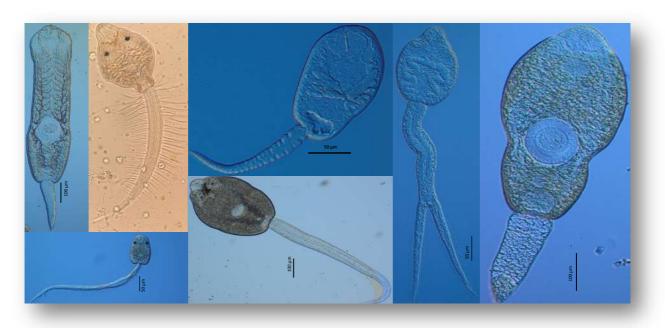
# Trematode parasites of mollusc hosts from marginal seas and their sensitivity to warming



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### **Summary**

Parasites, like the diverse Digenean trematodes, are crucial to ecosystems, influencing everything from ecological speciation to food webs and host behavior. However, knowledge deficits persist regarding their biodiversity, life cycles, and geographical distribution, which complicates our broader understanding of ecosystems and response to the crisis in biodiversity. Some parasites risk extinction before they are discovered, especially in rapidly warming Marginal Seas such as the Persian Gulf and Baltic Sea, as a result of the ongoing global warming accelerated by human activity. Therefore, it is imperative to investigate the effects of warming on host-parasite systems in these regions and to comprehend the thermal performance of parasites. This is especially true as basic information such as parasite biodiversity is still missing in some regions, while the impact of environmental change on parasite performance remains largely unknown even in well-studied areas.

Within four chapters, **this thesis aimed** to explore the yet unknown trematode biodiversity in the Persian and Oman Gulfs via molecular identification of trematodes infecting the most abundant mud snail *Pirenella cingulata*. Next, we investigate the thermal sensitivity of two prevalent trematodes (*Acanthotrema tridactyla* and Cyathocotylidae gen. sp) infecting *P. cingulata*. Lastly, this thesis investigates the combined effect of warming and parasitism on mussel performance using *Renicola roscovita* infecting *Mytilus edulis* as the host-parasite system in the Baltic Sea.

In *Chapter 1*, the diversity and distribution patterns of trematodes along the Persian and Oman Gulfs were assessed using molecular identification methods. A total of 1969 mud snails, P. cingulata, were collected from 8 localities along the study area and examined for trematode infections. The results from this chapter suggest species-rich trematode fauna comprising 29 species belonging to 10 families, demonstrating a unique species composition infecting P. cingulata in this study system. Some of these trematodes could only be identified up to the genus or family level due to the paucity of GenBank comparable isolates, highlighting the need for further investigations regarding trematode biodiversity in understudied areas. Despite the lack of available sequences to molecularly identify many trematodes to the species level, it was possible match identify human-related schistosome like and trematodes Ornithobilharzia canaliculata and Austrobilharzia sp. within our samples.

In *Chapter 2*, for the first time, we matched genomic data from adult and larval stages of *O. canaliculata* from the Persian and Oman Gulfs and worldwide from a potamidid snail host contributing to the knowledge on the life cycle of this particular species.

In *Chapter 3*, the thermal sensitivity of *Acanthotrema tridactyla* and Cyathocotylidae gen. sp trematodes infecting the horn snail *P. cingulata* was evaluated. This chapter's findings imply that *A. tridactyla* and Cyathocotylidae genus have the hottest-ever known thermal optima for cercarial emergence of aquatic trematodes (32.0 °C and 33.5 °C, respectively). The cercarial emergence of *A. tridactyla* followed a nocturnal pattern, a time-dependent decrease, and had a greater number of cercariae than Cyathocotylidae's, for which no significant time or light effect on cercarial emergence was detected. Our results indicate that Persian Gulf trematodes may be among the most heat-tolerant marine trematode species, suggesting a possibility for dispersal by maritime fleet and migrating birds to places that will continue to warm in the future.

Finally, in *Chapter 4*, the influence of *R. roscovita* on the physiological response of its second intermediate host *M. edulis* under mild temperature (16.0 °C) and acute heat ramp (30.5 °C) was investigated. The results from this chapter suggest a parasite-induced decline, albeit not statistically significant, of approximately 11 % in the filtration rate of *M. edulis* at a mild temperature for both small and large mussels (2 and 4 cm). However, an enlarged reduction of ca 37 % in filtration rate was found after short-term heat exposure indicating a greater impact of warming and trematode infection combined. The relationship between filtration rate and metacercarial intensity in mussels was a fascinating side finding in this chapter, showing that mussels with naturally higher feeding rates are prone to have a higher intensity of trematode infection.

This thesis concludes by elucidating the crucial function of parasites, specifically trematodes, in biodiversity assessments and their significant impact on host performance. This study contributes to a broader and more nuanced understanding of parasitology by disclosing the distinct diversity and thermal sensitivity of trematodes in the Persian and Oman Gulfs. The findings highlight the adaptability of these trematodes to exceptionally high temperatures, suggesting the possibility of their future dispersal to regions experiencing a warming trend.

However, our exploration of the complex world of parasitology is far from complete. Despite the progress made in comprehending trematode diversity and thermo sensitivity, this study reveals new research avenues for the future. Due to the scarcity of comparable GenBank isolates, many trematodes could only be identified to the genus or family level. This demonstrates the urgent need for increased efforts in trematode taxonomy and biodiversity research, particularly in regions that have been little explored.

Furthermore, the intricate interactions between parasitism and global warming require additional study. The amplified effects of these two factors on the filtration rate of the bivalve *Mytilus edulis* under conditions of high temperature, for example, provide compelling evidence

of their cumulative effect. Unraveling these complex relationships may yield new insights into how climate change may affect parasitic lifecycles and host-parasite interactions.

This thesis has essentially opened up new avenues for the study of trematode biodiversity and adaptation to changing environmental conditions. It emphasizes the significance of incorporating parasitology into our global biodiversity conservation and climate change mitigation strategies. The journey of discovering the ecological functions and future of trematodes in the context of global warming continues, necessitating additional exhaustive and multidisciplinary studies.

# Zusammenfassung

Parasiten, wie die verschiedenen einheimischen Trematoden, sind für Ökosysteme von entscheidender Bedeutung und beeinflussen alles von der ökologischen Artbildung bis hin zu Nahrungsnetzen und dem Verhalten des Wirts. Es bestehen jedoch weiterhin Wissensdefizite hinsichtlich ihrer Artenvielfalt, Lebenszyklen und geografischen Verteilung, was unser umfassenderes Verständnis von Ökosystemen und die Reaktion auf die Krise der Artenvielfalt erschwert. Einige Parasiten sind vom Aussterben bedroht, bevor sie entdeckt werden, insbesondere in sich schnell erwärmenden Randmeeren wie dem Persischen Golf und der Ostsee, da die globale Erwärmung durch menschliche Aktivitäten beschleunigt wird. Daher ist es unerlässlich, die Auswirkungen der Erwärmung auf Wirt-Parasit-Systeme in diesen Regionen zu untersuchen und die thermische Leistung von Parasiten zu verstehen. Dies gilt insbesondere, da in einigen Regionen noch grundlegende Informationen wie die Artenvielfalt der Parasiten fehlen, während die Auswirkungen von Umweltveränderungen auf die Leistung der Parasiten selbst in gut untersuchten Gebieten weitgehend unbekannt sind.

Ziel dieser Dissertation ist es, innerhalb von vier Kapiteln bestehende Wissenslücken zur Ökologie der marinen Trematoden zu füllen. Die Biodiversität bisher unbekannter Trematodenarten, die die Schlammschnecke *Pirenella cingulata* befallen und im Persischer Golf und Oman vorkommen, werden mit Hilfe von molekularer Identifizierung erforscht. Anschließend wird die thermische Empfindlichkeit von den Trematodenarten *Acanthotrema tridactyla* und Cyathocotylidae gen. sp. untersucht. Schließlich wird in dieser Arbeit die kombinierte Auswirkung von Erwärmung und Parasitismus auf die Leistung von Muscheln untersucht, wobei *Renicola roscovita*, der *Mytilus edulis* infiziert, als Wirts-Parasit-System in der Ostsee verwendet wird.

Kapitel 1 widmet sich der Vielfalt und Verbreitungsmuster von Trematoden entlang des Persischen und des Omanischen Golfs. Insgesamt wurden 1969 Schlammschnecken (*P. cingulata*) an acht Probestellen des Untersuchungsgebiets gesammelt und auf Trematodenbefall untersucht. Die Ergebnisse dieses Kapitels deuten auf eine artenreiche und einzigartige Trematodenfauna hin, die 29 Arten umfasst, die zu 10 Familien gehören. Einige dieser Trematoden konnten aufgrund des Mangels an GenBank-vergleichbaren Isolaten nur bis zur Gattungs- oder Familienebene identifiziert werden, was den Bedarf an weiteren Untersuchungen zur Trematoden-Diversität in unterstreicht. Trotz des Mangels an verfügbaren Sequenzen war es möglich, Schistosomen-Trematoden, die den Menschen infizieren können, in den Untersuchungsgebieten zu identifizieren.

Während der in **Kapitel 2** beschriebenen Arbeit waren wir erstmals in der Lage, Genomdaten von adulten und larvalen Stadien von *O. canaliculata* aus dem Persischer Golf und Oman sowie weltweit von einer Potamididien-Schnecke abzugleichen, was zum Wissen über den Lebenszyklus dieser speziellen Art beiträgt.

In **Kapitel 3** wurde die thermische Empfindlichkeit von *Acanthotrema tridactyla* und Cyathocotylidae gen. sp. Trematoden, die die Hornschnecke *P. cingulata* infizieren, untersucht. Die Ergebnisse dieses Kapitels deuten darauf hin, dass *A. tridactyla* und Cyathocotylidae gen. sp. die bekannten thermischen Optima für das Auftreten von Zerkarien aquatischer Trematoden überschreiten (32,0 °C, bzw. 33,5 °C). Das Auftreten von Zerkarien bei *A. tridactyla* folgte einem nächtlichen Muster und einem zeitabhängigen Rückgang. Außerdem waren die Zerkarien in einer größeren Anzahl als bei Cyathocotylidae zu finden, für die kein signifikanter Zeit- oder Lichteffekt auf das Auftreten von Zerkarien festgestellt wurde. Unsere Ergebnisse deuten darauf hin, dass die Trematoden des Persischen Golfs zu den hitzetolerantesten marinen Trematodenarten gehören, was auf eine mögliche Ausbreitung durch die Schifffahrt und Zugvögel an Orte, die sich in Zukunft weiter erwärmen werden, hindeutet.

Schließlich wurde in **Kapitel 4** der Einfluss von *R. roscovita* auf die physiologische Reaktion seines zweiten Zwischenwirts *M. edulis* bei milder Temperatur (16,0 °C) und akuter Hitzewelle (30,5 °C) untersucht. Die Ergebnisse dieses Kapitels deuten darauf hin, dass die Filtrationsrate von *M. edulis* bei milden Temperaturen sowohl bei kleinen als auch bei großen Muscheln (2 und 4 cm) um etwa 11 % abnimmt. Eine stärkere Verringerung der Filtrationsrate um ca. 37 % wurde nach kurzzeitiger Wärmeeinwirkung festgestellt, was auf eine größere Auswirkung von Erwärmung und Trematodeninfektion zusammen hinweist. Die Beziehung zwischen der Filtrationsrate und der Anzahl der Metazerkarien in Miesmuscheln war eine faszinierende Nebenerkenntnis dieses Kapitels, die zeigt, dass Muscheln mit einer von Natur aus höheren Fütterungsrate dazu neigen, eine höhere Intensität der Trematodeninfektion aufzuweisen.

Diese Arbeit schließt mit der Aufklärung der entscheidenden Funktion von Parasiten, insbesondere Trematoden, bei der Bewertung der Biodiversität und ihrem signifikanten Einfluss auf die Wirtsleistung. Diese Studie trägt zu einem breiteren und differenzierteren Verständnis der Parasitologie bei, indem sie die ausgeprägte Diversität und thermische Empfindlichkeit von Trematoden im Persischen und Oman-Golf offenlegt. Die Ergebnisse unterstreichen die Anpassungsfähigkeit dieser Trematoden an außergewöhnlich hohe Temperaturen und legen die Möglichkeit ihrer künftigen Ausbreitung in Regionen nahe, in denen ein Erwärmungstrend herrscht.

Unsere Erforschung der komplexen Welt der Parasitologie ist jedoch noch lange nicht abgeschlossen. Trotz der Fortschritte beim Verständnis der Trematodenvielfalt und Thermosensitivität zeigt diese Studie neue Forschungswege für die Zukunft auf. Aufgrund der Knappheit vergleichbarer GenBank-Isolate konnten viele Trematoden nur auf Gattungs- oder Familienebene identifiziert werden. Dies zeigt die dringende Notwendigkeit verstärkter Anstrengungen in der Trematodentaxonomie und der Biodiversitätsforschung, insbesondere in Regionen, die noch wenig erforscht sind.

Darüber hinaus erfordern die komplizierten Wechselwirkungen zwischen Parasitismus und globaler Erwärmung weitere Untersuchungen. Die verstärkten Auswirkungen dieser beiden Faktoren auf die Filtrationsrate der Muschel *Mytilus edulis* beispielsweise unter Bedingungen hoher Temperatur liefern überzeugende Beweise für ihre kumulative Wirkung. Die Aufklärung dieser komplexen Beziehungen könnte neue Erkenntnisse darüber liefern, wie sich der Klimawandel auf die Lebenszyklen von Parasiten und die Interaktionen zwischen Wirt und Parasit auswirken kann.

Diese Dissertation hat im Wesentlichen neue Wege für die Untersuchung der Biodiversität von Trematoden und deren Anpassung an sich ändernde Umweltbedingungen eröffnet. Es betont die Bedeutung der Einbeziehung der Parasitologie in unsere globalen Strategien zum Schutz der biologischen Vielfalt und zur Eindämmung des Klimawandels. Die Reise zur Entdeckung der ökologischen Funktionen und der Zukunft von Trematoden im Kontext der globalen Erwärmung geht weiter und erfordert zusätzliche umfassende und multidisziplinäre Studien.

## **General Introduction**

"Most biological studies, especially in ecology and evolution, have been done on free-livers. That is, the great bulk of our knowledge of biology comes from studying the minority of species!" (Windsor, 1998)

#### Host-parasite systems are an important component of marine ecosystems

Aquatic ecosystems can contain several systems of closely interacting species, including host-parasite systems. Since it is built on close links between two or more species, the relationship between parasites and their hosts is considered a sort of symbiosis. In parasitic partnerships, as opposed to other symbiotic systems (such as mutualism and commensalism), the host is typically damaged while the parasite benefits. However, the definition of parasitism is frequently challenged among authors and research disciplines; so, to avoid confusion, I define it as follows based on the following work on this definition by Poulin (2007).

"... parasite is an organism living in or on another organism, the host—feeding on it, showing some degree of structural adaptation to it, and causing it some harm (when the harm incurred by the host invariably leads to its death, the parasite is often referred to as a parasitoid)."

Parasitism is one of the most common life strategies to obtain nutrients ((Thompson, 1994; Hechinger and Lafferty, 2005) and references therein), with half of the animal species being parasitic at least during a certain period of their life history (Goater et al., 2014). Moreover, all metazoan species can serve as hosts to at least one parasite species (most species contain many more) (Marcogliese, 2004; Poulin, 2014). Parasites (*sensu stricto*) are highly diverse and represent members of distinct phyla: Apicomplexa, Amoebozoa, Excavata, Ciliata, Microsporidia, Myxozoa, Platyhelminthes, Acanthocephala, Hirudinea, Nematoda, and Arthropoda (Goater et al., 2014). All of these have different transmission strategies and have managed to circumvent the host's immune system adapting and evolving over time step by step after adaption (Schmid-Hemoel, 2008). This never-ending host-parasite coevolution has resulted in a successful system and an intrinsic part of marine ecosystems and biodiversity.

Parasites play an important role in marine ecosystems by affecting the hosts' physiological processes and their interactions with the environment. To name a few, parasites can mechanically impact the host's ability to acquire nutrients (Attia et al., 2021), alter the composition and diversity of the host gut microbiome (Fredensborg et al., 2020; Hahn et al.,

2022) and references therein) and induce the production of reactive oxygen species such as hydrogen peroxide and nitric oxide (Hahn et al., 2001b, 2001a). Parasitic infections can modify the expression of host genes that regulate behaviour in order to improve or ensure trophic transmission (van Houte et al., 2013; Grecias et al., 2020; Will et al., 2020). Some acanthocephalans, for instance, make their amphipod host less photophobic and persuade the host to swim toward the water surface where it can be preyed on by the final host (Bauer et al., 2000). Infection with the cestode Schistocephalus solidus causes its fish host, the three-spine stickleback Gasterosteus aculeatus, to swim closer to the surface (Barber and Wright, 2006; Poulin, 2018), and to avoid shoaling with other uninfected conspecifics, increasing their vulnerability to predation by birds (Barber and Caira, 1995). Other examples could be vigorous parasite infestation in mussels that can weaken the byssus-thread production and hinder mussels' attachment to substrates (Lauckner, 1984) and parasitic infection in cockles that obstacles the flow of haemolymph into the foot, impairing cockle's ability to re-burrow in the sediments (Mouritsen, 2002). Overall, such physiological and behavioural (phenotypic) changes can facilitate transmission and continuation of the parasite's life cycle (Moore, 2002; Hughes et al., 2012; McElroy and De Buron, 2014).

In a relationship that is generally harmful to hosts, parasite infections may confer little sidebenefits to the host. For instance, the microbial endolithic parasite responsible for the white discoloration of Mytilus edulis shell makes infected bivalves more resistant to the harmful effects of heat (Gehman and Harley, 2019). Protozoan and helminth parasites, for instance, can enrich host's diet. Moreover, parasites such as acanthocephalans and nematodes can serve as pollution sinks by accumulating higher metal concentrations than their host (Malek et al., 2007; Vidal-Martinez et al., 2010; Nachev and Sures, 2016; Sures et al., 2017; Younis et al., 2020). When there are few side-benefits on the sub-organismal level in addition to negative effects, it becomes more difficult to draw conclusions about the role of parasites in marine ecosystems. Nonetheless, in aquatic environments, the detrimental impacts of parasites on the functional features of their hosts can alter species interactions, community structure, and food web dynamics (Thompson et al., 2013). Regarding trophic interactions, it is generally accepted that parasites largely increase the density and connectivity of nodes in food-webs (Dunne et al., 2002, 2013; Lafferty et al., 2008; Sukhdeo, 2012; Morton et al., 2021). This is not surprising considering that parasite biomass is estimated to be higher than top predators and they constitute an important energy pathway in ecosystems (Kuris et al., 2008; Thieltges et al., 2008). Consequently, parasites are an essential component of marine biological processes.

#### Host-trematode systems are of particular importance in marine ecosystems

When it comes to marine ecological dynamics, trematode parasites are of particular importance, due to their abundance, diversity, complex life cycle, and remarkable effects on the hosts' physiology and behaviour (Mouritsen and Poulin, 2002a). They have adapted to persist in almost all types of environments (Goater et al., 2014). The class Trematoda is divided into two subclasses: Aspidogastrea (~80 species) (Rohde, 2005) and Digenea (~25,000 species) (Esch et al., 2002). Aspidogastreans have simple lifecycles involving only a mollusc and either a facultative or a compulsory vertebrate host. In contrast, the digeneans have surprisingly diverse life cycles involving multiple developmental stages (eggs, miracidia, cercariae, metacercariae, adults) and a wide variety of habitats and ecosystems (Choudhury et al., 2016; Faltýnková et al., 2016; Scholz et al., 2016; Soldánová et al., 2017). Digeneans can infect a vast variety of hosts (both invertebrates and vertebrates). Despite the great heterogeneity of trematode life cycles, a typical digenean life cycle always contains parthenogenetic and hermaphroditic generations. In their complex life cycle, digeneans often use two or three hosts, while one or four hosts are rarely observed (e.g., Hemiuroidea, Plagiorchiidae, Strigeidae). The majority of digeneans utilize a mollusc as their first intermediate host (with some exceptions utilising annelids). Depending on the parasite species, the second intermediate host can be an invertebrate or vertebrate (e.g., mollusc, crustaceans, fish, amphibians) and the definitive host is often a vertebrate (e.g., seabirds, fish, mammals) (Galaktionov and Dobrovolskij, 2003).

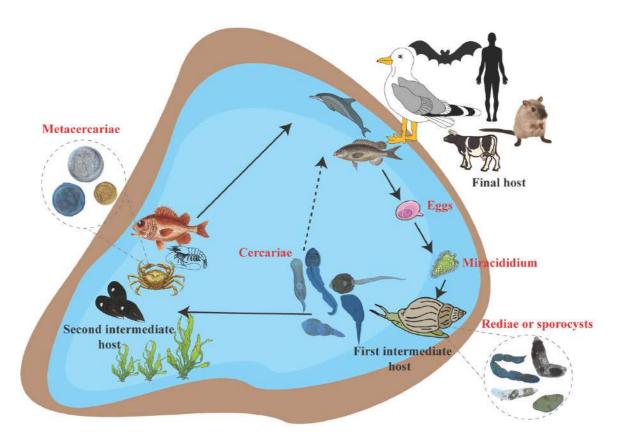
#### Trematode life cycle

Digeneans reproduce sexually in the definitive host by producing eggs, which are released into the environment with faeces. Each egg holds a miracidium which can actively or passively infect the first intermediate host (often a mollusc). For active-infective miracidia, hatching begins almost instantly after the eggs are immersed in water due to a sudden shift in the tonicity of the environment (Galaktionov and Dobrovolskij, 2003). The miracidium is a motile, short-lived, non-feeding, ciliated larva. Active swimming miracidia possess a combination of locomotion, sensing, and penetration organs that allow them to search for, identify, and eventually pierce their molluscan host. In the case of passive-infective miracidia, the egg is accidentally ingested by the first intermediate host. The larva within the egg is completely motionless while in its surrounding environment. Nevertheless, 10–15 minutes after entering the digestive tract of a suitable mollusc, the miracidium —both with active and passive infection strategies—uses chemical and mechanical means to penetrate the intestinal wall of the

molluscan host. Once the metamorphic transformation is completed, the young individual migrates to the desirable organ which varies among species. The miracidium undergoes metamorphic transformation and turns into a mother sporocyst which is the first intramolluscan generation.

A germ cell lineage, which is undifferentiated remaining cells from the zygote in the mother sporocyst, asexually produces daughter sporocysts or rediae. Sporocysts absorb food directly through the tegument, whereas rediae have mouth, pharynx and a short saccular gut. Apart from the difference in their feeding strategy, both are sac-like structures which asexually produce hundreds of free-living cercariae. The creation of hundreds of cercariae as a result of infection with a single egg represents an evolutionary advantage for Digenea *versus* Aspidogastrea, which has no asexual reproduction. Cercariae do not feed (known as lecithotrophy) (Barber et al., 2016), and their short functional lifespan (less than 24 h for most cases) is restricted by a non-renewable endogenous glycogen supply (Young et al., 1984; Nanduri et al., 1991). In response to abrupt temperature and light changes, cercariae emerge from the mollusc host in an open environment and infect the second or definitive host.

Having a brief lifespan, cercariae have acquired a variety of morphological and behavioural adaptations that enhance their infection efficacy. In two-host life cycles, cercariae infect the final host directly (dashed arrow in Figure 1). For instance, cercariae of the Schistosomatidae family can adhere to the host's skin, detach their tail, penetrate and enter the circulatory system, and mature into adults. Other types of cercariae (e.g., Bivesiculidae) are eaten directly or as cysts and mature in the ultimate host's gastrointestinal tract (Rohde, 2005). In contrast, cercariae of three-host trematodes are either eaten by the second intermediate host or penetrate it via well-developed glands and structures (e.g., stylet). In the interim, they shed their tail and penetration glands secret proteolytic enzymes and hyaluronidase to facilitate penetration and migration to their preferable site in the host's tissue. After penetration, cercariae undergo morphological modifications and encyst as metacercariae. The metacercaria is a transient stage between dispersive larvae and the adult stage. The metacercaria is trophically transmitted through ingestion of the second intermediate host by the definitive host. In the definitive host, the larva emerges from the metacercaria, matures into the adult stage, and then reproduces sexually. Eggs are then released into the environment, and the first intermediate host is infected (Figure 1).



**Figure 1.** Schematic representation of typical (two and/or three host) digenean life-cycles. Three host life cycle contains six distinct stages (adult, egg, miracidium, redia or sporocyst, cercaria and metacercaria).

#### Digenean impacts on the host

Digenean pathogenicity varies depending on the parasite's species and the life cycle stage. In this section, we discuss the overall influence of digeneans on the host during various stages of their life cycle.

#### Impacts on first intermediate host

In the first intermediate host, the asexual reproduction has a destructive impact on the mollusc host. Since the intramolluscan stage (sporocyst or rediae) almost entirely replaces the digestive/gonadal complex tissue (Fretter and Graham, 1962; Smyth and Halton, 1983). Infections are sometimes lost (Fried and Graczyk, 1997; Soldánová et al., 2012). However, most of the time molluscs remain infected for the rest of their lives and may become fully castrated or have their reproductive output significantly decreased (Hughes and Answer, 1982; Huxham et al., 1993). At the individual level, castration implies reorganizing energy utilization and allocation (Dawkins, 1982). At the population level, parasitic castration suggests that the animal will no longer create offspring, or its contribution to the population subsistence is

significantly reduced if the reproductive output is decreased rather than halted (Sorensen and Minchella, 2001). In the first intermediate host, infections are also known to hijack energy reserves from the host and affect feeding rates (Wood et al., 2007; Faro et al., 2013).

#### Impacts on second intermediate host

In the second intermediate host, encysted or unencysted metacercariae (mesocercariae) in the tissue have relatively little pathogenicity because no reproduction is involved, and their development at the host's expense is modest (absorption through the cyst wall rather than browsing and destructive impact on host tissue). However, when the metacercariae intensity is high and additional stressors are present, there can be significant pathogenicity. The metacercarial stage of trematodes of the genus Euhaplorchis, for instance, affects the sensory organ and central nervous system of the fish host resulting in conspicuous movements and altering swimming patterns, putting them at greater risk of predation (Lafferty and Morris, 1996; Fredensborg and Longoria, 2012). Metacercarial infection of fish eyes with diplostomoid trematodes induces the use of risky microhabitats, enhancing the host's visibility to piscivorous birds (Ruehle and Poulin, 2020) and dietary shifts towards bigger, more visible prey (Vivas Muñoz et al., 2021). Other forms of harm might be a decrease in the host's performance and reproductive output or an increase in the probability of predation, for instance, aberrant escape behaviour in infected gammarid with microphallid metacercariae. Encysted metacercariae of trematode *Himasthla elongata* in the *Mytilus edulis* foot impairs its ability to form proper byssus thread and increases the chance of dislodgment and predation (Lauckner, 1983).

#### Impacts on the definitive host

In the definitive (final) host, most digeneans live in the host's digestive tract, where they can feed on mucus, epithelial cells, gut content, and blood. No substantial pathogenicity has been associated with digeneans in the definitive host because their diet is innocuous, the worms are tiny compared to their host, and their movement prevents them from causing permanent damage at a single location. Nonetheless, the blood flukes can cause significant pathogenicity to their host via interference with blood flow and erosion of the blood vessel walls. Also, their eggs usually cause inflammatory reactions while passing through the host tissue when released. Schistosomatids (parasitic in marine birds) and spirorchiid (in marine turtles) frequently cause an inflammatory response in the liver, brain, and gut wall. Eggs of sanguinicolids (in marine fishes) usually block the gills, thus interfering with respiration and ultimately suffocating the fish (Rohde, 2005).

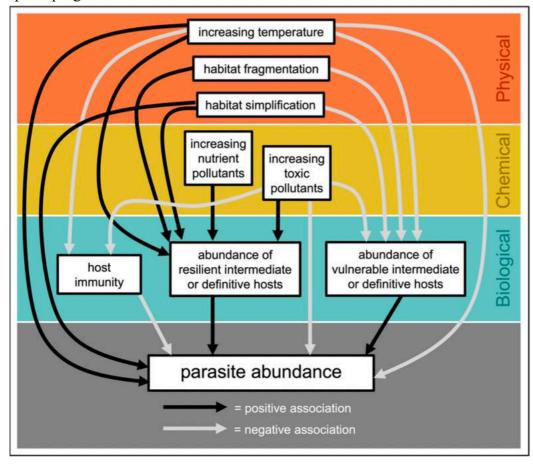
#### Climate change may impact marine trematode-host systems: some knowledge gaps

A trematode and its host may have distinct microhabitats while sharing similar marine (macro) habitats characterized by physicochemical or environmental conditions such as temperature, salinity, oxygen, pH, and nutrients. Changes in these external conditions can affect host-parasite interactions by altering parasite-free (or potential) hosts, free-stage parasites, or both, in addition to infected hosts. Each life stage and process in the life history of hosts and parasites can have specific environmental susceptibility, which complicates inferences about the overall impact of environmental change on host-parasite systems. Numerous host-digenean systems inhabit marine shallow-water regions characterized by environmental variability of various causes and scales, ranging from natural climatic events such as the North Atlantic Oscillation, El Niño or La Niña (Soares et al., 2014; Ma et al., 2020) to weather conditions such as in seasonal, daily or shorter fluctuations in temperature due to earth's tilted spin axis and rotation, upwelling and downwelling events, tides and waves.

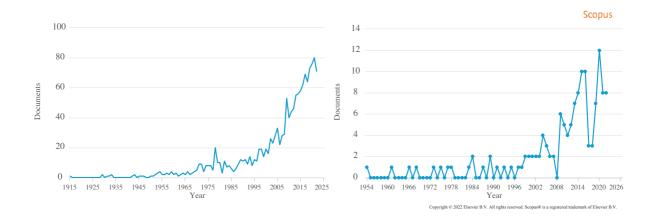
Indeed, host-parasite systems have been adapted to natural environmental regimes, as have other components of marine ecosystems. Nevertheless, humans have profoundly altered marine environments as part of the Anthropocene. Especially, the boosted release of greenhouse gases (i.e., CO<sub>2</sub>, CH<sub>4</sub>, N<sub>2</sub>O) and their accumulation in the atmosphere has resulted in unusual global environmental trends (known as ocean warming, acidification, and deoxygenation) and intensification of extreme environmental events such as heatwaves (Holbrook et al., 2019). These global environmental changes have altered the biogeographic distribution of species and brought about some extinction events (Barry et al., 1995; Sagarin et al., 1999; Bellard et al., 2012; Albert et al., 2021). Yet, predicting the fate of host-parasite systems in the face of climate change is not trivial, as environmental changes can have complex and multidirectional impacts on both hosts and parasites (Figure 2).

Priority should be placed on comprehending the effects of highly influential environmental drivers. Specifically, **temperature** is a global driver of physiological process rates. However, we have just begun to understand the temperature effects on marine or aquatic trematodes (and

infected hosts), as shown in Figure 3, which compares the results of Scopus search analyses of the temporal progression of research.



**Figure 2.** How does global change affect the abundance of parasites? This route diagram demonstrates some of the mechanisms through which physical, chemical, and biological changes to ecosystems can result in a shift in parasite abundance. The colour of each arrow shows whether the link between two variables is positive (black) or negative (grey). This figure was prepared by Wood and Vanhove (2022). Please refer to the original reference for more details.



**Figure 3.** Results of Scopus search analyses of the temporal progression of research. The left-side plot displays the number of research on trematodes in aquatic regions, whereas the right-side plot shows the number of research on the effect of temperature on trematodes.

Temperature can alter the physiology, behaviour, and geographic range of hosts and parasites (Byers, 2021 and references therein). In terms of physiology, warming has been shown to decrease the immunocompetence of fish hosts (Boltana et al., 2018), induce compensatory metabolism in invertebrate hosts, and in extreme cases, can be lethal to ectothermic hosts, especially when infected with trematodes (McDaniel, 1969; Fredensborg et al., 2005; Paull and Johnson, 2011; Arundell et al., 2019). Regarding behavior, warming can prompt organisms to search for microhabitats with milder temperatures (e.g., deeper waters in ponds, shaded areas on land (Hunt et al., 2016; Megía-Palma et al., 2020)), while it can also induce feeding in ectotherms to suffice energy requirements for protective mechanisms (Vivas Muñoz et al., 2018). More broadly, warming can induce poleward shifts in species seeking shelter from extreme temperatures in lower latitudes (Poloczanska et al., 2016). Secondly, warming effects on parasites include increases in hatching, maturation, and development rates. In contrast, decreases in the survival of free-living stages and declines in the longevity of larvae and adults in response to warming have been reported (Marcogliese, 2008). Thirdly, warming may affect transmission rates by inducing phenological changes by, for instance, accelerating reproduction and extending the reproduction and transmission period (i.e., longer summers and warmer winters) (Poulin and Mouritsen, 2006; Marcogliese, 2008; Studer et al., 2010). From another perspective, parasites can also modulate or shift the direction of warming effects to favour the hosts' fitness. For instance, infection may enhance the thermal tolerance of the host. The trematode Maritrema novaezealandensis has been observed to manipulate the snail's (Zeacumantus subcarinatus) thermal sensitivity in a way that, upon acute exposure to heat, infected snails are more active and recover faster from heat stress compared to their uninfected counterparts (Bates et al., 2011). This could be due to a parasite-induced alteration in the host's metabolic or stress response pathways, allowing the host to cope more effectively with temperature stress. Selbach et al. (2020) also found a beneficial impact of trematode infections on mussels' survival rate, where a higher infection intensity nullified the detrimental effects of temperature stress compared to milder infections.

*Gaps in knowledge*: Although mounting evidence suggests that transmission of certain parasites is facilitated by warming, causing a parasite-induced decline in host populations due to high virulence (Mouritsen et al., 2018), others contradict this perception suggesting a decrease in infectious diseases (Lafferty and Mordecai, 2016; Gehman et al., 2018). All these contradictions suggest that anthropogenic environmental perturbations may favour the increase of some parasites and the decrease of others.

#### Lack of knowledge on parasite biodiversity – molecular integration in trematode taxonomy:

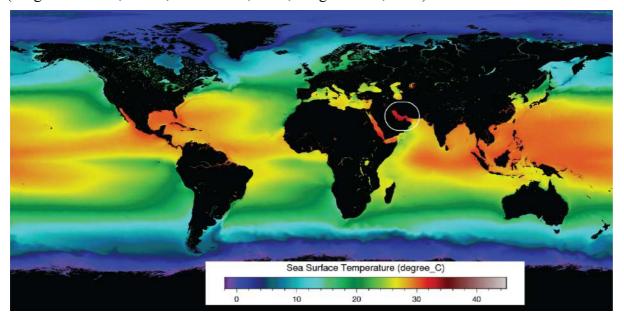
Knowledge of the world's biodiversity and geographic distribution is important for understanding how natural systems are characterized and how they function in response to natural and anthropogenic-induced changes. The ecological and medical relevance of trematodes underscores the importance of investigating snail-trematode diversity in aquatic ecosystems (Brant and Loker, 2013). Over 25,000 trematodes species have been described. Nevertheless, the number of species described until now is expected to be an underestimation of the real trematode diversity. This underestimation can be partly attributed to the lack of proper and thorough species descriptions, insufficient morphological characterizations, and a dwindling number of experts (Poulin and Presswell, 2022). Molecular and taxonomic work is important to fill the gap regarding global trematode biodiversity (Blasco-Costa et al., 2016). Integration of genetic data in the taxonomy and systematics of digenean trematodes will allow us to (i) accurately identify the adult stage although having interspecific phenotypic plasticity and interspecific variation (Cribb et al., 2022), (ii) elucidating the life cycle by linking the genetic data of larval stages with the respective adults from distinct time and locations, (iii) infer phylogeny (iv) allow an exploration of the geographical variations (Blasco-Costa et al., 2016).

*Bias in studied regions:* The underestimation in trematode diversity is not merely due to improper molecular and taxonomical descriptions but also to biases in the regions studied. There is a bias toward greater information on temperate and high latitudes compared to tropical and subtropical regions (Poulin and Morand, 2005). Moreover, to assess the impact of warming on host-parasite interactions, it is important to address those regions where warming impacts are severe and already noticeable.

Therefore, this thesis focused on two marginal seas with distinct physical and biological properties. However, both with extreme conditions regarding warming effects: the Persian Gulf—an understudied region regarding trematode diversity with extreme temperature conditions—and the Baltic Sea—where host-parasite systems are well-known and advanced warming conditions are rampant.

