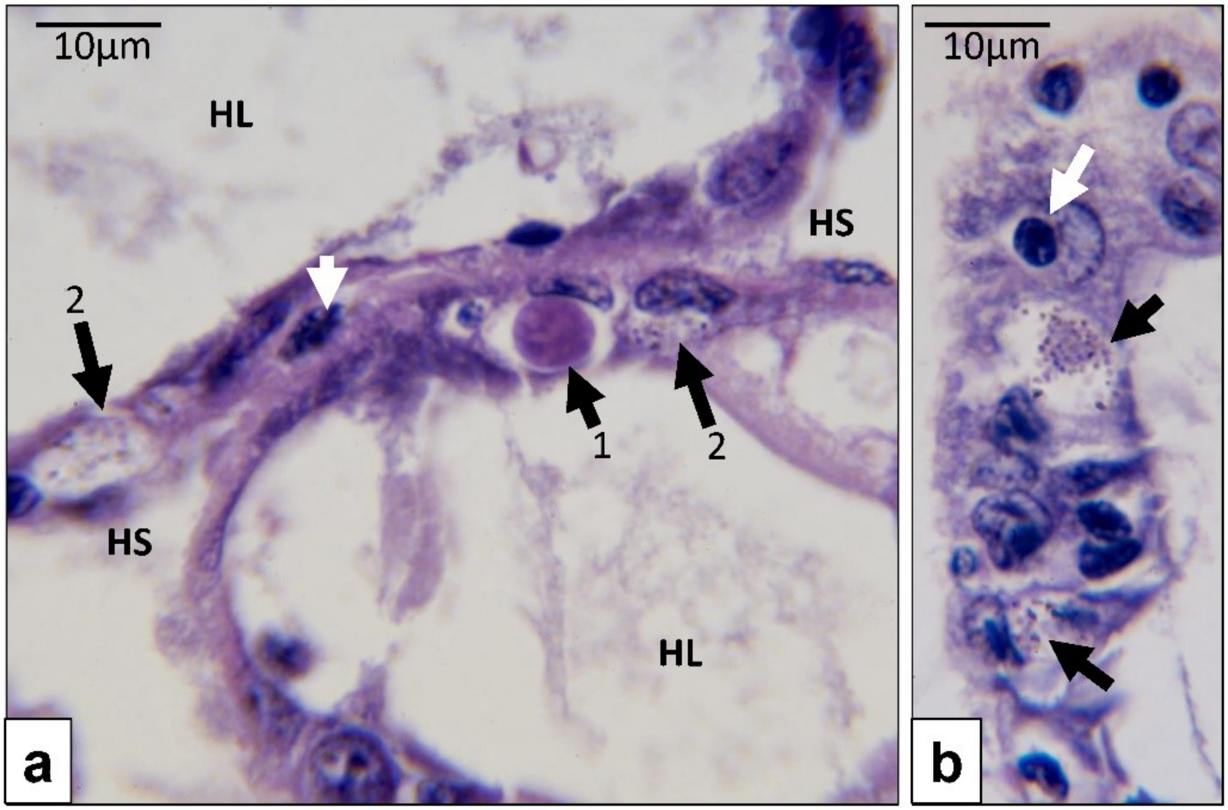
Fig. 4 Histopathological images of the infected hepatopancreas of *Neocaridina davidi*, revealing cytoplasmic inclusions in the hepatopancreatic epithelial alpha and beta cells. A) The relative morphology of the cytoplasmic inclusions reflects different development stages (reflecting the putative sporophorous vesicle, arrow 1; and infective spore stages, arrow 2). B) Cytoplasmic inclusions of microsporidian spores (black arrows) within the hepatopancreatic epithelia. The white arrows indicate example host nuclei. HL – hepatopancreatic tubule lumen, HS – haemal sinus.

