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Parasitic halacarid mites (Acari, Halacaridae): survey on literature

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Keywords Parasitism, Halacaridae, Aquatic Mites, Host

Abstract The article deals with a survey on literature of parasitic halacarid mites. Exact nature of relationship between mites and their invertebrate hosts is unknown for most of these associations, as live observations not found. Some species are assumed to be parasitic.

Pasożytniczne wodopójki z rodziny Halacaridae: przegląd literatury

Słowa kluczowe pasożytnictwo, Halacaridae, wodopójki, gospodarz

Streszczenie Artukuł zawiera przegląd literatury dotyczącej pasożytniczych wodopójek z rodziny Halacaridae. W większości przypadków pasożytniczych wodopójek i ich gospodarzy do dzisiaj nie jest znany dokładny charakter interakcji, ponieważ brak obserwacji osobników żywych. W przypadku części gatunków nie jest jasne nawet czy są pasożytami zwierząt bezkręgowych na których zostały znalezione.

Introduction

Halacarid mites (Acari, Halacaridae) live in a variety of substrata – within tufts of algae, on large fronds, in and on colonies of sponges, hydrozoans, bryozoans, barnacles, mussels, polychaetes, crustaceans etc. to sediments, flocculent rock crevices and coarse and fine sands (Chatterjee, Sarma 1991; Bartsch, 2006a). Exact nature of relationship between mites and their invertebrate hosts is unknown for most of these associations, as live observation of living material has not been executed. Some species are assumed to be parasitic or commensals or may have been transported accidentally on their hosts (Bartsch, 1987). A brief survey of parasitic halacarid mites was made by Newell (1956) and Bartsch (1987; 1988). In present paper a survey on the literature was made to get an updated overview of parasitic halacarid mites.

Methods

The present article is prepared based on records published by the end of June 2020. Different author's views about ecological nature of those species were also added.

Results and Discussion

Halixodes chitonis (Bruker, 1897) was reported based on larvae and protonymphs attached to the gills of mollusc (Mollusca: Polyplacophora) *Cryptochonchus porosus* (=*Acanthochiton porosus*) from New Zealand (Brucker, 1897; 1899). Later a subspecies *Halixodes chitonis stoutae* was reported based on protonymphs in the mantle cavity of tidal gastropods *Sigapatella novaezelandiae* from New Zealand (Stout, Viets, 1959). Chilton (1883) reported *Halixodes truncipes* (Chilton, 1883) based on adults (females) collected between tide marks in New Zealand and found not attached to a host. It was assumed that further investigation may prove the *Halixodes chitonis chitonis/ Halixodes chitonis stoutae* to be juveniles of *Halixodes truncipes* (Newell, 1956; Bartsch, 1986). Bartsch (1986) reported *Halixodes novaezelandiae* Bartsch 1986 based on female and deutonymphs collected from crevice systems between gravel and colonies of barnacles and mussels (in *Chamaesipho columna* and *Crassostrea* encrustations), Northern Auckland, Newzealand. Two pairs of barbs found at the end of the rostrum in female and deutonymph. The first leg is not stronger; the leg segments are slender and cylindrical; the ventral setae are small and slender; not specialized to catch and hold prey with the first leg. On the other side, the mite is well equipped with the two pairs of barbs at the end of the rostrum to clasp tightly to host tissue (Bartsch, 1986). 'One might speculate that *Halixodes* feeds rarely, but very substantially. After feeding *Halixodes* might leave its host and moult, hidden in crevices' – Bartsch (1986).

Astacopsiphagus parasiticus Viets, 1931 was reported as parasite on the fresh water crayfish *Euastacus spinifer* (Heller) (=*Euastacus serratus*; =*Astacopsis serratus*) (Crustacea: Decapoda) from Moran's Creek, Roberts Plateau, MacPherson Range, Queensland National Park in Queensland, Australia, south of Brisbane based on nymphs attached to the gills of the host by the chelicerae (Viets, 1931). Womersley (1943) reported adult male and female which were collected by process of dissecting out the adult inside advanced nymphs found attached to the gill chambers of fresh water crayfish *Euastacus sulcatus* Clark from Lamington National Park, Queensland, Australia.

Viets (1938) reported an intestinal parasitic halacarid species *Enterohalacarus minutipalpis* Viets, 1938 found in the gut of a sea urchin, *Plesiodiadema indicum* at a depth of 430 m off the west coast of Halmahera Island, Strait of Molucca, Sulu Sea. The species is characterised by reduced palps.

Newell (1956) reported *Copidognathus matthewsi* Newell, 1956 on the gill chamber of decapods crustacean *Parribacus antarcticus* based on nymph and adults from Oahu, Hawaii islands, Kaneohe Bay (Tropical Eastern Pacific Ocean).The species apparently lives as a parasite on the gills of the host. The filaments of gills infested by the mite sometimes show necrotic spots apparently due to feeding activities of the mite (Newell, 1956).

Laubier (1960) reported *Parahalixodes travei* Laubier, 1960 in association with the nemertean *Cerebratulus hepaticus* from Mediterannean Sea. The species was supposed to be ectoparasite and the enlarged, pointed rostrum and chelicerae are apparently used for piercing the hosts (Laubier, 1960). Newell (1971) reported *Parahalixodes chilensis* Newell, 1971 based on a protonymph at

210 m depth off Robinson Crusoe Islands, Jaun Fernandez Islands, Chile. *Parhalixodes* is believed to be a parasitic genus (Bartsch, 2006).

Genera *Halixodes* and *Parhalixodes* have rostra with barbs at the tip; these barbs may enable the mite to stay fixed to the host.

Bartsch (1976) reported *Copidognathus stevcici* Bartsch, 1976 between the egg mass of crab *Maja squinado* from eastern North Atlantic and Mediterranean Sea and the species assumed as a parasitic. This species was reported walking on the eggs of Maja squinado collected at Plymoth, England, UK (Green, MacQuitty, 1987). Green & MacQuitty (1987) also reported that this species found in the 'Michael Collection at the British Museum Natural History' and collection containing lebel 'Portgwarra, spider crab'. This species was also found associated with adult females of the spider crabs *Maja brachydactyla* and *M. squinado* under laboratory conditions; mites were observed alive on their host for extended periods and direct evidence of feeding on crab's eggs was also provided (Guerao et al 2011).

Copidognathus gasconi (Gil, Garzón, 1979) (=*Velardoacarus gasconi*) was reported from gill chamber of a marine crab *Peltarion spinulosum* (White) (Crustacea: Decapoda) in Uruguayan coast (Gil, Garzón, 1979).

Australacarus inexpectatus Bartsch 1987 described from shallow water in Strait of Magellan, Chile, South Atlantic characterised by the stylet rostrum, dorsoventrally widened palpal segment forming a trough and the slender gnathosoma and assumed to be correlated with life as a parasite (Bartsch, 1987). After discovery of the genus *Australacarus*, another four species were described under this genus viz. *A. pustulatus* Bartsch, 1993, *A. longipalpus* Otto, 2000, *A. mesaktanus* Otto, 2000, *A. zagorskisae* Otto, 2000 from Australia among sand and sediments (Bartsch, 1993; Otto, 2000a). The genus is characterised by the palps forming a trough, enclosing the needle-like rostrum and chelicerae and is believed to be a temporary parasite (Bartsch, 2006a). Otto (2000a) commented that feeding has not been observed and a predatory or algivorous lifestyle cannot be ruled out.

Thalassophthirius auster Bartsch, 1988 was reported from sublittoral waters (13–34 m depth) off Staten Island, South Atlantic Ocean. The species is suspected of being parasitic in habit (Bartsch, 1988).

Bartsch (1989) reported *Copidognathus* sp. from northeast of Lifou, 1420 m depth and expected it might be ectoparasitic in habit.

Colobocerasides koehleri (Trouessart, 1896) was reported from 1410 m depth in Gulf of Gascogne, North Sea (Troessart, 1896). Bartsch (1998) redesrbied this species and also reported a new species *C. auster* Bartsch 1998 associated with Polychaeta from Scuthern Atlantic and opined that both species are thought to be temporary ectoparasitic in habit.

Some halacarids were collected from sponges mentioned in early literature (eg. Trouessart, 1914; Viets, 1939; Andre, 1959; Newell, 1984). Majority of them are not only bound on sponges, but also found on other substrates. They are casuals or inhabitants on hydrozoans, bryozoans, algae and sediment contaminating the sponge sample (Bartsch, 1995).

Bartsch (1995) reported a halacarid *Halacarellus obsoletus* Bartsch, 1995 collected from hexactinellid sponges (*Rossella antarctica, R. nuda, R. racovitzae,* and *Scolpnastra joubini*) in eastern Weddell Sea, Antarctica. The mites were found within the sponge tissue only, and not found on the surface or in the central cavity of the sponges. Adult mites (males and females) and juveniles were extracted from the hexactinellids. *Halacarellus obsoletus* is thought to spend all its life within the sponge tissue. The halacarid mites may feed both on sponge tissue and on microfauna within the sponges (Bartsch, 1995).

Halacarus spongiphilus Kishida, 1927 reported from dictyonine sponges in Sagami Bay, Japan (Kishida, 1927; Abé, 2010). Nature of association, whether mite is a parasite on its sponge host, or just a commensal making use of the sponge for habitation are not clear (Abe, 2010).

Otto (2000b) described a new species *Spongihalacarus longiscutus* associated with 'sponge weed' (a symbiosis between red algae *Ceratodictyon spongiosum* and the sponge *Halicona cymaeformis*) from Great Barrier Reef lagoon in Australia This species is characterised by an almost complete reduction of the palps and possibly a parasitic halacarid (Otto, 2000b).

