DOCTORAL (PhD) DISSERTATION

WAN MUHAMMAD HAZIM BIN WAN SAJIRI

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PARASITIC INFESTATION AND CONTROL IN RECIRCULATING AQUACULTURE SYSTEMS (RAS): FOCUSING ON EUROPEAN CATFISH *Silurus glanis* Linnaeus, 1758 INFESTED WITH *Thaparocleidus vistulensis* (SIWAK, 1932)

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DEDICATION

This dissertation is dedicated to:

My beloved parents,

Noriza Binti Othman and Wan Sajiri Bin Wan Hassan,

my wife, the queen of my heart,

Nur Nadhirah Syafiqah Binti Suhaimi,

my dearest parents-in-law,

Sharifah Suzana Binti Syed Sheh and Suhaimi Bin Ismail,

my cherished siblings and in-laws,

whose unconditional love, support, and sacrifices have been the pillars of my success.

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LIST OF ABBREVIATIONS AND ACRONYMS

AIC	-	Akaike information criterion
BIO	-	Biokos
BLAST	-	Basic Local Alignment Search Tool
bp	-	base pair
dpi	-	Days post-infection
dpo	-	Days post-oviposition
GAR	-	Garlic
GIN	-	Ginger
H&E	-	Haematoxylin and eosin
hpo	-	Hours post-oviposition
INSDC	-	International Nucleotide Sequence Database Collaboration
ITS	-	Internal transcribed spacer
LSU	-	Large subunit
NCBI	-	National Central for Biotechnology Information
MBZ	-	Mebendazole
MEGA	-	Molecular Evolutionary Genetics Analysis
ML	-	Maximum likelihood
mtDNA	-	Mitochondrial deoxyribonucleic acid
NMB	-	Neem bark
OECD	-	Organization for Economic Cooperation and Development
PCR	-	Polymerase chain reaction
pi	-	Post-isolation
pop	-	Post-oviposition
PZQ	-	Praziquantel
RAS	-	Recirculating aquaculture system
RASOPTA	-	Safeguarding Future Production of Fish in Aquaculture Systems with Water Recirculation
rDNA	-	Ribosomal deoxyribonucleic acid
rRNA	-	Ribosomal ribonucleic acid
SEM	-	Scanning electron microscope
SPSS	-	Statistical Package for the Social Sciences
SSU	-	Small subunit
t	-	Tonnes
WoRMS	-	World Register of Marine Species

CHAPTER 1

INTRODUCTION

1.1. Research background

This dissertation is part of a European networking research project funded by the European Union's Horizon 2020 research and innovation program under Marie Skłodowska-Curie Actions. The project, titled 'Safeguarding Future Production of Fish in Aquaculture Systems with Water Recirculation' (RASOPTA), consists of three closely interconnected work packages focusing on water quality, off-flavour issues, and the health and welfare of fish reared in recirculating aquaculture systems (RAS). This initiative integrates academic expertise with hands-on experience from the RAS industry, ensuring a multidisciplinary approach to addressing key challenges in modern aquaculture. A central objective of the RASOPTA project is the development of a novel DNA chip designed for the RAS industry, aimed at improving monitoring and management practices. However, as part of the RASOPTA network, the present dissertation specifically focuses on studying only ectoparasitic infections, which pose a significant risk in RAS environments. The European catfish *Silurus glanis* Linnaeus, 1758 is used as the model species, providing valuable insights into parasite-host dynamics for designing potential mitigation strategies within intensive RAS production systems.

Global aquaculture production reached a new record 130.9 million tonnes in 2022, highlighting its vital and progressively increasing role in the global food industry (FAO, 2024a). However, the intensifying demand for food has led to highly escalated aquaculture practices, occasioning numerous diseases that cause significant damage. Among these diseases, parasitic infestations show remarkable impacts, as they mainly cause substantial economic losses for the aquaculture industry (Thoney & Hargis, 1991; Shinn et al., 2015). Fish parasites may directly affect fish populations and their nutritional value by deceasing large number of fish, or becoming inedible as a consequence of parasitic diseases (Hoffman, 1998). Economic losses due to these parasite infections are not only caused by fish mortality but also by the treatment expenses and low growth

performance during and after the parasite outbreak, which impede aquaculture expansion (Omeji et al., 2011).

In Hungary, the total fisheries and aquaculture production in 2022 was 23,545 tonnes, comprising 18,948 tonnes from aquaculture and 4,597 tonnes from capture fisheries (FAO, 2024b). Notably, European catfish (197.78 tonnes) ranked sixth in aquaculture production, following carps and several other freshwater species, highlighting its economic and ecological significance in Hungary according to data from FishStatJ (FAO, 2024b) (**Figure 1**). European catfish have been cultivated extensively in Central and Eastern Europe for over 100 years (Linhart & Proteau, 1993). This species is important as they have a high economic value due to rapid growth and excellent flesh (Tinkir et al., 2021). Subsequently, the increasing demand for European catfish has led to intensive farming practices. Breeding the species in high density provides more opportunity for provoking outbreaks by a specific gill monopisthocotylean parasite called *Thaparocleidus vistulensis* (Siwak, 1932), one of the common and significant parasitic threat encountered in the intensively cultured European catfish industry (Molnár et al., 2019). Moreover, this parasite has been reported to cause a fatal infection in different age groups of fish, especially in populations of European catfish fry (Molnár, 1980; Molnár et al., 2019).

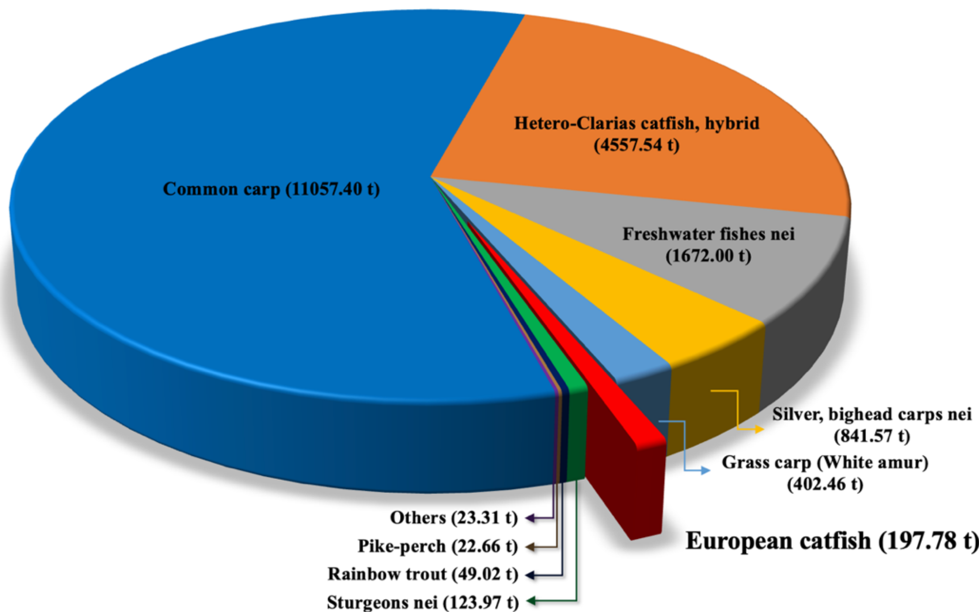

Figure 1. Top 10 aquaculture species produced in Hungary. Data extracted from FishStatJ (FAO, 2024b).

To effectively control infections caused by this ectoparasite, it is essential to understand its epidemiological patterns and biological parameters, as well as explore suitable treatment options. The use of chemical-based treatments has been expanding for decades, particularly in the field of aquaculture (Buchmann & Bresciani, 2006). However, in line with the increasing emphasis on environmental sustainability and the welfare of farmed fish, alternative therapeutic agents and environmentally friendly methods must be explored in greater depth. Furthermore, environmental factors that have the potential to regulate and reduce monopisthocotylean infestations in their hosts must also be thoroughly investigated. This dissertation analyzes and discusses viable solutions to the challenges faced by European catfish farmers, ultimately enhancing disease management strategies and improving aquaculture productivity in more sustainable and effective manner.

1.2. Problem statement and significance of study

Monopisthocotylean parasites possess conspicuous sclerotized organs (e.g., anchors and marginal hooks), together with some parts of the male copulatory organ, which are decisive for species differentiation (Řehulková et al., 2018). Although morphological studies form the basis of correct species identification, molecular data is vital in supporting a complete and conclusive taxonomic classification (Franceschini et al., 2018). To date, the description of *T. vistulensis* is primarily based on morphological characteristics, as it was made before molecular methods became widely used. Only limited molecular data are available, including ITS1, partial 18S rRNA, and partial 5.8S rRNA gene sequences reported by Šimková et al. (2003), and a partial 28S rRNA gene sequence published by Šimková et al. (2006). The sequence information available in the International Nucleotide Sequence Database Collaboration (INSDC) remains scarce. This study reevaluates morphometric data with a specific focus on the haptoral and copulatory sclerites, and presents new molecular data and scanning electron microscope (SEM) studies, extending current knowledge on the species.

Histopathological investigations regarding the effects of monopisthocotyleans on gills have already been done on various important freshwater fish in Europe, for instance, pike perch

Sander lucioperca (see Molnár et al., 2016), European eel *Anguilla anguilla* (see Abdelmonem et al., 2010; Buchmann, 2012), grass carp *Ctenopharyngodon idella* (see Molnár, 1972), and common carp *Cyprinus carpio* (see Jalali & Barzegar, 2005). However, Paladini et al. (2008) and Reading et al. (2012) reported about pathogenic effects associated with *T. vistulensis* on its hosts, noting a lack of previous histopathological studies. Notably, Molnár (1980) conducted a study that seems to have been overlooked by the above authors and highlighted the need for further investigation to validate and expand knowledge of the histopathological impacts of *T. vistulensis* infections in European catfish.

Due to the adaptive benefits of having a direct life cycle, without intermediate hosts, the monopisthocotyleans can proliferate exponentially in the host populations (Buchmann & Bresciani, 2006), and cause high morbidity and mass mortality, especially in confined fish farm systems like RAS, leading to significant economic losses for aquacultural enterprises (Thoney & Hargis, 1991). Fundamental knowledge and understanding of the host dynamics of these parasites may assist the development of management tools, which can be used to control the disease (Buchmann, 1988a). The present study investigates the infection dynamics and details life cycle parameters (oviposition, egg development, hatching, and survival of oncomiracidia, developing juvenile and adult parasites) for *T. vistulensis* currently eliciting disease in European catfish farms. This study is significant as it provides an essential understanding of the reproductive strategies of *T. vistulensis*, offering valuable data for the development of effective control strategies in aquaculture systems.

For most monopisthocotyleans, developmental rates, including the embryonation period, oncomiracidia longevity, age at sexual maturity, and adult lifespan, are influenced by environmental factors like water temperature, light intensity, salinity, and host species (Bauer et al., 1973; Gannicott & Tinsley, 1997; Buchmann & Bresciani, 2006). Nevertheless, research on *T. vistulensis* has primarily focused on its geographical occurrence (Soylu, 2005; Sobecka et al., 2010), morphology (Siwak, 1932; Bychowsky & Nagibina, 1957; Paladini et al., 2008), infection

dynamics, and life cycle in optimal water temperatures (Molnár, 1968), as well as the gill histopathological alterations during the infection (Molnár, 1980). As there have been no previous studies about the effects of changing different environmental factors on various life stages of *T. vistulensis*, studies of these processes are crucial for evaluating potential consequences on the host species. In this study, the effects of light-dark cycles and various water temperatures on the fecundity, hatching rate, and survivability of *T. vistulensis* were investigated through controlled laboratory experiments. This study enhances our understanding of *T. vistulensis* and assists in developing targeted control measures for aquaculture settings.

Despite environmental concerns and public opposition to the chemical treatment of commercial edible fish, conventional anthelmintics including praziquantel (PZQ) and mebendazole (MBZ), remain the main tools for controlling monogenean infections in aquaculture. The efficacy of these antiparasitic agents against *T. vistulensis* infecting gills of European catfish was investigated previously by *in vivo* studies at several concentrations and durations (Székely & Molnár, 1990). The treatments reduced but did not eradicate all parasites attached to the gills suggesting that various developmental stages of this parasite possess different drug sensitivity. Therefore, this study addressed the effectiveness of PZQ and MBZ in more detail and explored potential improvements in their efficacy through *in vitro* and *in vivo* investigations. In addition, the present study also evaluated, for the first time, the antiparasitic activity of the surfactant *Pseudomonas* H6, Biokos (BIO) against *T. vistulensis*. Moreover, alternative therapeutic agents using several herbal extracts, including garlic (GAR), ginger (GIN), and neem bark (NMB), were investigated for the first time. Hence, the present study examined the efficacy of several drugs and herbal antiparasitic agents against all life stages of *T. vistulensis* (eggs, oncomiracidia, developing juvenile, and adult parasites) both *in vitro* and *in vivo*. Thus, this study provides the first comprehensive evaluation of these treatments against *T. vistulensis*, contributing valuable data for the development of current and alternative therapeutic strategies in aquaculture systems.

1.3. Research aim and objectives

For the reasons mentioned above, the main aim of this work is to investigate options for controlling monopisthocotylean *T. vistulensis* infection in European catfish. Following this general point, this study specifically embarks on the following objectives:

1. To perform the molecular characterization of *T. vistulensis* – provide for the first time, complete characterization of the gene data including small subunit (SSU) ribosomal ribonucleic acid (rRNA) gene, partial sequence; internal transcribed spacer (ITS) 1, 5.8S rRNA gene, and ITS2, complete sequence; and large subunit (LSU) rRNA gene, partial sequence.
2. To study the morphology of adult *T. vistulensis* using light microscopy and SEM – provide morphometric measurements for the sclerotized structures including anchors and male copulatory organ.
3. To evaluate the pathological effects of *T. vistulensis* infection on the gills of European catfish – provide the condition of infected gills using histopathological and SEM studies.
4. To investigate the reproduction strategies of *T. vistulensis* infecting European catfish – provide the infection dynamics and detail life cycle parameters *in vitro* including oviposition, egg development, hatching, and survival of oncomiracidia, developing juvenile and adult parasites.
5. To study the influence of the light-dark cycle and various water temperatures on *in vitro* fecundity, hatching and survival rate of the *T. vistulensis* – provide for the first time, insights into the reproductive ability of *T. vistulensis* under different controlled environments.
6. To explore *in vitro* and *in vivo* efficacy of antiparasitic treatments against *T. vistulensis* infection in European catfish – provide for the first time, the use of bacterial-derived lipopeptide *Pseudomonas* H6 Biokos and the use of herbal therapeutic antiparasitic agents against *T. vistulensis*.

CHAPTER 2

LITERATURE REVIEW

2.1. European catfish (*Silurus glanis* Linnaeus, 1758)

European catfish (also known as sheatfish or wels) is the largest European freshwater fish, widespread from Central to Eastern Europe and Western Asia. This Siluriformes fish species is common from Germany eastwards through to Poland, up to southern Sweden and down to southern Turkey and north Iran, stretching through the Baltic States to Russia (Greenhalgh, 1999) and to the Aral Sea of Kazakhstan and Uzbekistan (Phillips & Rix, 1988; Copp et al., 2009). They are aggressive predatory fish that hunt at night or even during the day in muddy waters, relying on their non-visual perceptions (Antognazza et al., 2022; Sáez-Gómez & Prenda et al., 2022). With its excellent senses and high hemoglobin levels, this species is well-adapted to low water quality and exhibits notable tolerance to pollution (Ivanović et al., 2016) and extended durations of hypoxia (Massabuau & Forgue, 1995). It prefers still-water habitats (Copp et al., 2009), such as large, warm lakes and deep, slow-flowing rivers, where it can find shelter in holes and among sunken trees. They can tolerate a wide range of temperatures (3 to 30 °C), physiological optimum is between 25 and 28 °C (Hilge, 1985; Linhart et al., 2002), but mating pairs spawn at lower temperature ranging from 18 to 20 °C (Souchon & Tissot, 2012).

European catfish have been cultivated extensively in Central and Eastern Europe for over 100 years (Linhart & Proteau, 1993). The species possesses a high economic value because of its rapid growth and high-quality flesh (Tinkir et al., 2021). Although the majority of European catfish production originates from fishing, its aquaculture is expanding quickly (Linhart et al., 2002). The increasing trend of catfish farming is due to several factors including the flavorable taste of the flesh (Panicz et al., 2017; Lyach, 2021; Bergström et al., 2022), usefulness in polyculture fisheries (Vejřík et al., 2019; Linhart et al., 2020), and being popular for sport fishing (Rees et al., 2017; Lyach & Remr, 2019; Říha et al., 2022).

2.1.1. Species characterization and taxonomy of European catfish

In the past few decades, the largest individual European catfish recorded was 2.73 m and 130 kg (Boulêtreau & Santoul, 2016), with reports from previous centuries of fish caught was at least 5 m long and weighed 306 kg (e.g., Stone, 2007; Copp et al., 2009; Alp et al., 2011, Gkenas et al., 2015). They can be distinguished by their appearance, where the body is generally dark along its back, with marbled-patterned sides and a greyish-white belly. The skin is scale-less, covered with mucus, and contains sensory cells that aid in respiration through oxygen absorption and carbon dioxide secretion (Mihálik, 1955; Davies et al., 2004). European catfish has an elongated body that is laterally decompressed behind its broad triangular-shaped head (Černý, 1988), with a rounded and flattened snout together with widely-spaced nostrils anterior to the olfactory cavities (Mihálik, 1995). They have a pair of small eyes and a large mouth, with long, slender, and flexible cartilaginous barbs on the upper jaws, along with four short, flexible barbs, extending from the lower jaw (Davies et al., 2004).

European catfish also possess a pair of pectoral fins (with 18 rays), a pair of ventral fins (with 1 hard ray and 12–17 soft rays), and a pair of small pelvic fins located near the anal opening (with 10–13 soft rays). The anal fin is the longest, starting from the anal opening to the caudal fin (with 90–92 soft rays and 73–106 sturdy rays), about half of the total body length. The caudal fin is rounded and appears cut off at the end (with 17–19 soft rays), while a very small caudal fin located on the back (with 3–5 rays, the first is hard and another four are soft) (Mukhamediyeva & Sal'nikov, 1980; Maitland & Campbell, 1992; Greenhalgh 1999; Davies et al., 2004).

2.1.2. Pathogens and parasitic diversity in Silurus glanis

In recent decades, studies on diseases affecting European catfish have mainly focused on viral and bacterial infections related to aquaculture or experimental conditions. However, the interaction of pathogens, hosts, and the environment in aquaculture settings often does not fully resemble natural conditions (e.g., differences in stocking density and water quality) (Dohoo et al., 2003). Therefore, extrapolating the results of studies about diseases in aquaculture to wild European catfish

populations needs essential caution. Notably, some diseases are unique to European catfish, such as the European sheatfish virus (ESV), an Iridovirus infection from the genus *Ranavirus* that causes severe mortality in aquaculture populations (Ahne et al., 1990). Furthermore, European catfish are susceptible to viruses from the family Rhabdoviridae, such as spring viraemia of carp (SVC), which also has significant impacts on salmonid species (Fijan et al., 1984; Békési et al., 1987). Other viral infections include Circoviruses infection from the genus *Circovirus*, specifically European catfish circoviruses CfCV-H5 and CfCV-H6 (Lőrincz et al., 2012); Herpesvirus infections that trigger pathological changes resembling carp pox, such as skin lesions (Lucky, 1970; Békési et al., 1981); and Papillomavirus infections caused by *Nunpapillomavirus siluri*, that cause multiple, papilloma-like, epidermal hyperplasia on the skin (Surján et al., 2021).

Several common bacterial species can be found in aquatic environments, which may cause morbidity and mortality in farmed European catfish, including *Flexibacter columnaris* (Farkas & Oláh, 1980) and *Vibrio* spp. (Farkas & Malik, 1986). Some other bacterial infections in aquaculture also were reported, including outbreaks associated with *Aeromonas* species (Farkas & Oláh, 1982); *Flavobacterium* species (Farkas, 1985), a *Pasteurella*-like bacterium (Farkas & Oláh, 1984) and *Edwardsiella tarda* (Caruso et al., 2002).

At least 96 parasites have been discovered in European catfish (See **Table S1**), though this number is likely underestimated. These pathogens are generally spread through fish stocking, movement, and aquaculture practices, pointing to the significance of human activities in the process. The diversity of parasites in European catfish is consistent with its broad Eurasian distribution, encompassing anthropochorous dispersals from Asia to Europe (Bauer, 1991; Copp et al., 2009). Moreover, dispersion also occurred with parasites co-introduced within the European region, such as the introduction of *T. vistulensis* from Central Europe to Italy (Galli et al., 2003; Galli et al., 2005; Galli et al., 2007). Host switch of parasite was also reported when *Leptorhynchoides plagicephalus*, which is common in sturgeons (Dezfuli et al., 1990), infected European catfish (Bauer et al., 2002).

Mortality caused by listed parasites is very rare among fish over the fry stage, and the pathological reports associated with these infestations are limited due to a focus on taxonomic studies and survey data (Copp et al., 2009). However, there are monopisthocotylean infections, such as *T. vistulensis*, which alone can cause serious outbreaks in adult European catfish (Papp, 1955; Molnár, 1980), or mass mortality in fry population (Molnár et al., 2019).

2.2. Parasitic platyhelminths: General overview

Platyhelminthes Claus, 1887 (flatworms) is one of the major phyla of helminth parasites, along with Nematoda Diesing, 1861 (roundworms) and Acanthocephala Kölreuter, 1771 (thorny-headed worms). It consists of Neodermata (Nordmann, 1832), that composed of three classes of parasitic flatworms, including Monogenea van Beneden, 1858 (also classified as Monopisthocotyla and Polyopisthocotyla; see Brabec et al., 2023), Trematoda Rudolphi, 1808 (flukes) and Cestoda Rudolphi, 1808 (tapeworms). However, all flatworm groups harbour symbiotic representatives, Neodermata clade is exclusively composed of obligate parasites that infect all major vertebrate groups, with an estimated 60,000 species (Appeltans et al., 2012; Caira & Littlewood, 2013). Neodermata is characterized by increased evolutionary adaptation to parasitism, obligate parasites covered by an unciliated syncytial tegument or neodermis, which provides high resistance against host immune defences and enables an efficient mechanism for food intake (Hooge, 2001; Mulvenna et al., 2010).

2.2.1. Monogenea Carus, 1863

Monogeneans are typical ectoparasitic flatworms that infect the gills, skin, or fins of fish and lower aquatic invertebrates in various environments with a broad range of water temperatures. Approximately 4000 to 5000 monogeneans species have been identified in fishes of the world, from both fresh and saltwater (Reed et al., 2012). Monogeneans commonly move freely on fish's body surface, feeding mucus and epithelial cells of the skin and gills. Some adult monogeneans permanently attach to the same spot on their host for their entire lives. Besides the body surface, some monogenean species could occationally invade the internal sites such as the nasal cavity

(e.g., *Empruthotrema dasyatidis* in *Orectolobus maculatus*), urogenital system (including cloaca – e.g., *Calicotyle palombi* in *Mustelus mustelus*, and rectal cavity – e.g., *Calicotyle stossichi* in *Mustelus mustelus*), ureter (e.g., *Acolpenteron ureteroecetes* in *Micropterus salmoides*), inner wall of the body cavity (e.g., *Dictyocotyle coeliaca* in *Raja radiata*), and even the vascular system (Chisholm et al., 2001; Reimschuessel et al., 2011; Reed et al., 2012; Derouiche et al., 2019). Monogeneans are considered host-specific parasites, restricted to one or several closely related host species. They are known as niche-specific species, limited to special microhabitats within the same host species (Kearn, 1998; Whittington et al., 2000; Thatcher, 2006; Ŝimková et al., 2006). Monogeneans can cause significant epizootics with severe consequences, particularly among farmed fish (Thoney & Hargis, 1991; Woo, 2006).

2.2.2. Historical review and current classification

The nomenclature of this group of Platyhelminthes has been a subject of debate. However, a resolution in 1978 adopted during the Fourth International Congress of Parasitology (ICOPA IV) in Warsaw, Poland, officially established “Monogenea” as the name of the class, rather than “Monogenoidea” (see Wheeler & Chisholm, 1995). There is a persistent doubt regarding the monogenean subclasses (Justine, 1998; Perkins et al., 2010; Littlewood & Waeschenbach, 2015), which classically comprise either (i) Monopisthocotylea (possess a single opisthaptor) (**Figure 2A–D**) and Polyopisthocotylea (possess a more complex opisthaptor) (**Figure 2E–H**) based on both the spermiogenesis and spermatozoon ultrastructure and the morphology of adult’s attachment organs (Justine, 1991), or (ii) Polyonchoinea Bychowsky, 1937 and Heteronchoinea Boeger and Kritsky (1993) based on the larval attachment organ (Justine, 1998).

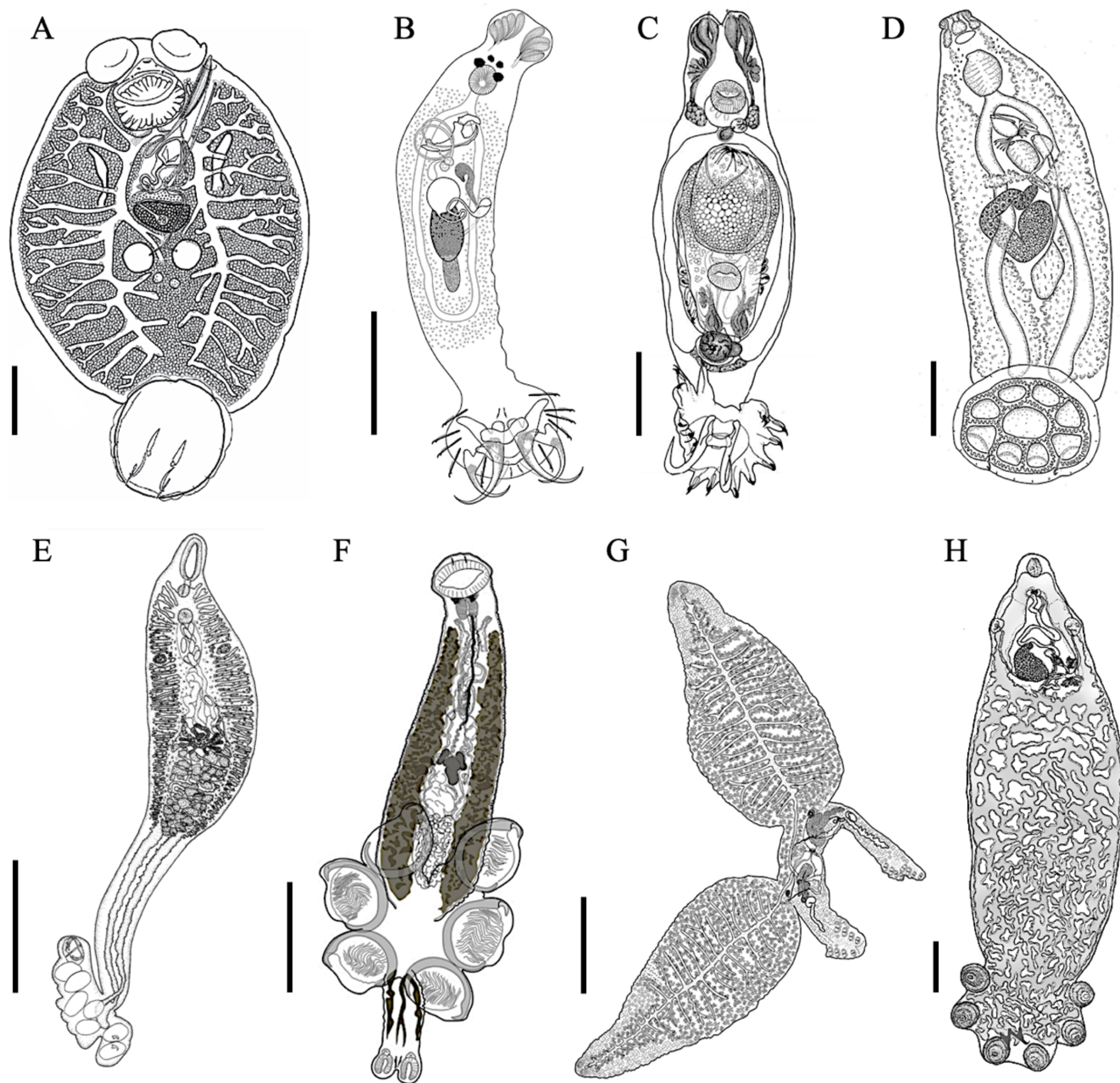

Figure 2. A diversity of ectoparasitic monogenean families, highlighting the various attachment organs in the Monopisthocotylea (A–D) and Polyopisthocotylea (E–H) subclasses. (A) *Benedenia akajin* Ogawa, Mizuochi, Yamaguchi, Shirakashi, Asai and Agawa, 2021 (Capsalidae) (Adapted from Ogawa et al., 2021); (B) *Diaphorocleidus magnus* Zago, Franceschini, Abdallah, Müller, Azevedo and da Silva, 2021 (Ancyrocephalidae) (Adapted from Zago et al., 2021); (C) *Gyrodactylus anaspidus* Vianna and Boeger, 2019 (Gyrodactylidae) (Adapted from Vianna & Boeger, 2019); (D) *Heterocotyle whittingtoni* Chisholm and Kritsky, 2020 (Monocotylidae) (Adapted from Chisholm & Kritsky, 2020); (E) *Chimaericola ogilbyi* Beverley-Burton, Chisholm and Last, 1991 (Chimaericolidae) (Adapted from Beverley-Burton et al., 1991); (F) *Narcinecotyle longifilamentus* Torres-Carrera, Ruiz-Escobar, García-Prieto and Oceguera-Figueroa, 2020 (Hexabothriidae) (Adapted from Torres-Carrera et al., 2020); (G) *Paradiplozoon iraqense* Al-Nasiri and Balbuena, 2016 (Diplozoidae) (Adapted from Al-Nasiri & Balbuena, 2016); (H) *Polystoma knoffi* du Preez and Domingues, 2019 (Polystomatidae) Scale bar: 1000 μm (Adapted from du Preez & Domingues, 2019). Scale bars represent (A) 300 μm, (B) 100 μm, (C) 50 μm, (D) 200 μm, (E) 1000 μm, (F) 800 μm, and (G) 1000 μm.

Analyses based on the phylogenetic of morphological data, ultrastructural spermiogenesis, and spermatozoa of the taxon Rhabdocoela (Platyhelminthes) suggested Monogenea is a group of monophyletic, which is more closely related to tapeworms than other platyhelminths (Justine, 1991; Zamparo et al., 2001). However, molecular data analyses (18S or 28S rDNA sequences) do not support the monophyly of the Monogenea, and Mollaret et al. (1997) suggested Monogenea as a paraphyletic group. But later, molecular phylogenies contradicted to the previous results and confirmed the monophyly of each of the two lineages (Polyopisthocotylea and Monopisthocotylea, Polyonchoinea and Heteronchoinea) as members of Neodermata, alongside Cestoda and Trematoda (Littlewood et al., 1999; Mollaret et al., 2000; Egger et al., 2015).

The class of Monogenea also were classified into 3 subclasses, including Polyonchoinea, Oligonchoinea Bychowsky, 1937, and Polystomatoinea Lebedev, 1986 (Boeger & Kritsky, 1993), which based on shared traits (synapomorphies) that suggests they all evolved from a common ancestor like possessing two pairs of eyespots for adults and larvae (oncomiracidium), equipping haptor with 16 marginal hooks and one pair of anchors, and having three rows of ciliary epidermal bands in the oncomiracidium (Brooks, 1989; Boeger & Kritsky, 1993). The monophyly of Polyonchoinea is supported by mouth on the ventral surface, less numbers of subsurface sperm microtubules, and haptor of oncomiracidium and adults possess 14 marginal hooks and two central hooks (Boeger & Kritsky, 1993). For Oligonchoinea, they have a hook-like structure in the clamp of the haptor, the presence of a pair of lateral sclerites, four pairs of haptoral suckers, and intestine walls with diverticula (pouches) (Boeger & Kritsky, 1993). The monophyly Polystomatoinea is supported by the absence of eggs filament. They are the sister group to Oligonchoinea, that sharing 6 traits such as having more than two testes, the presence of gastrointestinal canal, adults possess a haptoral suckers together with hooks, three parts of the haptoral suckers, and the presence of two lateral vaginal ducts (Boeger & Kritsky, 1993). The phylogenetic analysis by Mollaret et al. (2000) supported Boeger and Kritsky’s (1993) suggestion to include both Polystomatoinea Lebedev, 1986

(tetrapod monogeneans) and Oligonchoinea Bychowsky, 1937 (Polyopisthocotylea) within Heteronchoinea, and they formed a sister group with Polyonchoinea.

This debate has continued until the recent study by Brabec et al. (2023) that proved Monogenea as a non-monophyletic group on previous phylogenetic studies including mitochondrial deoxyribonucleic acid (mtDNA) (Perkins et al., 2010) and ribosomal deoxyribonucleic acid (rDNA) (Laumer & Giribet, 2014). Transcriptomic data studied by Brabec et al. (2023) showed robust and consistent signals in the two non-monophyly monogenean lineages (subclasses: Monopisthocotylea and Polyopisthocotylea). Despite the term use of conventionally recognized as class "Monogenea" being common in the nomenclature of phylum Platyhelminthes, Brabec et al. (2023) proposed to suppress the term and promote the previously subclasses to the class level as Monopisthocotyla new class and Polyopisthocotyla new class.

To reflect this proposed revision and avoid confusion in the present manuscript, the term "Monopisthocotylea" is used to specifically refer to the focal parasite species (i.e., *Thaparocleidus vistulensis*), while "Monogenea" is retained solely to refer to the group in its broader, traditional sense when necessary. This approach provides clarity and aligns with current molecular phylogenetic insights.

Based on extant database (2025a) of the World Register of Marine Species (WoRMS), the subclass Monopisthocotylea currently consists five orders including Capsalidea Baird, 1853, Dactylogyridea Bychowsky, 1933, Gyrodactylidea Beneden and Hesse, 1864, Monocotylidea Taschenberg, 1879, and Montchadskyellidea Bychowsky, Korotajeva and Nagibina, 1970. Meanwhile, the subclass Polyopisthocotylea also comprises five orders including Chimaericolidea Brinkmann, 1942, Diclybothriidea Bychowsky and Gussev, 1950, Lagarocotylidea Khotenovsky, 1985, Mazocraeidea Price, 1936, and Polystomatidea Lebedev, 1988.

2.2.3. Life cycles of Monogenea

Monogeneans (i.e., mono- and polyopisthocotyleans) have a direct, single-host, water-based life cycle without intermediate hosts (Hutson et al., 2018). Adult monogeneans are hermaphrodites (having both male and female reproductive structures), except protandrous species (having male reproductive organs before maturity and turning into a female) like *Benedenia seriolae* and *Zeuxapta seriolae* (Tubbs et al., 2005). Some monogeneans reproduce by laying shedding (settling) eggs in the water (oviparous) (**Figure 3**), and others live-bearing (viviparous), which releases live offspring larvae immediately attached to the same or new host. The eggshell of oviparous monogeneans is physically hard, with a detachable lid or operculum, allowing the infective oncomiracidium to escape from the eggs. Oncomiracidia can be either ciliated, free-swimming larvae that use their limited reserve energy to reach and infect a specific host before the energy is depleted, or non-ciliated larvae, which typically passively transmit to a new host, though some species can crawl (Whittington et al., 2000). This life stage ensures the completion of the life cycle by infecting the host population, then the parasites develop into adults, and the process repeats.

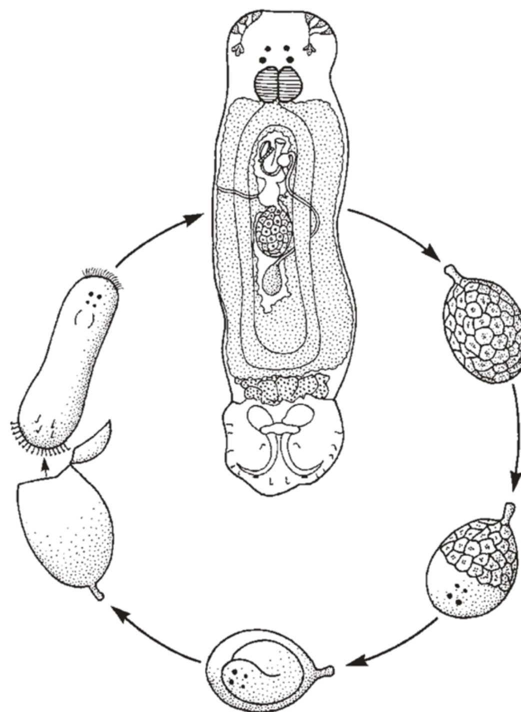

Figure 3. Life cycle of the oviparous monogenean *Pseudodactylogyrus anguillae*. Drawing by Beth Beyerholm. Adapted from Buchmann and Bresciani (2006).

2.2.4. *Morphological adaptations*

Monogeneans resemble endoparasitic digeneans, showing a leech-like looping locomotion (Kearn & Evans-Gowing, 1998; Whittington & Cribb, 2001). The anterior end of the monogenean body, called the prohaptor, contains various feeding and adhesive pad structures that facilitate attachment (Buchmann & Bresciani, 2006). Some monogenean species have a series of glands with cephalic openings at the prohaptor to secrete adhesive compounds (Whittington et al., 2000). The mouth is located in the ventral, cephalic region of the body and is connected to a muscular pharynx. The posterior end part consists of an attachment structure, opisthaptor differentiated highly in terms of shape and size, which is taxonomically important for the identification of Monogenea (Gussev, 1985). It bears an adhesive apparatus equipped with diverse sclerotized structures, like hooks, anchors, connective bars, clamps, suckers, and squamodiscs (Roberts & Janovy, 2009; Řehulková et al., 2018).

As previously mentioned in subsection 2.2.2, the structure of the haptor has been used for characterizing the major phylogenetic lineages of the monogeneans into two main groups, Monopisthocotylea (single posterior sucker – simple haptor) and Polyopisthocotylea (several posterior suckers – complex haptor, with several clamps or suckers) (Chisholm & Whittington, 1998), other than the sclerites within the reproductive organs that is crucial for species-level identification (Pouyaud et al., 2006; Messu Mandeng et al., 2015). The haptor usually has a disc-like form. Its center often contains one to three pairs of anchors, which are hook- or clamp-like structures supported by ventral and/or dorsal transverse bars or accessory sclerites, which offer stabilization and/or attachment.

2.2.5. *Impact of Monogenea on host*

Most monogeneans are ectoparasites, which substantially impact their hosts in terms of host health and population dynamics. The diversity in morphology, physiology, and the most crucial feeding behaviour of Monopisthocotylea and Polyopisthocotylea has remarkable implications for their impact on their host. Monopisthocotyleans primarily feed epithelial cells and mucus by grazing on

the body surface. Most of them can move in a leech-like manner, and use the hooks and prohaptoral adhesive secretions for the attachment. Whereas the Polyopisthocotyleans are sanguinivorous (blood-feeder) and significantly less motile, typically infecting gills, as well as the branchial and buccal cavities (Buchmann & Bresciani, 2006; Rubio-Godoy, 2007). These behaviours let them spread quickly within a population facilitated by their direct life cycle and short-generation time as well (Bychowsky, 1957; Turgut & Akin, 2003).

The impact on the host is minimal or moderate when parasite numbers are low, depending on the age of the fish, but when high monogenean burdens lead to notable adverse consequences, including tissue damage, inflammation, physiological stress, and ultimately, mortality. In many cases, mortality is not a direct result of the monogenean infestations but rather secondary (bacterial) infections associated with them as the feeding habits of Monopisthotyleans compromise the integrity of the skin and its defense mechanisms (Thoney & Hargis, 1991; Buchmann, 1999; Buchmann & Bresciani, 1997). Infestations of *Gyrodactylus* spp. led to thinning of the epidermis, vacuolar degeneration and infiltration of mononuclear cells, and on the gills, hypertrophy, interlamellar necrosis and fusion of secondary lamellae of filaments (Kristmundsson et al., 2006; Grano-Maldonado et al., 2018). Additional damage occurs through the insertion of anchors and marginal hooks into the epithelium, punching numerous tiny holes (Buchmann, 1999; Buchmann & Bresciani, 1997), which create an opportunity to enter bacteria and other infective agents (Williams & Jones, 1994).

Monopisthocotyleans have a broad geographical distribution and are pathogenic to diverse hosts across a range of phylogenetic groups. For example, *Gyrodactylus salaris*, a monogenean parasite that originates from Baltic Sea Atlantic salmon (*Salmo salar*), is an important pathogen that could trigger massive mortality in *S. salar* populations in Norway (Olstad et al., 2006; Mo, 2024). However, despite originating from Baltic Sea salmon, it does not pose a significant threat to salmon stocks in that region (Mo, 2024).

Members of genera like *Benedenia* and *Neobenedenia* pose threats to marine aquaculture (Rubio-Godoy, 2007). *Benedenia seriolae* can infect Japanese amberjack *Seriola quinqueradiata*, yellowtail amberjack *S. lalandi*, and greater amberjack *S. dumerili* (Egusa, 1983; Whittington et al., 2001), and *Neobenedenia girellae* infecting greater amberjack, yellowtail amberjack, tiger puffer *Takifugu rubripes*, spotted halibut *Verasper variegatus*, red seabream *Pagrus major*, olive flounder *Paralichthys olivaceus*, chub mackerel *Scomber japonicus*, tilapia *Oreochromis mossambicus*, barramundi *Lates calcarifer*, and cobia *Rachycentron canadum* (Ogawa & Yokoyama, 1998; Hirazawa et al., 2004; Ogawa et al., 2006; Ohno et al., 2008; Yamamoto et al., 2011; Yudarana et al., 2022). *Dactylogyrus* and *Cichlidogyrus* species are known to cause diseases in carp and tilapia in Africa (Paperna, 1996). Other monopisthocotyleans like *Pseudodactylogyrus anguillae* and *P. bini* are known to cause morbidity and mortality of young European eels (*Anguilla anguilla*), and have an adverse impact on the eel production in Europe (Buchmann et al., 2011).

Polyopisthocotyleans have a complex attachment organ (haptor) with several suckers which provide stable but looser adhesion that anchors or hooks of monopisthocotyleans. Their sanguinivorous behaviour can cause anaemia, emaciation and even host mortality (Rubio-Godoy, 2007). Few cases have been documented about severe problems or mortality associated with them including *Discocotyle sagittate* - in salmonids farms in the Isle of Man (Gannicott, 1997), *Zeuxapta seriolae* (syn. *Z. japonica*) - in amberjacks farms worldwide (Grau et al., 2003; Montero et al., 2004; Ogawa, 2015; Sicuro & Luzzana, 2016; Sepúlveda et al., 2017; Cavaleiro et al., 2018; Vivanco-Aranda et al., 2019), *Microcotyle sebastis* - in rockfishes *Sebastes melanops* (Thoney, 1986) and *S. schlegeli* (Kim et al., 2002), and *Paradeontacylix grandispinus* and *P. kampachi* - among greater amberjack (Ogawa & Fukudome, 1994).

In some cases, the anaemia could be linked to the polyopisthocotyleans infection, when negative correlations between worm burdens and haematocrit detectable, in greater amberjack (Montero et al., 2004) and yellowtail amberjack (Mansell et al., 2005) infected with *Z. seriolae*, *Heterobothrium okamotoi* infecting tiger puffer (Ogawa & Inouye, 1997), *Neoheterobothrium*

hirame in olive flounder (Yoshinaga et al., 2001; Mushiake et al., 2001; Nakayasu et al., 2002), *Heteraxine heterocerca* infecting Japanese yellowtail (Mooney et al., 2008), and *D. sagittate* infecting rainbow trout *Oncorhynchus mykiss* and brown trout *Salmo trutta* (Lewisch et al., 2021). Additional host responses on the gills infected by either monopisthocotyleans or polyopisthocotyleans include increased mucus production, hyperplasia, lamellar deformation, haemorrhage, aneurysms, and secondary bacterial or fungal infections, which impair or halt gas exchange (see Buchmann & Bresciani, 2006). Some monogeneans can also function as vectors of other pathogens, including bacteria and viruses (Cusack & Cone, 1986), triggering co-infections, leading to a further mortality rate increase.

2.2.6. Environmental factors impacting Monogeneans

Given the huge diversity of monogeneans and their varying reproductive strategies, it is extremely unlikely to make general statements about the majority of monogenean species and their interactions with hosts (Whittington & Chisholm, 2008). Beyond this, the interaction of the host with the environment also plays a significant role in perpetuating the monogenean life cycle. As a large number of parasites is detrimental to the hosts' fitness and resilience, it is important to understand the parasites' reproductive and survival dynamics under different environmental conditions (Caminade et al., 2019). For most monogeneans, development rates are influenced by natural factors like water temperature, light intensity, salinity, and the host species (Bauer et al., 1973; Gannicott & Tinsley, 1997; Buchmann & Bresciani, 2006).

Water temperature is a key element that has been widely investigated as a regulator in the reproduction of monogeneans (Kashkovskii, 1982; Buchmann, 1988b, 1990; Cecchini, 1994; Cecchini et al., 1998, 2001; Gannicott & Tinsley 1998a, 1998b; Lackenby et al., 2007; Hirazawa et al., 2010; Maciel et al., 2017; Zhang et al., 2015, 2022). Temperature increases within an optimal range can facilitate their development and reproductive success in fish hosts, while extreme temperatures could highly reduce their survival ability (Hirazawa et al., 2010; Zhang et al., 2015). The low water temperature results in decelerated development compared to the optimal

temperature (Hoai, 2020). For example, *Urocleidus adspectus* is unable to lay eggs at 4 °C, and larvae infecting yellow perch *Perca flavescens* host do not appear until seasonal water temperature rises to an optimal level, and the egg-laying activities resume (Cone & Burt, 1981). With further water temperature increases, some monogeneans (e.g., *Neobenedenia girellae*) exhibit a shortened life span (Hirazawa et al., 2010).

Water temperature greatly influences the egg development of monogenea. It was proven when Ernst et al. (2005) reported that *Benedenia* sp. eggs took 16 days to develop at 14 °C, but only 5 days at 28 °C. Similarly, egg hatching for *B. seriolae* on *Seriola lalandi* took 22, 11, and 9 days at temperatures of 13, 17.5, and 21 °C, respectively. Water temperature also affects the sexual maturation of parasites, which varies by host and region. Tubbs et al. (2005) discovered that *B. seriolae* matured on *S. lalandi* in 48, 25, and 20 days at 13, 18, and 21 °C, respectively. According to Brazenor & Hutson (2015), *Neobenedenia* sp. exhibited a quicker life cycle in warmer (10–13 days at 26–32 °C) than in colder conditions (15–16 days at 22–24 °C).

The photoperiod, or day length, also plays an important role in the reproductive strategies of monogeneans (Kearn, 1963, 1973, 1982; Mooney et al., 2008; Hoai & Hutson, 2014). Alterations in light conditions regulate the circadian rhythms of organisms, influencing parasites' life processes such as egg-laying and host-seeking (Macdonald & Jones, 1978; Mooney et al., 2006, 2008; Hoai & Hutson, 2014). These behavioral patterns indirectly maximise the larvae's chances of avoiding predation by other organisms, especially filter feeders (Euzet & Raibaut, 1960; Kearn, 1974). Environmental factors play a crucial role in the life cycle of monogenean parasites, particularly due to the shortening lifespan of most oncomiracidia, typically ranging from 24 to 48 hours. It means that the timing of the hatching rhythm is essential for increasing the chances of oncomiracidia finding an appropriate host on time (Tsutsumi et al., 2002). For instance, Kearn (1982) observed that *Entobdela diaderma* hatches more rapidly when exposed to light, while *E. soleae* eggs hatch immediately after being stimulated by light following a dark period (Kearn, 1963). On the other hand, polyopisthocotylean monogeneans like *Discocotyle sagittata* exhibit a

pronounced nocturnal hatching rhythm, with a peak two hours after darkness (Gannicott & Tinsley, 1997). A similar pattern was observed by Mooney et al. (2008), who found that nearly half of the eggs of *Heteraxine heterocerca* and *Benedenia seriolae* hatched during the first three hours following the onset of darkness. In contrast, some marine monogeneans, such as *Entobdella solea* (Kearn, 1973), *Diclidophora spp.* (Macdonald, 1975), and *Neobenedenia sp.* (Hoai & Hutson, 2014), display increased hatching rates in the presence of light. Some species show even more complex rhythms, as described by Euzet and Raibaut (1960), Macdonald and Jones (1978), Ernst et al. (2005), and Hirazawa et al. (2010). Understanding the interaction between biotic and abiotic factors in parasitic infections is crucial for identifying vulnerable stages in the parasite's life cycle. These insights could provide targeted and effective management strategies to control parasitic infestations (Dent, 2000; Clausen et al., 2015).

2.3. Current status of studies on the parasite *Thaparocleidus vistulensis* (Siwak, 1932)

Based on WoRMS (2025b), a total of 121 species within the genus *Thaparocleidus* Jain, 1952 are currently recognized as valid. Among the listed, Lim et al. (2001) recorded three species that were identified in causing specific infections on European catfish, including *T. siluri* (Zandt, 1924), *T. vistulensis*, and *T. magnus* (Bychowsky & Nagibina, 1957). *T. vistulensis* is one of the most pathogenic ectoparasites of European catfish in aquaculture, while the congeneric species *T. siluri* and *T. magnus* mainly occur only in wild populations (Molnár, 1968, 1980; Székely & Molnár, 1990; Molnár et al., 2019).

2.3.1. Classification and origin of Thaparocleidus sp. infecting European catfish

The three congeneric monogenetic gill parasites species mentioned above were reported in parasitizing Siluriformes, European catfish, belonging to the genus *Thaparocleidus* Jain, 1952 – which was formerly known as genus *Ancylodiscoides* Yamaguti, 1937 (Ancylodiscoididae: Monopisthocotylea), has been most recently included by Lim et al. (2001) in the list of known dactylogyridean monogenean genera of Old World siluriform fishes. The *T. siluri* and *T. vistulensis* were described originally from the European catfish by Zandt (1924) and Siwak

(1932) as *Ancyrocephalus siluri* and *A. vistulensis*, respectively. Yamaguti (1937) established the genus *Ancylodiscoides* for them, which was later supplemented with *Ancylodiscoides magnus* by Bychowsky and Nagibina (1957). In the meantime, Roman (1953) suggested that *A. vistulensis* could be a synonym of *A. siluri*, that was later re-emphasised by Roman-Chiriac (1960). However, Yamaguti (1963) rejected this classification and kept them as two distinct species. Although Akhmerov (1964) listed (temporarily) these three species into the *Parancylodiscoides* Caballero and Bravo Hollis, 1960 genus, Gussev (1973) revitalized the genus to *Ancylodiscoides*. Thenceforth, *Ancylodiscoides* has been used for the parasitic monogenean infecting European catfish before Gussev (1985) created them a new genus *Silurodiscoides* Gussev, 1976. Finally, Lim et al. (2001) compiled and listed the parasites infecting European catfish into the genus *Thaparocleidus* Jain, 1952. In the present dissertation, the most recent classification nomenclature is adopted, and the parasite is referred to as *Thaparocleidus*. Since the present study focuses on *T. vistulensis*, its taxonomy is outlined as follows (WoRMS, 2025c): Platyhelminthes (Phylum); Rhabditophora (Subphylum); Neodermata (Superclass); Monogenea (Class); Monopisthocotylea (Subclass); Dactylogyridea (Order); Ancylodiscoididae Gussev, 1961 (Family); *Thaparocleidus* Jain, 1952 (Genus); *T. vistulensis* (Siwak, 1932) (Species).