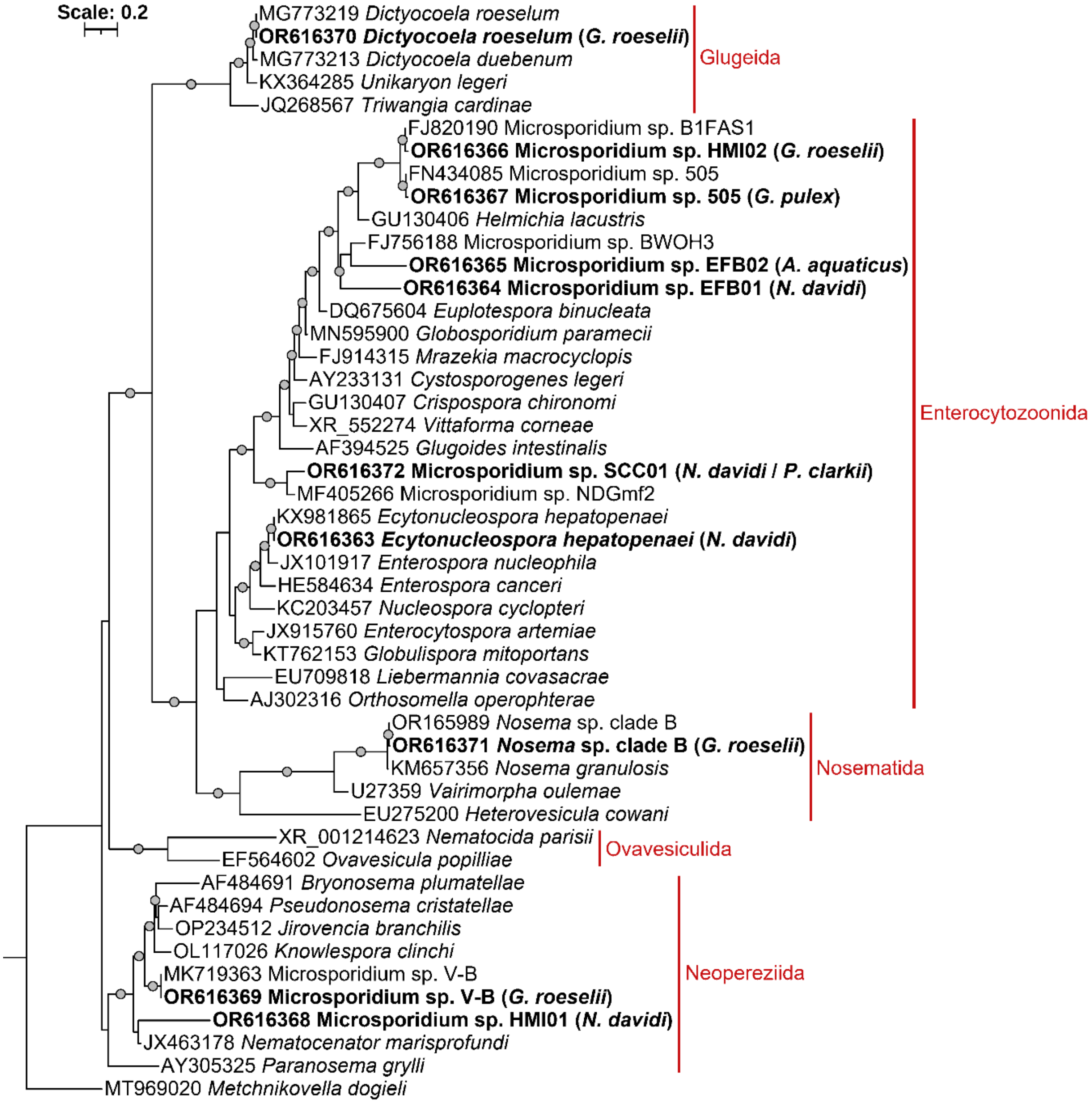
Fig. 5 Maximum likelihood phylogenetic tree of microsporidians identified in this study. Grey dots represent bootstrap support values above 90%. Sequences obtained in this study are indicated in bold, and the outgroup sequence in grey. The names and circumscriptions of microsporidian clades sensu Bojko et al. (2022) are indicated in red.


1) Erft catchment
N
Rhine
Erft
Gillbach
Finkelbach
Kleine Erft
10 km
3
1
2
4
6
5
400 km
2) Blies catchment
Klinkenbach
Water garden
Sinnerbach
Blies
2 km
3) Dolna Odra
Ciepły canal
0.5 km
4) Čepčínsky Creek
Čepčínsky Creek
0.5 km
5) Városliget Park
Szent István thermal spring
0.5 km
6) Miskolctapolca
Hejő
Békás pond
1 km


a)
N
54°N
52°N
50°N
48°N
46°N
44°N
Latitude
5°E
10°E
15°E
20°E
25°E
Longitude
1
2
3
4
5
6
300 km
Ndh1
Ndh2
Ndh3
Ndh4
Ndh5
b)
Ndh3
Ndh2
Ndh5
Ndh1
Ndh4
10 samples
1 sample
Erft catchment (DE)
Blies catchment (DE)
Miskolctapolca (HU)
Dolna Odra (PL)
Városliget Park (HU)
Čepčínsky Creek (SK)
Pet trade (PL)
Pet trade (DE)