Caeres-Martinez et al (2000) reported *Copidognathus* sp. found associated with the mussel *Mytilus galloprovincialis* from Ensenada, Baja California, NW Mexico, Ensenada Bay.The species was found on the mantle and gills of the host. There was no reported damage to branches or gills resulting from the presence of this halacarid mite. This *Copidognathus* species was more abundant in the surrounding environment and was scarce inside the mussels. They also commented that this species may be considered as an occasional invader, which seems to be innocuous to their occasional host (Caeres-Martinez et al., 2000). Pepato, Santos, Tiago, (2005) reported two species Copidognathus libiniensis and C. menippensis associated with crabs. Pepato et al. (2005) commented that both mite species have chelicerae with broad movable digits that could be used to pierce the delicate exoskeleton present at the ventral side of the host abdomen of males of Libinia spinosa or the egg membrane of Menippe nodifrons. Pepato et al. (2005) also commented that no direct evidence of feeding on crabs or crab's eggs was found in the study.

Porolohmannella violacea (Kramer, 1879) was found on variety of substrata, sand, flocculent ooze, vascular plants, mosses, algae etc from standing surface waters, swamps, ponds and lakes to high altitude water bodies and was also reported from gill chambers of crayfish *Astacus astacus*, *Astacus leptodactylus* (= *Potamobius leptodactylus*) and *Orconectes limosus* (Viets, 1927; Bartsch, 2006b; Wiszniewski, 1939; Zawal, 1998). This is an example of accidental or occasional invader of crayfish.

List of halacarid mites associated with decapods crustaceans has been provided in Norman et al (2013): majority of the association (from the list) are probably commensals or occasional invader in nature and only few species like *Astacopsiphagus parasiticus*, *Copidognathus gasconi* and *C. matthewsi* are asumed to be parasitic.

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Ectoparasitic species of the genus Trichodina (Ciliophora: Peritrichida) parasitizing Macedonian freshwater fish

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Keywords protozoa, *Trichodina* sp., grass carp, common carp, aquaculture

Abstract Trichodinids are probably the most commonly encountered protozoan parasites on fishes in marine and freshwater environments. They are typically found on the gills, skin and fins of fish. The aim of this study was to evaluate the prevalence and mean intensity of *Trichodina* sp. in freshwater fish from cyprinid aquaculture facilities in Macedonia. This study was carried out by seasons, in a period of three years. A total of 578 specimens of common carp (*Cyprinus carpio*, Linnaeus 1758), 76 specimens of grass carp (*Ctenopharyngodon idella*, Valenciennes 1844), 47 specimens of silver carp (*Hypophthalmichthys molitrix*, Valenciennes, 1844) and 53 specimens of bighead carp (*Hypophthalmichthys nobilis*, Richardson, 1845) from the most significant and larger cyprinid aquaculture facilities in Macedonia, including fish farms and reservoirs, were examined for parasitological investigations. Infestation with *Trichodina* sp. was found in 206 specimens of common carp in all aquaculture facilities examined, in all seasons, as well as, in 9 specimens of grass carp from two fish farms, only in autumn season. Total, the prevalence with *Trichodina* sp. was 11.84% (grass carp) and 35.67% (common carp), while the, mean intensity was 7.56 (grass carp) and 4.56 (common carp). The prevalence with *Trichodina* sp. in common carp by season was as following: spring – 11.82%, summer – 3.44%, autumn – 7.85%, winter – 7.05%, while the mean intensity was: spring – 4.55; summer – 4.67; autumn – 4.40; winter – 4.72. Our findings of *Trichodina* sp. in grass carp in the present study is considered as the first record in Macedonia. Also, this fish species is regarded as new host for *Trichodina* sp. in Macedonian waters.

Pasożyty zewnętrzne ryb słodkowodnych Macedonii z rodziny Trichodina (Ciliophora: Peritrichida)

Słowa kluczowe pierwotniaki, *Trichodina* sp., amur biały, karp, akwakultura

Streszczenie Trichodinidae są prawdopodobnie najczęściej spotykanymi pierwotniakami pasożytującymi na rybach słodkowodnych i morskich. Zazwyczaj spotykane są na skrzelach, skórze i płetwach ryb. Celem artykułu jest określenie częstotliwości i intensywności zarażenia ryb karpiowatych przez *Trichodina* sp. Badania trwały trzy lata. Łącznie przebadano pod kątem parazytologicznym 578 osobników karpia (*Cyprinus carpio*, Linnaeus 1758), 76 amurów białych

(*Ctenopharyngodon idella*, Valenciennes 1844), 47 tołpyg białych (*Hypophthalmichthys molitrix*, Valenciennes, 1844) i 53 tołpyg pstrych (*Hypophthalmichthys nobilis*, Richardson, 1845). Zakażenie *Trichodina* sp. stwierdzono u 206 osobników karpia we wszystkich badanych stawach i okresach, oraz w okresie jesiennym u 9 amurów białych z dwóch stawów hodowlanych. Łącznie zarażonych *Trichodina* sp. było 11.84% amurów białych oraz 35.67% karpi, średnia intensywność zarażenia amurów białych wynosiła 7.56, a w przypadku karpia – 4.56. W okresie wiosennym zarażonych było 11.82% karpi, latem – 3.44%, jesienią – 7.85%, a zimą – 7.05%. Odpowiednio średnia intensywność zarażenia wynosiła wiosną – 4.55; latem – 4.67; jesienią – 4.40 I zimą – 4.72. Stwierdzenie *Trichodina* sp. pasożytującego na amurze białym jest pierwszym przypadkiem zaobserwowanym w Macedonii. W związku z tym gatunek ten jest nowym gospodarzem dla *Trichodina* sp. w wodach Macedonii.

Introduction

The largest genus of the family Trichodinidae is the genus *Trichodina* (Ehrenberg, 1838), ciliate protists well known as the causative agent of trichodiniasis in numerous aquatic animals (Marcotegui et al., 2018; Martins et al., 2015), with more than 200 species described from fish (Asmat et al., 2005). According Tang and Zhao (2011; 2012), to date about 300 nominal *Trichodina* species have been reported from different environments in the world.

Trichodinids are ciliate protozoans widely known as ectocommensals and are probably the most commonly encountered protozoan parasites on fishes in marine and freshwater environments (Martins, Ghiraldelli, 2008). According Huh et al. (2005) and Khan (2004) trichodina can serve as a facultative ecto-parasite and can proliferate and invade hosts during unfavorable conditions in environments, such as poor water quality and food deficiency. Lom (1995) concluded that the trichodinid are able to invade their hosts within a short period because of their direct transmission, especially fish with less condition. Lom and Dykova (1992) considered that the determination of *Trichodina* species is difficult because many species are morphologically variable and show low host specificity. In the taxonomy of trichodinids, the exact number, shape and arrangement of the cytoskeletal denticles is critical for determining taxonomic relationships. These characters are usually revealed by silver nitrate staining of microscope slides, which stains the cell cytoplasm black and leaves the denticles white. Trichodinids are best known as ecto-parasites of skin, fin and gill of the fish with most of the species reported from freshwater environments.

According to data from parasitological research in Macedonia, Hristovski et al. (2006; 2012) determined *Trichodina* sp. at common carp (*Cyprinus carpio*) from Prespa Lake, with a prevalence of 1.32%. In addition, the same authors also established *Trichodina* sp. in *Leuciscus cephalus prespensis*. *Chondrostoma nasus prespensis*, *Alburnus alburnus belvica* and *Rutilus rubilio prespensis* from Prespa Lake.

According to literary reviews from the Balkan countries, *Trichodina* sp. at *Cyprinus carpio* in Bosnia and Herzegovina was first established by Žitnan et al. (1969) in the fish farming "Vučijak" – Prnjavor. Data on the presence of this parasite in waters in Serbia is published by Cakić (1992), while in Croatia, Tomec et al. (1995) in the fish farms "Narta" and "Blatnica" and Valić et al. (2005) in a fish population in the Krka River.

In the cyprinid fish farms in Romania, *Trichodina* sp. was established on gills of *Cyprinus carpio* by Lazar (2009), while on the fins, gills and body cavity by Radu et al. (2008). In the cyprinid fish farms in the Czech Republic, Ondračková et al. (2012) determined the presence of *Trichodina* sp. in two years old carp. In Turkey, data on the presence of *Trichodina* sp. at *Cyprinus carpio* in the Seyhan River was reported by Cengizler et al. (2001).

According to literary reviews from the world, Ali et al. (1988) and Mansoor & Al-Shaikh (2010) determined the presence of *Trichodina domerguei* in waters in Iraq, while Subasinghe (1992) established *Trichodina* sp. in juveniles and adult carp in cyprinid fish farms in Sri Lanka. Tantry et al. (2016) reported first findings of trichodinid fauna at *Cyprinus carpio* collected from various fish ponds and farms in Jammu division of Jammu and Kashmir state.

Regarding grass carp (*Ctenopharyngodon idella*), species of the genus *Trichodina* are identified by Ali et al. (1988) in fish farms in Iraq and Bozorgnia et al. (2012) in fish farms in Iran.

Materials and methods

The aim of this study was to evaluate the prevalence and mean intensity of Trichodina sp. in freshwater fish from cyprinid aquaculture facilities in Macedonia. This study was carried out by seasons, in a period of three years. A total of 578 specimens of common carp (Cyprinus carpio, Linnaeus 1758), 76 specimens of grass carp (Ctenopharyngodon idella, Valenciennes 1844), 47 specimens of silver carp (Hypophthalmichthys molitrix, Valenciennes, 1844) and 53 specimens of bighead carp (Hypophthalmichthys nobilis, Richardson, 1845) from the most significant and larger cyprinid aquaculture facilities in Macedonia, including fish farms and reservoirs, were examined for parasitological investigations. These aquaculture facilities included fish farms Zhabeni, Bukri, Dolneni and Zhelezara, as well as fish cage culture systems on reservoirs Tikvesh, Mladost, Globochica and Gradche. The specimens were placed in plastic containers and transferred alive to the laboratory.