2.3.2. Distribution of Thaparocleidus vistulensis

Thaparocleidus vistulensis was first discovered and described by Siwak in 1931, collected from the wild European catfish in Vistula River, Poland, with the name of *Ancylocephalus vistulensis* (Siwak, 1932). Since then, it has been detected widespread across Europe and several Asian countries (See **Table S2**). Considering their strict host specificity, the distribution of *T. vistulensis* is restricted to the Eurasian region (Copp et al., 2009).

In Hungary, Papp (1955) was the first to report the occurrence of gill parasitic infections in European catfish farms, and identified the causative agent as *Ancylodiscoides siluri*, demonstrating its potential to cause significant economic losses in broodstocks. However, the differentiation between *Ancylodiscoides siluri* and *A. vistulensis* species had not yet been established at the time

of her study. Later, Molnár (1963) clarified that both *A. siluri* and *A. vistulensis* could be found in natural waters, but only *A. vistulensis* was present in farmed European catfish. Additionally, the distribution of *T. vistulensis* in countries such as Italy (Galli et al., 2005) and the UK (Reading et al., 2012) is attributed to the introduction of the non-native European catfish (*Silurus glanis*) along with the parasite. Although *T. vistulensis* is considered a highly host-specific parasite, it has been reported in nine different fish species in Iraq (Mhaisen & Abdul-Ameer, 2019; Mhaisen et al., 2013). However, these findings are questionable (Mhaisen et al., 2013) and may be the consequence of misidentification, as this parasite is known to infect only silurid fishes (Lim et al., 2001; Pugachev et al., 2010).

2.3.3. General morphology and life cycle of Thaparocleidus vistulensis

In general, the morphology of *T. vistulensis* highly resembles that of most parasitic Dactylogyrids, characterized by a simple, dorsoventrally flattened, and elongated body. The parasite body is divided into an anterior (front) and posterior (rear) region, specialized for attachment to the host. The anterior region contains four cephalic lobes, that contain cephalic glands. Dorsally, two pairs of eyes are present, with one pair being smaller than the other. The mouth is located at the head, directly below the genital opening. The body is translucent, and under a microscope, internal organs such as the digestive tract, male and female copulatory organs, and nervous system can be seen in the parenchyma. The body narrows posteriorly, forming a cylindrical shape, terminating in a caudal disc slightly broader than the rest of the body. The haptor, located at the posterior end, is equipped with two pairs of hooks (dorsal and ventral), each connected by a transverse bar; the dorsal hooks being larger than the ventral ones. Siwak (1932) initially reported the presence of 16 marginal hooks around the edge of the opisthaptor, later Bychowsky and Nagibina (1957) corrected their number to 14. Various studies have contributed to the detailed morphometric characterization of this species, especially focusing on sclerotized structures like anchors, as well as other internal organs, notably the male copulatory organ (Siwak, 1932; Bychowsky & Nagibina,

1957; Soylu, 2005; Paladini et al., 2008; Mancheva et al., 2009). The morphological features of these parts are essential in the taxonomy and functional adaptations.

The life cycle of *Thaparocleidus vistulensis*, like other monopisthocotylean species, consists of several developmental stages, including eggs, oncomiracidia, juvenile, and adult forms (**Figure 4**). Despite its importance, research on the life cycle of this parasite remains limited; however, Molnár (1968) provided a comprehensive analysis. According to him, the total duration of the life cycle, from egg to egg-laying adult, is approximately 13 to 14 days at temperatures ranging from 20–23°C. The eggs hatch within 2.5 to 3 days at temperatures ranging from 20 to 25°C. The oncomiracidia remain active and capable of infecting a host for 1 to 1.5 days at 20–21°C. Additionally, he also reported that oncomiracidia can infect their hosts for 4 to 4.5 days at temperatures ranging from 21 to 23°C. After infestation, the larvae develop into adults, and the mature adults produce eggs, repeating the entire life cycle. The adult parasites were observed to lay 6 to 10 eggs in 3 to 5 hours in pure water *in vitro* (Molnár, 1968). Further details regarding the morphological characteristics and life cycle of *T. vistulensis* will be presented in the results section and thoroughly discussed in this dissertation.

2.3.4. Effect of Thaparocleidus vistulensis on hosts

Monopisthocotyleans infect economically important fish including anguillids, salmonids, cyprinids, ictalurids, clariids, fundulids, poeciliids, gasterosteids, cyclopterids, cichlids, and pleuronectids (Thoney & Hargis, 1991). Among monopisthocotyleans, *T. vistulensis* is one of the most pathogenic ectoparasites of European catfish in the aquaculture (Papp, 1955; Molnár, 1980). Their viviparous mode of reproduction can lead to the rapid development of epizootics in short periods. Although they are often found in mixed infections with other parasites (Soylu, 2005; Akmirza & Yardimci, 2014; Roohi et al., 2014), *T. vistulensis* is capable of causing mortalities of European catfish alone (Papp, 1955; Molnár, 1968; 1980). Similar to most monopisthocotyleans, *T. vistulensis* uses its opisthaptor as the primary attachment apparatus during infection of the host (Molnár, 1980).

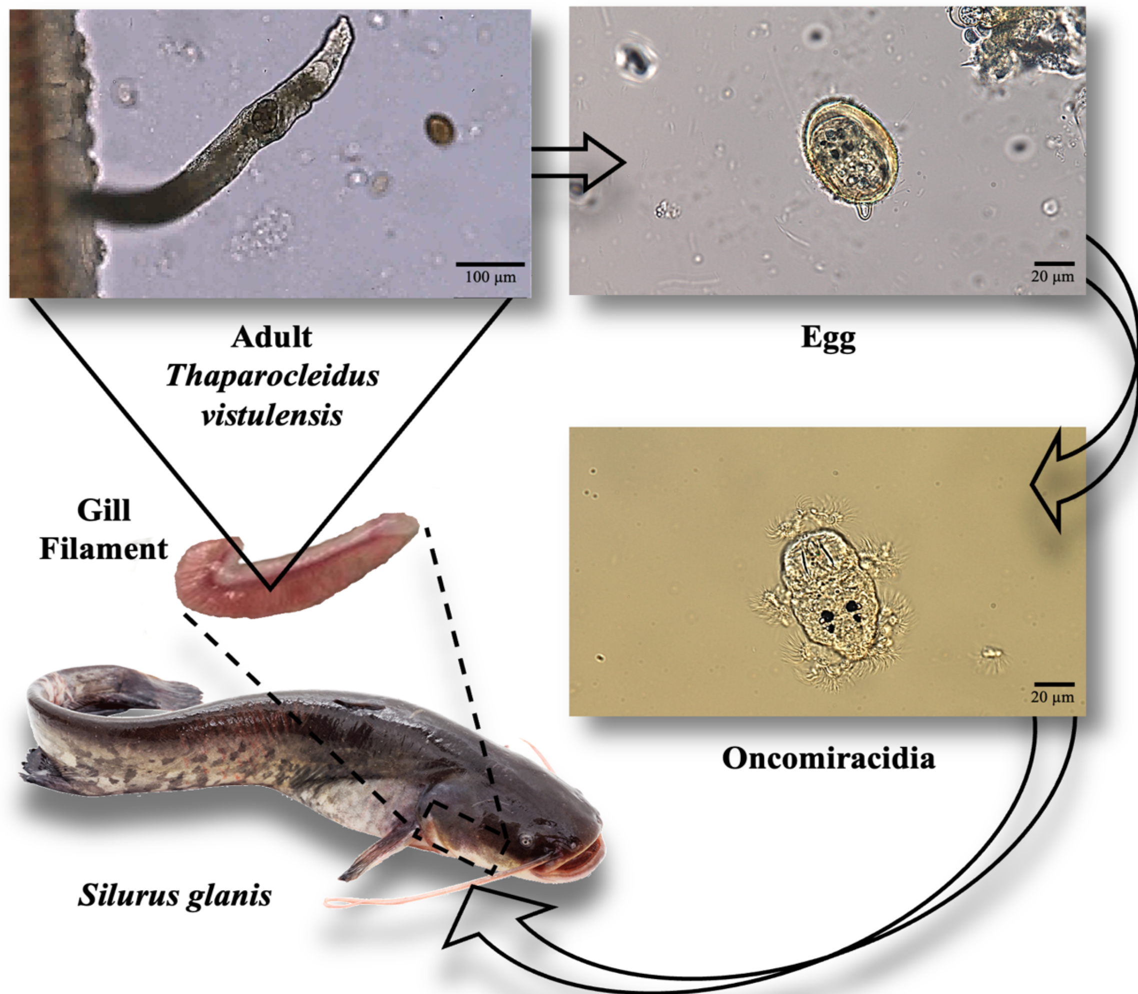

Figure 4. The life cycle of *Thaparocleidus vistulensis*.

The infection of *T. vistulensis* is considered fatal in hosts, ultimately causing death, based on the first reported on the pathogenic effects of *T. vistulensis* (reported as *Ancylocephalus siluri* – misidentified with *Ancyolocephalus vistulensis*) infecting the European catfish in Poland. She observed various developmental stages on the gills and attributed the death of a 30 cm long catfish, captured from the wild, to this infection (Siwak, 1932). After few decades, Papp (1955) reported significant losses in fingerling stocks from pond farms in Hungary caused by a gill monogenean initially identified as *T. siluri* (reported as *Ancylodiscoides siluri*). However, Molnár (1963) later confirmed that the species was *T. vistulensis* (reported as *Ancylodiscoides vistulensis*) as he found that the congeneric species *T. siluri* and *T. vistulensis* occur in natural waters, while *T. vistulensis* is the only species present in pond farms. The affected fingerlings (0.9–3.9 g weight 2.5–5 cm

length), harbouring between 20 and 286 worms, exhibited severe damage to their gill structures, ultimately leading to their mass mortality (Papp, 1955).

A few decades after the first reported mortality, Molnár (1980) corroborated Papp's (1955) findings, his experimental studies revealed that *T. vistulensis* could lethally infect two-week-old, 2 cm long European catfish fry, when oncomiracidia kill the hosts within a day due to extensive tissue destruction. The less intensive infections caused damages characterized by regressive and progressive lesions on the gills. A detailed description of the pathological effects of this parasitic infection was provided by Molnár (1980) using histopathological methods.

2.4. Prevention and control of Monogeneans

Parasitic infections are a major challenge in intensive fish farming, adversely affecting host health, growth, and overall productivity. The management and control of outbreaks are complicated by the limited availability of authorised therapeutic agents with varying efficacy (Morales-Serna et al., 2018; Zuskova et al., 2018). A wide range of methods for treating diseases caused by monogeneans has been discussed including mechanical, biological, and chemical approaches, as well as treatments using freshwater and saltwater (Buchmann & Bresciani, 2006; Whittington & Chisholm, 2008).

In aquaculture, treatments are commonly administered through oral delivery, injection, baths, or dip methods (See Buchmann & Bresciani, 2006; Whittington & Chisholm, 2008; Norbury et al., 2022). For the control of ectoparasites, bath treatments are widely used, as they are considered the standard approach for managing external parasitic infestations in fish (Levy et al., 2015). This method allows for direct exposure of the parasites to therapeutic agents, ensuring rapid and uniform distribution across the fish's body surface. Additionally, bath treatments are particularly effective against monogeneans and other skin or gill parasites due to their direct contact mode of action.

2.4.1. Chemical-based treatments

Various chemicals that disrupt the life functions of monogeneans are currently used for therapy in aquaculture practices, including copper sulfate, formaldehyde, potassium permanganate, sodium chloride, levamisole, praziquantel, oxidizers (e.g., sodium percarbonate and hydrogen peroxide), organophosphates (e.g., metrifonate, dichlorvos, and trichlorfon), and benzimidazoles (e.g., albendazole, fenbendazole, flubendazole, luxabendazole, mebendazole, oxfendazole, oxibendazole, parbendazole, thiabendazole, triclabendazole) (See Buchmann & Bresciani, 2006; Whittington & Chisholm, 2008). However, it is important to note that several monogenic parasites and their various fish hosts may have different sensitivities to the effects of these chemicals, making it challenging to find suitable substances that can be used broadly.

Praziquantel (PZQ) is a wide-ranging synthetic pyrazino-isoquinoline anthelmintic that is effective against all forms of schistosomiasis, and trematodes in humans (Andrews et al., 1983). Along with its important role in treating flatworm infections in humans, it is widely used in veterinary medicine (Andrews et al., 1983; Dayan, 2003; Doenhoff et al., 2009; Scala et al., 2016). PZQ is also known for its ability to control infestations of parasitic Platyhelminthes in the aquaculture industry (reviewed by Norbury et al., 2022). The impact of PZQ on parasites is noticeable and very quick, but its exact mechanism is not fully understood. Scientists consider PZQ interacts with calcium channels on the parasite's membrane, altering its permeability, which allows calcium to influx in and disrupts the calcium homeostasis (Bais & Greenberg, 2018; Park et al., 2019, 2021; Park & Marchant, 2020).

Another widely applied anthelmintic agent, Mebendazole (MBZ), was first used against *Gyrodactylus elegans* in goldfish (Goven & Amend, 1982). This ingredient is considered to disrupt the microtubules' structure and cell transport functions by binding to the microtubule subunit protein and tubulin in the cytoskeleton of the parasites (Buchmann & Bresciani, 2006; Whittington & Chisholm, 2008). However, this anthelmintic agent is ineffective for some species, such as *Dactylogyrus vastator* (Goven & Amend, 1982) and *Microcotyle* sp. (Katharios et al., 2006).

Moreover, prolonged exposure can lead to monogeneans resistant to mebendazole (Buchmann et al., 1992) and damage fish gills (Führ et al., 2012). The right dosage for effective treatment against a broad range of parasites is not specified (Whittington & Chisholm, 2008).

Both anthelmintic agents, PZQ and MBZ, have been widely implemented for the treatment of monopisthocotylean parasites in farmed fish (See **Table S3**). Even though concerns about the long-term sustainability of these treatments have been raised since the increasing development of resistance to these compounds, and the potential environmental impact of chemical treatment, applying these agents is still authorized. Due to the lack of effective vaccines, these traditional anthelmintic treatments continue to be the primary tool for controlling monogenean infections in aquaculture (Katharios et al., 2006; Schelkle et al., 2009; Woolley et al., 2022).

Recently, a novel substance, a bacterial-derived lipopeptide, called Biokos (BIO), was isolated and developed to treat parasitic infections in fish. This antiparasitic agent, released by the bacterium *Pseudomonas* H6, is highly efficient in eliminating external life cycle stages of the ciliate *Ichthyophthirius multifiliis* eliciting white spot disease in freshwater fish (Al-Jubury et al., 2018; Korbut et al., 2022; Li et al., 2022; Marana et al., 2023) and *Cryptocaryon irritans* in marine fish (Watanabe et al., 2023). The compound also has effective attack points on other species of ciliates within the genera *Ambiphrya*, *Chilodonella*, *Tetrahymena*, and *Trichodina*. In addition, killing effects were also detected in some non-ciliated parasite species within the flagellate genus *Ichthyobodo* and the amoeba genus *Vannella* (Jensen et al., 2020), and even for oomycetes such as *Saprolegnia* sp. (Liu et al., 2015). BIO by its strong surfactant properties, disrupts and destroys the cell membrane integrity in some parasites (Marana et al., 2023), which is achieved even in monogeneans (Holzer et al., 2020).

2.4.2. Herbal-based treatments

Over the last two decades, the usage of herbal remedies as effective and potent treatments for diseases in aquaculture has gained significant attention. Most studies have focused on the

immunostimulatory effects of herbal substances that improve fitness, enhance immune responses, and help the fight against infections (see Doan et al., 2020). Herbal-based products exhibit a range of advantageous therapeutic properties, including antibacterial, antiviral, and antiparasitic effects via bioactive compounds such as alkaloids, terpenoids, saponins, and flavonoids (Pandey et al., 2012; Reverter et al., 2014). However, studies investigating the anthelmintic effects of herbal-based treatments in fish remain relatively scarce. Despite this, some studies have demonstrated the positive impact of herbs, particularly in their role as anti-monopisthocotylean agents (See **Table S4**).

The specific mechanisms of garlic *Allium sativum* (GAR) in suppressing the monogeneans are unknown. However, studies have confirmed that GAR has positive immunological effects, improving the fish's immune response against parasites. Herbal treatment by GAR is well-known, one of its immunologically most active components called allicin, is capable of inhibiting RNA and protein synthesis in bacteria. It also could enhance the host immunological response by increasing the phagocytic activity (Nya & Austin, 2009), raising activation of scavenging hydroxyl radicals, superoxide anions (Kim et al., 2001), glutathione peroxidase, superoxide dismutase, and catalase (Metwally, 2009). Besides, S-allyl cysteine sulfoxide, an antioxidant found in GAR, effectively controls lipid peroxidation by boosting the antioxidant capacity (Augusti, 1977). Additionally, lectin, a protein found in abundance in GAR, can activate the fish's complement cascade and boost phagocytic activity by attaching to bacterial cells (Lee & Gao, 2012). Such positive immunological effects of GAR compounds are considered to play a significant role in countering parasitic infections through the putative inhibition of RNA and protein synthesis in parasite cells similar to their antibacterial activity (Nya & Austin, 2009). Nevertheless, some studies indicated that high dosages and/or extended treatments may negatively affect fish health and behaviour, highlighting the need to optimize dosage for routine GAR administration, keeping in mind specific host-parasite relation (Chitmanat et al., 2005; Schelkle et al., 2011).

Ginger *Zingiber officinale* (GIN) contains polyphenolic compounds including eugenol, gingerdiols, gingerols, shogaols, and zingerone, that may contribute to its therapeutic properties such as antibacterial, antioxidant, anti-inflammatory, anti-tumor-promoting, and anti-angiogenic activities (Kim et al., 2005; Singh et al., 2008). GIN has been proven to be a medicinal plant that can be used to deal with aquaculture diseases. El-Sayed and El-Saka (2015) reported that GIN has substantial anthelmintic activity against *Toxocara canis*, *Angiostrongylus cantonensis*, *Dirofilaria immitis*, *Hymenolepis nana*, *Schistosoma mansoni*, *Anisakis simplex* both *in vitro* and *in vivo*, and antiprotozoal activity against *Giardia lamblia*, *Blastocystis* sp., *Trypanosoma brucei* and *Toxoplasma gondii*. The efficacy of GIN solution against different fish parasites was studied using oral and bath treatments (Abo-Esa, 2008; Khalil & Houseiny, 2013; Levy et al., 2015; Fu et al., 2019). Unfortunately, studies describing the effectiveness of this herb against monogeneans are very limited. Trasviña-Moreno et al. (2019) found that GIN has superior efficacy compared to other herbal treatments against all life stages of *Neobenedenia* sp. under *in vitro* conditions. Similar results were reported by Levy et al. (2015), where GIN demonstrated anthelmintic activity against *G. turnbulli* under *in vitro* and *in vivo* conditions. Such anthelmintic activity by GIN may be partly attributed to polyphenolic compounds, though further research is needed to assess the mechanisms of these active biological substances that affect monogeneans in fish.

Neem *Azadirachta indica* is another herb that can be powerfully applied to strengthen resistance against parasite infections in aquaculture (Banerjee et al., 2014; Kumari et al., 2019). Each part of the neem plant such as fruits, seeds, leaves, bark and roots, are rich in bioactive compounds such as azadirachtin A and B, nimbin, salannin and similar chemicals. They have proven antiseptic, antibacterial, antiviral, antipyretic, anti-inflammatory, antiulcer, antifungal and antiparasitic effects (Isman, 2006; Girish & Bhat, 2008; Aslam et al., 2009; Kumar et al., 2012a, 2012b; Kavitha et al., 2017). The neem extract has been used as an antiparasitic drug in fish treatment for infestations by *Argulus* sp. (Kumari et al., 2019, 2021; Banerjee et al., 2014), *Lernaea* sp. (Raghavendra et al., 2020), leeches (Venmathi Maran et al., 2021), *Caligus* sp. (Khoa et al., 2019).

While no studies have examined the usage of Neem bark (NMB) as an anti-monogenean agent, aqueous extracts derived from the plant leaves were investigated. Neem leaf extract was able to control the infestation of *Dactylogyrus* sp. in Nile tilapia *Oreochromis niloticus* (Radwan et al., 2024). Also, it showed high and moderate antiparasitic efficacy against *Diplectanum* sp. in *Dicentrarchus labrax* (Aly et al., 2022), and *Dactylogyrus* sp. and *Gyrodactylus* sp. in *O. niloticus*, respectively (Suryani & Arya, 2017).

2.4.3. Treatments against Thaparocleidus vistulensis

Since Papp (1955) first reported the emergence of *T. vistulensis* on European catfish in Hungary, and Molnár (1968, 1980) proved its pathogenicity experimentally and described the histological impact of the infections; however, no effective treatments have been determined to prevent *T. vistulensis* infection. The only notable treatment reported was by Antalfi (1958), who published the use of potassium bichromate ($K_2Cr_2O_7$) and ammonium hydroxide (NH_4OH) solution in a short bath treatment for infected catfish.

Székely and Molnár (1990) explored several perspective methods for controlling *T. vistulensis* infections. For instance, they employed a bath treatment with Jaczó's Solution, consisting of 100 mg/L $K_2Cr_2O_7$ and 62.5 mg/L NH_4OH, for one minute. This mixture revealed promising results, reducing infection prevalence by 80%. However, upon applying components of the solution separately, only ammonium hydroxide effectively reduced the parasite number, while potassium bichromate had no effect. Furthermore, Székely and Molnár (1990) investigated the efficacy of antiparasitic agents such as PZQ and MBZ against *T. vistulensis* at various concentrations and treatment durations. Unfortunately, these chemicals were not fully effective in eliminating all parasites attached to the gills of European catfish. Other related drugs, including albendazole sulfoxide, levamisole, and toltrazuril, had no significant effect on *T. vistulensis*. To date, no herbs have been evaluated for efficacy against *T. vistulensis*.

CHAPTER 3

MATERIALS AND METHODS

3.1. Source of fish and parasites

European catfish specimens, naturally infected with *Thaparocleidus vistulensis*, were obtained from a local commercial fish farm, served a source of parasites for the experiments. Fish were transported in oxygenated water to the Veterinary Medical Research Institute, Budapest (HUN-REN VMRI) and kept in 60-L flow-through tank system at 23±1 °C. On arrival, the infected catfish were anaesthetized with clove oil (Javahery et al., 2012), and small samples of the gills were taken using surgical scissors at the first gill arch to confirm the presence of gill parasite, *T. vistulensis* and obtain a rough estimate of their abundance (Gussev, 1983; Bognár et al., 2024). Some monopisthocotyleans were freshly recovered and studied alive. Adult parasites were identified as *T. vistulensis* following morphological criteria as described by Siwak (1932) and Bychowsky and Nagibina (1957). Some infected catfish were sacrificed, and the excised gill arches were preserved in 80% ethanol and 5% formalin, for further use.

3.1.1. Maintaining Thaparocleidus vistulensis in laboratory

Infection was maintained by several methods including 1) Co-habitation, 2) Egg collection by Petri dishes, and by 3) Frame-stretched mesh. Co-habitation were performed by cohabit the naive fish in separated cage with the heavily infected fish in same water body for 2–3 days, then they were transferred into a parasite-free new aquarium, according to the method of Hutson et al. (2018) (**Figure 5**). For the second method, glass Petri dishes were deployed in a tank with highly infected fish for overnight (**Figure 6**). Then these Petri dishes were removed from the tank. The water inside the dishes were replaced with filtered (22 µm pore size) tank water, and eggs that were attached to the bottom of the plate were observed. When the eggs hatched, the oncomiracidia were transferred to the new tank with naive catfish. The final method utilized frame-stretched mesh based on a concept similar to using a glass Petri dish (**Figure 7**). The 70 µm mesh was attached to a square frame, enhancing its capacity to capture eggs.

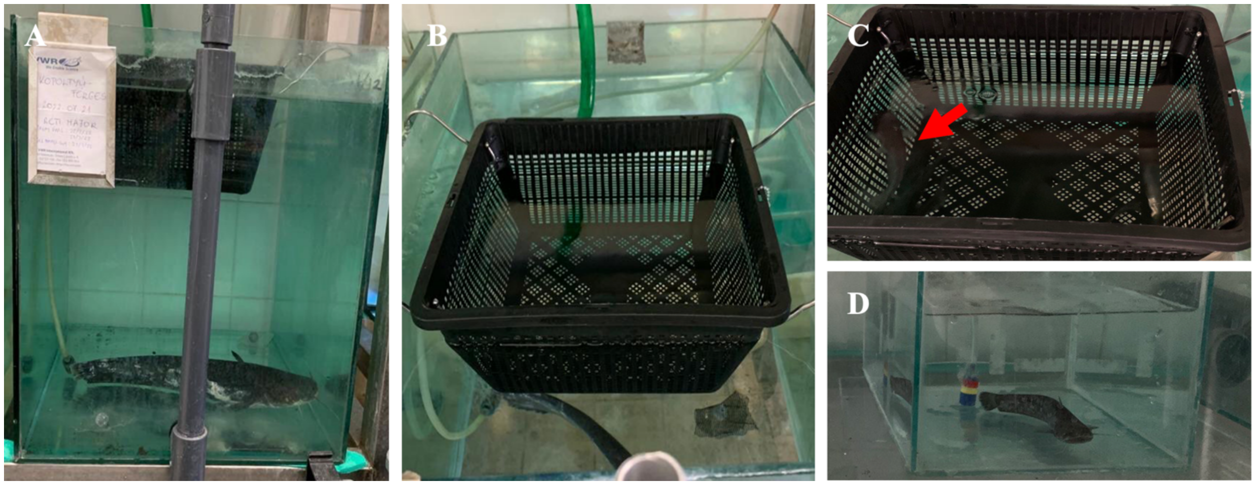

Figure 5. Maintaining *T. vistulensis* laboratory stock using co-habitation method. (A) Heavily infected fish; (B) Empty cage; (C) Naive catfish inside cage (red arrow); (D) Newly infected catfish.

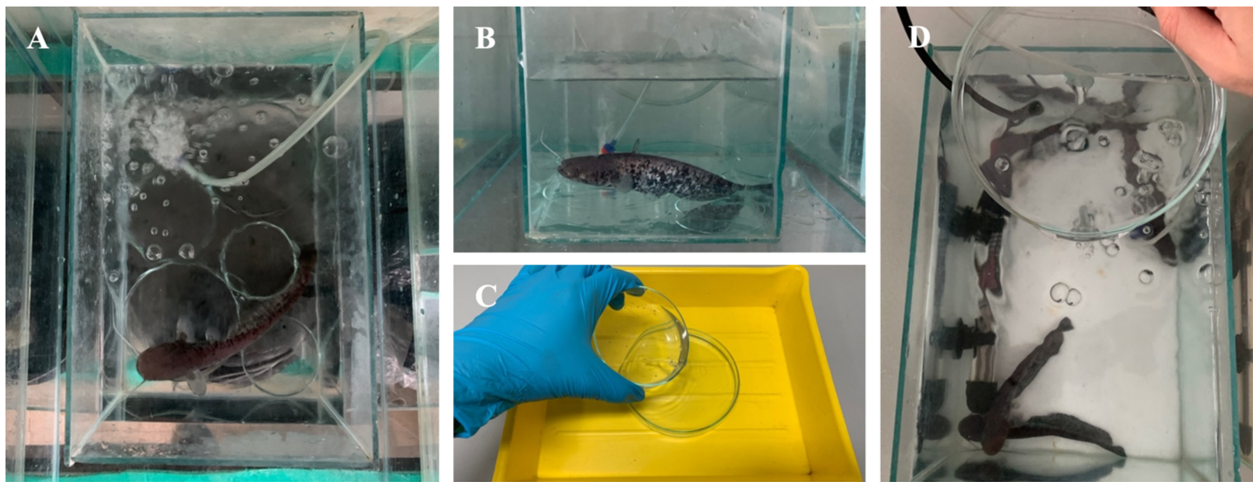

Figure 6. Maintaining *T. vistulensis* laboratory stock using glass Petri dish method. (A) Petri dishes deployed in a heavily infected fish tank; (B) Side view; (C) Water change in Petri dish after overnight incubation in the infected tank; (D) Infecting naive catfish with oncomiracidia.

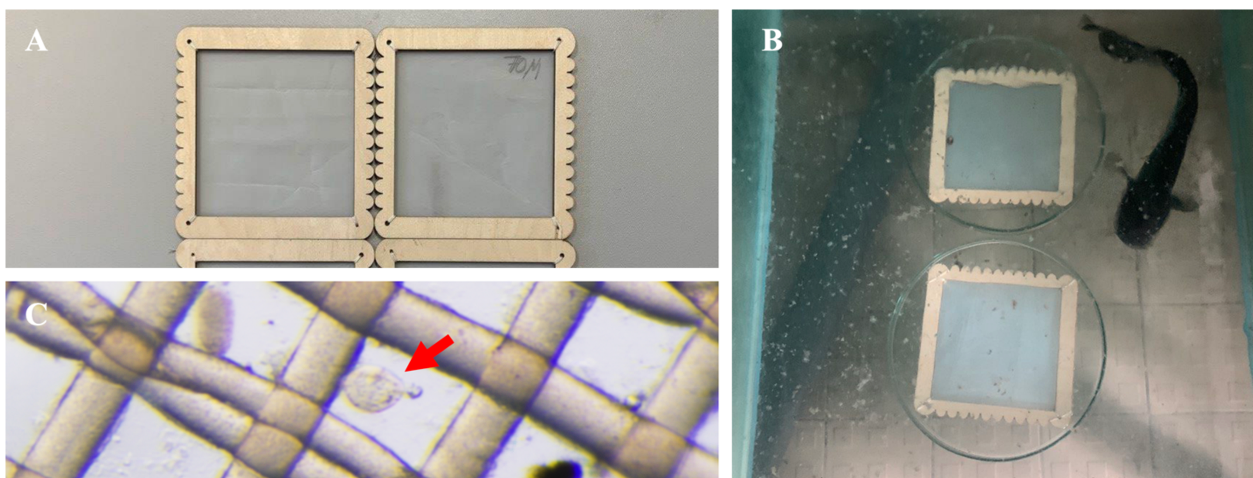

Figure 7. Maintaining *T. vistulensis* laboratory stock using frame-stretched mesh. (A) Frame-stretched mesh attached with 70 µm mesh net; (B) Frame-stretched mesh deployed in heavily infected catfish tank; (C) Parasite egg attached to the mesh net (red arrow).

3.2. Molecular identification

To confirm the morphological-based taxonomic classification of the parasite species being studied was correct, molecular investigation was performed through the polymerase chain reaction (PCR) and sequencing. Adult worms were directly obtained from the gills of the host using surgical scissors and separated using modified insect pins under a stereo microscope (Olympus SZ40, Olympus Optical, Tokyo, Japan).

3.2.1. DNA isolation, PCR and sequencing

Adult monopisthocothyleans that were preserved in 80% ethanol were used. The DNA of the specimen was extracted using a QIAamp® DNA Mini Kit (250) (cat. no. 51306, Qiagen, Denmark) according to the manufacturer's protocol with a final elution volume of 50 µl. The extracted DNA (2 µl) was subsequently quantified using a NanoDrop (ND-1000) spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The Internal Transcribed Spacer (ITS) ribosomal DNA (rDNA) fragment encompassing ITS1, 5.8S, ITS2 and flanked with 18S and 28S rDNA genes were amplified with primers PDG_18S_F5 (5'— CGA TAA CGA ACG AGA CTC—3') (in house primer) and NLR1270 (5'—TTC ATC CCG CAT CGC CAG TTC—3') (Bartošová et al., 2009). The PCR amplification was performed on a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) with a total volume of 60 µl reaction mixture containing 6 µl sample DNA, 6 µl for each primer (10 mM), 6 µl 10 × NH_4 buffer, 1.8 µl $MgCl_2$ (50 mM), 0.6 µl DNA polymerase 5 U/µl (cat. no BIO-21060, Nordic BioSite, Denmark), 6 µl dNTP's (10 mM) (cat. no. 4303442 Thermo Fisher Scientific, Denmark), and UltraPure™ DNase/RNase-Free Distilled Water (cat. no. 10977049, Thermo Fisher Scientific, Denmark) (Zuo et al., 2021). The PCR reaction conditions were 5-min at 94 °C for initial denaturation, followed by 45 cycles of denaturation at 94 °C for 30 sec, annealing at 54 °C for 30 sec, extension at 72 °C for 2.5 min, with a final extension step at 72 °C for 7 min, and an indefinite hold at 4 °C. The PCR product was estimated to be 2694 bp. The PCR product was separated by gel electrophoresis in 1.5% agarose (cat. no. 10264544, Thermo Fisher Scientific, Denmark) Tris–acetate-EDTA (TAE) gel

containing ethidium bromide stain alongside 5 µl of a 50 bp DNA Hyperladder™ (cat. no BIO-33040, Nordic BioSite, Denmark), and the amplified DNA fragment was visualized under Azure 200 Gel Imaging Workstation (Azure Biosystems, Dublin, California, USA). The PCR product was purified using Illustra™ GFX™ PCR and Gel band purification kit (VWR International, Denmark), and sequencing was performed at Macrogen Europe BV (Amsterdam, Netherlands) using the same PCR primers. Sequences obtained were analyzed using CLC Main Workbench v20.0.4 software (Qiagen, Denmark). Afterwards, the sequence was queried by BLAST analysis at the National Center for Biotechnology Information (NCBI) platform. The sequence was then submitted to GenBank.

3.2.2. Phylogenetic analysis

For the phylogenetic analysis and molecular comparison, 20 sequences of related *Thaparocleidus* spp. were selected based on their availability in the International Nucleotide Sequence Database Collaboration (INSDC) (**Table S5**). Sequences were aligned using ClustalW algorithm in Molecular Evolutionary Genetics Analysis (MEGA) 12 (Kumar et al., 2024). The phylogenetic tree was performed by the maximum likelihood (ML) method using the general time-reversible (GTR + G + I) substitution model according to the Akaike information criterion (AIC) in MEGA 12 (Nei & Kumar, 2000). Bootstrap analysis with 1000 replicates was applied to estimate nodal support. The analysis involved 25 nucleotide sequences with a total of 706 positions in the final data sets. Sequences of *Ligophorus llewellyni* (JN996858), *Ligophorus chabaudi* (JN996868), and *Ligophorus macrocolpos* (JN996855) were used to root the phylogenies. Level of sequence variation based on uncorrected pairwise distance (*p*-distance) was calculated using MEGA 12 (Kumar et al., 2024).

3.3. Morphological analysis

To study the detailed morphology of the parasites, morphometric measurements were conducted on *T. vistulensis* that were preserved in 80% ethanol. Some other parasites that preserved in 5% formalin were processed for scanning electron microscopy (SEM) to further study the external

morphology of the species. The morphological analysis also included an assessment of parasite-induced tissue damage through the histopathological investigation. All measurements were measured in micrometers (µm).

3.3.1. Parasite preparation

Some monopisthocotyleans were softened and cleared in a mild enzymatic digestion proteinase K, modifying the method described by Harris and Cable (2000), before mounted individually in 1–2 drops of glycerine-ammonium-picrate on a slide (depending on the size of specimens) (Malmberg, 1957). The preparation was then covered with a coverslip. Some specimens were stained using hematoxylin (Harris' modified solution, Sigma-Aldrich HHS32), mounted on a glass slide using AQUA-TEX® (cat. no. HC568794, Merck), and covered by a coverslip. Photomicrographs were performed using a digital camera (Leica MC170 HD) with LAS V4.12 software equipped with a light microscope (Leica DM5000B Microscope W/CTR5000 Controller).

3.3.2. Morphometric measurements

Line drawings of the parasite's important part (e.g., the sclerotized structure of the haptor and the male copulatory complex) were made based on the photomicrographs using Inkscape (Free software Foundation, Inc., MA, USA). The measurements were made and analyzed based on the captured images using the scientific image analysis tool – ImageJ 1.53t software (RRID: SCR_003070). The morphological parameters of *T. vistulensis*, including the sclerotized structure and male copulatory organ, were measured as proposed by Gussev (1962) and Paladini et al. (2008) (**Figure 8** and **9**). These measurements (**Table S6**) were presented as mean with standard deviation followed by the range in parenthesis, provided in µm. Measurements were conducted of adult parasites: total body width and length (n = 10), each of attachment structures (n = 20), marginal hooks (n = 40), and copulatory organs (n = 5). Structures of oncomiracidia (n = 5) (except for marginal hooks) were measured as well. Data obtained from this study were compared with the previous descriptions by Siwak (1932), Bychowsky and Nagibina (1957) and Paladini et al. (2008).

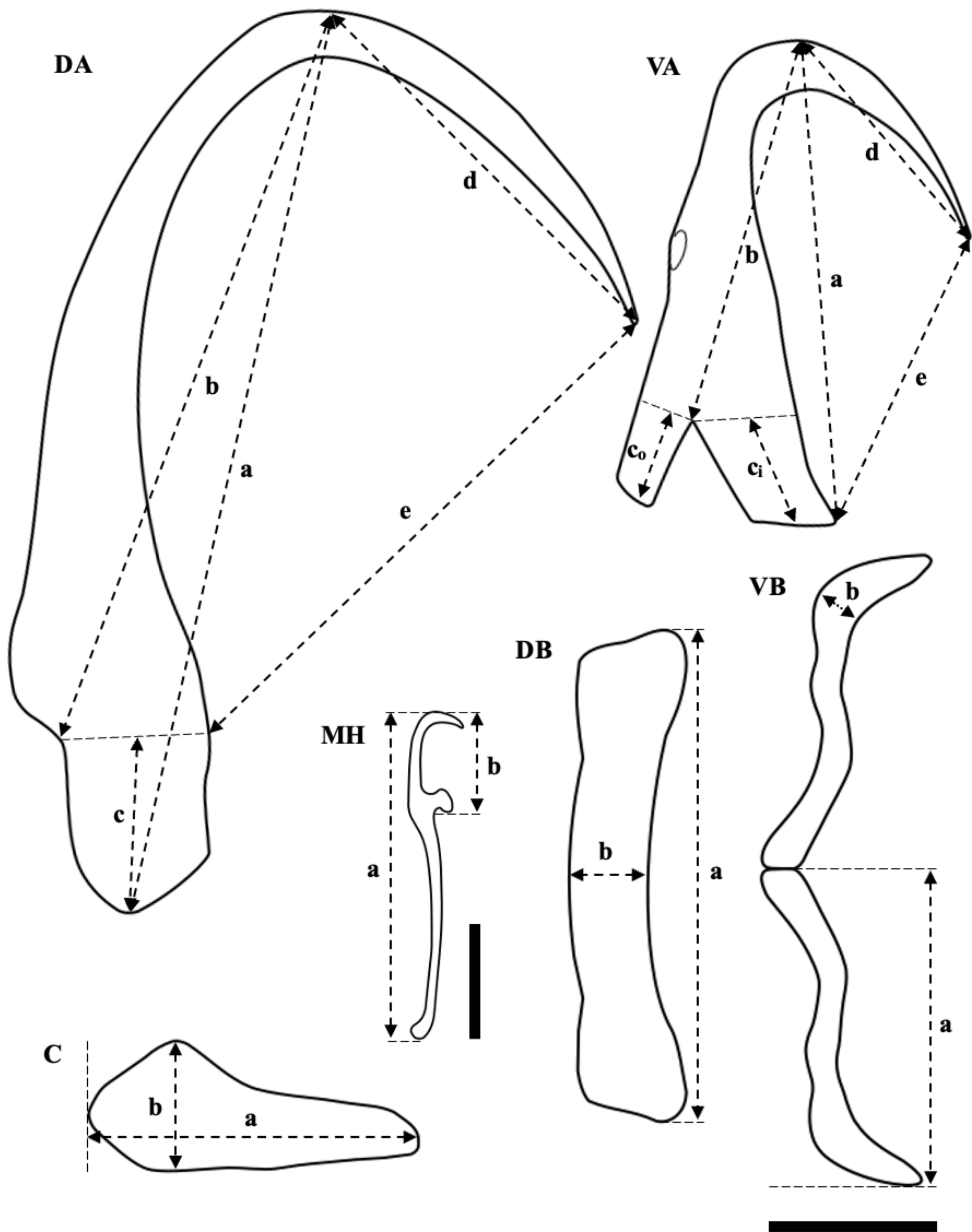

Figure 8. Metric parameters of the *T. vistulensis* attachment apparatus used in this study. Abbreviations: C, cuneus (a, total length; b, largest width); DA, dorsal anchor (a, total length; b, shaft length; c, root length; d, point length; e, aperture); DB, dorsal bar (a, total length; b, width in the middle); MH, marginal hooks (a, total length; b, sickle length); VA, ventral anchor (a, total length; b, shaft length; ci, inner root length; co, outer root length; d, point length; e, aperture); VB, ventral bar (a, length of one branch; b, largest width). All parts of anchors refer to a scale bar of 10 µm, except for the marginal hooks, with a scale bar of 5 µm. The terminology and methodology of measurements are according to Gussev (1985), Lim et al. (2001) and Paladini et al. (2008).

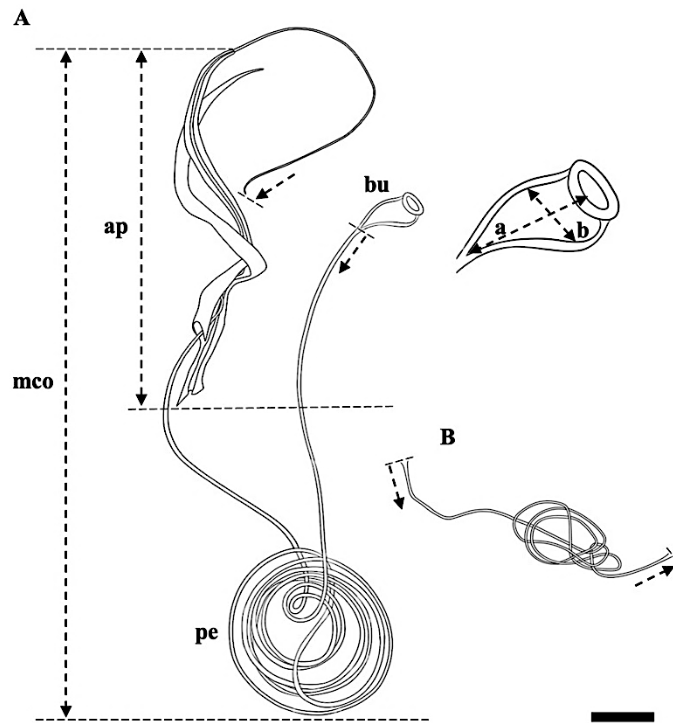

Figure 9. Measurements of the male copulatory organ. (A) Male copulatory organ; (B) Vaginal duct. Abbreviations: ap, total length of accessory piece; bu, bulbous base (a, total length; b, largest width); mco = distal end points of the male copulatory organ; pe, total length of penis (|→ = starting point, →| = ending point). Scale bar represents 20 µm.

3.3.3. Scanning electron microscope (SEM)

The parasites that were previously preserved in 5% formalin were transferred in Karnovsky's fixative (4% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer) for 15 min, then rinsed in 0.1 M sodium cacodylate buffer two times for 20 min, before immersed in demineralized water two times for 15 min. The samples were then dehydrated through the ascending concentrations of ethanol series (20, 30, 50, 70, 90, 90, 100, and 100%) for 15 min per treatment, except 100% (30 min per treatment). Samples were subsequently dried by passing into 100% hexamethyldisilazane (HMDS) (cat. no. 52620, Fluka) for 30 min and set aside in a fume hood overnight. The dehydrated samples were attached to a strip of carbon conductive double-sided tape that was fixed to an SEM aluminum stub. Then, the samples were sputter coated with gold in Leica EM ACE200 Vacuum Coater (Leica, Wetzlar, Germany) with a thickness of 5–10 nm and examined in an FEI Quanta 200 SEM (FEI Company, Hillsboro, Oregon, United States) operating at 3–8 kV acceleration voltage, using xT Microscope Control software.

3.3.4. Pathological effects of Thaparocleidus vistulensis infection on the gills

This subsection examined the attachment of *T. vistulensis* on European catfish gills using SEM, as described in subsection 3.3.3, while also documenting the histopathological effects of *T. vistulensis* on the gill tissues. Quantitative data analysis was not performed due to the descriptive nature of the research. Observations were documented using imaging and descriptive techniques to provide a comprehensive account of the parasite and its impacts.

3.3.4.1. Histology

Formalin-fixed gill arches were processed using standard histology techniques: they were dehydrated in a graded ethanol series (70, 96, 100%), cleared in xylene and embedded in paraffin, which was then sectioned using a Leica RM 2135 (Nussloch, Germany) microtome at 3–5 µm. The paraffin-embedded sections were placed on glass slides and dried at 40 °C for 24 h. After deparaffinizing in xylene and rehydration in a graded ethanol series, the sections were stained with haematoxylin and eosin (H&E) and Masson–Goldner trichrome staining before mounted in a DPX histology medium. Stained sections were examined using different magnifications under the light microscope (Olympus BX53) and photomicrographs were captured with a digital camera (Olympus DP74).

3.4. Reproductive strategies of *Thaparocleidus vistulensis*

To study the reproductive strategies of the parasitic monopisthocotyleans, eggs and oncomiracidia life stages were obtained following the methods described in subsection 3.1.1, while developing juvenile, and adult parasites were isolated directly from the sacrificed infected host's gills using modified insect pins under a stereo microscope (Olympus SZ40). Parasite-free fingerling catfish were obtained from a commercial fish farm, and the absence of the monopisthocotyleans (infestation) in these fish were confirmed by parasitological examination before experimental start. Infection and experimental studies were performed at 23 ± 1 °C, and all life stages of the parasite were collected and maintained in filtered (22 µm pore size) fish tank water.

3.4.1. Infection dynamics

A total of 30 European catfish fingerlings with a body weight of 6.35 ± 1.84 g and body length of 9.53 ± 1.16 cm were used in three exposure studies. Fingerlings catfish (*n* = 10/trial) were cohabited with infectious donor fish (*n* = 1/trial) (with different infection levels – roughly estimated by the Gussev, 1983 method). The estimated number of monopisthocotyleans on the gills of donor fish for the First Trial, Second Trial, and Third Trial were <100, <200, and >500, respectively. To determine the intensity of *T. vistulensis* infection throughout the course of infection, two fish were sacrificed every two days during the 10-day experimental period. The fish were weighed and euthanized by decapitation. The branchial baskets of the sacrificed fish were removed from both sides, and the gill arches were separated. The flukes found on all lamellae were counted individually under a stereo microscope at 6.7–40× magnification. The number of monopisthocotyleans found was recorded, and photos were taken using a high-resolution microscope equipped with a digital camera (Olympus DP74).

3.4.2. Parasite egg development

Monopisthocotyleans were isolated from the gill filaments as described earlier. Gravid specimens of *T. vistulensis* were observed releasing eggs when they were gently disturbed by vigorous shaking in a cavity block containing filtered water from a fish tank. The eggs were observed and the size were measured (*n* = 30) in µm. Thereafter, the eggs were randomly distributed in groups (*n* = 5–10) on concave glass microscope slides, 200 µL of filtered water from the fish tank was added, a cover-slip applied whereafter the slide was stored at 23 ± 1 °C in a humid chamber to prevent evaporation. The time of oviposition was set to "Time 0". The time periods are indicated below as hours post-oviposition (hpo) or days post-oviposition (dpo). The eggs were observed daily under a compound microscope (Olympus BX53) until hatching. Any changes in their morphology were documented and photographed using a high-resolution digital camera (Olympus DP74) mounted on the compound microscope.

3.4.3. *In vitro hatching rates*

The hatching rate was determined as an important parameter of the egg development process. *T. vistulensis* eggs were collected as previously described on glass Petri dishes by placing them overnight into tanks of heavily infected fish stock. A total of 445 eggs were gathered with a modified butterfly needle and glass Pasteur pipette and immediately distributed into the wells of a 96-well microtiter plate, each containing 100 µL of filtered fish tank water, and subsequently observed daily under a stereo microscope. A quick observation that involved checking the signs of viability (i.e., shape and colour of eggs) was confirmed prior to the transfer, and abnormal eggs were excluded. Water exchange or agitation was not applied (Whittington & Kearn, 1988). Hatching rates were recorded daily. These periods are expressed below as days post-oviposition (dpo). Hatching success (the percentage of hatched eggs of all the observed eggs) was determined by counting the number of empty eggshells with the operculum open. The assay was terminated 24 h after the last eggs hatched.

3.4.4. *In vitro survival rates*

In these trials, the *in vitro* survival rates of *T. vistulensis* in different life cycle stages (oncomiracidium, developing juvenile and adult) (without host) were observed. To achieve this, oncomiracidia (time periods are indicated below as days post-oviposition (dpo)) were obtained by hatching eggs in glass Petri dishes, while developing juvenile (4–6 days post-infection (dpi)) and adult (>10 dpi) monopisthocotyleans were collected directly from the separated gills arches with modified insect pins and butterfly needle. Developing juveniles (n = 204), adults (n = 153) and oncomiracidia (n = 135) were transferred into the wells of a 96-well microtiter plate and maintained at 23 ± 1 °C. The wells contained 50–100 µL of filtered (22 µm pore size) fish tank water. Intact parasites were carefully selected, and those that died or presented any signs of trauma within 1 h of distribution were eliminated prior to the assay. The microtiter plates were held under a stereo microscope, and the activity of the monopisthocotyleans, including death was observed and recorded daily. Active monopisthocotyleans were defined as those that swam (for

oncomiracidia), showed normal movements with their anterior end, or exhibited a regular vigorous longitudinal contraction. These parasites were transparent and not swollen. Moribund monopisthocotyleans rarely moved spontaneously, but responded to stimulation (when gently touched with the side of a dissecting needle) with slow contractions. Immobile monopisthocotyleans, which occasionally were swollen, and repeatedly failed to respond to small nudges with a needle (Grano-Maldonado et al., 2011), were considered dead.

3.5. Influences of environmental conditions against *Thaparocleidus vistulensis*

To study the effect of environmental conditions against different life stages of *T. vistulensis*, a light-dark cycle and varying water temperatures were applied. The light-dark cycle was set to mimic natural day-night transitions, while water temperatures were adjusted to simulate different environmental conditions.

3.5.1. Study of the influence of light-dark cycle

Throughout the experiments for observations low-intensity light was used under the stereo microscope to minimize photic interference, and exposure durations were limited to approximately 30 sec to reduce the additional impact of light on reproductive processes.

3.5.1.1. Fecundity and survival rates of adult parasites

To investigate the impact of light and darkness on fecundity and survival rate, adult *T. vistulensis* specimens ($n = 20$; 2 × [10 wells × 1 individual/well]) were collected and divided into two groups. One group was subjected to natural light exposure (16:8 [L:D]), while the other group was kept in constant darkness (0:24 [L:D]). Due to the small experimental volumes, the constant temperature of 23 °C was maintained and stabilized by room heating and monitored by measuring the ambient temperature with a thermometer. The parasites were then distributed individually in wells of the 24-well microtiter plates containing 2 ml of filtered (22 µm pore size) fish tank water. The number of eggs laid was recorded at intervals of 1, 2, 3, 4, 5, 6, and 24 h under a stereo microscope (Olympus SZX16). The survival ability of the adult *T. vistulensis* was observed at 24-h intervals

until all the individuals died. Individuals that remained motionless, consistently failed to respond to gentle nudges with a needle and occasionally appeared swollen were considered dead (Grano-Maldonado et al., 2011).