Scale: 0.1
AB300185 *Neocaridina davidi* (feral USA)
MG816764 *Neocaridina davidi* (feral PL)
OR610863 *Neocaridina davidi* Ndh2
MG816765 *Neocaridina davidi* (pet trade PL)
MG734262 *Neocaridina davidi* (TW)
OR610862 *Neocaridina davidi* Ndh5
MG734258 *Neocaridina davidi* (TW)
OM468123 *Neocaridina davidi* (feral DE)
OR610860 *Neocardina davidi* Ndh1
OR610861 *Neocaridina davidi* Ndh3
MG734280 *Neocaridina davidi* (TW)
LC324766 *Neocaridina davidi* (feral JP)
OM214466 *Neocaridina davidi* (feral HU)
OR610865 *Neocaridina davidi* Ndh4
MN336444 *Neocaridina davidi* (feral IL)
MN336443 *Neocaridina daividi* (feral IL)
MN336447 *Neocaridina davidi* (feral IL)
MN336479 *Neocaridina davidi* (feral IL)
MN336472 *Neocaridina davidi* (feral IL)
LC324764 *Neocaridina denticulata* (JP)
AB300191 *Neocaridina denticulata* (JP)
LC324769 *Neocaridina palmata*
LC324768 *Neocaridina koreana* (pet trade TW)
LC324771 *Neocaridina ikiensis*
AB300180 *Neocaridina ketalagan*
AB300178 *Neocaridina saccam*
MN701610 *Neocaridina hofendopoda*
AB300188 *Neocaridina spinosa*
AB300189 *Caridina formosae*


10µm
HL
HS
2
1
2
HS
HL
a
10µm
b


Scale: 0.2
MG773219 Dictyocoela roeselum
OR616370 Dictyocoela roeselum (G. roeselii)
MG773213 Dictyocoela duebenum
KX364285 Unikaryon legeri
JQ268567 Triwangia cardinae
Glugeida
FJ820190 Microsporidium sp. B1FAS1
OR616366 Microsporidium sp. HMI02 (G. roeselii)
FN434085 Microsporidium sp. 505
OR616367 Microsporidium sp. 505 (G. pulex)
GU130406 Helmichia lacustris
FJ756188 Microsporidium sp. BWOH3
OR616365 Microsporidium sp. EFB02 (A. aquaticus)
OR616364 Microsporidium sp. EFB01 (N. davidi)
DQ675604 Euplotespora binucleata
MN595900 Globosporidium paramecii
FJ914315 Mrazekia macrocyclopis
AY233131 Cystosporogenes legeri
GU130407 Crispospora chironomi
XR_552274 Vittaforma corneae
AF394525 Glugoides intestinalis
OR616372 Microsporidium sp. SCC01 (N. davidi / P. clarkii)
MF405266 Microsporidium sp. NDGmf2
KX981865 Ecytonucleospora hepatopenaei
OR616363 Ecytonucleospora hepatopenaei (N. davidi)
JX101917 Enterospora nucleophila
HE584634 Enterospora canceri
KC203457 Nucleospora cyclopteri
JX915760 Enterocytospora artemiae
KT762153 Globulispora mitoportans
EU709818 Liebermannia covasacrae
AJ302316 Orthosomella operophterae
Enterocytozoonida
OR165989 Nosema sp. clade B
OR616371 Nosema sp. clade B (G. roeselii)
KM657356 Nosema granulosis
U27359 Vairimorpha oulemae
EU275200 Heterovesicula cowani
Nosematida
XR_001214623 Nematocida parisii
EF564602 Ovavesicula popilliae
Ovavesiculida
AF484691 Bryonosema plumatellae
AF484694 Pseudonosema cristatellae
OP234512 Jirovencia branchilis
OL117026 Knowlespora clinchi
MK719363 Microsporidium sp. V-B
OR616369 Microsporidium sp. V-B (G. roeselii)
OR616368 Microsporidium sp. HMI01 (N. davidi)
JX463178 Nematocenator marisprofundi
AY305325 Paranosema grylli
Neopereziida
MT969020 Metchnikovella dogieli