Gills, fins and skin were examined using stereomicroscopes "Zeiss" – Stemi DV4 and „MBS 10", as well as microscopes "Zeiss" – PrimoVert and "Reichart" were used. All parasites found in each fish were identified and enumerated. Parasites on native smears were observed under a light microscope and magnification ×200 and ×400.

Classical epidemiological variables (prevalence and mean intensity) were calculated according to Bush et al. (1997).

The parasite specimens were identified using reference keys of Lom and Dykova (1992)

Results and discussion

A total of 578 specimens of common carp, 76 specimens of grass carp, 47 specimens of silver carp and 53 specimens of bighead carp from the most significant and larger cyprinid aquaculture facilities in Macedonia were examined for parasitological investigations. Infestation with *Trichodina* sp. was found in 206 specimens of common carp in all aquaculture facilities examined, in all seasons, as well as, in 9 specimens of grass carp from two fish farms, only in autumn, as following:

- on gills in common carp from fish farm Zhabeni, in summer and autumn,
- on gills in common carp from fish farm Bukri, in spring and summer,
- on fins in common carp from fish cage culture system on Tikvesh reservoir, in autumn,
- on gills in common carp from fish cage culture system on Mladost reservoir, in spring, autumn and winter,
- on fins and gills in common carp from fish cage culture system on Globochica reservoir, in spring and winter,
- on fins and gills in common carp from fish farm Dolneni, in autumn,

– on gills and liver in common carp from fish cage culture system on Gradche reservoir, in spring and winter,
– on gills in common carp from from fish farm Zhelezara, in spring,
– on fins in grass carp from fish farm Zhabeni, in autumn,
– on gills in grass carp from fish farm Bukri, in autumn,

Data on fish examined, fish infected, as well as the prevalence and mean intensity with *Trichodina sp.*, total and by seasons, are given in Table 1 and Table 2.

Table 1. Prevalence and mean intensity with *Trichodina sp.* in common carp (*Cyprinus carpio*) and grass carp (*Ctenopharyngodon idella*) from cyprinid aquaculture facilities in Macedonia

Fish species	Number of examined fish	Number of infected fish	Mean intensity	Prevalence (%)
Common carp (*Cyprinus carpio*)	578	206	4.56	35.67
Grass carp (*Ctenopharyngodon idella*)	76	9	7.56	11.84

Table 2. Prevalence (E) and mean intensity (I) with *Trichodina* sp. in common carp (*Cyprinus carpio*) from cyprinid aquaculture facilities in Macedonia, by seasons

Parasite species	Spring		Summer		Autumn		Winter	
	I	E (%)	I	E (%)	I	E (%)	I	E (%)
Trichodina sp.	4.55	11.82	4.67	3.44	4.40	7.85	4.40	7.05

Total, the prevalence with *Trichodina* sp. in common carp was 35.67 %, while the mean intensity 4.56. The prevalence with *Trichodina* sp. in common carp by season was as following: spring – 11.82%, summer – 3.44%, autumn – 7.85%, winter – 7.05%, while the mean intensity was: spring – 4.55; summer – 4.67; autumn – 4.40; winter – 4.40.

The prevalence with *Trichodina* sp. in grass carp was 11.84 %, while the mean intensity 7.56.

Our findings of *Trichodina* sp. in grass carp in the present study is considered as the first record in Macedonia. Also, this fish species is regarded as new host for *Trichodina* sp. in Macedonian waters.

Trichodinids are usually found on the skin, fins and gills of marine and freshwater fish. The presence of a ring of interlocking cytoskeletal denticles is their characteristic and it provide cell support and adhesion to surfaces including fish tissue. There are two surfaces, one of them is cytostome which is on the part that faces away from the host (oral surface) and the other is aboral surface, attaches to the skin of the host or other substrate.

Trichodinids have a simple direct life cycle. They have a single host and do not use alternation of generations or mass asexual replication of the host. Transmission occurs by direct contact between infected and uninfected hosts, and also by active swimming of trichodinids from one host to another.

According to Abowei et al. (2011), the representatives of the genus *Trichodina* are uniformly ciliated protozoan parasites whose body has the shape of a plate. Trichodinids are attached to the body of the fish by special fixative apparatus – an adhesive disc. The parasite moves through the water with the help of lashes that are arranged in two rows along the edges of the upper and lower side of the body. These parasites infest the skin, gills and fins of fish, causing tissue irritation. One of the symptoms is a general impairment of fish health, anxiety, loss of appetite and dyspnea.

According to Basson and Van as (2006), trichodinids generally have a low degree of specificity to a single host. Most species are commensals, which cause little or no damage to their hosts, while many are important ecto-parasites, which, if present in large numbers, can cause serious epithelial damage to the skin and gills in fish.

According to Lom and Dykova (1992), the rapid development and direct life cycle of *Trichodina* sp. can be problematic in both recirculation systems and low quality water systems. Trichodinids never occur in large numbers in fish with good condition. The most sensitive are the young categories of fish aged 4 days to 1 year, compared to the older categories which do not get sick, but can be parasite carriers. Despite the natural components of the ecosystem, the presence of these ectoparasites in debilitated fish may be an indicator of deteriorating environmental conditions.

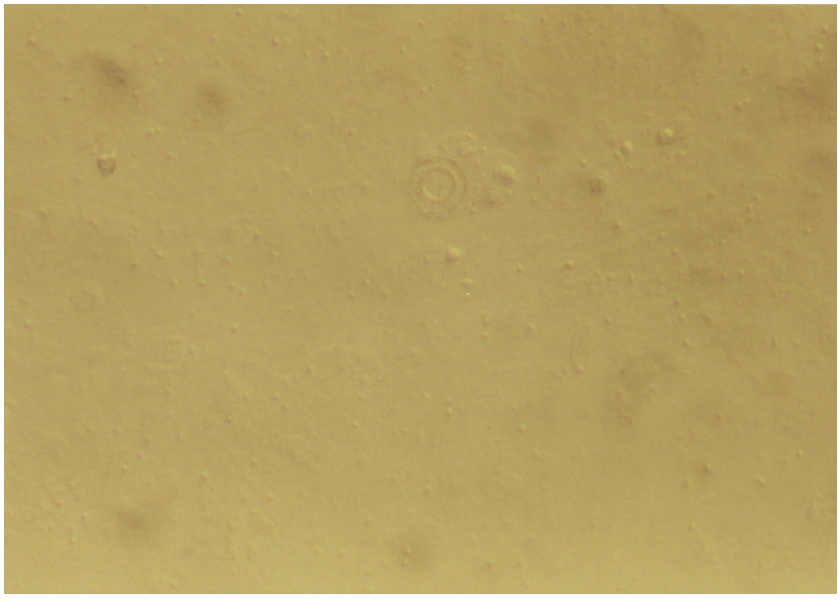


Figure 1. *Trichodina* sp. on gills of *Ctenopharyngodon idella* from fish farm Bukri

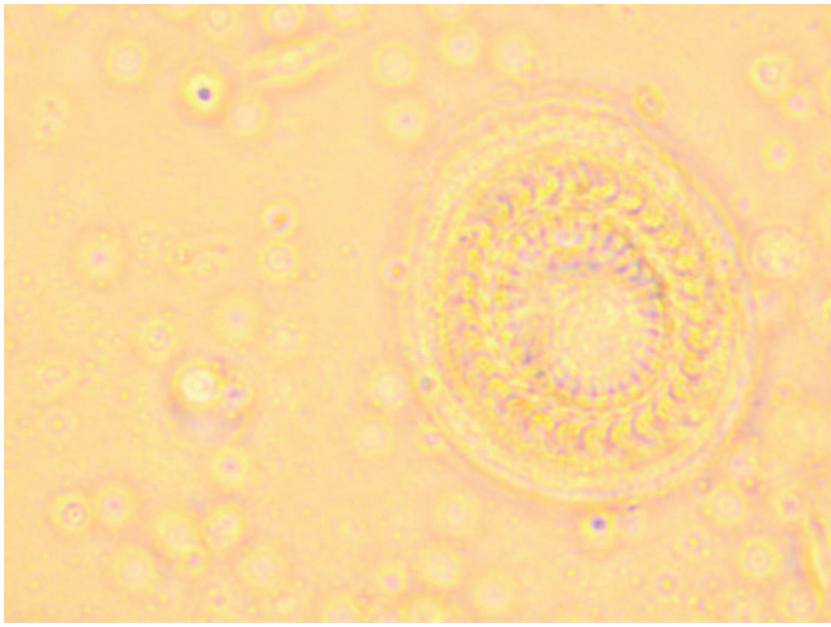


Figure 2. *Trichodina* sp. on liver of *Cyprinus carpio* from fish cage system on reservoir Gradche