#### The Persian Gulf

The Persian Gulf is a shallow waterbody (average depth: 36 m) with an area of 226,000 km² positioned between subtropical arid lands. It has a limited water exchange with an estimated 90 % flushing time of 5.5 years via the 60 km Strait of Hormuz to the open ocean (Riegl and Purkis, 2012b) (Figure 3). As a result, this region is ranked as the warmest sea in summer and undergoes significant temporal and spatial variations in temperature, salinity, carbonate chemistry, and nutrients (Kleypas et al., 1999; Riegl 2001). Salinity ranges between 36–41 PSU (Ibrahim et al., 2020), and the sea surface temperature can exceed 35 °C and fall below 13 °C (Coles, 2003), with heatwave events frequently exceeding 36 °C (Alosairi et al., 2020) (Figure 3). Moreover, *in situ* temperature measurements along the northern Persian Gulf coastline show considerable diurnal variations (9–55 °C) (Bordbar et al. unpublished data), suggesting that ectotherm intertidal inhabitants of this area are experiencing some of the world's most extreme environmental conditions and referred to as natural laboratory for climate change research (Riegl and Purkis, 2012a; Bento et al., 2016; Bargahi et al., 2020).



**Figure 4.** Global 4 km MODIS-Aqua SST seasonal climatology satellite image (processing level: 11  $\mu$ ; daytime) for summer 2002-2021. According to remote sensing data, the Persian Gulf had routinely recorded the highest SST globally, surpassing 36.0 °C during a major heatwave (NASA Ocean Colour https://oceancolor.gsfc.nasa.gov/l3/#&gid=1&pid=1).

Besides, this ecosystem suffers from many human-induced environmental perturbations such as overfishing, desalination plants, petrochemical industries, and discharge of ballast water from large ships and oil tankers (Devlin et al., 2019; Al-Yamani et al., 2020; Bakhtiar et al., 2020; Saleh et al., 2021). These anthropogenic stressors combined with Persian Gulf's harsh climatic/weather conditions, generate a stressful situation across this region, pushing many

species from various ecosystems (including coral reefs, mangrove swamps, and seagrass beds) close to their upper thermal limits (Sheppard and Loughland, 2002; Burt et al., 2012, 2019; Vajed Samiei et al., 2014; Bargahi et al., 2020; Brandl et al., 2020; Moghaddam et al., 2021).

Despite the stressful environment of the Persian Gulf, it is home to a wide variety of marine life, including five species of sea turtles, 42 shorebirds, 14 marine mammals, more than 270 invertebrates, and over 1,000 fish species. (Saeidpour, 2004; Al-Yamani et al., 2009; Al-Jufaili et al., 2010; Braulik et al., 2010; George, 2012; Ghasemi et al., 2012; Behrouzi-Rad, 2014; Campbell et al., 2017; Eagderi et al., 2019; Vaughan et al., 2019).

The Persian Gulf's host-trematode systems can be among the world's most heat-tolerant gene pools, with the potential to be transferred and thrive in future warmed-up ecosystems around the globe. This speculation warrants serious consideration as many migratory birds, potential trematodes' final hosts, utilize the Persian Gulf islands and its coastline as a temporary resting, foraging and/or breeding ground (Figure 5) (Delany et al., 2009; Bom and Al-Nasrallah, 2015; de Fouw et al., 2017). In addition, ships may contribute to biological invasions due to the high volume of travel to and from this marine area (Feary et al., 2013). Despite the high abundance and richness of birds in this region, metazoan parasites remain understudied.

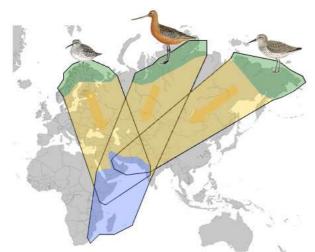


Figure 5. A representation of the migratory pathways of three dominant long-distance migratory birds that winter in The Persian and Oman Gulfs, with varying breeding areas (from left to right): *Calidris falcinellus* breading in the European Arctic and sub-Arctic (in Scandinavia), *Limosa lapponica* - in central Siberia, and *Calidris tenuirostris* - with eastern Siberian breeding ground. The Persian Gulf is a principal wintering and stopover site for a wide range of waterfowl and shorebirds, and thus an important site for parasite transmission. Map taken from (de Fouw et al., 2017).

Research on the metazoan parasites in the Persian Gulf is largely lacking. Only few studies have been carried out so far. Karimi et al. (2011) reported a prevalence of 12.7 % for trematodes, 0.2 % for nematodes, 21 % for nematomorphs infections in the sea snail *Tylothais savignyi* (Deshayes, 1844). Most of the publications written in Persian/Arabic languages treated the infection of economically valuable fishes with adult stages of trematodes, nematodes, and cestodes (Saoud et al., 1986a, 1986b, 1988; El-Naffar et al., 1992; Al Kawari et al., 1996; Sey et al., 2003; Peyghan et al., 2006; Ebrahim et al., 2015), while information on the diversity of

trematodes in the first intermediate hosts and their interactions with environmental conditions is still lacking.

Since most trematodes use molluscs as their first intermediate host, examining the digenean diversity in gastropods can enable us to capture the diversity and abundance of trematodes and their final host in a particular environment. One of the most abundant gastropods of the Persian Gulf pertains to the genus *Pirenella*. This genus is known to tolerate a wide range fluctuation of both salinity (1.2-60 PSU) (Plaziat and YounisR., 2005) and temperature (5-45 °C) (Reid and Ozawa, 2016). Members of the genus are found in densities of up to 2,100 ind.m<sup>-2</sup> in intertidal mudflats (Basson et al., 1977) and are known to serve as first intermediate host of a number of trematode parasites (Taraschewski, 1985; Chong-ti, 1990; Al-Kandari et al., 2012). Pirenella cingulata (Gmelin,1791) (Gastropoda: Potamididae; known as a horn snail or mudwhelk), is the most abundant and widespread species on sandy-muddy substrates along the Persian and Oman Gulfs (Vahidi et al., 2020). This macrobenthic invertebrate species relies on microbial mats (diatoms and cyanobacteria filaments) as a source of nutrition (Al-Zaidan et al., 2006). It is a major keystone species and plays an essential ecological role in the structure and function of marine intertidal zone by supporting critical ecological activities such as bioturbation and nutrient recycling, as well as significantly contributing to the transfer of energy from basic organic sources to higher trophic levels (Tumbiolo and Downing, 1994; Bolam and Eggleton, 2014; Saulnier et al., 2019). Given the high abundance, tolerance, and ecological role of this species, it is of high relevance to elucidate the trematode fauna parasitizing this gastropod and the thermal profile of the most abundant trematode species.

#### The Baltic Sea

Physical characteristics: the Baltic Sea is a landlocked marginal sea of the North Atlantic Ocean, located in a temperate region, has a surface area of 349,644 km², and an average depth of 55 m (Kullenberg and Jacobsen, 1981). It has limited water exchange with the North Sea through the Danish Skagerrak and Kattegat straits. The Baltic Sea is characterized by a strong salinity gradient and persistent halocline (highly stratified water column) (Snoeijs-Leijonmalm et al., 2017). The sharp reduction in salinity from marine (30-20 PSU) in the Kattegat to near freshwater (3-1 PSU) in the Bothnian Bay is regulated by the surplus of riverine freshwater, vertical mixing, and intermittent inputs of the North Sea's waters (Reissmann et al., 2009). Through the depth increase, the salinity increases exponentially while the temperature reduces and thus forming a boundary layer (Kullenberg and Jacobsen, 1981). Thus, the mixing of surface

and deep waters is severely restricted, resulting in oxygen deficiency in the depths (Fonselius, 1981).

The Baltic Sea's ecosystem is largely influenced by major anthropogenic pressures such as warming, acidification, nutrient pollution, and deoxygenation that most coastal regions could only face in the future, making it a time machine for climate change research (Reusch et al., 2018). The Baltic Sea is one of the fastest-warming marginal seas, with exceptional SST change over the previous decades, including a 1.35 °C increase between 1982–2006, which is seven times higher than the global rate (Belkin, 2009). Surface warming enhances the water column stratification, reducing vertical mixing and increasing marine heat waves. The Baltic Sea can be separated into five distinct regions with similar SST patterns: the Bothnian Bay, the Bothnian Sea, the eastern and western Baltic proper, and the southwestern Baltic Sea (Dutheil et al., 2022). In our study system, the shallow (about 1 m depth) coastal zone of the southwestern Baltic Sea, temperature fluctuations of 1–6 °C are frequent, with upwelling episodes causing temperature oscillation of up to 8 °C over days to weeks (Franz et al., 2019). The average sea surface temperature in the Baltic Sea is projected to rise by 1.5–4 °C by the end of the 21st century (Meier et al., 2012). Furthermore, it is one of the most well-studied marine environments, with many long-term data series, thus making it an ideal location for conducting multi-stressor experiments (Carstensen et al., 2014; Huang et al., 2015; Liu et al., 2015; Cloern et al., 2016).

In the case of host-parasite systems, the basic knowledge about trematode's diversity and their respective life cycles is available for most of the species in the Baltic Sea (Werding, 1969; Zander, 1998; Køie, 1999; Zander et al., 2000; Zander and Reimer, 2002). Moreover, ecological studies have been done to better understand the host-parasite system by either including the parasite as a biotic stressor in ecological studies or assessing the influence of abiotic factors (e.g., temperature and salinity) on host fitness, and parasites' emergence and transmission success (Thieltges and Rick, 2006; Bommarito et al., 2020). One of the well-established host-parasite systems in the Baltic Sea consists of a trematode parasitizing snails, mussels, and shorebirds. The trematode *Renicola roscovita* uses *Littorina littorea* as the first intermediate host, *Mytilus edulis* as the second intermediate host and shorebirds as the final host. *R. roscovita* and *Himasthla elongata* were subject of several studies (Thieltges and Rick, 2006; Fokina et al., 2018; Bakhmet et al., 2019; Díaz-Morales et al., 2022). Bommarito et al. (2021) utilised the regional salinity gradient (from 13 to 22 PSU) in the western Baltic Sea, to study the infection pattern in first (*Littorina littorea*) and second (*Mytilus edulis*) intermediate hosts of *H. elongata* and *R. roscovita*. They found that the abundance of *H. elongata* in the second host (*M. edulis*)

is correlated with salinity and density of infected first host (L. littorea), however, in the case of R. roscovita infection, mussel size is an additional factor explaining the parasite abundance in the second intermediate host (M. edulis). They concluded that a decrease in trematode transmission with a potential decrease in host density is plausible under a possible freshening scenario for the Baltic Sea. In another study, Bommarito et al. (2020) found that salinity as the main driver of cercarial activity and infectivity and mussel's susceptibility to infection. In contrast, warming had a significant detrimental effect on cercarial longevity. The results of this study suggest that freshening rather than global warming might induce a decline in marine trematodes. In another study, it was found that *Littorina littorea* infected with *H. elongata* had greater thermal sensitivity with substantial mortality at 22 °C, which is the projected summer temperature for the western Baltic Sea by the end of the century (Díaz-Morales et al., 2022). Although they found 22 °C as an optimum temperature for cercarial emergence and infectivity, cercarial survival was reduced, implying that effective mussel infection has a shorter time window. Moreover, they observed that over time, temperature decreased cercarial selfpropelling capacity. Their findings suggest that warming might hamper the possibility of the trematode to flourish.

The aforementioned host-parasite system also inhabits European coastal environments like North Sea, White Sea, and Wadden Sea, and have been the subject of several field and laboratory studies (Mouritsen et al., 1999; Nikolaev et al., 2006; Bakhmet et al., 2017; Liddell et al., 2017; Selbach and Mouritsen, 2020). For instance Thieltges (2006) found that *R. roscovita* infected *M. edulis* had lower growth compared to uninfected counterparts. Moreover, the findings indicated that tidal height had a greater influence on mussels' growth than trematode infection. Stier et al., (2015) reported a reduction in filtration rate of infected *M. edulis* with *R. roscovita* particularly in large mussels (shell length 4 cm). Nevertheless, the combined effect of temperature fluctuation and trematode infection on filtration and respiration of *M. edulis* was not investigated and remains to be elucidated.

#### Aim, objectives, and findings

This thesis first aimed to provide insights into the trematode biodiversity along the Persian Gulf coastline, using molecular analyses of the most abundant host snail, *Pirenella cingulata* (Chapter 1). In light of the first chapter's findings, it then aimed to elucidate the life cycle of a clinically important trematode, *Ornithobilharzia canaliculata* (Rudolphi, 1819) Odhner, 1912 and putative new species of *Austrobilharzia* Johnston, 1917, (Chapter 2), and to characterize thermal sensitivity of cercarial emergence for two abundant trematodes infecting *P. cingulata* 

(Chapter 3). These three chapters not only establish an essential baseline data for the trematode diversity of an understudied yet abundant snail at the world's warmest sea but also provide information on the temperature-dependence of cercarial emergence, important to infer about the possibility of future flourishment in other sea areas.

Finally, this thesis also aimed to evaluate the combined effect of trematode infection and acute warming on the metabolic trait of blue mussel *M. edulis* infected with *R. roscovita* from the Baltic Sea (Chapter 4). This last chapter serves as another stepping stone in understanding global warming effects on the Baltic Sea, a water body already experiencing warming conditions projected for other regions.

In **Chapter 1**, a comprehensive examination of the diversity of trematodes in the horn snail, *Pirenella cingulata*, is conducted. Snails were collected from eight locations along the Northern Persian and Oman Gulfs, individually incubated, and examined for the presence of patent infections. Recovered parasites' intramolluscan stages were preserved, and DNA was extracted from representative samples. Then, the genes for mitochondrial cox1 and nuclear 28S rRNA were amplified and sequenced. This enabled molecular identification and phylogenetic reconstruction, revealing 29 distinct species belonging to ten families. These trematodes, which are frequently neglected, have significant ecological effects and can also be of medical importance.

Chapter 2: In snails of the species *P. cingulata*, a molecular analysis revealed the presence of two schistosome species, *Ornithobilharzia canaliculata* and a putative novel species of *Austrobilharzia*. Two locations were sampled for snails, and their infections were determined using 28S and cox1 DNA sequences. This is the first time that *Ornithobilharzia* has been discovered in the Persian Gulf and in a potamidid snail host. It increased our knowledge of the distribution and host range of important agents of cercarial dermatitis and cast light on host-parasite relationships.

The thermal sensitivity of cercarial emergence was examined in **Chapter 3**. The focus of the investigation was on the two most common trematode species that infect *P. cingulata* snails. To determine the temperature dependence of cercarial emergence, infected snails were maintained at varying temperatures in the laboratory. This study uncovered the highest thermal optimum temperatures ever recorded for aquatic trematode species and established a maximum emergence temperature for these organisms. This provides crucial information regarding how these organisms may react to global warming scenarios.

**Chapter 4** tests the hypothesis that *Renicola roscovita* metacercarial infections affect the filtration and/or respiration of *M. edulis* as well as its acute heat sensitivity. The study revealed

that infection diminishes the filtration response of *M. edulis*, with the effect becoming more pronounced in response to temperature fluctuations. This chapter also investigates the connection between filtration rate and infection severity. The findings shed light on the intricate dynamics of host-parasite interactions under environmental stress.

Chapters and contributions of authors

Chapters and contributions of authors

Parts of this doctoral thesis have been published or submitted for publication:

Chapter 1:

Authors: Maral Khosravi\*, David W. Thieltges, and Simona Georgieva\*

Status: In preparation

<u>Contributions</u>: M.K. and SG conceived the research and performed field data collection. M.K. obtained most of the field data, performed the sequencing, and contributed to data analyses and writing the first draft. S.G. participated in the data acquisition, analysed the results and wrote the manuscript. D.W.T. and J.S. reviewed and edited the manuscript. All authors read and approved the final manuscript.

**Chapter 2:** 

Authors: Maral Khosravi, David W. Thieltges, Jebreil Shamseddin and Simona Georgieva

Status: Published in Scientific Reports

<u>Contributions:</u> MK and SG: conceived the study, obtained samples, carried out the sequencing, performed analyses prepared the first draft of the manuscript. MK, DT, JS, SG discussed the results and helped draft the manuscript. All authors read and approved the final manuscript.

**Chapter 3:** 

<u>Authors:</u> Maral Khosravi, Dakeishla M. Díaz-Morales, David W. Thieltges, Martin Wahl and Jahangir Vajedsamiei

Status: Published in Scientific Reports

<u>Contributions:</u> MK, JV, DWT, MW. designed the study. MK and DD ran the experiments, MK, JV, analysed the data, MK and DD wrote the first draft of the manuscript. All co-authors discussed the results, as well as reviewed and contributed to the final manuscript.

**Chapter 4:** 

<u>Authors:</u> Maral Khosravi, David W. Thieltges, Dakeishla M. Díaz-Morales, Claudia Bommarito and Jahangir Vajedsamiei

Status: Published in International Journal for Parasitology: Parasites and Wildlife

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<u>Contributions:</u> MK, JV, DWT designed the study; MK, JV ran the experiments, analysed data and wrote the first draft; DD, CB, and DWT helped in writing and revising the manuscript. All co-authors discussed the results, reviewed and contributed to the final manuscript.

# Chapter 1

Phylogenetics reveal trematode diversity in a dominant marine gastropod (*Pirenella cingulata*) in the Persian Gulf and the Gulf of Oman

# Phylogenetics reveal trematode diversity in a dominant marine gastropod (*Pirenella cingulata*) in the Persian Gulf and the Gulf of Oman

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#### **Abstract**

The Persian Gulf is among the highest anthropogenically impacted regions in the world. Harsh environmental condition, such as extreme seasonal temperatures and salinity fluctuations select for species with high tolerance and adaptability to short-term changes. Marine biodiversity data from the region are still scarce mainly covering larger taxa, while a substantial portion of the invertebrates remain poorly known. In this study we applied an integrative taxonomy approach to assess the trematode diversity in one of the most abundant mollusc species, the marine mud snail *Pirenella cingulata* along the coast of Iran. Examination of 1,696 *P. cingulata* at eight locations along the coast of Iran revealed diverse trematode fauna accounting for a total of 29 species. Component community analysis revealed distinctive trematode community composition at the different sampling locations. Higher overall prevalence of infection was detected along the northern coast which is characterized with lower temperature and salinity levels. Predominant part of the species recovered were parasites of birds with nearly third of major etiological significance. Our results highlight the importance of reference database in biodiversity surveys and provide a solid baseline for future studies on the shift tolerance, biodiversity and distribution of the larval trematodes.

Keywords: Persian Gulf, Larval trematode diversity, Pirenella cingulata

#### Introduction

The Persian Gulf and the Gulf of Oman are peripheral seas of the Northwest Indian Ocean that represent economically and ecologically important regions of the world's ocean (Farhadi and Anderson 2021). Both Gulfs are connected water basins characteristic with specific environmental features (Sheppard, Price, and Roberts 1992), including high air and water temperatures, intense evaporation and consequently high salinities that are most prominent during the summer season (Carpenter, Krupp, and Jones 1997; Sanders and Morgan 1989). Located in one of the most arid regions of the world, the Persian Gulfs is further characterized by the greatest seasonal temperate range in the world and the highest annual sea temperature (Sheppard et al. 2010). These environmental extremes have enabled the evolvement of a unique biota with high levels of endemism (Sheppard et al. 1992). In particular the landlocked Persian Gulf, known as the warmest marine ecosystem on the planet, harbours unique biota adapted to high salinity levels and water temperature (Farhadi and Anderson 2021). However, its marine fauna is considerably impoverished as a result of the harsh environmental conditions and its young geological age (Sheppard et al. 2010). Additionally, both basins are subject to severe anthropogenic stress, including rapid coastal urbanization, dumping of untreated sewage, release of brine from desalination plants, reduction in riverine influx, oil and related industries including high maritime activities for oil and gas transportation (Al-Said, Sarkar, and Madhusoodhanan 2021; Farhadi and Anderson 2021). Due to this, both Gulfs are known as the most anthropogenically impacted marine regions on the planet (Farhadi and Anderson 2021). Despite the high ecological value of the Gulfs and increased economic interest in the region, both marine basins are poorly studied in terms of biodiversity (Farhadi and Anderson 2021). Marine biodiversity data are still scarce, mainly covering vertebrate and some of the more common invertebrate taxa, while a substantial portion of the invertebrate fauna remains poorly known (Jawad 2021). Among the better-known taxa in the Gulfs are molluscs which are key organisms in aquatic communities that exhibit essential roles in ecosystem functioning such as decomposition and nutrient cycle and food source of high trophic level taxa. Due to these characteristics, they are commonly used as indicator taxa in biomonitoring surveys in the region (Astani et al. 2012; Mohammad Karami et al. 2014). In contrast, little is known on the parasite fauna inhabiting molluscs in the Gulfs. Molluscs commonly serve as obligate hosts for trematodes, a diverse and functionally important group of metazoan parasites in aquatic ecosystems (Esch, Barger, and Fellis 2002). Due to the sequential use of different invertebrate and vertebrate hosts for the completion of their life cycles, trematode parasites are recognised as key players in ecosystem functioning, e.g., being important regulators of host populations as well as contributors to ecosystem biodiversity, energetics and food web structure (Huspeni and Lafferty 2004; Lafferty 1997; Shea et al. 2012; Vidal-Martinez et al. 2010). While their immense significance in ecosystem functioning is widely acknowledged, there is a huge discrepancy in respect to the currently available knowledge on their true diversity, distribution and host associations. This is partially due to the fact that data on trematode parasite distribution and complex host associations are heavily biased towards specific sampling locations, research efforts and limited host-parasite systems studied. Further, increasingly limited taxonomic expertise (Poulin and Presswell 2022) frequently results in a poor estimation of trematode diversity which represents a prime obstacle for studies on ecology, ecosystem functioning, and subsequently affects conservation strategy implementation.

DNA-based approaches are promising tools to help overcome our poor knowledge on trematode diversity as they have generally accelerated the pace and accuracy of biodiversity studies and monitoring (Deiner et al. 2017; Euclide et al. 2021; Ji et al. 2013; Keck et al. 2017; Keck, Couton, and Altermatt 2022). They have become a critical tool in the biodiversity sciences, especially in respect to taxonomic groups comprising species complexes with high levels of interspecific homogeneity of morphological characters as often observed in parasitic organisms (Blaxter 2004; Creer et al. 2016; Criscione, Poulin, and Blouin 2005; Galan et al. 2018; Georgieva et al. 2013; Keck et al. 2022; de León and Nadler 2010; Pedersen et al. 2015). However, DNA-based approaches rely exclusively on reference databases that link genetic sequence data to specific taxa. Current taxonomic coverage and reference database quality often represent a serious pitfall that is geographically and taxonomically biased (Weigand et al. 2019). Phylogenetic analyses are essential in this respect allowing for sequence data matches of multiple hosts and distinct areas. Furthermore, they are a methodological tool in the assessment of relationships between traits and play a key role in analyses of spatial patterns in biodiversity. Taxonomy is at the root of our understanding about the richness of life on Earth and a centre of all biodiversity knowledge. Modern integrative taxonomy encompassing the discovery, description, circumscription, naming and documenting of species embraces multifaceted approaches in a phylogenetic framework (Matzke 2022). Phylogeny-based research has a major implication in respect to measuring the biodiversity and encountering how it changes in time and across space. Applying a phylogenetic approach frequently reveals "cryptic" speciation by the means of very young lineages that have diverged in an evolutionarily short period of time. Assessing the phylogenetic diversity in a region is assumed to represent a more stable and useful measurement of the biodiversity. Mapping phylodiversity adds a historical dimension to studies on the processes that shape biodiversity and its distribution. Joint

estimation of individual gene phylogenies along population phylogenies has become more frequently applied as it scales up by considering large population sizes and small time between population-divergence events.

In this study, we investigated trematode diversity along the Iranian coastline in the Persian Gulf and Gulf of Oman based on DNA- and phylogenetic approaches. We present the first known estimates of the trematode diversity, transmission pathways and host associations in one of the most abundant and heat tolerant marine mollusc species along the Iran coast, the mud snail *Pirenella cingulata*. The application of DNA-based approaches and the integration of morphological and ecological data have led to the discovery of exceptionally high trematode species richness in one of the world's most extreme marine environments. We provide a molecular framework and established trematode diversity baselines that will enable consistent identification and exploration of the local host-parasite systems at temporal scales in the future.

## Results

#### **Prevalence of infection**

A total of 1,696 *P. cingulata* was collected from eight different sites along the coast of Iran between December 2019 and January 2020 (Fig. 1; see Table 1 for details on sample sizes). Of these, 399 were recovered infected (23% overall prevalence). The prevalence of infection ranged considerably among the sampled locations and ranged from 3.0% (Dylam) to 69.0% (Shif) (Table 1). Samples from the southern locations, situated at the Strait of Hormuz revealed lower prevalence of infection (6.2–23%; average 14.8%) than the northernmost east cost of the Persian Gulf (3.0–69%; average 33.5%).

#### Phylogenies based on sequence data

Molecular data was successfully generated for representatives of the trematode species sampled. A total of 371 cox1 and 86 28S rDNA sequences were obtained and the position for each species was determined. Species delimitation was based on the comparison of molecular data including family level phylogenies and genetic distances. Individual phylograms illustrate species level relationships yielded from Bayesian inference and maximum likelihood analyses which allow for a visual comparison of the relative rates of evolution among the clades. Only representative partial 28S rDNA sequences for the recovered species were used in the final analyses. The cox1 datasets included all available sequences of the corresponding groups and were evaluated under the maximum likelihood analyses after excluding redundant sequences. Figures 2–7 depict the results of individual gene analyses.

In total, three cyathocotylids, two schistosomatids, and 24 plagiorchiideans of five suborders (Echinostomata, Haplosplanchnata, Hemiurata, Opisthorchiata and Pronocephalata) were recovered in *P. cingulata*. Single gene phylogenies were constructed for restricted datasets of the Cyathocotylidae (Fig. 2), Himasthlidae and Philophthalmidae (Fig. 3), Haplosplanchnata (Fig. 4), Hemiuroidea (Fig. 5), Heterophyidae (Fig. 6), and Microphalloidea (Fig. 7). A single case of a notocotylid infection was detect at the Northern location Genaveh. The newly generated partial *cox*1 sequence was compared against the published data available in GenBank. The BLASTn search indicated 72.44% identity with *Ogmocotyle sikae* (KR006934; two gaps, 100% query cover). We recovered only three snails infected with avian schistosomes, two from Genaveh and a single infection at Jask. Our molecular analyses inferred from *cox*1 and 28S sequences revealed presence of two schistosome species, *Ornithobilharzia canaliculata* (Rudolphi, 1819) and a putative new species of *Austobilharzia* Johnston, 1917. Detailed description of the findings is published separately (see Khosravi et al., 2022) reporting on the first confirmed elucidation of the life cycle of *O. canaliculata* and first report of its intermediate host.

### Composition and richness of the trematode fauna

A total of 29 trematode species belonging to 10 families was recovered: Cyathocotylidae Mühling, 1898 (three spp.), Haplosplanchnidae Poche, 1926 (one sp.), Hemiuridae Looss, 1899 (one spp.), Heterophyidae Leiper, 1909 (five spp.), Himasthlidae Odhner, 1910 (four spp.), Microphallidae Ward, 1901 (four spp.), Notocotylidae Lühe, 1909 (one sp.), Philophthalmidae Looss, 1899 (five spp.), Renicolidae Dollfus, 1939 (three spp.), Schistosomatidae Stiles & Hassall, 1898 (two spp.); (see Table 1 for species names, taxonomy and summary of hosts involved in their life cycles). Species identification was based on molecular data and general cercarial morphology. However, due to the scarce exiting molecular data on marine larval trematodes, only three species could be identified to species level, namely Acanthotrema tridactyla, Cloacitrema narrabeenensis, and Ornithobilharzia canaliculata. As the taxonomy of the Trematoda is based on the morphology of adult stages, formal identification of these isolates awaits until the corresponding adult stages are matched and/or recovered. Some of the species found in the course of this study may represent new species to science. Matching sequence data for different stages of the subjected trematode groups led to the partial life cycle elucidations of five taxa for which little or no information on the intramolluscan stages currently exists, i.e., Cloacitrema narrabeennensis, O. canaliculata, Pseudohaplosplanchnus, Saturnius and Nephromonorcha.

Trematode species richness varied considerably between the sampling sites ranging from 3 (Dylam) to 14 (Geneveh). Along the northern coast of the Persian Gulf, a total of 12 species were recovered: Heterophyidae gen. sp. 1, Heterophyidae gen. sp. 2, Himasthlidae gen. sp. 1, Maritrema sp. 2, Microphallidae gen. sp. 2, Microphallidae gen. sp. 3, Microphallidae gen. sp. 1, Notocotylidae gen. sp., O. canaliculata, Philophthalmus sp., Renicola sp. 1, and Saturnius sp.). Across the southern locations, a total of 11 species was unique to this region: Acanthoparyphium sp. 2, A. tridactyla, Austrobilharzia sp. (Iran), Galactosomum sp., Heterophyes sp., Mesostephanus sp. 1, Microphalidae gen. sp. 2, Philophtalmidae gen. spp. 1-3, and Renicolidae gen. sp., were present only, while six other species observed in the north also occurred in the south: Acanthoparyphium sp. 1, C. narrabeenensis, Cyathocotylidae gen. sp., Himasthlidae gen. sp. 2, Nephromonorcha sp., Pseudohaplosplanchnus sp. Out of the 29 species detected were present across both the northern and southern locations sampled along the coast of Iran. Overall, the total species richness was higher across the northern coast with a total of 20 versus 17 species detected along the southern coast. The most abundant species were Acanthotrema tridactyla (n = 52 infections), Cytacotylidae gen. sp. (n = 48), Saturnius sp. (n = 21), Heterophyidae gen. sp. 2 (n = 13), Heterophyes sp. (n = 12), and Pseudohaplosplanchnus sp. (n = 11).

#### Life cycle strategies

Trematode life cycles were reconstructed based on literature data. The predominant part (69%) of the trematode species recovered exhibited a typical three-host life cycles, seven species (24.1%) used an encystment in the environment as a transmission strategy to the definitive host, and only 2 species (6.9%) had a two-host life cycles with a direct infection of the definitive host. With the exception of Cyathocotylidae, Microphallidae, and Notocotylidae which can infect mammals as definitive hosts, all remaining species mature either in birds or fish and utilise at least six different life cycle pathways with respect to the combination of host groups used at each step of their life cycle: (i) snail-snail-bird (Himasthlidae); (ii) snail-snail/fish-fish (Renicolidae, Haplosplanchnidae); (iii) snail-fish-bird/mammal (Cyathocotylidae, Heterophyidae); (iv) snail-crustacean-bird (Microphallidae; Hemiuridae); (v) snailbird/mammal (Schistosomatidae); and (vi) snail-vegetation-fish/bird/mammal (Notocotylidae, Philophthalmidae, Haplosplanchnidae). Overall, the majority of the species (n = 22) fall within a transmission guild of trematodes of fish-eating birds that use fishes as second intermediate host. However, a predominant part (n = 13) of them belonged to a generalist guild that have incorporated mammalian hosts in their life cycles, typically in parallel with bird hosts. The remaining parasite species consisted of two species of gulls with a two-host life cycles, a single species of mammals with two-host life cycle, and two species parasitic in fish hosts with a typical three-host life cycles (see Table 1 for details).

At least six of the recovered species in *P. cingulata* are of zoonotic importance, namely, *Acanthoparyphium* spp. 1 and 2, *A. tridactila, Heterophyes* sp., *O. canaliculata*, and *Austrobilharzia* sp. Iran. The former four species are causatives of foodborne trematodiases (Keiser and Utzinger 2009; Sripa et al. 2010). while the latter two are known as important waterborne causatives of the human cercarial dermatitis (Khosravi et al. 2022). Given the taxonomic affiliation and life cycle pathways, the remaining 23 trematode species recovered in the mud snail along the Iranian coast are likely of no direct public health relevance.

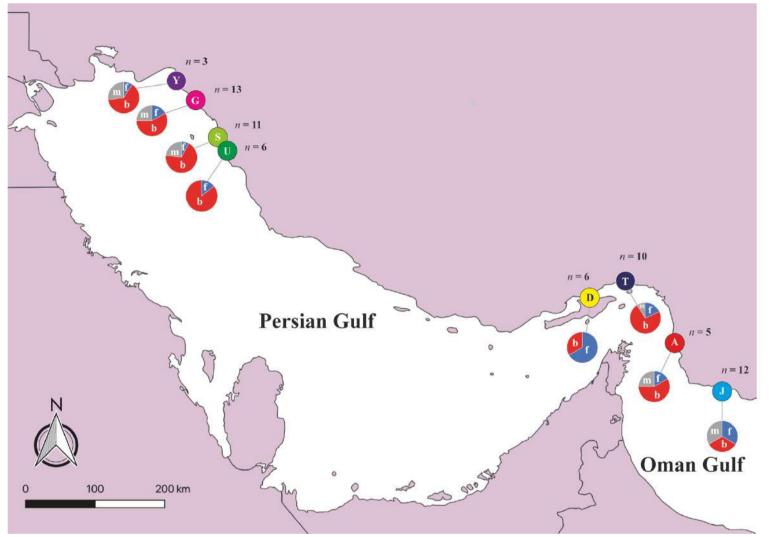


Fig. 1 Map of the Persian Gulf with the eight sampling localities along the coast of Iran indicated by round coloured circles and a letter coding. The pie-chart indicate the species richness (n) at each location. Colour code assigned for bird is red, for fish it's blue, and for mammal it's grey. The pie-charts indicate the definitive host groups (b, birds; f, fish; m, mammals) as a proportion for the trematode species recovered.

**Table 1** Summary of the species recorded in *P. cingulata* along the coast of Iran and their respective intermediate and definitive host groups. Each locality has been abbreviated as follows: Genaveh (G), Deylam (Y), Bushehr (B), Shif (S), Jusk (J), Bandar Abbas (T), Azini (A), and Dargahan (D).

(D).	North			_	South				Second IH	Definitive host
Locality	G	Y	В	S	J	T	A	D		
Totals	200	200	200	200	226	266	195	200		
No. of infected snails	101	6	23	138	39	62	12	18		
Mean shell height ± SD	$20.22 \pm 1.4$	$17.45 \pm 0.9$	$19.76 \pm 0.9$	$20.35 \pm 1.43$	$25.36 \pm 1.4$	$20.89 \pm 1.4$	$21.35 \pm 1.2$	$22.23 \pm 1.2$		
Overall prevalence	50.5	3.0	11.5	69.0	17.3	23.3	6.2	9.0		
Suborder Diplostomata										
Superfamily Diplostomoidea Poirier, 1886										
Cyathocotylidae Mühling, 1898									F, A, AqInv	R, B, M
Cyathocotylidae gen. sp.	✓			✓	✓	✓		✓		
Mesostephanus sp. 1			✓							Mainly B, M
Mesostephanus sp. 2					✓	✓		✓		Mainly B, M
Superfamily Schistosomatoidea Stiles & Hassall, 1898										
Schistosomatidae Stiles & Hassall, 1898									_	В
Austrobilharzia sp.					✓					В
Ornithobilharzia canaliculata (Rudolphi, 1819)	✓									В
Suborder Haplosplanchnata										
Haplosplanchnidae Poche, 1926									EE	F
Pseudohaplosplanchnus sp.	✓	✓	$\checkmark$	✓	✓	✓	✓	✓		F
Suborder Echinostomata										
Superfamily Echinostomatoidea										
Himasthlidae Odhner, 1910									G, Bi, Po	В
Himasthlidae gen. sp. 1	✓		✓	✓						В
Himasthlidae gen. sp. 2	✓			✓	✓	✓		✓		В
Acanthoparyphium sp. 1						✓				В
					I					

Acanthoparyphium sp. 2						✓				В
Philophthalmidae Looss, 1899									EE	Mainly B, Ma
Cloacitrema narrabeenensis Howell & Bearup, 1967	✓		✓		✓	✓	✓	✓		Mainly B, M
Philophtalmidae gen. sp. 1					✓	✓				Mainly B, M
Philophtalmidae gen. sp. 2					✓	✓				Mainly B, M
Philophtalmidae gen. sp. 3					✓					Mainly B, M
Philophtalmidae gen. sp. 4	✓									Mainly B, M
Suborder Hemiurata										
Superfamily Hemiuroidea Looss, 1899										
Hemiuridae Looss, 1899									C	F
Saturnius sp.		✓	✓	✓						F
Suborder Opisthorchiata										
Superfamily Opisthorchioidea Looss, 1899										
Heterophyidae Leiper, 1909									F	$F^b$ , B, M
Acanthotrema tridactyla (Martin & Kuntz, 1955)	✓	✓		✓	✓	✓	✓	✓		Mainly B, M
Heterophyes sp.	✓						✓			Mainly B, M
Galactosomum sp.					✓					Mainly B, M
Heterophyidae gen. sp. 1				✓						
Heterophyidae gen. sp. 2				✓						
Suborder Xiphidiata										
Superfamily Microphalloidea Ward, 1901										
Microphallidae Ward, 1901									C	Mainly B
Maritrema sp.	✓									Mainly B
Microphallidae gen. sp. 1	✓					✓				
Microphallidae gen. sp. 2			✓	✓						
Microphallidae gen. sp. 3				✓						
Renicolidae Dollfus, 1939									Bi, F	В
Nephromonorcha sp.	✓			✓	✓					В
					ı					

Renicola sp.	✓		✓						В	
Renicolidae gen. sp.							✓			
Suborder Pronocephalata										
Pronocephaloidea Looss, 1899										
Notocotylidae Lühe, 1909									EE	
Notocotylidae gen. sp.	✓									B, M
Number of harboured trematode species	14	3	7	11	12	11	5	6		

Abbreviations: A, amphibians; AqInv, aquatic invertebrates; B, birds; Bi, bivalves; C, crustaceans; F, fish; G, gastropods; M, mammals; Po, polychaetes;

*Note*: the lack of second intermediate host is indicated either with EE (encystment in the environment), or with a dash, when a direct infection of the definitive host is present.