Supplementary information

PCR settings for COI amplification with the LCO1490 and HCO2198 primers were as follows: initial denaturation at 94 °C for 60 s, five cycles of denaturation at 94 °C for 60 s, annealing at 45 °C for 90 s and extension at 68 °C for 90 s, followed by 40 cycles at 94 °C for 60 s, 50 °C for 90 s, 68 °C for 60 s, with a final extension at 72 °C for 5 min. Those used for the LCO1490-JJ and HCO2198-JJ primers were as follows: initial denaturation at 94 °C for 60 s; five cycles at 94 °C for 30 s, 45 °C for 90 s and 72 °C for 60 s, followed by 35 cycles with 94 °C for 30 s, 51 °C for 90 s and 72 °C for 60 s, with a final extension at 72 °C for 5 min. PCR settings for SSU rRNA amplification of microsporidians with the universal primers V1F and Micuni3R were as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles at 94°C for 35 s and 68°C for 40 s, with a final extension at 68 °C for 5 min. Those for the EHP-specific primers ENF779 and ENR779 were as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles at 94°C for 20 s, 58°C for 20 s, 72°C for 45 s, with a final extension at 72°C for 5 min. The PCR setting for the amplification of the spore wall protein of EHP (SWP1F - SWP1R and SWP2F - SWP2R primer pairs) were as follows: first step PCR with initial denaturation at 95°C for 5 min followed by 30 cycles at 95°C for 30 s, 58°C for 30 s, 68°C for 45 s, with a final extension at 68°C for 5 min; second step initial denaturation at 95°C for 5 min followed by 20 cycles at 95°C for 30 s, 64°C for 30 s, 68°C for 20 s, with a final extension 68°C 5 min.

General Discussion

Freshwater ecosystems are vulnerable to biodiversity loss resulting from multiple stressors such as climate change, pollution, flow alteration, habitat destruction or degradation, and the release of invasive species (Bellard et al., 2012; Birk et al., 2020; Dudgeon et al., 2006). Mounting efforts to reverse biodiversity loss through ecosystem restorations are often hampered by complex interactions within living organisms and their responses to anthropogenic stressors (Birk et al., 2020; Lorenz et al., 2018). Thus, understanding less investigated aspects, such as host-parasite interactions, is particularly relevant when considering the staggering diversity of ubiquitous but neglected parasites in keystone and invasive species.

Amphipods are keystone species and the most common macroinvertebrates found across three German river catchments with different degradation histories: the Boye, the Kinzig, and the Rotbach. Amphipods were also fairly common in degraded environments across Europe, which the atyid shrimp *N. davidi* has recently invaded. The extremely low prevalence of macroparasites in these hosts suggested prevailing degradation in all investigated sites (Sures et al., 2023). Therefore, the focus of the thesis shifted toward more prevalent but understudied parasites, microsporidians. These were common across all environments and hosts. The finding presented in this thesis indicates that microsporidians are highly diverse, infecting more hosts than previously reported. The generalist nature of the observed microsporidians in terms of host use highlights the importance of host specificity in parasite persistence and dispersal across degraded and recovering ecosystems. The dispersal of host and parasites, including invasive ones, appears to be reciprocally modulated.

Globally, 284 microsporidian species have been formally described, and over 1,600 isolates have been identified. Among these, 30 described species and a tenth of the undescribed isolates have been found in amphipods (Bojko et al., 2022; Bojko & Ovcharenko, 2019), a number steadily increasing with each explorative investigation. Among the 27 microsporidians identified within the four chapters of this thesis, five were formally described species. The remaining 22 were undescribed isolates based on DNA sequences, of which nine were new to science. Five of these new microsporidian isolates were identified in native amphipods, three in the invasive *N. davidi,* and one in the native isopod *A. aquaticus*. Several microsporidians, including those previously identified by other studies, were detected in novel hosts, totaling 29 novel microsporidian-hosts associations (17 in Chapter I, five in Chapter II, two in Chapter III, and five in Chapter IV). These findings clearly indicate that microsporidians are more diverse and generalist than previously thought. The higher degree of host specialization observed in the current study is partly due to the fact that most studies investigating microsporidians in freshwater crustaceans have targeted single relatively common parasites or specific host populations at a restricted geographic scale, missing out on non-target hosts and rarer parasites. Moreover, among the most widespread amphipods are species complexes with extremely high intraspecific diversity (Lagrue et al., 2014; Wattier et al., 2020), which has often been overlooked in previous studies. Hence, when looking at host specificity, it is important to consider host intraspecific, often cryptic diversity, as amphipods ascribed to single species based on morphology may be phylogenetically highly divergent (Wattier et al., 2020) and therefore host different parasite communities (Grabner et al., 2015).