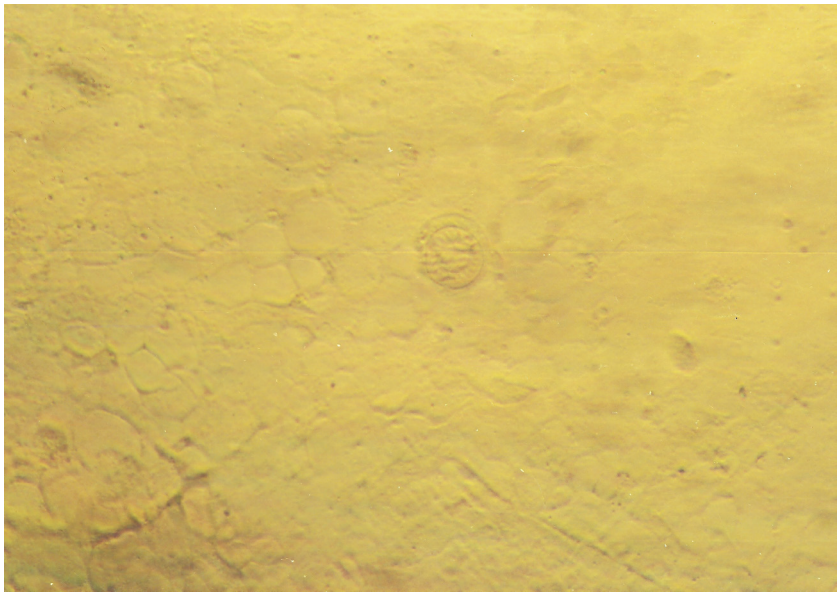


Figure 3. *Trichodina* sp. on fins of *Ctenopharyngodon idella* from fish farm Zhabeni

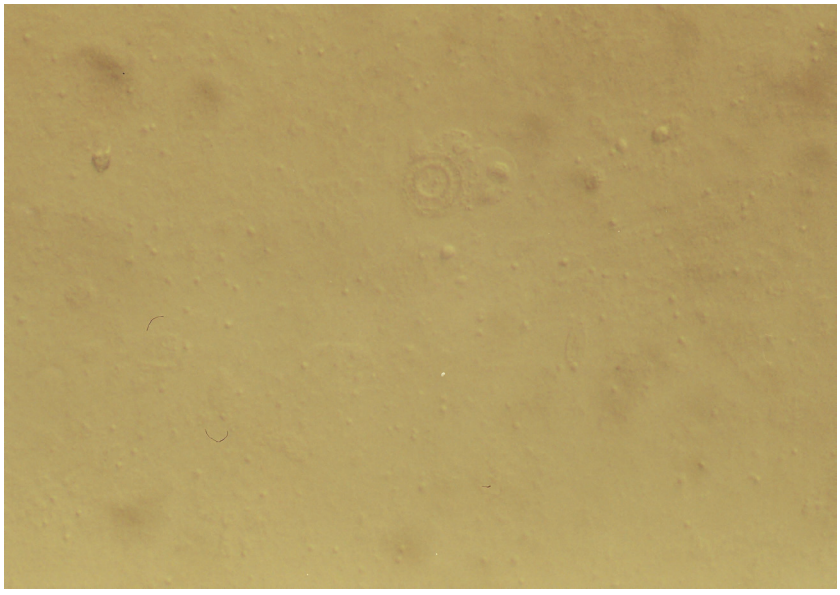


Figure 4. *Trichodina* sp. on gills of *Ctenopharyngodon idella* from fish farm Bukri

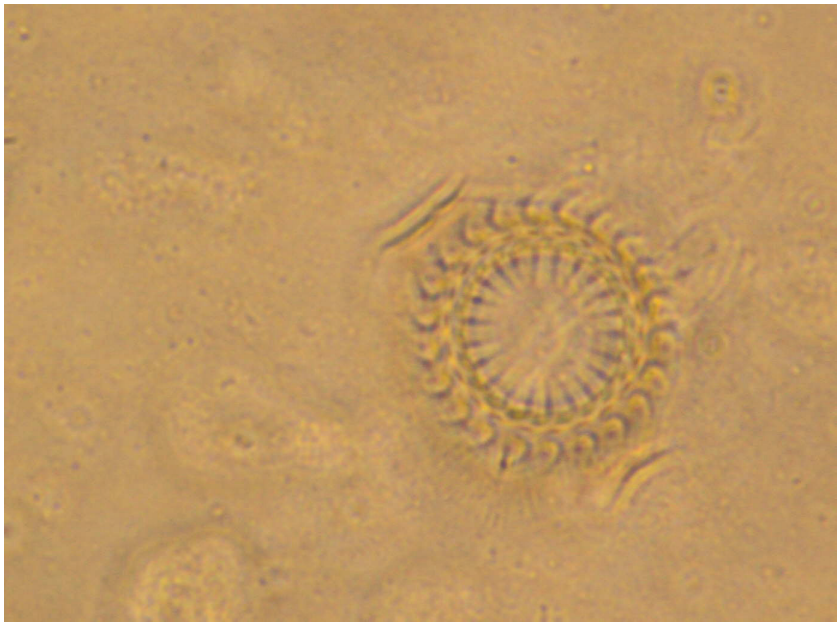


Figure 5. *Trichodina* sp. on gills of *Cyprinus carpio* from fish cage system on Globochica reservoir

Most trichodinids are ectocommensal and they use the fish only as a substrate for attachment, while they feed on suspended bacteria. High bacterial loads provide abundant food for trichodininds, which subsequently proliferate on hosts and then cause attachment-related pathologies. According Khan (2004) and Huh et al. (2005), most trichodinids are not pathogens, but when the relationship host/parasite/environment is misbalanced by nutritional deficiency, poor water quality, infectious and/or parasitic diseases, trichodinids may proliferate, being responsible for severe epidermal lesions and disease outbreaks.

Conclusions

The presence of this protozoan in common carp are determined throughout all seasons, but trichodins usually never occur in large numbers to seriously damage the health of the host, if other, non-specific factors do not act at the same time.

Our findings of *Trichodina* sp. in grass carp in the present study is considered as the first record in Macedonia. Also, this fish species is regarded as new host for *Trichodina* sp. in Macedonian waters.

Total, the prevalence with *Trichodina* sp. in common carp was 35.67%, while the mean intensity 4.56. The prevalence with *Trichodina* sp. in common carp by season was as following: spring – 11.82%, summer – 3.44%, autumn – 7.85%, winter – 7.05%, while the mean intensity was: spring – 4.55; summer – 4.67; autumn – 4.40; winter – 4.40.

The prevalence with *Trichodina* sp. in grass carp was 11.84%, while the mean intensity 7.56.

Clinical signs of Trichodiniasis in fish are slow movement, loss of appetite, black coloration, necrosis and ulcer on different parts of the body and excessive accumulation of mucous in gill filaments. If the trichodinids occur in aquaculture, it is usually indicates eutrophication or poor water quality.

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A note on centrifugation for improving freshwater invertebrate sample conservation

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Keywords live sorting, sampling trips, optimal sample conservation, benthos, streams

Abstract Sorting of freshwater invertebrate samples is best done while the animals are still alive. However, sorting in the field generally means less overall sampling can be done in the allotted time. Placing samples under cold conditions has been the traditional way to extend the life of the organisms contained within them. In this work, we evaluated whether the addition of a centrifugation step extends the survival time of organisms post-sampling, thus providing more time for sorting. Three salad spinners were tested for their capacity to extract water from samples. Additional observations were made on the compaction of samples due to excessive centrifugation. We conclude that adding a short centrifugation step of 30 seconds prolongs the life of several freshwater invertebrate taxa, including Hydrachnidia, Nematoda and Ostracoda. Furthermore, when fixation of samples in the field is necessary, centrifugation reduces the amount of fixative to be added to the samples to reach a certain concentration. Fixed samples can also be centrifuged, which may be particularly relevant when sampling abroad as inflammable liquids are often prohibited on planes. In our experience, centrifuging fixed samples does not compromise the fixed organisms, or their genetic material.

Wskazówka dotycząca wirowania w celu uzyskania lepszej jakości prób bezkręgowców słodkowodnych

Słowa kluczowe sortowanie przeżyciowe, wyprawy badawcze, optymalna ochrona próbek, bentos, strumienie

Streszczenie Sortowanie prób bezkręgowców słodkowodnych najlepiej wykonywać przeżyciowo, jednak sortowanie w terenie prowadzi na ogół do ograniczenia liczby wybieranych taksonów w wyznaczonym czasie. Umieszczanie próbek w niskich temperaturach jest tradycyjnym sposobem przedłużania życia zawartych w nich organizmów. W tej pracy oceniliśmy, czy dodanie etapu wirowania wydłuża czas przeżycia organizmów po pobraniu próbek, zapewniając tym samym więcej czasu na sortowanie. Trzy odsączarki do warzyw przetestowano pod kątem ich zdolności do ekstrakcji wody z próbek. Dodatkowe obserwacje dotyczyły zagęszczenia próbek w wyniku nadmiernego wirowania. Doszliśmy do wniosku, że dodanie krótkiego,

30-sekundowego etapu wirowania przedłuża życie kilku taksonów bezkręgowców słodkowodnych, w tym Hydrachnidia, Nematoda i Ostracoda. Ponadto, gdy konieczne jest utrwalenie próbek w terenie, wirowanie zmniejsza ilość środka utrwalającego dodawanego do próbek, aby osiągnąć określone stężenie. Utrwalone próbki można również odwirować, co może być szczególnie istotne w przypadku pobierania próbek za granicą, ponieważ płyny łatwopalne są często zabronione w samolotach. Z naszego doświadczenia wynika, że wirowanie utrwalonych próbek nie zagraża utrwalonym organizmom ani ich materiałowi genetycznemu.

Introduction

In any study of freshwater fauna, sorting of organisms is usually one of the more time consuming activity besides classification. The time required to sort organisms is dependent on their size, color and mobility. Ideally, sorting would occur in the field while the organisms are still alive and time is not limited. Under these circumstances, the organisms are often still moving around and, thus, are more easily detected. In addition, the most optimal fixative can be used according to the organism sorted (for a detailed list of preferred fixatives for different invertebrate taxa, see Table 4 in Valdecasas et al., 2010). However, sampling often does not occur under ideal conditions. Generally speaking, during a typical sampling effort, particularly those that occur abroad, several samples have to be taken in short periods of time and a generalized fixative must be used for sample preservation (Hauer, Resh, 2006).