<sup>&</sup>lt;sup>a</sup>The species have incorporated mammalian hosts in their life-cycles, typically in parallel with bird hosts.

<sup>&</sup>lt;sup>b</sup>Occasionally freshawater siluroid fish (Bray, 2008).

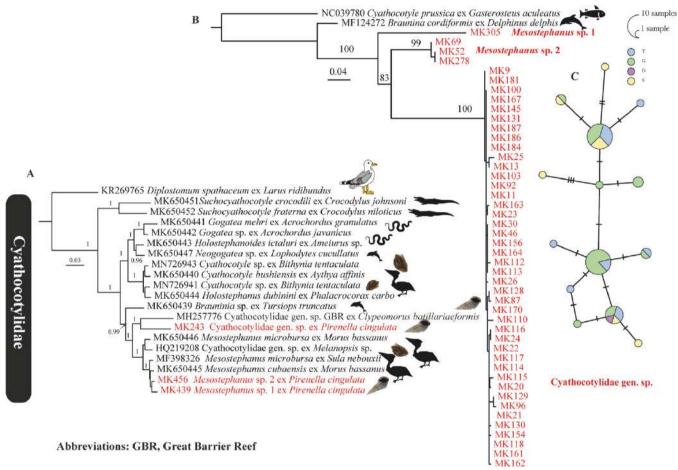


Fig. 2 A. Bayesian inference analysis of the 28S rDNA dataset for the family Cyathocotylidae constructed under the GTR+I+G model of nucleotide evolution. Host origin of the respective sequences are symbol indicated on the tree. Newly-generated sequence is colour indicated in red. The scale-bar indicates the expected number of substitutions per site. B. Maximum likelihood phylogram for the cox1 dataset constructed with RaxML under GTR+G model of evolution. Posterior probability ( $\geq 0.95$ ) and bootstrap support values ( $\geq 70\%$ ) are indicated on the nodes. C. Cox1 statistical parsimonious haplotype network of the most abundant species Cyathocotylidae gen. sp. Locations of haplotype origin are colour coded. Each vertex represents a unique haplotype with vertex size corresponding to haplotype frequency. Hatch-marks indicate inferred haplotypes not observed in the dataset.

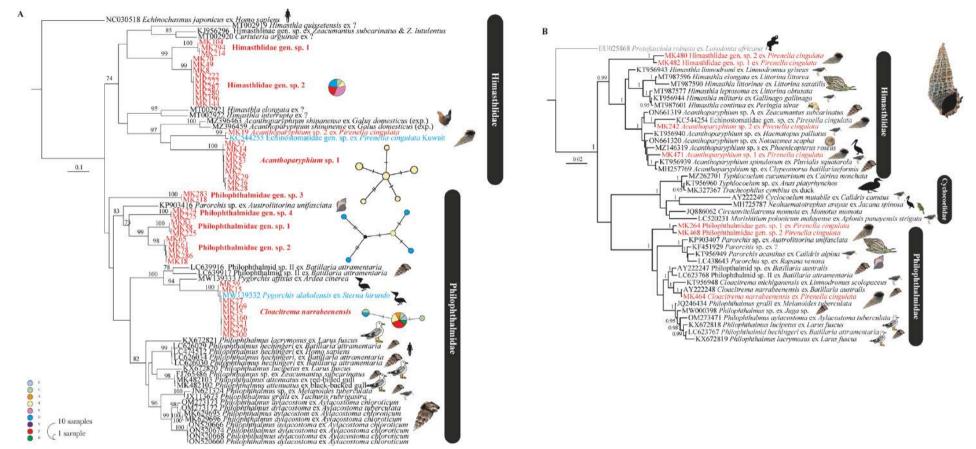
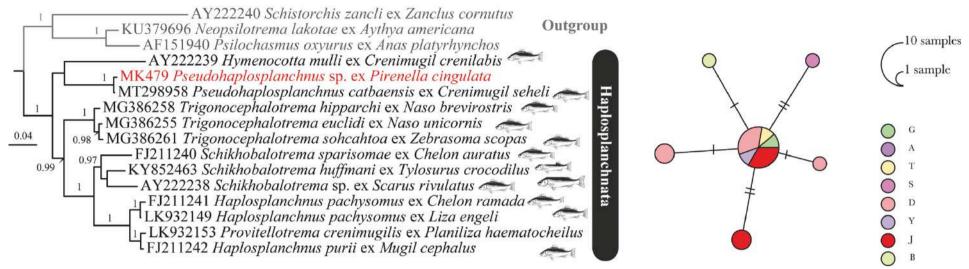
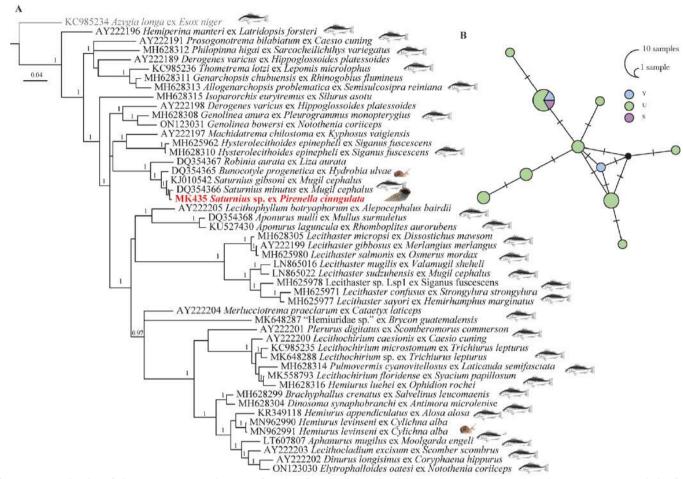


Fig. 3 A. Maximum likelihood phylogram for the cox1 dataset of the Himasthlidae + Philophthalmidae constructed with RaxML under GTR+GAMMA model of evolution. Cox1 statistical parsimonious haplotype networks for each of the species recovered is present next to the corresponding clade. Locations of haplotype origin are colour coded. Each vertex represents a unique haplotype with vertex size corresponding to haplotype frequency. Hatch-marks indicate inferred haplotypes not observed in the dataset. B. Bayesian inference analysis of the 28S rDNA dataset constructed under the GTR+I+G model of nucleotide evolution. Host origin of the respective sequences are symbol indicated on the tree. Newlygenerated sequences are colour indicated in red. Posterior probability ( $\geq 0.95$ ) and bootstrap support values ( $\geq 70\%$ ) are indicated on the nodes. The scale-bar indicates the expected number of substitutions per site.



**Fig. 4 A.** Bayesian inference analysis of the 28S rDNA dataset for the family Haplosplanchnidae constructed under the GTR+I+G model of nucleotide evolution. Newly-generated sequence is colour indicated in red. The scale bar indicates the expected number of substitutions per site. **B.** *Cox*1 statistical parsimonious haplotype network of *Pseudohaplosplanchnus* sp. The locations of haplotype origin are colour coded. Each vertex represents a unique haplotype with vertex size corresponding to haplotype frequency. Hatch-marks indicate inferred haplotypes not observed in the dataset.



**Fig. 5 A.** Bayesian inference analysis of the 28S rDNA dataset for the family Hemiuridae constructed under the GTR+G model of nucleotide evolution. Posterior probability values (≥ 0.95) are indicated on the nodes. Newly-generated sequence is colour indicated in red. The scale-bar indicates the expected number of substitutions per site. **B.** *Cox*1 statistical parsimonious haplotype network of *Saturnius* sp. Locations of haplotype origin are colour coded. Each vertex represents a unique haplotype with vertex size corresponding to haplotype frequency. Hatch-marks indicate a single mutational step, small black vertex indicates inferred haplotype not observed in the dataset.

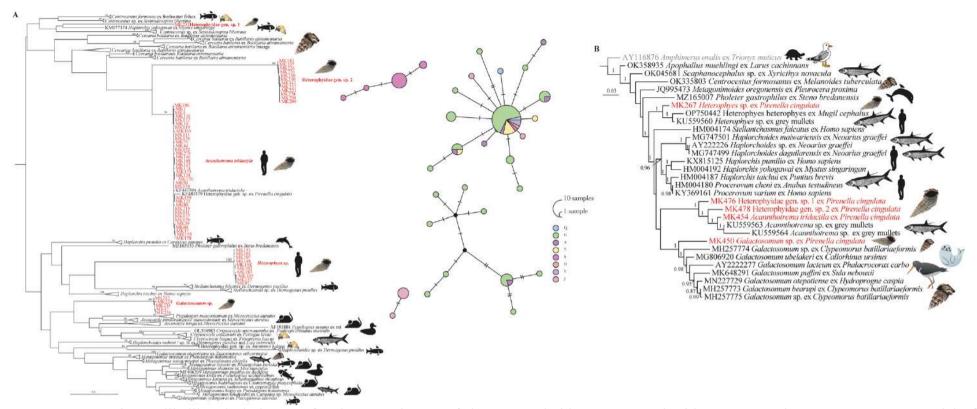


Fig. 6 A. Maximum likelihood phylogram for the cox1 dataset of the Heterophyidae constructed with RaxML under GTR+GAMMA model of evolution. Cox1 statistical parsimonious haplotype networks for each of the species recovered is present next to the corresponding clade. Locations of haplotype origin are colour coded. Each vertex represents a unique haplotype with vertex size corresponding to haplotype frequency. Hatch-marks indicate inferred haplotypes not observed in the dataset. B. Bayesian inference analysis of the 28S rDNA dataset constructed under the GTR+I+G model of nucleotide evolution. Host origin of the respective sequences are symbol indicated on the tree. Newly-generated sequences are colour indicated in red. Posterior probability ( $\geq 0.95$ ) and bootstrap support values ( $\geq 70\%$ ) are indicated on the nodes. The scale bar indicates the expected number of substitutions per site.

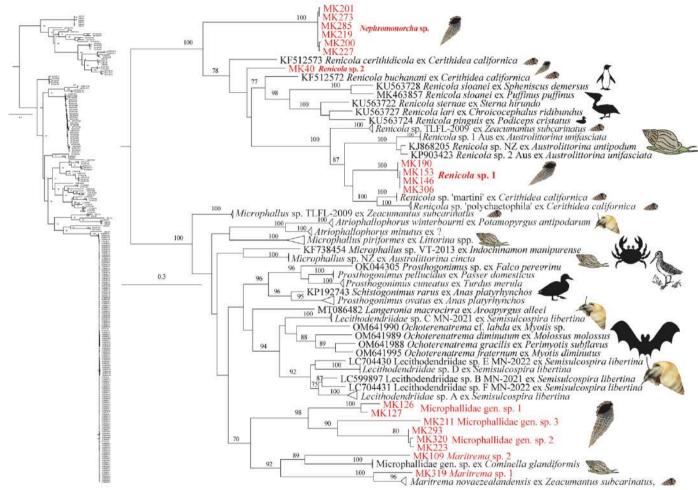


Fig. 7 A. Bayesian inference analysis for the Microphalloidea of the 28S rDNA dataset constructed under the GTR+I+G model of nucleotide evolution. Host origin of the respective sequences are symbol indicated on the tree. B. Maximum likelihood phylogram for the cox1 dataset of the Renicolidae constructed with RaxML under GTR+GAMMA model of evolution. Newly-generated sequences are colour indicated in red. Posterior probability ( $\geq$  0.95) and bootstrap support values ( $\geq$  70%) are indicated on the nodes. The scale-bar indicates the expected number of substitutions per site.

## **Discussion**

With our study we provide the first biodiversity inventory of the larval trematodes parasitising in the heat tolerant marine mud snail *P. cingulata* along the coast of Iran. Profiting from a large-scale sampling, we generated large sequence databases for the trematode intramolluscan stages recovered. Based on these sequence data, our analyses revealed considerably high digenean diversity accounting for a total of 29 distinct species of 10 families.

This is more than two times higher diversity found than previously reported from the region. A handful studies have reported on the larval trematode fauna of the mud snail from the coasts of Kuwait, Iraq and Iran indicating the existence of at least 12 species (Abdul-Salam and Al-Khedery 1992; Abdul-Salam and Bhaskaran 1995; Abdul-Salam and Sreelatha 1993; Abdul-Salam, Sreelatha, and Ashkanani 2000; Al-Kandari et al. 2015; Al-Kandari, Abdul-Salam, and Meakins 2000; Al-Kandari and Al-Bustan 2010; Kalat-Meimari, Shamseddin, and Salahi-Moghaddam 2018; Khosravi et al. 2022). Most of the studies reported only on the cercarial morphotype and just five have been identified to the genus or binominal species level (Austrobilharzia sp. (Kuwait), Austrobilharzia sp. (Iran), Ornithobilharzia canaliculata (Rudolphi, 1819), Probolocoryphe glandulosa (Sarkisian, 1957) Heard & Sikora, 1969 and Acanthotrema tridactyla Martin & Kuntz, 1955). However, the majority of the records represent otherwise unidentified cercarial morphs (Abdul-Salam and Al-Khedery 1992; Abdul-Salam and Bhaskaran 1995; Abdul-Salam and Sreelatha 1993; Abdul-Salam et al. 2000; Al-Kandari et al. 2015, 2000; Al-Kandari and Al-Bustan 2010; Kalat-Meimari et al. 2018; Khosravi et al. 2022) hampering further comparative surveys. The lack of consistency in assigning the cercarial morphotypes among the studies carried out represented an additional obstacle in comparing the trematode diversity across spatial and temporal scales in the region.

Linking mitochondrial (*cox*1) and nuclear (28S rDNA) sequences for isolates from intermediate and definitive hosts led to the elucidation of the life cycles of 5 species. Trematodes of birds dominated the community, with the echinostomids having the highest species richness (n = 9), followed by the microphallids (n = 7), heterophyids (n = 5), and cyathocotylids (n = 3). Only two of the species recovered, *Pseudohaplosplanchnus* sp. and *Saturnius* sp., use fish as definitive hosts. However, three of the represented groups, the Cyathocotylidae, Heterophyidae, and Renicolidae, use fish as second intermediate hosts for the completion of their life cycles. Despite the harsh environment, the region is known to harbour a diverse fauna accounting for more than 1,000 fish species, 42 shorebirds, 14 marine mammals, 5 species of sea turtles and over 270 invertebrates (Al-Jufaili et al. 2010; Al-Yamani et al. 2009; Behrouzi-Rad 2014; Braulik et al. 2010; Campbell et al. 2017; Eagderi et al. 2019; George 2012; Ghasemi et al.

2012; Saeidpour 2004; Vaughan, Al-Mansoori, and Burt 2019). Species richness, species heterogeneity and abundance of the final host are frequently positively correlated with these of the trematodes at their larval intramolluscan stages (Hechinger and Lafferty 2005). Only three out of the 29 species sequenced in the course of our study were identified to species level through the application of DNA-based approach. Inferred phylogenetic analyses for both gene markers sequenced helped us reach taxonomic resolution for most of the species to genus or family level.

The high species richness recovered herein was accompanied by a considerably high prevalence of infection across the localities sampled (accounting for up to 69% in Shif). Unfortunately, comparable studies on the larval trematode diversity in the snail intermediate hosts from extreme marine environments are currently lacking. The most comparable studies come from the closely related California horn snail, Cerithideopsis californica (Haldeman, 1840) (Haldeman 1840), from the Carpinteria Salt March in California. Although, from a distinct marine ecosystem a considerably lower trematode species richness, comprising a total of 19 species ((Hechinger 2019) and refences therein), an assessment based on long-term surveys covering three decades (Hechinger 2007; Huspeni and Lafferty 2004; Lafferty 1993a, 1993b; Whitney et al. 2007). Given the exceptionally high trematode species richness in the mud snail along the Iranian coast presumes that P. cingulata constitutes a keystone species in the trematode transmission in the region. Larval parasite communities of invertebrates are determined by a wide range of factors, such as their ecology, life history, phylogeny (Fredensborg, Mouritsen, and Poulin 2006; Hechinger and Lafferty 2005; Lafferty et al. 2008; Lafferty, Dobson, and Kuris 2006; Marcogliese 2003). Different hosts are acquired sequentially throughout the complex trematode life cycle and substantial part of the trematode transmission strategies relies on prereader-prey relationships. Therefore, host ecology including diet and habitat use drives first intermediate host use and play a key role in the parasite infection acquisitions (Louvard et al. 2022). Hence, larval trematode diversity and abundance are inherently linked to host diversity and abundance reflecting the food web dynamics at ecosystem level ((Hechinger and Lafferty 2005) and refence therein.).

Currently, significant range shifts are ongoing as a result of the climate change which are expected to exacerbate in the decades to come. Dramatic species turnovers of about 60% are predicted at a global scale as a result of climate change alone (Cheung et al. 2009). The importance of both sea temperature and salinity in determining marine biodiversity is immense. A main challenge in the attempts to assess change in the biodiversity, distribution and abundance of the biota in marine environment is the complete lack of historic a baseline data

widely known "shifting baseline concept" (Jackson et al. 2001; Knowlton and Jackson 2008). Studies suggest that biodiversity hotspots often occur in the most affected areas by climate change (Küpper and Kamenos 2018; Ramírez et al. 2017).

Matching sequence data from the cox1 and 28S datasets revealed taxonomic incongruences in the published sequence database. A taxonomic conflict was encountered between the published isolates of Cloacitrema narrabeenensis (AY222248; 28S) and Pygorchis alakolensis (MW139332; cox1) which clustered with isolate of a single species from our newly generated sequence dataset. Unfortunately, the sequences retrieved from GenBank originate from different publications and none of them provides data for the alternative gene marker. Additionally, we encountered that a number of published isolates exist in the GenBank database under distinct names. Thereby, our analyses helped update the nomenclature of published sequences, e.g., Pygorchis alakolensis (MW139332) should be considered as Cloacitrema narrabeenensis, Echinostomatidae sp. KWT (KC544266) as Acanthoparyphium sp. 2; and Heterophyidae gen. sp. WYAK-2013b (KF483579) as Acanthotrema tridactila. Another pitfall which we encountered comprised the lack of compatibility between the published studies in respect to the sequenced portions of the gene markers used (e.g., most prominent in the case of the microphallid dataset). Mislabeling, sequence and taxonomic conflicts, as well as missing taxa are just some of the current challenges of the current public repositories (Schols et al., 2020; Keck et al., 2022).

In summary, this study was conducted in order to obtain a first insights into the trematode parasite diversity in one of the most abundant snail species, *P. cingulata*, along the cost of Iran. We found a high parasite diversity and high prevalence in the snail intermediate across the sampled locations. The majority of the species recovered were parasites of birds with nearly third with potential etiological significance. Our results highlight the lack of data on parasites not only in the Gulf but worldwide. Future inventories on the larval trematode diversity are need to fully reveal the local parasite diversity that will allow revealing the ecological roles of the parasite communities in this extreme environment that is of considerable interest in respect to environmental change and anthropogenic pressure.

### Materials and methods

## Study area

The Iranian coastline spans almost 2,250 km along the Persian Gulf (PG) and the Gulf of Oman (GO), connected water bodies at the North-western edge of the Indian Ocean that are of immense economic and ecological importance (Farhadi and Anderson 2021). PG is a young shallow basin that has been in existence of about 10,000–14,000 years (Lambeck 1996). It is a semi enclosed shallow basin with less than 60 m depth (except at the Straits of Hormuz with c.200 m depth; (Reynolds 1993)), surrounded by a desert belt (Riegl and Purkis 2012) and located in one of the most arid regions of the world. The physical characteristics of the PG make it one of the world's most extreme marine environment with highly fluctuating conditions. The water temperature extremes reported range from 31.5 to 36.2 °C (Coles and Fadlallah 1991; Downing 1985). The relatively constrained water exchange with the open ocean, high level of evaporation and limited annual freshwater input (in average 110 km3/year) have led to exceptionally high salinity levels (>40 PSU in average). The PG is connected to the GO through the 56 km long Strait of Hormuz. The GO has remarkably different physical characteristics, having water higher depths (1,000 m on average) and receiving infusion of cool water from the Arabian sea's upwellings. The GO has a relatively more stable physical conditions with temperature hights of 22–32 °C and salinity levels of up to 32-37 PSU (Piontkovski et al. 2019). All this has led to the formation of characteristic habitats in both basins that host unique biodiversity.

The gastropod fauna along the coasts of the Persian Gulf and Gulf of Oman is relatively well documented, comprising a total of 850 species of 129 families, of which 383 species occur in both marine basins ((Amini-Yekta and Dekker 2021) and references therein). *Pirenella cingulata* (Gmelin,1791) (Gastropoda: Potamididae), widely known as the horn snail or mud whelk, is the most abundant and widespread species on sandy-muddy substrates (Vahidi et al. 2020). It is a deposit feeder and an important keystone species in the coastline ecosystem that is known to tolerate a wide range of temperature and salinity levels (15–45 PSU) (Reid and Ozawa 2016).

#### Sampling and examination procedures

Comprehensive field sampling from eight locations along the coast of Iran was accomplished between December 2019 and February 2020 (Fig. 1). A total of 1,969 mud snails (*P. cingulata*) was sampled comprising a minimum of 195 specimens from each sampling location (see Table 1 for n per location). Four of the sampling sites, Dylam (Y; 26°57'59.6"N, 56°03'02.0"E),

Genaveh (G; 29°19'53.1"N, 50°18'12.3"E), Shif (S; 29°02'37.0"N, 50°53'17.4"E), and Bushehr (U; 28°56'22.8"N, 50°48'33.4"E) were situated in the northern coast of the PG, two, Dagrahan (D; 26157'59.6"N, 56103'02.0"E), Bandar Abbas (T; 27°11'11.5"N, 56°20'26.0"E) at the Strait of Hormuz – the only sea passage from the PG to the GO, and the last two, Azini (A; 26°18'27.6"N, 57°07'05.3"E) and Jask (J; 25°43'53.2"N, 57°45'42.7"E) were located in the most north-eastern coast of the GO.

Snails were sampled haphazardly by hand from mud substrata at low tide, transferred alive to the laboratory, measured (length, width and weight), and labelled with a unique code assigned to each specimen. To stimulate cercarial emergence, the snails were placed individually in 50 ml beakers filled with filtered seawater and placed under a light source for at least 3–4 hours. Beakers were screened under a stereo microscope for the presence of patent infections and the released cercariae were preliminary identified based on general morphology to the lowest possible taxonomic level. Subsamples of the released cercariae from each infected snail were preserved in molecular grade ethanol for subsequent molecular studies. All snails were dissected after the 3rd day of light stimulation in order to check for prepatent infections. When such were detected, the respective intramolluscan stages (sporocysts and rediae) were dissected out of their host, washed with distilled water and preserved in molecular grade ethanol for DNA extraction and sequencing in order to achieve further species identification.

#### **Sequencing generation**

Total genomic DNA of the trematode parasites was isolated from ethanol-fixed samples using 5% Chelex (BT Chelex® 100 Resin BIO-RAD, Hercules, USA) suspension and 0.1 mg/ml proteinase K (20 mg/ml). The samples were incubated at 56 °C on a thermomixer overnight, vortexed for 20 seconds, boiled for 8 min at 90 °C, centrifuged at 14,000 rpm for 10 min while cooling at 4 °C, and stored at -20 °C.

Partial fragments of the mitochondrial cytochrome c oxidase subunit 1 (*cox*1) gene and the ribosomal 28S rRNA gene were amplified using the following primer combinations: (i) JB3 (forward: 5'-TTT TTT GGG CAT CCT GAG GTT TAT-3') and JB4.5 (reverse: 5'-TAA AGA AAG AAC ATA ATG AAA ATG-3') (Bowles, Blair, and McManus 1995) or COI R-Trema (reverse: 5'-CAA CAA AAT CAT GAT GCA AAA GG-3') for longer sequence reads (Miura et al. 2005) in the case of *cox*1 for the trematode samples; (ii) dig12 (forward: 5'-AAG CAT ATC ACT AAG CGG-3') and1500R (reverse: 5'-GCT ATC CTG AGG GAA ACT TCG-3;Tkach et al. 1999). PCR reactions were carried out in a final volume of 20 μl containing 5 μl of the template DNA, 2μl of each primer (5 pmol), 10 μl 2× MyFiTM Mix Bioline ® Master Mix and 1.8 μl nuclease-free water. In the case of 28S rDNA, the PCR reactions consisted of 2

 $\mu$ l template DNA, 1.6  $\mu$ l of each primer (5 pmol), 10  $\mu$ l 2× MyFiTM Mix Bioline ® Master Mix and with nuclease-free water accounting for the remaining volume (4.8  $\mu$ l).

The thermocycling profiles used were as follows: (i) *cox*1: initial denaturation at 95 °C for 3 min, followed by 35 cycles of amplification (95 °C for 50 sec, 50 °C for 50 sec and 72 °C for 60 sec) and final extension at 72 °C for 10 min; and (ii) 28S rRNA gene: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 55 °C for 30 sec, extension at 72 °C for 2 min, and final extension was at 72 °C for 7 min. PCR reactions were visualised on 1 % agarose gel, stained with GelRed (1 µl/100 ml), subjected to electrophoresis at 100 V for 45 min, and observed in an ultraviolet transilluminator. PCR products of interest were purified using QIAquick PCR purification kit (Qiagen Ltd, Hilden, Germany) according to the manufacturer's protocol and sent to Eurofins Technologies, Germany for Sanger sequencing using the same primer combinations as for the amplification reactions.

## Alignments and phylogenetic analyses

Contiguous sequences were assembled and inspected for errors in MEGA 7 (Kumar, Stecher, and Tamura 2016). Newly-generated and published sequences for each gene and/or taxonomic group were aligned together with MAFFT v.7 (Katoh, Rozewicki, and Yamada 2019; Kuraku et al. 2013). Cox1 alignments were checked for stop codons using the echinoderm and flatworm mitochondrial code (translation table 9; (Telford et al. 2000)), and trimmed in order the first base to correspond to the first codon position. Phylogenetic reconstructions were carried out to confirm species affiliations of the novel isolates sampled. Analyses were conducted on singlegene datasets using Bayesian inference (BI) and maximum likelihood (ML) analyses. Prior to the phylogenetic analyses, the best-fitting models of molecular evolution were estimated based on the Bayesian information criterion (BIC) in jModelTest v. 2.1.4 (Darriba et al. 2012). BIC analyses were carried out using MrBayes v. 3.2.7 (Ronquist et al. 2012) on the CIPRES Science Gateway v.3.3 (Miller, Pfeiffer, and Schwartz 2011) running Markov chain Monte Carlo (MCMC) searches on two simultaneous runs of four chains for 107 generations, and sampling trees every 103 generations. The "burn-in" determined by stationarity of lnL assessed with Tracer v.1.5 (Rambaut and Drummond 2009) was set for the first 25% of the trees sampled. Consensus tree topologies and nodal support were estimated as posterior probability values (Huelsenbeck et al. 2001) from the remaining trees. Maximum likelihood analyses were conducted independently on the cox1 datasets using RaxML on XSEDE. The resulted trees were visualized and finalised in FigTree v. 1.4.4 (Rambaut and Drummond 2012).

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#### **Author contributions statement**

M.K. and SG conceived the research and performed field data collection. M.K. obtained most of the field data, performed the sequencing, and contributed to data analyses and writing the first draft. S.G. participated in the data acquisition, analysed the results and wrote the manuscript. D.W.T. and J.S. reviewed and edited the manuscript. All authors read and approved the final manuscript.

## Additional information

To include, in this order: Accession codes (where applicable);
All data are available in the main manuscript or additional supporting files.

# **Competing interests**

The authors declare no competing interests.

## Chapter 2

Schistosomes in the Persian Gulf: novel molecular data, host associations, and life-cycle elucidations

# scientific reports

#### **OPEN**

# Schistosomes in the Persian Gulf: novel molecular data, host associations, and life-cycle elucidations

Maral Khosravi<sup>1⊠</sup>, David W. Thieltges<sup>2</sup>, Jebreil Shamseddin<sup>3</sup> & Simona Georgieva<sup>4,5</sup>

Avian schistosomes, comprise a diverse and widespread group of trematodes known for their surprising ability to switch into new hosts and habitats. Despite the considerable research attention on avian schistosomes as causatives of the human cercarial dermatitis, less it is known about the diversity, geographical range and host associations of the marine representatives. Our molecular analyses inferred from cox1 and 28S DNA sequence data revealed presence of two schistosome species, Ornithobilharzia canaliculata (Rudolphi, 1819) Odhner, 1912 and a putative new species of Austrobilharzia Johnston, 1917. Molecular elucidation of the life-cycle of O. canaliculata was achieved for the first time via matching novel and published sequence data from adult and larval stages. This is the first record of Ornithobilharzia from the Persian Gulf and globally the first record of this genus in a potamidid snail host. Our study provides: (i) new host and distribution records for major etiological agents of cercarial dermatitis and contributes important information on host-parasite relationships; (ii) highlights the importance of the molecular systematics in the assessment of schistosome diversity; and (iii) calls for further surveys to reach a better understanding of the schistosome diversity and patterns of relationships among them, host associations, transmission strategies and distribution coverage.

Avian schistosomes comprise a diverse and widespread group known for their surprising ability to switch into new hosts and habitats¹. Their cercariae are recognised as important causative agents of the waterborne allergic disease cercarial dermatitis (² and references therein). However, the current systematics and taxonomy of the group is exclusively based on morphological characters of the adults. Difficulties in the identification of their larval stages and the lack of suitability of experimental approaches in large-scale screening studies of natural infections in intermediate hosts, has hindered the real assessment of their diversity, host and distributional ranges³. Often larval and adult stages from natural infections in snails and birds have been assigned to belong to the same species with the lack of further evidence linking their conspecificity. The discovery of avian schistosome diversity, their life-cycle elucidations and taxonomy has largely benefited from molecular phylogenetics studies (².⁴ and references therein). To date, a total of 13 genera of avian schistosomes with about 70 species and 20 species-level genetically distinct lineages are known around the globe⁴.⁵. Based on the habitat where their life-cycles take place, avian schistosomes consist of freshwater and marine representatives.

Marine schistosomes represent a small group of widely distributed digeneans that are parasitic as adults in the vascular systems of various birds⁶. A predominant part of the extant marine schistosomes is known to parasitise charadriiforms (gulls and/or terns) with a few records in spheniscids⁵.⁶. Currently, four genera, Austrobilharzia Johnston, 1917, Gigantobilharzia Odhner, 1910, Marinabilharzia Lorenti, Brant, Gilardoni, Díaz & Cremonte, 2022 and Ornithobilharzia Odhner, 1912 are known to have marine-based life-cycles⁴.⁵. Of these, Ornithobilharzia Odhner, 1912 and Austrobilharzia Johnston, 1917 were recognised as an earlier diverging group which

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gave rise to all existent schistosomes<sup>7</sup>. Although, schistosomes represent a well-circumscribed monophyletic group, monophyly for the avian representatives has been rejected<sup>4,7</sup>. Phylogenetic hypotheses, revealed a basal switch from marine to freshwater environment which has occurred along a switch from caenogastropod to heterobranch snails<sup>1</sup>. A secondary switch from freshwater to marine environments has been suggested to have occurred with colonisation of heterobranch snails from the families Haminoeidae Pilsbry, 1895 and Siphonari- idae Gray, 1827<sup>8–10</sup>.

Ornithobilharzia canaliculata was first described by Rudolphi (1819) as Distoma canaliculatum, the first schistosome species reported from the intestine of terns ("Sternae species brasilianae") in Brazil<sup>11</sup>. In 1912, Odhner<sup>12</sup> erected the genus Ornithobilharzia and defined D. canaliculatum as the type-species. Despite the wide range of known definitive hosts including marine birds of six genera (Larus L., Sterna L., Chlidonias Rafinesque, Hydroprogne Kaup, Puffinus (Manxsherwater), and Thalasseus F. Boie), and a wide geographical range across the Holarctic and Neotropics<sup>13</sup>, only a single marine gastropod species, Lampanella minima (Gmelin), has been assigned as the intermediate host in the Gulf of Mexico<sup>14</sup>. However, experimental elucidation of the life-cycle has never been carried out and a formal description of the cercaria of O. canaliculata is still lacking. Under the current taxonomic treatment, the genus includes three species: O. amplitesta Gubanov & Mamaev in Mamaev, 1959; O. canaliculata (Rudolphi, 1819) Odhner, 1912; and O. lari McLeod, 1937. The closely-related genus Austrobilharzia Johnston, 1917 currently comprises 4 species: A. odhneri (Faust, 1924) Farely, 1971; A. penneri Short & Holliman, 1961; A. terrigalensis Johnston, 1917; and A. variglandis (Miller & Northup, 1926) Penner 1953. The genus was erected by Johnston (1917) to accommodate A. terrigalensis, a species found in the intestine of Larus novae-hollandiae shot at Terrigal, New South Wales, Australia. Caenogas- tropod snails have been reported as the natural intermediate hosts<sup>15–18</sup>. However, a combination of identification and taxonomic problems, have led to the biological paradox of a single species, A. terrigalensis, occurring at three distinct geographical regions and utilising different species of caenogastropod and bird hosts. Based on the geographical distribution, A. terrigalensis was assumed to occur in Larus novae-hollandiae and Batillaria australis in Australia; A. valisineria, Mergus serrator L., Aythya affinis Eyton, 1

Caenogastropods are one of the most diverse groups of gastropods comprising about 60% of the known species with predominantly marine forms<sup>19</sup> and are known as intermediate hosts for a variety of trematode parasites<sup>15,20,21</sup>. Members of the genus *Pirenella* J. E. Gray are abundant inhabitants of intertidal sedimentary shores with wide geographical distribution ranging from the western Pacific and Indian Ocean to the eastern Mediterranean Sea. A recent study reported a total of 16 valid species within the genus, with some species known as inhabitants of extreme environments, from brackish estuaries to hypersaline lagoons and inland lakes<sup>22</sup>. *Pirenella cingulata* (Gmelin, 1791) is the most abundant caenogastropod species in the Persian and Oman Gulfs. It is known for its tolerance to environmental extremes and ability to flourish in intertidal muddy or sandy substrates, as well as mudflats adjacent to mangrove forests<sup>23,24</sup>.

As part of an ongoing study aiming to characterise trematode diversity in the horn snail (*Pirenella cingulata*) along the coast of Iran, we here report on the diversity of avian schistosomes associated with marine life-cycles using *cox*1 and 28S rDNA sequence data. The present study is the first to molecularly elucidate the life-cycle of the first ever described schistosome, *O. canaliculata*, and further reports on a putative new species of *Austrobilharzia*. Both species recovered are of the largely understudied marine schistosomes known for their implication as causative agents of cercarial dermatitis. This is the first unambiguous documentation that the potamidid snail

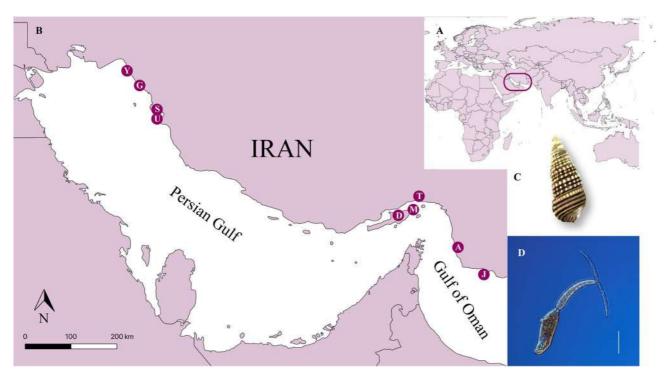
 $P.\ cingulata$  is the natural snail host for  $O.\ canaliculata$ . The evolutionary relationships and host-parasite associations among the avian schistosomes are further revisited.