The generalist nature and diversity of microsporidians and the extremely low prevalence of macroparasites indicate that the investigated freshwater environments, including restored ones, are experiencing some form of degradation. As suggested in Chapter I, host diversity, and environmental parameters did not influence the persistence and dispersal of generalist microsporidians in environments that experienced anthropogenic disturbance. Moreover, parasite communities of amphipods inhabiting a more natural stream near the Boye catchment showed a lower microsporidian diversity but a high prevalence and diversity of macroparasites (Grabner, 2017). This aligns with the assumption that monoxenous generalist parasites are more likely to persist in degraded environments due to simple life cycles and host plasticity (Lafferty, 2012; Poulin & Morand, 2004).

In contrast to heteroxenous and specialist parasites, monoxenous generalist parasites may benefit from multiple stressors as these might render the host more susceptible to infections (Coors et al., 2008; Sures et al., 2023). Stressors can also affect the stability of host populations and reduce their diversity, indirectly impacting heteroxenous parasites communities. Heteroxenous parasites are generally bound to stable host populations, as exemplified by higher trematode diversity in abundant keystone snail species (Selbach et al., 2020). However, host abundance and composition are often altered in the presence of multiple stressors where more tolerant species replace less tolerant ones. These changes may prevent life cycle completion in parasites relying on multiple hosts and in specialist parasites that cannot switch from a declining to a more abundant host (Sures et al., 2023).

Losing less tolerant hosts might relieve more tolerant hosts from resource competition, resulting in increased densities (Arfè et al., 2019). An increase in host densities coupled with a decrease in host diversity will likely enhance the transmission of monoxenous generalist parasites (Marcogliese, 2005; Sures et al., 2023). Furthermore, even if multiple stressors do not impact host populations, they can have stronger direct effects on the free-living stages of heteroxenous parasites than monoxenous ones, effectively hindering their establishment in degraded and recovering ecosystems (Sures et al., 2023). Accordingly, all the formally described microsporidians identified within the four chapters of this thesis are monoxenous and generalist. Nevertheless, the life cycle of undescribed microsporidian isolates remains unknown, and the persistence and dispersal of generalist microsporidians might depend on more complex mechanisms such as production of resistant spores, host switching, and host dispersal acting individually or conjointly.

Dispersal as one of those potential factors was studied in more detail in the context of this thesis. Dispersal is a prominent feature of most animals, including parasites, and has an important role in shaping ecological and evolutionary patterns in natural populations (Ronce, 2007). The findings of Chapter II indicate that the dispersal of host and parasites varies seasonally and appears to be reciprocally modulated with larger hosts harboring the bulk of infections. Host size explained seasonal variation in the prevalence and composition of microsporidians in drifting amphipods, suggesting that either larger host are more dispersive or that higher parasite prevalence drive directly or indirectly host dispersal.

While a positive correlation between host size and parasite prevalence is commonly observed in aquatic organisms such as fish and snails (Poulin, 2020; Soldánová et al., 2010), recent empirical evidence suggests that, at least for certain host-parasites systems, parasite infection can induce size-dependent host dispersal (Terui et al., 2017). From a fitness perspective, individuals with high dispersal ability (i.e., larger individuals) may benefit from moving away from infected areas as this may reduce exposure to parasites and thus prevent further infections. Dispersal costs for individuals with limited dispersal ability (i.e., smaller individuals) and minimal parasite load may, on the other hand, outweigh the derived benefits (Terui et al., 2017). Nevertheless, dispersal might also result from host manipulation or side effects of parasites.

Potential dispersal alterations in microsporidian-infected *G. pulex* were observed in the present study. Irrespective of the host size and season, *G. pulex* infected with two microsporidian isolates, Microsporidium sp. 505 and Microsporidium sp. 515, consistently drifted primarily during the day. On the other hand, the drift of uninfected *G. pulex* and those infected with other microsporidians mainly occurred during the night, as commonly observed in this amphipod species

(Elliott, 2002). A change in drift behavior from night to day in *G. pulex* infected with Microsporidium sp. 505 and Microsporidium sp. 515 suggests altered host phototaxis.