Commonly suggested methods for the conservation or fixation of freshwater benthic samples include, among others, filling a glass or plastic jar no more than 1/3 of its capacity and keeping it cold until reaching the lab (Schwoerbel, 1970) or adding 40% formaldehyde to a sample until reaching a final concentration of 1% (e.g. Tachet, 2000) or 4% (e.g. Andreu, Camacho, 2002). Notably, some authors explicitly mention the carcinogenic properties of formaldehyde, whereas other do not. In both cases, it was suggested that the fixative be exchanged for ethanol upon arrival to the lab or, at most, within a couple of days. Another common fixation strategy is to add 95% ethanol to a sample jar until a concentration of approximately 75% (Hauer, Resh, 2006). In many other publications, fixatives are not mentioned and animals were sorted in the lab, supposedly close to the sampling sites or without a special treatment (e.g. Stryjecki et al., 2018). Nowadays, ethanol is the favored fixative for all kinds of organisms as ethanol-preserved samples can be processed for molecular analyses (e.g. DNA extraction and sequencing).

The study of some aquatic micro- and macroinvertebrates, however, may require them to be alive for certain analyses in the laboratory. In addition, duplicates are often required for morphological and molecular studies of some taxa like Turbellaria. Transporting large volumes of benthic aquatic invertebrate samples to the laboratory is not only cumbersome but also problematic due to changes in water temperature and damage to specimens. Invertebrates confined to small spaces tend to interact, resulting in the loss of structures such as gills and legs, or they simply die in transit. On the other hand, sorting in the field is very time consuming, which may restrict a greater sampling effort.

For the last several years, we have been using a centrifugation method that is simple, effective, and inexpensive. This method also saves on the amount of generalized fixatives needed to be brought to the field, in the event samples need to be fixed. For samples not fixed in the field, it increases the chance that many more organisms will arrive to the lab alive, and generally with better body conditions. Our method uses a salad spinner to squeeze out sample liquid. We hypothesize that, based on the higher thermal transmission of water than air, greatly reducing

the volume of water in a sample jar may allow the freshwater invertebrate organisms to survive longer post-sampling, which may help prolong the time available for their sorting.

In this note, we evaluate parameters of three types of salad spinners, and additional materials and methods used for the centrifugation of benthic microinvertebrate samples in the field, including centrifugation time. We also assess the post-sampling survival of several invertebrate taxa over time.

Material and methods

Types and evaluation of salad spinners

There are four main types of salad spinners: pull cord, push bottom, crank operated, and electric, and for each category, there are different sizes and volumes. For this study, we excluded electric spinners as they are not practical for field work. Figure 1 shows the three spinners analyzed plus additional material used in the study.



A – pull-cord spinner; B – crank-operated spinner; C – push-bottom spinner; D – two metal sieves with a mesh size of 3.2 mm and 0.250 mm, respectively; E – sponge; F – 125-ml plastic jar; G – 0.200 mm nylon mesh bag and spoon.

Figure 1. The three types of salad spinners and additional material used in the study

The power of a spinner to extract liquid can be evaluated by the g force it applies over its contents. The g force is dependent on spinner speed and the radius of the rotating part. The diameter of the rotating part of the spinners tested was 18 cm (r = 9) for the pull cord, 14 cm (r = 7) for the push bottom and 20 cm (r = 10) for the crank operated. The following equation was used to calculate the g force of each spinner:

$$g = (1.118 \times 10^{-5})\, r \times RPM^2$$

where r is the radius of the rotating part and RPM is the revolutions per minute. To compare the three spinners, we used the maximum RPM, which was measured with a Pyle PLT26 laser tachometer.

We then used a set of equivalent Scotch-Brite sponges (green fiber scourer) to test the liquid extraction abilities of the different spinners, taking into account the g force of each spinner. For each spinner, five similarly sized pieces of sponges (15 in total) were repeatedly soaked in water. They were then allowed to drain freely without pressure before being put into the spinner. Each spin was carried out in two steps: sponges were spun for 30 seconds, then the water was removed from the spinner before another 30-second spin. Each sponge piece was weighed before and after spinning on a Smart Weight balance, model TZ 5000. The water extraction parameter was expressed as the percentage difference in weight between wet and spun sponges.

To statistically compare results from the three spinners, an ANOVA was carried out online at the VassarStats website (http://vassarstats.net/anova1u.html, accessed 22.05.2020).

Procedure in the field

Five benthic samples were taken from a gravel bottom mountain stream by kick sampling using a circular net with a 30-cm mouth diameter, 0.250-mm mesh, and 80-cm net length (Figure 2). The samples were filtered first through a 3.2 mm metal sieve followed by a 0.250 mm sieve.



Figure 2. Field sampling site, Canencia stream (Madrid)

The organisms in the upper sieve were easily visible to the naked eye and were not centrifuged. The contents of the finer sieve were divided in two approximately equal parts. One part was transferred to a plastic jar and placed inside a cool box with ice. The second part was transferred to a 200-µm nylon monofilament bag, placed into a pull-cord spinner, and spun twice for 30 sec each time, after which the contents of the bag were transferred to a 125-ml plastic jar. All the

samples were kept cold in a cool box while in the field prior to being transferred to a fridge once in the lab. Maximum and minimum temperatures were measured in the cool box during the trip and in the fridge during the post-sampling analysis with a max/min digital thermometer. Both sets of samples, non-centrifuged and centrifuged, were checked daily: a small amount from each was transferred to a petri dish filled with river water up to height of 5 mm and the contents viewed under a Nikon stereomicroscope. Live and dead specimens of the different animal groups were counted and categorized as either 1) almost all alive; 2) more alive than dead; 3) more dead than alive; 4) all dead or 5) not observed.

Each petri dish was reviewed several times under different backgrounds (black and white) and powers (10× and 18×).

To evaluate whether a single short spin would affect sample compaction, another two samples were each split into two nearly equal parts and one part was centrifuged with the pull-cord spinner and the other with the push-bottom spinner, both for only 30 seconds. Finally, as a control, two additional samples served as centrifuged and non-centrifuged replicates kept at ambient temperature. These samples were collected, split into two parts and treated as described above for the five benthic samples.

Results

Spinner type evaluation

We measured several parameters, including the mean RPM, g force and percentage of water extraction, for each of the spinners, i.e. pull cord, push bottom, and crank operated (Table 1).

Table 1. Parameters of the three spinners evaluated in the study

	A	B	C
Radius (cm)	9.00	7.00	10.00
Mean RPM	1,162.00	999.00	679.00
G force	135.86	78.10	51.50
Percent Water Extraction	77.06	70.50	70.30

A – pull-cord; B – push bottom and C – crank operated. RPM, revolutions per minute.

The mean RPM value for each spinner was calculated as the mean of three measurements taken of each empty spinner. The mean percentage of water extraction for the three spinner types was 77.0% for the pull cord, 70.5% for the push bottom, and 70.3% for the crank operated. There was a significant effect of the pull-cord spinner over the other two ($F(5.5) = 5.83$, $p = 0.017$). The percentage of water extraction for the push-bottom and crank-operated spinners were not significantly different from each other.

Field samples

Figure 3 summarizes the survival of the different taxa found in the five centrifuged or non-centrifuged samples 24 h up to 96 h post-sampling.

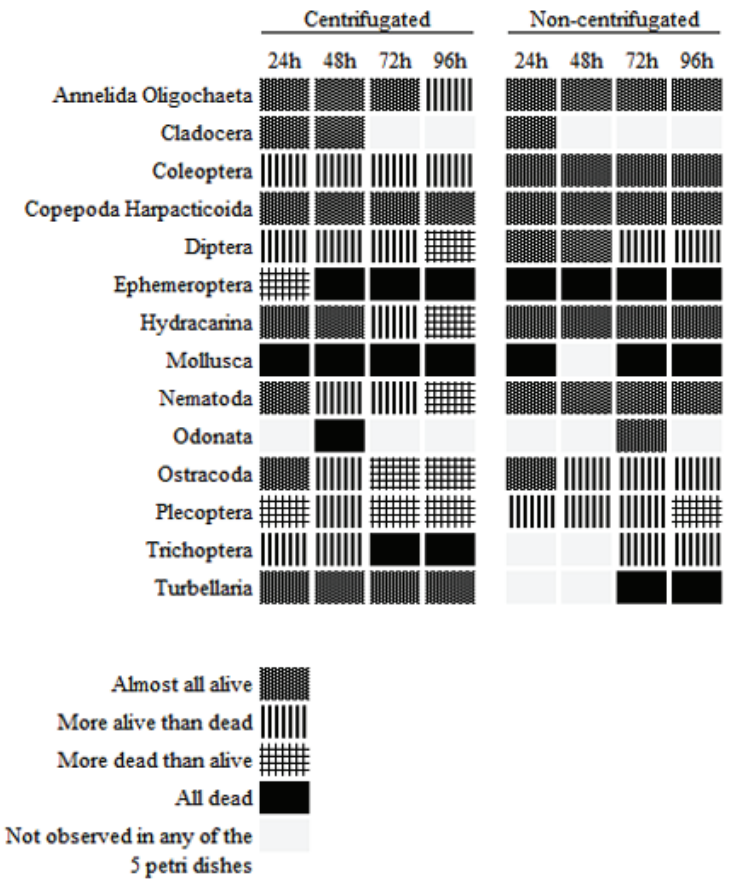


Figure 3. Summary of the differential survival of taxa in centrifuged versus non-centrifuged samples kept at 6ºC over a period of 4 days

These samples were all kept at about 6ºC in both the field and the lab. As shown in Figure 3, there is a substantial improvement in the overall survival of benthic invertebrate taxa with centrifugation, except for Ephemeroptera and Mollusca, which had all died in the first 24 hours. Oligochaeta, Coleoptera (larvae and adults), Harpacticoida, Hydrachnidia (water mites), and Nematoda were the most robust taxa over the 96-h post-sampling period. Of these, Hydrachnidia and Nematoda showed the most improvement, in terms of survival, whereas the others were relatively hardy in both sets of samples. In fact, water mites were one of the most resistant taxa analyzed in our study as plenty of live animals were seen in the centrifuged samples 312 h post-sampling. This enabled us to sort for live specimens for a longer period of time compared with the other taxa. Survival of Diptera and Ostracoda was also enhanced with centrifugation. Live Turbellaria were found in the non-centrifuged samples over the duration of the post-sampling period; however, in the centrifuged samples, they were either all dead or not observed. By contrast, in the samples centrifuged only once for 30 seconds, Turbellaria were found alive and

wandering (data not shown), suggesting that the higher compaction of the longer centrifugation was detrimental to their survival. In summary, we observed that the morphological conservation of most taxa, excluding Turbellaria which required a shorter centrifugation time, was better with centrifugation (either long or short).