#### Results

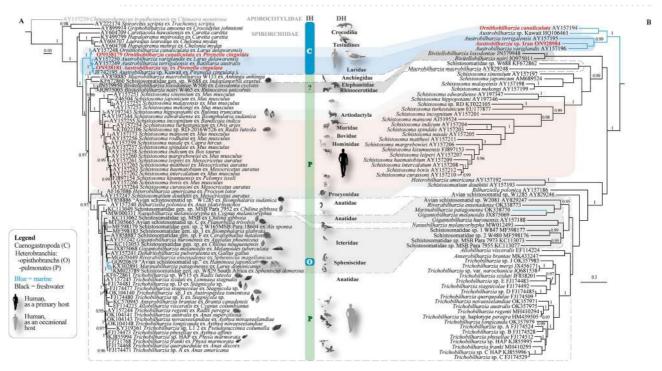
Three out of the 1,745 examined *P. cingulata* were infected with avian schistosomes. The infected snails were collected at two distinct localities named Genaveh (n = 2; prevalence = 1%) and Jask (n = 1; prevalence = 0.4%) (see also Fig. 1 for sampling locations). Successful amplifications were achieved for 28S and cox1 for all three isolates. The yielded sequences were 1254–1285 bp (28S rDNA) and 344–730 bp long (cox1). The two isolates from Genaveh shared an identical 28S rDNA sequence with a published isolates for *Ornithobilharzia canaliculata* from the USA ex *Larus delawarensis* and *L. occidentalis* (AF167085, AY157248, KP734309), while the isolate from Jask differed by 2.3% (29 bp) from the former ones. A BLASTn search indicated that the latter isolate belonged to the genus *Austrobilharzia*. The novel isolate from Iran differed by 12–19 bp (0.9–1.8%) from the published representatives of the genus. The closest relative was an otherwise unidentified isolate from the same host species, *P. cingulata*, from off Kuwait (12 bp, 0.9% genetic difference). *Cox*1 sequence divergence between our two isolates of *O. canaliculata* from Iran was 9 bp (2.6%). In contrast to the identical

Cox1 sequence divergence between our two isolates of *O. canaliculata* from Iran was 9 bp (2.6%). In contrast to the identical 28S sequences between the novel and published isolates for *O. canaliculata*, cox1 sequences differed substantially, ranging between 27 and 32 bp (7.9–9.3%). The single isolate from Jask differed by 1.66–1.82% (49–55 bp; 16.3–18.2%) from the novel isolates for *O. canaliculata*, and by 59 bp (20.1%) from *Austrobilharizia* sp. from Kuwait. Interspecific sequence divergence within *Austrobilharzia* was within the range of 21–63 bp (9.7–20.1%). However, the intergeneric divergence between the isolates for *Ornithobilharzia* and *Austrobilharzia* was somehow lower that the interspecific divergence for *Austrobilharzia*, i.e., 25–61 bp (7.7–18.4%). A single cox1 isolate for *Austrobilharzia variglandis* ex *Larus* sp. from Canada was not included in the sequence comparisons as it covers a distinct region of the cox1 gene and did not align with the remaining published isolates.

published isolates. The aligned 28S dataset consisted of 76 terminals (2 newly-sequenced) and it was 1370 bp long, 78 of which were excluded prior to analyses. The *cox*1 dataset comprised 66 terminals and it was 1031 bp long. Analyses of the individual genes resulted in well-resolved trees (Fig. 2). The 28S rDNA hypothesis, presented in Fig. 2A, included representatives of all named and molecularly characterised species-level lineages except for the monotypic



**Figure 1.** (**A**) General view map, generated using QGIS version 3.4 (http://www.qgis.org) $^{52}$ , and (**B**) sampling localities along the Persian Gulf and the Gulf of Oman off Iran. Points correspond to the sampling localities. *Abbreviations*: A, Azini; D, Dargahan; G, Genaveh; J, Jask; M, Geshm; S, Shif; T, Bandar Abbas; Y, Deylam; U, Bushehr. (**C**) Snail intermediate host *Pirenella cingulata* (Gmelin, 1791). (**D**) Cercaria collected from *P. cingulata. Scale-bar* = 100 μm.



**Figure 2.** Bayesian analyses of the **(A)** 28S rDNA and **(B)** cox1 datasets constructed using MrBayes v. 3.2.3 under the GTR+I+ $\Gamma$  model of sequence evolution. Analyses were run for 10,000,000 generation and 25% discarded as "burn-in". Posterior probability values are given above the branches; values. Nodes with < 0.95 posterior probability support have been collapsed. Branch length scale-bar indicates number of substitutions per site. Newly-generated sequences are indicated in colour indicated red and bold. Hosts of origin of individual sequences are indicated after the specimen's host name. Branches in blue indicate schistosomes with marine life- cycle. Shaded areas and taxa outlined with doted lines reflect on the current taxonomic framework of the family and also given on the right.

Jilinobilharzia as molecular data currently do not exist (the single species, J. crecci Liu & Bai, 1976, has not been reported since its original description). Therefore, the ingroup taxa consisted of representative sequences of the families Schistosomatidae and the closely related Spirorchiidae (see Supplementary Table S1). The outgroup comprised representative of the Aporocotylidae and it was informed from previous phylogenies<sup>25</sup>. Our phylogenetic hypothesis recovered the spirorchiids in freshwater crocodilian and testudine hosts as the earliest diverging lineage. Spirorchiids with marine life-cycle clustered in a distinct clade basal to the Schistosomatidae. Members parasitic in marine testudines were identified as a distinct clade sister to all remaining schistosomes parasitic in birds and mammals. Schistosomes clustered into four distinct lineages: (i) an earlier diverging and strongly sup- ported clade comprising the marine Ornithobilharzia and Austrobilharzia (ii) Macrobilharzia—a genus known from suliform birds which was resolved as a distinct lineage basal to the freshwater schistosomes, and two strongly-supported multi-taxa sister clades predominantly of (iii) mammalian and (iv) avian schistosomes. The mammalian schistosomes were further recovered as three distinct lineages: (i) Bivitelobilharzia—a genus including species parasitic in elephants and rhinoceros were recovered in a strongly-supported sub-clade sister to the main clade of mammalian schistosomes; (ii) a sub-clade of Schistosoma spp. with South East Asian dis-tribution; and (iii) a clade comprising African representatives of Schistosoma. The North American mammalian representatives, Heterobilharzia and Schistosomatium were resolved as closer relatives to the large clade of avian schistosomes (Trichobilharzia + Marinabilharzia + Dendritobilharzia + Cigantobilharzia + Nasusbilharzia + Rivera-bilharzia). The remaining avian schistosomes clustered in two sister monophyletic clades with generally strong support for the major nodes. Bilharziella

The cox1 tree was well-resolved and received strong support for most of the internal nodes (Fig. 2B). Taxa largely grouped in consistence with the 28S solution. Ornithobilharzia and Austrobbilharzia clustered into two distinct strongly-supported sister clades. The newly-sequenced isolate from Jask clustered in a clade with A. variglandis and A. terrigalensis; however, the isolate for O. canaliculata clustered with otherwise unidentified isolate labelled as Austrobilharzia sp. from Kuwait indicating a possible misidentification of the latter one. This was further confirmed by the high levels of genetic divergence in comparison with the other isolates of Austro- bilharzia as indicated above.

#### **Discussion**

The present study is part of an effort to document the trematode diversity in *P. cingulata* (Gmelin, 1791), one of the most abundant snail species along the Iranian coast<sup>23,24,26</sup>. Sequence data for two species of marine avian schistosomes, *Ornithobilharzia canaliculata* (Rudolphi, 1819) and a putative new species of *Austrobilharzia* Johnston, 1917, are represented in a phylogenetic context together with other members of the family Schistosomatidae. This is the first report and molecular evidence for *Ornithobilharzia canaliculata* (Rudolphi, 1819) infecting *P. cingulata* as an intermediate host and it is the first partial molecular elucidation of its life-cycle. Our study adds to the diversity, host associations and phylogeny of the avian schistosomes with marine-based life-cycles, a group of schistosomes with great etiological importance.

Cercariae of the marine schistosomes are recognised as important etiological agents of human dermatitis<sup>27–29</sup>. Despite their importance to the public health, still very little is known about their diversity and evolution<sup>7</sup> as a consequence of largely under surveyed marine habitats for schistosomes worldwide<sup>4</sup>. This is in sharp contrast with the wealth of knowledge gathered about the mammalian and avian schistosomes with freshwater-based life-cycles, and information concerning the natural history of most marine schistosomes is scarce. The slow rates in recovering marine schistosomes, low species richness recorded in snail hosts and the convoluted taxonomy of the group, including separate taxonomic treatments of the distinct life-cycle stages, reflects the scarcity of data<sup>30</sup>. Matching sequence data for different life-cycle stages and across distant localities has accelerated life-cycles elucidations and host-parasite associations<sup>4,5</sup>. Although, the molecular systematics has had a major impact for the recent increase in discoveries and species delimitation, it has led to a plethora of putative new species and lineages of avian schistosomes for which only molecular data for their cercarial stages exist. Most of these putative species/species level lineages are of considerable importance due to their etiological significance. Their formal descriptions await as reliably identified adult stages are needed to help infer on their respective life-cycles and host-parasite associations.

Ornithobilharizia canalicata was originally described from Sterna galericulata in Brazil<sup>11</sup>. Later the species was reported from a wide range of gulls and terns across the Americas, Europe, Asia and New Zealand (see Table 1 for details). Larus dominicanus Lichtenstein and L. maculipennis Lichtenstein serve as the main hosts in the southern hemisphere; Larus delawarensis Ord, and L. occidentalis Audubon have been reported as hosts in North America and a total of 22 species of gulls and terns were reported as hosts across Europe and the Middle East. Despite the large number of definitive hosts, thus far the species was reported only from a single mollusc species, Lampanella minima (Gmelin), in North America. However, an experimental infection linking larval and adult stages has never been conducted. An important result from our study is the molecular confirmation of the conspecificity of our isolate from P. cingulata with the published isolate of an adult worm from North America. Matching sequence data for isolates from different life-cycle stages collected from disparate locations and times, provides unambiguous link between adult and larval stages from natural infections and accelerates species circumscription. The intercontinental distribution and the rather narrowly defined clade of gulls is instructive for studies on the transmission of avian zoonoses and the epidemiology of human cercarial dermatitis. The trans-continental distribution of Ornithobilharzia across the America, Europe and Asia is an explicit example that species dispersal is determined by the most vagile, bird host, involved in the trematode life-cycle. It is widely accepted that the distribution of the definitive host governs the larval trematode recruitment in the snail (first)

Species	Host	Locality	References
	Batillaria australis (Quoy & Gaimard)	Australia: Iron Cove, Sedney Harbour	Lockyer et al. <sup>68</sup>
	Batillaria australis (Quoy & Gaimard)	Australia	Walker <sup>69</sup>
	Batillaria australis (Quoy & Gaimard)	Australia: Swan Estuary	Appleton <sup>70</sup>
	Batillaria australis (Quoy & Gaimard)	Australia: Swan Estuary	Appleton <sup>71</sup>
	Batillaria australis (Quoy & Gaimard)	Australia	Johnston <sup>72</sup> sensu Farley <sup>13</sup>
	Batillaria australis (Quoy & Gaimard)	Australia	Appleton <sup>73</sup>
	Batillaria australis (Quoy & Gaimard)	Australia: Narrabeen Lagoon	Bearup <sup>74</sup>
	Cerithideopsis scalariformis (Say)	North America	Holliman <sup>17</sup>
	Cerithideopsis scalariformis (Say)	North America	Short and Holliman <sup>18</sup>
	Ilyanassa obsoleta (Say)	USA	Miller and Northup <sup>36</sup> sensu Farely <sup>13</sup>
	Ilyanassa obsoleta (Say)	USA	Camishion et al. <sup>75</sup>
	Ilyanassa obsoleta (Say)	USA: New Jersey	Zibulewsky et al. <sup>76</sup>
	Ilyanassa obsoleta (Say)	USA: Atlantic coast	Bacha et al. <sup>77</sup>
	Ilyanassa obsoleta (Say)	USA: Atlantic coast	Wood and Bacha <sup>78</sup>
	Ilyanassa obsoleta (Say)	USA: North Carolina	Sindermann <sup>79</sup>
	Ilyanassa obsoleta (Say)	USA: California	Grodhaus and Keh <sup>28</sup>
Austrobilharzia terrigalensis Johnston, 1917	Littorina pintado (W. Wood)	US: Hawaii	George et al. <sup>80</sup>
	Littorina pintado (W. Wood)	Hawaii	Chu and Cutress <sup>37</sup> sensu Farely <sup>13</sup>
	Planaxis sulcatus (Born)	Australia: GBR, Heron Island	Rohde <sup>81</sup>
	Planaxis sulcatus (Born)	Australia	Rohde <sup>81</sup>
	Anous minutus (L.)	Australia: GBR, Heron Island	Rohde <sup>81</sup>
	Arenaria interpres (L.)	Hawaii	Chu and Cutress <sup>37</sup> sensu Farely <sup>13</sup>
	Arenaria interpres (L.)	US: Hawaii	George et al. <sup>80</sup>
	Aythya affinis (Eyton)	USA: Massatchusetts	Price <sup>82</sup> sensu Farely <sup>13</sup>
	Aythya valisineria (Wilson)	Canada	McLeod <sup>83</sup> sensu Farley <sup>13</sup>
	Larus californicus Lawrence	USA: Wyoming	Keppner <sup>84</sup>
	Larus novaehollandiae Stephens	Australia	Johnston <sup>85</sup> sensu Farley <sup>13</sup>
	Larus novaehollandiae Stephens	Australia: Swan Estuary	Appleton <sup>86</sup>
	Larus novaehollandiae Stephens	Australia: Swan Estuary	Appleton <sup>73</sup>
	Mergus serrator L	USA	Penner <sup>87</sup> sensu Farley <sup>13</sup>
	Egretta sacra (Gmelin)	Australia	Rohde <sup>81</sup>
	Egretta sacra (Gmelin)	Australia: GBR, Heron Island	Rohde <sup>81</sup>
	Larus novaehollandiae Stephens	Australia	Johnston <sup>85,88</sup> , Appleton <sup>73</sup>
	Larus novaehollandiae Stephens	Australia: Terrigal, near Sydney	Johnston <sup>85</sup>
	Larus novaehollandiae Stephens	Australia: GBR, Heron Island	Rohde <sup>81</sup>
	Larus novaehollandiae Stephens	Australia	Rohde <sup>81</sup>
	Larus hemprichii Bruch	Red Sea	Witenberg and Lengy <sup>89</sup>
	"Canary" (exp.)	USA: California	Grodhaus and Keh <sup>28</sup>
Continued		·	•

Species	Host	Locality	References
	Ilyanassa obsoleta (Say)	USA: Delaware estuaries	Curtis <sup>90</sup>
	Ilyanassa obsoleta (Say)	USA: Delaware estuaries	Curtis and Tanner <sup>91</sup>
	Ilyanassa obsoleta (Say)	USA: Mumford Cove, Connecticut	Barber and Caira <sup>92</sup>
	Ilyanassa obsoleta (Say)	North America	Grodhaus and Keh <sup>28</sup> , Curtis <sup>90</sup> , Leighton et al. <sup>93</sup>
	Ilyanassa obsoleta (Say)	USA: Little Egg Inlet, New Jersey	Ferris and Bacha <sup>94</sup>
	Littorina pintado (W. Wood)	USA: Hawaii	Chu and Cutress <sup>37</sup>
	Anous stolidus pileatus (Scopoli)	USA: Hawaii	Chu and Cutress <sup>37</sup>
	Arenaria interpres (L.)	USA: Hawaii	Chu and Cutress <sup>37</sup>
4 . 121 1 P. O.C. 0	Aythya affinis (Eyton, 1838)	USA: Eastern part	Price <sup>82</sup>
<i>Austrobilhariza variglandis</i> (Miller & Northup, 1926)	Branta canadensis (L.)	USA: Mumford Cove, Connecticut	Barber and Caira <sup>92</sup>
	Larus argentatus Pontoppidan	USA: Mumford Cove, Connecticut	Barber and Caira <sup>92</sup>
	Larus argentatus Pontoppidan (exp.)	USA	Stunkard and Hinchliffe <sup>95,96</sup>
	Larus delawarensis Ord	USA: Mumford Cove, Connecticut	Barber and Caira <sup>92</sup>
	Larus delawarensis Ord	USA: Delaware	Lockyer et al. <sup>68</sup>
	Larus marinus L	USA: Mumford Cove, Connecticut	Barber and Caira <sup>92</sup>
	Larus marinus L	North America	Keppner <sup>84</sup> , Barber and Caira <sup>92</sup>
	Larus novaehollandiae Stephens	Australa: Heron Island	Rohde <sup>81</sup>
	Mergus serrator L	USA	Penner <sup>87</sup>
	Mergus serrator L	North America: Hawaii	Penner <sup>97</sup>
	Phalacrocorax auritus (Lesson)	USA: Mumford Cove, Connecticut	Barber and Caira <sup>92</sup>
	Phalacrocorax auritus (Lesson)	North America	Barber and Caira <sup>92</sup>
	Sterna fusccata oahuensis (L.)	USA: Hawaii	Chu and Cutress <sup>37</sup>
Austrobilharzia odhneri (Faust, 1924) Farley, 1971	Numenius arquata (L.)	China	Faust <sup>98</sup>
	Cerithideopsis scalariformis (Say)	North America	Holliman <sup>17</sup>
Austrobilharzia penneri Short & Holliman, 1961	Cerithideopsis scalariformis (Say)	North America	Short and Holliman <sup>18</sup>
1901	Cerithidea scalariformis and "parakeets, chickens and pigeons (exp.)"	USA: Florida, Northern Gulf coast	Short and Holliman <sup>18</sup>
	Cerithideopsis californica (Haldeman)	USA: Bolinas Lagoon, in central California	Sousa <sup>99</sup>
	Cerithidia sp.	North America	Martin <sup>16</sup>
	Littorina pintado Wood	North America: Hawaii	Chu <sup>100</sup>
	Pirenella cingulata (Gmelin)	Kuwait: Kuwait Bay	Al-Kandari et al. <sup>15</sup>
	Pirenella cingulata (Gmelin)	Kuwait Bay	Al-Kandari et al. <sup>15</sup>
	Nassarius (Hinia) reticulatus (L.)	Italy	Canestri-Trotti et al. <sup>101</sup>
Austrobilhariza sp.	Littorina keenae Rosewater	North America	Penner <sup>102</sup>
	Planaxis sulcatus (Born)	Kuwait: Kuwait Bay	Abdul-Salam and Sreelatha <sup>103</sup>
	Anous minutus Boie	North America: Hawaii	Chu <sup>37</sup>
	Gavia immer (Brünnich)	North America	Kinsella and Forrester <sup>104</sup>
	Larus dominicanus Lichtenstein	South Africa	Appleton <sup>105,106</sup>
	Larus dominicanus Lichtenstein	South Africa: Umgeni Estuary	Appleton <sup>105</sup>
	Onychoprion fuscatus L	North America: Hawaii	Chu <sup>37</sup>
	Pelecanus occidentalis L	North America	Courtney and Forrester <sup>107</sup>
Continued	·	•	

Species	Host	Locality	References
	Lampanella minima (Gmelin)	North America	Penner <sup>14</sup> , Morales et al. <sup>108</sup>
	Lampanella minima (Gmelin)	USA: Florida	Morales et al. <sup>108</sup>
	Lampanella minima (Gmelin)	Brazil	Travassos et al. <sup>109</sup>
	Chlidonias hybrida (Pallas)	Caspian Sea	Saidov <sup>110</sup> , Bykhovskaya <sup>111</sup>
	Hydroprogne caspia (Pallas)	Black Sea, Central Europe	Leonov <sup>112</sup> , Macko <sup>113</sup>
	Hydroprogne caspia (Pallas)	West Siberia	Bykhovskaya <sup>114</sup>
	Ichthyaetus melanocephalus Temminck	Calabria, Southern Italy	Santoro et al. <sup>115</sup>
	Larus fuscus L	Red Sea	Witenberg and Lengy <sup>89</sup>
	Larus argentatus Pontoppidan	West Siberia	Bykhovskaya <sup>114</sup>
Ornithobilharzia canaliculata (Rudolphi, 1819)	Larus cachinnans Pallas	Spain, Galicia	Sanmartín et al. <sup>116</sup>
	Larus canus L	Black Sea	Popova <sup>117</sup> , Bykhovskaya <sup>111</sup>
	Larus delawarensis Ord	Canada	McLeod <sup>83</sup>
	Larus delawarensis Ord	USA: Donley County, Texas	Lockyer et al. <sup>68</sup>
	Larus delawarensis Ord	USA: Texas	Snyder and Locker <sup>7</sup>
	Larus dominicanus Lichtenstein	Brazil	Travassos <sup>118</sup>
	Larus dominicanus Lichtenstein	New Zealand	Rind <sup>119</sup>
	Larus dominicanus Lichtenstein	Argentina	Szidat <sup>120</sup>
	Larus maculipennis Lichtenstein	Argentina	Szidat <sup>120</sup>
	Larus fuscus L	North Russia, Red Sea	Shygin <sup>120</sup> , Bykhovskaya <sup>111</sup> , Witenberg and Lengy <sup>89</sup>
	Larus fuscus L	Sweden	Odhner <sup>12</sup>
	Larus hemprichii Bruch	Red Sea	Witenberg and Lengy <sup>89</sup>
	Larus ichthyaetus Pallas	Black Sea	Leonov <sup>112</sup>
	Hydrocoloeus melanocephalus (Temminck	Italy	Parona and Ariola <sup>121</sup>
	Hydrocoloeus minutus (Pallas)	Caspian Sea	Saidov <sup>110</sup> , Bykhovskaya <sup>111</sup>
	Larus occidentalis Audubon	USA	Jothikumar et al. <sup>122</sup>
	Larus ridibundus L	North Russia, Caspian Sea	Shigin <sup>123</sup> , Saidov <sup>110</sup> , Bykhovskaya <sup>111</sup>
	Larus ridibundus L	West Siberia	Bykhovskaya <sup>114</sup>
	Puffinus kuhli (Boie)	Red Sea	Witennberg <sup>124</sup>
	Sterna galericulata	Brazil	Rudolphi <sup>11</sup>
	Sterna hirundo L	Czech Republic	Kolářová et al. <sup>51</sup>
	Sterna sandwichensis Latham	Black Sea	Leonov <sup>112</sup>
Ornithobilharzia sp. (?canaliculata)	Eudocimus albus (L.)	North America	Bush and Forrester <sup>125</sup>
	Larus argentatus Pontoppidan	Canada. Nova Scotia	McLeod <sup>83</sup>
Ornithobilharzia lari McLeod, 1937	Larus delawarensis Ord	Canada. Nova Scotia	McLeod <sup>83</sup>
	Larus philadelphia (Ord)	Canada. Nova Scotia	McLeod <sup>83</sup>
<i>Ornithobilharzia amplitesta</i> Gubanov & Mamaev in Mamaev, 1959	Tringa glareola L	Russia	Mamaev <sup>126</sup>

 $\textbf{Table 1.} \ \ \textit{Records of } \textit{Ornithobilharzia spp. and } \textit{Austrobilharzia spp.}$ 

intermediate host (31 and references therein). Resolving the relative roles of both host ecology and phylogeny in respect to the parasite transmission dynamics over evolutionary times would require further concerted efforts. Phylogenetic studies based on denser and wide taxon sampling including diverse intermediate and definitive hosts is crucial for building up an improved framework and better interpretation of the schistosome biology<sup>3</sup>. Further, good documentation and re-

evaluation of the morphological charters of the respective larval stages is urgently needed. Successful transmission of parasites with complex life-cycles requires an overlap of all hosts involved. The invertebrate first intermediate host has been recognised as one of the keys to the evolutionary expansions of the digenean trematodes. All schistosomes (marine and freshwater) are known to develop in gastropods. The basal position of the marine schistosomes (*Austrobilharzia* and *Ornithobilharzia*) has been considered as an indication for a successful ancestral marine-transmitted bird parasite transmission in colonising both freshwater snails and mammals<sup>25</sup>. The schistosomes emerging from marine heterobranch snails (*Haminea* and *Siphonaria*) and also recorded in penguins are a well-known example of secondary colonisation of marine habitats by the schistosomes<sup>30,32,33</sup>. Considering the snail intermediate hosts, in at least two instances, even congeneric schistosomes depend on markedly divergent gastropod lineages, i.e., pulmonates *versus* opisthobranchs or caenogastropods, indicative for an extensive host switching within the molluscan hosts<sup>34</sup> and references therein).

Avian schistosomes are known to have colonized a wide range of snail hosts with representatives from 15 snail families: (i) caenogastropods from both marine (Potamididae, Batilariidae, Nassariidae, and Littorinidae $^{14,15,35-37}$  and freshwater environments (Thiaridae, Ampullariidae, Hydrobiidae, and Semisulcospiridae $^{35,38-41}$ ; (ii)

heterobranchs from marine (Haminoeidae<sup>8,9</sup>, and freshwater (Valvatidae<sup>42</sup>; and (iii) pulmonates from marine (Siphonariidae<sup>10</sup>) and freshwater (Physidae, Lymnnaeidae, Planorbidae, and Chilinidae<sup>1,3,35,43,44</sup>. Reports of avian schistosomes from distantly related snail intermediate hosts are not rare and invoke questions on the proper identification of the respective parasites. *Dendritobilharzia pulverulenta*<sup>45</sup> Skrjabin, 1924 has been reported from two distinct planorbid snails *Gyraulus* Charpentier, 1837 and *Anisus vortex* (L.)<sup>46</sup>. *Gyraulus* has been reported as a natural host of the species in North America and New Zealand, while *Anisus* and *Planorbis planorbis* (L.) have been reported as hosts in Europe. *Trichobilharzia jequitibaensis* Leite, Costa, & Costa, 1978 is known to infect both lymnaeid and physid snails<sup>47</sup>. *Austrobilharzia terrigalensis* has been considered to utilise distinct snail hosts across its distributional range in Australia (*Batillaria australis* (Quoy & Gaimard)), North America (*Cerithideopsis scalariformis* (Say), and *llyanassa obsoleta* (Say) and the Pacific (*Littorina pintado* (W. Wood)). Intercontinental and trans-hemispheric distribution has been recently reported for *Trichobilharzia querquedule*<sup>48,49</sup>, however the species is known as a parasite specific to *Physa* spp. as an intermediate host. In respect to their definitive hosts, a predominant part of the schistosomes is known as parasitises in birds. Currently a total of 13 genera are known as parasites in birds: *Austrobilharzia* Johnston, 1917 (6 species), *Allobilharzia* (1 species), *Bilharziella* (1 species), *Dendritobilharzia* (2 species) *Gigantobilharzia* (1 species), *Jilinobilharzia* (1 species), *Macrobilharzia* Travassos, 1922 (2 species), *Ornithobilharzia* Odhner, 1912 (3 species); *Nasusbilharzia* Flores<sup>50</sup> (1 species), *Marinabilharzia* have been subject of the most intensive research due to their recognition as leading etiological agents of human cercarial dermatitis (<sup>51</sup> and references there

schistosomes, yet remain largely unstudied with fragmentary data on their diversity, biology and ecology. This is especially true in respect to the marine schistosomes for which life-cycle information lags behind their freshwater relatives. Despite the great efforts made so far in building up a comprehensive framework for the study of schistosome diversity, still considerable data are needed for assessing their true diversity due to the difficulty of directly relating larval and adult stages. Consistent efforts towards the use of integrative approach including collecting novel data from diverse host species and combining them thorough morphological examination, traditional systematics, classical taxonomy and phylogenetics have been proven as most valuable practice providing important information to the better understanding of the biodiversity and evolutionary relationships of the group<sup>2-4</sup>.

Our study strongly suggests that the biodiversity of the marine schistosomes is underestimated. The extensive discovery-based studies about schistosome diversity during the last two decades has revealed an immense diversity of avian schistosomes. However, unravelling their true diversity across hosts and geographic areas have been hindered by the difficulties of matching distinct life-cycle stages. There is still a need for more records of identifiable adult and larval schistosomes. Our study is a crucial step towards better understanding of important properties of marine schistosome biology and ecology, their patterns of diversification and distribution.

#### Materials and methods

**Host and parasite collection.** A total of 1745 adult *P. cingulata* (Gmelin, 1791) were sampled from 9 distinct locations along the Iranian coastline between December 2019 and February 2020 (Fig. 1). Samples comprised a minimum of 200 individual snails per locality, which were opportunistically collected by hand at the low tide from the intertidal zone. Snails were transferred alive to the laboratory, where they were measured (length and weigh) and labelled with a unique code given to each specimen. Each snail was then placed in an individual 50 ml beaker filled with filtered seawater and exposed to a warm light source for 3–4 h to simulate cercarial emergence. Beakers were screened under a stereomicroscope for the presence of cercariae indicating patent infections in the snail host. Prepatent infections were detected with snail dissections, which were conducted on the 3rd day of light stimulation. Both the released cercariae and schistosome sporocysts recovered from the host's tissue were washed with distilled water and preserved in molecular grade ethanol for DNA isolation and sequencing.

**Sequence generation.** Ethanol-preserved samples of pooled cercariae were subjected to DNA extraction and sequencing. Partial cox1 and 28S rDNA sequences were generated for the schistosome parasites recovered in order to achieve molecular identification and carry out reconstruction of their evolutionary relation- ships using published primers (28S (digl2 +  $1500R^{53,54}$ ; ECD2 +  $900F^{55,56}$  as internal sequencing primers; cox1: JB3 + JB4.5<sup>57</sup> or CO1-R<sup>58</sup>). Contiguous sequences were aligned with MAFFT v.7<sup>59,60</sup> as an online execution. After alignment, sequences for cox1 were checked for stop codons using the echinoderm and flatworm mitochondrial code (translation table  $9^{61}$ ). All sequences were trimmed in order the first base to correspond to the first codon position in order to simplify position-coding in the downstream analyses.

**Phylogenetic analyses.** Phylogenetic analyses were performed on individual gene datasets using Bayesian inference (see Supplementary Table 1 for details on the taxa included in the analyses). Prior to analyses, the 'best- fitting' models of nucleotide substitution were estimated based on the Bayesian information criterion (BIC) in jModelTest v.  $2.1.4^{62}$ . BI analysis was carried out with MrBayes v.  $3.2.7^{63}$  on the CIPRES Science Gateway v.3.3<sup>64</sup> using Markov chain Monte Carlo (MCMC) searches on two simultaneous runs of four chains for  $10^7$  generations, sampling trees every  $10^3$  generations. The "burn-in" determined by stationarity of lnL assessed with Tracer

v.1.5<sup>65</sup> was set for the first 25% of the trees sampled, and a consensus topology and nodal support estimated as posterior probability values<sup>66</sup> were calculated from the remaining trees. Phylogenetic trees were visualized and finalised in FigTree v. 1.4.4<sup>67</sup>. The newly-generated sequences were deposited in GenBank under accession

numbers: ON928982-ON928984 (cox1), ON938179-ON938181 (28S) in the case of avian schistosomes, and ON911910 (cox1), ON911912 (28S)—for the snail host.

#### Data availability

All data are available in the main manuscript or additional supporting files.

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#### **Author contributions**

M.K. and S.G.: conceived the study, obtained samples, carried out the sequencing, performed analyses prepared the first draft of the manuscript. M.K., D.T., J.S., S.G. discussed the results and helped draft the manuscript. All authors read and approved the final manuscript.

#### Competing interests

The authors declare no competing interests.

#### **Additional information**

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# Supplementary Material for "Schistosomes in the Persian Gulf: novel molecular data, host associations, and life-cycle elucidations"

Table S1. Summary data for cox1 and 28S sequences retrieved from GenBank.