Altered phototaxis has been observed in amphipods and other aquatic and terrestrial hosts infected by acanthocephalans, cestodes, fungi, nematomorphs, and trematodes (Andriolli et al., 2019; Bethel & Holmes, 1973; Casalins et al., 2015; Obayashi et al., 2021). It is suspected to enhance trophic transmission to the final hosts or force hosts to habitats suitable for parasite life cycle completion (Andriolli et al., 2019; Obayashi et al., 2021). The possibility that microsporidians may alter host phototaxis and, consequently, their dispersal is ecologically significant, considering how ubiquitous microsporidians are. Alteration of drift and other forms of dispersal may play a role in shaping ecological and evolutionary patterns in natural populations with far-reaching consequences for entire ecosystems, particularly in those subject to abiotic disturbance (Bowler & Benton, 2005; Gilliam & Fraser, 2001; Hansson & Åkesson, 2014). Moreover, the finding of a closely related amphipod infecting microsporidian in fish guts suggests that fish and potentially other predators might act as vectors contributing to microsporidian dispersal.

Dispersal of microsporidians may also be modulated by the arrival of invasive species, particularly in degraded environments. Accordingly, freshwater habitats experiencing degradation are prone to biological invasions (Casatti et al., 2006; Gherardi, 2007). Biological invasions, especially those involving warm-adapted species, are bolstered by increasing water temperatures (Bellard et al., 2012). Non-native species are often more resilient towards increased water temperature and habitat degradation than their native counterparts and may, considering the results of Chapter I, potentially act as hosts or vectors for native host generalist microsporidians. This would allow native microsporidians to persist in degraded environments even in the absence of native hosts. However, invasive species might also bring novel parasites that can severely affect native species or enhance the host's invasive capabilities (Galbreath Slothouber et al., 2004; Prenter et al., 2004). In some cases, introduced parasites may indirectly benefit native biota by acting as a controlling agent for non-native species (Bojko et al., 2015). Likewise, parasite transmission from native to non-native species might reduce the impact of invaders (Dunn et al., 2009).

Microsporidians infecting native amphipods and isopods were not detected in invasive *N. davidi* during the explorative investigation presented in Chapters III and IV. However, *N. davidi* hosted four unreported microsporidians, one of which was also detected in another invasive species, the crayfish *P. clarkii*. Among the identified microsporidians, the host generalist *E.*

hepatopenaei appears to be established in degraded European environments, albeit seemingly constrained to *N. davidi*. The presence in Europe of this non-native parasite of tropical marine and brackish origin highlights the environmental adaptability of host generalist microsporidians and the key role played by thermal and saline pollution. Considering the observed range expansion of *N. davidi* from thermally polluted to colder water and the parasite's ability to infect a wide range of organisms spanning several taxonomical orders (Kumar Dewangan et al., 2023), concerns are raised over its ecological importance, transmission potential, and pathogenicity. This and other non-native parasites may, for instance, hinder the ability of native species to disperse and colonize restored environments, even in the absence of other stressors.

Future research direction

As shown by the results presented in this thesis, much work still needs to be done to unravel the ecological role of microsporidians and, more generally, neglected parasites. The gap between described species and undescribed microsporidian isolates clearly indicates that basic research in this field is lacking. Information regarding parasite diversity, host-specificity, life cycle, pathogenicity, and spore environmental resilience are largely missing. Unfortunately, such gaps are not unique to microsporidians, as most parasites that are not of medical or veterinary importance remain understudied (Poulin et al., 2019). Consequently, due to a general lack of molecular and morphological characterization, the diversity of parasites is likely to be underestimated (Schwelm et al., 2021; Selbach et al., 2020). Explorative investigations such as non-targeted large-scale molecular screening of parasites might shed more light on their diversity and host specificity.

Since temperate freshwater ecosystems remain the primary focus of parasitological investigations (Poulin et al., 2019), such explorative study should ideally include less studied regions like tropical and polar ones. This is particularly relevant in the face of global warming, widespread ecosystem degradation, and biological invasions. As shown by the results presented in this thesis, global warming and habitat degradation might facilitate biological invasions and the exchange of ecological and economically relevant parasites between native and invasive host species. Furthermore, to better understand the combined impact of non-native parasites and multiple stressors on native host communities and their recovery in restored sites, conducting a broader range of infection experiments, including non-target hosts and stressors such as temperature and salinity, is recommended.