3.5.1.2. Egg hatching rates

To determine whether light and darkness affect the hatching rates of *T. vistulensis* eggs, the eggs (*n* = 300) were collected and evenly distributed into wells of 96-well microtiter plates containing 200 µl of filtered water from the fish tank. Two experimental groups (2 × [5 wells × 10 eggs/well, in triplicate]) were set up for hatching observation; one was exposed to a natural photoperiod cycle, and the other was kept in constant darkness. Both groups were maintained at a constant room temperature of 23 °C, with no water exchange or agitation applied (Whittington & Kearn, 1988). The eggs were observed under a stereo microscope at 24-h interval. The hatching rates were recorded daily until no further hatch events occurred within 24 h of the last observed hatching. Hatching rates were determined by counting the number of empty eggshells with an operculum open.

3.5.1.3. Survival rates of oncomiracidia

To evaluate the influence of light and darkness on the survival rate of oncomiracidia, *T. vistulensis* larvae (*n* = 90; 2 × [5 wells × 3 individuals/well, in triplicate]) were harvested and allocated into wells of 96-well microtiter plates containing 100 µl of filtered water from the fish tank. The experimental conditions were the same as described in the subsection 3.5.1.2. The observations were done under a stereo microscope at a 24-h interval until all individuals had died. The criteria for determining the death of oncomiracidium were the same as described for the adult parasites.

3.5.2. Study of the influence of water temperature

The experiments for the study of the effects of water temperature on oviposition, hatching, and survival of oncomiracidia and adult parasites were investigated under constant dark conditions to exclude any potential masking effects of light exposure. The temperature was varied between 5

and 35 °C, and observations were executed at regular intervals of 1, 2, 3, 4, 5, 6, and 24 h for fecundity and at every 24 h for egg hatching and the survival rates of adults and oncomiracidia.

3.5.2.1. Fecundity and life span of adult parasites

To assess the effect of water temperature on egg production, adult *T. vistulensis* (*n* = 70; 7 × [10 wells × 1 individuals/well]) were collected and divided into seven groups, with each group being exposed to different water temperatures (5, 10, 15, 20, 25, 30, and 35 °C) for the experiment. The parasites were then distributed into wells of 24-well microtiter plates containing 2 ml of filtered (22 μm pore size) fish tank water. The plates were preconditioned with a Peltier cooled incubator (IPP 30, Memmert, Germany) set to the appropriate water temperatures. The number of eggs laid was recorded at intervals of 1, 2, 3, 4, 5, 6, and 24 h under a stereo microscope. The survival rate of the adult *T. vistulensis* was observed and recorded at a 24-h interval until all the individuals in the group had died.

3.5.2.2. Egg hatching rates

To evaluate the egg hatching rate of *T. vistulensis* at different water temperatures (5, 10, 15, 20, 25, 30, and 35 °C), eggs (*n* = 525; 7 × [5 wells × 5 eggs/well, in triplicate]) were collected and allocated into wells of 96-well microtiter plates containing 200 μl of filtered water from the fish tank. The egg hatching rate was monitored and recorded at a 24-h interval under a stereo microscope, and the observation was finished 24 h after the last eggs hatched.

3.5.2.3. Survival rates of oncomiracidia

To investigate the influence of water temperature on the survival rate of *T. vistulensis* oncomiracidia, free swimming larvae (*n* = 210; 7 × [5 wells × 2 individuals/well, in triplicate]) were harvested and evenly distributed into wells of 96-well microtiter plates containing 100 μl of filtered water from the fish tank. The plates were kept at predetermined water temperatures (5, 10, 15, 20, 25, 30, and 35 °C), and were observed at a 24-h interval under a stereo microscope until all individuals had perished.

3.6. Treatment trials against *Thaparocleidus vistulensis*

All parasite life stages (egg, oncomiracidium, developing juvenile, and adult) were collected as previously mentioned. They served as a source for different parasitic stages tested in the *in vitro* tests and infecting fish in the *in vivo* trials. For preventive *in vivo* experiments, naive fish were obtained from the RAS system of a local fish farm and maintained in the same conditions as the infected ones. Parasite-free status of fish was confirmed upon arrival.

3.6.1. Antiparasitic agents

Six chemicals as potential antiparasitic agents were utilised *in vitro*; Herbs – Garlic (GAR), Ginger (GIN), and Neem bark (NMB); and Drugs – Biokos (BIO), Praziquantel (PZQ), and Mebendazole (MBZ). Only PZQ and MBZ were used for *in vivo* studies. The treatment compounds were obtained from several companies: Garlic and Ginger powder were commercially purchased from grocery stores (Lucullus products, Szilasfood Kft., Hungary), aqueous Neem bark extract (Sigma-Aldrich, Lot: BCBF6855V, Austria), Biokos powder (Biokos®, Sundew ApS, Denmark), Praziquantel powder (Hisun Pharmaceutical Co. Ltd., China), and Mebendazole tablet (Vermox, Gedeon Richter Ltd., Hungary).

3.6.1.1. Anthelmintic stock solutions for treatments

Stock solutions for the treatments were prepared the day before or on the same day of the experiment, using ambient temperature filtered water (22 μm pore size) from the fish tank. The herbal (GAR and GIN) stocks and working solutions were prepared as follows modified method based on Goswami (2021). Briefly, 0.2 g powder were dissolved in 2 ml of distilled water. The mixture was shaken at room temperature (≈20 °C) for 1 h in sterile 2 ml Eppendorf tubes. Subsequently, the tubes were heated in a 65 °C water bath for 4 h and allowed to cool at room temperature overnight, allowing the insoluble particles to settle to the bottom. The herbal solution was carefully separated from the settled particles by centrifugation at 5000 g for 5 min. Then, the supernatant was filtered through 0.45 μm filter and the filtrate was stored at 4 °C until further use. For NMB, the stock solution was directly prepared from the chemical bottle. Serial dilutions of

each stock solution were made with filtered water to dilutions of 1:10, 1:50, and 1:100 for experimental use, the control group consisting only of the filtered tank water. The working solution was initially prepared at twice the required final dilution level and was then diluted to the desired concentration when added to the wells of the microtiter plate containing parasites and water.

Stock solutions of drugs were prepared as follows: BIO (10 mg BIO powder, 950 µL ddH_2O, 50 µL 0.2 M NaOH; 10000 mg/L), PZQ (2 mg PZQ powder, 10 mL ddH_2O; 200 mg/L), and MBZ (30 mg MBZ powder, 50 mL ddH2O; 600 mg/L). All subsequent concentrations (0, 1, 5, 10, 20, 40, 60, 80, and 100 mg/L) of the stock solutions were made with filtered (22 µm pore size) tank water.

3.6.2. In vitro antiparasitic efficacy assays

3.6.2.1. Egg

The eggs used for each assay originated from the same batch. They were collected by glass Petri dishes placed overnight in a tank with heavily infected fish as mentioned previously. A total of 900 eggs (4 concentrations × [5 wells × 5 eggs/well, in triplicate] × 3 treatments) for herbal treatments, and 2025 eggs (9 concentrations × [5 wells × 5 eggs/well, in triplicate] × 3 treatments) for drug treatments, were manually transferred into the wells of a 96-well microtiter plates, using a modified butterfly needle. Each well contained 200 µL final volume of the specific compound solutions. Experiments were conducted at 23 °C. The experimental conditions and observation methods were similar to those described in subsection 3.4.3, with the exception that observations focused on changes (e.g., deterioration or hatching), which were recorded daily.

3.6.2.2. Oncomiracidia, developing juvenile and adult flukes

For herbal treatments: active oncomiracidia (*n* = 540; 4 concentrations × [5 wells × 3 individuals/well, in triplicate] × 3 treatments), juveniles (*n* = 360; 9 concentrations × [5 wells × 2 individuals/well, in triplicate] × 3 treatments), and adults (*n* = 360; 9 concentrations × [5 wells × 2 individuals/well, in triplicate] × 3 treatments), and drug treatments: oncomiracidia (*n* = 1215; 9

concentrations × [5 wells × 3 individuals/well, in triplicate] × 3 treatments), juveniles (*n* = 810; 9 concentrations × [5 wells × 2 individuals/well, in triplicate] × 3 treatments), and adults (*n* = 810; 9 concentrations × [5 wells × 2 individuals/well, in triplicate] × 3 treatments) were distributed into the wells of 96-well microtiter plate using a modified butterfly needle and a glass Pasteur pipette, where each well contained 100–200 µL final volume of the specific treatment solutions and incubated at 23 °C. The experimental conditions and observation methods were similar to those described in subsection 3.4.4, except for the observation interval, which was monitored and recorded every 15 min for the first 2 h, then every 30 min for the next hour, the once in the following hour, and every 24 hours post-treatment (hpt).

3.6.3. Toxicity test on Silurus glanis

The treatment that exhibited promising antiparasitic properties *in vitro* results (MBZ) and widely used agent for eliminating monopisthocotyleans (PZQ) were selected for toxicity test prior to further *in vivo* studies against *T. vistulensis*. The toxicity of MBZ and PZQ was estimated using a healthy juvenile European catfish adhering to the limit test protocol outlined by the Organization for Economic Cooperation and Development (OECD) (2019). Toxicity tests were performed using 60 juvenile European catfish (weight: 18.63 ± 5.88 g, length: 14.78 ± 2.34 cm) distributed in 2 experimental groups (2 drugs × 3 concentrations [including control group] in duplicates) in 60-L tanks (*n* = 5/tank). The experimental concentrations were 10, and 20 mg/L for both antiparasitic treatments. Control groups were maintained under the same test conditions. The glass tanks were filled with aerated and antiparasitic-treated water at 23.7 ± 0.5 °C, with a pH of 7.9 ± 0.1, and a dissolved oxygen content of 7.4 ± 0.5 mg/L (87.4% saturation). The water parameters were monitored using a portable dissolved oxygen meter (Hanna Instruments, Rhode Island, USA) and a pH meter (Adwa AD 12, Romania). After 24 h of bath treatment, the fish were transferred to a new aerated tank with a flow-through system. Fish were not fed during the toxicity test. The experiment was terminated once fish exhibited adverse behavioural signs of toxicity, including 1) lethargy; 2) loss of equilibrium – sinking to the bottom and tilting to one side; 3) abnormal

ventilatory (respiratory) function – hyperventilation, including increased frequency of opercular ventilatory movements, open mouth and extended operculum; and 4) abnormal skin pigmentation (lightened) – pallor (OECD, 2019). The fish condition has been observed daily until 14 dpt.

3.6.4. In vivo antiparasitic efficacy assay

The applied concentrations of MBZ and PZQ treatment for *in vivo* study were selected based on toxicity tests (see subsection 3.6.3) and were executed (1, 5, 10 mg/L) on the juvenile European catfish. The control group of fish were kept in the same condition without treatment. *In vivo* assays were performed by using 144 juvenile European catfish (weight: 22.29 ± 13.47 g, length: 15.31 ± 3.94 cm) distributed in 2 experimental groups (2 drugs × 4 concentrations [including control group] in triplicates) in 60-L tanks (*n* = 6/tank) in which artificial infection was induced with oncomiracidia (*n* = 300/tank). The infected fish were kept in aerated glass tanks with a static water system. The mean water temperature in the tanks was 22.7 ± 0.5 ºC, with a pH of 8.0 ± 0.1, and a dissolved oxygen content of 8.2 ± 0.6 mg/L (95% saturation).

The treatments were employed after 3 dpi, and no food was provided during the treatment period. The fish were subjected to a 24 h bath treatment before being transferred to a new aerated tank with a flow-through system. Two individuals from the treatment and control groups were euthanised at 1, 7, and 14 days post-treatment (dpt). The gills (all 8 arches) of the sacrificed fish were carefully excised and observed under a stereo microscope, where the number of parasites present was quantified. The estimated efficacy percentage of the treatment was calculated using the equation (Onaka et al., 2003):

$$EF\ (\%) = \left(\frac{MNPCG - MNPTG}{MNPCG}\right) \times 100$$

Where,
EF = Efficacy
MNPCG = mean number of parasites in the control group
MNPTG = mean number of parasites in the treated groups

3.7. Statistical analysis

3.7.1. Influences of environmental conditions against different life stages

Under varying light conditions and water temperatures, biological parameters such as egg production, hatching rates, and survival rates were analyzed. The data are presented as the mean ± standard deviation. Given the non-normal distribution of the experimental data groups, the Mann–Whitney test was conducted to compare egg production and hatching rates under light and dark conditions. The Kruskal–Wallis test, another nonparametric approach, was used to discern significant differences in egg production and hatching rates across various water temperatures. The Kaplan–Meier survival analysis was used to estimate the cumulative survival probability of *T. vistulensis* under different light and water temperature conditions, providing estimations of survival rates at various time intervals. The log-rank (Mantel–Cox) test was used to compare the survival curve obtained from the Kaplan–Meier survival analyses for different water temperatures and light conditions to identify significant differences in survival between groups. The statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) software, version 29.0 (IBM Corp., Armonk, NY, USA), with a P value of < 0.05 considered statistically significant.

3.7.2. Treatment trial against Thaparocleidus vistulensis

In vitro studies on egg hatching and survival rates, and *in vivo* studies on the reduction of parasites on gills were analysed. For *in vivo*, drugs and herbs treatment analysis were performed separately. The efficacy of the various treatments was compared by survival analysis in the R environment (version 4.1.2) (R Core Team, 2021) using the packages “survival 3.2.13” (Therneau, 2021) and “survminer 0.4.9” (Kassambara et al., 2021). The model used the treatment type and the number of surviving parasite individuals or egg hatching at continuous time points as predictor and response variables, respectively. Since some eggs failed to hatch, the model was used with censoring (Crawley, 2015). In case of significant differences between treatments, a pairwise multiple comparison with Benjamini-Hochberg correction (Benjamini & Hochberg, 1995) was

performed using R environment (version 4.1.2) (R Core Team, 2021). For the *in vivo* study, the analysis was conducted at three stages: 1) Compare the efficacy of the overall treatment to assess their impact on parasite reduction; 2) Evaluate the differences among concentration levels across the time points for each anthelminthic treatment; 3) Analyse parasite counts across the three time intervals at varying concentrations. Normality tests were conducted at each stage to determine the appropriate statistical method, using parametric tests (e.g., one–way ANOVA, unpaired t-test) for normally distributed data and non-parametric tests (e.g., Kruskal–Wallis H test, Mann–Whitney U test) otherwise. Post hoc pairwise comparisons were conducted using Tukey HSD and Dunn's test with Bonferroni correction selected based on their statistical appropriateness, using SPSS software, with a P value of < 0.05 considered statistically significant.

CHAPTER 4

RESULTS

4.1. Molecular analysis and phylogenetic tree

In the present study, two identical 2694 bp rDNA fragments (partial 18S, ITS1, 5.8S, ITS2 and partial 28S) were amplified from *Thaparocleidus vistulensis*. As both sequences were found to be 100% identical, only one has been deposited in GenBank (OR916383), while the other is retained in-house for internal reference. The obtained sequences were then compared with previously deposited sequences of the genus *Thaparocleidus*. The current sequence shares 96.04% identity with the *T. vistulensis* sequence identified in European catfish from the Czechia (AJ490165) and exhibits a 94.27% similarity with *T. siluri* isolated from the same host species and geographical location (AJ490164) (Šimková, et al., 2003).

The ML tree, constructed based on the rDNA sequence, robustly supports a well-defined clade that includes the previously identified *T. vistulensis* sequence (**Figure 10**). *Thaparocleidus vistulensis* forms a sister group with *T. siluri*, another parasitic species specific to European catfish. Both *T. vistulensis* and *T. siluri* clustered with *T. varicus* and *T. mutabilis* in a distinct branch, and high bootstrap values strongly support this grouping. The sequence variabilities (uncorrected *p*-distance) within genus/species group with *T. vistulensis* were 86.1–99.8% (**Table S7**). Molecular analyses provide compelling evidence that the monopisthocotylean species examined in this study can be confidently attributed to *T. vistulensis*.

4.2. Morphological description

4.2.1. External morphology

The body is elongated and assumes a cylindrical form but tapers towards the posterior end and terminates with a slightly wider and non-segregated caudal disc (**Figure 11A** and **12A–B**). The anterior part of the body is extended and flattened, with two pairs of eyespots dorsally, and four pairs of head organs with cephalic glands (**Figure 11B–C**). The eyes, an aggregation of dispersed

pigment spots, are located along the vertices of a trapezoid. Adjacent to these pigment spots, positioned at a slightly oblique angle, transparent and intensely refractive corpuscles are discernible (**Figure 11C**). The mouth opening is subterminal, and the pharynx, is roundish or short oval, located ventrally in the region behind the eyes (**Figure 11C**). The widening haptoral region (**Figure 11G**) has two pairs – dorsal and ventral – of strong anchors (**Figure 11H–I** and **12C–D**). The dorsal ones have well-developed inner roots and less prominent outer roots. The ventral anchors have a smaller size, having well-developed inner and outer roots. The dorsal anchors are connected with a straight dorsal bar, while the ventral ones are connected with a V-shaped ventral bar. A small sheet, called a cuneus, was observed joining the inner roots of the dorsal anchors. Both dorsal and ventral anchors are pointed in opposite directions (**Figure 11K–L** and **12A–C**). The haptor comprises 14 small marginal hooks (**Figure 11J** and **12E**). All the haptoral sclerites are provided with fine chitinous stirrups. The haptoral sclerites gripping the gill lamellae of their host can be seen in **Figure 13A–D**.

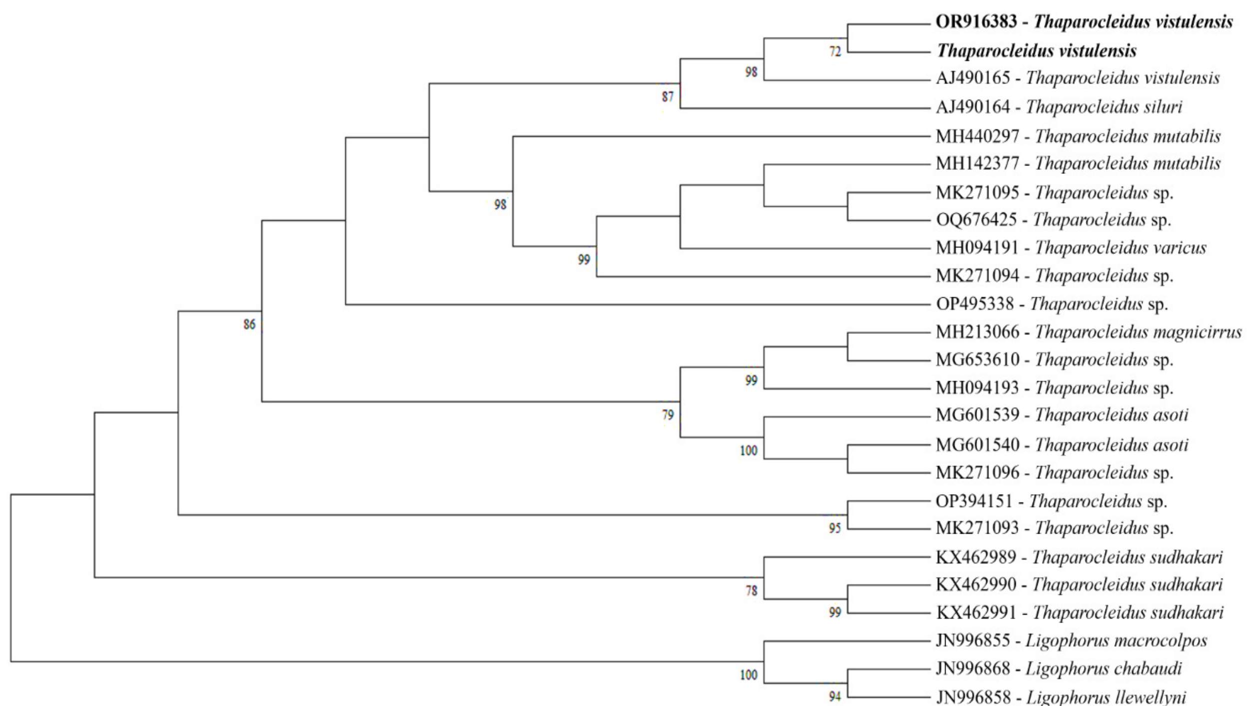

Figure 10. A phylogenetic tree based on the rDNA sequences demonstrating the positions of *T. vistulensis* with other *Thaparocleidus* species. The tree was generated by the ML method and rooted to *Ligophorus* spp. as an outgroup. Numbers at nodes indicate ≥70% bootstrap values (1000 replications). Species names are listed along the INSDC accession numbers. Species examined in this study are shown in bold.

Oncomiracidia measure 167.3 ± 7.6 (157.5–177.6) in length and 72.5 ± 4.2 (65–78.4) in width. They have 14 marginal hooks with a length of 15.7 ± 0.7 (14.1–16.7), which are about the same size as those of matured worms. The sickle length was 5.1 ± 0.2 (4.7–5.6). Only an underdeveloped pair of ventral anchors was found in the attaching disc. Their length proved to be 21 ± 1.3 (19.2–22.7), with 2.6 ± 0.1 (2.5–2.8) inner root and 2.6 ± 0.2 (2.4–2.8) outer root.

4.2.2. Internal morphology

Vitellaria are densely dispersed throughout the trunk except in the region of reproductive organs. The testicle is located at the posterior part of the body and is connected with the vas deferens to the seminal vesicle. The seminal vesicle is single, blind-bottomed. The male copulatory organ starts with a flask-shaped bulb usually facing to the right of the ventral body position and connected to a long sclerotized penis tube (**Figure 11D**). The flask-shaped bulb has 13.9 ± 0.6 (13.4–15.0) in length and 8.2 ± 1.1 (7.2–10.0) in width. The penis usually has 5–7 loops in the middle of its length before joining the penis accessory. The total length of the penis was 837.4 ± 95.9 (703.6–940.9). The accessory piece of the copulation organ is a sclerotized, open V-shaped structure, composed of a trough-like basal part which receives the penis. Its total length measures 97.0 ± 7.8 (92.5–110.9). In its middle where the accessory part turns to be V-shaped, it splits into two parts. One supports the penis, while the other has an elongated, slightly bent, and somewhat hook-like part that runs parallel before curving back towards the first. It was often observed the penis running free on a short section after leaving the accessory piece. The germarium (**Figure 11E**) is anteroventral to the testis (**Figure 11F**). Inside the germarium, large ovules with clear nuclei and nucleoli are present in the anterior part, while smaller cells can be seen in the posterior region. The size of the testis and germarium were not measured in the present study.

On the ventral side of the body, the vaginal opening is sinistral (**Figure 12A** and **13E**) and located above the germarium. The vaginal duct, with a measurement 358.1 ± 39.7 (323.2–409.1), consists of irregular convolutions, connecting to the uterine pore, often forming several loops, and ends at

the opening of the seminal receptacle. The terminal part of the vagina forms a muscular chamber that encloses a small chitinous plaque.

Remarks

Morphometric measurements of the sclerotized parts of organs in general corresponded to data from Siwak (1932), Bychowsky and Nagibina (1957) and Paladini et al. (2008) (**Table S6**). Of them (Siwak, 1932), remarked that in young *T. vistulensis* specimens, the pair of ventral anchors appear and grow faster than the dorsal ones, it was accepted that anchors seen in oncomiracidia belonged to the future ventral anchors. *Thaparocleidus vistulensis* resembles *T. magnus* in the shape of a tube base with a flask-shaped, very long penis with several tubular loops in the middle of the penis, but differs in an accessory piece, where *T. magnus* has the appearance of a tuberous groove with a strongly swollen anterior end, forming three teeth of different shapes (Ergens & Lom, 1970). The taxonomic characterization was completed with the addition of SEM and histological figures, especially with the morphological description of the oncomiracidia of *T. vistulensis*.

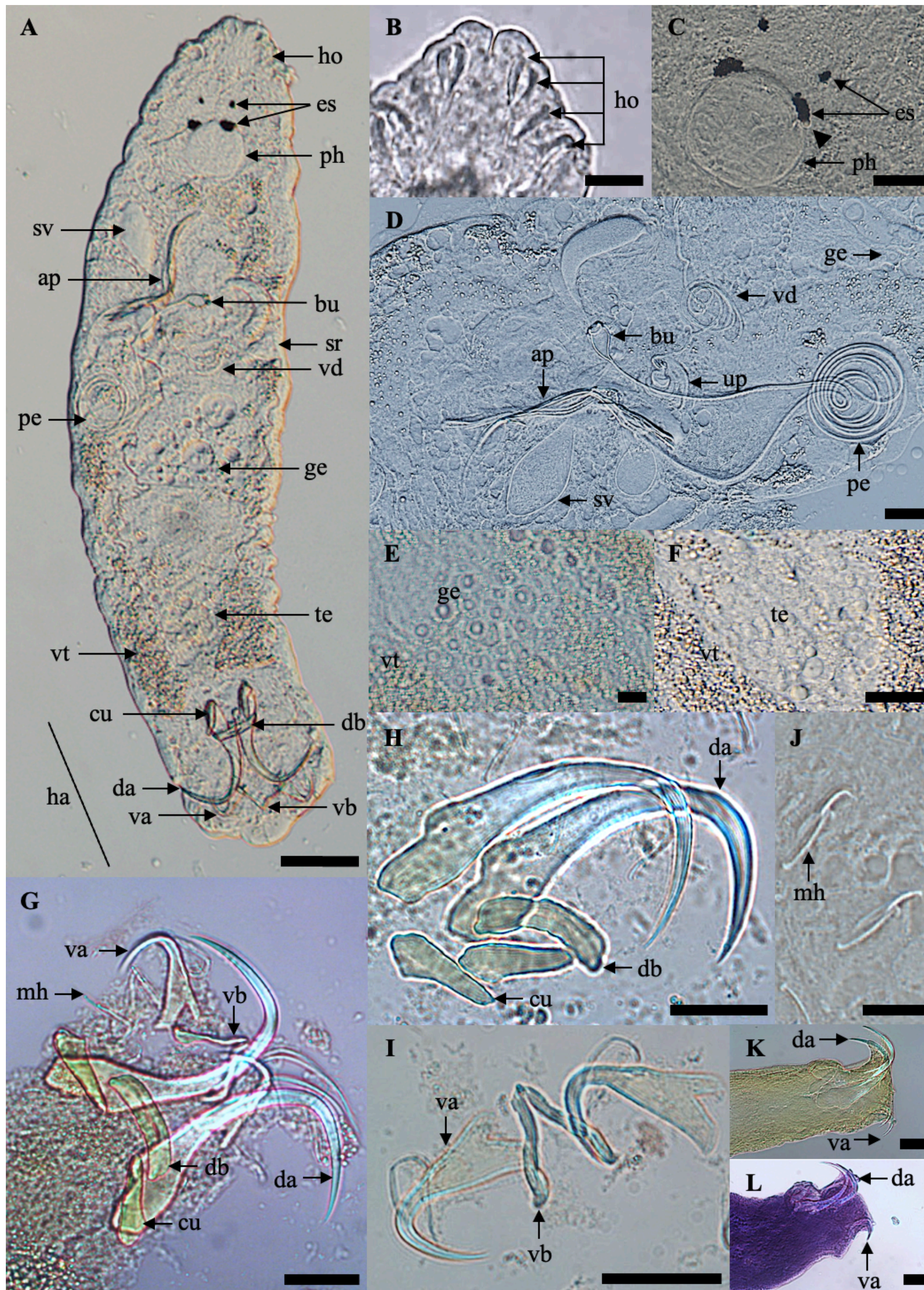

Figure 11. Photomicrograph of *T. vistulensis*. (A) Whole mount—dorsal view; (B–C) Anterior region; (D) Complex internal organ; (E) Germarium; (F) Testis; (G) Opisthaptor; (H) Dorsal anchor with cuneus and dorsal bar; (I) Marginal hooks; (J) Ventral anchor and ventral bar; (K–L) Haptor—lateral view. (A–F) Fresh samples; (G–J) Softened with proteinase K and mounted in glycerine-ammonium-picrate; (K) Mounted in glycerine-ammonium-picrate; (L) Stained with hematoxylin. Abbreviations: ap, accessory piece; bu, bulbous base; cu, cuneus; da, dorsal anchor; db, dorsal bar; es, eye spots; ge, germarium; ha, haptor; ho, head organs; mh, marginal hooks; pe, penis; ph, pharynx; sr, seminal receptacle; sv, seminal vesicle; te, testis; up, uterine pore; va, ventral anchor; vb, ventral bar; vd, vaginal duct; vt, vitellaria. ▲, transparent and intensely refractive corpuscles. Scale bars represent 20 μm, except (A) 100 μm and (J) 10 μm.

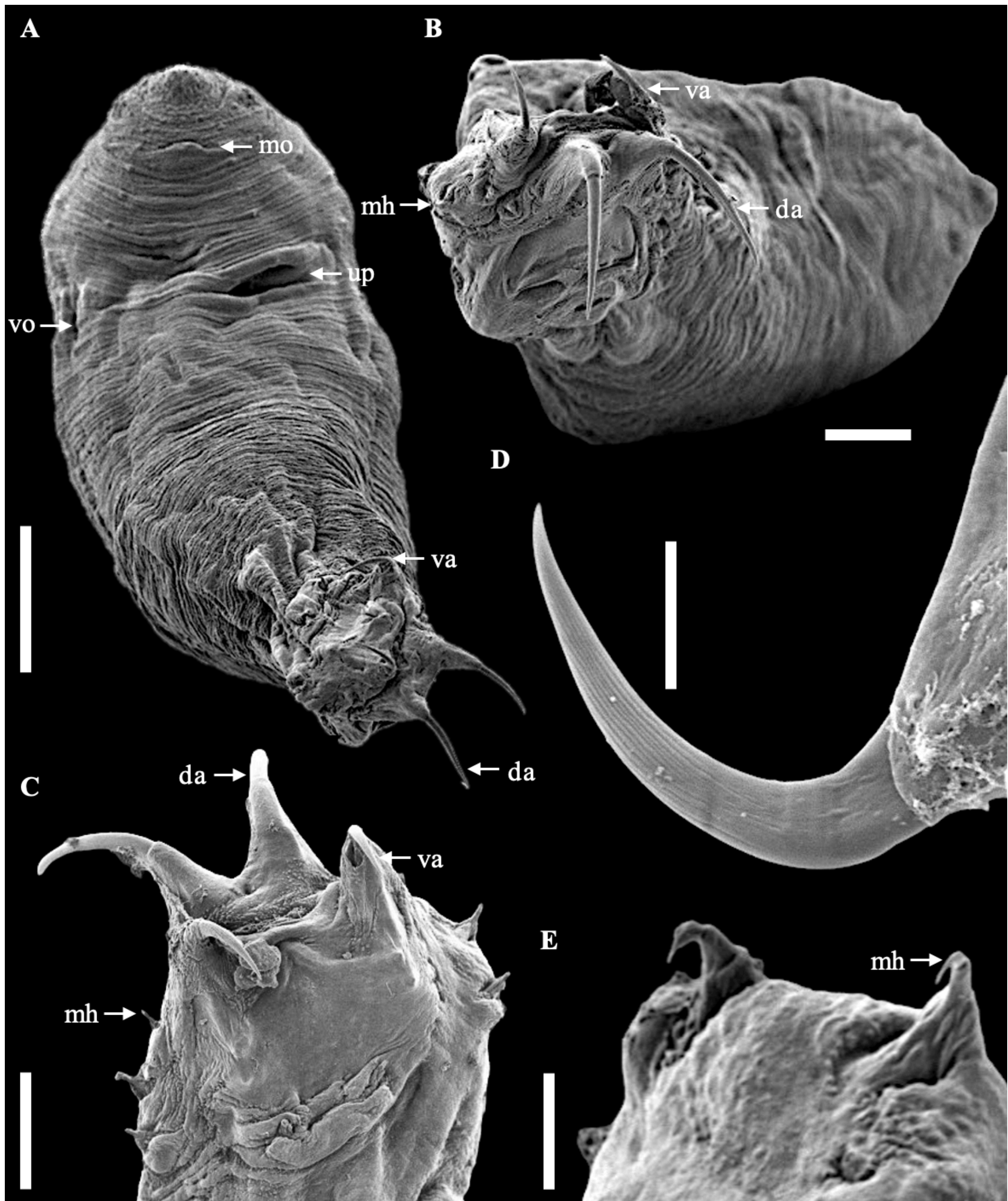

Figure 12. SEM micrographs of *T. vistulensis*. (A) Whole body—ventral view; (B) Whole body—posterior view; (C) Opisthaptor; (D) Dorsal anchor; (E) Marginal hooks. Abbreviations: da, dorsal anchor; mh, marginal hooks; mo, mouth opening; up, uterine pore; va, ventral anchor; vo, vaginal opening. Scale bars represent (A) 50 µm, (B–C) 20 µm, and (D–E) 5 µm.

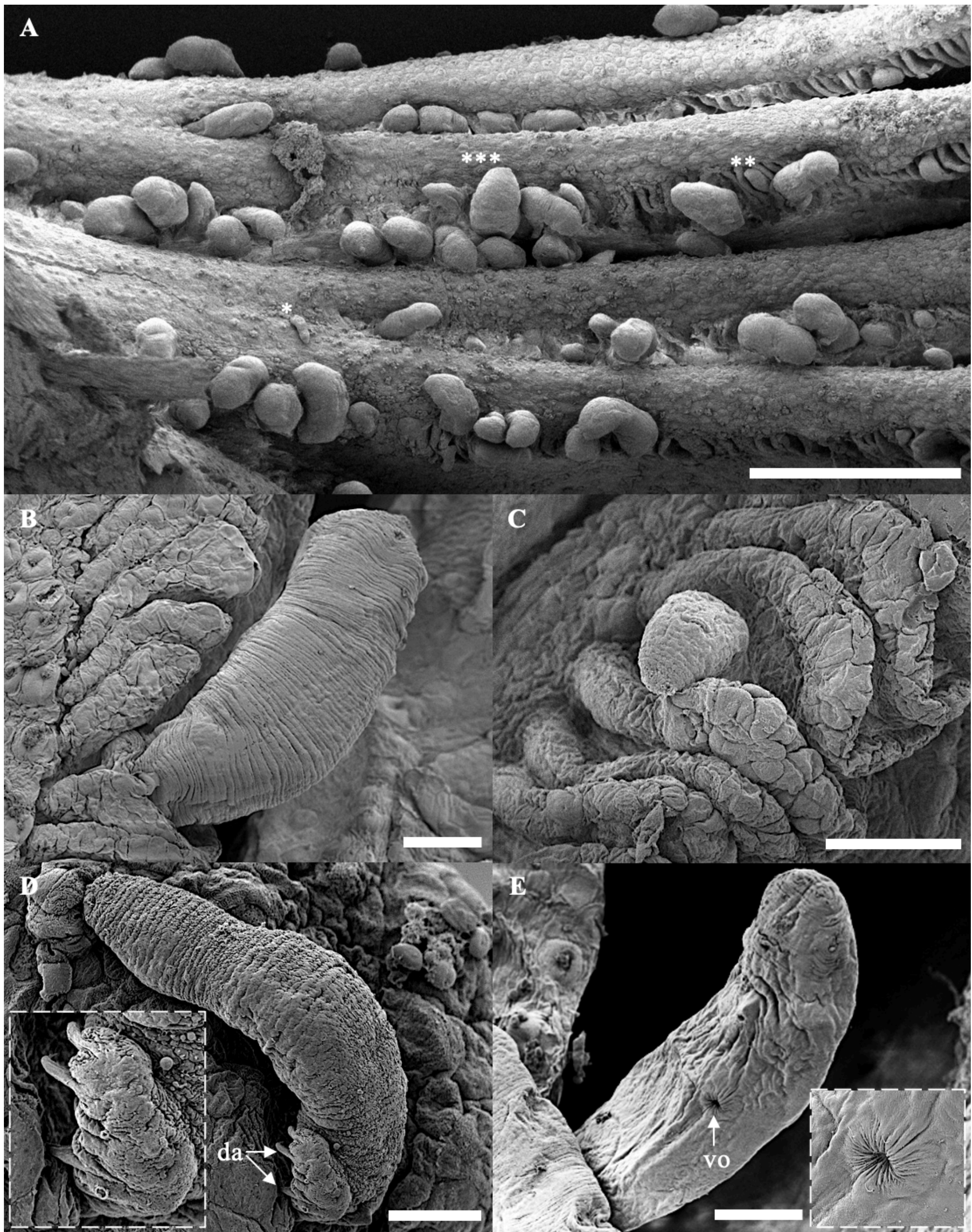

Figure 13. SEM micrographs of *T. vistulensis* are attached to gills lamellae. (A) Extensive hyperplasia of gill filament heavily infected by *T. vistulensis*; (B) Adult *T. vistulensis* with posterior part surrounded by gill tissue—lateral view; (C) Young *T. vistulensis* with posterior part in between gill lamellae—ventral view; (D) Adult *T. vistulensis* penetrating the gill filament—dorsal view; (E) Vaginal opening—ventral view. Abbreviations: da, dorsal anchor; vo, vaginal opening. Asterisks represent (*) Larvae, early stage of attachment, (**) Young, and (***) Adult T. vistulensis. Scale bars represent (A) 500 µm, (B, C, E) 50 µm, and (D) 20 µm.

4.3. Pathological effects of *Thaparocleidus vistulensis* infection on the gills

4.3.1. Attachment of Thaparocleidus vistulensis on gills

The primary attachment apparatus of *T. vistulensis* is the opisthaptor, with both dorsal and ventral anchors are pointed in opposite directions penetrating between adjacent gill lamellae (**Figure 14**). This attachment was further reinforced by marginal hooks that secured a firm attachment at the margin of the opisthaptor. Meanwhile, the prohaptor facilitate temporary attachment. The opisthaptor was commonly attached superficially (**Figure 15A**) as well as basally to the filaments (**Figure 15B**), causing deep concave cup-like depressions on the surface of the gill lamellae.

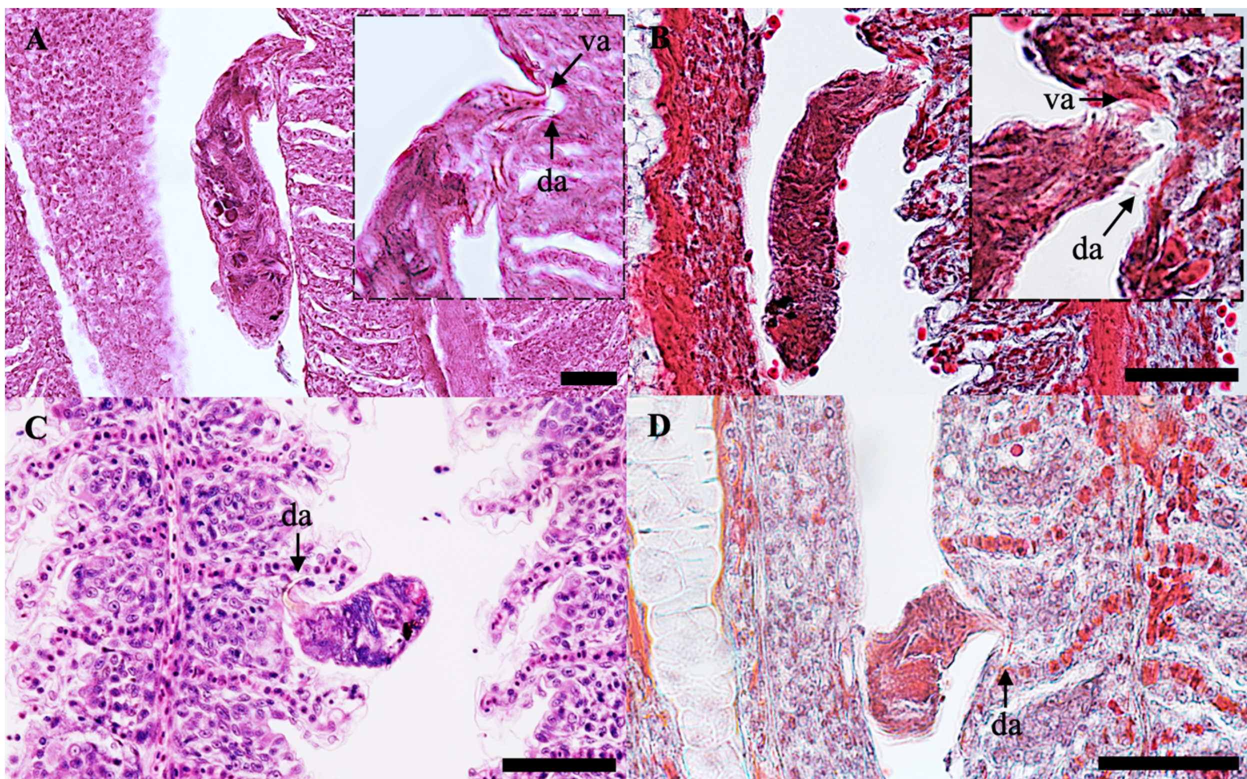

Figure 14. Histological sections of gills attached by *T. vistulensis*. (A–B) Adult *T. vistulensis* with posterior part—anchor inserted and pierced in gill lamellae in the opposite direction; (C–D) Young *T. vistulensis*. Staining method (A, C) Stained with H & E; (B, D) Stained with Masson–Goldner trichrome staining. Abbreviations: da, dorsal anchor; va, ventral anchor. Scale bars represent 50 µm.

4.3.2. Histopathological effects of Thaparocleidus vistulensis infection on gills

The histopathological examination revealed significant cell proliferation and diffuse epithelial hyperplasia, leading to lamellar fusion and subsequent extravasated erythrocytes in the gill lamellae. Occasionally, the epithelial disintegration led to desquamation, and released erythrocytes

were observed within the excess mucus between filaments in certain areas (**Figure 16**). In heavily infected gills, a row or a cluster of filaments with clubbing at the tips was commonly observed (**Figure 17**), and free sera occasionally with coagulated blood cells between the gill filaments (**Figure 18**). The presence of eosinophilic granular cells was commonly detected, particularly at the sites where the anchor of the parasite penetrates the gill lamellae (**Figure 19**).

Infection by *T. vistulensis* causes depression of the epithelium due to anchor penetration, and in some cases, the parasite's body exerts pressure on the gill lamellae (**Figure 20A**). Piercing of gill lamellae caused by anchors and marginal hooks also leads to rupture of epithelial tissue, loss of definitive cellular characteristics, and lamellar integrity. The parasite damages and disrupts gill lamellae through feeding. Tissue debris and epithelial cells could be identified in the oral cavities and intestines of parasites (**Figure 20A**). Sometimes, deeper anchor penetration affects the extracellular cartilaginous matrix, and occasionally it reaches the core of the matrix, touching the chondrocytes of the gill rays and causing distortion primarily in the extracellular cartilaginous matrix (**Figure 20B**).

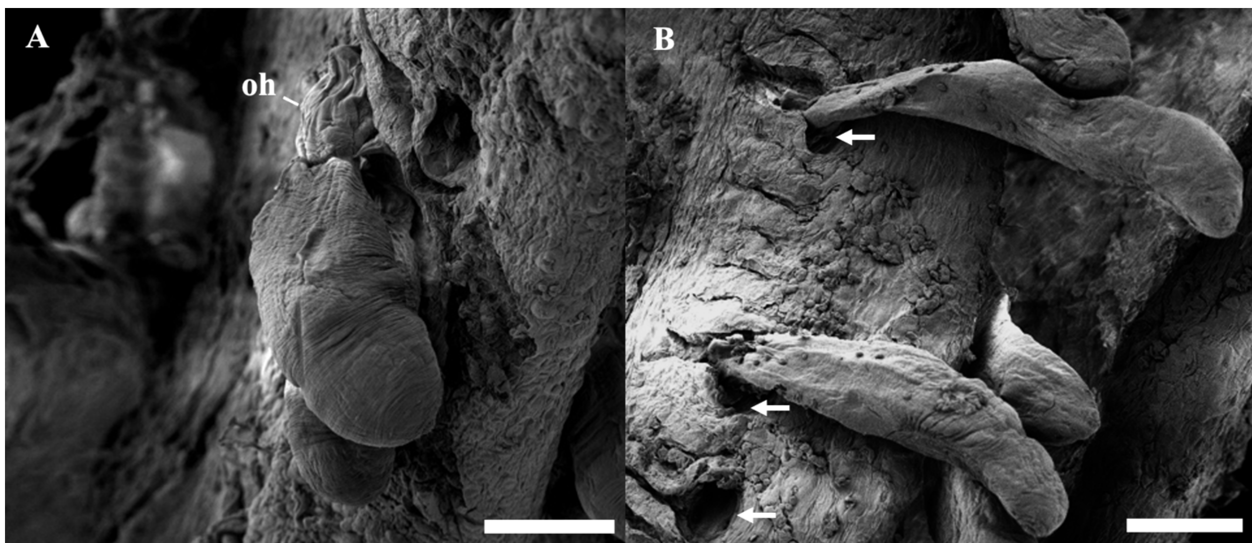

Figure 15. SEM of *T. vistulensis* attachment on gills using opisthaptor (oh) causing concave cup-like deep hollows and deformation on the surface of the gill lamella of European catfish (arrow). (A) Superficial attachment; (B) Deep attachment. Scale bars represent 100 μm.

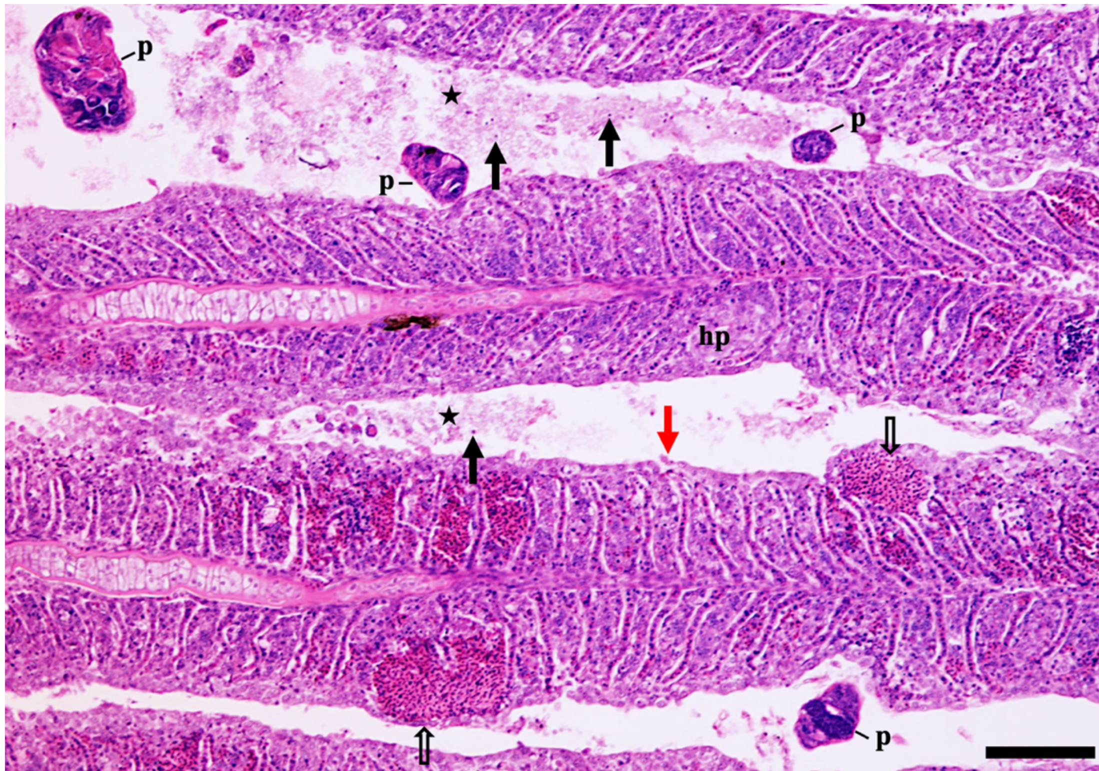

Figure 16. Histological longitudinal section of gills infested by *T. vistulensis* (p) showing lamellar proliferation, diffuse epithelial hyperplasia (hp), extravasated erythrocytes in the lamella capillaries (hollow arrow), excess mucus (star) and extravascular erythrocytes escaping from the circulatory system (arrow), and epithelial desquamation (red arrow). Haematoxylin and eosin (H&E) staining. Scale bar represent 100 µm.

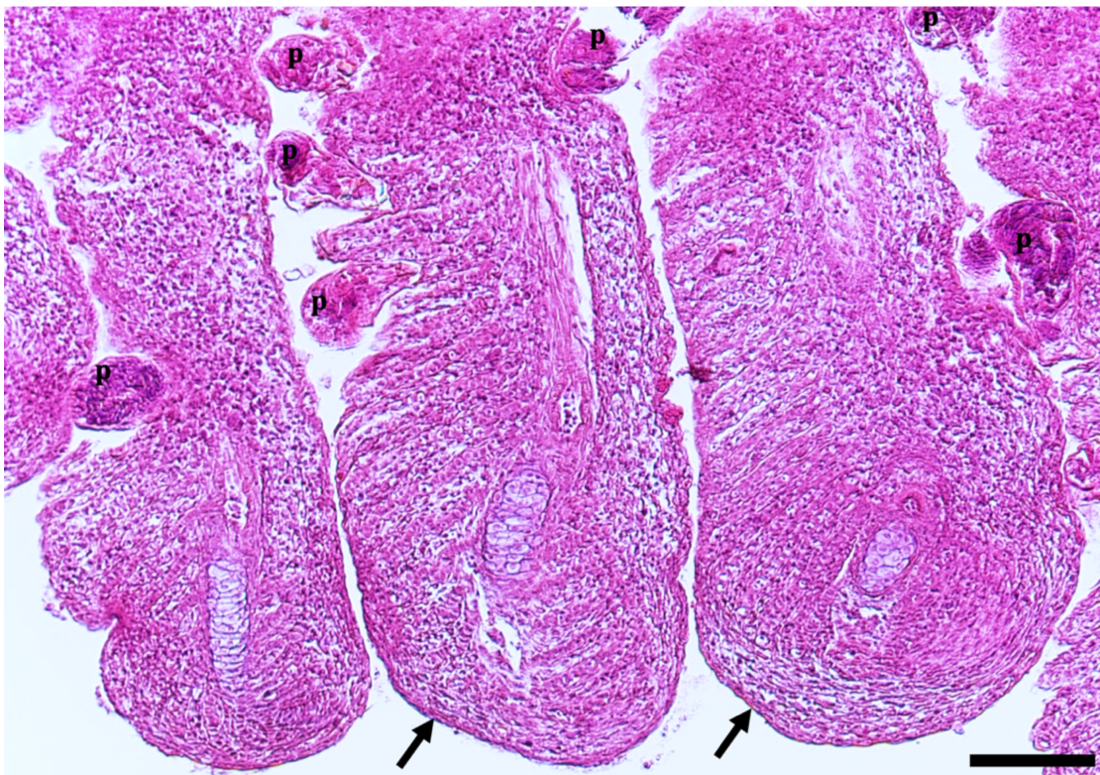

Figure 17. Histological section of gills infested by *T. vistulensis* (p) illustrating clubbed filament tips with lamellar fusion (arrow). H&E staining. Scale bar represent 100 µm.