	Life-cycle					
Species	stage	Host	Loclaity	28S	cox1	Reference
Allobilharzia visceralis Allobilharzia visceralis	adult	Cygnus columbianus	USA		EF114224	Brant et al. (2007) Skírnisson & Kolářová
W262tuswNM	adult	Cygnus columbianus	USA	EF114223		(2008)
Anserobilharzia brantae Anserobilharzia brantae = "Avian	cercaria	Gyraulus sp.	USA: Michigan Lake Victoria, Kenya Fisheries		MK433247	Rudko et al. (2019)
schistosomatid sp. W2081"  Anserobilharzia brantae	cercaria	Ceratophallus natalensis	Association landing site, Kisumu, Kenya		AY829247	Brant et al. (2006)
MSB:Para:7984	adult	Branta canadensis	Canada	KC570945		Brant et al. (2013)
Austrobilharzia sp.	cercaria	Pirenella cingulata	Iran: Jask	MK484	MK284	Present study
Austrobilharzia sp. Kuwait	cercaria	Pirenella cingulata	Kuwait	JF742195	HQ106461	Al-Kandari et al. (2012)
Austrobilharzia terrigalensis	cercaria	Batillaria australis	Australia	AY157249	AY157195	Lockyer et al. (2003)
Austrobilharzia variglandis Avian schistosomatid sp. C BAM-	adult	Larus delawarensis	USA	AY157250	AY157196	Lockyer et al. (2003)
2020 Avian schistosomatid sp. SVB-	cercaria	Planorbella trivolvis	Canada	MT305665		McPhail et al. (2021)
2009W214HaminoeaCA	cercaria	Haminoea japonica	USA Lake Victoria, Kenya Fisheries	GQ920619		Brant et al. (2010)
Avian schistosomatid sp. W1285	cercaria	Biomphalaria sudanica	Association landing site, Kisumu, Kenya Lake Victoria, Kenya Fisheries	AY858886		Brant et al. (2006)
Avian schistosomatid sp. W1285	cercaria	Biomphalaria sudanica	Association landing site, Kisumu, Kenya Lake Victoria, Kenya Fisheries		AY829246	Brant et al. (2006)
Avian schistosomatid sp. W2081	cercaria	Ceratophallus natalensis	Association landing site, Kisumu, Kenya	AY858887		Brant et al. (2006)
Bilharziella polonica	adult	Anas platyrhynchus	Ukraine	AY157240	AY157186	Lockyer et al. (2003)
Bivitellobilharzia loxodontae W500	eggs	Loxodonta cyclotis Rhinoceros unicornis,	Central African Republic	JN579950	JN579948	Brant et al. (2013)
Bivitellobilharzia nairi W465	miracidia	Elephas maximus	Nepal	JQ975005	JQ975011	Devkota et al. (2014)
Carettacola hawaiiensis	adult	Chelonia mydas	USA: Pacific Ocean, Hawaii	AY604709		Snyder (2004)

Chimaerohemecus trondheimensis	adult	Chimaera monstrosa	Norway	AY157239		Lockyer et al. (2003)
Dendritobilharzia pulverulenta	adult	Gallus gallus	USA	AY157241		Lockyer et al. (2003)
Dendritobilharzia pulverulenta	adult	Gallus gallus	USA	11113/211	AY157187	Lockyer et al. (2003)
Dendritobilharzia sp. W499	cercaria	Gyraulus parvus	USA		KX302892	Brant et al. (2017)
-	adult	, .	USA	AY157242	AY157188	•
Gigantobilharzia huronensis		Agelaius phoeniceus			A113/100	Lockyer et al. (2003)
Gigantobilharzia melanoidis	cercaria	Melanoides tuberculata	United Arab Emirates	JX875068		Schuster et al. (2014)
Gigantobilharzia melanoidis	cercariae	Melanoides tuberculata	United Arab Emirates		JX875069	Schuster et al. (2014)
Griphobilharzia amoena	adult	Crocodylus johnstoni	Australia	AY899914		Brant & Loker (2005)
Hapalotrema mehrai	adult	Chelonia mydas	USA: Pacific Ocean, Hawaii	AY604708		Snyder (2004)
Hapalotrema mistroides	adult	Caretta caretta	Italy: Latium	KY499799		Santoro et al. (2017)
Heterobilharzia americana	adult	Procyon lotor	USA	AF167086		Snyder (2000)
Heterobilharzia americana	adult	Mesocricetus auratus	USA		AY157192	Lockyer et al. (2003)
Learedius learedi Macrobilharzia macrobilharzia	adult	Chelonia mydas	USA: Pacific Ocean, Hawaii Louisiana, Ascension Parish, Sorrento	AY604707		Snyder (2004)
W133	adult	Anhinga anhinga Chroicocephalus	Timberton Hunt Club	AY858885	AY829248	Brant et al. (2006)
Marinabilharzia patagonense Nasusbilharzia melancorhypha	adult	maculipennis	Argentina: Puerto Madryn		OK338770	Lorenti et al. (2022)
"Schistosomatidae gen. n. sp. n."	1.1.			NAW 000221	MW01249	El (1 (2021)
SB-2020W822	adult	Cygnus melancoryphus	Argentina	MW000331	3	Flores et al. (2021)
Ornithobilharzia canaliculata	adult	Larus delawarensis	USA	AY157248		Lockyer et al. (2003)
Ornithobilharzia canaliculata	adult	Larus delawarensis	USA	3.577.6.5	AY157194	Lockyer et al. (2003)
Ornithobilharzia canaliculata	cercaria	Pirenella cingulata	Iran: Gevanesh	MK265	MK98	Present study
Ornithobilharzia canaliculata	cercaria	<b>Pirenella cingulata</b> Chroicocephalus	Iran: Gevanesh	MK472	MK172	Present study
Riverabilharzia ensenadense	adult	maculipennis	Argentina: La Plata		OK338773	Lorenti et al. (2022)
Schistosoma bovis	adult	Mus musculus	Tanzania	AY157266	AY157212	Lockyer et al. (2003)
Schistosoma curassoni	adult	Mesocricetus auratus	Senegal	AY157264	AY157210	Lockyer et al. (2003)
Schistosoma edwardiense	cercaria	Biomphalaria sudanica	Uganda: Lake Edward	AY197344	AY197347	Morgan et al. (2003)
Schistosoma haematobium	adult	Mesocricetus auratus	Mali	AY157263	AY157209	Lockyer et al. (2003)

Schistosoma hippopotami	cercaria	Bulinus truncatus	Uganda: Lake Edward	AY197343	AY197346	Flores et al. (2015)
Schistosoma incognitum	adult	Bandicota indica	Thailand	AY157255	AY157201	Lockyer et al. (2003)
Schistosoma indicum	adult	Bos taurus	Bangladesh	AY157258	AY157204	Lockyer et al. (2003)
Schistosoma intercalatum	adult	Mus musculus	Sao Tome and Principe	AY157262	AY157208	Lockyer et al. (2003)
Schistosoma japonicum	adult	Mus musculus	China	Z46504		Lockyer et al. (2003)
Schistosoma japonicum	adult	Bubalus sp. Pelomys isseli, National	China:Yueyang, Hunan Province		AM689524	Liu. et al. (Unpublished Data)
Schistosoma kisumuensis MSB:Para:164	adult	Museum of Kenya: 167811	Kenya: Nyabera Swamp, Kisumu	FJ897154	FJ897153	Hanelt et a.,l (2009)
Schistosoma leiperi	adult	Mesocricetus auratus	South Africa	AY157261	AY157207	Lockyer et al. (2003)
Schistosoma malayensis	adult	Mus musculus	Malaysia	AY157252	AY157198	Lockyer et al. (2003)
Schistosoma mansoni	adult	na	Senegal		AJ519524	Kane et al. (2003)
Schistosoma mansoni	adult	Mus musculus	experimental infection; isolate NHM-3454/5/6.	AY157173		Lockyer et al. (2003a)
Schistosoma margrebowiei	adult	Mus musculus	Zambia	AY157260	AY157206	Lockyer et al. (2003)
Schistosoma mattheei	adult	Mesocricetus auratus	Zambia	AY157265	AY157211	Lockyer et al. (2003)
Schistosoma mekongi	adult	Mus musculus	Laos	AY157253	AY157199	Lockyer et al. (2003)
Schistosoma nasale	adult	Capra hircus	Sri Lanka	AY157259	AY157205	Lockyer et al. (2003)
Schistosoma rodhaini	adult	Mus musculus	experimental infection; lab strain (NHM)	AY157256		Lockyer et al. (2003a)
Schistosoma sinensium	adult	Mus musculus	China	AY157251	AY157197	Lockyer et al. (2003)
Schistosoma sp. RD-2016W526	cercaria	Radix luteola	Nepal	KT022106		Devkota et al (2016)
Schistosoma sp. RD-2016W803	cercaria	Radix luteola	Nepal		KT022105	Devkota et al (2016)
Schistosoma spindale	adult	Mus musculus	Sri Lanka	AY157257	AY157203	Lockyer et al. (2003)
Schistosoma turkestanicum	adult	Ovis aries	Iran	AY157254		Lockyer et al. (2003)
Schistosoma turkestanikum	adult	"sheep"	China		EU177877	Li et al. (2008)
Schistosomatidae sp. 1 W847 Schistosomatidae sp. 1	cercaria	Biomphalaria glabrata	Brazil		MF598177	Pinto et al. (2017)
W848MSB:Para:25515 Schistosomatidae sp. 2	cercaria	Biomphalaria glabrata	Brazil	MF598183		Pinto et al. (2017)
W165MSB:Para:18604	adult	Aix sponsa	USA	MF598179		Pinto et al. (2017)

Schistosomatidae sp. 2 W480	cercaria	Gyraulus parvus	USA		MF598176	Pinto et al. (2017)
Schistosomatidae sp. CRAM2127 Schistosomatidae sp. ex penguin	eggs	Spheniscus magellanicus	Brazil: Rio Grande	MG670449		Vanstreels et al. (2018)
JA-2014153/12	eggs	Spheniscus demersus	South Africa	KM023789		Aldhoun & Horne (2015)
Schistosomatidae sp. MSB Para 7952	cercaria	Chilina gibbosa	Argentina	KC113049		Flores et al. (2015)
Schistosomatidae sp. MSB Para 7955 Schistosomatidae sp. MSB Para	cercariae	Chilina gibbosa	Argentina		KC113077	Flores et al. (2015)
7957	cercaria	Chilina gibbosa	Argentina	KC113062		Flores et al. (2015)
Schistosomatidae sp. MSB Para 7970 Schistosomatidae sp. MSB Para	cercariae	Chilina neuquenensis	Argentina	KC113053		Flores et al. (2015)
7973	cercariae	Chilina dombeiana	Argentina		KC113073	Flores et al. (2015)
Schistosomatidae sp. W217	cercaria	Haminoea japonica	USA		KX302895	Brant et al. (2017)
Schistosomatidae sp. W636	sporocyst	Siphonaria lessonii	Argentina	KX302889		Brant et al. (2017)
Schistosomatidae sp. W640	sporocyst	Siphonaria lessonii	Argentina	KX302890		Brant et al. (2017)
Schistosomatidae sp. W688	cercaria	Indoplanorbis exustus	Nepal	KF672860	KF672862	Devkota et al. (2013)
Schistosomatidae sp. W829	sporocyst	Siphonaria lessonii	Argentina		KX302898	Brant et al. (2017)
Schistosomatium douthitti	adult	Mesocricetus auratus	USA	AY157247		Lockyer et al. (2003)
Schistosomatium douthitti	adult	Mesocricetus auratus Trachemys scripta	USA		AY157193	Lockyer et al. (2003)
Spirorchis scripta	adult	scripta	USA	AY222174		Olson et al. (2003)
Trichobilharzia australis	adult	Anas superciliosa	New Zealnad	OK104141		Davis et al. (2021)
Trichobilharzia australis	adult	Anas superciliosa	New Zealnad		OK357975	Davis et al. (2021)
Trichobilharzia franki	adult	Anas platyrhynchos	Iran: Guilan Province		MH410295	Ashrafi et al. (2018)
Trichobilharzia franki sbb	cercariae	Physa marmorata	Germany	FJ711768		Brant & Loker (2009)
Trichobilharzia longicauda	adult	Aythya novaeseelandiae	New Zealnad	OK104148	OK357979	Davis et al. (2021)
Trichobilharzia mergi	adult	Mergus serrator	Iceland: Botsvatn Lake		JX456171	Kolářová et al. (2013)
Trichobilharzia novaeseelandiae	adult	Aythya novaeseelandiae	New Zealnad	OK104143		Davis et al. (2021)
Trichobilharzia novaeseelandiae	adult	Aythya novaeseelandiae	New Zealnad		OK357971	Davis et al. (2021)
Trichobilharzia physellae	adult	Aythya affinis	USA		FJ174512	Brant & Loker (2009)

Trichobilharzia physellae W193lescNM	adult	Aythya affinis	USA	FJ174473		Brant & Loker (2009)
Trichobilharzia querquedulae Trichobilharzia querquedulae	adult	Anas clypeata	Canada		FJ174509	Brant & Loker (2009)
W137blteLA	adult	Anas discors	USA	FJ174468		Brant & Loker (2009)
Trichobilharzia regenti	cercaria	Radix peregra	Czech Republic	AY157244		Lockyer et al. (2003)
Trichobilharzia regenti	adult	Anas platyrhynchos	Iran: Guilan Provinc		MH410294	Ashrafi et al. (2018)
Trichobilharzia regenti	adult	Anas platyrhynchos	Iran: Guilan Province		MH410294	Ashrafi et al. (2018)
Trichobilharzia sp. A SVB-2009 Trichobilharzia sp. A SVB-	adult	Anas americana	USA		FJ174524	Brant & Loker (2009)
2009W192amwi	adult	Anas americana	USA	FJ174471		Brant & Loker (2009)
Trichobilharzia sp. B SVB-2009	adult	Anas americana	USA		FJ174528	Brant & Loker (2009)
Trichobilharzia sp. C HAP 2014	adult	Aix sponsa	USA		KJ855996	Pinto et al. (2014)
Trichobilharzia sp. C SVB-2009	adult	Lophodytes cucullatus	USA		FJ174529	Brant & Loker (2009)
Trichobilharzia sp. D SVB-2009	cercaria	Stagnicola sp.	Canada		FJ174485	Brant & Loker (2009)
Trichobilharzia sp. E SVB-2009	cercaria	Stagnicola sp.	Canada		FJ174483	Brant & Loker (2009)
Trichobilharzia sp. E SVB- 2009W332StagnicolaMB 1 Trichobilharzia sp. HAP	cercaria	Stagnicola sp.	Canada	FJ174480		Brant & Loker (2009)
2013W701 Trichobilharzia sp. haplotype	cercariae	Physa marmorata	Brazil	KJ855994	KJ855995	Pinto et al. (2014)
peregra	eggs	Anas clypeata	France		HM439505	Jouet et al. (2010)
<i>Trichobilharzia</i> sp. J	cercaria	Austropleplea tomentosa	New Zealnad	OK104140		Davis et al. (2021)
<i>Trichobilharzia</i> sp. J	cercaria	Austropleplea tomentosa Pseudosuccinea	New Zealnad		OK357985	Davis et al. (2021)
<i>Trichobilharzia</i> sp. L1 2 <i>Trichobilharzia</i> sp. var.	cercaria	columella	USA	KY319361		Adema et al. (2009)
narochanica	cercariae	Radix ampla	Belarus: Naroch Lake		JQ681538	Chrisanfova et al. (2009)
Trichobilharzia sp. W515 Trichobilharzia stagnicolae	cercaria	Radix luteola	Nepal	KF672861		Devkota et al. (2013)
W164StagnicolaMN	cercaria	Stagnicola sp.	USA	FJ174477		Brant & Loker (2009)
Trichobilharzia szidati	cercaria	Lymnaea stagnalis	Czech Republic	AY157245		Lockyer et al. (2003)
Trichobilharzia szidati	cercariae	Lymnaea stagnalis	Russia: Moscow, Altufyevo ponds		JF838201	Korsunenko et al. (2012)

## Chapter 3

Thermal optima of cercarial emergence in trematodes from a marine high-temperature ecosystem, the Persian Gulf

# scientific reports

#### **OPEN**

# Thermal optima of cercarial emergence in trematodes from a marine high-temperature ecosystem, the Persian Gulf

Maral Khosravi<sup>12</sup>, Dakeishla M. Díaz-Morales<sup>12</sup>, David W. Thieltges<sup>13</sup>, Martin Wahl<sup>14</sup> & Jahangir Vajedsamiei<sup>1</sup>

Global warming may alter the dynamics of infectious diseases by affecting important steps in the transmission of pathogens and parasites. In trematode parasites, the emergence of cercarial stages from their hosts is temperature-dependent, being highest around a thermal optimum. If environmental temperatures exceed this optimum as a consequence of global warming, this may affect cercarial transmission. However, our knowledge of cercarial emergence patterns of species from high temperature environments is currently very limited. Here, we investigated the effect of temperature on the emergence of two common trematode species from an abundant mud snail *Pirenella cingulata* in the Persian Gulf, the warmest sea on Earth. Infected snails were incubated in the laboratory at 6 temperatures from 10 to 40 °C for 3 days. We found an optimal temperature for cercarial emergence of 32.0 °C and 33.5 °C for *Acanthotrema tridactyla* and Cyathocotylidae gen. sp., respectively, which are the warmest recorded thermal optima for any aquatic trematode species.

Emergence of both species dropped at 40 °C, suggesting upper thermal limits to emergence. Overall, Persian Gulf trematodes may be among the most heat-tolerant marine trematode species, indicating a potential for dispersing to regions that will continue to warm in the future.

Global warming is expected to have profound effects on biological systems at every level of their organization, from molecules to whole ecosystems<sup>1,2</sup>. These effects include, but are not limited to, altering organismal function- ing and life history, population dynamics, and species dispersal<sup>3-6</sup>. In addition to affecting free-living species, it is anticipated that global warming will alter pathogen and parasite outbreaks, particularly through effects on transmission dynamics<sup>7-9</sup>. Studies have demonstrated, for instance, that warming can directly boost infection levels in hosts by increasing the production of infective stages of parasites<sup>10</sup>, expanding seasonal infection windows<sup>11</sup>, elevated movement activity of cercariae thus increasing chances to find a host<sup>12</sup>, and intensifying the susceptibility of hosts by reducing host immune response<sup>13-16</sup>. Since the effects of warming on parasite trans- mission dynamics can have far-reaching ecological and economic consequences for hosts and ecosystems<sup>9,17</sup>, it is important to understand the effects of temperature on parasite transmission as a pre-requisite for predicting climate change effects on the biosphere.

Trematodes are an excellent example of parasites for which temperature effects are well known to affect crucial steps in the transmission process from one host to another  $^{18,19}$ . Their life cycle usually involves three different hosts: a mollusc as first intermediate host, invertebrates or vertebrates (depending on the species) as second intermediate host, and vertebrates as final host  $^{20}$ . Apart from trophic transmission to final hosts, trematodes transmit via free-living larval stages, which are at the mercy of the external environment  $^{21}$ . Cercarial stages are responsible for the transmission from the first to the second intermediate host, and their emergence from the first intermediate hosts is temperature dependent  $^{10,22}$ . Cercarial emergence usually occurs within a specific tempera- ture window in which the conditions are optimal for cercariae functional activity appropriate for transmission to the downstream host  $^{23,24}$ . Within this window, cercarial emergence is often very low at the lower temperature end, then increases towards an optimum, and often declines again at the higher end of the temperature window  $^{10,24,25}$ .

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In general, the optimal temperature ranges for cercarial emergence usually exhibit a latitudinal decline from 20 to 30 °C at low latitudes ( $\leq 35^{\circ}$  of latitude) to 15–25 °C at mid-latitudes ( $36-60^{\circ}$ )<sup>22</sup>. Potential temperature limits to cercarial emergence would be highly relevant for anticipating climate change effects on cercarial transmission dynamics. However, our knowledge of cercarial emergence patterns of species from high-temperature environments at low latitudes is very limited and largely restricted to a few freshwater trematode species of medical or veterinarian importance<sup>22</sup>. Likewise, little is known about how cercarial emergence in high-temperature environments. ronments interacts with other abiotic factors such as light<sup>26</sup>, which can sometimes lead to distinctive circadian rhythms, with some species showing higher cercarial emergence during the day and others at night <sup>25,27,28</sup>.

An ideal system to conduct research on the effects of temperature on cercarial emergence in species from hightemperature environments is the Persian Gulf, the warmest sea on Earth. Due to its shallow depth and restricted water exchange with the open ocean, the sea surface temperature (SSTs) in its various locations can fall as low as 13 °C in the winter<sup>29</sup> and reach to 35 °C in the summer. The frequency of heatwaves surpassing 36 °C has increased 19-fold since 1997<sup>30</sup>, with a recent extreme record of 37.6 °C in SSTs on 30th July 2020<sup>31</sup>. Thus, hosts and parasites inhabiting the intertidal zones of the Persian Gulf are eurytherms with a broad temperature tolerance window. Among the trematode hosts are gastropod species from the genus *Pirenella* which can occur in densities of up to 2100 ind.m<sup>-232</sup> and endure a wide range of salinity (1.2–60)<sup>33</sup> and temperature (5–45 °C)<sup>34</sup>. *Pirenella* species serve as the first intermediate host for several trematode species<sup>35–37</sup>. The most abundant *Pirenella* species along the intertidal zone of the Persian Gulf is *Pirenella cingulata*, a species that is currently found to inhabit the Indo-West Pacific from India and Sri Lanka to Papua New Guinea, the northern coast of Japan and south to central Queensland (Supplementary Fig. S1). P. cingulata can be infected by 28 trematode species belonging to 10 families along the Persian Gulf coastline (Khosravi et al. unpublished data). From these, the most common trematode species are *Acanthotrema tridactyla*<sup>38</sup> (Sohn et al. 2003)<sup>39</sup>, a cercaria with parapleurolophocercous morphology and a prevalence of up to 18%, and the morphospecies (still pending formal description) 'Cyathocotylidae gen. sp.', a cercaria with furcocercous morphology and a prevalence of up to 12% (Khosravi et al. unpublished data). A. tridactyla pertains to the family Heterophyidae, has a median dorso-ventral fin-fold on the tail, is an active swimmer, uses eye spots to locate the next host (the fish *Aphanius dispar*<sup>40,41</sup>, and has rediae as the intra-gastropod stage (parthenitae). Metacercariae of A. tridactyla have been found under the scale of laboratory-infected Gambusia and in the connective tissue of the head and visceral region of naturally infected *Aphanius fasciatus* in Red Sea, Egyptian intertidal coasts<sup>38</sup>. Final host of genus *Acanthotrema* (previously *Stictodora*) are piscivorous birds and mammals, including humans<sup>38-44</sup>. 'Cyathocotylidae gen. sp.' belongs to the Cyathocotylidae family. Although the life cycles of the manufacture of this family recognition of the cycles the members of this family remain largely unresolved, they are known to use fish, amphibians, or crustaceans as second intermediate host, and reptiles, birds or mammals, as final host 45,46. Yet, it's cercariae is an active swimmer, has a bifurcated tail, no eyespots, and has sporocysts as parthenitae. The thermal biology of these trematodes is unknown, and progress in this area can shed light on their future transmission dynamics in the warming Persian Gulf and surrounding regions.

This study sought to determine the temperature dependence of cercarial emergence of two trematode species (A. tridactyla and Cyathocotylidae gen. sp.) infecting P. cingulata snails in the Northern Persian Gulf. In our laboratory experiment, we measured the emergence of cercariae across a broad range of experimental temperatures which were based on sea surface temperatures measured at our sampling site (Genaveh, located at 29° 33′ 14.022″ N, 50° 30′ 34.0416″ E in the Northern Persian Gulf) over the last twelve years (2010–2022). The experimental temperatures ranged from 10 to 34 °C, with an additional extreme temperature scenario of 40 °C. In addition, we investigated whether these emergence patterns were affected by circadian rhythms (exposure to day and night). Our research contributes to our extremely limited knowledge of cercarial emergence in marine high-temperature

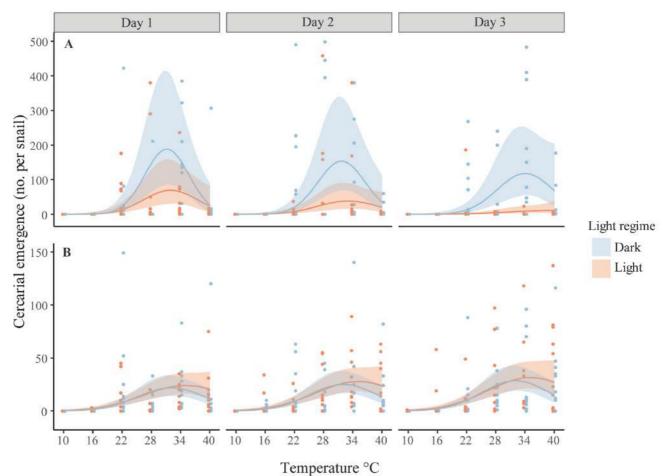
environments at low latitudes.

Cercarial emergence in response to temperature, light regime, and time. The best-fit models adequately explained variation in cercarial emergence of *Acanthotrema tridactyla* ( $R^2c = 0.82$ ) and Cyathocotylidae gen. sp. ( $R^2c = 0.82$ ) = 0.61). Both species of trematode exhibited temperature-dependent (curvilinear) cercarial emergence under all experimental days and light regimes (Fig. 1). *A. tridactyla* showed a generally more abundant emergence than Cyathocotylidae gen. sp. which decreased over time and exhibited a clear circadian pattern (Fig. 1, Table 1). In contrast, Cyathocotylidae exhibited a less abundant cercarial emergence independent of light or time (Fig. 1, Table 1). The results of the Wald tests for the significance of the models' estimated parameters representing the primary and interactive effects of *temperature*, *light regime*, and *time* on cercarial emergence are presented in Table 1. The overall effect of temperature on cercarial emergence was significant for both trematode species (Table 1; Fig. 2).

A. tridactyla and Cyathocotylidae gen. sp. had the highest mean cercarial emergence rates of 94 and 26 cercariae per snail, respectively, at temperatures of 32.0 °C and 33.5 °C (Fig. 2). At temperatures above these optima, there was a trend for cercarial emergence to decline for both species.

Based on raw data, for both species, 10 °C was the only temperature with zero emergence, thus representing MDTT, whereas 16 °C was the METT with less than 20 emerged cercariae per snail for both parasite species. During the 3day incubation experiment, the highest number of emerged cercariae from a single snail within 24 h was 2798 at 28 °C for *A. tridactyla* and 520 at 34 °C for Cyathocotylidae gen. sp.

For A. tridactyla, the non-linear effects of temperature on cercarial emergence significantly changed in response to the light regime (p = 0.018; Table 1). In addition, the main effect of the light regime on cercarial emergence was marginally insignificant (p = 0.078; Table 1) as the species tended to have increased cercarial emergence during dark or night time (Fig. 1). Based on the model prediction, the absolute effect size was 2.4 times higher emergence in dark compared to light. However, at the optimal temperature range (ca. 28-34 °C),



**Figure 1.** Generalized linear mixed models' predictions for the mean (lines) and 95% confidence intervals (shaded areas) of cercarial emergence in response to *temperature*, *light regime*, and *time*, presented for two studied trematode species *Acanthotrema tridactyla* (**A**) and Cyathocotylidae gen. sp. (**B**). The number of cercariae emerged from snails was counted in 12 h intervals (*dark* and *light* periods) over 3-day experiments, represented by blue (*dark*) and orange (*light*) points.

the emergence was up to 2.8 times higher in dark than light. Besides, cercarial emergence significantly decreased with time (p = 0.008; Table 1). The interactive effect of light regime and time on emergence was also significant (p < 0.001; Table 1), represented by an increased influence of light regime with time (Fig. 1). The non-linear effect of temperature also significantly changed over time (p = 0.013; Table 1).

In the case of Cyathocotylidae gen. sp., a change in the non-linear effect of *temperature* in response to the *light regime* was found marginally insignificant (p = 0.052; Table 1). The main effect of *light regime* on emergence was insignificant (p = 0.069; Table 1), while the effect of *light regime* combined with quadratic effects of *temperature* was marginally insignificant (p = 0.052). A trend of increase in emergence over time was also found marginally insignificant (p = 0.054, Table 1). Based on the model prediction, the interaction between light and temperature was also marginally insignificant (p = 0.052; Table 1) (Fig. 1). The data are accessible in supplementary material.

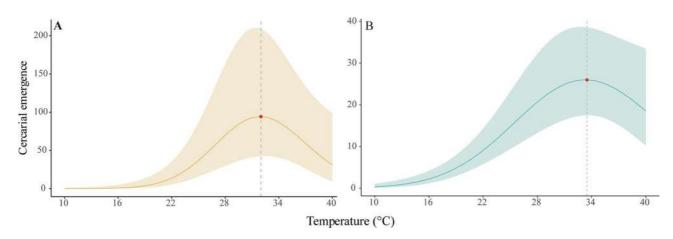
#### Discussion

Cercarial emergence from the first mollusc host is an important step in transmission, dispersion and continuation of trematode life cycles and, as a result, disease dynamics. Cercarial emergence is temperature-driven and optimized at temperatures representing thermal optima for cercariae functioning<sup>11,47</sup>. Considering ongoing global ocean warming, understanding the thermal sensitivity of cercarial emergence can help us predict the future transmission dynamics of trematode species. However, this knowledge is largely lacking for marine trematodes living in high-temperature environments.

**High thermal optima of cercarial emergence.** Temperature facilitates the transition of parthenitae from the formation of one type of embryo to another, speeding up the embryogenesis process. This acceleration of embryogenesis involves the increase in the appearance of both young rediae and/or sporocysts of different generations that ultimately release cercariae<sup>20</sup>. Cercarial emergence commonly increases up to an optimum temperature above which it declines<sup>10,22,48</sup>. This thermal optimum usually varies due to genetic adaptation of the trematode and snail host and can depend on their acclimating (micro)habitat conditions<sup>22,49</sup>. Previous studies have shown that the temperature optimum for cercarial emergence decreases with latitude, from 20 to 30 °C at

(A) Acanthotrema tridactyla	Estimate	Std. Error	z value	Pr(> z )
(Intercept)	1.173	0.506	2.317	0.020**
Light8	0.671	0.382	1.759	0.078
Poly (Temperature, 2)1	40.777	10.607	3.844	0.000***
Poly (Temperature, 2)2	-20.582	8.288	- 2.483	0.013*
Poly (Day, 2)1	-11.276	4.282	- 2.633	0.008**
Poly (Day, 2)2	- 3.965	3.767	- 1.053	0.292
Light8: Poly (Temperature, 2)1	14.870	8.486	1.752	0.079
Light8: Poly (Temperature, 2)2	-14.106	5.969	- 2.363	0.018*
Light8: Poly (Day, 2)1	12.876	2.941	4.378	0.000***
Light8: Poly (Day, 2)2	3.761	2.734	1.376	0.169
Poly (Temperature, 2)1: Poly (Day, 2)1	-41.871	82.977	- 0.505	0.613
Poly (Temperature, 2)2: Poly (Day, 2)1	122.798	56.55	2.171	0.029*
Poly (Temperature, 2)1: Poly (Day, 2)2	8.389	76.118	0.110	0.912
Poly (Temperature, 2)2: Poly (Day, 2)2	20.906	53.340	0.392	0.695
(B) Cyathocotylidae gen. sp	Estimate	Std. Error	z value	Pr(> z )
(Intercept)	1.984	0.184	10.760	0.000***
Light8	-0.342	0.188	- 1.814	0.069
Poly (Temperature, 2)1	25.326	3.672	6.897	0.000***
Poly (Temperature, 2)2	- 10.989	3.235	- 3.397	0.000***
Poly (Day, 2)1	2.070	1.075	1.925	0.054
Poly (Day, 2)2	-0.055	1.027	- 0.054	0.957
Light8: Poly (Temperature, 2)1	2.641	4.148	0.637	0.524
Light8: Poly (Temperature, 2)2	- 6.353	3.278	- 1.938	0.052

**Table 1.** Wald test results for the significance of quasipoisson linear mixed models' parameter estimates which are linked to main and interactive effects of *temperature*, *light regime*, and *time* on cercarial emergence, for trematode species *Acanthotrema tridactyla* (A) and Cyathocotylidae gen. sp. (B). The significant effects have p-value < 0.05. *light regime* had two levels: *Light20* and *Light8* representing 12-h light and dark periods, respectively. *Poly (predictor, 2)1* and *Poly (predictor, 2)2* represent linear and quadratic effects of the predictor, respectively. Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 '' 1.



**Figure 2.** Overall effects of the temperature on cercarial emergence of *Acanthotrema tridactyla* (**A**) and Cyathocotylidae gen. sp. (**B**). Generalized linear mixed models' predictions of the mean (lines) and 95% confidence intervals (shaded areas) were averaged for each temperature. The red dots and grey dashed lines depict cercarial emergence optimal temperatures (i.e., 32.0 °C and 33.5 °C for *A. tridactyla* and Cyathocotylidae gen. sp., respectively).

low latitudes (35°) to 15 to 25 °C at mid-latitudes (36–60°)<sup>22</sup>. The trematode species studied herein, *A. tridactyla* and Cyathocotylidae gen. sp., exhibited a maximum emergence at 32.0 °C and 33.5 °C, respectively, which are the warmest recorded thermal optima so far for any aquatic trematode species<sup>22</sup>. These findings suggest that both parasite species are adapted to the extreme thermal habitats in the Persian Gulf. In the sampling region (northwest Persian Gulf), the monthly average SST has varied between 28 and 36 °C, with an average of ca. 32.0 °C during the summers of 2010–2022 (Supplementary Fig. S2; Data courtesy of NOAA coast watch).

Therefore, summer might be the peak season for the cercarial emergence of these two species infecting  $P.\ cingulata$ . Nevertheless, these intertidal habitats can also experience wide diurnal temperature ranges in summer (e.g.,  $\sim 30$ –55 °C; Bordbar et al. unpublished data) $^{50$ –52</sub>, which, in combination with tidal aerial exposures in sum- mer, might be restrictive for cercarial emergence. Moreover, although seasonality was not directly assessed in this study, many studies suggest that seasonality plays a major role in cercarial emergence. In fact, some studies suggest that seasonality might have a stronger influence on emergence and transmission than temperature itself. For instance, de Montaudouin et al. For experted seasonality in the accumulation of  $H.\ quissetensis$  in cockles at Oualidia, Morocco, where the temperature fluctuates slightly along the year. Therefore, we suggest future studies tackling the seasonality of the Persian Gulf trematodes to weigh the influence of seasonality and temperature on trematode transmission. Information on the seasonality of trematodes from the Persian Gulf is not available, however, since closely-related species with similar hosts, transmission strategies and searching behaviours share similar emergence rhythms certain inferences can be drawn based on other described species. Trematodes of the family Heterophyidae, such as  $A.\ tridactyla$ , are known to have active rediae throughout the year, while for Cyathocotylidae gen. sp., the phenology of intramolluscan stages had not yet been described. However, for Diplostomidae, a better-studied and closely related family to Cyathocotylidae<sup>54</sup>, parthenita (sporocyst in this case) activity is not arrested during winter (i.e., at colder temperatures)<sup>55</sup>. This resembles the seasonal cercarial emergence patterns of Heterophyids. The similarity in the overwintering capacity of the larval stages of both species studied herein is supported by the minimum emergence temperature threshold (METT) values, which was ca. 16 °C (based on

Notably, the Persian Gulf's SST has warmed up, on average, by 0.04 °C year<sup>-1</sup> since the 1980s, which is double the global average<sup>31,56</sup>, and by the year 2100, the SST is projected to increase by another 2–4 °C, depending on the global emission scenario<sup>30,31,57</sup>. Despite the fact that we reported a high thermal optimum for cercarial emergence (32.0 and 33.5 °C for *A. tridactyla* and Cyathocotylidae gen. sp. respectively), our results demonstrate a drop in the emergence around 40 °C, which might indicate the onset of beyond-optimal conditions. There-fore, the seasonality of cercarial release in the Persian Gulf may change in the future, in the absence of further heat adaptation, shifting from summer to spring or fall in the Persian Gulf. Furthermore, the warming of the Indian ocean and other marine regions may provide new habitats for warm-adapted trematodes such as Persian Gulf trematodes. By now, *A. tridactyla* has been only reported from intertidal habitats in the Egyptian Red Sea, which also experiences high temperatures in summer (up to ca. 30 °C)<sup>38</sup>. The establishment and subsequent flourishment of trematodes outside their current range will depend on the presence of the host species needed to complete the life cycle. While knowledge about other intermediate hosts of the studied trematodes is limited, the first intermediate host *P. cingulata* of both trematode species and the second intermediate host of *A. tridactyla*, the fish *A. dispar*<sup>40,41</sup>, are both eurytherms with vast geographic distributions<sup>34,58,59</sup> (see the Supplementary Fig. S1). Many migrating birds, which may be the trematodes' ultimate hosts, use the Persian Gulf islands and shoreline for resting, feeding, and/or shipping to and from this maritime area<sup>63</sup>, ships may also contribute to the co-invasion of trematodes along with their hosts<sup>64</sup>.

Other important aspects of cercarial emergence. Successful transmission from the first to second intermediate host is a challenge in the life cycle of trematodes. Given the short life span of cercariae (< 24 h), they have adapted their emergence patterns to optimize successful rendezvous transmission to the next host<sup>65</sup>. In general, even at optimal temperature, cercarial emergence will not stay constant because it depends on the developmental intramolluscan stage of trematode. Cercariae of some trematodes continuously emerge from the host, while in other cases, cercariae emerge in pulses followed by a period of recovery<sup>20</sup>. We observed a significant decline in emerged cercariae for *A. tridactyla* over time (see Table 1 and Fig. 1), suggesting that this parasite's redial maturation may follow a pulsed-emergence strategy, potentially followed by a regeneration period. This pulsed-emergence strategy is characteristic of trematodes having second-intermediate hosts in low abundances, and, generally, such trematodes show high synchrony with the host spatial distribution and timing of activities<sup>20</sup>. In contrast, our result showed a marginally insignificant increase in cercarial emergence over time for Cyathocotylidae gen. sp., which may suggest that the emergence and sporocyst maturation is rather continuous and random. For diplostomoids, such as some members of Cyathocotylidae, the second-intermediate host is usually fish<sup>66</sup>. Such random and continuous cercarial emergence can provoke the accumulation of metacercariae in fish, a pattern that has been observed in other diplostomoid-fish systems<sup>67</sup>. However, because the life cycle of members of the Cyathocotylidae family is largely unknown, this remains speculative. The different temporal emergence pattern and the higher number of emerged cercariae of *A. tridactyla* compared to Cyathocotylidae gen. sp. might be because the downstream host of the latter is more abundant than the former. Nevertheless, it is important to keep in mind that this study monitored cercar

Circadian cercarial emergence rhythms are genetically controlled<sup>65</sup>, and correspond with the activity of the second intermediate hosts "host-time" while avoiding contact with the regular predators<sup>68–73</sup>. The rhythm is often linked to rough transition between periods of light and darkness rather than minor influence of intensity or quality of light<sup>74</sup>. Prokofiev et al.<sup>53</sup> and Uspenskaya<sup>75</sup> observed a similar phenomenon in which crabs were entering the intertidal zone in the evening and presented a rather quiescent behaviour during the night, the time at which *Microphallus similis* emerged and actively searched for its second intermediate host. Some cercariae can detect light by the use of photoreceptors (eye spots) that allow them to swim towards (positive phototaxis; e.g., *Cryptocotyla lingua*) or away from the light (negative phototaxis; e.g., *Trichobilharzia szidati*)<sup>76,77</sup>. The results

of the present study showed a clear nocturnal pattern in cercarial emergence for *A. tridactyla*. The cercaria of this species has eye spots that, as described above, work as photoreceptors for orientation and host finding. Its second intermediate host is the fish *Aphanius dispar*. *A. dispar* is a eurythermal and euryhaline fish species of coastal lagoons. The wide range of temperature and salinity tolerance enables it to also exist in freshwater river systems, hot sulfur-rich springs, and hypersaline conditions<sup>78–80</sup>. Individuals of *A. dispar* are active during the day and relatively quiescent during the night<sup>78</sup>. A higher emergence during the host's resting time over the night might offer an advantage since the fish is conglomerated in the same place for a limited time and provide a convenient situation for the parasite by providing a point-static target for the cercariae to penetrate its skin. Therefore, it makes sense that a high number of cercariae are shed at once to maximize infection intensity in the next host. There are four hypotheses concerning how cercariae react to light changes: (1) The snail hosts act as mediators (e.g. via behavioural alterations) between the external environment and intra-molluscan larval stage of parasite; (2) The cercariae themselves are sensing and reacting to the light; (3) The daughter sporocysts (or rediae) are causing the rhythmic phenomenon; and (4) Both cercariae and daughter sporocysts are involved, separately or consecutively, with two possibilities of information transmission: from cercariae to sporocyst or vice versa<sup>65</sup>. Notably, our study did not describe the exact timing of cercarial release. This information will be needed to test the above hypotheses regarding the relationship between circadian rhythm of cercarial release, cercaria phototaxis, and host behaviour.