Compaction of samples was evaluated qualitatively by eye when we were preparing the sample subsets for observation in petri dishes. In the double centrifuged samples, we observed some compaction. Lumps were split either with needles or by agitating the sample subset with a small amount of water in a vial prior to observation. No lumps were observed in the samples centrifuged only once with either the pull-cord or the push-bottom spinner. Even more, Turbellaria, which formed lumps and were dead in the double centrifuged samples, were seen wandering around in the single centrifuged samples.

The macroinvertebrates in the benthic samples kept at ambient temperature, which reached a maximum of 24ºC, were almost all dead, regardless of whether they were centrifuged or not.

Discussion

Classic and recent publications on the sampling and sorting of aquatic invertebrates emphasize the convenience of sorting live organisms (Anderson et al., 2013). Small organisms can be more easily detected when moving. Indeed, the human eye can see particles smaller than 0.04 mm if they are in motion (Kalloniatis, Luu, 2007). Size and color (at least, those that contrast with the background) are additional factors that aid detection. The dilemma is how to balance time spent sampling versus sorting in the field. More time spent sampling in the field often necessitates taking live samples to the lab for sorting. This is particularly true when a sampling trip involves sampling several different localities before returning to the lab.

This work deals with sample conservation prior to sorting, which itself has been treated extensively in the specialized literature (e.g. Valdecasas et al., 2010 and references therein). Given that live, mobile organisms are easier to detect than dead ones, we tested whether centrifuging samples in the field prior to sorting improves the survival of the organisms contained within them, which, in turn, would extend the time available for sorting live organisms. In both centrifuged and non-centrifuged samples, keeping them at a low temperature appears to be a critical factor for survival as evidenced by the poor survival of samples kept at ambient temperature.

We suggest that the main reason for the difference in the survival of organisms between centrifuged and non-centrifuged samples is the higher thermal conductivity of water versus air (approximately 0.6 vs 0.025 W/(mK); https://www.engineeringtoolbox.com/thermal-conductivity-d_429.html, accessed on 25.05.2020). In this sense, centrifugation helps to better preserve the specimens in cold temperatures. We did not measured putrefaction, but the non-centrifuged samples kept at a low temperature smelled bad earlier than the centrifuged ones, suggesting they had started to decay first. Also, consistent with the idea that samples should be kept cold, organisms in the centrifuged and non-centrifuged samples kept at ambient temperature, which was relatively high during the post-sampling period (about 24ºC), all died within the first 24 hours.

A long centrifugation could be counter-productive as it may excessively compact the substrate in the sample. According to the parameters of the spinners analyzed, either of the two spinner sizes tested with the short centrifugation (i.e. the pull cord or push bottom) would be adequate for use in the field. In our many years of experience, the smaller spinner size (e.g. r = 7) has proven sufficient for our purposes. Given that baggage space is usually limited on many sampling trips, its smaller size is particularly relevant. We recommend a single centrifugation step of about

30 seconds in order to extract a substantial amount of water from the sample without excessive compaction. The centrifugation time may be modulated depending on the specific taxa of interest.

Below, we provide a brief summary of the applications of centrifugation of benthic freshwater invertebrate samples from our experience:

1. We have used this method with success mainly on samples collected in mountain streams and flowing water with mixed gravel sediments. In our experience, it is not ideal for samples taken from the muddy bottom of ponds or pools, or from water that has an abundance of filamentous floating algae, as the samples become highly compacted after centrifugation.
2. The sieve and bag mesh size to use during sampling depends on the organisms and/or life cycle stages under study. We typically work with a 250-µm mesh stream net, but smaller mesh sizes may be used, if desired.
3. Notably, there is a big difference between centrifugation, which gently compresses the material and organisms contained in a sample, and squeezing the nylon bag by hand, which usually distorts or breaks the organisms' body structures.
4. When samples need to be fixed in the field with ethanol, a centrifugation step will save on the amount of fixative needed to reach the recommended ethanol concentration, which is usually 70% or more.
5. An interesting and relevant consideration is the transportation of samples by plane. Many airlines prohibit transporting ethanol at concentrations greater than 70% in checked baggage. The spinners allow the alcohol to be drawn out from the sample before departure. The samples can then be refilled as soon as reaching lab. We have not observed detectable differences in the extractability of genetic material (DNA) using this method (García-Jiménez et al., 2017).

Conclusions

Centrifugation of benthic invertebrate samples has the following advantages:

– it is less damaging for delicate animals compared with hand squeezing samples in nylon monofilament bags,
– organisms in centrifuged samples kept at a low temperature usually live longer,
– it is a simple and inexpensive yet efficient method,
– centrifuging samples may reduce the amount of ethanol needed per sample either for routine fixing or to avoid transporting inflammable liquids on planes.

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Species composition and diversity of morphotypes in epilithic biotopes of Zhytomyr region, Ukraine

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Keywords naked amoebae, morphotypes, epilithic biotopes, Zhytomyr region

Abstract 16 species of naked amoebae are recorded in the eplithic biotopes of Zhytomyr region, Ukraine: *Saccamoeba* sp. (1), *Saccamoeba* sp. (2), *Saccamoeba* sp. (3), *Cashia limacoides* Page, 1974, *Thecamoeba striata* (Penard, 1890) Schaeffer, 1926, *Thecamoeba* sp., *Stenamoeba stenopodia* (Page, 1969) Smirnov et al., 2007, *Mayorella cantabrigiensis* Page, 1983, *Mayorella* sp., *Korotnevella* sp., *Vexillifera* sp., *Vannella* sp., *Ripella platypodia* Smirnov, Nassonova, Chao et Cavalier-Smith, 2007, *Acanthamoeba* sp. (1), *Cochliopodium* sp., *Vahlkampfia* sp. (1). The frequency of occurrence in samples is highest for *Thecamoeba* sp., *Mayorella* sp., *Vahlkampfia* sp. (1), *Korotnevella* sp., *Vannella* sp., and is lowest for *Saccamoeba* sp. (1), *C. limacoides*, *M. cantabrigiensis*, *Cochliopodium* sp., *S. stenopodia*, *R. platypodia*, *T. striata*, *Saccamoeba* sp. (2). The species and morphotype diversity of naked amoebae of the epilithic mosses and lichens is influenced by the humidity and the height above soil of the substrate. The species and morphotype diversity of naked amoebae decreases with the increase in height above soil and the decrease of humidity of substrate. We recorded naked amoebae of 9 morphotypes: monotactic, dactylopodial, fan-shaped, lens-like, lingulate, mayorellian, striate, acanthopodial, and eruptive. Amoebae of the lens-like and fan-shaped morphotypes disappear with the increase of height of substrate above soil.

Skład gatunkowy i zróżnicowanie morfotypów w biotopach epilitycznych regionu żytomirskiego na Ukrainie

Słowa kluczowe ameby nagie, morfotypy, biotopy epiliptyczne, region Zhytomyr

Streszczenie W eplitycznych biotopach rejonu Żytomierza na Ukrainie odnotowano 16 gatunków nagich ameb: *Saccamoeba* sp. (1), *Saccamoeba* sp. (2), *Saccamoeba* sp. (3), *Cashia limacoides* Page, 1974, *Thecamoeba striata* (Penard, 1890) Schaeffer, 1926, *Thecamoeba* sp., *Stenamoeba stenopodia* (Page, 1969) Smirnov et al., 2007, *Mayorella cantabrigiensis* Page, 1983, *Mayorella* sp., *Korotnevella* sp., *Vexillifera* sp., *Vannella* sp., *Ripella platypodia* Smirnov, Nassonova, Chao et Cavalier-Smith, 2007, *Acanthamoeba* sp. (1), *Cochliopodium* sp., *Vahlkampfia* sp. (1). Frekwencja w próbach była najwyższa dla *Thecamoeba* sp., *Mayorella* sp., *Vahlkampfia* sp. (1), *Korotnevella* sp., *Vannella* sp., a najniższa dla *Saccamoeba* sp. (1), *C. limacoides*, *M. cantabrigiensis*, *Cochliopodium* sp., *S. stenopodia*, *R. platypodia*, *T. striata*, *Saccamoeba* sp. (2).

Na różnorodność gatunkową i morfotypową nagich ameb mchów epilitycznych i porostów ma wpływ wilgotność i wysokość podłoża nad ziemią. Zróżnicowanie gatunkowe i morfotypowe nagich ameb zmniejsza się wraz ze wzrostem wysokości nad ziemią i spadkiem wilgotności podłoża. Odnotowano 9 morfotypów nagich ameb: monotaktyczne, daktylopodialne, wachlarzowate, soczewkowate, jęczmienne, majorelijskie, prążkowane, akantopodialne i wyrzynające się. Ameby soczewkowatych i wachlarzowatych morfotypów zanikają wraz ze wzrostem wysokości podłoża nad glebą.