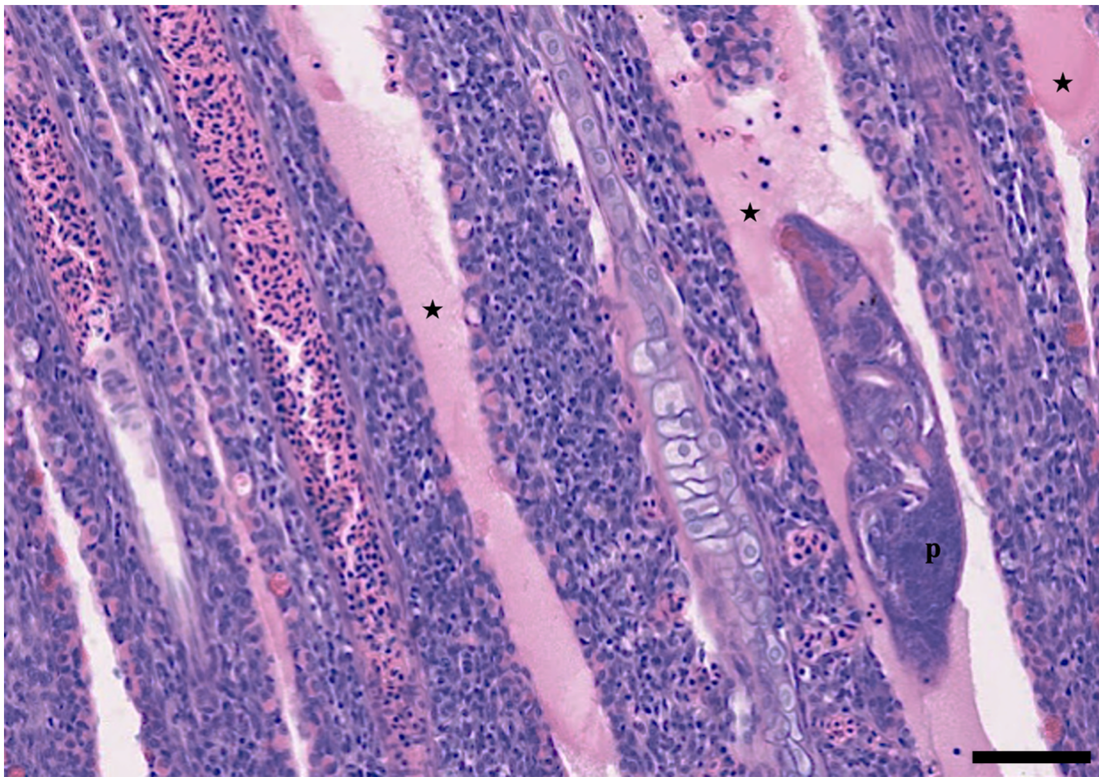

Figure 18. Histological section of gills infested by *T. vistulensis* (p), with serum coagulating between gill filaments (star). H&E staining. Scale bar represent 50 µm.

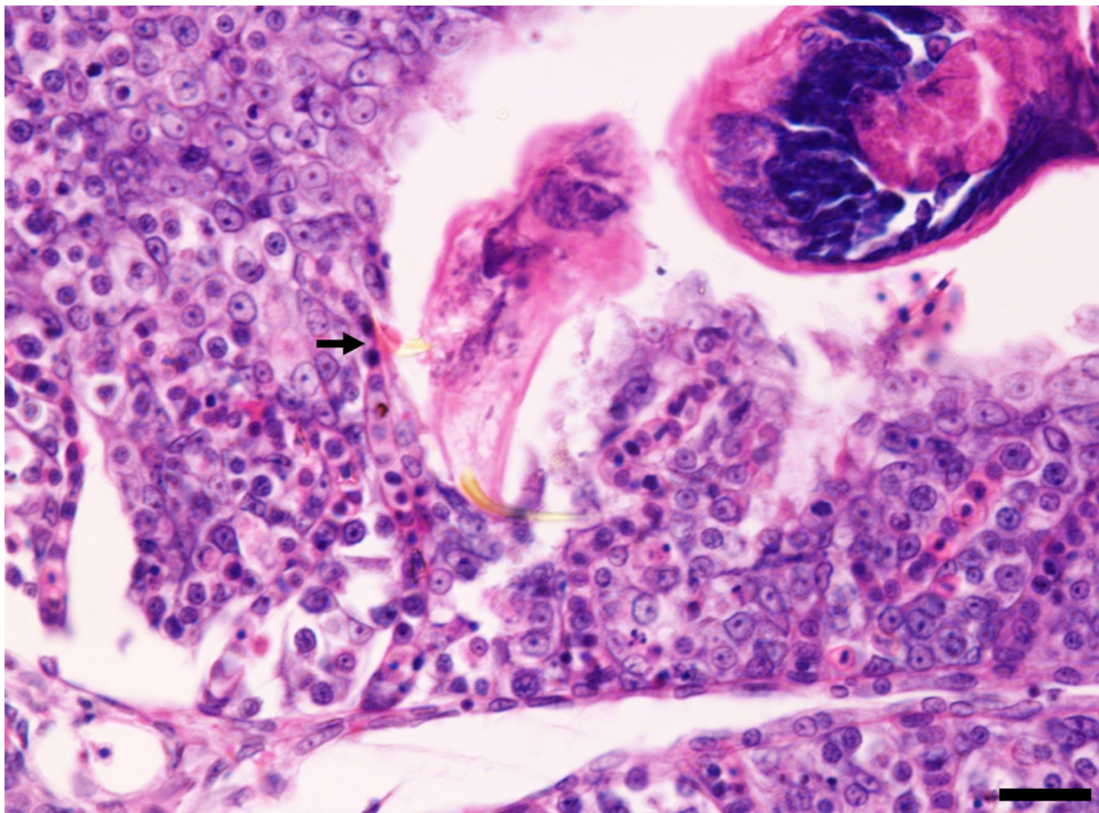

Figure 19. Histological section of gills infested by *T. vistulensis* showing eosinophilic granular cell at the anchoring sites (arrow). H&E staining. Scale bar represent 20 µm.

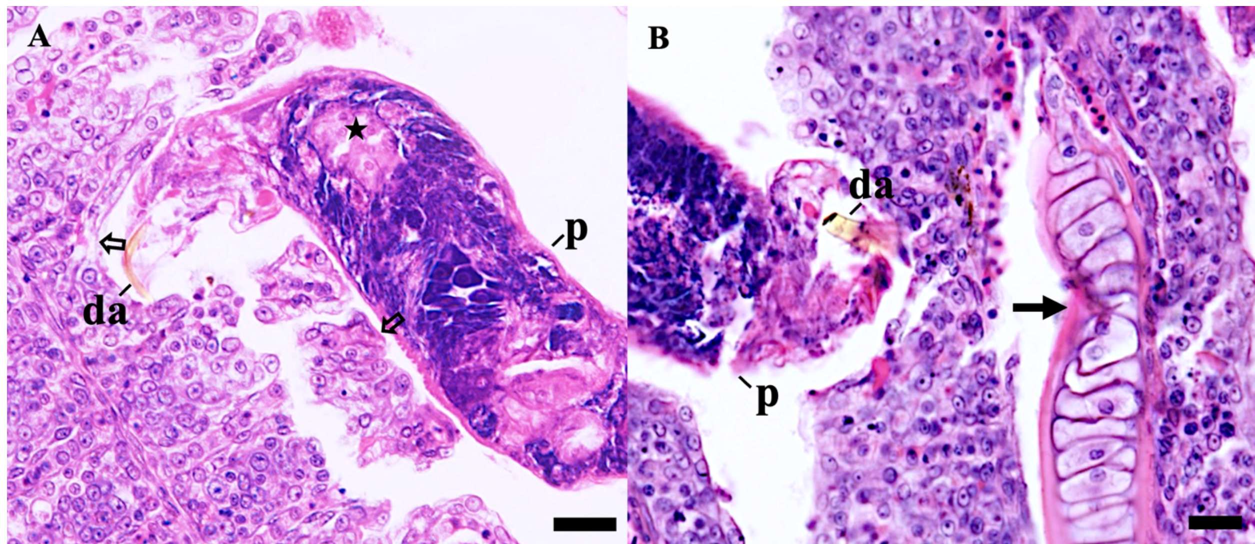

Figure 20. Histological section of gills infested by *T. vistulensis* (p) showing dorsal anchors (da) penetrating basally between adjacent secondary lamellae. (A) Depression on lamellae due to *T. vistulensis* anchor and body (hollow arrow), and gill debris inside the parasite is highlighted (star); (B) Damaged extracellular cartilaginous matrix (arrow). H&E staining. Scale bars represent 20 μm.

4.4. Reproductive strategies of *Thaparocleidus vistulensis*

4.4.1. Infection dynamics

Following experimental exposure of European catfish to *T. vistulensis*, a significant increase in the number of gill monopisthocotyleans over the 10-day observation period was observed. The intensity was dependent on the initial infection level of the donor fish (**Figure 21**). Fish were affected by the higher infection level and showed equilibrium disturbances (position upside down in a near vertical position on the 10th day of co-habitation in the Third Trial), where the estimated number of monopisthocotyleans attached to the gills were up to 17,000–18,000 individuals. These fish clearly exhibited pathological changes of the gill structure, whereas the lower infection of the fish in the First and Second Trials showed fewer gross pathological changes of the filaments. Thus, the Third Trial showed gills heavily populated by monopisthocotyleans (**Figure 22A–B**), which was associated with various levels of colour changes (from anaemic via pale colour to places to areas with bright red appearance) and lamellar fusion (**Figure 22C–D**). Mature monopisthocotyleans with an egg inside the body could be observed from 8 to 10 dpi for all trials (**Figure 22C–D**).

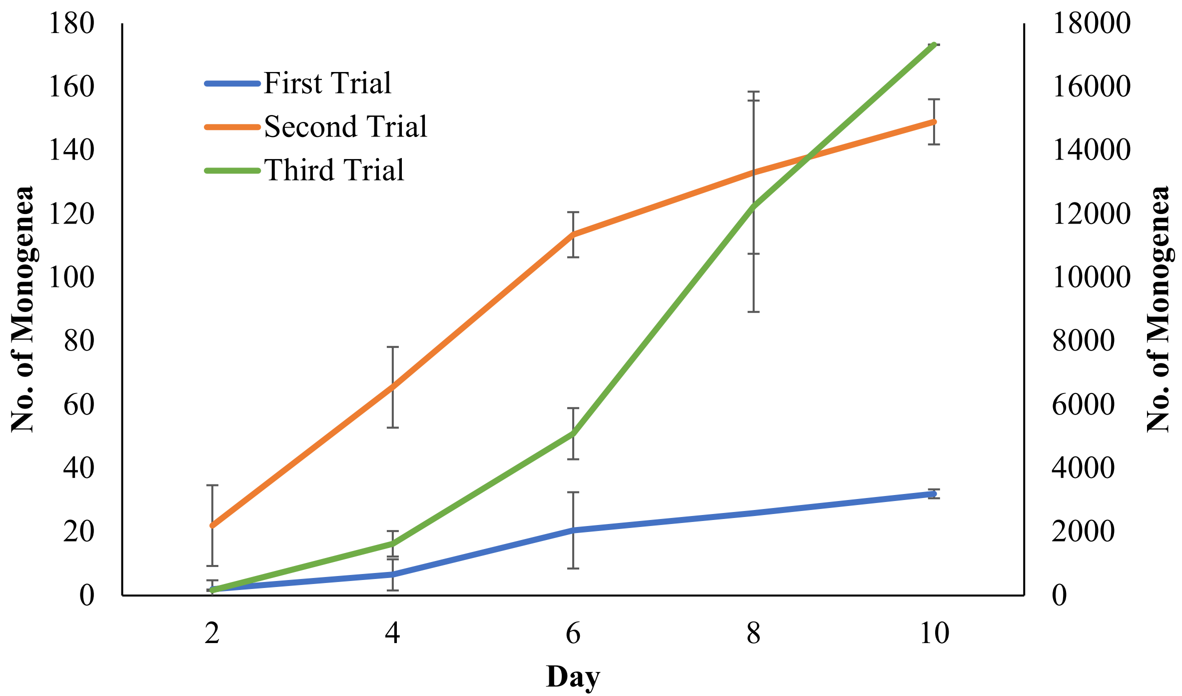

Figure 21. Average infection dynamics of *T. vistulensis*. The First Trial and Second Trial refer to the primary axis (left side), while the Third Trial refers to the secondary axis (right side).

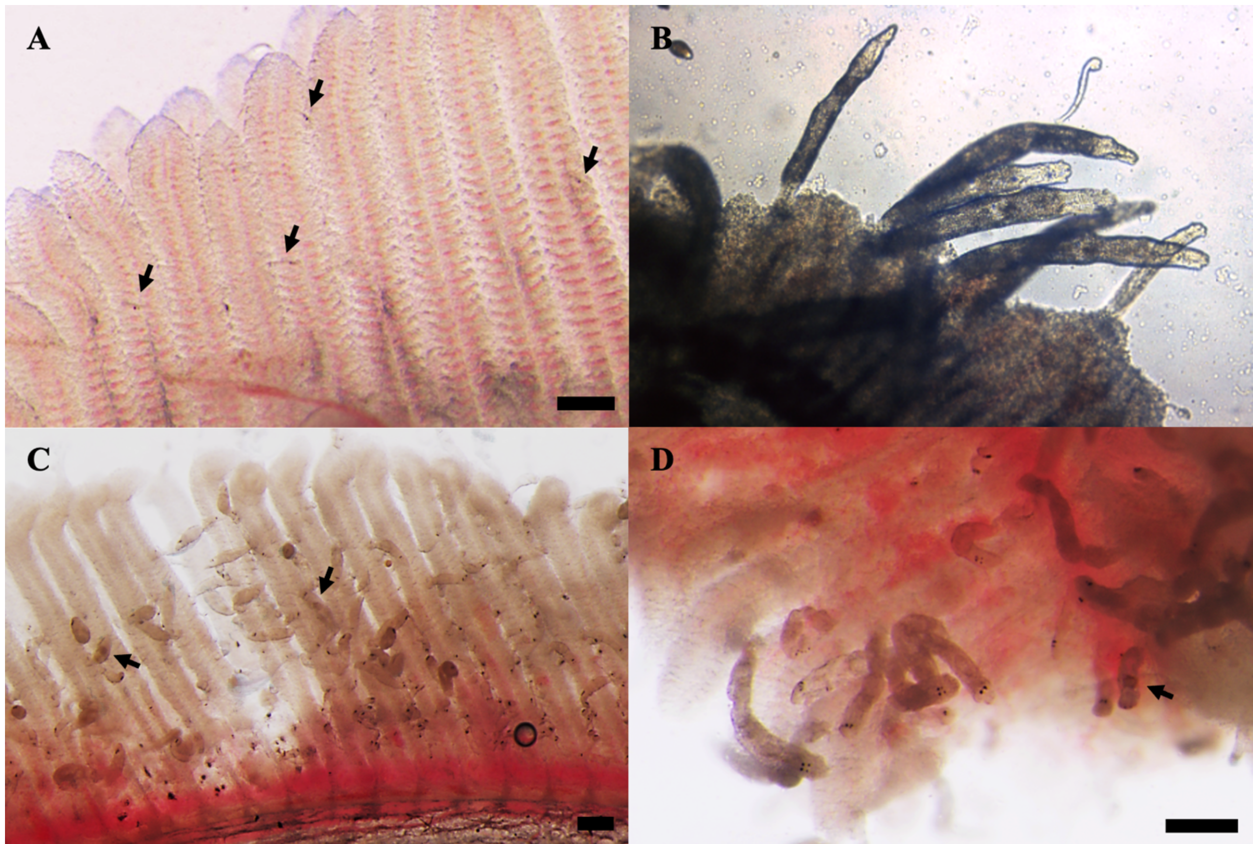

Figure 22. The gills of infected fingerling European catfish by *T. vistulensis*. (A) Developing *T. vistulensis* attached to the normal gill filaments (arrows) at 2 dpi; (B) Abundance of *T. vistulensis* on the gill at 10 dpi; (C–D) Sexually mature monogenean with egg inside the body (arrows) situated on the heavily injured gill at 10 dpi. Scale bars represent 200 µm.

4.4.2. Egg development

Eggs were spheroidal in shape; 72.35 ± 5.03 (65.40–82.78) in length and 56.20 ± 4.14 (47.46–62.44) in width, with a short and hooked polar filament 42.31 ± 6.87 (55.07–31.06) facilitating attachment to substrate. Mature monopisthocotyleans deposited eggs individually. The viable eggs were light brown, while the infertile eggs were darker and sometimes deformed. The development of eggs until the first hatching 3 to 4 dpo showed a uniform pattern. Shortly after oviposition, the eggs, were full of vitelline material (**Figure 23A**) and the embryo could not be distinguished (**Figure 23B**). Vitelline material was displaced to the periphery of the egg when an organized embryonic mass could be seen inside the egg with a central circle (**Figure 23C**). After 24 hpo, the embryo became visible in the center of the egg, surrounded with dispersed vitelline material (**Figure 23D**), which gradually decreased during the period of observation. The progressive development of the embryo was detected (**Figure 23E**) within 48 hpo. Before the larva was fully developed, two pairs of primordial eyespots were visible as small and scattered accumulation of pigment that gradually condensed into well-defined eyespots. The appearance of a primordial hamulus was also positioned between 48 and 72 hpo of development (**Figure 23F–G**). During this time, primordia of aligned ciliated cells were visible (anterior, lateral, and posterior). The anterior part (head) of the oncomiracidium was directed towards the operculum, while the posterior part (haptor) folded backwards. The movement of larva in the egg was limited in this phase. After 72 hpo, the sclerotized structures (central anchor and marginal hooks) were completely formed (**Figure 23H–I**). In this phase, the lively movement of the larva were observed, with the oncomiracidium retracting and elongating in the egg. Ciliary beating was seen in the anterior, lateral, and posterior ciliated cell groups. Prior to hatching vigorous movements of the oncomiracidium along with the ciliated activity accelerated until the operculum opened, whereafter the larva could escape by contraction of the body and propulsion by the cilia (**Figure 23J**). Empty eggs shells were observed between 72 and 96 hpo (**Figure 23K**), and the number of free swimming oncomiracidium emerged in the water column (**Figure 23L**).

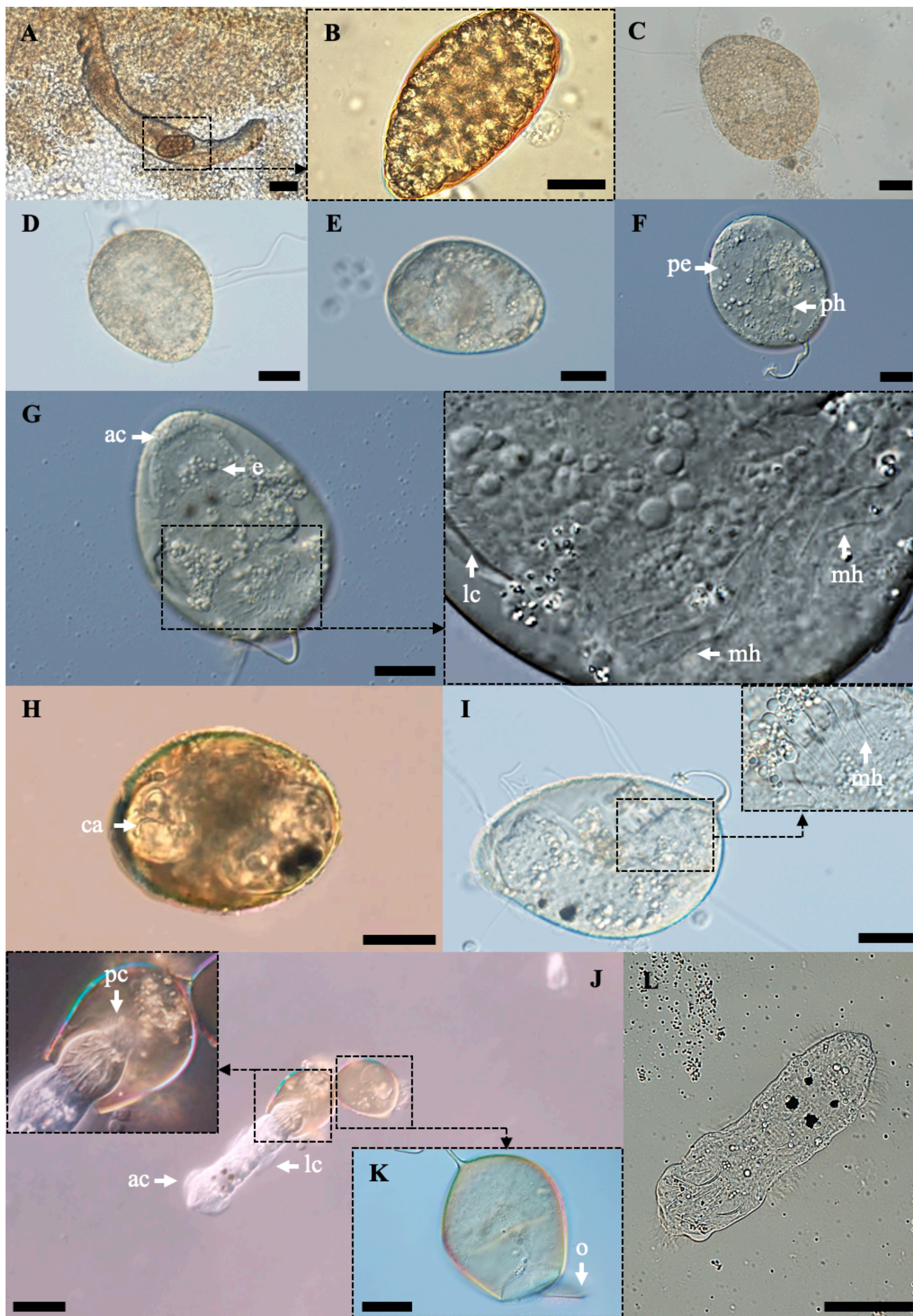

Figure 23. Egg development of *T. vistulensis*. (A) Adult *T. vistulensis* with an egg inside its body; (B) Egg right after oviposition; (C) Egg after 6 hpo; (D) Egg after 24 hpo; (E–F) Eggs between 24 and 48 hpo: (E) The whole embryo, (F) Larva with primordia of scattered pigment of eyespots and primordia of hamulus; (G) Eggs between 48 and 72 hpo: Developing larva with marginal hooks and ciliated cells, ventral view; (H–I) Eggs after 72 hpo: (H) Developed larva before eclosion with anchors and (I) marginal hooks, lateral view; (J) Moment of eclosion; (K) Empty egg shell with opened operculum; (L) Recently hatched oncomiracidium. Abbreviations: ac, anterior cilia; ca, central anchor; e, eyespot; lc, lateral cilia; mh, marginal hooks; o, operculum; pc, posterior cilia; pe, primordial eyespot; ph, primordia of hamulus. Scale bars represent 20 µm except for (A), (J), and (L) 50 µm.

4.4.3. In vitro hatching rates

The average time for *T. vistulensis* egg hatching was 3 days and the overall hatching success was 89.7%. The first hatching was detected on 3 dpo at a rate of 12.5% whereafter hatching ended at 84% on 5 dpo (**Figure 24**). Most of the eggs hatched between 3 and 4 dpo, resulting in a 72.2% increase in cumulative hatching success, which raised only a further 5% by 5 dpo. No newly hatched eggs were observed after 5 dpo. The unhatched eggs were stuck at some stage of the developmental process, and their colour changed to dark brown.

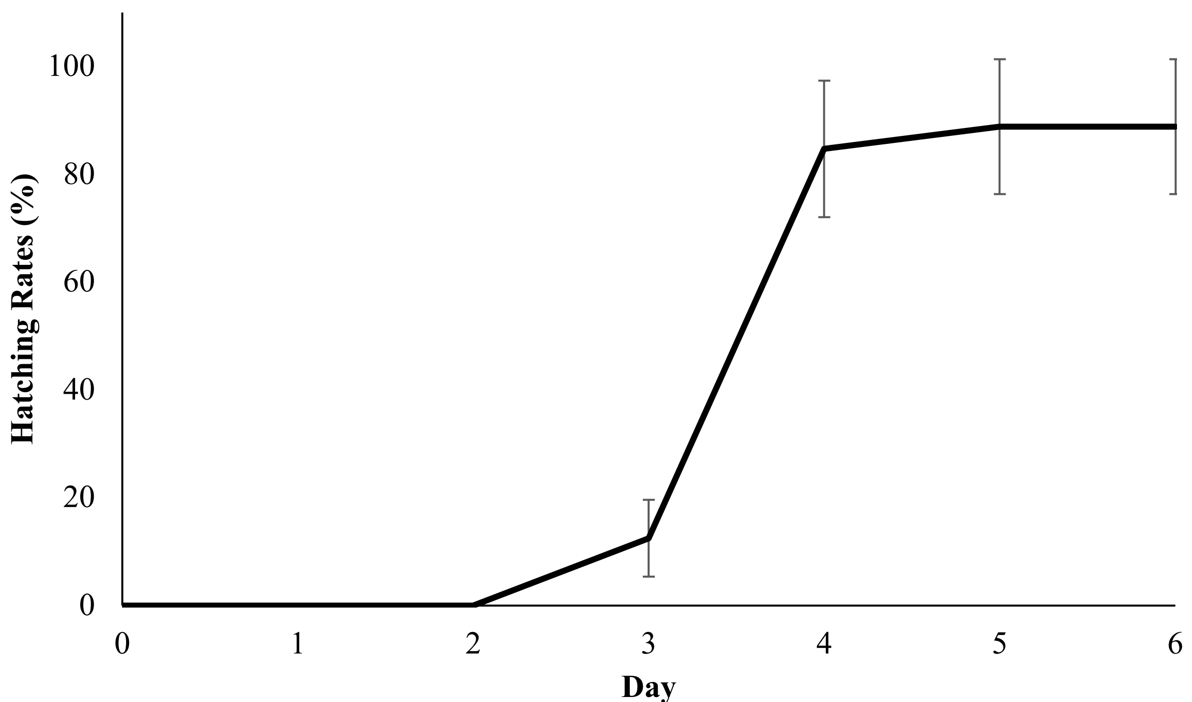

Figure 24. Average *in vitro* hatching rates of *T. vistulensis* eggs.

4.4.4. In vitro survival rates

Isolated parasites (larvae, developing juveniles, adults) were observed *in vitro*, without host. The survival rates of the monopisthocotyleans at different life cycle stages differed considerably. While the oncomiracidia could survive for up to 5 days (7.4%), the developing juvenile and adult monopisthocotyleans stayed alive for up to 3 days (0.9% and 1.6%, respectively) (**Figure 25**). The mobility rate gradually decreased over time and the monopisthocotyleans were considered dead when they showed no physical response to an external stimulus. Sometimes their bodies were swollen or opaque white.

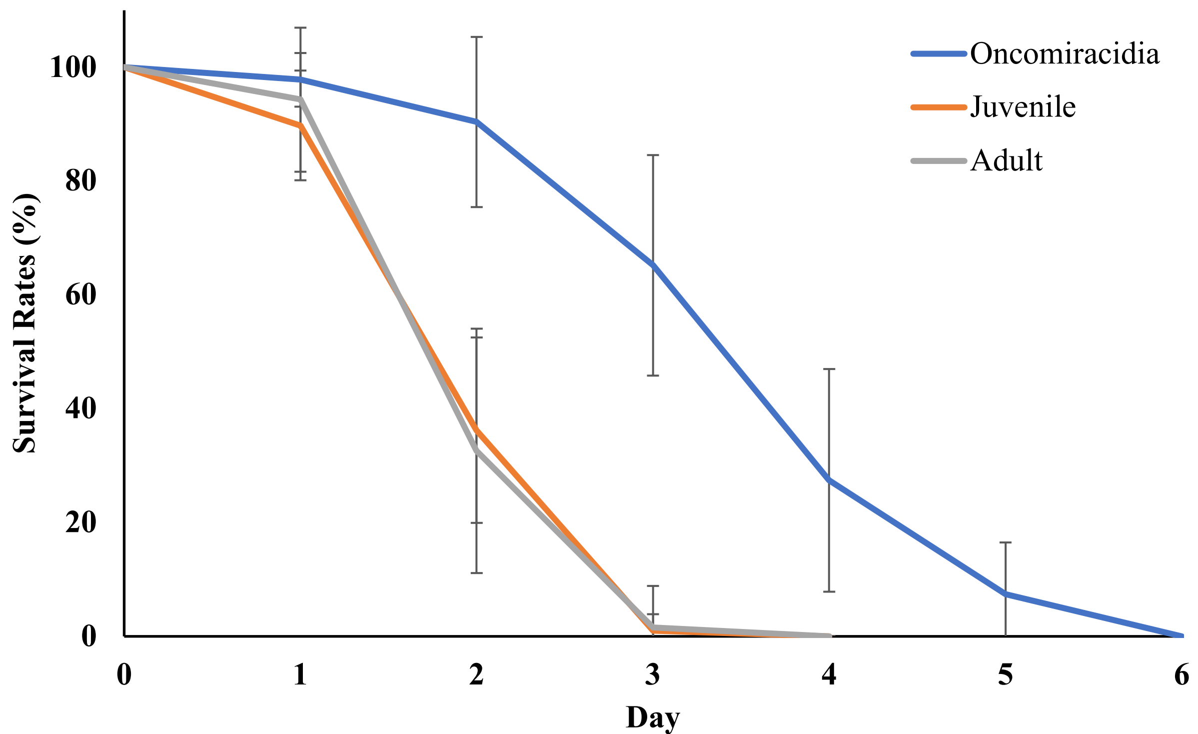

Figure 25. Average *in vitro* survival rates of *T. vistulensis* at different life stages.

4.5. Influences of environmental conditions against different life stages

4.5.1. Fecundity of Thaparocleidus vistulensis in relation to light and darkness

The impact of light and darkness on *T. vistulensis* egg production was assessed by observing it in natural and constant dark photoperiod conditions (**Figure 26**). The number of eggs laid by the adults were three times higher in the light (96 ± 3.5 eggs), compared to those in darkness (30 ± 1.15 eggs), which was a statistically significant difference ($P < 0.001$).

4.5.2. Hatching rates of Thaparocleidus vistulensis in relation to light and darkness

The hatching rates of *T. vistulensis* kept at a normal photoperiod, and constant darkness showed a similar tendency of hatching (**Figure 27**). The eggs from both groups started to hatch on day 3 post-oviposition (pop), and ended on day 5 pop, with a hatching success of 88.0% and 88.7% in light and dark conditions, respectively. There was no statistically significant difference in hatching rates between the groups ($P > 0.926$).

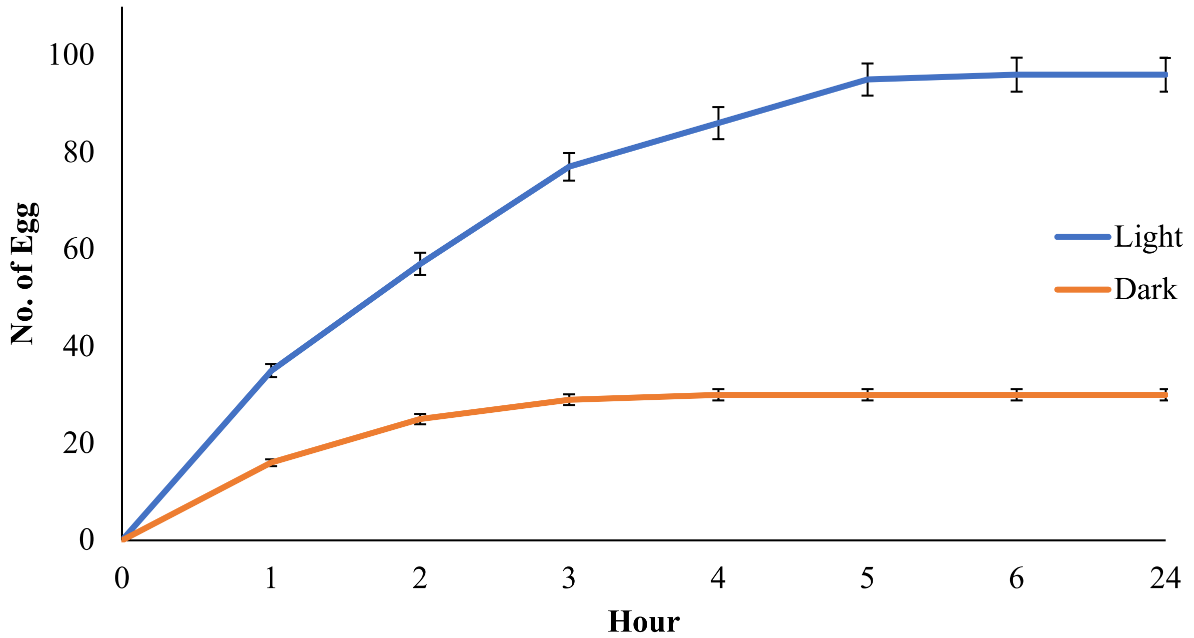

Figure 26. Cumulative egg production of *T. vistulensis* under light-dark conditions.

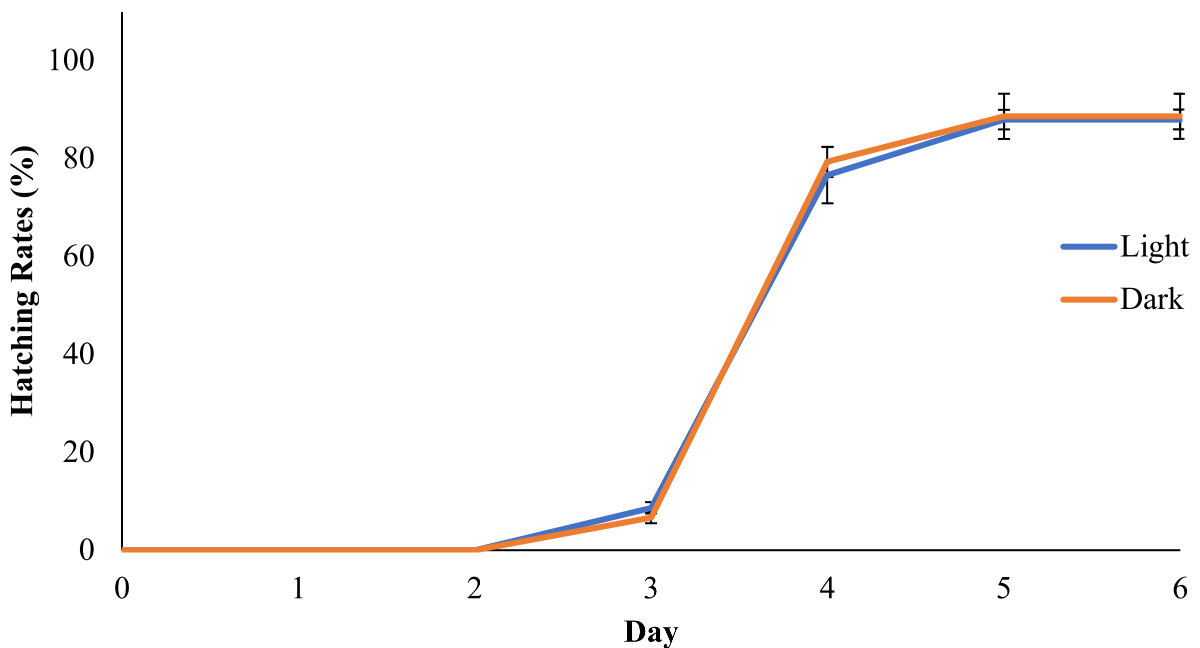

Figure 27. The impact of light-dark cycle on the cumulative egg hatching rates of *T. vistulensis.*

4.5.3. Survival rates of Thaparocleidus vistulensis adults and oncomiracidia in relation to light and darkness

The survival rates of *T. vistulensis* for adult and oncomiracidium groups were very similar in both normal and dark conditions (**Figure 28** and **29**). For the adult parasites, the group exposed to

darkness had its first mortality on day 1 post-isolation (pi), while the normal photoperiod exposed group recorded mortality on day 2 pi. Individuals survived for less than 3 days in both groups (**Figure 28A**). The survival curves of adult *T. vistulensis* for both conditions showed no significant differences (**Figure 28B**), which was confirmed by a log-rank test ($P > 0.142$).

Oncomiracidia survived up to 5 days in both groups; their host-seeking movement was stochastic and did not show detectable phototaxis (**Figure 29A**). The first death in the dark-exposed group was recorded on day 3 pi, while the non-exposed normal group had its first death on day 4 pi. All individuals in both groups perished by day 6 PI. The survival curves of oncomiracidia *T. vistulensis* differed significantly in the two conditions (**Figure 29B**), as confirmed by a log-rank test ($P < 0.001$).

4.5.4. Fecundity of Thaparocleidus vistulensis at different water temperatures

The fecundity of *T. vistulensis* was evaluated at different water temperatures (**Figure 30**). Maximum egg production was observed at 15 °C (52 ± 1.40 eggs), which showed a statistically significant difference compared to the data recorded at the other water temperatures ($P < 0.001$). At 20 °C, 25 °C, and 30 °C, the number of eggs produced was 39 ± 1.10, 30 ± 1.15, and 26 ± 0.97, respectively. The number of eggs produced at 20 °C was also statistically significant compared to the other water temperatures ($P < 0.001$). However, no statistical difference was observed between the egg production at 25 °C and 30 °C ($P > 0.583$). The egg production at 35 °C (6 ± 0.70) and 10 °C (11 ± 0.88) was low, but there was no statistically significant difference between them ($P > 0.051$). No eggs were produced at 5 °C, which was statistically significant compared to the other water temperatures ($P < 0.001$).

4.5.5. Hatching rates of Thaparocleidus vistulensis at different water temperatures

The hatching rates of *T. vistulensis* were monitored across various water temperatures (**Figure 31**). The total hatching success at 10 °C, 15 °C, 20 °C, 25 °C, and 30 °C was 80%, 81.3%, 89.3%, 89.3%, and 88%, respectively. Notably, no hatching was observed at 5 °C and 35 °C, and no

statistical difference was recorded between the two water temperatures (P = 1.000). The observations were terminated on day 30 pop and day 10 pop, respectively, due to the deterioration of the experimental medium. The shortest hatching times for the eggs were recorded at 30 °C, followed by 25 °C. The hatching started as early as day 2 pop, and the last eggs were hatched on day 3 pop and day 4 pop, respectively. However, data at these two water temperatures showed no statistically significant difference ($P > 0.528$). At 20 °C, the first eggs hatched on day 3 pop and continued until day 5 pop. Egg hatching was initiated on day 5 pop and ended on day 9 pop at 15 °C. The longest hatching period occurred at 10 °C, spanning between day 12 pop and day 19 pop. Egg hatching at 5 °C and 35 °C significantly differed ($P < 0.001$) compared to egg hatching at 10 °C, 15 °C, 20 °C, 25 °C, and 30 °C.

4.5.6. Survival rates of Thaparocleidus vistulensis adults and oncomiracidia at different water temperatures

To assess the survival rates of *T. vistulensis*, adults and oncomiracidia were studied at several water temperatures (**Figure 32** and **33**). The trend was found to be similar for adults and oncomiracidia, and the survival period shortened when the water temperature decreased. For adult *T. vistulensis* (**Figure 32A**), the group exposed to 35 °C died quickly, with all of them perishing within 24 h. Death began after day 1 pi in the groups at 30 °C and 25 °C, and lasted until day 2 pi and day 3 pi, respectively. Mortality commenced on day 3 pi for the 20 °C group, and continued until day 4 pi, while for the 15 °C group, it started on day 4 pi and persisted until day 6 pi. A higher survivability was observed in the lower water temperature groups, 10 °C and 5 °C, where the adult monopisthocotyleans survived up to day 8 pi and day 12 pi, respectively. The survival curves of adult *T. vistulensis* differed significantly at all water temperatures (**Figure 32B**), with a log-rank test confirming their statistical significance ($P < 0.001$). For oncomiracidia of *T. vistulensis*, the larvae could only survive for less than 1 day at extreme water temperatures (35 °C and 5 °C) (**Figure 33A**). At 30 °C and 25 °C, oncomiracidia stayed alive for 3 and 4 days, respectively. The survival trends for 20 °C and 15 °C were similar, starting on day 3 pi and day 4 pi, with the last

oncomiracidia surviving until day 7 pi. The survival period of the parasite larvae was the longest in the 10 °C group, where the first death happened on day 4 pi, and the last oncomiracidium stayed alive until day 9 pi. A log-rank test confirmed the statistical significance of all survival curves ($P < 0.001$).

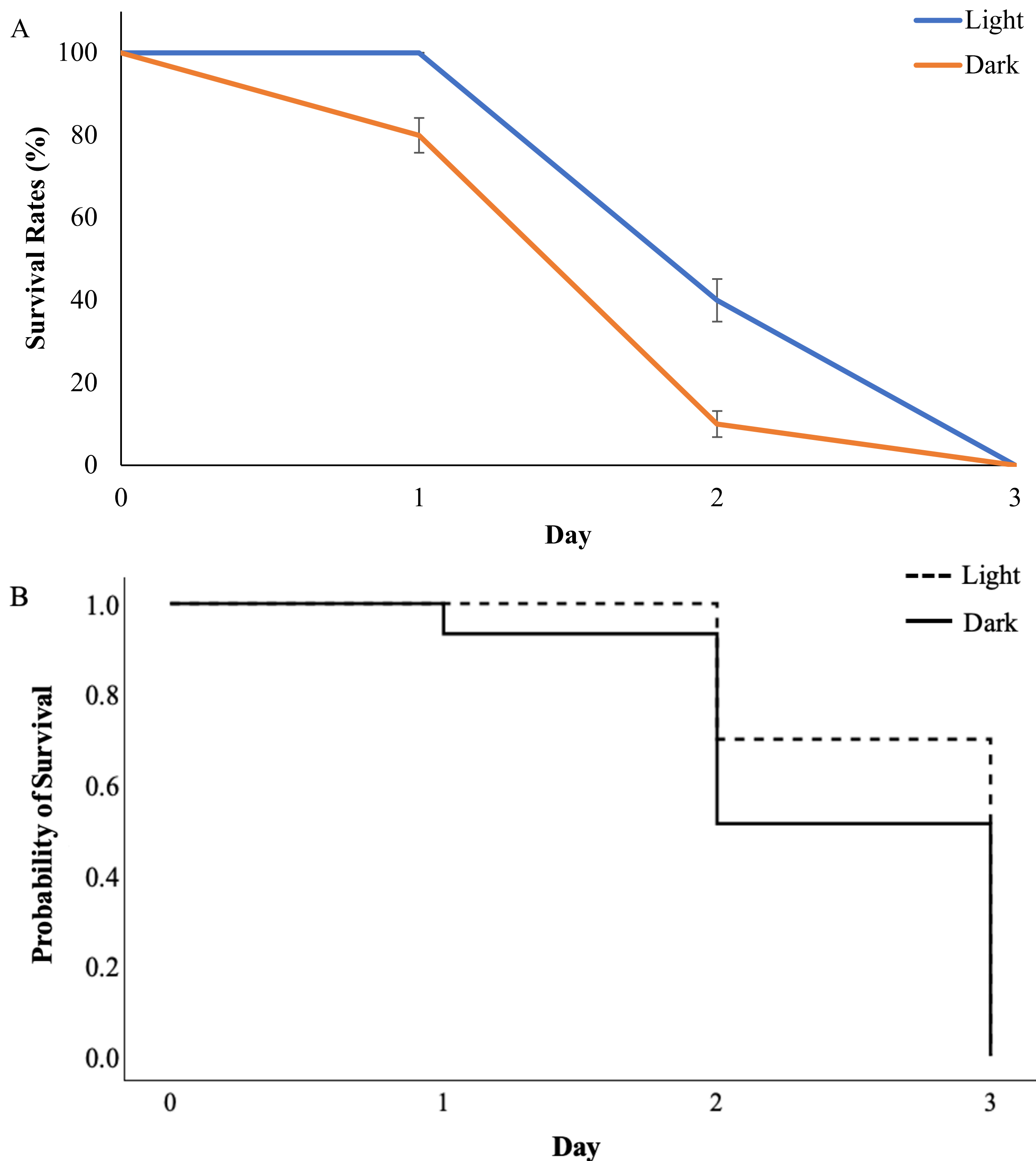

Figure 28. The influence of light-dark cycle on adult *T. vistulensis*. (A) Survival rates; (B) Survival curve.

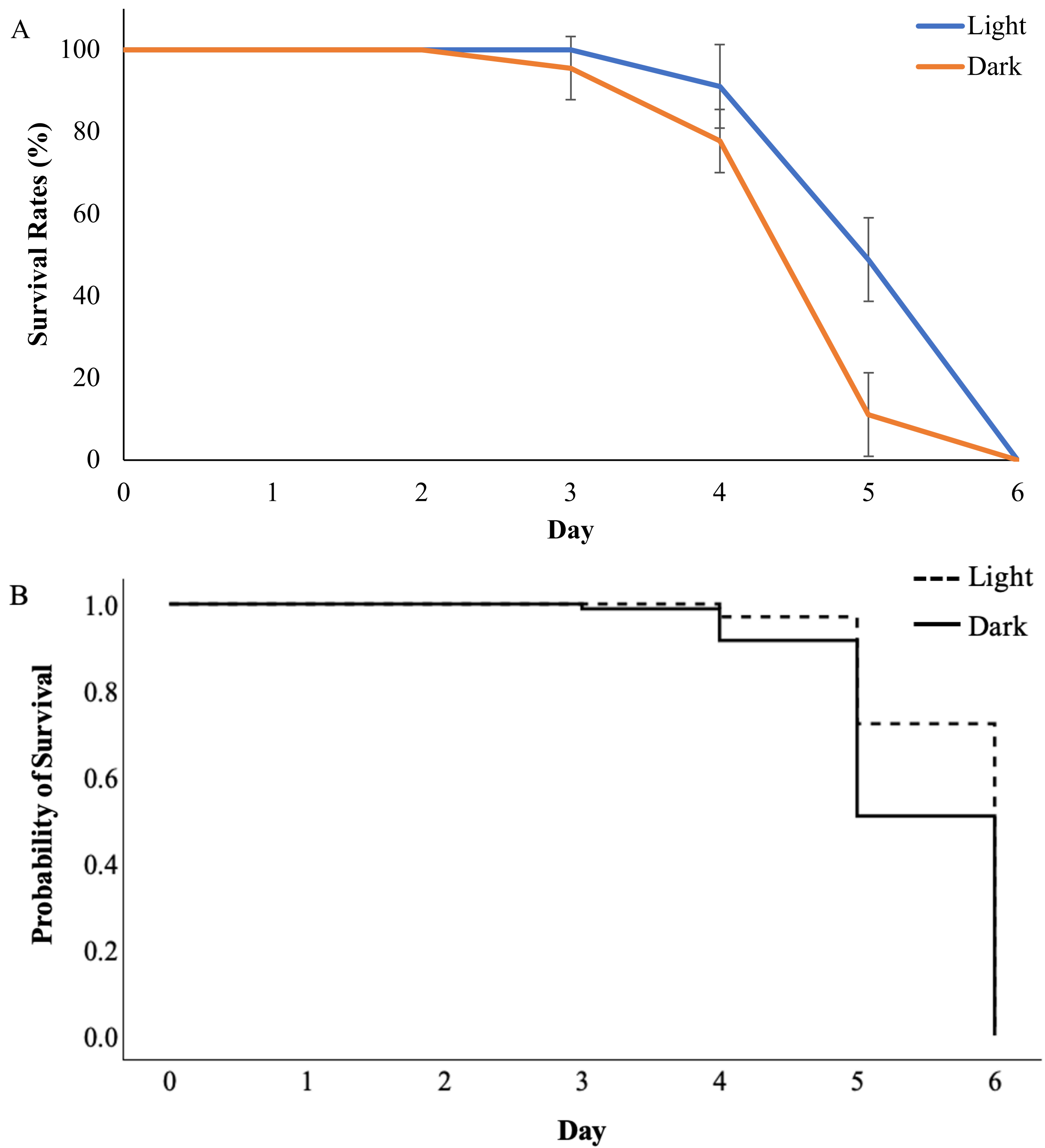

Figure 29. The impact of light-dark conditions on oncomiracidia of *T. vistulensis*. (A) Survival rates; (B) Survival curve.

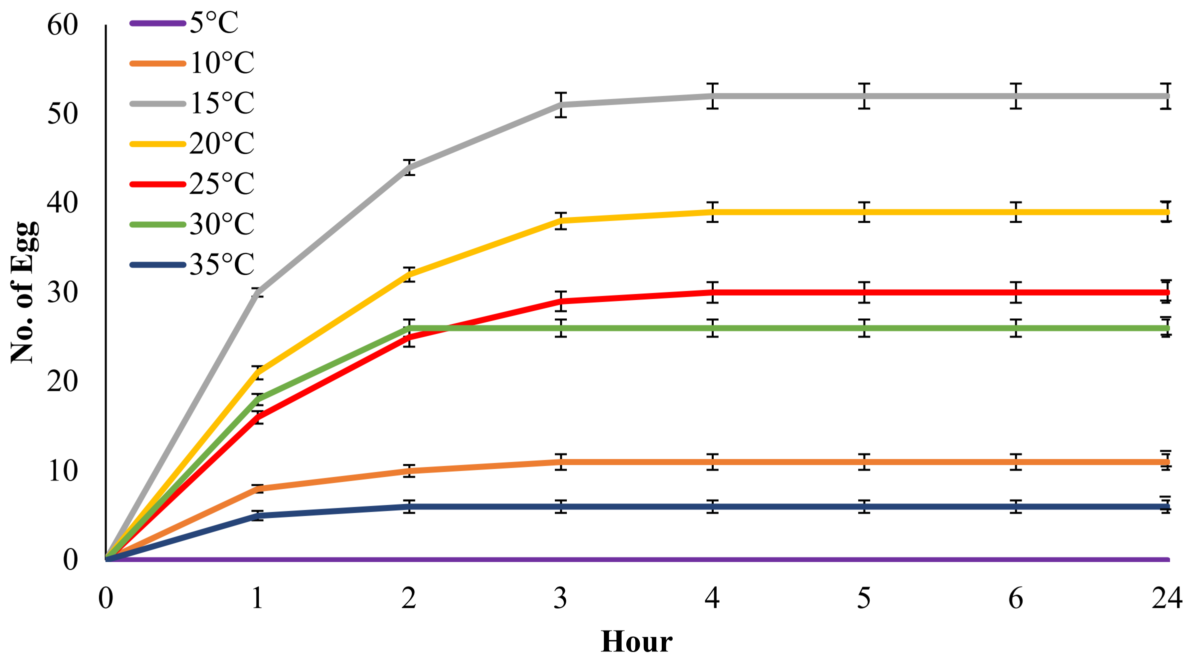

Figure 30. Cumulative egg production of *T. vistulensis* across various water temperatures.