Variability in cercarial emergence among and within trematode species. The considerable variance in cercarial production can be attributed to the sizes of both the parasites and the hosts<sup>81</sup>. In our study, Cyathocotylidae gen. sp. cercariae (ca. 1000 μm body length) are two times larger than the ones of *A. tridactyla* (ca. 500 μm body length; Supplementary Fig. S3). The former also had 3.6 times lower cercarial emergence than the latter at their optimal temperature. When comparing trematode species infecting the same host species of equal size (as done in this study), usually trematodes with smaller cercarial sizes may have more space in the host to produce more cercariae and thus may show higher emergence<sup>82</sup>. In contrast, trematodes with larger cercariae (i.e., Cyathocotylidae gen. sp.) will inherently have fewer cercariae emerging from the host. Our results are consistent with prior research that revealed a negative correlation between daily cercarial output and cercariae size for 12 trematode species in the Barents and White Seas<sup>53</sup>. The abundance of emerged cercariae may also depend on the release pattern and might be linked to the abundance of the downstream host<sup>83</sup>. In terms of cercarial production and activity, parasite genotypes may respond differently to temperature<sup>84</sup>. The size and physiological state of the snail host at the time of infection may affect cercarial output by determining available resources. The bigger the snail host at the moment of infection, the more developed its gonad, resources, and space. Due to variations in inherent immunity, certain snail genotypes may be easier for the parasite to exploit<sup>81</sup>. We randomly collected snails from the field in our investigation and chose those with patent infections (emerging cercariae). Therefore, we did not know about these factors and the age of infection; as such, its contribution to the data variance would be speculative.

#### **Conclusions**

The Persian Gulf is the Earth's warmest sea regarding the maximum seasonal temperature and its host-parasite systems are adapted to high temperatures, as suggested by the results of this study. The trematode species investigated here adopt highly tolerant intermediate hosts (*P. cingulata* and *A. dispar*), which are highly warm tolerant and widely distributed, suggesting that both trematode species could expand to new habitats under ongoing global warming. Further research is needed to determine the heat sensitivity of additional trematode stages, such as cercariae survival and infection success in this extreme environment. To understand disease dynamics, this research must also incorporate cercarial infectivity and survival, the infected downstream host's survival, and the effects of additional abiotic stressors like desiccation, hyper salinity, and eutrophication on trematode transmission.

#### Materials and methods

Host and trematode collection. On a single occasion in February 2020, several hundreds of *Pirenella cingulata* host snails were haphazardly collected by hand at low tide from the upper intertidal zone of a site known from previous research to have high trematode infection levels (Genaveh in the Northern Persian Gulf; 29°33′14.022″N, 50°30′34.0416″E; (Khosravi et al. unpublished data)). The samples were transported by flight to GEOMAR Helmholtz Center for Ocean Research Kiel, Germany, where they were kept in groups of 100 individuals in 2 L tanks for two weeks before starting the experiment. Each tank was filled with aerated reconstituted seawater (37 ± 1) (mixing Red Sea Salt® and deionized water) and kept in a thermo-bath set to 18 °C with a 12:12 photoperiod (start of sunrise at 6:00, maximum light intensity at 12:00 (50% of the maximum possible intensity with irradiance ~ 445 µmol m⁻² s⁻¹, and complete sunset at 18:00). The light:dark cycles were made using Smart Reef LED (Hydra® 32HD). Snails were fed ad libitum with dried powder of *Chlorella vulgaris* (Algomed®), and the water of each tank was changed every other day. In order to detect snails with patent infections (infections presenting developed cercarial emergence), the snails were placed individually in 6-well plates filled with 10 mL of aerated reconstituted seawater at 25 ± 2 °C under constant illumination for 3–5 h. Each well plate was inspected for emerged cercariae under a stereo-microscope (Nikon, SMZ1000 body, C-PS160 stand). Infected individuals (identified by emerged cercariae) were kept in separate containers based on their infection status in the same conditions explained above for 4 days before starting the experiment.

**Experimental design.** A total number of 20 snails, half infected by *A. tridactyla* or Cyathocotylidae gen. sp., were assigned to each experimental target temperature (10, 16, 22, 28, 34, 40 °C). The first five temperatures

represented normal to extreme SSTs observed over the past decade (Supplementary Fig. S2), and the last one represented an end-of-century heatwave temperature, given a projected 4 °C increase<sup>85</sup> in the baseline temperature of a typical heatwave<sup>30</sup>.

The snails were first acclimated from the baseline temperature of 18 °C to the target temperatures by increments or decreases of 2 °C per hour in a way that all samples reached the target temperature at the same time. The acclimation protocol lasted 12 h. During the warm season, littoral molluscs in the study site (and other intertidal regions of the Persian Gulf) experience daily temperature ranges as large as 10 to 20 °C (Bordbar et al. unpublished data). Since cercarial emergence is likely to be triggered by these gradual temperature changes, we monitored cercarial emergence during the acclimation period. For this, one day before starting the acclimation, snails were individually placed in 50 mL beakers filled with 40 mL of aerated seawater and 2000  $\mu$ l of a *C. vulgaris* solution (2.5 mg-algae mL<sup>-1</sup>) for a final concentration of 125  $\mu$ g-algae mL<sup>-1</sup>. During the acclimation, two thermo-bathes (DC10, Thermo Scientific) were used for each target temperature, one as holding tank and the other one set to follow up temperature (2 °C lower or higher). Every 2 h, snails were transferred to the newly set thermo-bath and new beaker filled with 40 mL of seawater pre-equilibrated to the temperature corresponding to the following temperature change and containing dried *C. vulgaris* as a food source (125  $\mu$ g-algae mL<sup>-1</sup>). The water from the old beaker was placed in a 50 mL falcon tube with 7 mL of 99% ethanol to preserve cercariae. All preserved samples were kept in a fridge at 4 °C, and cercariae were counted at a later stage under a dissecting microscope (Nikon, SMZ1000 body, C-PS160 stand).

PS160 stand). After the acclimation phase, the main experiment was conducted over a 3-day period under the same photoperiod described above (start of sunrise at 8:00, maximum white light intensity at 12:00 (50% of the maximum possible intensity), and complete sunset at 20:00. Emerged cercariae were collected at two time points per day: at 8:00 representing the number of released cercariae over the dark period and at 20:00 representing those emerged during the light hours, resulting in a total of 6 cercariae collection points. Sample preservation was performed as described above. During the main experiment we utilize one thermo-bath per temperature.

For both species we reported the minimum emergence temperature threshold (METT), the temperature at which the number of emerging cercariae drops to a level just above zero $^{86,87}$ . Previously, this threshold was deemed to be between 20 and 50 cercariae per snail each day. In general, METTs of specific species appear to be 2–3 °C higher than the minimum development temperature threshold (MDTT) where intramolluscan development ceases $^{86,87}$ .

**Statistical analysis.** All analyses were conducted in R (version 4.1.0) and RStudio© 1.4.1717 (2021R Rstudio, PBC)<sup>88</sup>. The general version of the R scripts can be found in the Supplementary Script.

**Acclimation phase emergence of cercariae.** First, we utilized ANOVA and pairwise t-tests and checked that, for each trematode species, the total number of cercariae that emerged during the acclimation phase were not significantly different between samples of various target temperatures (Supplementary Fig. S4).

**Experimental phase emergence of cercariae.** Then, for each trematode species, a generalized linear mixed model (GLMM) was developed to explain variation in experimental phase emergence potentially caused by the main and interactive effects of *temperature* (second-order polynomial term), *light regime* (intercept term; two levels: *light* and *dark*), and *time* (second-order polynomial term). In the GLMMs, we also considered the random intercept effect of *snail identity* to account for longitudinal data collection (temporal residual dependence). The GLMMs were fitted using *glmmTMB* function from *glmmTMB* package<sup>85,89</sup>, assuming a quasipoisson residual distribution. Afterward, the model selection was performed using *dredge* function from *MuMIn* package<sup>90</sup>, which applied the Second-order Akaike Information Criterion (AICc) to rank the subsets of the GLMMs (<sup>91</sup>; selected GLMMs are presented in the Supplementary Script). The Wald test was used to evaluate the significance of parameter estimates associated with the selected GLMMs' assumed main and interactive effects. For all GLMMs, assumptions were validated using residual diagnostics tool of the *DHARMa* package<sup>92</sup> and explained variance (R<sup>2</sup>c) was calculated using *r.squaredGLMM* function from *MuMIn* package<sup>90</sup>. Finally, using *predict* function from *car* package<sup>93</sup>, we predicted the cercarial emergence responses (mean and confidence intervals) to all predictor combinations, which were plotted using *ggplot2*<sup>94</sup>. In addition, the predictions were averaged for each temperature level to plot the overall thermal performance curves and define the optimal temperature for cercarial emergence of each trematode species. Selected model formula for *Acanthotrema tridactyla* and Cyathocotylidae gen. sp. were respectively as followed:

```
glmmTMB \ Emergence \sim Light + poly \ Temperature, 2 + poly \ Day, 2 + Light * poly \ Temperature, 2 + 1 | Sample\_ID , family = nbinom1) glmmTMB(Emergence \sim Light + poly \ Temperature, 2 + poly \ Day, 2 + Light * poly \ Temperature, 2 + 1 | Sample\_ID , family = nbinom1) + 1 | Sample\_ID , family = nbinom1)
```

#### Data availability

All data generated or analysed during this study are included in this published article [and its supplementary information files].

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#### **Author contributions**

M.K., J.V., D.W.T., M.W. designed the study. M.K. and D.D. ran the experiments, M.K., J.V., analysed the data, M.K. and D.D. wrote the first draft of the manuscript. All co-authors discussed the results, as well as reviewed and contributed to the final manuscript.

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#### Competing interests

The authors declare no competing interests.

#### Additional information

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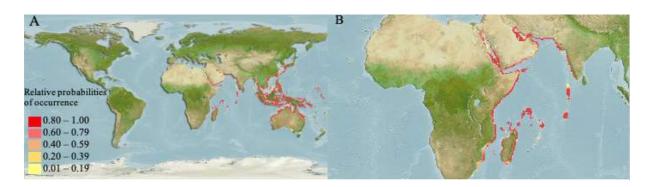
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Supplementary Material for "Thermal optima of cercarial emergence in trematodes from a marine high-temperature ecosystem, the Persian Gulf"

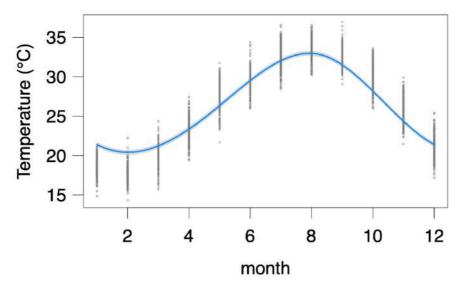
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**Supplementary Figures Supplementary R Scripts** 

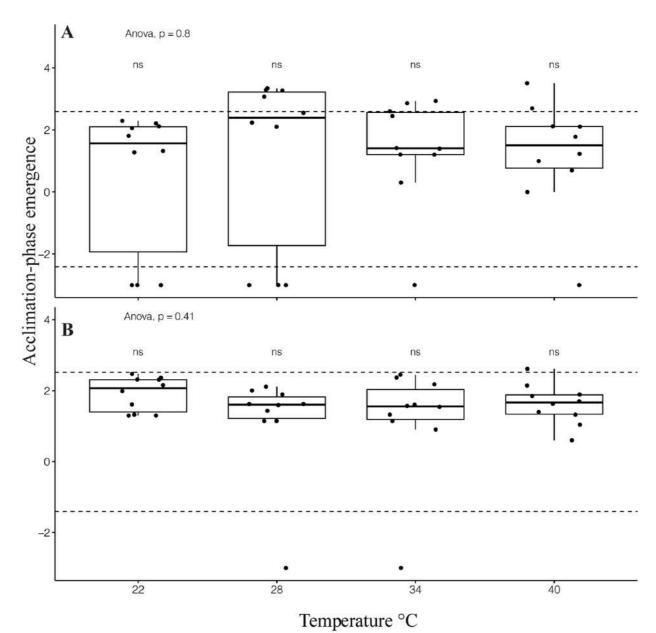
#### **Supplementary Figures**



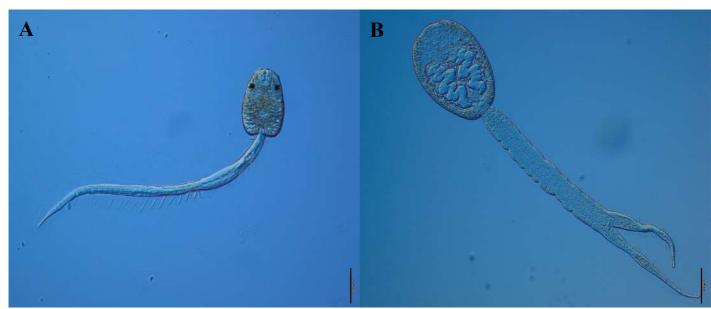
**Supplementary Figure S1.** Native habitat of *Pirenella cingulata* (A) and *Aphanius dispar* (B) based on IPCC RCP8.5 emission scenario Retrieved from https://www.aquamaps.org.



**Supplementary Figure S2.** Averaged monthly Aqua MODIS SST of the sampling locality (Genaveh in the Northern Persian Gulf; 29°33'14.022"N, 50°30'34.0416"E) from 2010-2022 (Data courtesy of NOAA coast watch (<a href="http://coastwatch.pfeg.noaa.gov/erddap">http://coastwatch.pfeg.noaa.gov/erddap</a>)).



**Supplementary Figure S3.** Box plot compares the total number of the cercarial emergence during the acclimation phase in different temperature for *Acanthotrema tridactyla* (A) and Cyathocotylidae gen. sp. (B). Horizontal dash lines represent standard deviation.



**Supplementary Figure S4.** Picture of *Acanthotrema tridactyla* (A) and Cyathocotylidae gen. sp. (B) scale bar 50μm and 100μm, respectively.

#### **Supplementary R Scripts**

```
R Scripts
###Import data frame----
address main =
address for saving = paste()
shedding total h = read excel(paste(address main, "/shedding total h.xlsx", sep=""))
# Inspecting and revising the data frame
df <- shedding total h
df <- as.data.frame(df)
head(df)
str(df)
df$Day <- format(df$Date time, format="%d")
# subset data set separate acclimation from the experiment----
df accl = subset(df, total hour <= 12)
df accl P = subset(df, total hour <= 12& Ptype == 'P')
df accl F = subset(df, total hour <= 12& Ptype == 'F')
#sum of emerged cercariae based on Tank during the acclimation----
sum_accl_P = aggregate( shedding ~Sample ID, data = df accl P, FUN = "sum", simplify =
TRUE, drop = TRUE)
colnames(sum accl P) <- c('Sample ID','shedding accl')
sum accl F = aggregate( shedding ~Sample ID, data = df accl F, FUN = "sum", simplify =
TRUE, drop = TRUE)
colnames(sum accl F) <- c('Sample ID','shedding accl')
head(sum accl F)
df incu P = subset(df, total hour >= 24 & Ptype == 'P' & total hour <= 84)
df incu F = \text{subset}(df, \text{total hour} >= 24 \& \text{Ptype} == 'F' \& \text{total hour} <= 84)
tail(df incu P)
###
df incu P = left join(df incu P, sum accl P, by= 'Sample ID')
df incu F = left join(df incu F, sum accl F, by= 'Sample ID')
###----
df incu P$Day <- format(df incu P$Date time, format="%d")
number day P = aggregate( shedding ~ Day, data = df incu P, FUN = "sum", simplify =
TRUE, drop = TRUE)
head(df incu P)
df incu F$Day <- format(df incu F$Date time, format="%d")
number day F = aggregate( shedding ~ Day, data = df incu F, FUN = "sum", simplify =
TRUE, drop = TRUE)
head(df incu F)
#Revising variable types
# factors:
df incu P$Sample ID <- as.factor(df incu P$Sample ID)
df incu P$Tank<- as.factor(df incu P$Tank)
df incu P$Light<- as.character(df incu P$Time)
df incu P$Light<- as.factor(df incu P$Light)
df incu P$Day<- as.factor(df incu P$Day)
df incu P$Day<- as.numeric(df incu P$Day)
unique(df incu P$Light)
```

```
##FORCO
df incu F$Sample ID <- as.factor(df incu F$Sample ID)
df incu F$Tank<- as.factor(df incu F$Tank)
df incu F$Light<- as.character(df incu F$Time)
df incu F$Light<- as.factor(df incu F$Light)
df incu F$Day<- as.numeric(df incu F$Day)
unique(df incu F$Light)
str(df incu F)
## test correlation of acclimation-phase emergence and exp emergence for each temperature
(3 plots for each species)----
head(df incu P)
df P = aggregate(shedding \sim Sample ID + shedding accl + Temperature, data = df incu P,
FUN = "sum", simplify = TRUE, drop = TRUE)
head(df P)
dir file name = paste(address for saving, "cor accl incub para", ".pdf", sep="")
pdf(file = dir file name, width = 10, height = 8)
par(mfrow=c(3,2), mar = c(2,3,2,2), cex=0.7,
  mgp = c(1, 0.7, 0.1), las=1)
for(i in unique(df P$Temperature)){
 df P T = subset(df P, Temperature == i)
 print(i)
 plot(df_P_T) = (x_a + y_b) = (x_b + y_b)
 lines(df P T$shedding accl, predict(lm(df P T$shedding ~ df P T$shedding accl)), col =
'grey')
 title(main=paste('T =',i,'°C', ' R-squared =', round(cor(df P T$shedding,
df P T$shedding accl), 2), 'p-value =', round(cor.test(df P T$shedding,
df P T$shedding accl)$p.value, 2)))
dev.off()
ggscatter(subset(df P, Temperature >=22), x = "shedding accl", y = "shedding", add =
"reg.line",
      ylab = 'Experimental emergence', xlab = 'Acclimation-phase emergence') +
 stat cor(aes(label = paste(..rr.label.., ..p.label.., sep = "~','~")),
      label.x = 3)
ggsave("scatter para>22.pdf", width = 12, height = 12, units = "cm")
df P mean = aggregate(list(df P$shedding, df P$shedding accl), by=
list(df P$Temperature), mean)
colnames(df P mean) = c('Temperature', 'shedding', 'shedding accl')
ggscatter(subset(df P mean, Temperature >=22), x = "shedding accl", y = "shedding", add =
"reg.line",
      ylab = 'Experimental emergence', xlab = 'Acclimation-phase emergence') +
 stat cor(aes(label = paste(..rr.label.., ..p.label.., sep = "~', `~")),
      label.x = 3)
ggsave("scatter para mean>22.pdf", width = 12, height = 12, units = "cm")
head(df incu F)
df F = aggregate(shedding \sim Sample ID + shedding accl + Temperature, data = df incu F,
FUN = "sum", simplify = TRUE, drop = TRUE)
head(df F)
dir file name = paste(address for saving, "cor accl incub Forco", ".pdf", sep="")
pdf(file = dir file name, width = 10, height = 8)
```

```
par(mfrow=c(3,2), mar = c(2,3,2,2), cex=0.7,
  mgp = c(1, 0.7, 0.1), las=1)
for(i in unique(df F$Temperature)){
 df F T = subset(df F, Temperature == i)
 print(i)
 plot(df F T\$shedding \sim df F T\$shedding accl, ylab = ", xlab = ")
 lines(df F T$shedding accl, predict(lm(df F T$shedding ~ df F T$shedding accl)), col =
 title(main=paste('T =',i,'°C', ' R-squared =', round(cor(df F T$shedding,
df F T$shedding accl), 2), ' p-value =', round(cor.test(df_F_T$shedding,
df F T$shedding accl)$p.value, 2)))
}
dev.off()
ggscatter(subset(df F, Temperature >=22), x = "shedding accl", y = "shedding", add =
"reg.line",
      ylab = 'Experimental emergence', xlab = 'Acclimation-phase emergence') +
 stat cor(aes(label = paste(..rr.label.., ..p.label.., sep = "~`,`~")),
      label.x = 3)
ggsave("scatter Forco>22.pdf", width = 12, height = 12, units = "cm")
df F mean = aggregate(list(df F$shedding, df F$shedding accl), by=
list(df F$Temperature), mean)
colnames(df F mean) = c('Temperature', 'shedding', 'shedding accl')
ggscatter(subset(df F mean, Temperature >=22), x = "shedding accl", y = "shedding", add =
"reg.line",
      ylab = 'Experimental emergence', xlab = 'Acclimation-phase emergence') +
 stat cor(aes(label = paste(..rr.label.., ..p.label.., sep = "~', '~")),
      label.x = 3)
ggsave("scatter Forco mean>22.pdf", width = 12, height = 12, units = "cm")
## box plot of acclimation-phase emergence for each temperature----
accl df P = aggregate(shedding accl ~ Sample ID + Temperature, data = df incu P, FUN =
"mean", simplify = TRUE, drop = TRUE)
accl df P$log10 shedding accl = log10(accl df P$shedding accl+0.001)
head(accl df P)
accl df Pa = subset(accl df P, Temperature >=22)
boxplot P <-ggboxplot(accl df Pa, x = "Temperature", y = "log10 shedding accl",add =
"itter")+#, main = 'Para', ) +
 geom hline(yintercept = mean(accl df P$log10 shedding accl)+
1*sd(accl df P$log10 shedding accl), linetype = 2) + # Add horizontal line at base
 geom hline(yintercept = mean(accl df P$log10 shedding accl)-
1*sd(accl df P$log10 shedding accl), linetype = 2) + # Add horizontal line at base
 stat_compare_means(method = "anova", label.y = 5) + # Add global p-value
 stat compare means(label = "p.signif",method = "t.test", ref.group = ".all.", label.y = 4)
boxplot P
ggsave("Box plot accl Para.pdf", width = 24, height = 12, units = "cm")
accl df F = aggregate(shedding accl ~ Sample ID + Temperature, data = df incu F, FUN =
"mean", simplify = TRUE, drop = TRUE)
accl df F$log10 shedding accl = log10(accl df F$shedding accl+0.001)
head(accl df F)
```

```
accl df Fo = subset(accl df F, Temperature >=22)
boxplot F <-ggboxplot(accl df Fo, x = "Temperature", y = "log10 shedding accl", add =
"jitter") +#main = 'Forco',
 geom hline(yintercept = mean(accl df F$log10 shedding accl)+
1*sd(accl df F$log10 shedding accl), linetype = 2) + # Add horizontal line at base
 geom hline(yintercept = mean(accl df F$log10 shedding accl)-
1*sd(accl df F$log10 shedding accl), linetype = 2) + # Add horizontal line at base
 stat compare means(method = "anova", label.y = 4) + # Add global p-value
 stat compare means(label = "p.signif",method = "t.test", ref.group = ".all.", label.y = 3)
boxplot F
ggsave("Box plot accl Forco.pdf", width = 24, height = 12, units = "cm")
####PARA glmmTMB----
# full model
glmNBP XXX = glmmTMB(shedding \sim Light + poly(Temperature,2) + poly(Day,2) +
#poly(shedding accl,2) +
             Light*poly(Temperature,2)*poly(Day,2) + (1|Sample ID),
            family=nbinom1, data = df incu P, na.action = "na.fail")
summary(glmNBP XXX)
pdf("Diagnostic PARA Full glmNBP XXX.pdf",width=9, height=5)
s=simulateResiduals(fittedModel=glmNBP XXX,n=250)
s$scaledResiduals
par("mar")
plot(s)
dev.off()
acf(resid(glmNBP XXX))
pacf(resid(glmNBP XXX))
?trigamma
MuMIn::r.squaredGLMM(glmNBP XXX)
pdf("dredge full para.pdf")
dr=MuMIn::dredge(glmNBP XXX)
op <- par(mar=c(2,5,14,3))
plot(dr)
dev.off()
# best model based on AICc (dredge)
glmNBP XXX = glmmTMB(shedding ~ Light + poly(Temperature,2) + poly(Day,2) +#
poly(shedding accl,2) +
             Light*poly(Temperature,2) + Light*poly(Day,2) +
poly(Day,2)*poly(Temperature,2) +
             (1|Sample ID), family=nbinom1, data = df incu P, na.action = "na.fail")
#AICc(glmNBP XXX1,glmNBP XXX)
apatheme=theme bw(base size = 11.base family = "sans")+
 theme(panel.grid.major=element blank(),
    panel.grid.minor=element blank(),
    panel.border=element blank(),
    axis.line=element line())
```

```
P<-ggboxplot(df incu P, x = "Temperature", y = "shedding",color = "Light")+
facet wrap("Day")+
 scale color manual (values = c('#91bfdb', '#ef8a62'),
             breaks=c("8", "20"),
             labels=c("Dark", "Light"), name = "Light regime")+
 scale fill manual(values = c('#91bfdb','#ef8a62'),
           breaks=c("8", "20"),
            labels=c("Dark", "Light"),name = "Light regime") +apatheme
P
ggsave(file="Para_inc_box.pdf", width=20, height=10, dpi=1000, units = "cm")
summary(glmNBP XXX)
sink("Summary finalm PARA.txt")
print(summary(glmNBP XXX))
sink()
pdf("Diagnostic PARA FinalM.pdf",width=9, height=5)
s=simulateResiduals(fittedModel=glmNBP XXX,n=250)
s$scaledResiduals
#graphics.off()
par("mar")
\#par(mar=c(1,1,1,1))
plot(s)
dev.off()
MuMIn::r.squaredGLMM(glmNBP XXX)
### predict and back transform because of log link function of full model----
newdata2 \leq- expand.grid(Temperature = seq(10,40, by = 0.5),
              Light=unique(df incu P$Light),
              Day=unique(df incu P$Day),
              #shedding accl=0,
              Sample ID=unique(df incu P$Sample ID))
preds2 <- predict(glmNBP XXX, newdata2, se.fit=T, allow.new.levels = F,
          re.form=NA, type="link")
newdata2$pred = preds2$fit
newdata2$se = preds2$se.fit
newdata2$ulimit = exp(newdata2$pred + qnorm(0.975)*(newdata2$se))
newdata2$llimit = exp(newdata2$pred - qnorm(0.975)*(newdata2$se))
newdata2$pred = exp(newdata2$pred)
head(newdata2)
newdata2 <- as.data.frame(newdata2)</pre>
#take out the prediction for only temperature based on the model predict the max and plot it
df3=aggregate(x = newdata2[c('pred','se','ulimit','llimit')], by=list(newdata2$Temperature),
mean)#aggregate based on temperature
df3<- as.data.frame(df3)
head(df3)
### Plotting the full Model PARA----
apatheme=theme bw(base size = 11,base family = "sans")+
 theme(panel.grid.major=element blank(),
```

```
panel.grid.minor=element blank(),
          panel.border=element blank(),
          axis.line=element line(),
          axis.title.x=element blank(),
          axis.text.x=element blank(),
          axis.ticks.x=element blank())
Day.labs = c('20' = "Day 1", '21' = "Day 2", '22' = "Day 3")
\#names(Day.labs) =c('20' = "Day 1",'21'= "Day 2",'22'= "Day 3")
ggplot() +
  geom point(data = df incu P, aes(x = Temperature, y = shedding, colour=Light),
                alpha=1, shape= 16, size=1, position=position dodge(width=1)) +
  geom line(data= newdata2, aes(x = Temperature, y= pred, colour=Light), size=0.5) +
  geom ribbon(data= newdata2, aes(x = Temperature, y = pred, ymin = ulimit,
                                          ymax = llimit, fill = Light), alpha = .3, colour = NA) +
  v_{\text{lim}}(-10, 500) + \text{facet wrap}(\sim \text{Day, labeller} = \text{labeller}(\text{Day} = \text{Day.labs})) + \text{labeller}(-10, 500) + \text{lab
  scale x continuous(breaks=c(10,16,22,28,34,40))+
  scale_color_manual (values = c('#91bfdb','#ef8a62'),
                           breaks=c("8", "20"),
                           labels=c("Dark", "Light"), name = "Light regime")+
  scale fill manual(values = c(\#91bfdb',\#ef8a62'),
                         breaks=c("8", "20"),
                         labels=c("Dark", "Light"),name = "Light regime") +
  labs(y = 'Cercarial emergence (no. per snail)')+apatheme
ggsave(file="FinalM PARA.pdf", width=20, height=7, dpi=1000, units = "cm")
dev.off()
###plot the model for PARA only temperature----
#obtain maximum value from model
apatheme= theme bw(base size = 11,base family = "sans")+
  theme(panel.grid.major=element blank(),
          panel.grid.minor=element blank(),
          panel.border=element blank(),
          axis.line=element line(),
          legend.position = "none")
T opt = df3$Group.1[which.max(df3$pred)]#optimal temperature= 32°C
CE max = max(df3\$pred) \# max cercarial release on average = 94
plotemerg<-ggplot(df3, aes(x=Group.1, y=pred))+geom_line(color='#E1BE6A') +
  geom ribbon( aes(ymin = llimit, ymax = ulimit,
                       color=NULL), fill='#E1BE6A', alpha=0.25, size=0.5)+
  apatheme+ geom vline(xintercept=T opt, lty = "dashed", col = "slategray3")+
  geom point(aes(x=T opt, y= CE max), colour="red")+
  labs(x = 'Temperature (°C)', y = 'Cercarial emergence',
         title=NULL,colour="Temperature (°C)")+
  scale x continuous(breaks=c(10,16,22,28,34,40))
plotemerg
ggsave(file="Emerged cercariae para temp.pdf", width=16, height=10, dpi=1000, units =
"cm")
```

# **Chapter 4**

Filtration and respiration responses of mussels (Mytilus edulis) to trematode parasite infections (Renicola roscovita) and transient heat exposure



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# International Journal for Parasitology: Parasites and Wildlife





# Filtration and respiration responses of mussels (*Mytilus edulis*) to trematode parasite infections (*Renicola roscovita*) and transient heat exposure

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#### ABSTRACT

and resilience.

infections. Yet, the impact magnitude and potential interactions with environmental stressors remain largely unexplored. This study scrutinizes the effect of *Renicola roscovita* infections on mussel filtration and respiration. We first assessed performance in both uninfected and lab-infected mussels at a mild temperature (16 °C), following an acute heat ramp to 30.5 °C and subsequent cooling. The experiment revealed neither a significant direct impact of the infection on the mussels' performance, nor any significant interplay between the infection and temperature variations. To account for possible infection effects obscured by low sample sizes or mussel size disparities, we conducted a reassessment at 16 °C using both small and large mussels. Infection notably hampered filtration in large mussels, with a marginal impact on smaller ones. A positive correlation was found between infection intensity and mussel filtration capacity, though the infection had no discernible impact on respiration. Our consistent finding of an 11-12 % infection effect size across all experiments indicates a slight reduction in mussel filtration due to trematode infections. While the exacerbating effect of transient heat stress on the infection's impact on filtration was not statistically significant, future investigations should explore potential interactions with prolonged heat stress. Our findings underscore the nuanced ways in which parasitic infections

can influence marine bivalve physiology, emphasizing the need for more comprehensive studies that incorporate environmental stressors, such as heat stress, to fully elucidate the impact of parasitism on marine ecosystem health

The mussel Mytilus edulis, a host to various trematode species, experiences performance decrements due to these

### 1. Introduction

The bivalve mussel *Mytilus edulis* sensu lato is a species complex that forms epibenthic mussel bed ecosystems in shallow waters of the Northern Atlantic Ocean and the Baltic Sea (Stuckas et al., 2017; Larsson et al., 2017). Mussels are of high commercial importance as their aquaculture contributes billions of Euros to global food and non-food services each year (Seed and Suchanek 1992; Schatte Olivier et al., 2020; Avdelas et al., 2021). Furthermore, they are considered ecosystem engineers due to the high level of biodiversity that they support by providing habitat in the form of a mussel bed matrix (Borthagaray and Carranza 2007; Buschbaum et al., 2009; Zippay and Helmuth 2012). In addition, mussels provide important ecosystem functions and services

via their extensive filtration activity, which is not only essential for nutrient and energy cycling but also for trapping suspended particulate organic matter and contaminants and controlling the community structure of micro-planktonic producers and pathogens (Gili and Coma 1998; Widdows et al., 1998; Burge et al., 2016).

The filtration activity of mussels can be affected by various factors, among which temperature plays a pivotal role (Vajedsamiei et al. 2021a, 2021b). In particular, heatwaves, which are amplified by ocean warm- ing (Lima and Wethey 2012; Boyd et al., 2016), can have detrimental impacts on mussel performance (Vajedsamiei et al. 2021a, 2021b). In response to high critical temperatures, mussels suppress their metabolic rates and enter a 'metabolic depression' phase to control the heat-induced increase in ATP synthesis and consumption and protect the

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organism's energy reserves at high critical temperatures (McMahon et al., 1995; Sokolova et al., 2012a). This phenomenon results in the oscillation of filtration (feeding) and respiration rates of mussels during daily temperature fluctuations, including transient exposures to high critical temperatures in shallow habitats (Guppy and Withers 1999; Hui et al., 2020). The continuation of metabolic depression periods, which can occur during heatwaves, might be a more common phenomenon in the course of climate warming, resulting in lower growth rates of mussels. Prolonged heat waves with baseline temperatures of 27–28 °C are known to be lethal for *M. edulis* (Jones et al., 2009; Vajedsamiei et al., 2021b).

Heat stress responses of mussels may further be exacerbated by infections with parasites and pathogens. Mussels serve as hosts for a variety of metazoan parasitic taxa, including polychaetes, copepods, and trematodes (Lauckner 1983). One of these species is the trematode Renicola roscovita which represents one of the most abundant parasites of Mytilus edulis in northern Europe (Lauckner 1983; Goedknegt et al., 2019; Bommarito et al., 2021). In its complex life cycle, R. roscovita infects a first intermediate host, the marine gastropod Littorina littorea, in which it asexually produces thousands of cercariae (Werding 1969). For this asexual reproduction, the parasite invades a substantial part of the host tissue, provoking host castration, inducing high metabolic costs, and elevating mortality risk (Mouritsen et al., 1999; Sorensen and Minchella 2001; Mouritsen and Poulin 2002; Macleod and Poulin 2016). Cercariae then emerge from the snail into the external environment and enter their second intermediate mussel host through the mussel's inhalant siphon. Here, the parasite uses penetration glands along with a specialized penetration apparatus (stylet) to puncture the epidermis of the mussel and preferably encyst in gills and labial palps as meta-cercaria—a transitional stage between dispersive larval stages and adult stages in the definitive bird hosts. In general, metacercarial stages often negatively affect second intermediate hosts less than the first interme- diate host (Lauckner 1983; Bower et al., 1994). However, as the labial palps and gills where R. roscovita metacercariae encyst are critical functional structures of mussels, infections can result in lower growth rates and condition indices ultimately causing a reduced filtration ca-pacity, with more potent effects in larger mussels (Thieltges 2006; Stier et al., 2015). This reduced performance of infected mussels is likely to exacerbate some heat stress responses, but the potential synergistic ef- fects of the two stressors on mussel performance have not been inves- tigated to date.

The present study aimed to determine whether R. roscovita metacercarial infections affect the filtration and/or respiration and the acute heat sensitivity of the mussel M. edulis. We first recorded the responses of laboratory-infected and uninfected mussels under a constant optimum temperature followed by a 24-h thermal fluctuation to test the following hypotheses: (i) R. roscovita infection impacts M. edulis filtration and/or respiration, and (ii) the effect is exacerbated under temperature fluctuations that impose brief critically-warm exposures. As we observed statistically insignificant impacts of infections on mussel filtration rates due to high inter-individual variability in filtration, we conducted two more experiments to retest the first hypothesis, focused on the sole effect of parasites. For this, we used two mussel sizes of 20  $\pm$  2 mm and 40  $\pm$  2 mm to maintain consistency and comparability with earlier published research. We could then compare average impacts on filtration (effect sizes non-standardized by variance) between experiments. In addition to the two main hypotheses, the relationship between filtration rate and infection intensity were evaluated for infected mussels.

#### 2. Materials and methods

The hypotheses of this study were tested in three separate experiments. In Experiment 1, we tested whether the infection by the parasite *Renicola roscovita* impacted *Mytilus edulis* filtration and/or respiration responses during a 5-h constant mild temperature followed by a 24-h fluctuation between mild and critically high temperatures. In

Experiment 2 and 3, we performed additional tests with larger sample sizes to strictly assess the responses of infected versus uninfected mussels of two size classes, large and small, to constant mild temperature exposures lasting around 1 or 2 h.