As multiple stressors somehow seem to affect both degraded and restored sites, more effort should be put into investigating parasite diversity in less impacted areas (e.g., nature reserves) and museum specimens. This approach may give a better insight into which parasites should or should not be present in a given area (Wood et al., 2023). Follow-up comparisons between the current and inferred original parasite communities would provide a better understanding of species loss and acquisition after exposure to multiple stressors. Therefore, an integrated approach, including a wide range of habitats in different regions, historical samples, infection experiments, taxonomical, histological, ultrastructural, and molecular analyses, is recommended to generate a clear baseline picture on which other studies, including multiple stressors ones, can be built upon.

Conclusion

In conclusion, the presence of a large number of host generalist parasites with seemingly simple life cycles indicates that the investigated environments, including those undergoing recovery, are still under the influx of multiple stressors. Information on aspects such as microsporidian diversity, host-specificity, life cycle, pathogenicity, spore environmental resilience, and alteration of host behavior are largely lacking. Therefore, the content of the present thesis is of high ecological relevance as such information is crucial for understanding the ecological role of these parasites. Microsporidians infect keystone species; hence, the prospect that they may alter host dispersal behavior opens new possibilities for microsporidians to shape ecological and evolutionary patterns in natural populations, affecting entire ecosystems. Moreover, when it comes to biological invasions, microsporidians have been neglected in non-amphipod hosts. However, invasive hosts, as in the case of *N. davidi*, can harbor unreported non-native parasites with high ecological and economic impacts. The spread of invasive hosts and parasites is facilitated by habitat degradation and might hinder the recovery of native species following habitat restoration.

Although invasive and native microsporidians can influence the recovery of free-living communities, they do not appear to be a prime indicator of stream recovery. Their ability to infect different hosts and persist in degraded environments suggests they will remain in these ecosystems following restoration. Macroparasites, instead, seem to be better indicators of ecosystem recovery. However, interactions between microsporidians and macroparasites might affect the latter's establishment, irrespective of stream ecological status. For instance, host behavioral manipulation induced by acanthocephalans in amphipods can be weakened by microsporidians, reducing the acanthocephalans' chance of transmission to the final host (Haine et al., 2005). The outcome of such interactions is largely unknown and may depend on the relative prevalence of the different parasites in the host population; thus, investigations comprising all the parasites found in different key organisms like fish, snails, crustaceans, and the most important aquatic insects might be more insightful in tracking dynamics unfolding during ecosystem recovery. This approach was used in the current study. However, the data produced by screening fish, snails, and aquatic insects for parasites was not well suited for the present analyses. Snails were absent from several of the investigated areas, the number of fish allowed for screening was insufficient, and aquatic insects generally had a very low prevalence of parasites. Nevertheless, the unpublished information generated during the present study provides a baseline for future investigations.

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Curriculum vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

Declarations

Declaration:

In accordance with § 6 (para. 2, clause g) of the Regulations Governing the Doctoral Proceedings of the Faculty of Biology for awarding the doctoral degree Dr. rer. nat., I hereby declare that I represent the field to which the topic "*Effects of stream degradation and recovery on parasite communities: a multiple stressor approach"* is assigned in research and teaching and that I support the application of Sebastian Prati.

Essen, date ________ _______________________ __________________________________

Name of the scientific supervisor/member of the University of Duisburg-Essen	Signature of the supervisor/member of the University of Duisburg-Essen

Declaration:

In accordance with § 7 (para. 2, clause d and f) of the Regulations Governing the Doctoral Proceedings of the Faculty of Biology for awarding the doctoral degree Dr. rer. nat., I hereby declare that I have written the herewith submitted dissertation independently using only the materials listed, and have cited all sources taken over verbatim or in content as such.

Essen, date _________________ _______________________________

Signature of the doctoral candidate

Declaration:

In accordance with § 7 (para. 2, clause e and g) of the Regulations Governing the Doctoral Proceedings of the Faculty of Biology for awarding the doctoral degree Dr. rer. nat., I hereby declare that I have undertaken no previous attempts to attain a doctoral degree, that the current work has not been rejected by any other faculty, and that I am submitting the dissertation only in this procedure.

Essen, date _________________ _______________________________

Signature of the doctoral candidate