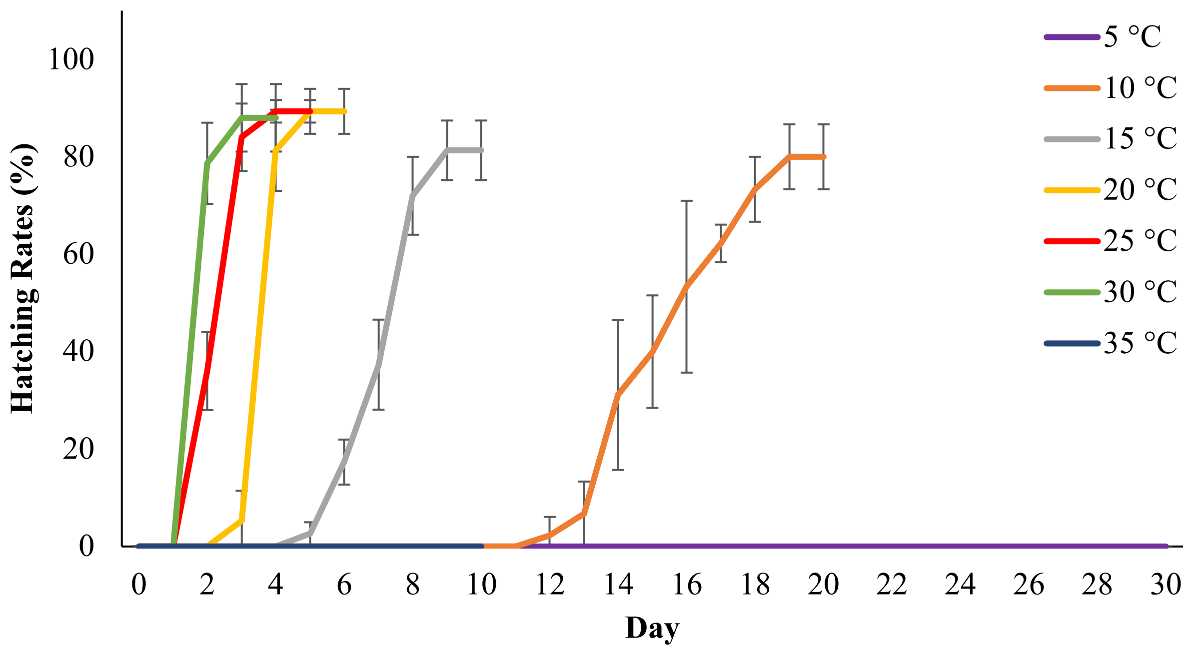

Figure 31. Cumulative egg hatching rates of *T. vistulensis* under different water temperatures.

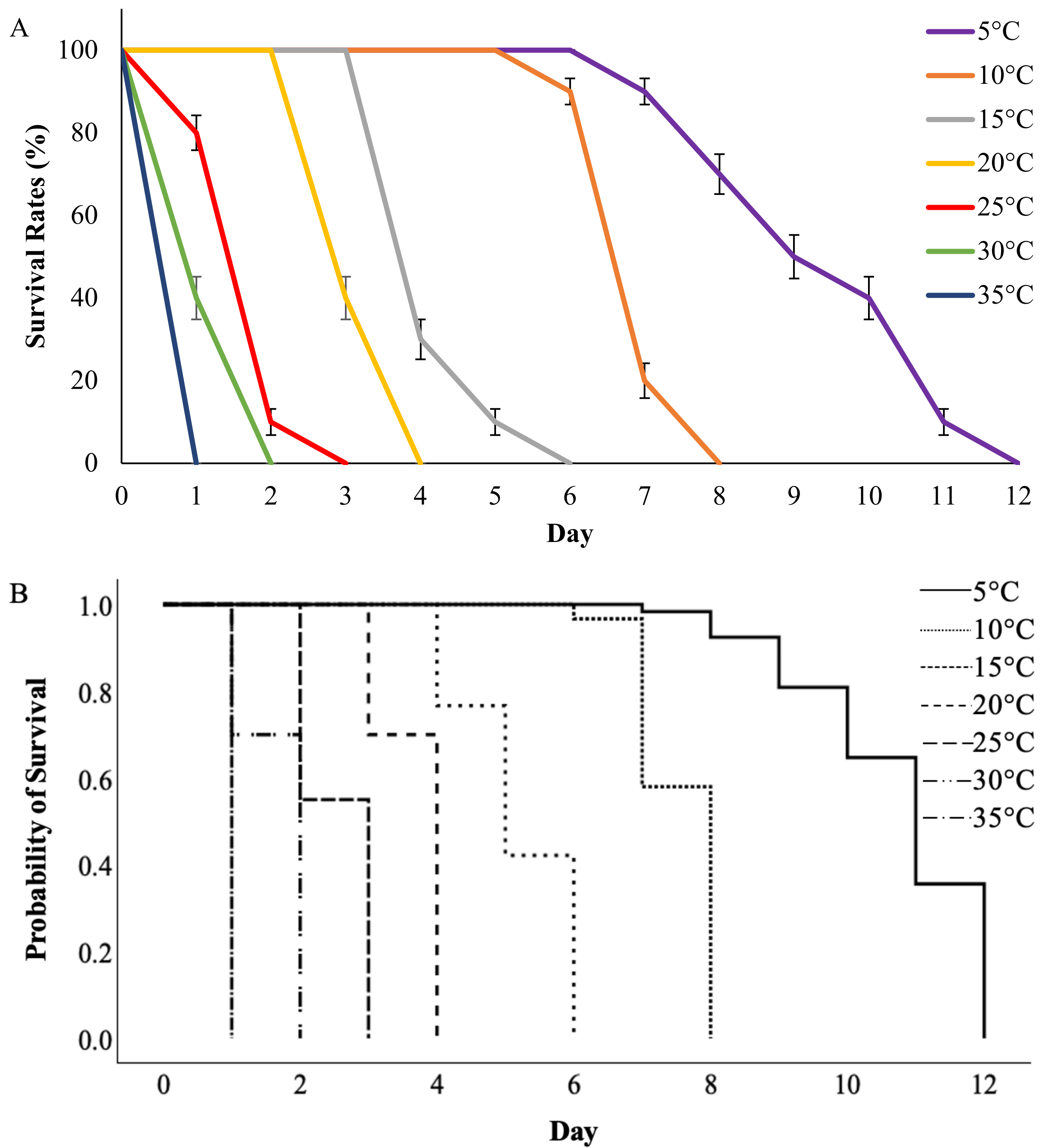

Figure 32. The influence of various water temperatures on adult *T. vistulensis*. (A) Survival rates; (B) Survival curve.

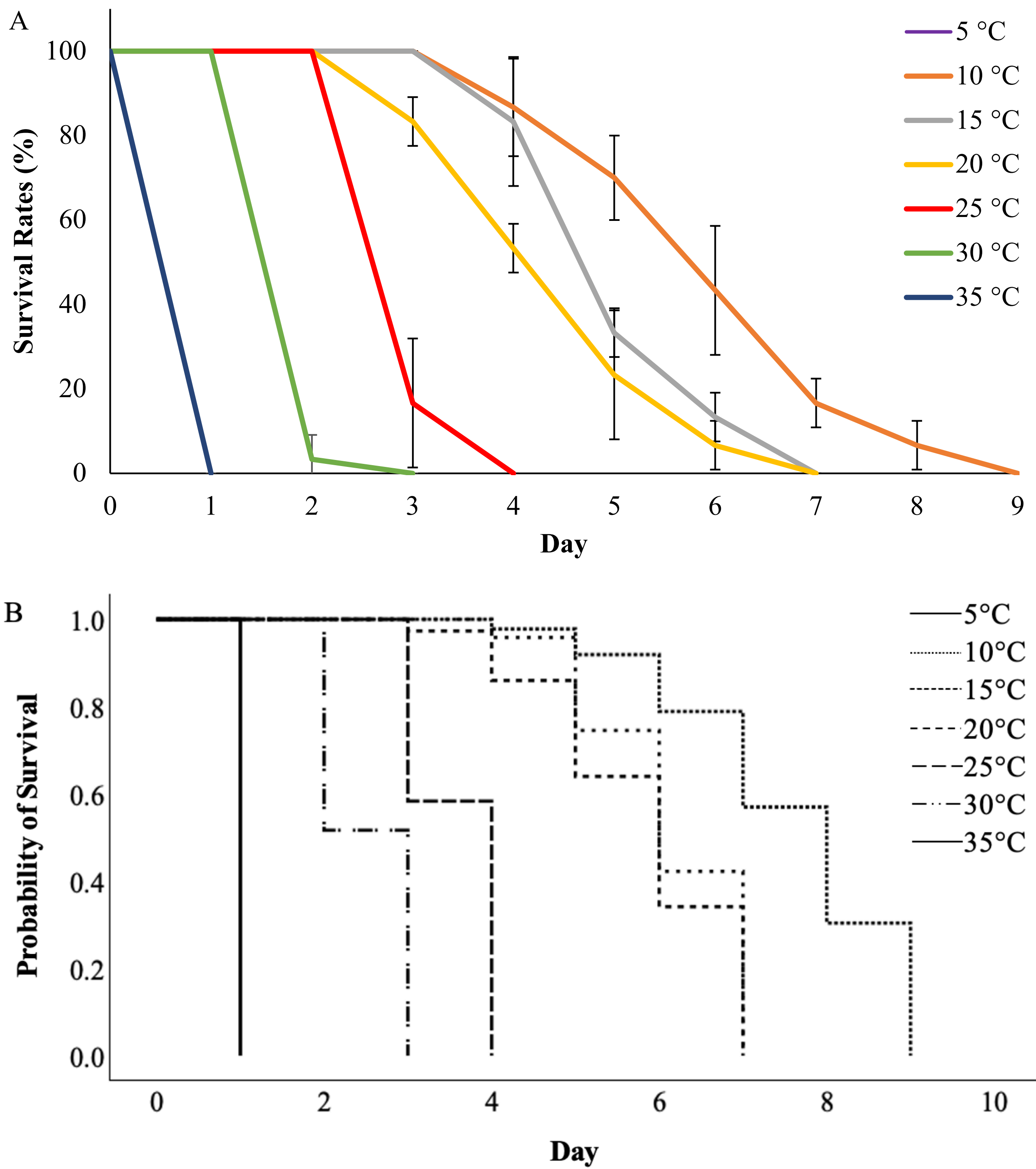

Figure 33. The influence of various water temperatures on oncomiracidia *T. vistulensis*.
(A) Survival rates; (B) Survival curve.

4.6. Treatment trial against *Thaparocleidus vistulensi*s

4.6.1. In vitro herbal treatment against Thaparocleidus vistulensis

4.6.1.1. Egg hatching

The egg hatching rates differed significantly between herbal treatments (χ^2 = 524, DF = 11, $P < 0.0001$). Generally, the GAR treatment showed the highest efficiency against *T. vistulensis* eggs compared to GIN and NMB, showing a significant difference ($P < 0.0001$) with the control (**Table S8**), where it totally suppressing the eggs development for all dilutions; eggs remained translucent brown until the experiment terminated, with 0% hatching success (**Figure 34** and **35A)**. Meanwhile, for GIN and NMB, only the lowest dilution (1:10) demonstrated their effectivities with 0% hatching rates. The hatching rates for GIN at 1:50 and 1:100 dilutions exhibited a similar pattern, with no significance difference observed ($P > 0.6651$) (**Table S8**), and the recorded hatching success were 70.67% and 72%, respectively (**Figure 34** and **35B**). Similarly, the hatching success rates for NMB at 1:50 and 1:100 dilutions showed no significant difference ($P > 0.1024$), with recorded hatching rates of 62.67% and 68%, respectively, although the 1:50 dilution showed a slightly lower hatching success on day 3 and 4 compared to 1:100 (**Figure 34** and **35C**).

4.6.1.2. Oncomiracidia longevity

The survival analysis showed a significant difference in the survival of oncomiracidia between the treatments (χ^2 = 1167, DF = 11, $P < 0.0001$). According to the pairwise multiple comparisons, the differences between each dilution within the treatment groups and the corresponding control groups for each treatment were significantly difference ($P < 0.0001$) (**Table S9**). Treatment using NMB appears to be the most effective herbal remedy for eliminating 100% of the oncomiracidia, even at the highest dilution (1:100), achieving complete eradication within 75 min (**Figure 36C**). Other than that, GAR was less effective compared to NMB, but significantly more effective than GIN, as it killed within 4 hpt at the highest dilution (1:100) (**Figure 36A**). For GIN, only the lower dilution showed effectiveness, killing all oncomiracidia within 24 hpt, while the higher dilutions (1:50 and 1:100) took longer to show similar results (**Figure 36B)**.

4.6.1.3. Lifespan of developing juvenile and adult flukes

Survival analysis of developing juvenile *T. vistulensis* indicated a significant difference in survival across treatments ($\chi^2 = 694$, DF = 11, P < 0.0001). Pairwise multiple comparisons showed that differences between dilutions within treatment groups and their corresponding control groups were statistically significant (P < 0.0001) (**Table S10**). The use of GAR was the best option for herbal treatment against juvenile *T. vistulensis*, eradicating all juvenile parasites within 3 hpt at the highest dilution (1:100) (**Figure 37A**). In contrast, NMB achieved complete eradication within 15 min only at the lowest dilution (1:10), while higher dilutions (1:50 and 1:100) required more time but remained effective within 24 hpt (**Figure 37C**). Longer period needed for GIN to achieve complete elimination even at the lower dilutions (1:10 and 1:50) compared to other treatments (**Figure 37B**).

At the same time, the effectiveness of treatments against adult *T. vistulensis* differs significantly between the treatment groups ($\chi^2 = 843$, DF = 11, P < 0.0001). Pairwise comparisons revealed significant differences between each dilution within the treatment groups and their respective control groups (*P* < 0.0001) (**Table S11**). Interestingly, the results for GAR and NMB treatments showed approximately similar pattern, where they could eliminate all adult *T. vistulensis* within 24 hpt (**Figure 38A** and **38C**). For the lowest dilution (1:10), NMB showed a better efficiency compared to GAR, as it required less than 15 min for completely eradicating the adult parasites, while 45 min for GAR treatment. Whereas, 4 hpt required for 1:50 dilution in both treatments for totally eliminating the parasites, although NMB recorded the first mortality earlier (1.5 hpt) compared to GAR (2.5 hpt). In contrast, complete mortality of adult *T. vistulensis* exposed to GIN was achieved within 24 hpt at the lower dilutions (1:10 and 1:50), while the highest dilution (1:100) did not show a clear effect (**Figure 38B**).

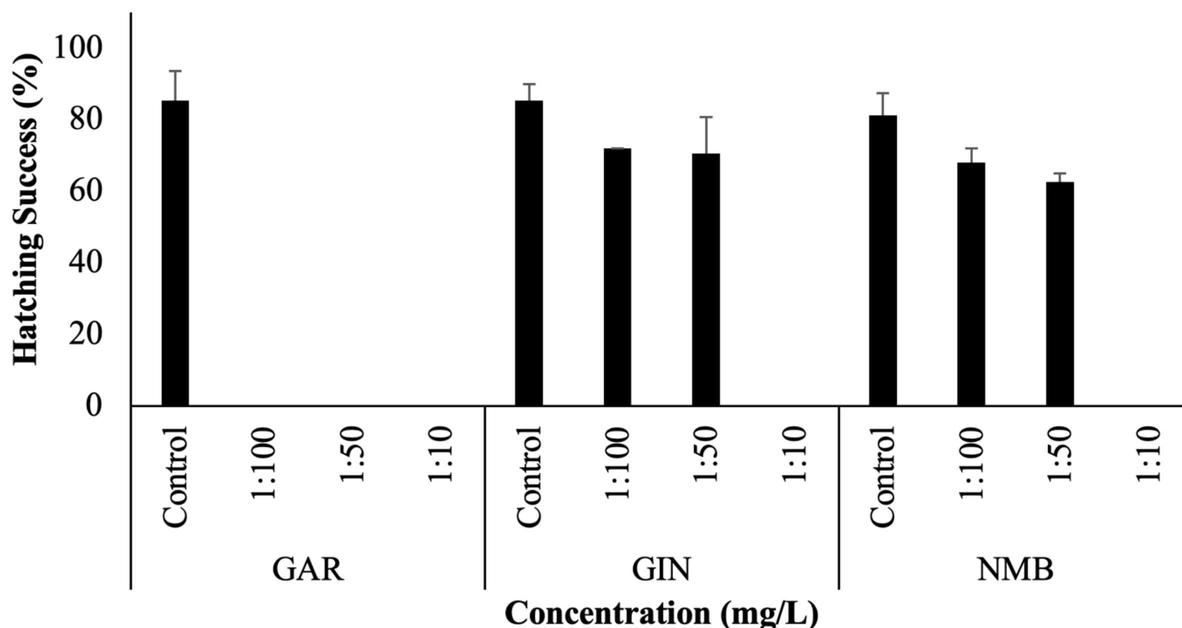

Figure 34. *In vitro* egg hatching success of *T. vistulensis* across various herbal treatments and dilutions.

4.6.2. In vitro drug treatments against Thaparocleidus vistulensis

4.6.2.1. Egg hatching

The egg hatching rates differed significantly between treatments ($\chi^2 = 1464$, DF = 26, $P < 0.0001$). The treatment MBZ had the highest efficacy against *T. vistulensis* eggs, completely inhibiting their development even at the lowest tested concentration, compared to PZQ and BIO (**Figure 39** and **40**). The hatching rates of eggs in the groups exposed to BIO were not significantly different than in the control group (**Figure 40A** and **Table S12**), and eggs in all concentrations exhibited at least 85.33% hatching success (**Figure 39**). Hatching success was significantly reduced in the PZQ treatment at higher dosages, with only 6.67% and 5.33% success recorded at 80 mg/L and 100 mg/L drug concentrations, respectively (**Figure 39** and **40B**). The eggs hatching treated with BIO and PZQ began on day 3, with most of them completed by days 4 and 5. No egg development was observed in the groups treated with MBZ at any dose, which represents a significant difference compared to the control group ($P < 0.0001$) (**Table S12**); the colour of eggs changed from translucent to dark brown towards the end of the observation period, indicating deterioration, with 0% hatching success (**Figure 39** and **40C**).

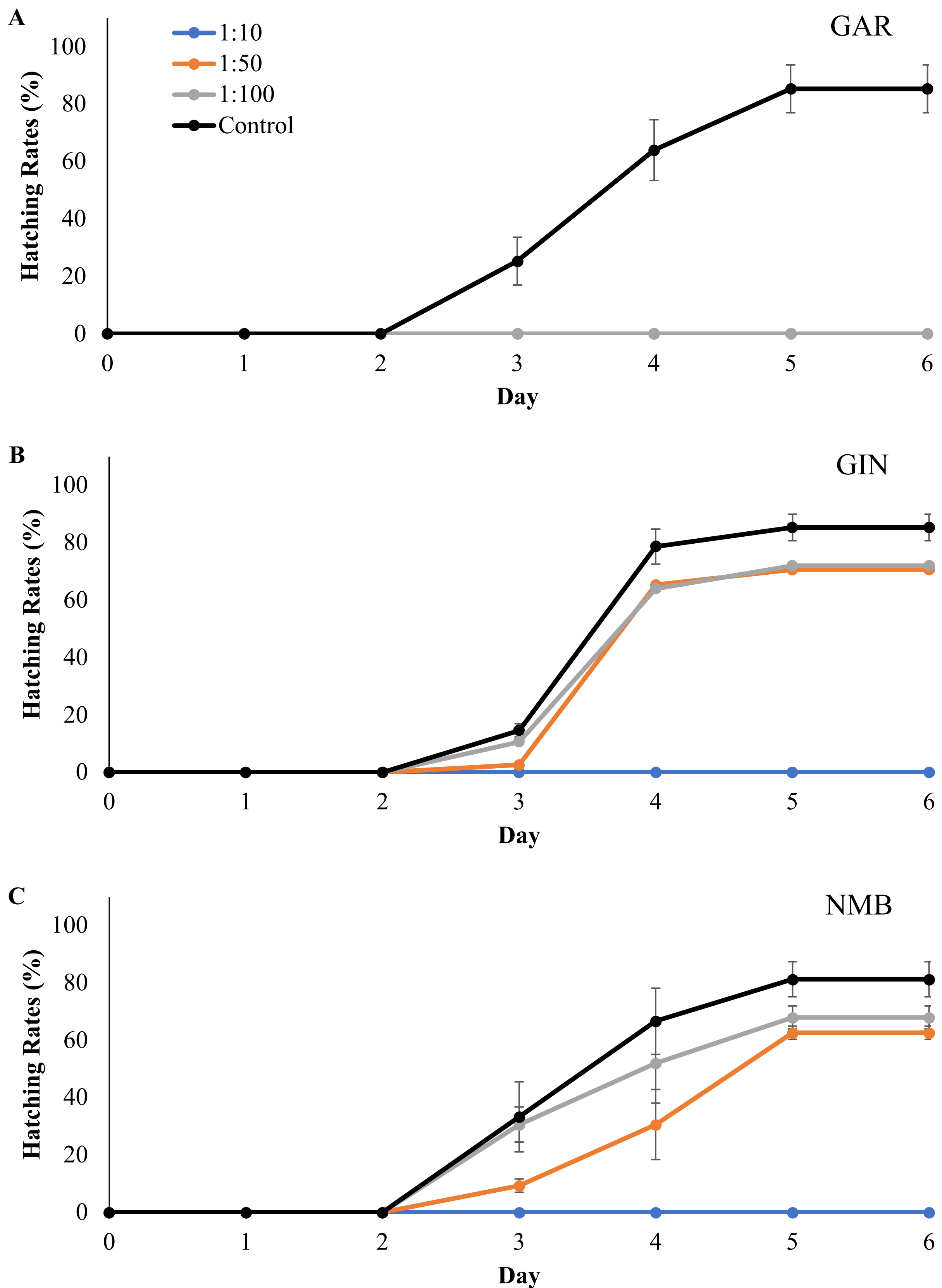

Figure 35. *In vitro* efficacy of different herbal antiparasitic agents against eggs of *T. vistulensis*. (A) Garlic; (B) Ginger; (C) Neem bark.

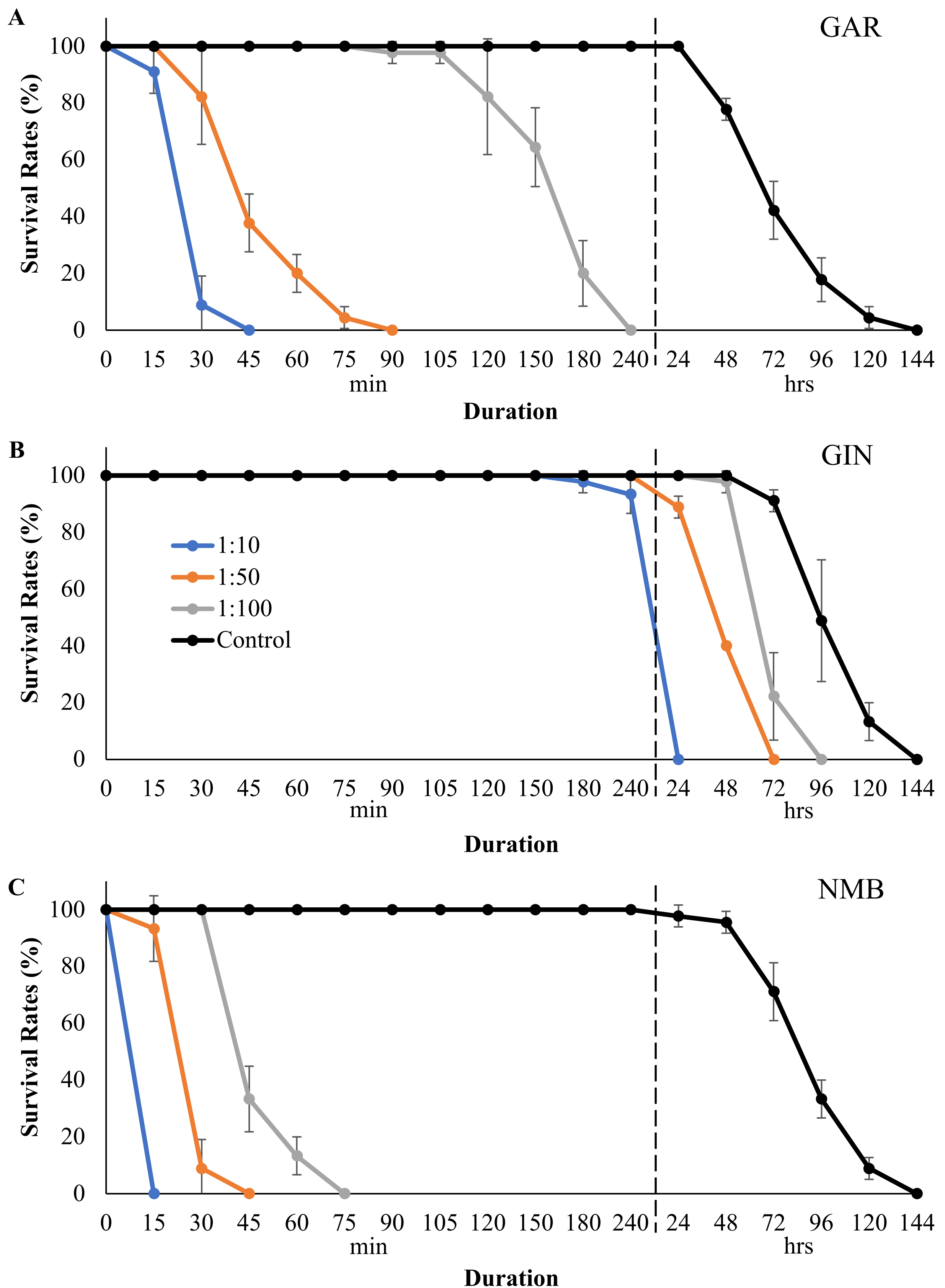

Figure 36. *In vitro* efficacy of different herbal antiparasitic agents against oncomiracidia of *T. vistulensis*. (A) Garlic; (B) Ginger; (C) Neem bark.

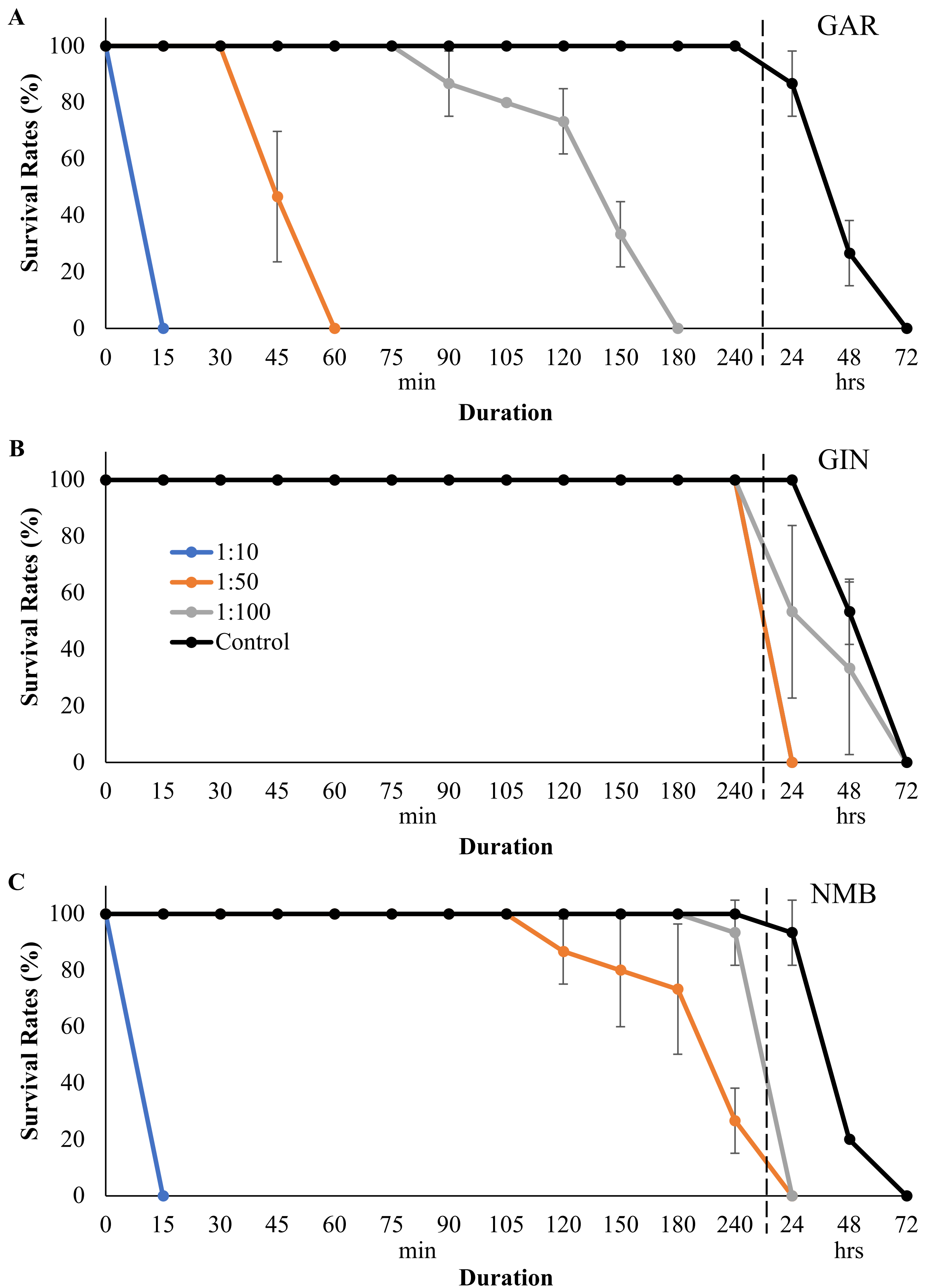

Figure 37. *In vitro* efficacy of different herbal antiparasitic agents against developing juvenile *T. vistulensis*. (A) Garlic; (B) Ginger; (C) Neem bark.

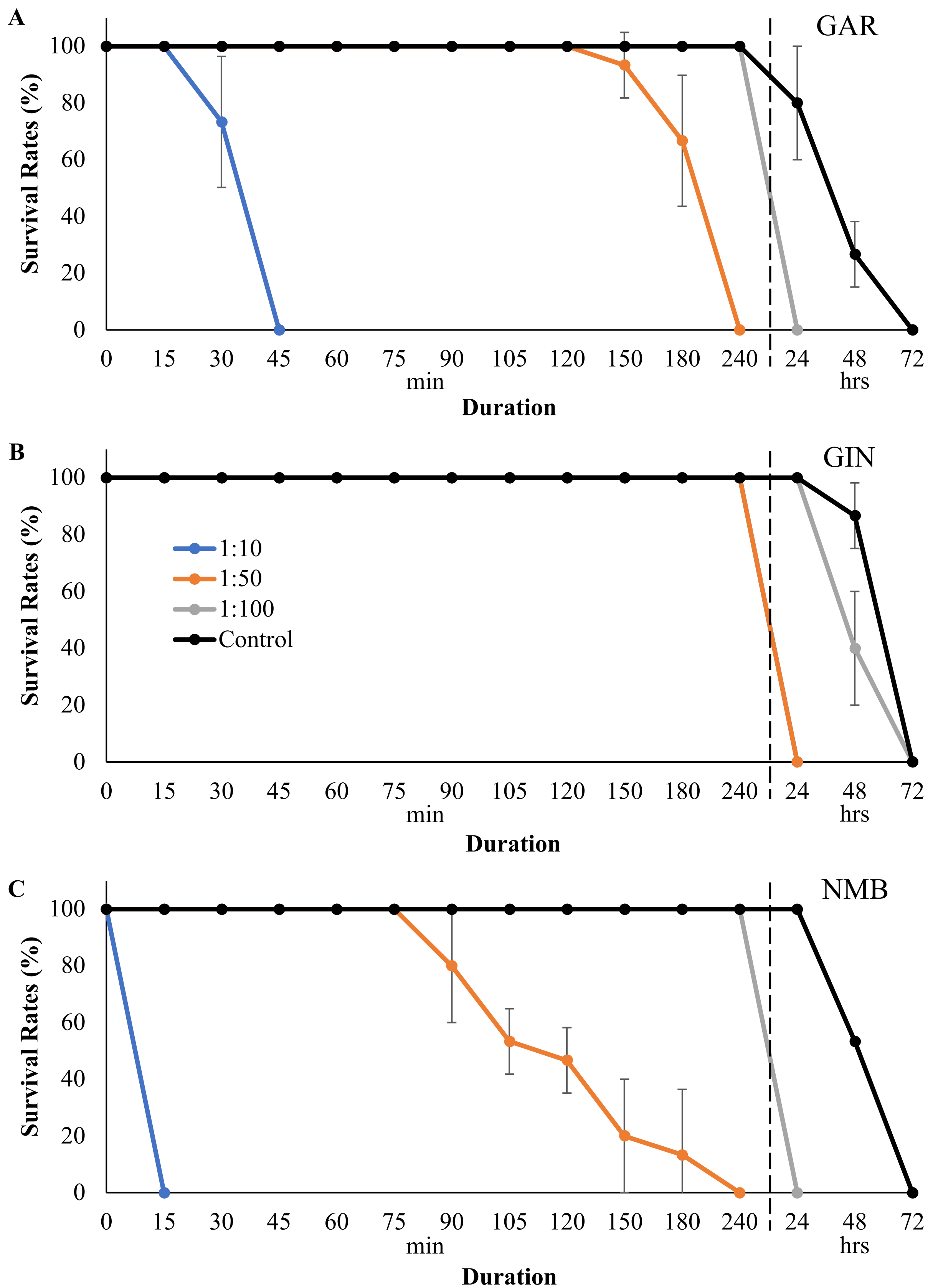

Figure 38. *In vitro* efficacy of different herbal antiparasitic agents against adult *T. vistulensis*. (A) Garlic; (B) Ginger; (C) Neem bark.

4.6.2.2. Oncomiracidia longevity

The survival analysis showed a significant difference in the survival of oncomiracidia between the treatments ($\chi^2 = 2812$, DF = 26, $P < 0.0001$). According to the pairwise multiple comparisons, the differences between each concentration within the treatment groups and the corresponding control group for each treatment were significant except for 1 mg/L ($P < 0.4801$), 5 mg/L ($P < 0.4027$) and 10 mg/L ($P < 0.4808$) for BIO (**Table S13**). The use of BIO was the most effective treatment against oncomiracidia, especially at higher dosages (≥40 mg/L), that could kill all the oncomiracidia within 3 hpt, compared to other treatments (**Figure 41A**). For PZQ, over 50% of oncomiracidia were dead by 72 hpt at the lowest concentration tested (**Figure 41B**). In contrast, higher dosages of MBZ (≥40 mg/L) were needed to achieve complete mortality within 72 hpt (**Figure 41C**).

4.6.2.3. Lifespan of developing juvenile and adult flukes

The survival analysis of juvenile *T. vistulensis* revealed a significant difference in their survival among treatments ($\chi^2 = 580$, DF = 26, $P < 0.0001$). Pairwise multiple comparisons indicated that the differences between each concentration within the treatment groups and the corresponding control groups were not statistically significant except for 60, 80, and 100 mg/L of BIO and PZQ, and 100 mg/L of MBZ (**Table S14**). The survival patterns of juvenile parasites treated with BIO and PZQ were almost similar, where nearly all juvenile *T. vistulensis* was eradicated within 48 hpt at higher concentrations of ≥60 mg/L (**Figure 42A** and **42B)**. Nevertheless, the effect of BIO was demonstrated faster, with the first mortality recorded within 75 min post-treatment at the highest dosage (100 mg/L), compared to PZQ, which required 3 hpt for the first mortality observed. In contrast, MBZ exhibited no significant effect on the juvenile *T. vistulensis*, except for 100 mg/L ($P < 0.01$) (**Figure 42C** and **Table S14**).

Meanwhile, the effect of treatments against adult *T. vistulensis* shows a significant difference between the treatments ($\chi^2 = 705$, DF = 26, $P < 0.0001$). However, pairwise comparisons could not be performed as some treatments showed no variance in survival times, with all parasites dying at the same time point. Different doses and time periods of effectiveness were demonstrated by the BIO, where complete mortality was achieved within 24 hpt at the highest dosage (100 mg/L), within 48 hpt (80 mg/L), and lower concentrations requiring up to 72 hpt (**Figure 43A**). In comparison to PZQ, lower concentrations (≥60 mg/L) were needed for exhibiting full efficacy by 24 hpt, at 20–40 mg/L by 48 hpt, and at concentrations ≤10 mg/L by 72 hpt (**Figure 43B**). In contrast, MBZ eliminated all adult *T. vistulensis* at concentrations ≥20 mg/L by 48 hpt, with concentrations ≤10 mg/L achieving complete mortality by 72 hpt (**Figure 43C**).

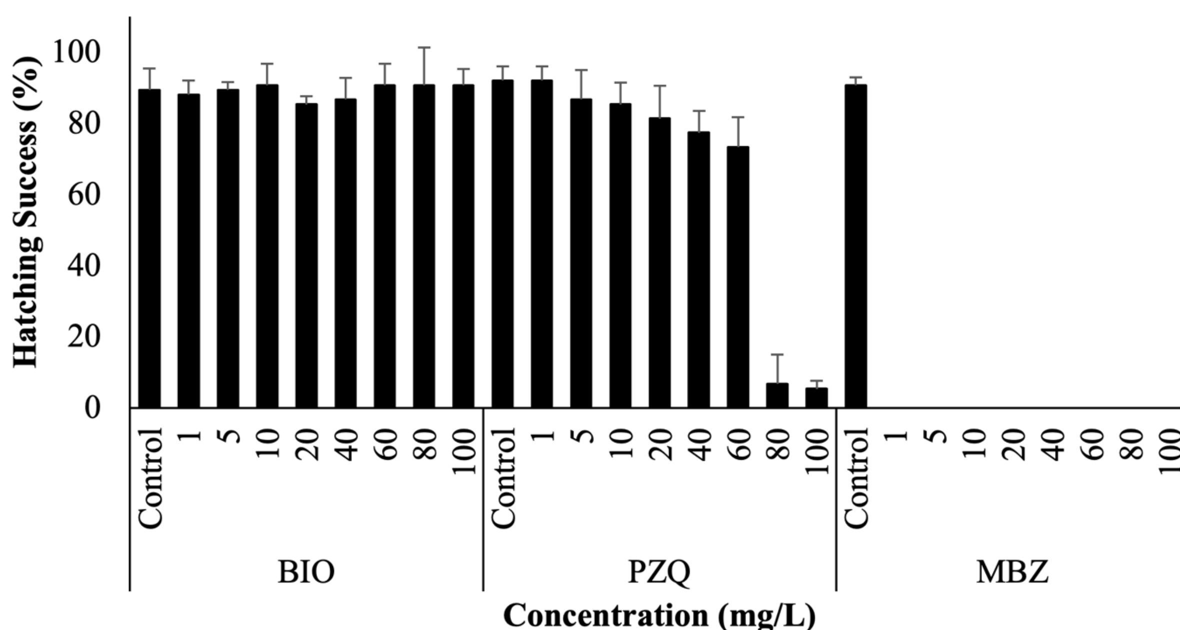

Figure 39. *In vitro* egg hatching success of *T. vistulensis* across various treatments and concentrations.

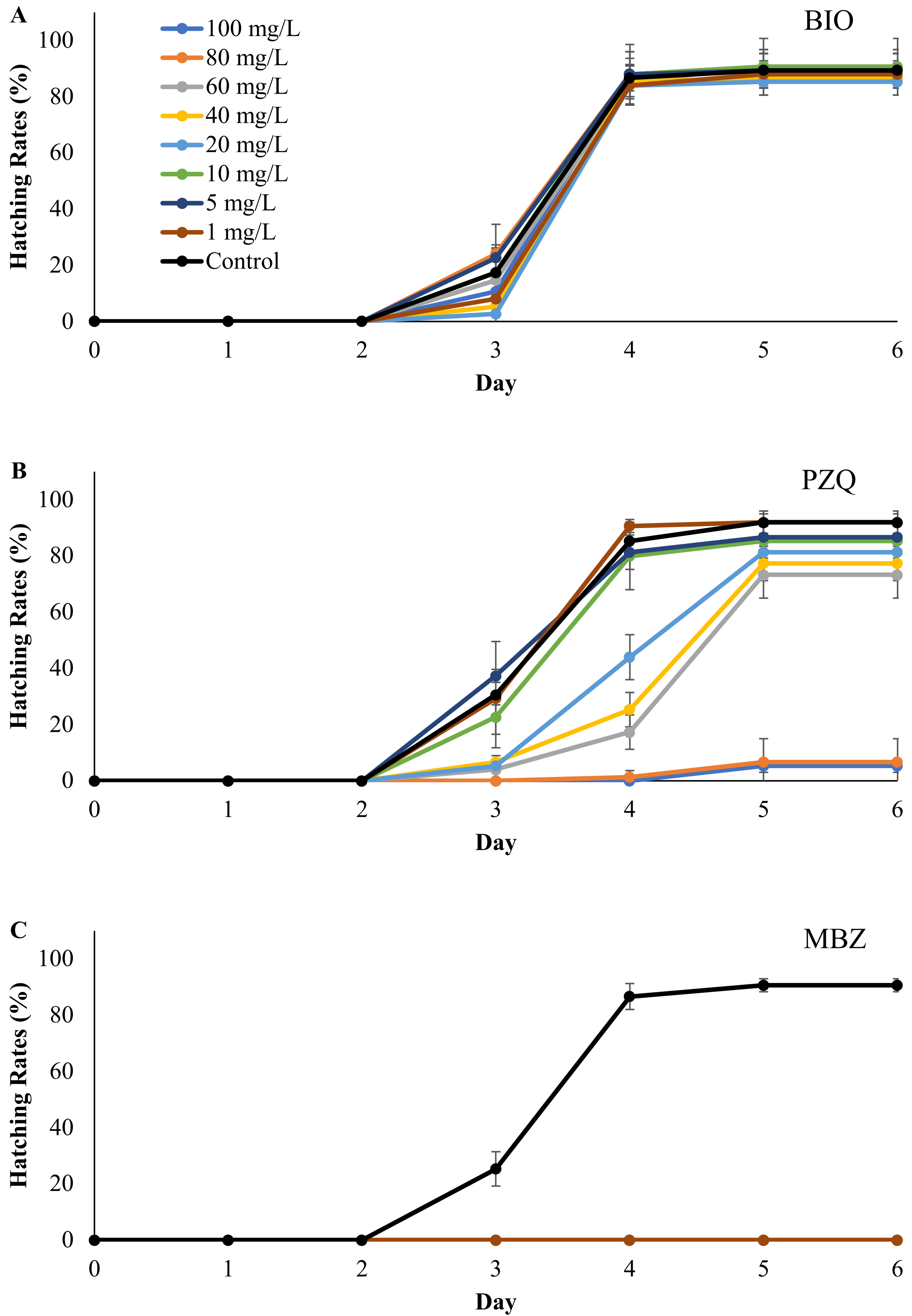

Figure 40. *In vitro* efficacy of different antiparasitic agents against eggs of *T. vistulensis*. (A) Biokos; (B) Praziquantel; (C) Mebendazole.

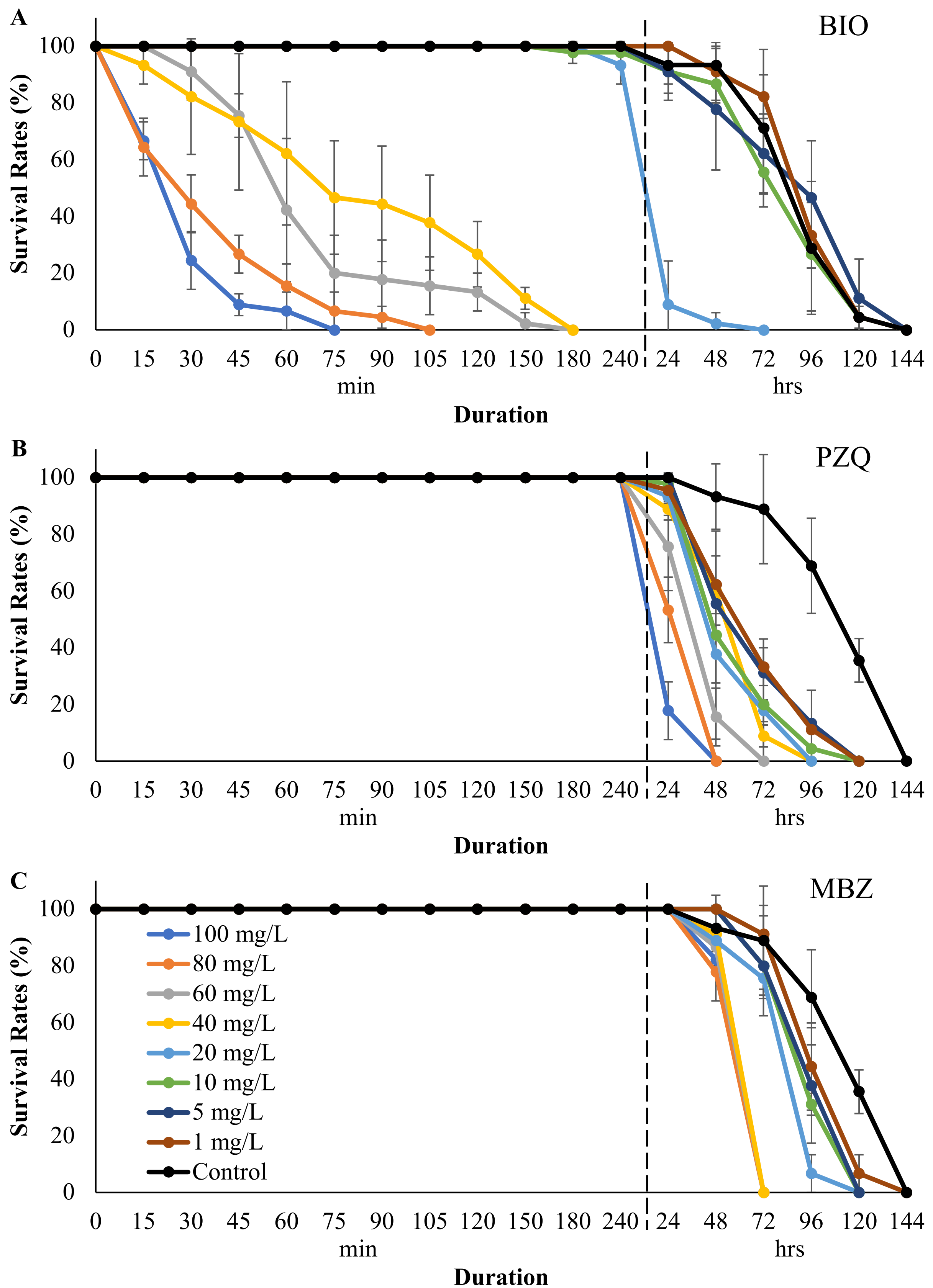

Figure 41. *In vitro* efficacy of different antiparasitic agents against oncomiracidia of *T. vistulensis*. (A) Biokos; (B) Praziquantel; (C) Mebendazole.

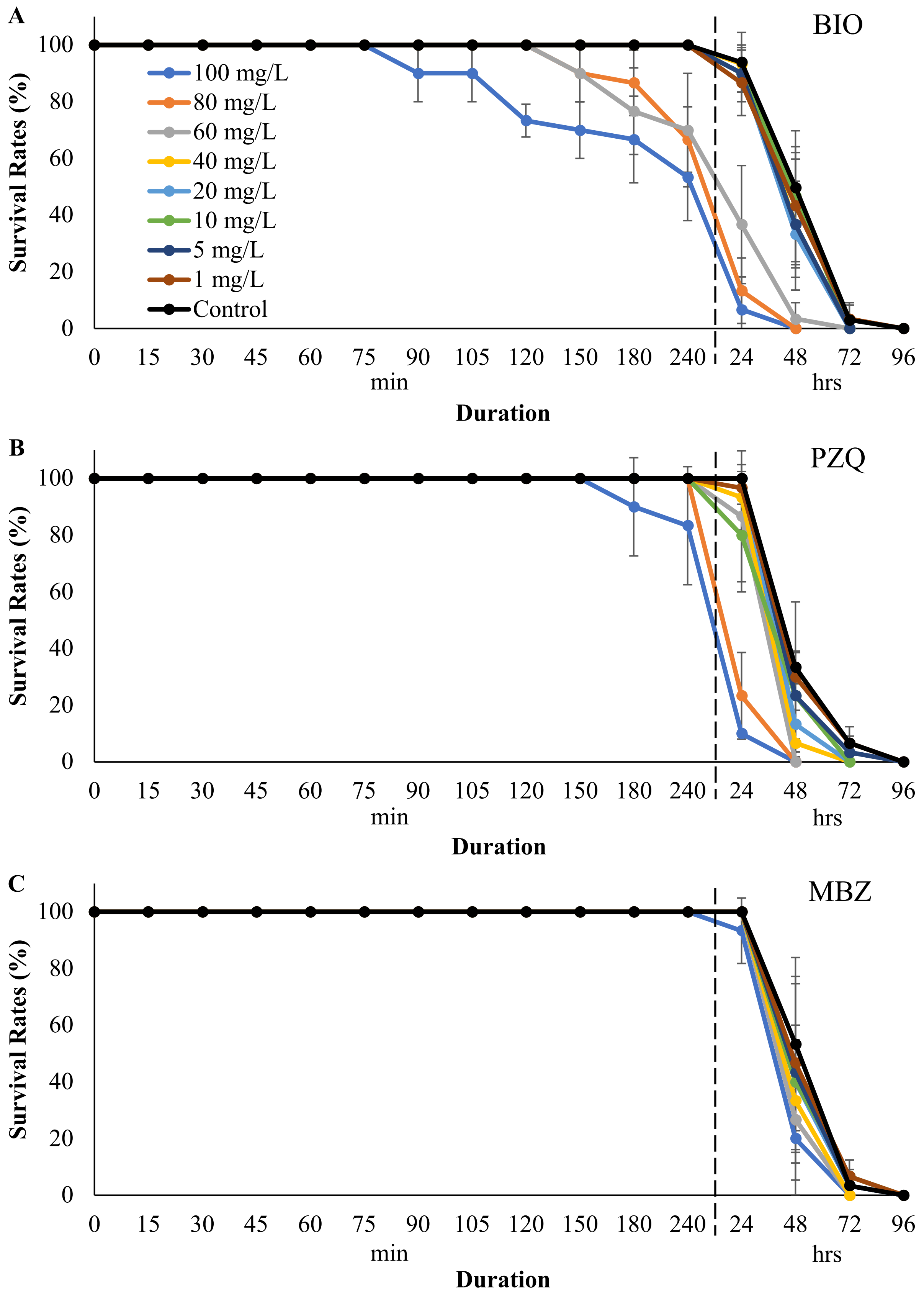

Figure 42. *In vitro* efficacy of different antiparasitic agents against developing juvenile *T. vistulensis*. (A) Biokos; (B) Praziquantel; (C) Mebendazole.