#### 2.1. Parasite and host sources

Specimens of the first intermediate host Littorina littorea snail were collected from a coastal site (54°21'32.3532" N 10°8'38.7168" E; area ca. 2 km<sup>2</sup> and depth ca. 0.5 m) located in the Kiel fjord in the southwestern Baltic Sea. Approximately 1000 snails were collected in October 2018 for Experiment 1 and 1500 snails in June 2020 for Experiment 2 and 3. Snails were brought to the laboratory in GEOMAR (Helmholtz Center for Ocean Research Kiel, Germany) and kept in a constant-temperature room at 15 °C inside mesh bags (10 L) submerged within a 100 L tank supplied by a flow-through of sand-filtered seawater from the Kiel Fjord. Snails were fed ad libitum with Ulva sp. and Fucus vesiculosus. On the following days, snails were incubated individually under warm illumi- nation to induce cercariae emergence following (Thieltges and Rick 2006). Afterward, the water in each beaker (snail-surrounding water) was screened with a stereomicroscope (Nikon, SMZ1000 body, C-PS160 stand) to detect the presence of cercariae. Morphological identification of emerged cercariae were done based on Werding (1969). From 1000 to 1500 snails initially screened for Experiment 1 and 2, respectively, a total of 15 and 35 snails released R. roscovita cercariae. These snails infected with R. roscovita were kept in a separate holding tank from snails that did not shed cercariae, and both were returned to the

flow-through system at 15  $^{\circ}$ C until controlled infections of the second intermediate host, M. edulis, began.

Mussels were collected from the mussel farm "Kieler Meeresfarm" in the Kiel fjord (54° 22′ 59.1″ N 10° 09′ 41.8″ E) at the same time as snails for each experiment (in October 2018 for Experiment 1 at 13.5 °C, and in June 2020 for Experiment 2 and 3 at 16.0 °C), and 30 individuals (randomly selected with different sizes) were inspected with a stereo-

microscope as whole soft body squash preparations to ensure that they were free of metacercariae. For all experiments, mussels were acclimated to laboratory conditions in one or two 5-litter containers (for Experiment 1 and Experiment 2 and 3 respectively) supplied with

seawater from a flow-through system at 15 °C and feed ad libitum with Rhodomonas salina for 4 weeks prior to "control infection" step. Then small or large mussels were individually kept for 2–3 days at 15 °C in a 100 mL or a 250 mL seawater-filled container, respectively. Mussels acclimated for Experiment 1 were of  $20 \pm 2$  mm shell length, and those of Experiment 2 and 3 were  $20 \pm 2$  mm and  $40 \pm 2$  mm (details specified below). Once a day, the water of all containers was changed, and small and large mussels were fed with 2 and 5 mL of a living R. salina solution (2 million cells mL<sup>-1</sup>) provided by Kiel Marine Organism Culture Centre at GEOMAR, KIMOCC.

#### 2.2. Controlled infections

Experiment 1 and 2 were conducted in November–December 2018 and July–August 2020, respectively. Before experiments, *R. roscovita* infected and uninfected *L. littorea* were individually distributed among 50 mL beakers and kept for 2 h under constant warm illumination. Cercariae less than 4 h old were considered viable and infective since Thieltges and Rick (2006) suggests that the functional longevity of

R. roscovita cercariae (the time when cercariae are no longer able to infect a host despite still being alive) is between 8 and 16 h, a time two to four times longer than the used in this study. Furthermore, laboratory infections with 4 h cercariae were successful in a study previously per-formed on Himasthla elongata (a trematode species with the same second-intermediate host and similar swimming behaviour) (Bommarito et al., 2020). All water containing viable and infective cercariae of R. roscovita was pooled together and well-mixed to generate a genetically mixed array of cercariae, then added to half of the mussel

containers (Studer and Poulin 2013). The other half of the mussel containers received water from uninfected snails. For Experiment 1, each mussel received 60 mL of snail water, and for Experiment 2 and 3, depending on the recipient mussel size, 30 mL or 80 mL of snail water was added to the mussel container. Immediately afterward, mussels were subjected to one dose of *R. salina* food suspension to induce their filtration activities and valve-opening response to increase the chance of cercarial entrance to mussels (Riisgård et al., 2011).

For Experiment 1, 24 mussels were used, and 12 out of 24 were successfully infected in a one-time infection endeavour using 750 mL of snail water containing ca. 35000 cercariae (the density of cercariae was determined by counting cercariae in a subsample of 50 ml and esti-mating the total number of cercariae in the water sample). For Experiment 2 and 3, 10 out of 20 small and 18 out of 36 large mussels were infected three times due to a shortage of emerged cercariae (ca. 1500 cercariae in 1750 mL snail water). After controlled infection, all the mussels were kept in a constant temperature room under the above- explained conditions for 7 days to ensure the full encystment of metacercariae.

# 2.3. Experiment 1: mussel filtration and respiration in response to parasite and heat stress

Experiment 1 tested the combined effect of infection and tempera-ture on mussels' filtration rate and respiration (oxygen consumption) and was composed of 8 temporally replicated trials with different mussels assigned to each trial using simple randomization in December 2018. In each trial, filtration and respiration rates of 3 different mussels, randomly selected from infected and uninfected mussels, were simul-

taneously recorded in 3 separated containers during a pre-heat stress phase (at  $16~^\circ\mathrm{C}$ ; on the time interval 14:00-5:00) followed by a 24 h temperature fluctuation phase (linear heating  $16-30.5~^\circ\mathrm{C}$  on 5:00-17:00 and subsequent cooling  $30.5-16~^\circ\mathrm{C}$  on 17:00-5:00) and a post-heat stress phase ( $16~^\circ\mathrm{C}$ ; 5:00-8:00). The temperatures 16~and  $30.5~^\circ\mathrm{C}$  represent a mild present-day and an end-of-century extreme summer

temperature, respectively (Gra"we et al., 2013; Franz et al., 2019).

All trials were performed using the Fluorometer- and Oximeter- equipped Flow-through Setup (FOFS) based on the protocol described by Vajedsamiei et al. (2021c). In FOFS, a phytoplanktonic (*R. salina*) food suspension was continuously pumped into 4 paths. Along each path, the food suspension passed from an oximetry (or incubation) cylinder and, subsequently, a fluorometry chamber. The filtration or respiration rate of 3 mussels placed in 3 incubation chambers at each time point was calculated based on the difference between chlorophyll (Chl) or dissolved oxygen concentrations taken from three flow-through paths, each containing one mussel, and the measurement taken from one mussel-free flow-through path of FOFS every 5 min (data cropped to 32-h long measurements for each mussel). The food concentration in the ambient of mussels was always kept within the optimal range (1000–7000 cells mL<sup>-1</sup>) for filtration activity (Supplementary Table 1) (Riisgård et al.,

# 2.4. Experiment 2 and 3: filtration and oxygen consumption rates of small and large mussels under constant temperature

2012).

Experiment 2 and 3 focused on testing the effect of infection on filtration and oxygen consumption rates of mussels from two size classes. These experiments were conducted in August 2020 and consisted of two sets of 7 and 12 trials using small and large mussels, respectively. In each experimental trial, filtration and respiration rates of 3 different mussel specimens were simultaneously recorded every minute for 2 h (for small mussels in the afternoon interim) and for 1 h (for large mussels on

morning and afternoon time intervals) at the constant mild temperature of  $17~^{\circ}$ C using FOFS. The collected data was then cropped to 96 or 40 min for small and large mussels, respectively.

#### 2.5. Post-experimental dissections

After Experiment 1 or 2, the length of mussels was measured with a digital Vernier calliper (0.01 mm). Subsequently, mussels were dissected, the whole body was flattened using a glass compressorium, and the number of metacercariae was counted with a stereomicroscope. The same procedure was used for uninfected mussels to confirm the uninfected status.

#### 2.6. Data analyses

Initial data processing was done using Python (Python Software Foundation) based on the scripts and the protocol described by Vajed-samiei et al. (2021c). Raw Chlorophyll (Chl) and oxygen measurements from each experimental trial were denoised using a robust estimation method, temperature corrected, and converted to units of interest. The delay in the Chl measurement, caused by the Chl sensor being post-positioned relative to the oxygen sensor in each path of FOFS, was addressed using linear differential modelling. The revised time series were then used to calculate filtration and respiration rates. Not all the trial's outputs were used because time series were lost or affected by technical problems (i.e., trapped air bubble covering fluorometer sensor), broken magnetic stirrer causing lack of mixture in oximetry chamber. From 24 mussels used in Experiment 1, data for 8 infected and

11 uninfected mussels were analysed. From 20 small and 36 large mussels used in Experiment 2 and 3, measurements of 9 infected and 9 uninfected small mussels and measurements of 18 infected, and 16 un-infected large mussels were analysed separately for two different size groups.

Data analyses were conducted using R (version 4.1.0) and RStudio© 1.4.1717 (Team, 2021). Using the *bam* function from the *mgcv* package (Wood et al., 2016; Wood 2017), we defined Generalized Additive Mixed Models (GAMMs) or Linear Mixed Model (LMM) separately for filtration and respiration response variables as functions of *time* and *infection status* or *infection intensity* (details as follows).

For Experiment 1, (i) filtration and respiration rates as functions of time and infection status were modelled as GAMMs, whereas the re-sponses over time were highly nonlinear due to temperature fluctuation. In each GAMM, time was a smooth-effect predictor that could have an effect with some degree of nonlinearity. Infection status was an ordered factor allowing the intercept (or the mean) and the degree of nonline- arity (effective degrees of freedom, edf) of the reference level smoother (uninfected) to be compared to zero and a straight intercept line, respectively. The treatment level smoother (infected) was compared to the reference level smoother. (ii) To determine whether infection intensity influenced the heat recovery potential of mussels, we used only postfluctuation phase (times >1700 min) filtration or respiration rate data from each infected mussel time series, scaled the values (dividing to the mean filtration or respiration before heat ramp (times <300 min)), and defined GAMMs using the scaled time series of infected mussels. Additionally, the difference in recovery potential during the post-warming period was measured by modelling the scaled filtration rate for both infected and uninfected mussels using GAMMs. The rationale behind scaling was that the level of recovery depends on the original level of response. In all GAMMs, the number of basis functions (knots) was chosen to optimize the k-index while balancing the non-linearity (degree of freedom) and goodness of fit of the models (Wood 2017).

For Experiment 2 and 3, (i) filtration and respiration rates as functions of *time* and *infection status* were models as LMMs, whereas the responses were highly consistent over time. Besides, (ii) GAMMs were defined to test whether filtration and respiration were affected by *infection intensity* and time, separately examined for small and large size mussels.

In all GAMMs and LMMs, mussel identity (*replicate*) was a random effect factor, and Restricted Maximum Likelihood (REML) was used for unbiased estimation of variance components (Wood et al., 2016).

Temporal autocorrelation of residuals was assessed for each model using *check\_resid* function from *itsadug* package (Wood 2017) and the lag-one autoregressive term was considered in the models (Wood 2017). The models' scripts can be found in the supplementary material. After testing for the significance of main and interactive effects, modelling assumptions regarding states of residuals were checked.

Our setup's limited capacity for simultaneous recording of the response of only three mussels at each trial imposed a sample size limitation (explained above). Thus, power analyses were performed using the *powerCurve* function from the *simr* package with 200 simulations, to determine sample sizes required for observing statistically significant effects of the infection-status on filtration rates ( $P \le 0.05$ ). Data from the preand post-fluctuation phases of Experiment 1 (times  $\le 355$  and  $\ge 1700$  min) and small-size mussel data of Experiment 2 were used in three separate power analyses. For the power analyses, LMMs were defined (using *lmer* from *lme4* package) with the same model designs as previous LMMs but without autocorrelation term.

#### 3. Results

Post-experimental dissections demonstrated that the laboratory mussel infections were successful. An infection intensity of 3030  $\pm$  1510 SD metacercariae mussel $^{-1}$  (n = 8) was attained for mussels of Experiment 1. Lab-exposed infections of Experiment 2 and 3 resulted in 166  $\pm$  46 SD metacercariae mussel $^{-1}$  (n = 9) and 584  $\pm$  312 SD metacercariae mussel $^{-1}$  (n = 18), for small mussels and large mussels, respectively. Mean relative parasite intensity was 143  $\pm$  72 SD metacercariae mussel $^{-1}$  mm-shell-length $^{-1}$  (n = 8) for mussels in Experiment 1 and 8  $\pm$  2 SD metacercariae mussel $^{-1}$  mm-shell-length $^{-1}$  (n = 9) for small and 15  $\pm$  7 SD metacercariae mussel $^{-1}$  mm-shell-length $^{-1}$  (n = 18) for large mussels in Experiment 2 and 3. None of the mussels were infected with other macro-parasites (i.e., polychaetes, copepods, or trematodes) and no *R. roscovita* metacercariae were found in uninfected mussels.

# 3.1. Experiment 1: mussel filtration and respiration in response to parasite and temperature fluctuation

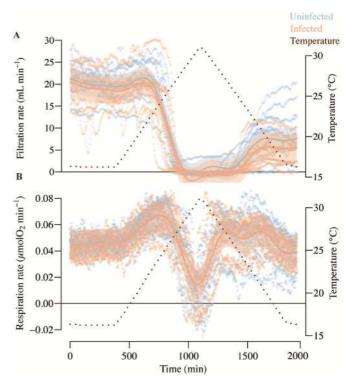
The deviance explained by the Generalized Additive Mixed Model (GAMM) was 86 % and 73 % for filtration and respiration rates, respectively. Considering the whole duration of Experiment 1, *R. roscovita* metacercariae infection did not significantly impact the filtration or respiration rate of the host *M. edulis* mussels, evaluated both in terms of the main and interactive effects (Fig. 1 A, B; Table 1). The distance between smoothers that modelled filtration or respiration time series of uninfected versus infected mussels was not significant during the pre-fluctuation phase when the temperature was constant and mild (16 °C), neither in the subsequent heating and cooling phases of the 24 h fluctuation, nor during the post-fluctuation phase (constant 16 °C) (see Fig. 1 A, B).

Both infected and uninfected mussels suppressed filtration to zero during the late-warming phase at > 25 °C, and filtration rate did not resume until lower temperatures were reached (Fig. 1A). The filtration

rate recovered to a maximum 50~% of its initial level, whereas the respiration recovered to almost 90~% of its initial rate.

When analysing the data of pre- and post-fluctuations phases sepa- rately using Linear Mixed Models (LMMs), we again found that the effect of infection on filtration or respiration was not significant (p > 0.05). Despite being non-significant, regarding the effect size non-standardised by variance, filtration rates of infected mussels were, on average 9 % lower than uninfected ones in the pre-fluctuation phase, and this effect size became 37 % (albeit still non-significant) in the post-fluctuation- phase (Fig. 1A; Table S4). In the pre-fluctuation phase of Experiment 1, the power analysis suggested that approximately 85 replicates per

1, the power analysis suggested that approximately 85 replicates per infection status were required to detect a statistically significant ( $p \le 0.05$ ) infection impact on filtration with approximately 80 % test power (Fig. S3). In the post-fluctuation phase, the required sample size was



**Fig. 1.** Mussel filtration and respiration responses during Experiment 1. Generalized Additive Mixed Models (GAMMs) of responses of small size mussels uninfected and infected with *Renicola roscovita* during exposure to a constant mild temperature (for 5 h) followed by a 24-h thermal fluctuation. Each point represents filtration or respiration measurement per 5 min (shaded areas represent 95 % CIs). Sample size for each group was 8 and 11 for infected and uninfected, respectively. The negative values recorded during the metabolic depression phase are due to extra random variation in the measurement, variability between individuals and the white noise of oximeter device.

estimated to be approximately 40 replicates per infection status (Fig. S3).

During the cooling period, mussels started to partially recover filtration capacity when the temperature was lowered to <20 °C after 1700 min (Fig. 1A). The capacity for post-heat recovery of filtration (or scaled filtration recovery) followed a dome shape pattern in relation to the infection intensity (Fig. 2A) while it was linear for the respiration rate (Fig. 2B). GAMMs explained 96.8 % and 87.3 % of filtration and respiration variance, respectively. Nevertheless, the effect of parasite intensity was not significant neither for filtration (edf = 1.666, F-value = 1.207, P = 0.389) nor for respiration (edf = 1.006, F-value = 0.06, P = 0.808) (Fig. 2).

# 3.2. Experiment 2 and 3: R. roscovita effects on filtration and respiration of mussels from two size classes

The LMMs explained 83 % and 97 % of the variance in filtration responses of small and large mussels, respectively. For the respiration response, the LMM explained 94 % of the variation in both small and large mussels. For large mussels (40 mm), the variation in filtration rate was significantly explained by infection status, as both the intercept (uninfected-infected) and fixed term (infected) were significant (P = 0.011; Table 2; Fig. 3 B). Infected large mussels filtered approximately 12 % less than uninfected mussels. For small mussels (20 mm), the infection effect on filtration was marginally insignificant (P = 0.055; Table 2; Fig. 3 A), with a non-standardised effect size of ca. 11 %. According to the power analysis, 25 replicates were needed to detect a statistically significant effect of infection on the filtration rate of small mussels. Moreover, the infection effect on respiration rate was 112

Table 1

Renicola roscovita metacercarial infection effects on mussel filtration and respiration rates over the whole experiment tested using Generalized Additive Mixed Models (GAMMs). The intercept (or the average) and the degree of nonlinearity (effective degrees of freedom, edf) of the reference level smoothers (Uninfected) are compared to zero and straight intercept lines, respectively. The treatment level smoothers (Infected) are compared to the reference level smoother. Parametric coefficients' estimates are intercept values or differences. Besides, the effects of random variance in time series intercept are tested as s(replicate). The

significant impact is considered for *p-value*  $\leq 0.05$ .

Filtration rate	C. parametric coefficients	Estimate	Std. Error	t-value	p-value
	Intercept (Uninfected)	10.5609	0.8256	12.7913	<0.0001
	Infected – Uninfected	-1.3624	1.2579	-1.0831	0.2788
	D. smooth terms	edl	Reldl	F-value	p-value
	s(Time).: Uninfected	9.9409	9.9993	195.2528	<0.0001
	s(Time): Infected	1.0281	1.0558	0.6242	0.4240
	s(replicate)	11.8251	17.000	2.2851	<0.0001
Respiration rate	A. parametric coefficients	Estimate	Std. Error	t-value	p-value
	Intercept (Uninfected)	0.0468	0.0031	15.3024	< 0.0001
	Infected – Uninfected	-0.0005	0.0047	-0.1143	0.9090
	B. smooth terms	edf	Ref.df	F-value	p-value
	s(Time).: Uninfected	11.7837	11.9918	50.3226	< 0.0001
	s(Time) : Intected	1.0181	1.0360	0.4693	0.4908
	s(Replicate)	15.2636	17.0000	8.7906	< 0.000

insignificant for both small and large mussels (P > 0.05; Table 2; Fig. 3 C and D).

A positive relationship between filtration rate and infection intensity was detected for both small and large mussels (Fig. 4). The intercept and the smooth effect of the number of parasites and time were significant for both the small and large mussels (P < 0.001). For small mussels: the smoother number of parasites was nearly a second-degree curve (edf = 1.9366, F-value = 102.5735), and so was the smoother of time (edf = 1.9686, F-value = 26.1321). For large mussels: the smoother number of parasites was slightly less curvilinear (edf = 1.6102, F-value = 36.1847), while the smoother of time was closer to a second-degree curve (edf = 1.9994, F-value = 65.6676). However, there was no significant correlation

between infection intensity and respiration rate for both small and

large mussels (P = 0.108, P = 0.57 for small and large mussels, respectively) (Fig. 2 and Table 3 in Supplementary).

#### 4. Discussion

This study analysed the effects of metacercarial infections of *Renicola roscovita*, a trematode common in the north-east Atlantic and Baltic Sea, on the filtration and respiration processes of Mytilus mussels and on their acute heat sensitivity. The infections consistently reduced filtration by a 9-11 % absolute effect size across three different experiments. Although nonsignificant, we found the effect size enlarging to 37 % at mussels' recovery from transient heat exposure. Additionally, size-related differences emerged as the larger mussels displayed a statistically significant drop-in filtration rate due to infection. However, these alterations did not affect the overall respiration rates. Interestingly, we observed a correlation between higher infection intensities and elevated filtration capacities in mussels. Our findings hint towards a subtle interplay between infection, physiological processes, and individual mussel characteristics. We further discuss these results, explore the study's limitations, and propose avenues for future research in this fascinating intersection of parasitology, physiology, and environmental stressors.

We initially expected R. roscovita infections to worsen the filtration and respiration responses of mussels to acute heat stress. The rationale behind our prior expectation was that: (i) metacercarial cysts (measuring ca. 150 µm in diameter) could have interfered with the beating of lateral cilia on gill filaments impacting their particle capture and water pump function (Werding 1969; Galaktionov and Dobrovokskij 2003); and (ii) the infection could have imposed an energic toll due to immunological reactions of mussels and haemolymph loss after cercarial injury. Notably, haemolymph loss was expected to be greater in the early stages of infection (first few hours) when hundreds of cercariae were penetrating mussel tissue with the stylet (i.e., a sword-like apparatus in the cercariae used for puncturing host tissue) (Werding 1969)

In general, if a major allocation of ATP to immunological and repair responses were occurring, infected mussels would have initiated their metabolic (feeding and respiration) depression responses at lower temperature limits.

Based on the findings in Experiment 1 (response to transient hours- long heat exposure), R. roscovita metacercarial main impact and its interactive effect with temperature (time) on mussel filtration or respi- ration were statistically not significant. Although not statistically sig- nificant, the enlarging trend of the absolute size of the infection effect on filtration was interesting: The difference in filtration between infected and uninfected mussels became more prominent during the post-heat (recovery) phase (from 9 to 37 %, with infected mussels having lower respiration rate than uninfected mussels). This can be explained by the 'metabolic depression' phase that Mytilus mussels enter to regulate heat- induced rises in ATP synthesis and consumption (McMahon et al., 1995; Sokolova et al., 2012b). This phase is typically followed by diminished

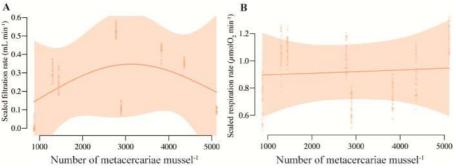


Fig. 2. Post-warming scaled mussel filtration (A) and respiration (B) in relation to infection intensity. Generalized Additive Mixed Models (GAMMs) predictions (lines) and 95 % CIs (shaded area) are conditioned on the average post-warming time points. Individual points represent filtration or respiration measured every 5 min and each stratum represents measurements of one mussel.

Table 2 Renicola. roscovita metacercarial infection effects on mussel filtration and respiration rates over Experiment 2 and 3 tested using linear mixed models. The intercept and the slope (over Time) of the reference level line (Uninfected) are compared to zero and the straight intercept line, respectively. The treatment level line (Infected) is compared to the reference level line. Parametric coefficients' estimates are intercept and slope values or the differences. Besides, the effects of random variance in time series intercept are tested as s(replicate). The significant impact is considered for p-value  $\leq 0.05$ .

		Small mussels				Large mussels			
Filtration rate	A. parametric coefficients	Estimate	Std. Error	t-value	p-value	Estimate	Std. Error	t-value	p-value
	Intercept (Uninfected)	24.183557	0.998530	24.219	< 0.0001	66.507585	2.339536	28.428	< 0.0001
	Intercept (Infected - Uninfected)	-2.707140	1.412135	-1.917	0.0554	-8.143140	3.215385	-2.533	< 0.0114
	Uninfected : Time	-0.012703	0.002792	-4.549	< 0.0001	0.137867	0.008742	15.770	< 0.0001
	Infected : Time	0.025056	0.003949	6.345	< 0.0001	0.016603	0.012015	1.382	0.1673
	B. smooth terms	edf	Ref.df	F-value	p-value	edf	Ref.df	F-value	p-value
	s(Replicate)	15.65	16	45.18	< 0.0001	31.9	32	324.2	< 0.0001
Respiration rate	A. parametric coefficients	Estimate	Std. Error	t-value	p-value	Estimate	Std. Error	t-value	p-value
	Intercept (Uninfected)	5.019e-02	2.551e-03	19.676	< 0.0001	1.848e-01	9.944e-03	18.581	< 0.0001
	Intercept (Infected - Uninfected)	-2.076e-03	3.607e-03	-0.576	0.565	-1.411e-02	1.367e-02	-1.032	0.302
	Uninfected: Time	-9.671e-06	6.927e-06	-1.396	0.163	5.168e-04	6.176e-05	8.368	< 0.0001
	Infected : Time	1.085e-05	9.796e-06	1.108	0.268	8.230e-05	8.488e-05	0.970	0.332
	B. smooth terms	edf	Ref.df	F-value	p-value	edf	Ref.df	F-value	p-value
	s(Replicate)	15.85	16	104.8	< 0.0001	31.61	32	81.45	< 0.0001

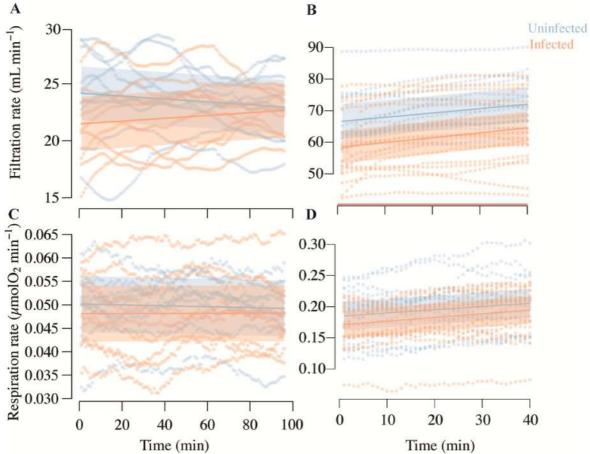


Fig. 3. Linear mixed models of filtration and respiration rates of small (A, C) and large (B, D) *M. edulis*, either uninfected (light blue) or infected (light pink) with *Renicola roscovita* under a constant temperature of 17 °C. The bottom red line in the subplot B indicates the interval of significant difference between smoothers. The shaded area represents 95 % CIs. Each point represents the filtration or respiration rate measured minutely. The sample size for each group of small or large mussels was 9–18 and 9–16 for infected and uninfected, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

feeding as a response to the oxygen debt induced by anaerobic metabolism (Po"rtner & Knust, 2007; Collins et al., 2020). Consequently, due to the energy expenditures related to immunological responses to infection, infected mussels might undergo a lengthier depression period and experience an intensified heat-induced oxygen (and energy) debt. This, in turn, could impair their post-heat recovery of energy-demanding

activities such as feeding.

In Experiments 2 and 3, we re-evaluated the harmful impact of R. roscovita metacercarial infection at a mild temperature in mussels of different sizes. Here, the infection impact on the filtration rate was statistically significant for larger mussels (P = 0.011) and marginally insignificant (P = 0.055) for small mussels. With regard to the

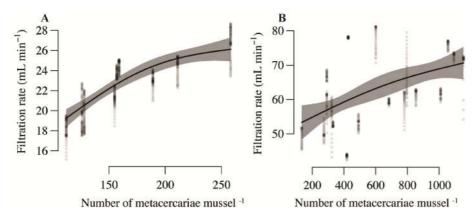


Fig. 4. Filtration rate in relation to infection intensity (metacercariae mussel<sup>-1</sup>) for small (A) and large (B) mussels. Generalized Additive Mixed Models (GAMMs) predictions (lines) and 95 % CIs (shaded area) are conditioned on the average time points. Each point represents filtration measurement per minute and each stratum shows temporal filtration of one mussel.

marginally insignificant p-value for small mussels, we would like to highlight that p-values often neglect such small-size effects (Sullivan and Feinn 2012), and one must pay attention to the consistency of absolute sizes of infection effects on mussel filtration rates across our three ex- periments (ca. 9, 11, and 12 %). This suggests that *R. roscovita* meta- cercarial infections can slightly decrease the ability of mussels to feed. However, because filtration rates vary widely between individuals, detecting these effects within a single experiment can be challenging. These absolute effect sizes are in accordance with previous experimental results indicating that the growth of mussels in intertidal and shallow subtidal mussel beds decreased by 5–14 % due to *R. roscovita* infection

 $(334 \pm 270 \text{ metacercariae mussel}^{-1})$  (Thieltges 2006). Our findings were also partially in accordance with Stier et al. (2015), who found a negative impact of *R. roscovita* on filtration rates of mussels (small: 19–22 and large: 46–49 mm shell length) with a stronger impact in large compared to small mussels (71 versus 42 % reduced filtration rate, respectively). In the study of Stier et al. (2015), the average infection intensity was much higher than in our experimental infections (1559 versus 166 metacercariae mussel<sup>-1</sup> and 3032 versus 584 metacercariae mussel<sup>-1</sup> for small and large mussels, respectively) but still within the extremes found in natural systems (i.e., up to 6000 metacercariae mussel<sup>-1</sup>) (Sva rdh and Thulin 1985; Zens 1999; Buck et al., 2005).

The effect of trematode infections on respiration rates was insignificant, with an absolute effect size of <5 % across all experiments. Disruption induced by metacercariae encystment, particularly on the gill and labial palp, may result in compromised filtration activity of gills, which might have no significant impacts on respiration since the organism takes up oxygen through diffusion (Jorgensen et al., 1986). Moreover, the feeding and digestion activities of M. edulis typically consume <20 % of the total mussel metabolic energy expenditure (Widdows and Hawkins 1989). Since, in our experiments, the absolute effect of parasites on filtration rate per se was small, its impact on respiration should have been minor. While the effect of infections on mussel respiration may be minor, one study suggests that this may not be a universal pattern for R. roscovita infections on bivalves. Magalha es et al. (2020) observed a significant ca. 40 % decrease in respiration rate of cockles infected by 10 versus 3 R. roscovita metacercariae. This surprising effect demand further studies resolving species-specific sensitivity of bivalve respiration to R. roscovita infections.

Finally, the results of Experiments 2 and 3 suggest that infection intensities in mussels can be higher in mussels with a higher filtration capacity, both among small and large size mussels. Such an evident correlation could not be found in Experiment 1, probably due to the higher availability of cercaria in the mussels' surrounding water during the labinfection procedure. This overdose of cercariae might have forced the chances of successful infection regardless of the mussels' innate filtration rate. A positive relationship between infection intensity

and filtration rate could result from two mechanisms. On the one hand, there is variation in filtration rates among mussel individuals (pheno-typic variation) (Steeves et al., 2020). Therefore, a mussel with a phenotype characterized by a higher filtration rate might be innately prone to higher metacercarial infection intensity due to a higher "inflow" of cercariae. Alternatively, higher metacercarial infection in-tensities could result in an upregulation of filtration rates to compensate for the additional energetic costs of infections for mussels. Given that we did not observe a positive correlation between respiration rates and infection intensity, the first mechanism that mussels with higher innate filtration rates are in danger of acquiring higher infection levels seems more plausible. Recent research supports our claim, suggesting that mussels with a lower filtration rate are more adept at avoiding trema- todes, resulting in a reduced infection intensity (Selbach et al., 2022). Although Mouritsen et al. (2022) showed that fear of parasite caused a reduction in filtration activity of M. edulis by 30 % to avoid Himasthla elongata infection, they also discovered a positive relationship between infection success and clearance rate, which is consistent with our find- ings. The existence of innate variation in mussel filtration rate is sug- gested as a possible explanation for the positive relationship between metacercariae intensity and filtration activity. This finding is also in accordance with (Nikolaev et al. (2006), who found that among M. edulis of the same age, higher infection intensities of the trematodes

H. elongata and Cercariae parvicaudata occurred in larger individuals. Those larger mussels were most likely mussels with higher filtration rate phenotypes which usually also have a higher growth rate (Prieto et al., 2018). Notably, the positive relationship of infection intensity to filtration capacity found in our study provides a possible explanation for Thieltges (2006) not detecting a negative relationship between long-term growth and infection intensity of R. roscovita infected mussels, i.e., mussels with higher infection intensity might be of the phenotype of faster filtration and growth.

#### 5. Conclusion

Overall, our results tentatively suggest that *R. roscovita* infections are benign in regard to mussel respiration, while they can slightly lower the ability of mussels to feed. Contrary to expectation, we did not observe significant interactions with short-term exposure to transient heat stress. Further series of experiments are required to investigate the effects of prolonged heat stress exposure. Also, measuring mussels' physiological performance before and after infection (e.g., using a before-after- control-impact design) must be considered in future studies using a setup that permits more replication. The power analysis results sug- gested that increasing the sample size from 8 to 40 mussels might have resulted in detecting a significant difference between infected and un- infected mussels' filtration recovery from heat exposure. Therefore, in

future experiments a larger sample size might be desirable. In addition, the role of mussel phenotypic variation in filtration capacity in driving infection levels and subsequent effects warrants further investigation to understand better the combined impacts of parasitism and heat stress on mussel performance and aquaculture in the context of ocean warming.

#### Authors' contributions

M.K., J.V., D.W.T. designed the study; M.K., J.V. ran the experiments, analysed data and wrote the first draft; D.M.D.M., C.B., and D.W.T. helped in writing and revising the manuscript. All co-authors discussed the results, reviewed and contributed to the final manuscript.

#### **Ethical statement**

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#### Declaration of competing interest

None.

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#### Appendix A. Supplementary data

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Supplementary Material for "Filtration and respiration responses of mussels (Mytilus edulis) to trematode parasite infections (Renicola roscovita) and transient heat exposure"

## **Content:**

Supplementary Tables
Supplementary Figures
Supplementary R Scripts

# **Supplementary Tables**

**Supplementary Table 1.** Average and standard deviation of the food concentration (*Rhodomonas salina* cells  $mL^{-1} \pm SD$ ) around the mussel during the experiments.

	Infected	Uninfected
Experiment 1	$3354.555 \pm 875.4777$	2936.058 ± 592.4666
Experiment 2 – Small	4989.427 ± 126.2731	$2306.182 \pm 273.8527$
Experiment 2 – Large	$6273.018 \pm 338.4091$	$5601.979 \pm 3050.871$

**Supplementary Table 2.** The difference in scaled filtration rate of infected mussels with *R. roscovita* metacercarial and uninfected mussels throughout the post-fluctuation phase (>1700 min) was investigated using Generalized Additive Mixed Models (GAMMs).

		Small mussels					
o	A. parametric coefficients	Estimate	Std. Error	t-value	p-value		
rate	Intercept (Uninfected)	0.3926	0.0802	4.895	< 0.0001		
	Infected – Uninfected	-0.1319	0.1236	-1.068	0.286		
filtration	B. smooth terms	edf	Ref.df	F-value	p-value		
	s(Time): Uninfected	1.890	1.988	7.422	0.001		
	s(Time): Infected	1.001	1.001	4.053	0.044		
Scaled	s(replicate)	16.918	17.000	206.540	< 0.0001		
Š	R <sup>2</sup>	0.95					

**Supplementary Table 3.** Generalized additive mixed models were used to model the relationship between respiration rate and parasite intensity in small and large mussels of Experiment 2 and 3.

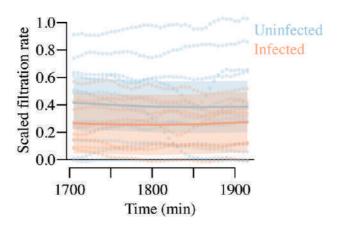
		Small mus	all mussels				Large mussels			
rate	A. parametric coefficients	Estimate	Std. Error	t-value	p-value	Estimate	Std. Error	t-value	p-value	
	Intercept	0.04820	0.00104	46.35	<2e-16	0.183161	0.003138	58.38	<2e-16	
Respiration	B. smooth terms	edf	Ref.df	F-value	p-value	edf	Ref.df	F-value	p-value	
)ira	S (Number of parasites)	1.002	1.003	2.594	0.10	1.548	1.795	0.514	0.57	
Se	s(total-min)	1.002	1.004	0.346	0.55	1.002	1.004	42.931	<2e-16	
<u> </u>	R <sup>2</sup>	0.445 0.0423								

**Supplementary Table 4.** Results of Liner Mixed Models used for three separate power analyses. LMMs were defined using data of pre- and post-fluctuation phases of Experiment 1 (times  $\leq$ 355 and  $\geq$ 1700 min) and small-size mussel data of Experiment 2.