Introduction

The naked amoebae are widely distributed in soils, freshwater and saltwater biotopes. For most protists, humidity is the limiting factor in terrestrial biotopes (Bonnet, 1973). The majority of known species of protists are small, which allows them to live in thin films on substrates (Bonnet, 1973; Schonborn, 1989; Korganova, 2003).

There have been no previous reports of the amoebic fauna of epilithic mosses and lichens. For Ukraine, there are only reports of the species compositions of amoebae in the water bodies and the soils (Patcyuk, Dovgal, 2012; Patsyuk, 2014–2020). Hence, any new data on species of these protists from various biotopes of Ukraine is valuable for science.

Materials and methods

Material was collected during two field trips in the forests of Zhytomyr region (near Zhytomyr city, Denyshi village, Novohrad-Volynsky district, and Olevsk district) in 2019. 162 samples were analyzed. The species composition of naked amoebae was studied in soils and in the mosses and lichens growing on the soils and the rocks near water bodies. *Sphagnum* spp. dominated in the moss cover, *Hypogymnia* spp. in that of lichens. The collected samples were stored in zip-lock bags and transferred to the laboratory. 5 g of substrate sample was placed in a flask of 150 ml with 100 ml of distilled water for 2 hours, after which the mixture was shaken. 10 ml of the mixture was evenly distributed in a Petri dish with non-nutrient agar. The amoebae were cultured after Page's method (Page, 1991).

The amoebae cultures were maintained at 20°C. Alive protist cells were observed in water droplets on slides, using Axio Imager MI light microscope with differential interference contrast.

The amoebae species were identified using the relevant sources (Page, 1988; Page, 1991; Smirnov, Goodkov, 2004; Smirnov et al., 2008; Smirnov et al., 2011).

Current methods do not allow to assess the abundance of amoebae, thus we've analyzed the frequency of occurrence of amoebae in the samples from the epilithic biotopes of Zhytomyr region (calculated as ratio of the samples containing amoebae to all samples). The substrate humidity was established by weight method.

The diversity of the amoeba species and morphotypes were analyzed using the Chekanovsky-Sørensen index, constructing the dendrogram with bootstrap support of nodes in PAST 1.18 (Hammer, 2001).

Results and discussion

In total, 16 species of the naked amoebae were found in the epilithic biotopes of Zhytomyr region (Tables 1, 2). The systematics of those protists is given below following the current systematics of eukaryotes (Adl et al., 2019).

Amoebozoa Luhe, 1913
Class Tubulinea Smirnov et al., 2005
Order Euamoebida Lepsi, 1960
Family Hartmannellidae (Volkonsky, 1931) Page, 1974
Genus *Saccamoeba* Frenzel, 1892
Saccamoeba sp. (1)
Saccamoeba sp. (2)
Saccamoeba sp. (3)
Genus *Cashia* Page, 1974
Cashia limacoides Page, 1974
Class Discosea Cavalier-Smith et al., 2004
Order Thecamoebida Schaeffer, 1926
Family Thecamoebidae Schaeffer,1926
Genus *Thecamoeba* Fromentel, 1874
Thecamoeba striata (Penard, 1890) Schaeffer, 1926
Thecamoeba sp.
Family Stenamoebidae Cavalier-Smith, 2016
Genus *Stenamoeba* Smirnov et al., 2007
Stenamoeba stenopodia (Page, 1969) Smirnov et al., 2007
Order Dermamoebida Cavalier-Smith, 2004
Family Mayorellidae Schaeffer,1926
Genus *Mayorella* Schaeffer, 1926
Mayorella cantabrigiensis Page, 1983
Mayorella sp.
Order Dactylopodida Smirnov et al., 2005
Family Paramoebidae Poche,1913
Genus *Korotnevella* Page, 1981
Korotnevella sp.
Family Vexilliferidae Page, 1987
Genus *Vexillifera* Schaeffer, 1926
Vexillifera sp.
Order Vannellida Smirnov et al., 2005
Family Vannellidae Bovee, 1979
Genus *Vannella* Bovee, 1965
Vannella sp.
Genus *Ripella* Smirnov, Nassonova, Chao et Cavalier-Smith, 2007
Ripella platypodia Smirnov, Nassonova, Chao et Cavalier-Smith, 2007
Order Centramoebida (Rogerson and Patterson, 2002) Cavalier-Smith, 2004
Family Acanthamoebidae Sawyer and Griffin, 1975
Genus *Acanthamoeba* Volkonsky, 1931
Acanthamoeba sp. (1)

Order Himatismenida Page, 1987
Family Cochliopodiidae De Saedeleer, 1934
Genus *Cochliopodium* Hertwig et Lesser, 1874
Cochliopodium sp.
Excavata Cavalier-Smith, 2002
Discoba Simpson in Hampl et al., 2009
Class Heterolobosea Page & Blanton, 1985
Family Vahlkampfiidae Jollos, 1917
Genus *Vahlkampfia* Chatton et Lalung-Bonnaire, 1912
Vahlkampfia sp. (1)

Hartmannellidae were represented by the maximum number of species (four). There were three species of Thecamoebidae, two of Mayorellidae and of Vannellidae, and one of Paramoebidae, Vexilliferidae, Acanthamoebidae, Cochliopodiidae, Vahlkampfiidae each.

By the frequency of occurrence in the epilithic biotopes of Zhytomyr region, the most common species were *Thecamoeba* sp. (89%), *Mayorella* sp. (83%), *Vahlkampfia* sp. (1) (67%), *Korotnevella* sp. (61%), *Vannella* sp. (50%); the least common were *Saccamoeba* sp. (1) (5.0%), *C. limacoides* (5.7%), *M. cantabrigiensis* (11%), *Cochliopodium* sp. (11.3%), *S. stenopodia* (13%), *R. platypodia* (22%), *T. striata* (28%), *Saccamoeba* sp. (2) (28%); *Acanthamoeba* sp. (1) (39%) and *Vexillifera* sp. (45%) were found with the average frequency of occurrence (Figure 1).

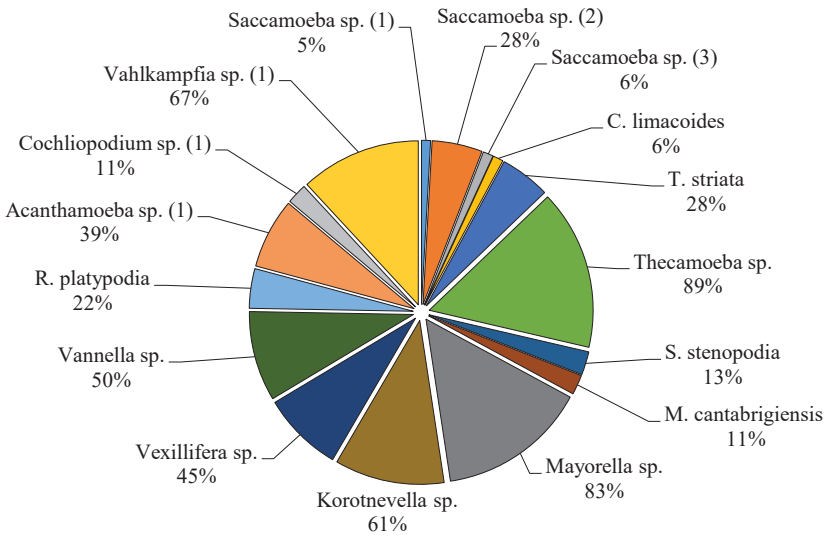


Figure 1. Frequency of occurrence of naked amoebae findings in epilithic mosses and lichens of Zhytomyr region (Ukraine)

We also analyzed the distance of epilithic biotopes to soil (height) as a factor influencing the species diversity of the naked amoebae. With increasing height, the species richness parameters decreased for those protists. The highest number of species was recorded in substrates at the soil

level (Table 2), including *Saccamoeba* sp. (1), *Saccamoeba* sp. (2), *Saccamoeba* sp. (3), *C. limacoides*, *Korotnevella* sp., *Vexillifera* sp., *Vannella* sp., *Cochliopodium* sp., *M. cantabrigiensis*, *Mayorella* sp., *T. striata*, *Thecamoeba* sp., *S. stenopodia*, *Acanthamoeba* sp. (1), *Vahlkampfia* sp. (1), 94% of all species identified in the study. By the frequency of occurrence in mosses, the most widely distributed amoebae were *Acanthamoeba* sp. (1) (95%), *Mayorella* sp. (94%), *Vexillifera* sp. (93%), *Vahlkampfia* sp. (1) (92%), *Thecamoeba* sp. (91%), *Korotnevella* sp. (84%), *T. striata* (70%), *Vannella* sp. (67%), 50% of all species under study. The most common amoeba species of the epilithic lichens included *Thecamoeba* sp. (84%), *Mayorella* sp. (80%), *Saccamoeba* sp. (2) (74%), *Korotnevella* sp. (70%), *Vahlkampfia* sp. (1) (67%), *Vexillifera* sp. (65%), *Vannella* sp. (60%), 44% of the total species diversity. The least common species in the epilithic mosses at soil level were *R. platypodia* (36%), *S. stenopodia* (36%), *M. cantabrigiensis* (35%), *C. limacoides* (33%), *Cochliopodium* sp. (32%); in the epilithic lichens, such species were *R. platypodia* (37%), *Saccamoeba* sp. (3) (36%), *Saccamoeba* sp. (1) (33%), *T. striata* (28%), a quarter of all found species of amoebae (Table 1).