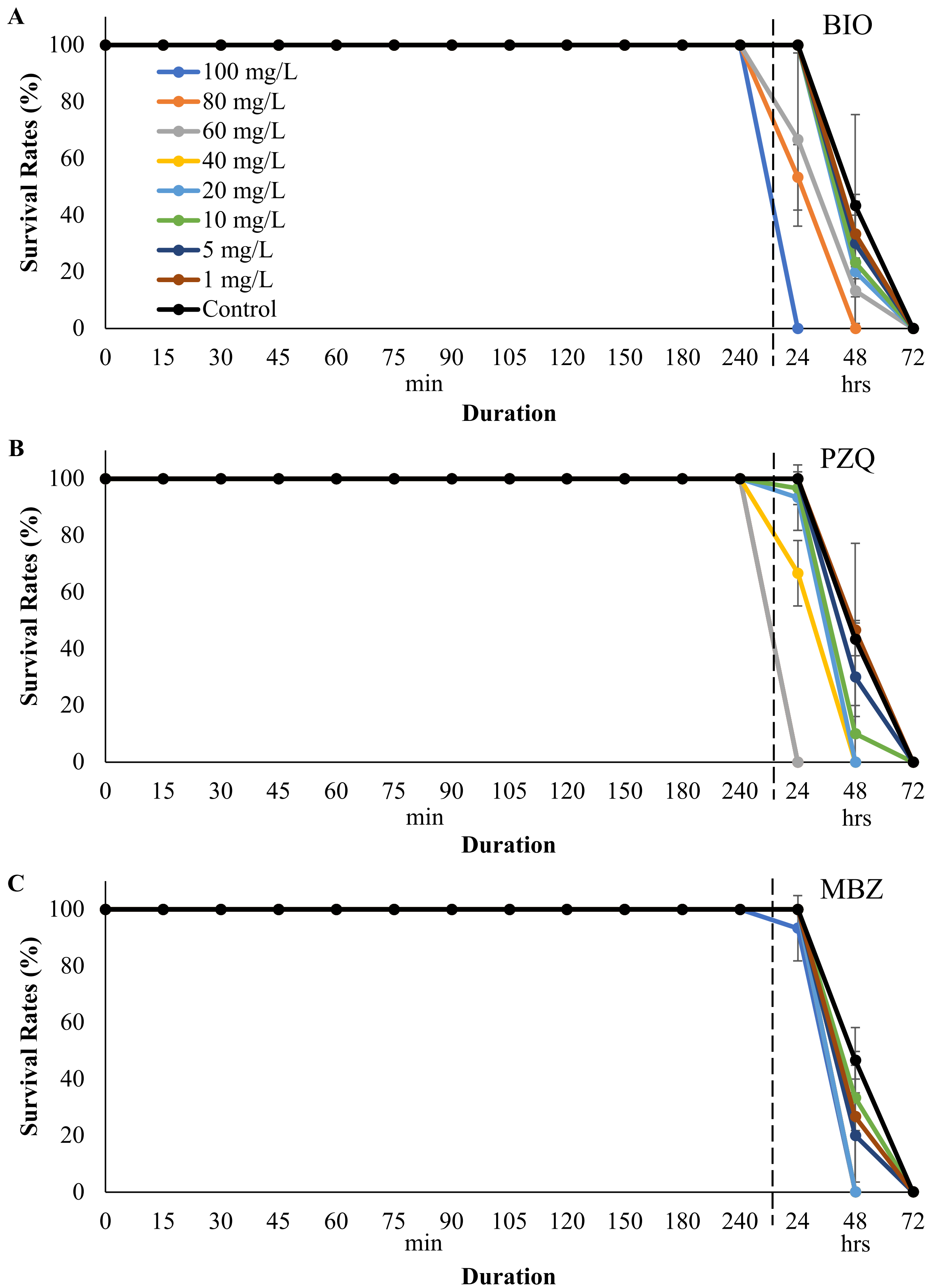

Figure 43. *In vitro* efficacy of different antiparasitic agents against adult *T. vistulensis*. (A) Biokos; (B) Praziquantel; (C) Mebendazole.

4.6.3. In vivo drug treatments against Thaparocleidus vistulensis

4.6.3.1. Toxicity test

A toxicity test was conducted using the treatments (in 10 and 20 mg/L concentrations) with the most promising *in vitro* results (MBZ) and the treatment that is widely used for eliminating monopisthocotyleans (PZQ) to evaluate the physiological stress effects of varying concentrations of a compound on juvenile European catfish (**Table 1**). All fish survived throughout the testing period except those exposed to 20 mg/L of MBZ, where the test was terminated within 10 min due to the immediate appearance of toxicity symptoms. Fish exposed to 20 mg/L of PZQ exhibited body paling within 1 h, which slowly turned to normal after several hours of 100% water change; however, no other toxicity signs were observed, and they survived for the entire testing period.

Table 1 Signs of toxicity in European catfish at different dosages.

Signs of Toxicity	Praziquantel (PZQ)			Mebendazole (MBZ)		
	0 mg/L	10 mg/L	20 mg/L	0 mg/L	10 mg/L	20 mg/L*
Lethargy	-	-	-	-	-	+
Loss of equilibrium	-	-	-	-	-	+
Abnormal ventilation	-	-	-	-	-	+
Abnormal skin pigmentation	-	-	+	-	-	+

* The tests were terminated within 10 minutes

4.6.3.2. In vivo treatment efficacy assessment

Antiparasitic treatment against *T. vistulensis* on the host revealed a significant difference between the control and treatment groups (Kruskal–Wallis, H = 44.015, $P < 0.001$). Post hoc Dunn's test for pairwise comparisons showed that the control group differed significantly from PZQ ($P < 0.001$) and MBZ ($P < 0.001$), but no significant difference was found between PZQ and MBZ ($P > 0.963$). Generally, increasing the treatment concentration increases the treatments' efficacy (**Figure 44** and **Table 2**). Evaluating based on the antiparasitic treatment PZQ against the *T. vistulensis*, statistical analysis confirmed significant differences in efficacy between the concentrations at each time point (Kruskal–Wallis, H = 41.113, $P < 0.001$), highlighting the

superior performance of the 10 mg/L of PZQ compared to the lower concentrations. The number of parasites counted after being treated with PZQ shows a significant difference, except between concentrations of 1 mg/L and 5 mg/L ($P > 0.109$) (**Table S15**). A significant difference in parasite counts was found across the concentrations at various days post-treatment (Kruskal–Wallis, H = 21.330, $P < 0.001$). Dunn's post-hoc test confirmed these differences, except for the comparison between 1 dpt and 7 dpt ($P = 1.000$) (**Table S15**). The most notable observation was that treatment with 10 mg/L of PZQ successfully eliminated all *T. vistulensis* from the gills by 1 dpt and maintained complete efficacy (100%) throughout the experiment, up to 14 dpt (**Figure 44** and **Table 2**). At 1 dpt, the group treated with 10 mg/L of PZQ achieved significantly higher efficacy compared to lower concentrations, with a 100% reduction in parasite count, followed by the 5 mg/L group with 65.6% efficacy and the 1 mg/L group with 33.6 % efficacy. This trend was consistent at 7 dpt and 14 dpt, where the highest efficacy was observed in the 10 mg/L group (100%), while the lowest efficacy for the 1 mg/L group ranged from 15.6% to 34.9%. Each PZQ concentration used in the current study exhibited varying levels of efficacy (more effective or less effective), with a statistically significant difference observed (**Table S16**), and resulted in reduced parasite counts compared to the control group. Nevertheless, while the parasite counts were maintained through 7 dpt, an unexpected reemergence of parasites was observed in these groups by 14 dpt, with both adult and juvenile monopisthocotyleans detected on the gill arches.

Focusing on the effects of the antiparasitic treatment MBZ, statistical analysis showed significant differences among the various concentration groups (Kruskal–Wallis, H = 7.989, $P < 0.018$) and time point groups (Kruskal–Wallis, H = 31.736, $P < 0.001$), with post hoc Dunn's test for pairwise comparisons varies (**Table S15**). Overall, treatment efficacy at different MBZ concentrations was practically ineffective at 1 dpt (one–way ANOVA, $F(2, 15) = 1.097$, $P > 0.359$) (**Figure 44** and **Table S16**). At 7 dpt, one–way ANOVA revealed a significant difference between the concentration groups ($F(2, 15) = 16.687$, $P < 0.001$). Post-hoc analysis using Tukey's HSD

revealed that all concentrations were significantly different from each other, except at 1 mg/L and 5 mg/L ($P > 0.979$). The number of parasite counts was maintained for both concentrations, 1 mg/L and 5 mg/L, but reductions were recorded after 7 dpt at 10 mg/L (**Figure 44** and **Table 2**). Interestingly, a delayed effect was observed across all concentrations, with more than 98% of parasites being removed by 14 dpt (**Table 2**).

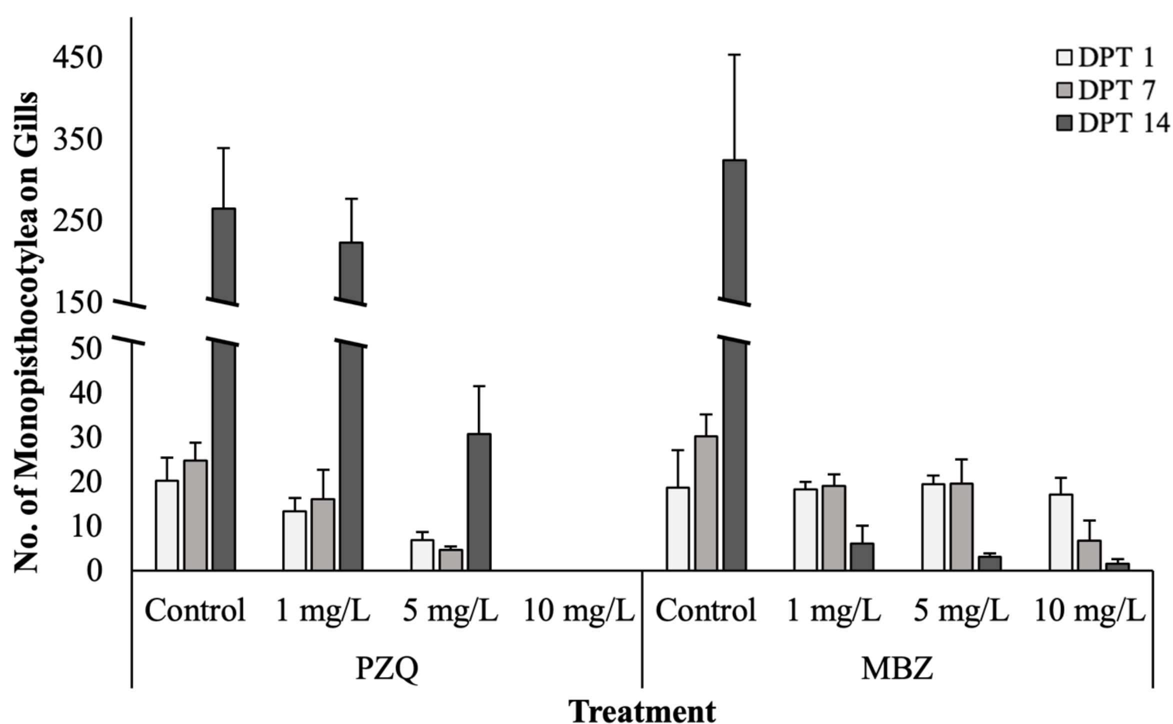

Figure 44. *In vivo* efficacy of PZQ and MBZ treatments against *T. vistulensis* over time.

Table 2 Mean number ± standard deviation of *T. vistulensis* in gills of European catfish, 1-, 7-, and 14-day post-treatments (dpt) with Praziquantel (PZQ) or Mebendazole (MBZ), and their respective efficacies (%).

Treat.	Conc. (mg/L)	1		7		14	
		No. of Parasites	Efficacy (%)	No. of Parasites	Efficacy (%)	No. of Parasites	Efficacy (%)
PZQ	0	20.33 ± 5.24	-	24.83 ± 4.07	-	265.50 ± 74.33	-
	1	13.50 ± 2.88	33.6	16.17 ± 6.59	34.9	224.00 ± 53.3	15.6
	5	7.00 ± 1.79	65.6	4.67 ± 0.82	81.2	30.83 ± 10.89	88.4
	10	0.00 ± 0.00	100	0.00 ± 0.00	100	0.00 ± 0.00	100
MBZ	0	18.83 ± 8.45	-	30.33 ± 4.97	-	324.50 ± 129.49	-
	1	18.33 ± 1.75	2.7	19.17 ± 2.64	36.8	6.17 ± 4.02	98.1
	5	19.50 ± 2.07	0	19.67 ± 5.47	35.1	3.17 ± 0.75	99
	10	17.17 ± 3.87	8.8	6.83 ± 4.49	77.5	1.67 ± 1.03	99.5

CHAPTER 5

DISCUSSION

5.1. Morphological characterization and molecular analysis

The three related species (*Thaparocleidus siluri*, *T. vistulensis*, and *T. magnus*) were described from the European catfish by Zandt (1924), Siwak (1932), and Bychowsky and Nagibina (1957), respectively. These congeneric species were distinguished by several unique features (Bychowsky & Nagibina, 1957). The essential attributes of sclerotized structures, encompassing the haptoral parts and copulatory organs – copulatory piece and vagina, remain of paramount importance for species identification and taxonomy (Gussev, 1985; Wu et al., 2005; Pouyaud et al., 2006; Mendlová et al., 2012; Řehulková et al., 2013; Khang et al., 2016). The morphological characteristics of the haptor are regarded as adequate for genus-level parasite identification, while the reproductive organ proves a more suitable clue for species-level discrimination, likely attributable to its higher rate of variability (Pouyaud et al., 2006; Wu et al., 2007; Wu et al., 2008). Therefore, it is vitally essential to describe these features and structures meticulously.

The body size of parasites in this study exhibits a broader range of length (507.1–1002.4 μm) and total body width (120.6–196.2 μm) compared to the literature. This variation can be attributed to the parasites being measured as early as day 10 after infection, as indicated in the result of infection dynamics study in the subsection 4.4.1, which considered them to be in a mature stage. Furthermore, the measured parasites were preserved in 80% ethanol for a period before assessment, potentially leading to a size reduction. Nevertheless, the dimensions of each sclerotized feature, specifically the attachment organ, were found to be approximately consistent with the previous study except smaller than those reported by Paladini et al., 2008 (**Table S6**). This attachment organ suggests that the rigid components of the parasite remain fixed and resistant to shrinking.

The male copulatory organ of *T. vistulensis* is a relatively large structure. Hitherto, only a few studies included the dimension size of the copulatory organ studied by light microscopy in whole-mounted specimens (Bychowsky & Nagibina, 1957; Abdullah & Shwani, 2010). Furthermore, although some of these authors have provided detailed descriptions of an excellent drawing, the exact measurement points are not mentioned, making it sometimes difficult to interpret their descriptions. The present report of the male copulatory organ is based on light microscopy with a detailed point of measurement on the organ's features, allowing a detailed analysis of the shape.

Based on published drawings, the male copulatory organ (i.e., a long thread-like sclerotized penis with coils) has been documented and described in at least eight *Thaparocleidus* species, similar to that of *T. vistulensis*. The characteristic, a long thread-like sclerotized penis with coils, is shared with several other species, as outlined in **Table S17**. The overall structure of the male copulatory organ bears a resemblance to *T. magnus* and *T. siluri*, characterized by a sclerotized penis tube structure with a flask-shaped bulb, coils in the middle of the length of the penis, and an accompanying accessory piece. However, *T. vistulensis* exhibits a closer affinity to *T. magnus*, but is easily distinguishable based on the male copulatory organ (i.e., distant points of the copulatory organ) other than the body size and attachment structure (Bychowsky & Nagibina, 1957).

In certain instances, taxonomic identification presents challenges at both generic and specific taxonomic levels. As a result, molecular identification emerges as a valuable tool for clarifying taxonomic issues, especially when morphological distinctions among monopisthocotylean genera or species groups are ambiguous (Pouyaud et al., 2006; Wu et al., 2007; Wu et al., 2008). The phylogenetic analysis clustered the *T. vistulensis* sequence obtained in this study (**Figure 10**) together with other *Thaparocleidus* spp. that have shown the highest nucleotide similarities via a BLAST search.

In the present study, a 2694 bp-long fragment, including partially 18S, ITS, 5.8S, and partially 28S rDNA of the monopisthocotylean (accession number OR916383), was successfully sequenced.

Sequences from selected gene regions were used for phylogenetic analysis. The sequences of *T. vistulensis* isolates from the current study and from a previous study (AJ490165), were found to be closely related to each other. In the phylogenetic tree, the isolates branched at a single nodal point and were well supported by a high bootstrap, proving they belong to the same species. *Thaparocleidus siluri* was found to be a sister species to *T. vistulensis*. This clade comprises *Thaparocleidus* species that have been reported to infect European catfish (Bychowsky & Nagibina, 1957; Pugachev et al., 2010).

5.2. Pathological effects of *Thaparocleidus vistulensis* infection on the gills

This part of the study extends the investigations of morphological characterization that highlighted the early development of the sclerotized anchor and identified critical characteristics of *T. vistulensis*, particularly in relation to the shape and size of the anchors and copulatory organs. This study investigated the effects of *T. vistulensis* infestation on European catfish's gills through histopathological observation. While many of the histopathological effects have been thoroughly explored by Molnár (1980), an advanced microscopy technique (i.e., SEM) was employed and discussed additional findings, providing a more detailed analysis of the parasite's impact on gill structure.

Thaparocleidus vistulensis causes damage to the host gills when it attaches and feeds, resulting in mechanical rupture and loss of gill tissue. The attachment of *T. vistulensis*, which typically anchors itself between the gill lamellae of the European catfish, is mainly achieved using the ventral and dorsal anchors and is further strengthened with the fixation of marginal hooks. Various monopisthocotyleans, for instance, *Macrogyrodactylus clarii* on *Clarias gariepinus* (Arafa et al., 2009), *Cichlidogyrus* spp. on *Oreochromis niloticus* (El-Naggar et al., 2001), *Paradactylogyrus* sp. on *Labeo rohita* (Kaur & Shrivastav, 2014), and *Cichlidogyrus philander* on *Pseudocrenilabrus philander* (Igeh & Avenant-Oldewage, 2020) insert their anchors into the interlamellar gill epithelium of their hosts. Anchors in some species may even penetrate and damage the extracellular cartilaginous matrix of the gills (Kaur & Shrivastav, 2014; Igeh &

Avenant-Oldewage, 2020). Molnár (1980) found that *Ancylodiscoides vistulensis* (syn. *T. vistulensis*) attaches between two adjacent lamellae, on one side with the dorsal pair of anchors, and to the other side with the ventral pair. In the same study, the author also reported that the anchors were often found sunk as deep as the cartilaginous supporting structure of the gill filament. The present study found similar dispositions to those reported by Molnár (1980). Additionally, the present study also revealed that structural damage to the gills occurred when the anchors penetrated deeply into the gill lamellae, reaching and degrading the (core) extracellular cartilaginous matrix. The SEM study supported the histopathological observation, which showed the opisthaptor of individuals *T. vistulensis* embedded entirely into the proliferating gills.

Thaparocleidus vistulensis can cause lesions, and Molnár (1980) extensively discussed the histopathological changes caused by *T. vistulensis* on the gills of the European catfish, including epithelial cell proliferation, diffuse epithelial hyperplasia of gill filaments, and capillary haemorrhages, which are in agreement with the current observations. The tissue damage observed in the present study predominantly occurs in areas where the anchors penetrate the gills, causing rupture of epithelial tissue and definitive cellular changes. This pathology is caused by the pressure and compression created by the penetration of the anchors and marginal hooks into the gill epithelium and the contact between the parasite's body and the secondary gill lamellae. Arafa et al. (2009) and Igeh and Avenant-Oldewage (2020) documented similar compression in the gills of *C. gariepinus* infected with *M. clarii*, and *P. philander* infected with *C. philander*. Arafa et al. (2009) noted that attachment by the haptor caused neighbouring gill lamellae to be compressed and tightly packed, leading to an abnormal appearance of the gills. The SEM findings revealed an atypical appearance of the gills, characterized by deep concave cup-like hollow surface deformations. The indentation formed by the opisthaptor on the gill lamellae may exert pressure on the blood vessel walls, potentially causing stasis and rupture. The destruction of lamellae and clubbing would diminish the gills' surface area available for gaseous exchange and decrease the fish's respiratory capacity.

The pathological changes observed in this study also resemble those documented by Molnár (1972) (*Dactylogyrus lamellatus* infecting *Ctenopharyngodon idella*), Buchmann (2012) (*Pseudodactylogyrus anguillae* and *P. bini* infecting *Anguilla anguilla*), Molnár et al. (2016) (*Ancyrocephalus paradoxus* infecting *Sander lucioperca*), Arya and Singh (2020) (*Mizelleus indicus* infecting *Wallago attu*), and Igeh and Avenant-Oldewage (2020) (*Cichlidogyrus philander* infecting *Pseudocrenilabrus philander*), who also reported hyperplasia of the epithelial cells and subsequently fusion of lamellae, which represent a key feature of the parasitic infection. The intensive proliferation of interlamellar epithelia thickened the secondary lamellae with damaged capillaries deeply embedded in the hyperplastic tissue. Clubbing at the tip of filaments of heavily infected gills was also observed where a row or group of filaments was affected. Clubbing manifested as extensive epithelial hyperplasia, infiltrated with numerous epithelioid cells and varying numbers of small mononuclear cells. These histopathological changes proved to be less frequent and severe than Molnár (1980) reported, where several filaments were fused. Buchmann (2012) also observed analogous pathological host responses in *Anguilla anguilla* infected with *Pseudodactylogyrus anguillae* and *P. bini*, including hyperplasia of mucous cells and gill epithelial cells, excess mucus production, and fusion of gill lamellae resulting in clubbing of filaments. These gill changes result from the host's defence mechanisms (Reda & El-Naggar, 2003; Vankara et al., 2022).

While most authors have stated the increase in the number of mucous cells (Buchmann, 2012; Arya & Singh, 2020; Igeh & Avenant-Oldewage, 2020; Vankara et al., 2022) in response to monopisthocotylean infection around the infected tissue filaments, the current observations reveal that, although mucous cells are present in the space between the filaments, other cells with goblet-like morphology seemed to be noticeably absent. This phenomenon is consistent with the findings of Molnár (1980), who observed the disappearance of such cells. Typically, when parasitic infection occurs on the host, the immune system often triggers an inflammatory response, including activation of the mucous cells. The infection may trigger these cells to produce more

mucins that act as a barrier to protect the epithelial surfaces from further damage and expel the parasites (Gomez et al., 2013). However, the absence of goblet-like mucous cells was not evident in the present study. It is hypothesized that some parasite-produced substances may specifically impede the function or regeneration of the mucous cells, resulting in their depletion or disappearance in the infected area (Xian et al., 1999) or consequence of the host's immune response (Bergstrom et al., 2008). Clarifying the underlying process and understanding the fundamental mechanisms that define mucous cell dynamics in the gills of European catfish will provide deeper insights into this biological system. However, it was agreed that the mucoid material observed mainly originates from the cytoplasmic remnants of disrupted cells and the destroyed vascular network, as previously described by Paperna (1964) and Molnár (1972). On the other hand, the present observations differed somewhat from those of Molnár (1980), especially with regard to eosinophilic granular cells. Although the author did not demonstrate an increase in the amount of these cells, the present observation indicated their enrichment, especially at the anchoring sites of *T. vistulensis*.

5.3. Reproductive strategies of *Thaparocleidus vistulensis*

Life cycle studies aiming at revealing the reproductive potential of monopisthocotyleans on fish provide a basis for understanding infection processes in fish farms and may eventually lead to the development of management and control methods. Due to the amazing adaptability of monopisthocotyleans to abiotic and biotic factors over time, it is advised that these studies be performed for specific systems at specific time periods. Thus, Molnár (1968, 1980) previously published well-supported data on the infection dynamics of *T. vistulensis* and its life cycle parameters, but some deviation was found in the present study focusing on the development of embryo in the egg, hatching rate, hatching success, and survival rates without host. It has previously been shown that the main abiotic factors influencing the monopisthocotylean parasite population are water temperature, light intensity and salinity (Bauer et al., 1973; Gannicott & Tinsley, 1997). The main biotic factor is the host species, but in addition, the microbiota in the fish

farm filters and water phase may affect the life cycle significantly (Buchmann & Bresciani, 2006). The present study showed that the *T. vistulensis* population on European catfish in fish tanks has a marked propagation potential and may reach devastating levels within 10 days, depending on the severity of the initial infection of the donor fish. This observation should be kept in mind when developing management protocols in fish farms at risk. Quarantine periods of fish before introduction into fish farms is a feasible way to prevent infection (Bauer et al., 1973). The pathology induced by the infection and the associated clinical signs were previously reported by Molnár (1980), but the present study indicates that these reactions differ considerably between fish. Thus, it may be suggested that the selection and breeding of fish strains with lower susceptibility to a parasitic disease (Jaafar et al., 2020) may be a promising approach to improving health in catfish farms.

An important epidemiological parameter is the generation time. The present observation indicated that some of the monogeneans became sexually mature and produced eggs after 8 dpi, which is a highly important factor to consider when forecasting the propagation of a parasite population in the farm and determining the correct time point for control method installment. Previous studies on *Pseudodactylogyrus* spp. parasites in recirculated eel farms also showed that adults developed within 7–8 days (Buchmann, 1988b).

The reproductive strategies of monopisthocotyleans causing diseases in finfish aquaculture have been well summarized by Hoai (2020). Unfortunately, apart from a study by Molnár (1968), there is a little comparative information on reproductive strategies for *Thaparocleidus* species. After the oviposition of a mature *T. vistulensis*, the eggs adhere to substrates and hatch to produce a free-swimming ciliated larva. The life cycle of *T. vistulensis* (egg to egg-laying adult) is completed approximately within 13–15 days at 23 ± 1 °C, which corresponds well with Molnár (1968), who showed that the entire life cycle took around 13–14 days at 20–23 °C. The developmental data align very well with other dactylogyrid monogeneans, and inspiration for future control methods may be found. One approach is to remove parasite eggs or larvae from the fish farm system. The

average size of the eggs (72.35 µm in length and 56.20 µm in width) observed in this study at 23 °C was morphologically consistent with those recorded by Molnár (1968) (67 µm in length and 52 µm in width) at 18 °C, while the average length of the filament was 42.31 µm and 41 µm, respectively. Nevertheless, the average size of the eggs of *T. vistulensis* was smaller than those of *T. gomtius* (84 µm in length and 75 µm in width) and of *T. sudhakari* (87 µm in length and 59 µm in width), which infected the catfish, *Wallago attu* (Verma et al., 2017). However, by using the obtained information on egg and larval size, it is possible to develop filtration systems in recirculated fish farms. Mechanical filters with a mesh size of e.g., 40 µm can catch eggs and oncomiracidia, thereby reducing the infection pressure (Buchmann & Bresciani, 2006).

A series of descriptions have been published on the embryonic development of various monopisthocotylean species, but none of them concerned members of the genus *Thaparocleidus*. Nevertheless, information on other monogeneans, including *Heterobothrium ecuadori* (Grano-Maldonado et al., 2011), *Sparicotyle chrysophrii* (Repullés-Albelda et al., 2012), and *Dawestrema cycloancistrium* (Maciel et al., 2017), suggests that the main process during egg development of *T. vistulensis* follows a similar pattern to that described for others. It comprises the appearance of primordial hooks and eyespots, followed by the development of the ciliated wreath, and finally, the formation of the operculum for the eclosion of the oncomiracidium (Repullés-Albelda et al., 2012). This process is consistent with the results of the present study, where the primordia of scattered pigment of eyespots and the hamulus became visible before the aligned ciliated cells appeared, and vigorous larval movement began. Unhatched, infertile eggs of *T. vistulensis* turned dark brown, in agreement with *D. cycloancistrium* described by Maciel et al. (2017), but in contrast to *H. ecuadori* (Grano-Maldonado et al., 2011), where the eggs became transparent or colorless. The eggs of *T. vistulensis* hatched spontaneously in the same, but filtered water that was used for maintaining the fish, and the process included intense larval movements exerting a direct pressure on the operculum. Once the operculum opened, eclosion occurred with the help of stretching and retraction of the body, and propulsion by the cilia. The ciliary wreath covered the anterior, lateral,

and haptoral zones of the oncomiracidia, as in other monopisthocotyleans as described by Whittington et al. (2000). In some cases, the larva hatched immediately after manipulation of the egg. Some studies suggested hatching to be hampered by environmental factors like clarity, darkness, shade or the presence of the host (Tinsley & Owen, 1975; Whittington & Kearn, 1988; Gannicott & Tinsley, 1997). Direct predation on eggs by invertebrates in the biofilm microbiota or even anaerobic zones in fish tank filters was suggested to inhibit the *Pseudodactylogyrus* egg development (Buchmann, 1988a).

The hatching dynamics of *T. vistulensis* eggs established in the present study agree with the previously published result of Molnár (1968) and showed great similarity with several other freshwater and marine water monopisthocotyleans species (**Table S18**). In contrast, the reproductive period of polyopisthocotyleans lasts longer on average (Gannicott & Tinsley, 1998a, 1998b). However, environmental factors, especially temperature, could significantly influence the cycle's duration and the parasites' reproductive pattern (Tinsley, 2004; Whittington & Chisholm, 2008). Kearn (1986) emphasized that the duration of development of most monogenean eggs is shortened at higher temperatures. Temperature has also been shown to influence hatching success, and there is probably an optimal hatching temperature for each parasite species (Ogawa, 1988; Gannicott & Tinsley, 1998a; Tubbs et al., 2005). A detailed discussion of the influence of temperature on egg hatching success, apart from fecundity and survival rates at different life stages, will be addressed in subsection 5.4.

The successful hatching of larvae and finding a suitable host are crucial factors for infection and the continuity of the life cycle (Kearn, 1973; Grano-Maldonado et al., 2011). The average survival ability for oncomiracidia of *T. vistulensis* without host was 5 dpo at 23 ± 1 °C, which predicted a relatively long period of infection. It poses a significant risk and should be considered when forecasting the parasite population increase in the fish farm. In systems with water contact between different fish sizes (ages), it is crucial for spreading and sustaining the parasite population because the incubation period of European catfish eggs at a similar temperature (21–23 °C) averages only

2.5–3 days (Molnár, 1968). The survival rate of the oncomiracidia in this study was higher than in most other monopisthocotyleans species (**Table S18**).

Once the oncomiracidia successfully attached to a host, they lost their ciliated cells, developed to the post-larval stage and eventually matured to the adult stage. In the present study, both developing juvenile and adult *T. vistulensis* showed a similar *in vitro* survival rate pattern. The survivability of developing monogeneans also can significantly impact the host, especially in high stocking density farmed fish such as European catfish. According to the present observation, both the developing juvenile and adult *T. vistulensis* could crawl by a vermiform movement at the bottom of the vessel. This leech-like movement enables immature monogeneans clinging to the body surface to reach the preferred site for permanent settlement (Reed et al., 2012). It means that the parasites can actively crawl longer distances to reach the host's gills. This peculiarity also provides them with the opportunity to find a new host in the rearing system where fish are in close contact, such as the European catfish, which is known for its sedentary behavior (Copp et al., 2009; Brevé et al., 2014; Slavík et al., 2014) with high site fidelity (Carol et al., 2007) and usually stay at the bottom of the pond/tank.

Documented data on the effective transmission of live adult monopisthocotyleans between hosts can also be found in the literature (Hutson et al., 2018). The survival rate of mature *T. vistulensis* is as important as that of developing juveniles, since detached parasites also produce eggs. Since the ability of monogenean parasites to anchor firmly to the host gills is usually quite strong due to their well-developed haptors consisting of central hooks and marginal hooks, displacement is relatively rare (Khang et al., 2016). Under specific circumstances, however, they can become detached by external disturbances such as strong water currents or violent movements by the host fish (Kearn, 2014). It can here be added that the high *T. vistulensis* infection levels observed in catfish in this study were associated with severe inflammation. Therefore, it is suggested that extensive immune reactions in host gills can induce the detachment of the parasites, which could survive in the fish tank for a short time and deliver parasite eggs. To date, several *in vitro* studies

have been conducted on the fecundity of mature monogeneans (Tubbs et al., 2005; Turgut, 2012; Hirazawa et al., 2010; Mooney et al., 2008; Maciel & Alves, 2020) showing that the adult parasites could survive for some time and produce eggs even after they have been removed from the gills. Nevertheless, *in vitro* studies for assessing adult parasite fecundity are debated, as starvation of monopisthocotyleans separated from their host leads to a progressive decline in egg production rate and quality (Whittington, 1997; Mooney et al., 2008). In order to complete the data set for epidemiological models in a fish farm system, it is recommended to perform egg deposition rate studies with adult parasites on the host fish as previously shown for *Pseudodactylogyrus* on eel gills (Buchmann, 1988b, 1990). Thus, egg deposition was carried out, and details regarding the fecundity of *T. vistulensis* are discussed in subsection 5.4.

5.4. Influences of environmental conditions against different life stages

In order to create the right control strategies, it is crucial to know the ecology and infection dynamics of the parasites, especially the impacts of environmental factors on their life cycles (Villar-Torres et al., 2018; Huston et al., 2020). Most research on monopisthocotyleans focuses on reproductive strategies (i.e., egg production, hatching, and survival rates), which are often studied *in vitro*, such as the present study. However, separating parasites from their host reduces their viability due to starvation and other factors (Whittington, 1997; Mooney et al., 2008). Although the present study may not reflect proper natural conditions, it serves as preliminary knowledge for the recognition of the biological and ecological features of the studied parasite, which are essential for further investigations to improve management and treatment in the field of aquaculture.

5.4.1. The influence of light-dark cycle against Thaparocleidus vistulensis

Several studies reported that the monopisthocotyleans' response to the daily light-darkness cycles (photoperiod) is one of the factors influencing their life cycles and reproductive strategies (Kearn 1963, 1973, 1982; Mooney et al., 2008; Hoai & Hutson, 2014). The present study exposed *T. vistulensis* at distinct life stages (oncomiracidia and adults) to natural and dark conditions of different durations to examine changes in biological parameters such as fecundity, egg hatching,

and survival rates. The present observations showed that continuous exposure to darkness resulted in lower egg production by adult *T. vistulensis* compared to natural conditions. A previous study revealed that parasitic activity is highly dependent on the host's lifestyle, and is specific to each host (Shirakashi et al., 2021).

The reduced oviposition of adult *T. vistulensis* may be related to the behavior of its host, the European catfish, which is mainly active and feeds at night (Boujard, 1995; Slavík et al., 2007). Increased nocturnal activity requires special adaptations for the parasite, as enhanced movement and water flow limit the egg attachment and ongoing development. In addition, the lifespan of hatched oncomiracidia, which is longer in *T. vistulensis* than in most monopisthocotyleans, as previously mentioned in subsection 5.3, is also crucial for the completion of the parasite's life cycle, which is terminated by finding the host. The extended life of oncomiracidia is important due to European catfish's spatial and temporal segregation, especially during the growing season, confirmed by the ontogenetically determined home range size and the agonistic behavior (Hedger et al., 2005; Kynard & Parker, 2005).

In contrast, previous studies have found that the majority of eggs are released in the dark (Macdonald & Jones, 1978; Mooney et al., 2008; Hoai & Hutson, 2014; Woo et al., 2024). Macdonald and Jones (1978) suggest that the behavior of *Barbus meridionalis* in its natural habitat, where it swims actively during the day, results in *Paradiplozoon homoion* producing fewer eggs during the day than at night. Some monogeneans keep their eggs in utero until light conditions are optimal for release (Mooney et al., 2006, 2008; Poddubnaya et al., 2017; Tinsley, 2017). For example, to increase the likelihood of its hatching larvae finding, a suitable new host, *Zeuxapta seriolae*, accumulates eggs in utero and releases them at dusk or in darkness due to the behavior of its specific host *Seriola lalandi*, which is active during the day and congregates around submerged structures at dusk (Mooney et al., 2006). This strategy demonstrates that these parasites can adapt to their hosts' light-regulated daily behavior, thereby enhancing and maximizing the transmission of their larvae to a new host.

The present results showed that the trends of egg hatching were similar in both light and dark conditions. The eggs of *T. vistulensis* are able to hatch through the regulation of their internal developmental cues, regardless of the influence of environmental factors such as changes in light conditions. An optimal egg hatching rate is essential for infection success, as it ensures the release of the maximum number of viable larvae. However, host behavior and physiology related to environmental conditions, especially light-dark cycles, may influence the hatching strategies of monopisthocotylean eggs (Ernst & Whittington, 1996; Whittington & Ernst, 2002). The results of normal and dark conditions on hatching rates in the present study pointed to the importance of further detailed examination of egg hatching rhythm under varying light conditions, as shown in several previous studies (Kearn, 1963, 1973, 1982; Macdonald, 1975; Whittington, 1987; Gannicott & Tinsley, 1997; Mooney et al., 2008; Hoai & Hutson, 2014).

Similar to egg hatching, the survival rates of adult *T. vistulensis* were also found to be independent of variations in light and dark conditions. The recorded maximum lifespan of adult *T. vistulensis* was less than 3 days under both conditions, although they began to die earlier in darkness. Interestingly, statistical analysis of the survival rates of oncomiracidia from days 3 to 5 pop revealed a significant difference between the two experimental settings, despite their maximum lifespan being the same in both conditions (less than 6 days). Nearly half of the oncomiracidia in the natural photoperiod group survived up to 5 days. This suggests that this species might have a higher chance of successfully infecting its hosts during the day when the host is less active, as previously mentioned. However, thorough studies should be done to investigate this. It is interesting to note that the life span of *T. vistulensis* oncomiracidia is relatively longer than that of other monopisthocotylean species at similar water temperatures (20–25 °C), allowing them more time to find a new host, whereas for most studied oncomiracidia, it usually does not exceed 2 days (**Table S18**). The prolonged lifespan of the oncomiracidium suggests that hosts leave their habitat frequently or for longer periods, resulting in the larvae needing more time to find their host. Even so, the European catfish is considered a notorious nocturnal predator, its diurnal activity highly

can change depending on the seasons and age (territorial hierarchy) (Slavík et al., 2007; Daněk et al., 2016).

5.4.2. The influence of temperature against Thaparocleidus vistulensis

Environmental factors, particularly water temperature, are crucial in modifying parasite reproduction patterns (Tinsley, 2004; Whittington & Chisholm, 2008). Studying the effect of water temperature on the reproductive strategies of parasites is extremely important, considering the current use of anthelmintics, which detach parasites from the gills by inducing paralysis (Burka et al., 1997; Watson, 2009) without necessarily killing them (Woo et al., 2024). These alive, isolated parasites could continue egg production using the nutrients they have taken up, and understanding the effect of water temperature on this process assists in determining the number and optimal time for treatments (Whittington, 1997).

The present study found that the optimal water temperature for adult *T. vistulensis* to lay eggs is 15 °C, like other monogenea such as *Microcotyle sebastis* (Woo et al., 2024). European catfish are reported to be "dormant" and inactive below 8–10 °C; their activity increases highly above this temperature range, and when 18–20 °C is reached, spawning begins (Souchon & Tissot, 2012), and water temperatures of 25–28 °C is optimal for growing (Hilge, 1985; Linhart et al., 2002). This study demonstrated that *T. vistulensis* is capable of laying a considerable number of eggs per individual, even within this water temperature range. The optimal water temperature for egg production for some of the other monopisthocotylean parasites is higher, such as *Pseudodactylogyrus anguillae* at 25 °C (Buchmann, 1990) and *Dactylogyrus vastator* at 35 °C (Zhang et al., 2015). The results showed that *T. vistulensis* was able to lay a minimal number of eggs at 10 °C and 35 °C, indicating their wide water temperature tolerance that should be carefully considered in efforts to eradicate them within aquaculture systems. The large drop in egg production at these extreme water temperatures is possibly due to a reduction in metabolic activity exploited for egg production as a consequence of adaptation, as reported by Woo et al. (2024). Nevertheless, no eggs could be observed at 5 °C, which is in line with the reported egg production

behavior of other monopisthocotyleans such as *Urocleidus adspectus* (Cone & Burt, 1981), *Pseudodactylogyrus bini* (Chan & Wu, 1984; Buchmann, 1988b), and *Dactylogyrus vastator* (Zhang et al., 2022). It points to the impact of extreme water temperatures on the life cycle of *T. vistulensis*, providing valuable insights into its biological responses under such conditions.

Water temperature also influences the hatching success of monopisthocotylean species (Tubbs et al., 2005). The observation of hatching rates of *T. vistulensis* in the present study revealed a high hatching success (>80%) across a wide range of water temperatures (10–30 °C). The present findings, which indicate that a decreased water temperature initiates a prolonged hatching process, align with a statement by Kearn (1986) that higher water temperatures shorten the developmental period of most monogenean eggs. The longer hatching period of *T. vistulensis* caused by decreased water temperature is consistent with previous findings for *Dactylogyrus vastator* (Zhang et al., 2015), *D. extensus* (Turgut, 2012), *Benedenia seriolae* (Tubbs et al., 2005), *Neobenedenia girellae* (Bondad-Reantaso et al., 1995), and *N. hirame* (Yoshinaga et al., 2000). According to Molnár (1968), the hatching period for *T. vistulensis* eggs varies with water temperature, taking 6 to 6.5 days at 15–17 °C, 3 days at 20–21 °C, and 2.5 days at 24–25 °C. The results of the present study align with previously defined time ranges, showing hatching periods of 5 to 9 days at 15 °C, 3 to 5 days at 20 °C, and 2 to 4 days at 25 °C.

The developmental and egg hatching intervals can vary widely among species (Kearn, 1986; Tubbs et al., 2005; Chen et al., 2010; Marchiori et al., 2015), demonstrating their adaptability. The present study showed that the eggs of *T. vistulensis* hatched at various temperatures (10–30 °C) with an average success rate of 85.58%, with the fastest hatching occurring on day 2 pop at 25 °C and 30 °C. The prolonged hatching and embryonic development period of *T. vistulensis* at 10 °C demonstrates its resilience to extreme environments, allowing it to maintain reproduction and parasitism, as stated in literature (Perry, 1989; Thompson, 2020; Marcus et al., 2023). The adaptability of *T. vistulensis* to adjust egg hatching rates based on temperature is a strategic response to host availability and environmental conditions. The hatching period, which is still long

(12–19 days) at 10 °C, becomes shorter depending on the simultaneous increase of activity of the host and the temperature (2–5 days at 20–25 °C).

The seasonal feeding and migratory behaviors of the European catfish also have a crucial role, especially during warmer months. In these periods, the catfish tend to spend more time in shallow (Říha et al., 2022), which simultaneously increases the possibility of (re)infection of the host and the spread of the parasite, assuming that the food-rich waters are favored equally by conspecifics, as well. This requires the parasite's dynamic adaptability to host behavior, synchronized with environmental fluctuations to ensure the emergence of infective larvae coincides with periods of higher host availability and movement, maximizing infection success and parasite survival. Furthermore, the present results show that extreme water temperatures (5 °C and 35 °C) terminate the development of *T. vistulensis* eggs, suggesting species-specific responses to temperature alterations, highlighting the importance of temperature control during fish rearing.

The survival rates of *T. vistulensis* (adults and oncomiracidia) negatively correlated with water temperature. Both adults and oncomiracidia exhibited considerably increased longevity as the water temperature decreased, consistent with other monopisthocotyleans species from previous studies (Brazenor & Hutson, 2015; Valles-Vega et al., 2019). Although all *T. vistulensis* adults and oncomiracidia died within 24 h at 35 °C in the present experiments, Zhang et al. (2015) found that *Dactylogyrus vastator* oncomiracidia could survive 42 h at this water temperature. Interestingly, some *in vivo* studies reported that at 34 °C, the oncomiracidia of *P. bini* (Buchmann, 1988b) and *P. anguillae* (Buchmann, 1990) could survive up to 14 days and 17 days, respectively. The present study also showed that the adult *T. vistulensis* could survive up to 12 days without a host at a lower water temperature of 5 °C. However, despite their extended survivability, the parasites could not produce eggs at this extreme water temperature, as previously mentioned. Additionally, oncomiracidia died rapidly at 5 °C, within less than a day. Careful adjustments of the water temperature can greatly enhance the effectiveness of treatments against *T. vistulensis* by

disrupting their life cycle. Since the study was conducted *in vitro*, its findings would benefit from validation under *in vivo* conditions.

5.5. Treatment trial against *Thaparocleidus vistulensis*

The management and control of monogenean infestations in fish farming is needed to secure fish health and welfare. Anthelmintic treatments may be necessary if pathogen-free production systems cannot be established. However, the success rate is rarely predictable due to the differential sensitivity of various life cycle stages (eggs, oncomiracidia, developing juveniles, adults) to the available compounds (Morales-Serna et al., 2018). Monopisthocotyleans are highly fecund and have short generation times with a direct life cycle (without intermediate hosts), resulting in exponential population growth, particularly within the high density of intensive fish production (Buchmann & Bresciani, 2006). Detailed knowledge of drug effects on each specific life cycle stage may allow the development of strategically timed treatments with a higher probability of disrupting the parasites' life cycle.

In vitro tests have widely been used to analyse parasites' tolerance to various antiparasitic agents. Exposing the monopisthocotylean ectoparasites to the anthelmintic agent *in vitro* simulate conditions of *in vivo* bath treatments of fish (Reimschuessel et al., 2011). The method is suitable for estimation of the effectiveness of potential agents before starting *in vivo* bath treatment. The following discussion will be discussed on the efficiency of three antiparasitic herbal treatments including garlic (GAR), ginger (GIN), and neem bark (NMB), and three antiparasitic drug agents including the novel biological compound Biokos (BIO), and the two conventional anthelmintics Praziquantel (PZQ) and Mebendazole (MBZ) by conducting *in vitro* effects on all life stages of monopisthocotylean *T. vistulensis* parasite. The promising treatment results from *in vitro* antiparasitic drug agents (MBZ) and the widely used in treating monogenea (PZQ), were further proceeded with toxicity tests on juvenile European catfish prior to the *in vivo* bath treatments.

5.5.1. In vitro herbal treatment against Thaparocleidus vistulensis

Herbal treatments against parasites are gaining increasing attention due to their potential as an alternative to chemical therapeutics. This study is the first to report the efficacy of herbal therapies against *T. vistulensis*. Among the tested herbs, GAR exhibited the highest antiparasitic activity, reinforcing its reputation as an "all-healing" herb widely employed in aquaculture due to its diverse beneficial properties, including antibacterial, antioxidant, antiviral, antiparasitic, immunostimulant, and growth-promoting effects. Furthermore, its positive impact on fish intestinal microbiota has been well documented (Lee & Gao, 2012; Valenzuela-Gutiérrez et al., 2021). The present findings highlight GAR's significant efficacy against multiple life stages of *T. vistulensis*, particularly its strong ovicidal effect. Notably, even at the highest dilution (1:100), GAR completely inhibited egg hatching, making it the only herbal treatment to prevent egg development in this study. This result is particularly significant given that monopisthocotylean eggs are known for their high resistance to external factors due to their protective proteinaceous shell (Whittington, 2012). In correlation with the observation of Militz et al. (2013), who demonstrated that continuous immersion in GAR containing more than 15.20 µL/L of allicin reduced the hatching rate of *Neobenedenia* sp. by only 5%. In addition to its ovicidal properties, GAR treatment significantly shortened the lifespan of *T. vistulensis* oncomiracidia and juveniles to less than 4 and 3 hours, respectively. These findings suggest that GAR has strong potential as an alternative antiparasitic agent.

Besides, NMB exhibited positive effects against *T. vistulensis*, although its efficacy was restricted to the oncomiracidia stage. Treatment with NMB demonstrated the highest effectiveness, eliminating all oncomiracidia within 75 min, even at the highest dilution, compared to GAR and GIN. The *in vitro* study also revealed that the aqueous extract of NMB displayed moderate antiparasitic efficacy against juvenile and adult *T. vistulensis*, with its effects observed within 24hours. Recent studies have highlighted neem extract as a promising strategy for successfully treating monopisthocotylean infections, including *Diplectanum* sp. in sea bass *Dicentrarchus*

labrax (Aly et al., 2022), as well as *Dactylogyrus* sp. and *Gyrodactylus* sp. infecting tilapia *Oreochromis niloticus*) (Suryani & Arya, 2017). The efficacy is likely attributed to the presence of bioactive triterpenoids, such as azadirachtin, in neem aqueous extracts. Previously, Mordue and Nisbet (2000) reported that the mortality of *Argulus* parasites was induced by oxygen deficiency in the water, caused by the presence of triterpenoids, leading to the death of eggs, larvae, and adult parasites under *in vitro* conditions. In another study, Martinez (2002) demonstrated the potential of aqueous neem leaf extract in controlling fish parasites and fish predators, such as dragonfly larvae. Nevertheless, though the present findings highlight the promise of aqueous extract of NMB against *T. vistulensis*, evaluating its effectiveness under *in vivo* conditions is essential for optimizing its application in aquaculture.

Antiparasitic agent GIN contains active components such as gingerols, shogaol, and curcumin (Lin et al., 2014), which allows it to be applied widely in infectious diseases and helminthiasis treatments (El-Bahy & Bazh, 2015). However, there is limited knowledge about the special mechanisms by which GIN's active components interact with monopisthocotylean parasites. In the present study, GIN exhibited the least pronounced effects among all the herbal treatments tested. In the *in vitro* treatment, there were affected all life stages of *T. vistulensis* only at the lowest dilution, and even then, the required exposure time was longer compared to previous studies, such as those by Levy et al. (2015) and Trasviña-Moreno et al. (2019). Nevertheless, the antiparasitic effects of GIN on other freshwater monopisthocotyleans have been demonstrated, including *Gyrodactylus turnbulli*, which infects guppies *Poecilia reticulata* (Levy et al., 2015), and *Dactylogyrus ctenopharyngodonis*, which infects grass carp *Ctenopharyngodon idella* (Fu et al., 2017). Additionally, its efficacy has been confirmed against the marine monopisthocotylean *Neobenedenia* sp., a parasite of yellowtail amberjack *Seriola lalandi* (Trasviña-Moreno et al., 2019). The aqueous extract of GIN evaluated in the present study was less effective against *T. vistulensis* most probably because of the instability or low concentration of bioactive compounds. Moreover, the use of different solvents, such as aqueous and ethanolic, in the

preparation of the GIN extract solution plays a crucial role, as Levy et al. (2015) reported significant differences in the effectiveness of water- and ethanol-extracted solutions.

5.5.2. In vitro and in vivo drug treatments against Thaparocleidus vistulensis

This study is the first to test the efficacy of BIO against Platyhelminthes. The use of BIO, a cyclic lipopeptide produced by the bacterium *Pseudomonas* H6, was reported for eradication of freshwater protozoon parasites, such as white spot disease agent *Ichthyophthirius multifiliis* (Al-Jubury et al., 2018) and more recently for effective treatment of the marine white spot disease, *Cryptocaryon irritans* (Watanabe et al., 2023). Although BIO showed no antiparasitic effects on eggs at any tested concentrations (1–100 mg/L), it killed more than 90% of free-swimming larvae within 24 hours of applying at 20 mg/L or higher concentrations. The exact mechanism of action of BIO is unknown, but evidence of disruption of the parasites' ciliated membranes has been presented. Thus, tomonts of *I. multifiliis* exposed to this surfactant resulted in deciliation (Marana et al., 2023). Oncomiracidia of *T. vistulensis*, after hatching, initially possess a multi-segmented ciliary crown, which allows their free-swimming movement, shortening the time to find a host in the relatively large water body. Upon exposure to BIO, the free-swimming monogenean larvae lost their motility or exhibited erratic swimming behaviours similar to those observed in *I. multifiliis*, which rotates in one place (Al-Jubury et al., 2018). The effect of BIO on *I. multifiliis* and *C. irritans* was associated with disruption of the membrane integrity, leading to an unregulated influx of water into the cytoplasm (Al-Jubury et al., 2018; Watanabe et al., 2023). Through time, the cilia surrounding the oncomiracidia body were gradually detached, revealing the dead larva as an opaque white body.