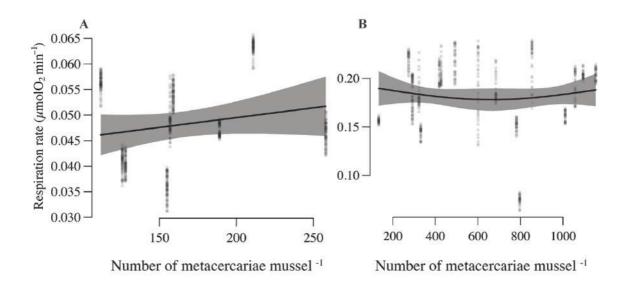
Fixed effects	Pre-fluctuation			Post-fluctuation		
	Estimate	Std. Error	t-value	Estimate	Std. Error	t-value
Intercept (Uninfected)	20.802	1.216	17.097	10.575	1.893	5.587
Intercept (Infected – Uninfected)	-1.829	1.875	-0.975	-4.019	2.917	-1.378
Uninfected: Time	-0.004	0.0007	-5.713	-0.001	0.0006	-2.362
Infected: Time	0.004	0.001	3.585	0.001	0.001	0.763

Fixed effects	Experiment 2 Small mussels					
	Estimate	Std. Error	t-value			
Intercept (Uninfected)	24.345	0.966	25.199			
Intercept (Infected – Uninfected)	-2.465	1.366	-1.804			
Uninfected: Time	-0.016	0.001	-11.518			
Infected: Time	0.027	0.002	13.417			

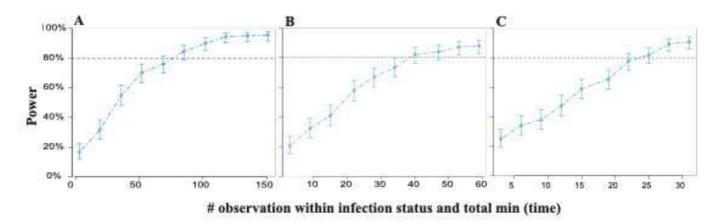
# **Supplementary Figures**



**Supplementary Figure 1.** Generalized Additive Mixed Models were used to model scaled filtration rate of mussels during post-warming phase. GAMMs explained 95% of variation. Each point represents filtration or respiration measurement per 5 minutes (shaded areas represent 95% CIs).



**Supplementary Figure 2.** Generalized additive mixed model were used to model the relationship between respiration rate and parasite intensity (number of metacercariae) in small (A) and large (B) mussels. Line represent model prediction and shaded band represents 95 % CIs conditioned on the average time points.



Supplementary Figure 3. The power analysis graphs demonstrate how changes in sample size could have affected the statistical test power to detect a significant effect of infection-status on filtration rates. In Experiment 1, we needed 85 replicates for the pre-fluctuation phase (A), 40 replicates for the post-fluctuation phase (B), and 25 replicates in Experiment 2 (on small mussels) to detect statistically significant (p < 0.05) impacts of the infection on filtration rates, considering the test power of 80% and the significance level of 0.05.

# **Supplementary R Scripts**

## **Experiment 1**

## Filtration rate:

```
# for autocorrelation consideration
df <- start event (df, column="total min", event=c("replicate"), order = TRUE,
label.event="Event")
# order factor
df\$infection status <- as.ordered(df\$infec status)
contrasts(df$infection status) <- "contr.treatment"
contrasts(df\$infection status)
#Model
simp bam RIS = bam (filt ml per min S \sim infection status + s (total min, k=11) +
s (total min, by=infection status, k=11) + s (replicate, bs='re'), family=gaussian, data=df,
method = "REML")
gam.check(simp bam RIS, rep=100)
#Consider autocorrelation of residuals
r1 <- start value rho (simp bam RIS, plot=T)
simp bam RIS AC <- bam (filt ml per min S ~ infection status + s (total min, k=11) +s
(total min, by=infection status, k=11) + s (replicate, bs='re'), family=gaussian, data=df,
method = "REML",rho=r1, AR.start=df$start.event)
summary(simp bam RIS AC)
gam.check(simp bam RIS AC, rep=100)
check resid (simp bam RIS AC, split pred="AR.start", ask=FALSE)
diagnostics (simp bam RIS AC, ask=FALSE)
```

# For respiration rate:

```
simp bam RIS resp = bam (resp ymolO2 per min S \sim infection status + s (total min, k=13)
+ s (total min, by=infection status, k=13) + s(replicate, bs='re'), family=gaussian, data=df,
method = "REML")
gam.check(simp bam RIS resp, rep=100)
# consider autocorrelation of residuals
r1 <- start value rho(simp bam RIS resp, plot=T)
simp bam RIS AC resp <- bam (resp ymolO2 per min S ~ infection status + s(total min,
k=13) +s(total min, by=infection status, k=13) + s(replicate, bs='re'), family=gaussian,
data=df, method = "REML",rho=r1, AR.start=df$start.event)
summary(simp bam RIS AC resp)
gam.check(simp bam RIS AC resp, rep=100)
check resid(simp bam RIS AC resp, split pred="AR.start", ask=FALSE)
diagnostics (simp bam RIS AC resp, ask=FALSE)
Recovery potential:
# Making reference scaled filt & resp
df total = data.frame()
for (i in unique(df$replicate)){
 print(i)
 df rep=df[(df$replicate==i), ]
 df_ref=df[(df$total_min<300), ]
 ref filt=mean(df ref$filt ml per min S)
 ref_resp=mean(df_ref$resp_ymolO2_per_min_S)
 df rep$filt ref scaled=df rep$filt ml per min S/ref filt
 df rep$resp ref scaled=df rep$resp ymolO2 per min S/ref resp
 df total <- rbind(df total,df rep)
}
```

```
head(df total)
df=df_total
#revising variable types
df$replicate = as.factor(df$replicate)
df\$infec status <- as.factor(df\$infec status)
df$temp = as.numeric(df$Temp C x)
df$total min = as.numeric(df$total min)
df$resp ymolO2 per min S = as.numeric(df$resp ymolO2 per min S)
df$filt ml per min S = as.numeric(df$filt ml per min S)
df\filt ref scaled = as.numeric(df\filt ref scaled)
df$resp ref scaled = as.numeric(df$resp ref scaled)
df paras = subset(df, df\$infec status == 1)
df paras <- as.data.frame(df paras)</pre>
df paras rec = subset(df paras, df paras$total min > 1700)
df paras rec <- as.data.frame(df paras rec)
df paras rec$replicate = as.character(df paras rec$replicate)
df paras rec$replicate = as.factor(df paras rec$replicate)
df paras rec <- start event(df paras rec, column="total min", event=c("replicate"),
order = TRUE, label.event="Event")
unique(df paras rec$replicate)
simp bam relative filt = bam(filt ref scaled \sim s(total min, k=3 + s(num par, k=3) +
s(replicate, bs='re'), family=gaussian, data=df paras rec, method = "REML")
r1 <- start_value_rho(simp_bam_relative_filt, plot=T)
simp bam relative filt AC<- bam(filt ref scaled ~ s(total min, k=3) +s(num par, k=3) +
s(replicate, bs='re'), family=gaussian, data=df paras rec, method = "REML", rho=r1,
AR.start=df paras rec$start.event)
gam.check(simp bam relative filt AC, rep=100)
```

```
summary(simp_bam_relative_filt_AC)
```

check resid(simp bam relative filt AC, split pred="AR.start", ask=FALSE)

### **Experiment 2**

## Filtration rate small and large:

```
simp_bam_RI_Filt = bam (filt_ml_per_min_S ~ infection_status*total_min + s (replicate, bs='re'), family = gaussian(), data=df, method = "REML")
```

# consider autocorrelation of residuals

r1 <- start\_value\_rho(simp\_bam\_RI\_k3\_Filt, plot=T)

 $simp\_bam\_RIS\_AC\_Filt <- bam (filt\_ml\_per\_min\_S \sim infection\_status*total\_min + s (replicate, bs='re'), family=gaussian(), data=df, method = "REML",rho=r1, AR.start=df$start.event)$ 

# Respiration rate small and large

```
simp_bam_RI_Resp = bam (resp_ymolO2_per_min_S ~ infection_status*total_min +s(replicate, bs='re'), family=gaussian(), data=df, method = "REML")
```

#consider autocorrelation of residuals

r1 <- start\_value\_rho(simp\_bam\_RI\_k3\_Resp, plot=T)

 $simp\_bam\_RIS\_AC\_Resp <- bam(resp\_ymolO2\_per\_min\_S \sim infection\_status *total\_min + s(replicate, bs='re'), family=gaussian(), data=df, method = "REML",rho=r1, AR.start=df$start.event)$ 

## # Filtration versus Number of parasite small /large

```
df paras = subset (df, df\$infec status == 1)
```

df paras <- as.data.frame(df paras)</pre>

 $simp\_bam\_RI = bam (filt\_ml\_per\_min\_S \sim s (num\_par, k= 3) + s (total\_min, k= 3), \\ family=gaussian(), data=df\_paras, method = "REML")$ 

r1 <- start value rho (simp bam RI, plot=T)

simp\_bam\_RIS\_AC <- bam (filt\_ml\_per\_min\_S~ s (num\_par, k=3) + s (total\_min, k=3), family=gaussian(), data=df\_paras, method = "REML", rho=r1, AR.start=df\_paras\$start.event) summary(simp\_bam\_RIS\_AC)

## # Respiration versus Number of parasite small / large

```
df_paras = subset (df, df$infec_status == 1)

df_paras <- as.data.frame(df_paras)

simp_bam_RI = bam(resp_ymolO2_per_min_S ~ s(num_par, k= 3) + s(total_min, k= 3),

family=gaussian(), data=df_paras, method = "REML")

r1 <- start_value_rho(simp_bam_RI, plot=T)

simp_bam_RIS_AC <- bam (resp_ymolO2_per_min_S ~ s (num_par, k=3) + s (total_min, k=3), family=gaussian(), data=df_paras, method = "REML", rho=r1,

AR.start=df_paras$start.event)

summary(simp_bam_RIS_AC)
```

## # Curve difference plots for large mussel

plot\_diff(simp\_bam\_RIS\_AC\_k3\_Filt, view="total\_min", comp=list(infection\_status=c("0", "1")),rm.ranef=T, xlab = ", ylab = "", main = "Difference Non-infected/ Infected",alpha=0.1, sim.ci=T, set.seed(100), hide.label = T)

# **General Discussion**

This thesis provides a fundamental overview of the taxonomy and distribution of trematodes from a common and abundant snail (i.e., *Pirenella cingulata*) from the Northern Persian Gulf (Chapter 1 and 2), the world's hottest sea. Acquiring knowledge about the temperature sensitivity of cercarial emergence of the trematodes with the highest infection prevalence in the first host was a second step in understanding the future performance of trematode species in a warming sea (Chapter 3). While all parasites can impact their hosts' performance, the severity of the impact and potential interactions with environmental stressors are still up for debate for many species. Therefore, the fourth chapter (Chapter 4) of this thesis was focused on investigating the effects of metacercarial infections on the metabolic rate of a common second intermediate host (i.e., the blue mussel *Mytilus edulis*) inhabiting the Baltic Sea—another marginal sea for which information regarding the diversity and distribution of host-parasite systems as well as past or future environmental regimes are better understood.

The main findings of these chapters were as followed:

- Chapter 1: A remarkably high trematode diversity, comprising 29 species across 10 families, was identified in the horn snail *Pirenella cingulata* from the Persian and Oman Gulfs off the coast of Iran.
- Chapter 2: First report of a potamidid snail (*P. cingulata*) serving as an intermediate host for two schistosome species, *Ornithobilharzia canaliculata* (Rudolphi, 1819) Odhner, 1912 and a putative new species of *Austrobilharzia* Johnston, 1917. Despite predominantly parasitizing birds, avian schistosomes are medically significant because they can cause cercarial dermatitis, also known as "swimmer's itch," in humans, demonstrating their importance to public health.
- Chapter 3: The thermal dependence assessment of cercarial emergence revealed optimal temperatures of 32 and 34 °C for *Acanthotrema tridactyla* and Cyathocotylidae gen. sp., respectively, which were the warmest ever-recorded thermal optima of aquatic cercarial emergence. Besides, we found that the time and light regime (circadian cycles) may potentially play a role in determining the temperature sensitivity of cercarial emergence, depending on the trematode species.
- Chapter 4: *Renicola roscovita* trematode infections were benign to the respiration rate of the second host, the blue mussel *Mytilus edulis*, while the infection slightly lowered the ability of the host to feed. Contrary to our expectation, we did not observe significant

interactions of infection with short-term exposure to transient heat stress. Besides, we found that mussels of higher baseline filtration rates could be infected by higher numbers of encysted metacercariae (infection intensity).

In the following, we will discuss and relate these findings in a broader context and provide a perspective to future research endeavors.

# Trematode parasites in the warmest sea the Persian Gulf: their biodiversity and relevance to humans

Research indicates that parasites account for between one-third and one-half of total species diversity (Goater et al., 2014), including an estimated 100,000 to 350,000 endo-helminth species, of which 85 to 95 percent remain undiscovered (Carlson et al., 2020). In the meantime, accelerated biodiversity loss caused by human activities makes it difficult to comprehend the extent of this loss, which varies across regions (Díaz et al., 2019; Bradshaw et al., 2021). We are in the middest of a 6th mass extinction, species—particularly hidden parasites—may vanish before they're even identified (Barnosky et al., 2011). It is anticipated that by 2070, 30% of helminths will be lost due to host coextinction (Carlson et al., 2017). In addition, climate change is anticipated to have a significant impact on biodiversity in polar and tropical regions (Hoberg and Brooks, 2015). This thesis seeks to assess the diversity of trematodes in the Persian Gulf, a subtropical region with high temperatures, heavy human impact, and limited research.

The latitudinal gradient in species diversity is one of the most fundamental distributional patterns in ecology, depicting a decrease in species richness from the equator to the poles across animal and plant groups in both terrestrial and aquatic ecosystems (Fine, 2015). However less is known about parasite richness and abundance over larger geographical scales (Poulin, 2014). Following the "diversity begets diversity" concept, the diversity of host species is a fundamental driver of parasite richness (Hechinger and Lafferty, 2005; Kamiya et al., 2014a; Johnson et al., 2016). In tropics there is more biodiversity of free-living species than temperate or polar regions, therefore, it is also expected to have higher diversity of parasites. According to a meta-analysis by Guernier et al. (2004), this holds true for human pathogens (i.e., bacteria, viruses, fungi, protozoa and helminths). This is not the case for parasites of other taxa (non-human) where no association between latitudinal gradients and diversity has been found, but rather a reverse latitudinal diversity gradient (Harris and Dunn, 2010; Kamiya et al., 2014b; Torchin et al., 2015; Dallas et al., 2018; Preisser, 2019). For instance, trematode infection in snails, helminth parasites of fishes, fleas on rodents and parasites of carnivores showed latitudinal diversity rise from tropical to temperate regions (e.g., (Choudhury and Dick, 2000; Lindenfors

et al., 2007; Torchin et al., 2015). Following the abovementioned pattern, the Persian and Oman Gulfs are located in subtropical area, where we expect to have low diversity of non-human parasites. Moreover, they are considered to have an impoverished Indo-Pacific fauna and flora due to their harsh environment and young age in the case of Persian Gulf (Sheppard et al., 2010). In order to gain an overview about trematode diversity in a particular area, it is ideal to investigate snails because they serve as a first intermediate host for several trematodes' species and the infection rate is a valid bioindicator of the distribution and population dynamics of the final host (Hechinger et al., 2007; Stout et al., 2022). This is particularly useful for surveying the diversity of trematodes which utilize birds and marine mammals as it is difficult to obtain collecting permits almost all around the world, making it practically challenging to survey parasites in such organisms. In our investigation, trematode diversity in *Pirenella cingulata*, we found a high diversity including of trematode, identifying 29 distinct species. The trematode species richness in this region exceeds that of many marine environments (Cable, 1963; Sousa, 1983; Kuris, 1990; Abdul-Salam, 1998), while the species composition is two times higher than trematode communities infecting the Cerithidae snail, Clypeomorus batillariaeformis, in biodiversity-rich locations such as the Great barrier reef (Huston et al., 2018). The high diversity of trematode infection found in *P. cingulata* in this ecoregion (Persian and Oman Gulf) rise two questions, Is the Persian Gulf a hot spot for trematode infection? Or is P. cingulata the key host for trematode transmission in this region? As we know the Persian Gulf is the stop over and wintering ground of many migratory shorebirds (Delany et al., 2009; Bom and Al-Nasrallah, 2015; de Fouw et al., 2017) and the final hosts of more than half of the trematode species observed in this study correspond to birds. Thus, the high diversity of trematode infection might be the result of infections brought to the area by migratory birds (Chapter 1 and 2). These findings correlate well with previous studies in other areas suggesting the strong association between migratory birds and occurrence of parasites (Smith, 2001; Hechinger and Lafferty, 2005; Fredensborg et al., 2006; Hechinger et al., 2007). To answer these questions comprehensively, additional research is required to investigate the trematode diversity not only in P. cingulata but also in other snail species (e.g., Cerithium scabridum, Cerithium caeruleum, Clypeomorus bifasciata (Amini-Yekta et al., 2019)) and during both cold and warm seasons. However, monthly sampling from more localities would be ideal to have a holistic picture on trematode infection dynamics and diversity. Although P. cingulata plays a substantial role in the trematode life cycles from this region, we cannot infer with certainty that it is the key host in trematode transmission overall since we do not know the trematode diversity in other snail species. However, trematodes have co-evolved with tolerant hosts to a wide variety of environmental factors to ensure effective asexual reproduction during their lifecycle (Poulin, 2007). Thus, *P. cingulata*, a tolerant snail, matches the criteria to consider it as potential key species in trematode life-cycles.

We used molecular methods to evaluate trematode diversity in the primary host because traditional morphological identification is hampered by cryptic species diversity, scarce morphological features, and phenotypic variations (Cribb et al., 2022). Due to a lack of resemblance, trematode taxonomy, which is traditionally based on adult morphology, struggles to link larval and adult stages (Blasco-Costa et al., 2016). Molecular techniques have improved species identification, revealing new trematode diversity and providing crucial insights into their complex life cycles, evolutionary processes, and ecological interactions (Criscione et al., 2005; de Leon and Nadler, 2010). Particularly in understudied regions, such as the Persian Gulf, where morphological identification markers are lacking, molecular methods proved indispensable, allowing us to identify 29 species by matching genomic data with GenBank sequences.

From all of these species with important roles and ecological implications, we also found human-related trematodes. Schistosomes, causing severe diseases like schistosomiasis and nonlethal allergic reactions such as cercarial dermatitis, hold significance for both ecology and human welfare (Colley et al., 2014; Horák et al., 2015). Their evolutionary ability to use diverse gastropods as first hosts enables them to thrive in both freshwater and marine settings. Avian schistosomes exemplify this versatility, known for their adaptability to various hosts and environments (Horák et al., 2015; Lashaki et al., 2020). Our molecular analysis revealed the existence of two bird schistosome species: Ornithobilharzia canaliculata (Rudolphi, 1819) Odhner, 1912 and a putative species of Austrobilharzia Johnston, 1917. This study reported for the first-time O. canaliculata from the Persian Gulf and globally the first record of this genus in a potamidid snail (P. cingulata) (Chapter 1). In Chapter 2 of this thesis, we zoomed deep into the evolutionary relationships and host-parasite associations among avian schistosomes. Our study highlights the importance of molecular systematics in assessing the disease risk based on occurrence, distribution and biology of bird schistosomes. Even though we discovered bird schistosomes in only two locations with a low prevalence (Genaveh: n = 2, prevalence = 1%; Jask: n = 1, prevalence = 0.4%), this is typical for bird schistosomes and relatively common in regions where swimmer's itch affects humans (Zbikowska, 2004). Despite these low prevalence, other investigations have demonstrated that human infections are possible considering the large number of cercariae (up to 30,000 cercariae per snail per day) that can be released in a short period of time from each infected snail host (Soldánová et al., 2016). The previous study also indicated the importance of migratory and non-resident bird contribution in the sustainability of avian schistosomes (Rudko et al., 2022).

Overall, molecular systematic investigations demonstrate a remarkable richness of trematodes in the Persian and Oman Gulfs. Future isolates from potential second intermediate or definitive hosts would be desirable and could be matched using the published molecular data to deduce the complete life cycles and fully define these species. Therefore, the first two chapters of this thesis set the stage for future ecological investigations of trematode diversity and distribution in the Persian and Oman Gulfs.

## Factors affecting trematode transmission and biodiversity

The variation in trematode prevalence found in the intertidal zones of the study system (Northern Persian and Oman Gulf) is attributed to the interplay of a number of biotic and abiotic variables. For instance, besides the abundance of the final host which plays a critical role, the distribution (uniform, random, aggregated), density and composition of intermediate hosts, as well as the particular features of the sensing and searching behavior of parasite larvae are equally significant (Prinz et al., 2011; Wilson et al., 2013). The highest number of harbored trematode species (13 species) in our study system belongs to the locality "Genaveh" which was accessible for terrestrial mammals (close to this beach it was dumping place which attracts cats, dogs and rodents) of these 13 species, three infects cats, dogs, and rodents.

Among abiotic factors: the exposure of the coastal zone to wave action has significant effects on successful transmission. High wave activity impedes host search and infection by free-swimming larvae and accelerates the clearance of parasite eggs and or cercariae, which are washed to deeper locations or inland (Galaktionov, 1993). In our study system, for example the locality "Shif" was beside the fish factory and was the most protected one from wave action thus it was not surprising to have the highest trematode prevalence 69% and the most cases of double infection. Our results are in agreement with previous studies stating that the prevalence of infected snails was high close to fish ports, fish factories and salmon farms where the gulls which serve as the final host are more abundant (Bustnes and Galaktionov, 1999; Bustnes et al., 2000). The same pattern was observed from analysis of databases on infection of mussels by trematode larvae in the North Atlantic and North pacific (Wilson et al., 2013). Another example is the gradual decrease of *Himastla elongata* parthenitae in *Littorina saxatilis* from the apex to the innermost part of a Yarnyshnaya bay (The Barrent Sea), with a prevalence ranging from 0% at the mouth of the bay, adjacent to open coast, to 26% at the apex (Galaktionov et al., 1997).

Global warming and related temperature fluctuations is one of the main factors expected to affect trematode diversity and distribution. Warming is expected to affect trematode transmission in many ways including the trigger of cercarial emergence under warming conditions or heat events. Given the ecological importance of the trematodes found in the Persian Gulf, and the striking warming events recorded in this region, we decided to take one step further beyond trematode diversity characterization and evaluate the thermal sensitivity of cercarial emergence for two model organisms (*Acanthotrema tridactyla* and Cyathocotylidae gen. sp.) using *P. cingulata* as first intermediate host (Chapter 3).

Cercarial emergence is a critical step in the transmission and continuation of trematode complex life cycles and, thus, disease dynamics (Galaktionov and Dobrovokskij, 2003). Temperature is known to have a pivotal effect on cercarial emergence; with rising temperature, cercarial emergence increases up to an optimal temperature, above which a decline in cercarial emergence is commonly observed (Poulin, 2006; Poulin and Mouritsen, 2006; Morley and Lewis, 2013). The optimal temperature of cercarial emergence varies depending on the trematode species, the snail host, their microhabitat, and the latitudinal location (Morley and Lewis, 2013; Galaktionov, 2016). A previous review done by Morley and Lewis (2013) reported ≈ 25 °C as the optimal temperature for cercarial emergence of low latitude regions. Nevertheless, in the low-latitude habitat examined herein, the optimal temperature for cercarial emergence of both species was shown to be higher (*A. tridactyla*: 32.0 °C and Cyathocotylidae gen. sp.: 33.5 °C) (Chapter 3). Given that the temperature in the intertidal zone of Persian Gulf can range from 9 to 55 °C (and specifically 30 to 55 °C in summer) (Bordbar et al. unpublished data), the high optima for cercarial emergence in this region are not astonishing.

In this extremely warm environment, trematodes and their free-living transmission stages (i.e., miracidia, cercariae) must develop some adaptations to survive and increase the chance of successful transmission to second intermediate hosts. (i) Some trematode species express circadian rhythms for cercarial emergence to maximize transmission success. Their emergence corresponds to rendezvous the second host "host-time" [time that target host is available and probably most active] while avoiding contact with the regular feeding cycle of predators. In schistosomes, for instance, the circadian rhythm is the dominant driver of cercarial emergence (Valle et al., 1973; Nojima and Sato, 1982). The maximum output of Schistosoma curassoni and S. bovis cercariae is during early morning, when the definitive hosts of these parasites, ruminants, come to drink water (Pages and Théron, 1990b; Mouchet et al., 1992). Those of S. haematobium, S. intercalatum, and S. mansoni cercariae have a diurnal rhythm, when humans are actively using freshwater resources (Théron, 1984; Pages and Théron, 1990a), and those of

S. rodhaini cercariae, are active during the evening dusk, when murine rodents, leave their shelters (Théron, 1989). In the case of Proterotrema edneyi, the daily emergence pattern coincides with the feeding behavior of certain fish species, thus enhancing transmission success since fish become infected by consuming cercariae as 'prey' (Lewis et al., 1989). All of these mechanisms and behaviors of cercariae depend on the next host's behavior (e.g., fast- or slowmoving, benthic, or living near the water surface or a shore) and presence (Haas, 1994; Morley, 2020). In our case, A. tridactyla had nocturnal emergence which matches the resting time of its second intermediate host, the fish Aphanius dispar (Plaut, 2000) when they could invade the fish's skin more efficiently (Martin and Kuntz, 1955). Although this differs from those trematodes synchronized to the host active time, resting time might provide a convenient situation for the parasite by providing a point-static target for the cercariae to penetrate its skin. (ii) The presence of morphological characteristics such as eye-spots enables cercariae to sense light beams and swim toward or away from the light to increase the likelihood of infection. In our study, A. tridactyla has eye-spots and presented nocturnal emergence. Since the secondintermediate host, A. dispar, is close to the bottom of the sea while resting during the night, the cercariae could benefit from light-sensitive eyespots to swim against the moon light towards the bottom where fish conglomerate. Nevertheless, this hypothesis requires further testing. Although cercarial emergence represents an essential step in trematode transmission, in order to construct prediction frameworks on the fate of trematode transmission in this area, future research must incorporate all transmission components, providing information on additional traits such as survival and infection success. According to several meta-analyses temperature can have effects on other cercarial performance traits (e.g., cercarial survival and infection) and other larval stages (miracidia performance) (Morley, 2011; Morley and Lewis, 2013, 2015). In the case of cercarial survival, the larvae are restricted by their limited energy storage (glycogen). Temperature can directly increase their metabolism and thus their glycogen utilization rate resulting in shorter lifespans (Morley, 2011). Warm adapted cercariae can increase their survival time through changes in physiology that lead them to rest more frequently and therefore save their limited energy stores (Morley, 2011). Moreover, cercariae thermal performance can be also affected by the thermal-history of their host. According to (Berkhout et al., 2014), higher proportion of cercariae remain active after 8 hours at 25 °C when emerging from a 20 °Cacclimated hosts than from a 15 °C-acclimated hosts.

# How could trematode diversity change under ongoing warming in extreme environments like the Persian Gulf?

The understanding of trematode diversity and thermal tolerance in the Persian Gulf is in its infancy. We are still lacking information on temperature effects on trematode and host performance while other snail hosts and their parasites remain largely unexplored. However, based on the results from the thermal limits of cercarial emergence derived in this thesis, trematodes in the Persian Gulf have optimum temperatures representing summer thermal averages at present (32.0-33.5 °C), while beyond this temperature cercarial emergence declines. Although cercarial emergence is not the only important parameter in trematode performance, this information suggests that a warming Persian Gulf reaching temperatures in the intertidal regions that surpass 50 °C, will result in an overall decline in trematode transmission rates. Therefore, with ongoing warming, trematode abundance would probably decrease particularly in summer time when temperatures exceed the thermal optimum for emergence. Like other organisms, parasites have certain limits of thermal performance. Under warming, hosts capable of adapting to new thermal conditions may escape parasitic infections through "thermal refugia" which is intolerable for parasites. Nevertheless, since we do not have enough information about the parasite's transmission window in the Persian Gulf, it is difficult to estimate whether there will be a shift in seasonality (in the case that seasonality applies to the species studied herein) or an overall decrease in trematode proliferation. Moreover, based on the patterns observed on free-living organisms (Meester et al., 2018), it would be important to understand the capacity of adaptation, acclimation or dispersal of these parasites to a changing world since this might dictate whether we would expect local gene adaptation, a shift in seasonality, or a shift in the range of these parasites. In the event that parasites from the Persian Gulf have a high capacity for dispersal and of establishing novel host-parasite interactions, these species could flourish in the future in adjacent or even more distant regions experiencing global warming. Finally, it is expected that warming might favor more generalist parasites which can infect a wide range of organisms versus specific parasites that are restricted to a particular genus or species (Cizauskas et al., 2017). Warming might as well favor parasites with shorter life cycles needing one or two hosts to complete the life cycle such as avian schistosomes (Poulin and Cribb, 2002). As a result, we may hypothesize that warming will reduce parasite diversity, while an increase in the population of tolerant species will make them dominate parasite community structures in the region (Cizauskas et al., 2017).

#### Perspectives for future research in the Persian Gulf

This thesis provides the baseline information on trematode diversity in the world's warmest Sea, the Persian Gulf. Moreover, this thesis highlights the importance of investigating the impact of warming on host, parasites and host-parasite systems in marginal seas, which are considered as natural laboratories and the "time machine" for climate change research (Riegl and Purkis, 2012a; Bento et al., 2016; Bargahi et al., 2020). However, a myriad of questions and hypotheses still need to be answered and tested. More specifically, present knowledge on the future of trematodes in a warming sea might benefit from the following research directions and aims: (1) comprehensive field-sampling surveys coupled with molecular identification of other snail species and potential second and definitive hosts must be executed in order to illustrate trematodes life cycles; (2) morphological identification keys for all trematode stages in the Persian and Oman Gulf might be useful for researchers interested in this particular study system; (3) periodical monitoring programs (monthly or bimonthly) of trematode diversity, prevalence and intensity would be beneficial to estimate potential seasonal shifts. In addition to this, it would be interesting to assess the differences in the diversity, prevalence and intensity of trematodes in the extreme environment of the Persian Gulf with more localities in the Oman Gulf which is at the same latitude but with more benign environmental conditions. Finally, in order to provide a holistic picture on trematode performance, the effect of temperature on cercarial survival and infection success must be assessed as well as the influence of trematode infection on host fitness and biochemical condition (e.g., energy reserves and stress responses such as heat shock proteins).

# Switching to the Baltic Sea: How metacercarial infection impact host feeding and temperature sensitivity?

While this thesis focused mostly on trematodes from the Persian Gulf, it is important to take into consideration other systems (in other latitudes) when attempting to understand trematode performance in a globally warming ocean. The Baltic Sea represents the perfect opportunity for understanding warming effects on trematode performance and, contrary to the Persian Gulf, parasite and host diversity has been thoroughly described (Werding, 1969; Zander, 1998; Køie, 1999). In addition to this, there are long-term datasets available for several abiotic variables including temperature. Therefore, the groundwork regarding parasite diversity has been already performed, allowing us to focus on temperature effects on parasites and their hosts.

The thermal sensitivity of *R. roscovita* and *H. elongata* as well as the combined effect of infection and temperature on their hosts has been previously assessed. Regarding the parasite,

the cercariae of these trematodes are well described in terms of the thermal performance. Optimal temperature for cercarial emergence was 20 and 23 °C for R. roscovita and H. elongata, respectively (Thieltges and Rick, 2006; Díaz-Morales et al., 2022). Cercariae half-life time (LD<sub>50</sub>) significantly decreased with increasing temperature and lasted ca. 20 and 38 h at the optimal temperature for cercarial emergence for R. roscovita and H. elongata, respectively, and cercarial infectivity was found to be at its optimum at 25 and 22 °C (Thieltges and Rick, 2006; Díaz-Morales et al., 2022). In terms of the first intermediate host, survival of infected L. littorea with *H. elongata* was significantly decreased at 22 °C, while non-infected gastropods started to die at 28 °C (Díaz-Morales et al., 2022). In the case of second intermediate host, the effect of parasite and temperature combined was found to exert greater biochemical alterations in infected Cerastoderma edule versus non-infected counterparts (Magalhães et al., 2018, 2020). For instance, the lipid peroxidation (LPO) assay, which evaluates the activity and the level of cellular damage, showed significantly higher levels in infected cockles at higher temperature (22 °C). Or in the case of catalase (CAT), a marker of stress, temperature treatment influenced CAT activity in infected cockles, with considerably lower CAT levels when cockles were subjected to the higher temperature (22 °C) than when they were exposed to the control temperature (17 °C). Within a 22 °C treatment, infection state had a substantial influence on CAT activity, with non-infected cockles exhibiting much greater CAT than infected cockles (Magalhães et al., 2018). However, for M. edulis, only information on the effect of H. elongata infection and temperature combined on the survival has been performed (Selbach et al., 2020). Selbach et al., (2020) found that infection decreased the survival of the host at 15 °C, while at 35 °C higher infection intensity nullified this impact. However, it is difficult to mechanistically explain these impacts on survival. Investigating the effect on metabolic traits (e.g., respiration and feeding) might help to understand what can happen physiologically with this bivalve when exposed to trematode and heat stress combined.

Therefore, we decided to measure metabolic rates of *M. edulis* infected with *R. roscovita*. They mainly encyst on gills, palps and in visceral mass (Lauckner, 1983) of *M. edulis* and could have deleterious impact on host's feeding rate (Stier et al., 2015) and growth (Thieltges, 2006). Therefore, in Chapter 4 of this thesis we investigated the filtration and respiration rates of *M. edulis* in response to *R. roscovita* metacercarial infection and acute heat ramp which often happens during heatwave events. Results from our initial experiment demonstrated a significant impact of temperature, notably diminishing both the filtration and respiration rates of the mussels. The effect of infection, however, was minimal on both responses. Although these effects were statistically non-significant, it's worth highlighting that, on average, infected

mussels exhibited a 9% lower filtration rate than their uninfected counterparts prior to the heat wave. This reduction increased to 37% after acute heat exposure during the recovery phase.

Focusing on the sole effect of infection on two mussel size class of 2 and 4 cm mussels under the benign temperature (16 °C) in second and third experiments suggested similar pattern of reduction in filtration capacity of infected mussels. However, the reduction was statistically significant only for larger mussels, mainly due to larger absolute values of feeding rate and lower relative variance compared to small mussels. Our results are in accordance with previous study where they found 71 versus 41% reduction in clearance rate for large and small mussel, respectively (Stier et al., 2015). These observations prompt further investigation, as they suggest that the impact of heat might become more pronounced over time. Additionally, capturing pre- and post-infection measurements of the same individuals could yield insightful results and help us understand the potential long-term effects of such infections.

#### Conclusion

This thesis shows that there is a hidden diversity in the Persian Gulf with 30 species of trematodes infecting a single snail species. Nevertheless, the study of trematode diversity in the Persian Gulf is still in its infancy and future studies should focus on other snail hosts and sampling sites to complete the picture illustrated herein. Furthermore, the optimal temperature for cercarial emergence was higher than other reported optimal temperatures in the literature. Regardless of the high optimal temperature for emergence, warming is expected to create challenging conditions for the parasites assessed in this thesis since future temperature regimes in the Persian Gulf will reach conditions which are exceeding the thermal optimal for cercarial emergence. Finally, study systems with more advanced knowledge on parasite and host diversity such as the Baltic Sea provide an excellent ground to pursue studies that evaluate the mechanistic aspects of warming effects on parasites and host performance. For future research directions it is essential to test available hypotheses in different systems and ecoregions as done herein.

### **References for General Introduction and Discussion**

This reference list contains all of the references used in the general introduction and general discussion. Individual article references are listed at the end of the corresponding articles.

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## **Declaration**

I, Maral Khosravi, hereby declare that the dissertation submitted, entitled "Trematode parasites of mollusc hosts from marginal seas and their sensitivity to warming" was written independently by me and only using the sources listed. The content and design of this thesis, apart from the supervisor's guidance, is my own work. The thesis has not been submitted either partially or wholly as a part of a doctoral degree to another examining body and is my first and only doctoral procedure. In time of defense, Chapter 1 of this thesis is under preparation, Chapter 2 and Chapter 3 of this thesis are published in *Scientific report*, Chapter 4 of this thesis is published in *International Journal for Parasitology: Parasites and Wildlife*. The authors' share of the manuscripts is explained in the subsection "Chapters and contributions of authors" (page 24 and 25). This work has been prepared respecting the Rules of Good Scientific Practice of the German Research Foundation. I have not been deprived of an academic degree.

Kiel, August 2023

Maral Khosravi