The effect of BIO on juvenile and adult *T. vistulensis* parasites was less pronounced. Detectable effects on these stages could only be observed in higher concentrations (≥60 mg/L), especially on juveniles. The moderate sensitivity of young individuals may be attributed to the bioactive properties of lipopeptide surfactants, such as BIO, which link to the cell membrane, compromising its integrity by reducing surface tension and dissolving its constituent components (Vandyke et al.,

1991). These findings confirm that BIO exhibits stage-dependent efficacy, highlighting the importance of exploring its interaction with cellular structures and its potential for broader therapeutic application.

Praziquantel (PZQ) has been used as an anthelmintic against a range of flatworms that infect a wide spectrum of host animals (invertebrates and vertebrates, including mammals). This synthetic pyrazino-isoquinoline induces calcium ions inflow across the worm tegument, causing an immediate muscular contraction (i.e., paralysis), quick vacuolisation, tegument integrity, and loss of sucker function (Bylund et al., 1977; Schmahl & Mehlhorn, 1985, Schmahl & Taraschewski, 1987; Redman et al., 1996; Hirazawa et al., 2000). Even though the presumable target of this drug is a calcium-permeable membrane channel, precise molecular mechanisms remain unknown (Köhler, 2001). The use of PZQ has been tested on a wide range of monogeneans, including both mono- and polyopisthocotylean parasites from various hosts, mainly in farm-cultured fish with bath or oral treatments (reviewed by Norbury et al., 2022). According to these studies, adult monogeneans were often completely eradicated, while some juveniles are less affected by PZQ treatment (Schmahl & Taraschewski, 1987; Chisholm & Whittington, 2002; Forwood et al., 2013a), and many monogenean eggs were refractory to this compound (Thoney, 1990; Chisholm & Whittington, 2002; Sharp et al., 2004; Sitjà-Bobadilla et al., 2006; Morales-Serna et al., 2018). *In vitro* treatment of *T. vistulensis* showed that PZQ has limited efficacy in eliminating the parasite. At high concentrations (≥80 mg/L), the hatching rate of eggs was significantly reduced (less than 7% of eggs to be hatched) and caused a moderate increase in mortality among oncomiracidia and juvenile flukes while at lower doses (60–20mg/L), the maturation process of eggs was temporarily halted and then compensated.

While the present study demonstrated concentrations of 80 and 100 mg/L of PZQ were highly effective in completely eradicating *T. vistulensis in vitro*, toxicity tests revealed signs of stress (i.e., abnormal skin pigmentation) on the host, juvenile European catfish at a concentration as low as 20 mg/L in less than 10 min. Indicating that concentrations above this threshold are unsuitable

for treatment, taking into account ethical and fish welfare regulations. Interestingly, positive results were obtained when a concentration of 10 mg/L was tested *in vivo*, all *T. vistulensis* detached within 1 dpt. Furthermore, this effect was sustained, as consistent results were observed after 14 dpt. The outcome is noteworthy, as most studies have reported an insufficiency of complete parasite elimination, often leading to progressive reinfection of the hosts and horizontal transmission within the facilities (Sitjà-Bobadilla et al., 2006). A previous study showed only a 15% reduction in the prevalence of parasites in an *in vivo* experiment with 10 mg/L of PZQ on fingerlings European catfish, after 3–4 dpt with a 5 h bath treatment (Székely & Molnár, 1990). The concentration applied in *in vivo* experiments was the same as in the previous study to obtain comparable results. This proved that a prolonged bath (24 h) treatment effectively eliminated (100%) *T. vistulensis* from the gills of European catfish, consistent with other literature data (Chisholm & Whittington, 2002; Sharp et al., 2004; Fridman et al., 2014; Buchmann et al., 2011; Hoai & Van, 2014).

Evaluation treatments in shorter periods or at lower doses is controversial as PZQ induces paralysis in monogeneans, which often leads to death after prolonged agony (Sharp et al., 2004; Hirazawa et al., 2013, Morales-Serna et al., 2018). Flukes detach from the host but survive for a limited time, depending on dose concentrations, and continue to produce viable eggs. Suboptimal PZQ concentrations result in a temporary decline followed by a recovery in population growth, facilitating subsequent reinfections. However, *in vitro* studies indicate that *T. vistulensis* can survive up to 1–3 dpt at ≥10 mg/L concentration, but the reinfection was only detected at lower concentrations (<5 mg/L) in *in vivo* study, where the parasite population increased again by 14 dpt. The discrepancy between *in vitro* and *in vivo* results highlights the effect of PZQ on muscular contraction, which interferes with the functionality of hooks and anchors, leading to reduce the ability for attachment to its host, as described in the earlier studies (Schmahl & Mehlhorn, 1985; Schmahl & Taraschewski, 1987; Hirazawa et al., 2000).

Mebendazole (MBZ) is another anthelmintic that has long been widely used to treat a number of parasitic worm infestations. However, it is highly effective against a broad spectrum of both larval and adult helminths. In the *in vitro* study, *T. vistulensis* exhibited resilience *in vitro* to MBZ (<40 mg/L) at most life stages. Nevertheless, MBZ treatment at all doses tested had an obvious inhibitory effect on egg development. The proteinaceous eggshell of monogeneans is highly resistant to external factors, including chemical and physical agents (Whittington, 2012), but this anthelmintic benzimidazole agent could penetrate it, exhibiting ovicidal activity even at the lowest concentration of 1 mg/L, as previously shown for *Pseudodactylogyrus* monogeneans (Buchmann & Bjerregaard, 1990). The exact mechanisms of action of MBZ are not fully understood; it is considered to bind to the β-tubulin molecule, disrupting cell functions like division and transport, which ultimately interfere with egg hatching by interfering with cellular processes (Lacey et al., 1987). The finding that MBZ inhibits egg development in helminths (Reynolds, 1989), is supported by the observation of Buchmann and Bjerregaard (1990), who reported that no reinfestation occurred in farmed European eel (*Anguilla anguilla*) two weeks after treatment against *Pseudodactylogyrus anguillae* and *P. bini*. However, very low concentrations of MBZ may not show ovicidal properties. Thus, Zhang et al. (2014) showed that hatching *Dactylogyrus vastator* eggs incubated in a concentration as low as 0.03 mg/L MBZ was considered a safe concentration for the host.

Additionally, MBZ at concentrations ≥40 mg/L reduced the lifespan of *T. vistulensis* oncomiracidia by half, presumably by inhibiting glucose uptake and impeding microtubule synthesis (Anderson & Waller, 1985; Reynolds, 1989; Martin, 1997). Juvenile and adult monopisthocotyleans proved insensitive. Compared to PZQ, the MBZ treatment showed obvious symptoms of toxicity at 20 mg/L concentration. Thus, 10 mg/L is considered the safe concentration threshold for juvenile European catfish at which no indications of stress were monitored. However, skin lesions in fingerling European catfish have been reported when exposed to 10 mg/L of MBZ for 26 hours (Székely & Molnár, 1990), referring to the size-dependent toxicity of therapeutic

agents, as the pigmented *A. anguilla* demonstrates more resistance to a 1 mg/L of MBZ concentration than the glass eel stage (Buchmann & Bjerregaard, 1990).

Findings *in vitro* assays with MBZ predicted the ineffectivity of this compound in the *in vivo* experiments. However, *in vitro* responses toward benzimidazoles *in vitro* do not always correlate with *in vivo* results (Santamarina et al., 1991; Tojo et al., 1992). The *in vivo* observations following 1 dpt showed no significant changes in the parasite load at 10 mg/L concentration compared with the control, consistent with the results recorded by Székely and Molnár (1990). However, a gradual decrease in the number of *T. vistulensis* on the host gills was detectable at all doses (1, 5, and 10 mg/L) applied 7 and 14 days after treatment. This delayed effect may be associated with the anthelmintic mechanism of benzimidazoles, which impair the parasite's sugar metabolism by impeding glucose uptake and digestion, leading to the slow depletion of energy sources and ultimately causing the death of the parasite (Ahmad & Nizami, 1987; Zhou et al., 2023). The process is mediated by MBZ via binding tubulin monomers in the parasite, disrupting microtubules in the cytoskeleton and impairing cellular transport functions (Buchmann & Bresciani, 2006; Whittington & Chisholm, 2008).

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

The present study highlighted a detailed morphological characterization of *Thaparocleidus vistulensis* using SEM and molecular analysis, confirming the identity of the studied monopisthocotylean species, that was previously widely reported to infect European catfish. This study also provides a redescription for *T. vistulensis*, particularly for the characterization of the male copulatory organ, combined with the molecular data for species identification. To better understand this monopisthocotylean parasite, further studies on different developmental stages of parasite specimens and additional genetic markers are needed.

The histopathological alterations caused by *T. vistulensis* on the European catfish have been discussed thoroughly. Although the majority of the histopathological effects were previously described by Molnár (1980), this study identified several distinct variations, including the presence of epithelial eosinophilic granular, which is commonly detected at the parasite attachment sites, and the distortion primarily in the extracellular cartilaginous matrix caused by the deep attachment sites that touching the chondrocytes of the gill rays. The pathological effects of *T. vistulensis* on the gills of the European catfish were mainly related to the attachment sites of the parasite to its host, but damage also affected adjacent structures. This study has also provided the impact of European catfish gills infected with the monopisthocotylean through the SEM for the first time.

The present study investigated the basic knowledge of infection dynamics and life cycle parameters at different life stages for *T. vistulensis*, which is currently eliciting disease in European catfish farms. Based on the observed dynamics, future control by use of life cycle management has been suggested. Knowledge of the reproductive strategies of the monopisthocotylean *T. vistulensis* is important, especially for the development of specific prophylactic and therapeutic methods in intensive aquaculture. Forecasting parasite population increases and developing

epidemiological models are needed in modern fish farming, and studies on life cycle parameters, as presented in the present study, are basic elements of this process.

In addition, the present findings elucidate the significant impact of photoperiodicity and water temperature on the life cycle and reproductive strategies of *T. vistulensis*, providing insight into the parasite's ecology and adaptability to environmental variables. The reproductive process of *T. vistulensis* is hardly affected by light-dark cycles, except with egg production, but is highly dependent on temperature conditions. Understanding the general biological features and behaviour of *T. vistulensis*, which infects the European catfish, is particularly relevant given the importance of this species in European fish farming. However, it is important to note that the present study was conducted under controlled laboratory conditions and focused solely on an *in vitro* experimental setup, which may not fully represent the natural behaviour of the parasite. In planning future research, this should be kept in mind, and upcoming studies should be directed toward *in vivo* investigations to gain a better understanding of the reproductive strategies of *T. vistulensis* in aquaculture systems.

The present study also investigates the *in vitro* effects of three widely effective antiparasitic herbal treatments. The findings highlight for the first time the potential of alternative herbal treatments. While GAR and NMB were proven as effective antiparasitic agents, GIN exhibited limited efficacy in *in vitro* assays against *T. vistulensis*. Overall, GAR shows the most effective alternative, even at the highest dilution, for eliminating the majority of monopisthocotyleans in the shortest time across nearly all life stages of *T. vistulensis*. However, future studies, particularly *in vivo* investigations, are essential to reveal potential side effects and evaluate the suitability of these promising herbal treatments. Additionally, exploring herbs as alternative treatments should be further investigated to effectively manage this specific monopisthocotylean parasitic disease in aquaculture. Such studies could significantly contribute to an environmentally friendly approach and simultaneously reduce the monopisthocotyleans burden in cultured European catfish populations.

Furthermore, aside from the *in vitro* study on herbal treatments, *in vitro* comparative assays of three drug parasiticides against *T. vistulensis* are presented. The findings pointed out that the effectiveness of different drugs is highly life stage dependent. The present study showed that the novel biological compound (BIO) has a potential therapeutic effect on *T. vistulensis* oncomiracidia and juvenile parasites but does not affect eggs and adult parasites. The effect of the conventional anthelmintic PZQ was manifested by a temporary blockage or pause of the early life stage, followed by unexpected regeneration even after very high treatment doses. The widely used conventional anthelmintics MBZ effectively inhibited egg development, however, to achieve efficacy against juvenile and adult *T. vistulensis,* higher concentrations were required. This is the first study to test BIO as an anthelmintic compound against a monopisthocotylean parasite, and it has proven to be effective in targeting the oncomiracidial life stage. Based on the differential effects of the three different compounds, the present study suggests a combination strategy in order to elevate the over-all efficacy of the treatments, whereby all life cycle stages are targeted. Thus, BIO targeted oncomiracidia, PZQ targeted juvenile parasites, and MBZ targeted mainly eggs. However, *in vivo* studies should be conducted to elucidate this combination strategy.

Although PZQ did not demonstrate significant efficacy *in vitro* assays, it significantly impacted *in vivo*, where a 24 h bath treatment completely eliminated parasites from the gills of European catfish. A similar effect was observed with MBZ, which reduced more than 98% parasite load at 14 dpt, even at the lowest concentration, indicating a delayed effect but a persistent antiparasitic agent. *In vitro* and *in vivo* findings of drug treatments highlight the potential of using established and novel treatments to manage aquaculture parasitic infections, especially within the European catfish farming population. While the current research is promising, optimising treatment protocols and evaluating their long-term effects on fish health and ecosystem sustainability remain crucial for enhancing their effectiveness.

At present, no proven treatment shows a complete antiparasitic effect against gill monogenean parasitosis caused by *T. vistulensis*. Therefore, the use of alternative or additional management

techniques is recommended. Quarantine involves transferring infected stock from one system to another, allowing time for the parasites to be eliminated from the water system. The duration of the quarantine should be at least as long as the combined time it takes for the last egg laid in the system to hatch, along with the lifespan of the oncomiracidium larvae. This ensures that any remaining eggs and larvae are killed before the fish enters the new environment. It is advisable to increase the water temperature in the system, as the parasites die faster. Adherence to this guideline minimizes the risk of (re)infection. However, infection control by moving infected fish stocks between water bodies at specified intervals (**Table S19**) alone provides limited effectiveness as *in vivo* study on heat treatment should be conducted to confirm the success of elimination of the parasites that attached to the host. Heat treatment of infected fish poses risks, particularly when it is close to its tolerance threshold. Even so, it remains an alternative option to be considered.

In general, this study underscores the importance of effective parasite management in sustainable aquaculture and lays the groundwork for future advancements in fish health research. This study will enhance the understanding of the infection dynamics of *T. vistulensis*, the factors influencing its life cycle, and the efficacy of more environmentally friendly alternative treatments. By comprehending these aspects, this study is expected to contribute to the development of more sustainable control strategies in closed aquaculture systems (i.e., RAS), thereby reducing reliance on synthetic chemicals. The findings of this study may also provide valuable guidance for fish farmers in managing fish health more effectively, ensuring the well-being of cultured species, and supporting a more sustainable aquaculture industry in the future.

For future directions, it is suggested that the findings be applied in RAS systems, following the recommended concentrations, as European catfish farming continues to grow. This farming system is gaining popularity among fish farmers, particularly for the cultivation of European catfish, thereby enhancing both the economy and the health of the fish. Further research into optimizing these treatments in RAS systems could contribute significantly to sustainable aquaculture practices.

NEW SCIENTIFIC RESULTS

1. For the first time, the present study provides extensive molecular characterization data of *Thaparocleidus vistulensis* (2964 bp), including partial sequences of the SSU rRNA gene, complete sequences of the ITS1, 5.8S rRNA gene, ITS2, and partial sequences of the LSU rRNA gene.
2. This study presents, for the first time, the external morphology and pathological effects of *T. vistulensis* using scanning electron microscopy (SEM) images.
3. This study demonstrates new insights into previously undescribed aspects of the life cycle and reproductive strategies of *T. vistulensis.*
4. This research delivers, for the first time, insights into the influence of environmental factors, including the light-dark cycle and various water temperatures, on different life stages of *T. vistulensis*.
5. This study introduces, for the first time, the use of bacterial-derived lipopeptide *Pseudomonas* H6 Biokos (BIO) and the use of herbal therapeutic antiparasitic agents against *T. vistulensis*.

LIST OF PUBLICATIONS

Peer-reviewed journal articles

1. **Wan Sajiri, W.M.H.**, Székely, C., Molnár, K., Buchmann, K., & Sellyei, B. (2023). Reproductive strategies of the parasitic flatworm *Thaparocleidus vistulensis* (Siwak, 1932) (Platyhelminthes, Monogenea) infecting the European catfish *Silurus glanis* Linnaeus, 1758. *International Journal for Parasitology: Parasites and Wildlife, 22,* 113–120. https://doi.org/10.1016/j.ijppaw.2023.09.010
2. **Wan Sajiri, W.M.H.**, Székely, C., Molnár, K., Kjeldgaard-Nintemann, S., Kania, P. W., Buchmann, K., & Sellyei, B. (2024). Molecular and SEM studies on *Thaparocleidus vistulensis* (Siwak, 1932) (Monopisthocotyla, Ancylodiscoididae). *Scientific Reports*, *14*(1), 10292. https://doi.org/10.1038/s41598-024-61032-3
3. **Wan Sajiri, W.M.H.**, Székely, C., Molnár, K., Buchmann, K., & Sellyei, B. (2025). Pathological effects of *Thaparocleidus vistulensis* (Siwak, 1932) infection on the gills of *Silurus glanis* Linnaeus, 1758. *Acta Veterinaria Hungarica*, *73*(1), 56–63. https://doi.org/10.1556/004.2025.01121
4. **Wan Sajiri, W.M.H.**, Székely, C., Sellyei, B. (2025). Influences of light-dark cycle and water temperature on in vitro egg laying, hatching and survival rate of the *Thaparocleidus vistulensis* (Dactylogyridea: Ancylodiscoididae). *Parasitology Research*. *124*(1), 4. https://doi.org/10.1007/s00436-024-08430-8
5. **Wan Sajiri, W.M.H.**, Székely, C., Czeglédi, I., Buchmann, K., & Sellyei, B. (2025). Comparative *in vitro* effects of novel and conventional parasiticides on *Thaparocleidus vistulensis* (Siwak, 1932) (Monopisthocotyla) parasitizing European Catfish (*Silurus glanis*). *Aquaculture Reports*, *43*, 102952. https://doi.org/10.1016/j.aqrep.2025.102952

Publications in progress

1. **Wan Sajiri, W.M.H.**, Székely, C., Buchmann, K., & Sellyei, B. *In vivo* efficacies of conventional parasiticides on *Thaparocleidus vistulensis* (Siwak, 1932) (Monopisthocotyla) parasitizing European Catfish (*Silurus glanis*). *Aquaculture Reports.*
2. **Wan Sajiri, W.M.H.**, Székely, C., Buchmann, K., & Sellyei, B. *In vitro* efficacy of herbal treatments targeting *Thaparocleidus vistulensis* (Siwak, 1932) in European Catfish (*Silurus glanis*). *Aquaculture Reports.*

Other related publication

1. **Wan Sajiri, W.M.H.**, Székely, C., & Sellyei, B. (2023). Survey of ectoparasite diversity in different rearing systems (fish ponds and RAS) at a fish farm. In: Hungarian Journal of Aquaculture and Fisheries Science, ISSN 3003-9797. (Vol. 9), No. 2, pp. 22 – 29. https://www.agrarlapok.hu/halaszat-tudomany-20232

Conference abstracts/ papers/ oral and poster presentations

1. **Wan Sajiri, W.M.H.**, Székely, C., & Sellyei, B. (2022). Advancement of my PhD work at the field and the laboratory: Parasitic infestation and feasible control against them in RAS. [Oral presentation]. In: Proceedings of Young Researchers' Day 2022, Budapest, Hungary, October 12, 2022.
2. **Wan Sajiri, W.M.H.**, Sellyei, B., & Székely, C. (2023). Reproductive strategies of the parasitic flatworm *Thaparocleidus vistulensis* (Platyhelminthes, Monogenea) infecting the European catfish (*Silurus glanis*). [Oral presentation]. In: Proceeding of Academical Days / Parasitology-Zoology-Fish Pathology Session, Budapest, Hungary, January 20–23, 2023.
3. **Wan Sajiri, W.M.H.**, Székely, C., & Sellyei, B. (2023). Reproductive strategies of the parasitic flatworm *Thaparocleidus vistulensis* (Monogenea) infecting the European catfish (*Silurus glanis*). [Oral presentation]. In: Proceedings of the 47th Hungarian Scientific Conference on Fisheries & Aquaculture (A XLVII. Halászati Tudományos Tanácskozás), Szarvas, Hungary, June 7–8, 2023.

4. **Wan Sajiri, W.M.H.**, Székely, C., Buchmann, K., & Sellyei, B. (2023). Reproductive strategies of the parasitic flatworm *Thaparocleidus vistulensis* (Platyhelminthes, Monogenea) infecting the European catfish (*Silurus glanis*). [Oral presentation]. In: Proceeding of the 21st International EAFP Conference on Diseases of Fish and Shellfish, Aberdeen, Scotland, September 11–14, 2023.
5. **Wan Sajiri, W.M.H.**, Székely, C., Molnár, K., Buchmann, K., & Sellyei, B. (2023). Reproductive strategies of the parasitic flatworm *Thaparocleidus vistulensis* (Platyhelminthes, Monogenea) infecting the European catfish *Silurus glanis* Linnaeus, 1758. [Poster presentation]. In: Proceeding of the 9th International Symposium on Monogenea, Lucknow, India, October 8–11, 2023.
6. **Wan Sajiri, W.M.H.**, Székely, C., & Sellyei, B. (2024). Introducing RASOPTA: Safeguarding future production of fish in aquaculture systems with water recirculation. [Oral presentation]. In: Scientific Meeting of the Hungarian Parasitological Society on Fish Parasitology, April 10, 2024.
7. **Wan Sajiri, W.M.H.**, Székely, C., & Sellyei, B. (2024). Introducing RASOPTA: Safeguarding future production of fish in aquaculture systems with water recirculation. [Oral presentation]. In: Proceedings of the 48th Scientific Conference on Fisheries & Aquaculture (A XLVIII. Halászati Tudományos Tanácskozás), Szarvas, Hungary, June 5–6, 2024.
8. **Wan Sajiri, W.M.H.**, Székely, C., Molnár, K., Kjeldgaard-Nintemann, S., Kania, P.W., Buchmann, K., & Sellyei, B. (2024). Molecular and SEM studies on *Thaparocleidus vistulensis* (Siwak, 1932) (Monopisthocotyla, Ancylodiscoididae). [Poster presentation]. In: Proceedings of the 48th Scientific Conference on Fisheries & Aquaculture (A XLVIII. Halászati Tudományos Tanácskozás), Szarvas, Hungary, June 5–6, 2024.

SUMMARY

The European catfish (*Silurus glanis*) is an important species with high economic value, and its growing demand has led to intensive farming practices for the species. However, this species is increasingly challenged in the aquaculture industry by parasitic infections, particularly from a specific gill monopisthocotylean parasite called *Thaparocleidus vistulensis*. The present study aimed to explore control options for monopisthocotylean infection in European catfish, with key objectives including molecular characterization and morphological study of *T. vistulensis*, evaluation of its pathological effects on gills, investigation of its reproductive strategies and environmental influences, and assessment of *in vitro* and *in vivo* efficacy of antiparasitic treatments.

Presenting new molecular and SEM features, this study gives additional data to the better knowledge of *T. vistulensis*. In addition, notes on the early development of sclerotized anchors are also provided. The main morphological difference of *T. vistulensis* from other congeneric species is associated with the male copulatory organ, which has 5–7 loops in the middle of the penis and a long open V-shaped sclerotized accessory piece that splits terminally to secure the penis tube's terminal. The present study provides for the first time molecular characterization data based on the 2694 bp long nucleotide sequence of rDNA submitted in GenBank with the accession number OR916383. The morphological characterization of the male copulatory organ and molecular data of *T. vistulensis* extends knowledge of this monopisthocotylean species and offers new insights for future phylogeny studies.

The pathological effects of *T. vistulensis* on the gills of the European catfish were examined through histopathological methods and SEM. The results were mainly related to the attachment sites of the parasite to its host, but damage also affected adjacent structures. The parasite attaches by the opisthaptor using anchors, adhering both superficially and deeply to the basal region between adjacent secondary lamellae. This causes deep, cup-like depressions on the gill lamellae

and occasional damage to the extracellular matrix. Epithelial hyperplasia leads to lamellar fusion and erythrocyte extravasation. Severe infections result in club-like structures at the gill tips and frequent eosinophilic granular cells at attachment sites. These pathological changes highlight the need for effective management strategies to control *T. vistulensis* in farmed European catfish.

The life cycle of *T. vistulensis* was investigated by detailed observation of infection dynamics, egg development, *in vitro* hatching and survival rates of the monopisthocotyleans at different life stages at 23 °C. The population of *T. vistulensis* on European catfish in fish tanks increased significantly within ten days, based on the initial infection intensity on fish donors. Eggs hatched 3-4 days post-oviposition, with a peak hatching rate of 89.7% on day five. The survival rate of oncomiracidia was 7.4% after five days while developing juvenile and adult parasites showed a higher dependence on host contact (survival rates of 0.9% and 1.6%, respectively, after three days). These findings help predict parasite-host dynamics and could improve control of gill diseases in farmed European catfish.

The present study also observed the fecundity, hatching rate, and survival ability of *T. vistulensis* under varying light-dark conditions and various water temperatures (5 to 35 °C). Light exposure increased egg production by adult *T. vistulensis* threefold compared to constant darkness. While light and dark conditions did not affect the hatching or survival of juveniles or adults, they significantly impacted the oncomiracidia survival rate. Optimal fecundity occurred at 15 °C, with eggs hatching fastest at 30 °C, and no hatching at 5 °C or 35 °C. Both oncomiracidia and adult survival rates decreased with higher water temperatures. These findings enhance a fundamental understanding of environmental impacts on the parasite's life cycle, providing a basis for managing *T. vistulensis* in farmed European catfish.

In vitro and *in vivo* efficacy of herbal (garlic (GAR), ginger (GIN), and neem bark (NMB)) and drug (Biokos (BIO), Praziquantel (PZQ), and Mebendazole (MBZ)) antiparasitic treatments against *T. vistulensis* infection in European catfish were assessed. For herbal *in vitro* treatment,

GAR was demonstrated to be the most effective in eliminating most of the life stages. NMB treatment exhibited the most efficient against oncomiracidia, while GIN was only effective at lower stock dilution treatments. Meanwhile, *in vitro* drug assays demonstrated that MBZ is the most effective in inhibiting the development of eggs with 100% efficacy. BIO treatment was proven effective in targeting the oncomiracidia life stage compared to the others. Higher concentrations are required for all antiparasitic treatments to show their effectiveness against developing juvenile and adult *T. vistulensis*. This suggests that strategic combinations of the parasiticides may elevate efficacy.

The toxicity test demonstrated that European catfish could endure 10 mg/L of PZQ and MBZ without recorded any stress signs. The *in vivo* long bath treatment revealed PZQ could eliminate 100% of parasites from the gills of juvenile European catfish within 1 dpt. Meanwhile, MBZ displayed a delayed effect, where the parasite reduction of at least 98% occurred at 14 dpt, even at the lowest concentration. Based on these results, it is possible to identify key moments during European catfish farming to apply the treatment to prevent disease outbreaks.

The study on *in vitro* herbal treatments for *T. vistulensis* demonstrates their potential as alternative treatments for controlling monopisthocotylean infections in European catfish farming. Nevertheless, *in vivo* treatment studies are needed to further understand their efficacy and long-term impact on parasite management. The observations presented for drug treatments in the *in vitro* study suggest that a combination of the three different compounds (BIO, PZQ, and MBZ) for the treatment of monopisthocotylean infection in catfish culture could enhance overall effectiveness. BIO was effective against oncomiracidia, PZQ was effective on juvenile parasites, and MBZ primarily affected eggs. Nonetheless, *in vivo* research is required to better understand the potential of this combined treatment approach.

ÖSSZEFOGLALÁS

A lesőharcsa (*Silurus glanis*) nagy gazdasági jelentőséggel bíró halfaj, melynek intenzív tenyésztésbe vonását, az iránta való megnövekedett kereslet, felgyorsította az utóbbi években. Ugyanakkor, a nagy egyedsűrűség mellett gyorsan terjedő parazitás fertőzések kordában tartása, mint amilyen a *Thaparocleidus vistulensis* (Monopistocotylea) okozta kopoltyúférgesség, nagy kihívást jelent a haltenyésztés számára. A jelen tanulmány célja a lesőharcsa gazdaspecifikus kopoltyúféreg fertőzésének féken tartására alkalmas lehetőségeinek feltárása. Ennek érdekében, elvégeztük a *T. vistulensis* molekuláris és morfológiai jellemzését, vizsgáltuk a parazita kopoltyúkra gyakorolt patológiai hatásait, az életciklusának alakulását bizonyos környezeti hatások változtatása mellett, valamint egyes parazitaellenes kezelések in vitro és in vivo hatékonyságát.

Az értekezés új pásztázó elektronmikroszkópiás (angolul: scanning electron microscope, SEM) és molekuláris adatok (a GenBank-ban OR916383 regisztrációs szám alatt elhelyezett 2694 bp hosszú rDNS nukleotidszekvencia) bemutatásával járul hozzá a *T. vistulensis* parazita faj jobb megismeréséhez. A Csáklyásférgek morfológiai elkülönítésében meghatározó jelentőségű szklerotizált struktúrák nagyfelbontású vizsgálata részletes adatokat szolgáltatott a rögzülésért felelős opisthator közép- és szegélyhorgainak korai fejlődésével, valamint a hím párzószerv szilárd részeinek felépítésével kapcsolatosan. A megfigyelések alapján, a pénisz középen 5-7 hurokkal és egy hosszú, nyitott, V-alakú, szklerotizált tartozékkal rendelkezik, amely terminálisan kettéválik ekként rögzítve a péniszcső végét.

A *T. vistulensis*, a lesőharcsa kopoltyúira gyakorolt szövetkárosító hatását hisztológiai módszerekkel és SEM segítségével egyaránt vizsgáltuk. A parazitát az opisthaptor horgai – a szegélyhorgok felületesen, míg a középhorgok mélyen belefúródva – rögzítik két szomszédos kopoltyúlemez között, közel azok bazális régiójában. A megtapadás helyén csészeszerű bemélyedések alakulnak ki, a horgok időnként a kopoltyúlemezek vázát adó porcot is elérik és

károsítják. A sérülés során kialakuló epitheliális hiperplázia a kopoltyúlemezek összeolvadásához, fúziójához és a vörösvértestek érhálózatból való kilépéséhez vezet. A súlyos fertőzések nyomán kopoltyúlemezek csúcsainak az összeolvadásával dobverőszerű struktúrák jönnek létre, a sérült szövetekben eozinofil szemcsés sejtek bevándorlása tapasztalható. Ezen súlyos, a légcserét jelentősen veszélyeztető elváltozások rávilágítanak arra, hogy hatékony kezelési stratégiákra van szükség a tenyésztett lesőharcsa *T. vistulensis* elleni megóvása érdekében.

A *T. vistulensis* fertőzés dinamikájának feltérképezése érdekében vizsgáltuk a peték fejlődését, kelési ütemét, és az egyes élet fázisok *in vitro* túlélési arányát 23 °C-on. A kísérleti populációban, a kezdeti dózis, vagyis a donorhal fertőzöttségi intenzitása függvényében tíz nap alatt lezajlott a fejlődés a petétől az ivarérett egyedekig és elterjedt a fertőzés. A peterakást követő 3.-5. napon a peték kikeltek. A szabadon úszó lárvák (onkomiracidia) túlélési aránya öt nap után 7,4%-ra csökkent. A fiatal és felnőtt paraziták gazdától való függősége jóval kifejezettebb volt, túlélési arányuk, három nap után, mindössze 0,9%, illetve 1,6% volt. Ezek a megfigyelések segítséget nyújtanak a parazita fertőzés dinamikájának előrejelzésében és a kopoltyúférgesség elleni védekezés időbeni stratégiájának megtervezésében.

A jelen tanulmány a különböző környezeti tényezők, mint a változó fényviszonyok és vízhőmérséklet (5-35 °C) a *T. vistulensis* termékenységére, kelési arányára és túlélési képességére tett hatásait is vizsgálta. A fényexpozíció háromszorosára növelte a kifejlett *T. vistulensis* peterakási intenzítását az állandó sötétségben tartott egyedekhez képest. Ugyanakkor a világos és sötét viszonyok változása nem befolyásolta a peték kelését vagy a fiatal és felnőtt férgek túlélését, de jelentősen befolyásolták az onkomiracidiumok élettartalmát. Az optimális kelési hőmérsékletnek a 15 °C bizonyult; a peték leggyorsabban 30 °C-on fejlődtek, míg 5 °C-on vagy 35 °C-on egyáltalán nem keltek ki. Mind az onkomiracidiumok, mind a felnőttkori túlélési arány csökkent a magasabb vízhőmérséklet hatására. Ezek az eredmények elősegítik a parazita életciklusára gyakorolt környezeti hatások alapvető megértését, alapot biztosítva a *T. vistulensis* kezelések optimális feltételeinek meghatározásához tenyésztett lesőharcsában.

Kísérleteket végeztek különböző gyógynövény kivonatok (fokhagyma (GAR), gyömbér (GIN) és ním fa kéreg (NMB)) és gyógyszer hatóanyagok (Biokos (BIO), praziquantel (PZQ) és mebendazol (MBZ)) parazitaellenes hatékonyságának megítélésére *T. vistulensis* fertőzés elleni leső harcsában *in vitro* és *in vivo* kezelések formájában. A gyógynövény kivonatok *in vitro* alkalmazása során a GAR bizonyult a leghatékonyabbnak a különböző életszakaszban lévő paraziták megfékezésére. Az oncomiracidiumok a NMB hatóanyagra voltak a leginkább érzékenységet, de alacsony higításban a GIN kivonat is hatásos volt. A gyógyszer hatóanyagok *in vitro* vizsgálatai azt mutatták, hogy az MBZ képes a peték fejlődését teljesen meggátolni. A BIO hatékonynak bizonyult az onkomiracidiumok aktivitásának korlátozásában. Azonban, a fiatal és felnőtt *T. vistulensis* férgekre a fenti parazita ellenes szerek csak magas koncentrációban képesek hatást kifejteni. A fenti megfigyelések arra utalnak, hogy a parazitaölő szerek megfelelő időben alkalmazott kombinációi növelhetik a kezelési hatékonyságot.

Az elvégzett *in vivo* toxicitási tesztek kimutatta, hogy a fiatal lesőharcsa 10 mg/l PZQ, illetve MBZ kezelést képes elviselni hosszú fürösztés formájában anélkül, hogy jelét adná bármely stressznek. A vizsgálatok kimutatták, hogy a PZQ esetében a paraziták teljesen eltűntek a fiatal lesőharcsa kopoltyúiból már a kezelést követő napon. Míg a MBZ hatása késleltetett, 14 nappal a kezelést követően a paraziták száma 98%-os csökkenést mutat még a legalacsonyabb alkalmazott koncentráció esetén is.

Bár a gyógynövény kivonatok hatásának in vivo kipróbálására nem nyílt lehetőség, az *in vitro* eredmények sejtetik, hogy ezek alternatív kezelési lehetőségek alkalmasak lehetnek a monopisthotylea fertőzések leküzdésében a lesőharcsatenyésztésben. Mindazonáltal szükség van az *in vivo* kezelési vizsgálatok elvégzésére, hogy jobban megértsük hatékonyságukat és hosszú távú hatásukat a parazitakezelésben.

A gyógyszer hatóanyagok *in vitro* vizsgálatból származó megfigyelések azt sugallják, hogy a három különböző vegyület (BIO, PZQ és MBZ) kombinációja javíthatja a kopoltyúféreg fertőzés

kezelés általános hatékonyságát. A BIO az onkomiracidia ellen, a PZQ a fiatal paraziták ellen, az MBZ pedig elsősorban a peték fejlődésére fejt ki gátló hatást. Mindazonáltal további *in vivo* vizsgálatokra van szükség ahhoz, hogy jobban megértsük e kombinált kezelési megközelítésben rejlő lehetőségeket.

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APPENDICES

Appendix A: Bibliography

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Appendix B: Supplementary Tables

Table S1. Eukaryotic parasites reported to infect European catfish.

Parasite	References
Protozoons	
Capriniana piscium (Buetschli, 1889)	Sobecka et al. (2010)
Chilodonella piscicola (Zacharias, 1894)	Sobecka et al. (2010)
Ichthyophthirius multifiliis Fouquet, 1876	Roohi et al. (2014)
Nosema tisae Lom and Weiser, 1969*	Lom and Dyková (1992)
Trichodina acuta Lom, 1961	Lom and Dyková (1992)
Trichodinella epizootica (Raabe, 1950)	Lom and Dyková (1992)
Trichodina nigra Lom, 1961	Lom and Dyková (1992)
Trichodina siluri Lom, 1970	Sobecka et al. (2010)
Trichodina sp.	Roohi et al. (2014)
Trichodinella sp.	Nash et al. (1988)
Trypanoplasma ninae-kohl-yakimovae Yakimov and Schokhor, 1917	Lom and Dyková (1992)
Trypanosoma markewitschii Lubinsky ex N.M.Zalewska, 1950	Lom and Dyková (1992)
Trypanosoma spp.	Reading et al. (2012)
Myxozoans	
Myxobolus exiguus Thélohan, 1895	Lom and Dyková (1992)
Myxobolus bondi Landsberg and Lom, 1991*	Lom and Dyková (1992)
Myxobolus miyairii Kudo, 1920	Lom and Dyková (1992)
Myxobolus sp.	Sobecka et al. (2010)
Sphaerospora schulmani (Rumyantsev, 1997)	Lom and Dyková (1992)
Sphaerospora siluri Molnar, 1993	Molnár (1993)
Monogeneans	
Dactylogyrus sp.	Nash et al. (1988)
Gyrodactylus sp.	Sobecka et al. (2010)
Thaparocleidus magnus (Bychowsky and Nagibina, 1957)*	Ondračková et al. (2004)
Thaparocleidus siluri (Zandt, 1924)*	Roohi et al. (2014)
Thaparocleidus vistulensis (Siwak, 1932)*	Roohi et al. (2014)
Trematodes	
Allocreadium siluri Osmanov, 1967*	Osmanov (1971)
Aphanurus stossichii (Monticelli, 1891)	Pazooki and Masoumian (2012)
Aspidogaster limacoides Diesing, 1834	Markevich (1951)
Asymphylodora kubanica Issaitschikov, 1923	Mikailov (1975)
Asymphylodora tincae (Modeer, 1790)	Scarlato (1987)
Azygia lucii (Müller, 1776)	Murvanidze et al. (2018)
Bolbophorus confusus (Krause, 1914)	Osmanov (1971)
Bucephalus polymorphus von Baer, 1827	Sobecka et al. (2010)
Bunocotyle cingulata Odhner, 1928	Pazooki and Masoumian (2012)
Bunodera luciopercae (Müller, 1776) Lühe, 1909	Mikailov (1975)
Bychowskycreadium schiliani Mikailov, 1967	Mikailov (1975)
Caudotestis skrjabini (Koval in Markevich, 1951)*	Scarlato (1987)
Clinostomum complanatum (Rudolphi, 1814)	Ibrahimov (1977)
Diplostomum chromatophorum (Brown, 1931)	Ibrahimova (2010)
Diplostomum huronense (La Rue, 1927)*	Ibrahimova (2010)
Diplostomum mergi Dubois, 1932	Ibrahimova (2010)
Diplostomum spathaceum (Rudolphi, 1819)*	Javad et al. (2014)
Hysteromorpha triloba (Rudolphi, 1819)	Osmanov (1971)
Ichthyocotylurus pileatus (Rudolphi, 1802)	Copp et al. (2009)
Ichthyocotylurus variegatus (Creplin, 1825)	Scarlato (1987)
Metagonimus yokogawai (Katsurada, 1912)	Moravec (2001)
Nicolla skrjabini (Iwanitzky, 1928)*	Yakhchali et al. (2012)
Orientocreadium pseudobagri Yamaguti, 1954*	Roohi et al. (2014)
Phyllodistomum folium (Olfers, 1816)	Kirillov et al. (2018)
Phyllodistomum petruschewskii Pigulewsky, 1953	Scarlato (1987)
Posthodiplostomum cuticola (von Nordmann, 1832)	Mikailov et al. (2001)
Pygidiopsis genata Looss, 1907	Scarlato (1987)
Rhipidocotyle campanula (Dujardin, 1845)	Ibrahimova (2010)
Sphaerostoma bramae (Müller, 1776)	Moravec (2001)
Tylodelphys clavata (von Nordmann, 1832)	Moravec (2001)
Cestodes	
Glanitaenia osculata (Goeze, 1782)*	Sefidkare-Langeroudi (1965)
Postgangesia inarmata de Chambrier, Al-Kallak and Mariaux, 2003	de Chambrier et al. (2003)
Schyzocotyle acheilognathi (Yamaguti, 1934)*	Yakhchali et al. (2012)
Silurotaenia siluri (Batsch, 1786)	Roohi et al. (2014)
Triaenophorus crassus Forel, 1868	Khara et al. (2005)
Nematodes	
Anisakis sp. larvae	Sefidkare-Langeroudi (1965)
Camallanus lacustris (Zoega in Müller, 1776)	Reading et al. (2012)
Camallanus truncatus (Rudolphi, 1814)	Kirjušina and Vismanis (2007)
Contracaecum microcephalum (Rudolphi, 1819)	Osmanov (1971)
Contracaecum rudolphii Hartwich, 1964*	Osmanov (1971)
Cucullanus heterochrous Rudolphi, 1802	Kirjušina and Vismanis (2007)
Cucullanus sphaerocephalus (Rudolphi, 1809)	Javad et al., 2014
Dichelyne (Cucullanellus) minutus (Rudolphi, 1819)*	Ibrahimov (1977)
Dioctophyme renales (Goeze, 1782)	Osmanov (1971)
Eustrongylides excisus Jägerskiöld, 1909	Ibrahimova (2010)
Eustrongylides mergorum (Rudolphi, 1809)	Sobecka et al. (2010)
Gnathostoma hispidum Fedtschenko, 1872	Osmanov (1971)
Goezia ascaroides (Goeze, 1782)	Scarlato (1987)
Hysterothylacium bidentatum (Linstow, 1899)	Kirillov et al. (2018)
Pseudocapillaria (Pseudocapillaria) tomentosa (Dujardin, 1843)	Mikailov (1975)
Raphidascaris acus (Bloch, 1779)	Kirillov et al. (2018)
Rhabdochona chodukini Osmanov, 1957	Osmanov (1971)
Rhabdochona denudata (Dujardin, 1845)	Sobecka et al. (2010)
Rhabdochona gnedini Skrjabin, 1946	Osmanov (1971)
Rhabdochona hellichi (Šrámek, 1901)	Osmanov (1971)
Schulmanela petruschewskii (Schulman, 1948)*	Moravec (2001)
Skrjabinisakis schupakovi Mozgovoy, 1951*	Volodina et al. (2016)
Acanthocephalans	
Acanthocephalus anguillae (Müller, 1780)	Moravec (2001)
Acanthocephalus clavula (Dujardin, 1845)	Dezfuli et al. (1990a)
Acanthocephalus lucii (Müller, 1776)	Roohi et al. (2014)
Corynosoma caspicum Golvan and Mokhayer, 1973	Mokhayer (1976)
Corynosoma strumosum (Rudolphi, 1802)	Sefidkare-Langeroudi (1965)
Leptorhynchoides plagicephalus (Westrumb, 1821)	Dezfuli et al. (1990a)
Neoechinorhynchus (Neoechinorhynchus) rutili (Müller, 1780)	Sobecka et al. (2010)
Pomphorhynchus laevis (Zoega in Müller, 1776)	Dezfuli (1992)
Pomphorhynchus perforator (von Linstow, 1908)	Sefidkare-Langeroudi (1965)
Arthropodes	
Argulus coregoni Thorell, 1865	Moravec (2001)
Argulus foliaceus (Linnaeus, 1758)	Reading et al. (2012)
Ergasilus sieboldi Nordmann, 1832	Reading et al. (2012)
Lamproglena pulchella Nordmann, 1832	Kurbanova et al. (2002)
Lernaea cyprinacea Linnaeus, 1758	Roohi et al. (2014)
Sinergasilus major (Markevich, 1940)	Dos Santos et al. (2021)

* Accepted nomenclature. Differs from the original name mentioned in the literature.

Table S2. Distribution of *T. vistulensis* in Europe and Asia.

Country	Locality	Host	Reference
Europe			
Bulgaria	Danube River	*Silurus glanis*	Mancheva et al. (2009)
Czechia	-	*S. glanis*	Ergens and Lom (1970)
	-	*S. glanis*	Moravec (2001)
	Morava basin	*S. glanis*	Šimková et al. (2003)
Georgia	Jandari Lake	*S. glanis*	Chiaberashvili (1959)
Hungary	Bicske	*S. glanis*	Papp (1955)
	Szarvas	*S. glanis*	Molnár (1963)
Italy	Ticino River	*S. glanis*	Galli et al. (2003)
	Po River	*S. glanis*	Paladini et al. (2008)
Poland	Vistula River	*S. glanis*	Siwak (1932)
	Odra River and Lake Dąbie	*S. glanis*	Sobecka et al. (2010)
Romania	Danube River	*S. glanis*	Cojocaru (2009)
Russia	Volga River, Samara	*S. glanis*	Kirillov et al. (2018)
Turkey	Lake Sapanca and Terkos	*S. glanis*	Öktener, 2003
	Terkos Lake	*S. glanis*	Soylu (2005)
	Lake Sapanca and Durusu	*S. glanis*	Soylu (2009)
	Atatürk Dam Lake	*S. glanis*	Öktener and Alaş (2009)
	Lake Sığırcı	*S. glanis*	Çolak (2013)
	Lake Gala	*S. glanis*	Soylu (2014)
	Sakarya River	*S. glanis*	Akmirza and Yardimci (2014)
	Büyükçekmece Dam Lake	*S. glanis*	Yardimci et al. (2018)
	Susurluk Basin	*S. glanis*	Aydogdu et al. (2024)
UK	Staffordshire, Hampshire, Kent and Essex	*S. glanis*	Reading et al. (2012)
	Northfield Main Pit	*S. glanis*	Rees (2020)
Asia			
Azerbaijan	Mingachevir Reservoir, Kura River	*S. glanis*	Mahmudova (2019)
Iran	Anzali Wetland	*S. glanis*	Roohi et al. (2014)
	Amirkelayeh Wetland	*S. glanis*	Khara and Sattari (2016)
Iraq	Tigris River, Baiji Town	*S. triostegus*	Abdul-Ameer (1989)
	Tigris River, Mosul	*S. glanis*	Al-Niaeemi (1997)
	Garmat Ali River, Basrah	*Leuciscus vorax**	Abdul-Rahman (1999)
		*Mesopotamichthys sharpeyi**	
		*Alburnus sellal**	
		Heteropneustes fossilis	
		*Planiliza abu**	
		*Mastacembelus mastacembelus**	
		*Mystus pelusius**	
		S. triostegus	
	Garmat Ali River, Basrah	*M. pelusius*	Adday (2001)
		S. triostegus	
	Darbandikhan Lake	*S. triostegus*	Abdullah (2013)
	Greater Zab River	*S. triostegus*	Abdullah and Shwani (2010)
	Lesser Zab River and Greater Zab River	*S. glanis*	Abdullah and Mhaisen (2004)
	Tigris River, Mosul	*S. glanis*	Rahemo and Al-Neemi (1999)
	Tigris River	*S. glanis*	Rahemo and Al-Niaeemi (2001)
	Lesser Zab and Greater Zab Rivers	*S. glanis*	Abdullah (2002)
	Euphrates River	*S. triostegus*	Al-Sa'adi (2007)
	Greater Zab River	*S. triostegus*	Shwani (2009)
	Darbandikhan Lake	*S. triostegus*	Abdullah and Abdullah (2015a,b)
	Darbandikhan Lake	*S. triostegus*	Al-Jawda and Asmar (2015)
	Euphrates River, Al-Musaib City	*S. triostegus*	Mhaisen et al. (2015)
	Greater Zab River	*S. triostegus*	Bilal (2016)
	Euphrates River, Samawa City	*S. triostegus*	Al- Helli (2019)
Syria	Lake Assad	*S. triostegus*	Al-Samman et al. (2006)
Uzbekistan	Amudarya River Delta	*S. glanis*	Kurbanova (2024)

*Accepted nomenclature. Differs from the original name mentioned in the literature.

Table S3. Chemical-based bath treatments with anthelmintic agents for monopisthocotylean control in aquaculture.

Parasites	Fish Species	Delivery	Dose (mg/L)	Efficacy	References
Against PZQ					
Anacanthorus penilabiatus	*Piaractus mesopotamicus*	30 min	500	68.3%, 7 dpt	Onaka et al. (2003)
Benedenia seriolae	*Seriola lalandi*	48 h, 7 w post-catch	2.5	100%	Sharp et al. (2004)
Benedeniella posterocolpa	*Rhinoptera bonasus*	90 min	20	100%, 2 dpt	Thoney (1990)
Clemacotyle australis	*Aetobatus narinari*	45 min	25	100%	Janse and Borgsteede (2003)
Dactylogyrus extensus	*Cyprinus carpio*	180 min	5	100%	Schmahl and Mehlhorn (1985)
Dactylogyrus intermedius	*Carassius auratus*	48 h	13.5	93.3%, 6 dpt	Zhang et al. (2013)
Dactylogyrus sp.	*Poecilia reticulata*	24 h	3	100%	Fridman et al. (2014)
Dactylogyrus sp.	*Ctenopharyngodon idella*	48 h	7.5	100%	Hoai and Van (2014)
Dactylogyrus vastator	*Cyprinus carpio*	180 min	5	100%	Schmahl and Mehlhorn (1985)
Diplectanum oliveri	*Argyrosomus japonicus*	2 h	20	100% of adults	Joubert (2012)
Gracilobenedenia lutjanid, G. rohdei	*Lutjanus carponotatus*	2 × 2 h, within 48 h	20	100%	Whittington and Ernst (2002)
Gyrodactylus sp.	*Oncorhynchus mykiss*	3 h	10	97.7%	Santamarina et al. (1991)
Gyrodactylus turnbulli	*Poecilia reticulata*	24 h	3	78–100%	Levy et al. (2015)
Haliotrema abaddon	*Glaucosoma hebraicum*	24 h	2	~97%	Stephens et al. (2003)
Lepidotrema bidyana	*Bidyanus* sp.	48 h	10	99% of adults, 84% of juveniles	Forwood et al. (2013a)
Lepidotrema bidyana	*Bidyanus* sp.	60 min	40	77%	Forwood et al. (2013b)
Pseudodactylogyrus anguillae	*Anguilla anguilla*	25 h	10	100%	Buchmann et al. (1993)
Pseudodactylogyrus anguillae	*Anguilla anguilla*	24 h	5	95–100%	Buchmann et al. (2011)
Thaparocleidus vistulensis	*Silurus glanis*	5 h	10	15% prevalence reduction, 3–4 dpt	Székely and Molnár (1990)
Against MBZ					
Dactylogyrus minutus	*Cyprinus carpio*	24 h	1	95% Adult	Buchmann et al. (1993)
Dactylogyrus vastator	*Carasslus auratus*	24 h	2	no effect	Goven and Amend (1982)
Dactylogyrus vastator	*Carassius auratus*	48 h	0.03	46.90%	Zhang et al. (2014)
Gyrodactylus elegans	*Carasslus auratus*	24 h	0.01	100%	Goven and Amend (1982)
Pseudodactylogyrus anguillae,	*Anguilla anguilla*	24 h	1	100%	Székely and Molnár (1987)
P. bini		10 min	100	100%	
P. anguillae, P. bini	*Anguilla anguilla*	72 h	1	100%	Buchmann and Bjerregaard (1990)
Pseudodactylogyrus sp.	*Anguilla anguilla*	24 h	1	100%, 4 dpt	Mellergaard (1990)
		10 - 30 min	50	100%, 7 dpt	
		10 - 30 min	100	100%, 7 dpt	
Thaparocleidus vistulensis	*Silurus glanis*	4 - 26 h	10 - 100	0% prevalence reduction	Székely and Molnár (1990)
Against BIO					
-					

Table S4. Herbal-based bath treatments with anthelmintic agents for monopisthocotylean control in aquaculture.

Parasites	Fish Species	Delivery	Dose (mg/L)	Efficacy	References
Against GAR					
Gyrodactylus spp.	*Orechromis niloticus*	Indefinite (crushed garlic clove)	300	68%, 7 dpt	Abd El-Galil and Aboelhadid (2012)
		1 h (oil)	3 ppt	55%, 7 dpt	
Gyrodactylus turnbulli	*Poecilia reticulata*	5 min (Freeze dried flakes)	1 ppt	96%	Schelkle et al. (2013)
		5 min (Freeze dried powder)	30	95%	
		5 min (Minced)	70	78%	
		5 min (Granules)	70	78%	
Gyrodactylus turnbulli	*Poecilia reticulata*	1 h (aqueous)	7.5 - 12.5 ml/L	87.7 - 93.9%	Fridman et al. (2014)
Against GIN					
Dactylogyrus ctenopharyngodonis	*Ctenopharyngodon idella*	Indefinite (renewed every day after water change)	4	100%, 28 dpt	Fu et al. (2017)
Gyrodactylus turnbulli	*Poecilia reticulata*	30 min (ethanolic)	7.5 ppt	100%	Levy et al. (2015)
Against NMB*					
Dactylogyrus sp.	*Oreochromis niloticus*	7 d (aqueous)	3 ppt	92.4%	Radwan et al. (2024)
Diplectanum sp.	*Dicentrarchus labrax*	12 h (aqueous)	150	67.85%	Aly et al. (2022)
Dactylogyrus sp.	*Oreochromis niloticus*	30 min	20 ppm	100%	Suryani and Arya (2017)
Gyrodactylus sp.	*Oreochromis niloticus*	30 min	20 ppm	100%	Suryani and Arya (2017)

Table S5. List of *Thaparocleidus* spp. sequences used in the phylogenetic analysis obtained from the International Nucleotide Sequence Database Collaboration (INSDC).

	Accession No.	Species	Length (bp)
1	AJ490165	*Thaparocleidus vistulensis*	830
2	AJ490164	*Thaparocleidus siluri*	829
3	MH094191	*Thaparocleidus varicus*	887
4	MH142377	*Thaparocleidus mutabilis*	849
5	MK440297	*Thaparocleidus mutabilis*	934
6	OP394151	*Thaparocleidus magnicirrus*	898
7	KX462990	*Thaparocleidus sudhakari*	1310
8	KX462991	*Thaparocleidus sudhakari*	1290
9	KX462989	*Thaparocleidus sudhakari*	815
10	OP394151	*Thaparocleidus magnicirrus*	898
11	MG601539	*Thaparocleidus asoti*	916
12	MG601540	*Thaparocleidus asoti*	925
13	OQ676425	*Thaparocleidus* sp.	848
14	MK271094	*Thaparocleidus* sp.	866
15	MK271095	*Thaparocleidus* sp.	854
16	OP495338	*Thaparocleidus* sp.	941
17	MK271093	*Thaparocleidus* sp.	838
18	MG653610	*Thaparocleidus* sp.	940
19	MH094193	*Thaparocleidus* sp.	870
20	MK271096	*Thaparocleidus* sp.	926

Table S6. Morphometric characteristics of *T. vistulensis* from the present study and relevant literature. The number of studied parasites and mean values of morphometric characters were not specified in Siwak (1932), and Bychowsky and Nagibina (1957). Mean ± Standard deviation, with range in parentheses. Measurements expressed in micrometers (µm).

Morphometric Characteristics	Siwak (1932)	Bychowsky and Nagibina (1957)	Paladini et al. (2008) (*n* = 20)	Present Study (*n* = 20)
Body Size *(*n* = 10)				
total body length	(740.0–1140.0)	(400.0–750.0)	1102.1 ± 167.6 (772.0–1325.6)	691.2 ± 163.0 (507.1–1002.4)
oncomiracidia	–	–	–	167.3 ± 7.6 (157.5–177.6)
total body width	(85.0–159.0)	(140.0–270.0)	308.5 ± 48.8 (202.6–360.7)	155.2 ± 27.2 (120.6–196.2)
oncomiracidia	–	–	–	72.5 ± 4.2 (65–78.4)
Dorsal Anchor				
total length	(70.0–79.0)	(70.0–77.0)	85.8 ± 2.4 (82.8–88.4)	66.2 ± 4.3 (57.6–73.9)
shaft length	–	(58.0–63.0)	71.7 ± 5.4 (66.2–81.6)	54.5 ± 3.5 (46.9–60.9)
root length	(18.0–22.0)	(16.0–19.0)	17.7 ± 4.1 (10.5–23.3)	13.9 ±1.5 (10.9–16.5)
point length	–	(31.0–35.0)	38.7 ± 3.2 (32.8–41.9)	31.4 ± 2.1 (27.3–35.0)
aperture	(59.0–68.0)	–	52.8 ± 2.4 (49.1–56.5)	42.8 ± 3.6 (36.4–50.4)
Cuneus				
total length	(22.0–27.0)	(24.0–28.0)	29.0 ± 2.1 (26.8–32.4)	23.4 ± 1.8 (19.7–26.5)
largest width	–	(7.0–8.0)	9.7 ± 1.5 (7.6–12.4)	7.8 ± 1.3 (5.8 –10.1)
Ventral Anchor				
total length	(27.0–30.0)	(25.0–28.0)	30.3 ± 2.1 (27.3–32.7)	25.8 ± 1.2 (23.7–27.6)
oncomiracidia *(*n* = 5)[a]	–	–	–	21 ± 1.3 (19.2–22.7)
shaft length	–	(21.0–22.0)	25.7 ± 1.3 (23.8–27.6)	21.1 ± 1.0 (19.3–22.6)
inner root length	–	7.0	8.7 ± 1.46 (7.0–11.2)	6.4 ± 0.6 (5.3–7.9)
oncomiracidia *(*n* = 5)[b]	–	–	–	2.6 ± 0.1 (2.5–2.8)
outer root length	–	–	–	5.3 ± 0.4 (4.5–6.0)
oncomiracidia *(*n* = 5)[b]	–	–	–	2.6 ± 0.2 (2.4–2.8)
point length	–	(14.0–16.0)	16.2 ± 1.2 (14.3–17.8)	14.9 ± 1.0 (12.1–16.5)
aperture	(18.0–22.0)	–	20.6 ± 1.6 (18.1–22.7)	18.1 ± 1.4 (16.0–21.2)

oncomiracidia *(n = 5)[b]	–	–	–	14.8 ± 0.3 (14.6–15.3)
Dorsal Bar				
total length	(32.0–37.0)	–	38.4 ± 2.6 (35.1–41.9)	31.6 ± 2.4 (27.1–35.8)
width in the middle	–	–	9.32 ± 0.7 (8.1–10.0)	6.7 ± 1.2 (5.2–9.9)
Ventral Bar				
length of one branch	(23.0–25.0)	(23.0–25.0)	25.7 ± 1.5 (23.3–27.5)	21.9 ± 1.4 (19.1–24.4)
largest width	–	3.0	5.3 ± 0.8 (4.3–6.8)	3.1 ± 0.5 (2.4–4.5)
Marginal Hook *(*n* = 40)				
total length	16.3	16.0	17.5 ± 0.5 (16.8–17.9)	15.8 ± 0.6 (14.9–16.9)
oncomiracidia	–	–	–	15.7 ± 0.7 (14.1–16.7)
sickle length	4.3	–	6.3 ± 0.4 (5.7–6.8)	5.3 ± 0.3 (4.8–5.9)
oncomiracidia	–	–	–	5.1 ± 0.2 (4.7–5.6)
Male Copulatory Organ *(*n* = 5)				
penis	–	640.0	–	837.4 ± 95.9 (703.6–940.9)
no. of loop	–	≥ 4	–	5–7
accessory piece	–	(68.0–71.0)	–	97.0 ± 7.8 (92.5–110.9)
farthest point	–	–	–	131.9 ± 20.4 (103.4–156.0)
bulbous base length	–	(14.0–16.0)	–	13.9 ± 0.6 (13.4–15.0)
bulbous base width	–	8.0	–	8.2 ± 1.1 (7.2–10.0)
Female Copulatory Organ *(*n* = 5)				
vaginal duct	–	≈ 200	–	358.1 ± 39.7 (323.2–409.1)

* Referred to the number of specimens examined in the present study
[a] Morphological part was measured following the anchor curve
[b] Morphological part was measured following ventral anchor parameters in **Figure 8**

Table S7. The sequence distances (in %) between taxa (*p*-distance) within genus/species group with *T. vistulensis*.

	Accession No. ID, Species and Host	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
1	**OR916383 *Thaparocleidus vistulensis* ex *Silurus glanis***																								
2	***Thaparocleidus vistulensis* ex *Silurus glanis***	100.0																							
3	AJ490165 *Thaparocleidus vistulensis* ex *Silurus glanis*	99.8	99.8																						
4	AJ490164 *Thaparocleidus siluri* ex *Silurus glanis*	98.1	98.1	98.2																					
5	OQ676425 *Thaparocleidus* sp.	95.8	95.8	96.0	96.3																				
6	MK271094 *Thaparocleidus* sp. ex *Silurus asotus*	95.8	95.8	96.0	96.3	100.0																			
7	MH094191 *Thaparocleidus varicus*	95.6	95.6	95.8	96.1	99.8	99.8																		
8	MK271095 *Thaparocleidus* sp. ex *Silurus asotus*	95.6	95.6	95.8	96.1	99.8	99.8	99.7																	
9	MH142377 *Thaparocleidus mutabilis*	95.6	95.6	95.8	96.1	99.8	99.8	99.7	99.7																
10	MK440297 *Thaparocleidus mutabilis* ex *Silurus asotus*	95.8	95.8	96.0	96.1	98.7	98.7	98.5	98.5	98.5															
11	OP495338 *Thaparocleidus* sp. ex *Silurus asotus*	96.5	96.5	96.3	96.1	95.5	95.5	95.5	95.3	95.3	95.3														
12	OP394151 *Thaparocleidus magnicirrus* ex *Silurus asotus*	94.4	94.4	94.2	94.4	93.9	93.9	93.7	93.7	93.7	94.7	94.8													
13	MK271093 *Thaparocleidus* sp. ex *Silurus asotus*	93.4	93.4	93.2	93.4	92.9	92.9	92.7	92.7	92.7	93.7	93.4	96.1												
14	KX462990 *Thaparocleidus sudhakari* ex *Wallago attu*	88.2	88.2	88.0	87.9	88.0	88.0	87.9	88.2	87.9	88.5	88.5	89.2	88.0											
15	KX462991 *Thaparocleidus sudhakari* ex *Wallago attu*	86.1	86.1	86.0	85.6	85.8	85.8	85.6	86.0	85.6	86.3	86.5	87.3	86.1	96.9										
16	KX462989 *Thaparocleidus sudhakari* ex *Wallago attu*	88.0	88.0	87.9	87.7	87.6	87.6	87.7	87.4	87.4	88.2	89.3	89.2	88.2	91.4	90.5									
17	OP394151 *Thaparocleidus magnicirrus* ex *Silurus asotus*	96.1	96.1	96.0	96.0	95.5	95.5	95.6	95.3	95.3	95.6	96.5	95.0	94.0	88.5	86.5	88.7								
18	MG653610 *Thaparocleidus* sp.	96.1	96.1	96.0	96.0	95.5	95.5	95.6	95.3	95.3	95.6	96.5	95.0	94.0	88.5	86.5	88.7	100.0							
19	MH094193 *Thaparocleidus* sp.	95.8	95.8	95.6	95.6	95.1	95.1	95.3	95.0	95.0	95.3	96.1	94.8	93.7	88.2	86.1	88.3	99.7	99.7						
20	MG601539 *Thaparocleidus asoti*	95.3	95.3	95.2	95.6	94.7	94.7	94.5	94.5	94.5	94.8	95.6	94.4	93.2	87.7	85.6	87.6	96.1	96.1	95.8					
21	MK271096 *Thaparocleidus* sp. ex *Silurus asotus*	95.3	95.3	95.2	95.6	94.7	94.7	94.5	94.5	94.5	94.8	95.6	94.4	93.2	87.7	85.6	87.6	96.1	96.1	95.8	100.0				
22	MG601540 *Thaparocleidus asoti*	95.3	95.3	95.2	95.6	94.7	94.7	94.5	94.5	94.5	94.8	95.6	94.4	93.2	87.7	85.6	87.6	96.1	96.1	95.8	100.0	100.0			
23	JN996868 *Ligophorus chabaudi* ex *Mugil cephalus*	41.6	41.6	41.6	41.9	42.3	42.3	42.4	42.3	42.1	42.1	41.3	40.5	41.5	41.5	41.5	42.0	41.1	41.1	41.1	41.5	41.5	41.5		
24	JN996855 *Ligophorus macrocolpos* ex *Liza saliens*	42.5	42.5	42.5	42.8	42.9	42.9	43.1	42.9	42.8	42.6	42.1	41.2	42.3	41.7	41.5	42.5	42.0	42.0	42.0	42.3	42.3	42.3	90.0	
25	JN996858 *Ligophorus llewellyni* ex *Liza haematocheilus*	41.4	41.4	41.4	41.4	42.2	42.2	42.4	42.2	42.1	41.9	41.1	40.5	41.4	41.3	41.3	42.0	40.9	40.9	40.9	40.8	40.8	40.8	93.5	90.1

Table S8. *P*-values from pairwise multiple comparisons of different herbal treatments against the egg hatching rate of *T. vistulensis*.

		GAR				GIN				NMB		
		0:1	**1:100**	**1:50**	**1:10**	**0:1**	**1:100**	**1:50**	**1:10**	**0:1**	**1:100**	**1:50**
GAR	**1:100**	7.4E-26	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	1:50	7.4E-26	1.0000	NA	NA	NA	NA	NA	NA	NA	NA	NA
	1:10	7.4E-26	1.0000	1.0000	NA	NA	NA	NA	NA	NA	NA	NA
GIN	**0:1**	1.0000	1.14E-25	1.14E-25	1.14E-25	NA	NA	NA	NA	NA	NA	NA
	1:100	0.0679	8.4E-20	8.4E-20	8.4E-20	0.0643	NA	NA	NA	NA	NA	NA
	1:50	0.01391	2.23E-19	2.23E-19	2.23E-19	0.0087	0.6651	NA	NA	NA	NA	NA
	1:10	7.4E-26	1.0000	1.0000	1.0000	1.14E-25	8.4E-20	2.23E-19	NA	NA	NA	NA
NMB	**0:1**	1.0000	1.89E-24	1.89E-24	1.89E-24	0.9434	0.0544	0.0095	1.89E-24	NA	NA	NA
	1:100	0.1024	1.31E-18	1.31E-18	1.31E-18	0.1442	1.0000	0.5832	1.31E-18	0.1065	NA	NA
	1:50	6.86E-05	3.1E-16	3.1E-16	3.1E-16	2.17E-05	0.0533	0.1214	3.1E-16	0.0002	0.1024	NA
	1:10	7.4E-26	1.0000	1.0000	1.0000	1.14E-25	8.4E-20	2.23E-19	1.0000	1.89E-24	1.31E-18	3.1E-16

Table S9. *P*-values from pairwise multiple comparisons of different drug treatments against the survival rate of *T. vistulensis* oncomiracidia.

		GAR				GIN				NMB		
		0:1	**1:100**	**1:50**	**1:10**	**0:1**	**1:100**	**1:50**	**1:10**	**0:1**	**1:100**	**1:50**
GAR	**1:100**	3.48E-22	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	1:50	2.9E-23	3.99E-23	NA	NA	NA	NA	NA	NA	NA	NA	NA
	1:10	3.48E-22	3.48E-22	1.29E-12	NA	NA	NA	NA	NA	NA	NA	NA
GIN	**0:1**	1.93E-05	3.48E-22	2.9E-23	3.48E-22	NA	NA	NA	NA	NA	NA	NA
	1:100	0.1317	3.48E-22	2.9E-23	3.48E-22	7.98E-13	NA	NA	NA	NA	NA	NA
	1:50	7.56E-08	3.48E-22	2.9E-23	3.48E-22	2.29E-19	3.11E-10	NA	NA	NA	NA	NA
	1:10	1.88E-20	1.75E-20	2.9E-23	3.48E-22	1.88E-20	1.88E-20	7.8E-17	NA	NA	NA	NA
NMB	**0:1**	0.0119	3.48E-22	2.9E-23	3.48E-22	0.0629	4.94E-07	3.11E-14	1.16E-19	NA	NA	NA
	1:100	2.62E-23	2.62E-23	0.8973	2.72E-17	2.62E-23	2.62E-23	2.62E-23	2.62E-23	2.62E-23	NA	NA
	1:50	3.48E-22	3.48E-22	1.36E-12	0.8205	3.48E-22	3.48E-22	3.48E-22	3.48E-22	3.48E-22	2.5E-17	NA
	1:10	6.2E-21	6.2E-21	6.2E-21	8.13E-18	6.2E-21	6.2E-21	6.2E-21	6.2E-21	6.2E-21	6.2E-21	1.48E-18

Table S10. *P*-values from pairwise multiple comparisons of different drug treatments on the survival rate of juvenile *T. vistulensis*.

		GAR				GIN				NMB		
		0:1	**1:100**	**1:50**	**1:10**	**0:1**	**1:100**	**1:50**	**1:10**	**0:1**	**1:100**	**1:50**
GAR	**1:100**	3.48E-22	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	1:50	2.9E-23	3.99E-23	NA	NA	NA	NA	NA	NA	NA	NA	NA
	1:10	3.48E-22	3.48E-22	1.29E-12	NA	NA	NA	NA	NA	NA	NA	NA
GIN	**0:1**	1.93E-05	3.48E-22	2.9E-23	3.48E-22	NA	NA	NA	NA	NA	NA	NA
	1:100	0.1317	3.48E-22	2.9E-23	3.48E-22	7.98E-13	NA	NA	NA	NA	NA	NA
	1:50	7.56E-08	3.48E-22	2.9E-23	3.48E-22	2.29E-19	3.11E-10	NA	NA	NA	NA	NA
	1:10	1.88E-20	1.75E-20	2.9E-23	3.48E-22	1.88E-20	1.88E-20	7.8E-17	NA	NA	NA	NA
NMB	**0:1**	0.0119	3.48E-22	2.9E-23	3.48E-22	0.0629	4.94E-07	3.11E-14	1.16E-19	NA	NA	NA
	1:100	2.62E-23	2.62E-23	0.8973	2.72E-17	2.62E-23	2.62E-23	2.62E-23	2.62E-23	2.62E-23	NA	NA
	1:50	3.48E-22	3.48E-22	1.36E-12	0.8205	3.48E-22	3.48E-22	3.48E-22	3.48E-22	3.48E-22	2.5E-17	NA
	1:10	6.2E-21	6.2E-21	6.2E-21	8.13E-18	6.2E-21	6.2E-21	6.2E-21	6.2E-21	6.2E-21	6.2E-21	1.48E-18

Table S11. *P*-values from pairwise multiple comparisons of different drug treatments on the survival rate of adult *T. vistulensis*.

		GAR				GIN				NMB		
		0:1	**1:100**	**1:50**	**1:10**	**0:1**	**1:100**	**1:50**	**1:10**	**0:1**	**1:100**	**1:50**
GAR	**1:100**	3.48E-22	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	1:50	2.9E-23	3.99E-23	NA	NA	NA	NA	NA	NA	NA	NA	NA
	1:10	3.48E-22	3.48E-22	1.29E-12	NA	NA	NA	NA	NA	NA	NA	NA
GIN	**0:1**	1.93E-05	3.48E-22	2.9E-23	3.48E-22	NA	NA	NA	NA	NA	NA	NA
	1:100	0.1317	3.48E-22	2.9E-23	3.48E-22	7.98E-13	NA	NA	NA	NA	NA	NA
	1:50	7.56E-08	3.48E-22	2.9E-23	3.48E-22	2.29E-19	3.11E-10	NA	NA	NA	NA	NA
	1:10	1.88E-20	1.75E-20	2.9E-23	3.48E-22	1.88E-20	1.88E-20	7.8E-17	NA	NA	NA	NA
NMB	**0:1**	0.0119	3.48E-22	2.9E-23	3.48E-22	0.0629	4.94E-07	3.11E-14	1.16E-19	NA	NA	NA
	1:100	2.62E-23	2.62E-23	0.8973	2.72E-17	2.62E-23	2.62E-23	2.62E-23	2.62E-23	2.62E-23	NA	NA
	1:50	3.48E-22	3.48E-22	1.36E-12	0.8205	3.48E-22	3.48E-22	3.48E-22	3.48E-22	3.48E-22	2.5E-17	NA
	1:10	6.2E-21	6.2E-21	6.2E-21	8.13E-18	6.2E-21	6.2E-21	6.2E-21	6.2E-21	6.2E-21	6.2E-21	1.48E-18

Table S12. *P*-values from pairwise multiple comparisons of different drug treatments against the egg hatching rate of *T. vistulensis*.

		BIO (mg/L)									MBZ (mg/L)									PZQ (mg/L)							
		0	1	5	10	20	40	60	80	100	0	1	5	10	20	40	60	80	100	0	1	5	10	20	40	60	80
BIO (mg/L)	1	0.3110	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	5	0.6887	0.1247	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	10	0.9446	0.2172	0.8139	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	20	0.0502	0.4191	0.0131	0.0288	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	40	0.1405	0.7569	0.0446	0.0889	0.6912	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	60	0.9724	0.3836	0.5861	0.8166	0.0685	0.1874	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	80	0.5027	0.0687	0.8757	0.6141	0.0058	0.0228	0.4148	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	100	0.7153	0.5455	0.3592	0.5708	0.1131	0.2918	0.8447	0.2327	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
MBZ (mg/L)	0	0.4603	0.0632	0.8149	0.5708	0.0054	0.0210	0.3859	1.0000	0.2168	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	1	3.34E-27	4.81E-27	5.37E-27	7.52E-28	1.3E-25	4.82E-26	4.97E-28	7.52E-28	7.52E-28	5.4E-28	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	5	3.34E-27	4.81E-27	5.37E-27	7.52E-28	1.3E-25	4.82E-26	4.97E-28	7.52E-28	7.52E-28	5.4E-28	1.0000	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	10	3.34E-27	4.81E-27	5.37E-27	7.52E-28	1.3E-25	4.82E-26	4.97E-28	7.52E-28	7.52E-28	5.4E-28	1.0000	1.0000	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	20	3.34E-27	4.81E-27	5.37E-27	7.52E-28	1.3E-25	4.82E-26	4.97E-28	7.52E-28	7.52E-28	5.4E-28	1.0000	1.0000	1.0000	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	40	3.34E-27	4.81E-27	5.37E-27	7.52E-28	1.3E-25	4.82E-26	4.97E-28	7.52E-28	7.52E-28	5.4E-28	1.0000	1.0000	1.0000	1.0000	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	60	3.34E-27	4.81E-27	5.37E-27	7.52E-28	1.3E-25	4.82E-26	4.97E-28	7.52E-28	7.52E-28	5.4E-28	1.0000	1.0000	1.0000	1.0000	1.0000	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	80	3.34E-27	4.81E-27	5.37E-27	7.52E-28	1.3E-25	4.82E-26	4.97E-28	7.52E-28	7.52E-28	5.4E-28	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	100	3.34E-27	4.81E-27	5.37E-27	7.52E-28	1.3E-25	4.82E-26	4.97E-28	7.52E-28	7.52E-28	5.4E-28	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	NA	NA	NA	NA	NA	NA	NA	NA	NA
PZQ (mg/L)	0	0.2158	0.0202	0.4590	0.2854	0.0012	0.0054	0.1724	0.6341	0.0833	0.6912	7.88E-29	7.88E-29	7.88E-29	7.88E-29	7.88E-29	7.88E-29	7.88E-29	7.88E-29	NA	NA	NA	NA	NA	NA	NA	NA
	1	0.1681	0.0107	0.4091	0.2174	0.0004	0.0023	0.1210	0.5708	0.0499	0.6341	4.97E-28	4.97E-28	4.97E-28	4.97E-28	4.97E-28	4.97E-28	4.97E-28	4.97E-28	1.0000	NA	NA	NA	NA	NA	NA	NA
	5	0.2969	0.0448	0.5452	0.3764	0.0040	0.0136	0.2705	0.7328	0.1350	0.8143	5.77E-27	5.77E-27	5.77E-27	5.77E-27	5.77E-27	5.77E-27	5.77E-27	5.77E-27	0.9531	0.9866	NA	NA	NA	NA	NA	NA
	10	0.9724	0.4696	0.6388	0.8321	0.1117	0.2458	1.0000	0.4603	0.8817	0.4148	7E-26	7E-26	7E-26	7E-26	7E-26	7E-26	7E-26	7E-26	0.1874	0.1707	0.2918	NA	NA	NA	NA	NA
	20	9.09E-05	0.0019	2.4E-05	2.39E-05	0.0172	0.0049	8.3E-05	6.71E-06	8.34E-05	7.97E-06	7.48E-24	7.48E-24	7.48E-24	7.48E-24	7.48E-24	7.48E-24	7.48E-24	7.48E-24	1.65E-06	2.69E-07	8.12E-05	0.0019	NA	NA	NA	NA
	40	7.03E-08	2.5E-06	1.78E-08	1.11E-08	4.81E-05	7.98E-06	4.87E-08	3.59E-09	3.42E-08	5.03E-09	1.74E-21	1.74E-21	1.74E-21	1.74E-21	1.74E-21	1.74E-21	1.74E-21	1.74E-21	9.38E-10	8.81E-11	2.66E-07	7.59E-06	0.1519	NA	NA	NA
	60	1.98E-10	9.41E-09	4.99E-11	2.18E-11	2.8E-07	3.42E-08	1.11E-10	7.99E-12	5.77E-11	1.26E-11	7.74E-20	7.74E-20	7.74E-20	7.74E-20	7.74E-20	7.74E-20	7.74E-20	7.74E-20	2.2E-12	1.36E-13	2.14E-09	6.93E-08	0.0147	0.3847	NA	NA
	80	3.15E-25	1.09E-24	4.19E-25	5.81E-26	5.19E-23	1.17E-23	2.9E-26	4.82E-26	5.27E-26	2.96E-26	0.0350	0.0350	0.0350	0.0350	0.0350	0.0350	0.0350	0.0350	2.42E-27	8.9E-27	1.34E-24	2.36E-23	2.32E-20	7.57E-18	4.27E-16	NA
	100	6.49E-26	1.76E-25	8.62E-26	1.14E-26	8.41E-24	2.06E-24	5.37E-27	9.38E-27	9.69E-27	5.77E-27	0.0616	0.0616	0.0616	0.0616	0.0616	0.0616	0.0616	0.0616	7.17E-28	2.7E-27	2.55E-25	4.53E-24	2.71E-21	7.85E-19	4.3E-17	0.8149

Table S13. *P*-values from pairwise multiple comparisons of different drug treatments against the survival rate of *T. vistulensis* oncomiracidia.

		BIO (mg/L)									MBZ (mg/L)									PZQ (mg/L)							
		0	**1**	**5**	**10**	**20**	**40**	**60**	**80**	**100**	**0**	**1**	**5**	**10**	**20**	**40**	**60**	**80**	**100**	**0**	**1**	**5**	**10**	**20**	**40**	**60**	**80**
BIO (mg/L)	**1**	0.4801	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	5	0.4027	0.7179	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	10	0.4088	0.1356	0.1598	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	20	6.28E-19	1.14E-20	1.15E-16	1.52E-16	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	40	2.3E-23	2.3E-23	2.3E-23	4.72E-23	2.3E-23	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	60	2.3E-23	2.3E-23	2.3E-23	2.3E-23	2.3E-23	0.0553	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	80	1.27E-23	1.27E-23	1.27E-23	1.27E-23	1.27E-23	1.36E-08	3.11E-06	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	100	2.3E-23	2.3E-23	2.3E-23	2.3E-23	2.3E-23	1.45E-11	1.77E-11	0.0777	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
MBZ (mg/L)	**0**	3.68E-05	0.0002	0.0029	6.61E-06	3.73E-21	2.3E-23	2.3E-23	1.27E-23	2.3E-23	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	1	0.0501	0.2029	0.5471	0.0073	5.87E-22	2.3E-23	2.3E-23	1.27E-23	2.3E-23	0.0038	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	5	0.4714	0.9890	0.6651	0.1203	1.5E-21	2.3E-23	2.3E-23	1.27E-23	2.3E-23	6.48E-05	0.1667	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	10	0.7494	0.6369	0.4598	0.2267	1.5E-21	2.3E-23	2.3E-23	1.27E-23	2.3E-23	1.75E-05	0.0592	0.6196	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	20	0.1206	0.0094	0.0319	0.7014	3.19E-20	2.3E-23	2.3E-23	1.27E-23	2.3E-23	2.04E-08	3.93E-05	0.0047	0.0180	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	40	5.37E-10	4.73E-12	2.39E-05	9.97E-06	1.32E-19	2.3E-23	2.3E-23	1.27E-23	2.3E-23	2.33E-14	3.84E-17	3.61E-14	3.61E-14	5.03E-10	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	60	3.98E-10	3.23E-12	1.38E-05	5.75E-06	4.45E-19	2.3E-23	2.3E-23	1.27E-23	2.3E-23	1.91E-14	4.31E-17	3.34E-14	3.34E-14	3.01E-10	0.5288	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	80	1.84E-10	1.25E-12	4.32E-06	1.8E-06	3.44E-18	2.3E-23	2.3E-23	1.27E-23	2.3E-23	9.68E-15	3.81E-17	2.08E-14	2.08E-14	9.61E-11	0.0955	0.2964	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	100	2.78E-10	2.06E-12	7.69E-06	3.24E-06	1.27E-18	2.3E-23	2.3E-23	1.27E-23	2.3E-23	1.42E-14	4.28E-17	2.74E-14	2.74E-14	1.75E-10	0.2385	0.5864	0.6196	NA	NA	NA	NA	NA	NA	NA	NA	NA
PZQ (mg/L)	**0**	1.6E-05	6.98E-05	0.0015	2.92E-06	3.73E-21	2.3E-23	2.3E-23	1.27E-23	2.3E-23	0.8140	0.0017	2.51E-05	6.76E-06	7.91E-09	2.33E-14	1.91E-14	9.68E-15	1.42E-14	NA	NA	NA	NA	NA	NA	NA	NA
	1	0.0004	1.61E-05	0.0004	0.0105	6.15E-16	2.3E-23	2.3E-23	1.27E-23	2.3E-23	1.47E-10	5.03E-08	4.63E-06	1.48E-05	0.0044	0.4368	0.2961	0.1203	0.1915	6.49E-11	NA	NA	NA	NA	NA	NA	NA
	5	0.0005	2.39E-05	0.0005	0.0113	8.65E-17	2.3E-23	2.3E-23	1.27E-23	2.3E-23	2.18E-10	9.57E-08	7.09E-06	2.18E-05	0.0056	0.8003	0.5934	0.2778	0.4169	9.6E-11	0.9806	NA	NA	NA	NA	NA	NA
	10	4.09E-07	5.1E-09	4.23E-06	5.62E-05	1.69E-15	2.3E-23	2.3E-23	1.27E-23	2.3E-23	1.56E-13	5.3E-12	6.17E-10	1.65E-09	1.21E-06	0.0903	0.1792	0.4923	0.3140	7.78E-14	0.1075	0.1216	NA	NA	NA	NA	NA
	20	5.96E-09	3.76E-11	1.74E-07	2.08E-06	4.25E-14	2.3E-23	2.3E-23	1.27E-23	2.3E-23	2.61E-15	2.33E-14	3.64E-12	8.82E-12	6.74E-09	0.0122	0.0320	0.1403	0.0720	1.43E-15	0.0128	0.0149	0.3766	NA	NA	NA	NA
	40	9.8E-10	8.58E-12	3.54E-07	1.33E-06	5.57E-14	2.3E-23	2.3E-23	1.27E-23	2.3E-23	3.95E-15	1.85E-15	4.77E-13	8.24E-13	9.14E-10	0.0364	0.0955	0.3645	0.2006	2.75E-15	0.0244	0.0422	0.6492	0.7098	NA	NA	NA
	60	4.34E-15	2.62E-17	4E-11	6.87E-12	2.49E-09	2.3E-23	2.3E-23	1.27E-23	2.3E-23	9.49E-19	1.71E-20	6.62E-19	6.62E-19	5.14E-16	1.32E-12	1.71E-11	1.46E-09	1.77E-10	9.49E-19	1.57E-07	2.39E-07	3.25E-05	0.0006	2.78E-05	NA	NA
	80	2.97E-17	3.44E-18	2.71E-13	8.15E-15	2.59E-05	2.3E-23	2.3E-23	1.27E-23	2.3E-23	9.43E-19	1.2E-20	1.2E-20	1.2E-20	1.24E-17	3.44E-18	4.06E-17	2.96E-15	3.82E-16	9.43E-19	1.35E-11	9.47E-12	7.16E-10	3.8E-08	6.43E-10	0.0029	NA
	100	5.49E-20	5.67E-21	1.4E-16	2.1E-17	0.1832	2.3E-23	2.3E-23	1.27E-23	2.3E-23	2.01E-21	5.57E-23	5.57E-23	5.57E-23	1.5E-20	5.67E-21	3.82E-20	1E-18	2.2E-19	2.01E-21	1.24E-15	3.01E-16	8.4E-15	3.29E-13	7.59E-14	3.6E-08	0.0006

Table S14. *P*-values from pairwise multiple comparisons of different drug treatments on the survival rate of juvenile *T. vistulensis*.

		BIO (mg/L)									MBZ (mg/L)									PZQ (mg/L)							
		0	**1**	**5**	**10**	**20**	**40**	**60**	**80**	**100**	**0**	**1**	**5**	**10**	**20**	**40**	**60**	**80**	**100**	**0**	**1**	**5**	**10**	**20**	**40**	**60**	**80**
BIO (mg/L)	**1**	0.4829	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	5	0.2140	0.7189	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	10	0.5025	0.9505	0.6228	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	20	0.1415	0.5948	0.8818	0.4913	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	40	0.2431	0.7801	0.9303	0.6996	0.7872	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	60	1.31E-07	6.68E-06	7.33E-06	2E-06	1.1E-05	3.16E-06	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	80	4.15E-10	1.14E-08	7.04E-09	3.85E-09	8.16E-09	2.94E-09	0.3156	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	100	3.56E-11	7.22E-10	4.27E-10	3.27E-10	4.64E-10	2.18E-10	0.0191	0.1294	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
MBZ (mg/L)	**0**	0.9473	0.4267	0.1625	0.4459	0.1016	0.1872	5.09E-08	1.67E-10	2.98E-11	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	1	0.9663	0.5110	0.2439	0.5547	0.1693	0.2823	1.27E-07	2.63E-10	2.98E-11	0.9075	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	5	0.7083	0.7350	0.4055	0.7889	0.2979	0.4544	1.93E-07	3.02E-10	2.98E-11	0.6290	0.7538	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	10	0.7370	0.7219	0.4106	0.7788	0.3032	0.4613	2.78E-07	3.27E-10	2.98E-11	0.6764	0.7845	0.9950	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	20	0.5812	0.8428	0.5025	0.9152	0.3861	0.5749	2.83E-07	3.27E-10	2.98E-11	0.4983	0.6387	0.8852	0.8780	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	40	0.2314	0.8030	0.8855	0.7033	0.7350	0.9663	6.15E-07	4.17E-10	2.98E-11	0.1705	0.2778	0.4544	0.4708	0.5850	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	60	0.0961	0.5749	0.8705	0.4459	0.9950	0.7644	1.2E-06	4.7E-10	2.98E-11	0.0619	0.1262	0.2373	0.2625	0.3213	0.6996	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	80	0.0961	0.5749	0.8705	0.4459	0.9950	0.7644	1.2E-06	4.7E-10	2.98E-11	0.0619	0.1262	0.2373	0.2625	0.3213	0.6996	1.0000	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	100	0.0187	0.2505	0.4267	0.1557	0.5460	0.3220	1.75E-05	5.06E-09	2.88E-10	0.0100	0.0253	0.0543	0.0678	0.0889	0.2461	0.4544	0.4544	NA	NA	NA	NA	NA	NA	NA	NA	NA
PZQ (mg/L)	**0**	0.5101	0.9152	0.6134	0.9950	0.4829	0.6996	5.94E-07	4.17E-10	2.98E-11	0.4453	0.5663	0.7845	0.7845	0.9113	0.7244	0.4544	0.4544	0.1528	NA	NA	NA	NA	NA	NA	NA	NA
	1	0.3620	0.9303	0.8074	0.8258	0.6996	0.9003	2.35E-06	1.39E-09	8.67E-11	0.2979	0.4051	0.5935	0.5977	0.7132	0.9473	0.6996	0.6996	0.2985	0.8030	NA	NA	NA	NA	NA	NA	NA
	5	0.1294	0.6387	0.9113	0.4949	0.9663	0.8030	1.61E-06	5E-10	2.98E-11	0.0882	0.1614	0.2821	0.3032	0.3744	0.7302	0.9950	0.9950	0.4704	0.4983	0.7350	NA	NA	NA	NA	NA	NA
	10	0.0145	0.1702	0.2979	0.1073	0.3842	0.2314	0.0003	2.58E-07	6.11E-09	0.0085	0.0186	0.0387	0.0442	0.0596	0.1669	0.3032	0.3032	0.7350	0.0945	0.1872	0.3032	NA	NA	NA	NA	NA
	20	0.0067	0.1872	0.3158	0.0911	0.4459	0.2314	3.44E-06	5.32E-10	2.98E-11	0.0028	0.0098	0.0217	0.0325	0.0392	0.1383	0.3156	0.3156	0.9473	0.0911	0.2314	0.3646	0.7370	NA	NA	NA	NA
	40	0.0067	0.1872	0.3158	0.0911	0.4459	0.2314	3.44E-06	5.32E-10	2.98E-11	0.0028	0.0098	0.0217	0.0325	0.0392	0.1383	0.3156	0.3156	0.9473	0.0911	0.2314	0.3646	0.7370	1.0000	NA	NA	NA
	60	2.11E-05	0.0044	0.0067	0.0009	0.0119	0.0026	0.0005	5.34E-08	1.44E-09	5.11E-06	2.42E-05	5.13E-05	0.0001	0.0001	0.0004	0.0014	0.0014	0.0426	0.0004	0.0029	0.0026	0.2821	0.0128	0.0128	NA	NA
	80	3.1E-09	2.54E-07	1.72E-07	6E-08	2.32E-07	5.34E-08	0.8119	0.0392	0.0002	9.96E-10	2.05E-09	2.83E-09	3.39E-09	3.39E-09	5.13E-09	7.07E-09	7.07E-09	1.61E-07	5.13E-09	2.45E-08	8.16E-09	1.33E-05	1.01E-08	1.01E-08	3.45E-06	NA
	100	3.1E-09	1.31E-07	7.97E-08	3.76E-08	9.9E-08	2.74E-08	0.6996	0.5193	0.0251	1.13E-09	1.95E-09	2.39E-09	2.86E-09	2.86E-09	3.59E-09	4.58E-09	4.58E-09	6.03E-08	3.69E-09	1.21E-08	5.13E-09	3.19E-06	5.87E-09	5.87E-09	8.27E-07	0.2314

Table S15. Post hoc Dunn's test for pairwise comparisons of treatments at different concentrations and time points of observations, using Kruskal–Wallis H statistical tests. Significant differences *P*-values are highlighted.

Treatments	**Variables**	**Comparison**	***P*-value***
PZQ	Concentrations	10 mg/L – 5 mg/L	**0.001**
		10 mg/L – 1 mg/L	**0.001**
		5 mg/L – 1 mg/L	0.109
	Time point (Day post-treatment)	14 DPT – 7 DPT	**0.001**
		14 DPT – 1 DPT	**0.001**
		7 DPT – 1 DPT	1.000
MBZ	Concentrations	10 mg/L – 5 mg/L	0.062
		10 mg/L – 1 mg/L	**0.031**
		5 mg/L – 1 mg/L	1.000
	Time point (Day post-treatment)	14 DPT – 7 DPT	**0.001**
		14 DPT – 1 DPT	**0.001**
		7 DPT – 1 DPT	0.993

* Significance value $P < 0.05$

Table S16. Statistical analysis of the effects of treatments Praziquantel (PZQ) and Mebendazole (MBZ) against the number of parasite counts at different days post-treatment (DPT). Results include overall comparisons using Kruskal–Wallis or One–Way ANOVA, pairwise comparisons using Mann–Whitney U or independent t-tests where applicable, and post-hoc tests (Dunn’s or Tukey’s HSD) to identify significant differences between groups. Significant *P*-values are highlighted, indicating differences between treatments.

Treatments	DPT	Comparison (mg/L)	Statistical Test	Test Statistic (Value)	*P*-value*	Post Hoc		
						Test	Comparison	*P*-value*
PZQ	1	1 vs 10	Mann–Whitney	U = 0.000	**0.002**	-	-	-
		5 vs 10	Mann–Whitney	U = 0.000	**0.002**	-	-	-
		1 vs 5	Independent t-test	t(10) = 4.695	**0.001**	-	-	-
	7	1 vs 10	Mann–Whitney	U = 0.000	**0.002**	-	-	-
		5 vs 10	Mann–Whitney	U = 0.000	**0.002**	-	-	-
		1 vs 5	Independent t-test	t(10) = 4.245	**0.002**	-	-	-
	14	1 vs 10	Mann–Whitney	U = 0.000	**0.002**	-	-	-
		5 vs 10	Mann–Whitney	U = 0.000	**0.002**	-	-	-
		1 vs 5	Independent t-test	t(10) = 8.698	**0.001**	-	-	-
MBZ	1	1 vs 5 vs 10	One–Way ANOVA	F(2, 15) = 1.097	0.359	-	-	-
	7	1 vs 5 vs 10	One–Way ANOVA	F(2, 15) = 16.687	**0.001**	Tukey's HSD	1 vs 5	0.979
							1 vs 10	**0.001**
							5 vs 10	**0.001**
	14	1 vs 5 vs 10	Kruskal–Wallis	H = 12.697	**0.002**	Dunn's Test	1 vs 5	0.224
							1 vs 10	**0.001**
							5 vs 10	0.224

* Significance value *P* < 0.05

Table S17. List of freshwater monopisthocotylean parasites with long penis and coils based on published drawings.

Species	Total Length (µm)	Total Coils	References
Genus *Thaparocleidus* Jain 1952			
T. armillatus Verma, Chaudhary and Singh, 2017	82–89	1.5	Verma et al. (2017)
T. devraji Gussev, 1976	93–133	1.5	Rajvanshi et al. (2014)
T. magnus Bychowsky and Nagibina, 1957	1400–1600	*	Bychowsky and Nagibina (1957)
T. malabaricus Gussev, 1976	*	3.5	Pandey et al. (2003)
T. seenghali Jain, 1961	*	2–3	Pandey et al. (2003)
T. siluri Zandt, 1924	390–420	2–3	Bychowsky and Nagibina (1957)
T. susanae Rajvanshi & Agrawal, 2013	193–198	3	Rajvanshi and Agrawal (2013)
T. wallagonius Jain, 1952	*	3–4	Verma et al. (2017)
Genus *Demidospermus* Suriano, 1983			
D. spirophallus Franceschini, Zago, Müller, Francisco, Takemoto & da Silva, 2017	193–230	2.5	Franceschini et al. (2018)
D. prolixus Franceschini, Zago, Müller, Francisco, Takemoto & da Silva, 2017	210–234	1.5	Franceschini et al. (2018)
D. anus Suriano, 1983	143–156	1–1.5	Franceschini et al. (2018)
Genus *Mastacembelocleidus* Kritsky, Pandey, Agrawal & Abdullah, 2004			
M. bam Tripathi, 1959	*	2	Kritsky et al. (2004)
M. heteranchorus Kulkarni, 1969	*	2	Kritsky et al. (2004)
Genus *Dactylogyrus* Diesing, 1850			
D. nasutai Narba, Matey, Agarwal & Tripathi, 2022	*	27	Narba et al. (2022)
D. pulcher Bychowsky, 1957	250	6–7	Pugachev et al. (2010)
D. simplicimalleata Bychowsky, 1931	340	*	Pugachev et al. (2010)
D. wuhuensis Lee, 1960	155–185	2.5–3	Pugachev et al. (2010)
D. falciformis Akhmerov, 1952	190–220	3	Pugachev et al. (2010)
D. procypris Ma, Li & Wang, 1981	360	*	Wu et al. (2000)

D. longivagina Zhang & Pan, 1988	410–610	6–8	Wu et al. (2000)
D. pseudoflagillicirrus Long, 1964	300	*	Wu et al. (2000)
D. luciosomis Zhang & Guo, 1981	80–140	*	Wu et al. (2000)
D. sphyrna Linstow, 1878	90–98	*	Wu et al. (2000)
D. onychocirrus Long, 1981	88 - 110	*	Wu et al. (2000)
D. lingualis Long, 1981	114–125	3	Wu et al. (2000)
D. rhychoideus Long, 1981	122	2	Wu et al. (2000)
D. spirovagina Long, 1981	71	2	Wu et al. (2000)
D. longquanensis Wu & Wang, 1983	199–282	1–2	Wu et al. (2000)
D. quadricurvitubus Zhang & Guo, 1982	165–235	4–7	Wu et al. (2000)
D. austrosinensis Zhang & Li, 1991	190–207	2	Wu et al. (2000)
D. strombus Tao & Long, 1981	340–660	4–11	Wu et al. (2000)
D. daojiensis Luo & Long, 1982	376	*	Wu et al. (2000)
D. pectinate Zhao & Ma, 1991	174–177	2	Wu et al. (2000)
D. ehrenbergii Yao & Wang, 1997	149–210	2	Wu et al. (2000)
D. garrae Ma & Long, 2000	167–185	7	Wu et al. (2000)
D. lianshanensis Ma & Long, 2000	120–207	*	Wu et al. (2000)
D. helicoides Yao & Wang, 1997	376–1455	4–10	Wu et al. (2000)
Genus *Pseudacolpenteron* Bychowsky & Gussev, 1955			
P. ignotus Gussev, 1955	190	2	Pugachev et al. (2010)
Genus *Ancyrocephalus* Creplin, 1839			
A. subaequalis Akhmerov, 1952	130–170	*	Pugachev et al. (2010)
A. pavlovskyi Gussev, 1955	140–160	*	Pugachev et al. (2010)
A. brevifilis Yao & Wang, 1997	248–348	*	Wu et al. (2000)
Genus *Dogielius* Bychowsky, 1936			
D. strombicinms Ma & Long, 2000	357	7	Wu et al. (2000)
Genus *Pseudancylodiscoides* Yamaguti, 1963			
P. panduriformis Zhang & Ma, 1997	116–149	1.5	Wu et al. (2000)

*Data not provided

Table S18. Aquaculture disease-causing monogeneans and their reproductive strategies.

Subclasses / Species	Host	Macro Habitat	Micro Habitat	Hatching time		Oncomiracidia longevity		Reach sexual maturation		Reference
				dpo	°C	dph	°C	dpi	°C	
Monopisthocotyleans										
Thaparocleidus vistulensis	European catfish (*Silurus glanis*)	Freshwater	Gills	3-5	23±1	5	23±1	8	23±1	Present study
T. vistulensis	European catfish (*Silurus glanis*)	Freshwater	Gills	2.5-3 6-6.5	20-25 15-17	1-1.5	20-21	-	20-23	Molnár (1968)
Pseudodactylogyrus bini	European eel (*Anguilla anguilla*)	Fresh and Brackish water	Gills	3-6	20-25	5-6h	19-26	-	-	Chan and Wu (1984), Buchmann (1988b)
P. anguillae	European eel (*Anguilla anguilla*)	Fresh and Brackish water	Gills	2-4.5	20-25	3-5h	20-25	7-9	25-28	Golovin and Shukhgalter (1979) Buchmann (1990)
Diplectanum aequans	Sea bass (*Dicentrarchus labrax*)	Marine water	Gills	3-6	20-25	-	-	-	-	Cecchini (1994)
Benedenia seriolae	Japanese Yellowtail (*Seriola quinqueradiata*)	Marine water	Skin, fins	5	23	1	-	14	22	Kearn et al. (1992b)
Neobenedenia girellae	Japanese flounder (*Paralichthys olivaceus*)	Marine water	Young: Body, skin, fins. Adult: Skin, mouth, eye regions	5-6	25	-	-	10-11	25	Bondad-Reantaso et al. (1995)
Neobenedenia sp.	Barramundi (*Lates calcarifer*)	Marine water	Skin	-	-	37h	25	-	-	Militz et al. (2014)
Dactylogyrus extensus	Common carp (*Cyprinus carpio*)	Freshwater	Gills	3	22-25	1-2	25	6-7	24-25	Prost (1963) Turgut (2012)
D. aristichthys	Bighead carp (*Hypophthalmichthys nobilis*)	Freshwater	Gills fillaments	2	30	2-11h	17-23	11-13	17-23	Musselius (1968)

D. vastator	Common carp Goldfish	Freshwater	Gills	2-3	24-28	<1	24-28	10	24-28	Bauer et al. (1973)
Dawestrema cycloancistrium	Piracucu (*Arapaima gigas*)	Freshwater	Gills, skin	3-4	28-32	50-58h	24-27	-	-	Maciel et al. (2017)
Polyopisthocotyleans										
Heteraxine heterocerca	Japanese Yellowtail (*Seriola quinqueradiata*)	Marine water	Gills	5	23	-	-	-	-	Kearn et al. (1992a)
Discocotyle sagittata	Rainbow trout (*Oncorhynchus mykiss*)	Freshwater	Gills, mainly secondary gill lamellae	20	18	26h	22	-	-	Gannicott and Tinsley (1998a), (1998b)
Heterobothrium okamotoi	Tiger puffer (*Takifugu rubripes*)	Marine and Brackish water	Branchial cavity wall, gill fillaments	5.3-11.8	15-25	4-9	15-25	-	-	Ogawa (1998)
H. ecuadori	Bullseye pufferfish (*Sphoeroides annulatus*)	Marine water	Gills	7-10	23±1	4-7	21±1	-	-	Grano-Maldonado et al. (2011), (2015)

dpo, days post-oviposition; dph, days post-hatching; dpi, days post-infection

Table S19. Estimated duration for parasite clearance in the system in relation to temperature variation.

Temperature (°C)	Egg hatching (day)	Life span of oncomiracidia after hatching (day)	Estimated parasite clearance interval in water system (day)*
5	Ø	<1	Ø
10	12–19	<9	>28
15	5–9	<7	>16
20	3–5	<7	>12
25	2–4	<4	>8
30	2–3	<3	>6
35	Ø	<1	Ø

* Water can be considered free of *T. vistulensis* after these intervals

Ø data cannot be interpret