Table 1. Frequency of occurrence of naked amoebae in epilithic mosses and lichens of Zhytomyr region (%)

No.	Species of amoebae	Mosses			Lichens		
		0 m	0–0.5 m	0.5–1.0 m	0 m	0–0.5 m	0.5–1.0 m
1.	*Saccamoeba* sp. (1)	0	0	0	33	0	0
2.	*Saccamoeba* sp. (2)	0	0	0	74	60	30
3.	*Saccamoeba* sp. (3)	0	0	0	36	0	0
4.	*C. limacoides*	33	0	0	0	0	0
5.	*T. striata*	70	30	0	28	38	0
6.	*Thecamoeba* sp.	91	76	67	84	93	95
7.	*S. stenopodia*	36	30	0	0	0	0
8.	*M. cantabrigiensis*	36	30	0	0	0	0
9.	*Mayorella* sp.	94	92	75	80	95	25
10.	*Korotnevella* sp.	84	89	41	70	30	33
11.	*Vexillifera* sp.	93	74	0	65	35	0
12.	*Vannella* sp.	67	33	0	60	62	58
13.	*R. platypodia*	36	30	0	37	28	0
14.	*Acanthamoeba* sp. (1)	95	38	0	40	30	29
15.	*Cochliopodium* sp.	33	0	0	0	33	0
16.	*Vahlkampfia* sp. (1)	92	89	50	67	67	32

The diversity of amoebae did not change significantly with the distance between soil and the epilithic mosses and lichens increasing to 0.5 m (Tables 1, 2). At that height, we recorded *Vahlkampfia* sp. (1), *T. striata*, *Thecamoeba* sp., *R. platypodia*, *Vannella* sp., *S. stenopodia*, *Mayorella* sp., *Korotnevella* sp., *Saccamoeba* sp. (2), *Cochliopodium* sp., *Vexillifera* sp., *Acanthamoeba* sp. (1). At the height of 1 m (above soil), species composition changed and the most common core species were not present. At that height, *Mayorella* sp. (75%), *Thecamoeba* sp. (67%), *Vahlkampfia* sp. (1) (50%), *Korotnevella* sp. (41%) were found in the epilithic mosses, and *Thecamoeba* sp. (95%), *Vannella* sp. (58%), *Korotnevella* sp. (33%), *Vahlkampfia* sp. (1) (32%), *Saccamoeba* sp. (2) (30%), *Acanthamoeba* sp. (1) (29%), *Mayorella* sp. (25%) in the epilithic lichens (Table 1).

Table 2. Distribution of naked amoebae in epilithic biotopes of Zhytomyr region (Ukraine)

No.	Species of amoebae	Neighborhood of Zhytomyr city						Novohrad-Volynsky district						Olevsk district					
		mosses			lichens			mosses			lichens			mosses			lichens		
		0 m	0.5 m	1.0 m	0 m	0.5 m	1.0 m	0 m	0.5 m	1.0 m	0 m	0.5 m	1.0 m	0 m	0.5 m	1.0 m	0 m	0.5 m	1.0 m
1.	*Saccamoeba* sp. (1)	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–
2.	*Saccamoeba* sp. (2)	–	–	–	–	+	–	–	–	–	+	–	–	–	–	–	+	+	+
3.	*Saccamoeba* sp. (3)	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–
4.	*C. limacoides*	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
5.	*T. striata*	–	–	–	–	–	–	+	+	–	+	+	–	+	–	–	–	–	–
6.	*Thecamoeba* sp.	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+
7.	*S. stenopodia*	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–
8.	*M. cantabrigiensis*	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–
9.	*Mayorella* sp.	+	+	–	+	+	–	+	+	+	–	+	–	+	+	+	+	+	+
10.	*Korotnevella* sp.	+	+	–	+	–	–	+	+	–	–	–	+	+	+	+	+	+	–
11.	*Vexillifera* sp.	+	–	–	+	–	–	+	+	–	–	–	–	+	+	–	+	+	–
12.	*Vannella* sp.	+	–	–	+	+	–	–	–	–	–	–	+	+	+	–	+	+	+
13.	*R. platypodia*	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	+	+	+
14.	*Acanthamoeba* sp. (1)	+	–	–	–	–	–	+	+	–	–	–	–	+	–	–	+	+	+
15.	*Cochliopodium* sp.	+	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–
16.	*Vahlkampfia* sp. (1)	+	+	–	+	+	–	+	+	+	+	+	+	+	+	–	–	–	–
Total		8	4	1	6	5	1	9	9	3	6	5	4	10	6	2	8	8	6
		8			7			9			10			10			8		
		9 species						14 species						12 species					

Nine of 16 species of amoebae were found at all heights: *Vahlkampfia* sp. (1), *T. striata*, *Thecamoeba* sp., *Vannella* sp., *Mayorella* sp., *Korotnevella* sp., *Saccamoeba* sp. (2), *Vexillifera* sp., *Acanthamoeba* sp. (1).

C. limacoides, *Saccamoeba* sp. (1), *Saccamoeba* sp. (3) were not numerous and preferred the epilithic mosses and lichens at the soil level. At the height of 0.5 m these species were not found.

The species diversity of amoebae from the epilithic biotopes of Zhytomyr region was compared to that in soil at the ground level and that of epigeic mosses in order to determine the biotope-specific features. The soil-living species included *Vahlkampfia* sp. (1), *C. limacoides*, *T. striata*, *S. stenopodia*, *M. cantabrigiensis*, *Vannella* sp., *Acanthamoeba* sp. (1), for all of them the frequency of occurrence did not exceed 38%. In the epigeic mosses, *Vahlkampfia* sp. (1) (15%), *Saccamoeba* sp. (1) (3.6%), *Acanthamoeba* sp. (1) (12%) were found. That points to the similarity of the species diversity of amoebae of the epilithic mosses and lichens and that of soils. Besides that, most of the species of our study have been found before in the water bodies of Ukraine (Patcyuk, Dovgal, 2012; Patsyuk, 2014–2020).

The similarity of species composition of the naked amoebae from various epilithic biotopes was analyzed using the Chekanovsky-Sørensen index. At the dendrogram, two clusters can seen (Figure 2). One of the clusters represented the species diversity of amoebae of the epilithic lichens at different height, and that of the epilithic mosses at 0.5 m above soil. The bootstrap support of that cluster is 47%. The other cluster (bootstrap support 100%) represented the amoeba species sampled at 1.0 m above soil. Its specifics were determined by *Vahlkampfia* sp. (1), *Thecamoeba* sp., *Mayorella* sp., and *Korotnevella* sp. The index of faunistic similarity was higher than 50% (0.50–0.96) for the species diversity of amoebae collected in the various epilithic biotopes of Zhytomyr region.

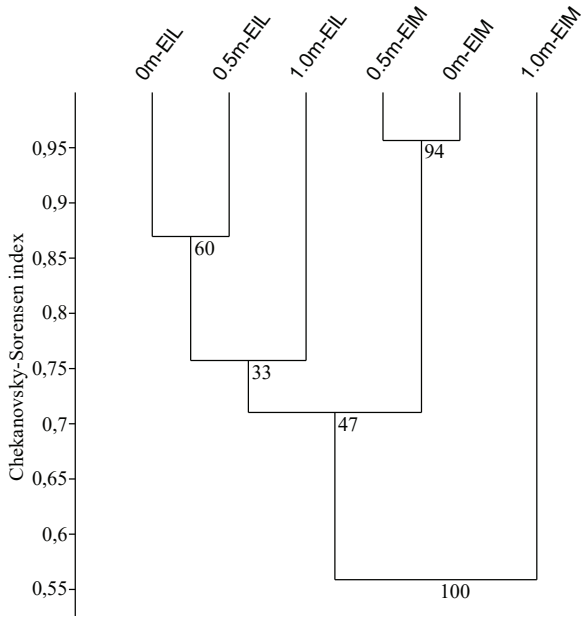


Figure 2. Similarity of species diversity of naked amoebae in epilithic mosses and lichens at different height above soil in Zhytomyr region (ElL – epilithic lichens, ElM – epilithic mosses)

The species richness of amoebae was defined by the levels of microbiotope humidity. It was relatively high in the moist mosses and lichens (68–78%). Low values of species richness were found in the driest conditions of the epilithic mosses and lichens (41–44%). The humidity levels of the epilithic biotopes decreased with the increasing height above soil.

Similar findings have been observed in the studies of relationship between the species composition of other invertebrates (testate amoebae, heterotrophic flagellates, mites, etc.) and the distance from soil (Miller et al., 2007; Lindo et al., 2008).

We have identified amoebae of nine morphotypes in the present study: monotactic (*Saccamoeba* sp. (1), *Saccamoeba* sp. (2), *Saccamoeba* sp. (3), *C. limacoides*); dactylopodial (*Korotnevella* sp., *Vexillifera* sp.); fan-shaped (*Vannella* sp., *R. platypodia*); lens-like (*Cochliopodium* sp.); lingulate (*S. stenopodia*), mayorellian (*M. cantabrigiensis*, *Mayorella* sp.), striate (*T. striata*, *Thecamoeba* sp.), acanthopodial (*Acanthamoeba* sp. (1)), eruptive (*Vahlkampfia* sp. (1)). Amoebae of the eruptive morphotype were the most common in the epilithic biotopes of Zhytomyr region (67%), in contrast to those of lingulate (5.5%) and monotactic (7.4%) morphotypes. Frequency of occurrence was 20.3% for fan-shaped amoebae, 22.2% for lens-like amoebae, 33.3% for mayorellian and acanthopodial amoebae, 35.2% for dactylopodial amoebae, and 40% for the striate amoebae.

All nine morphotypes of amoebae were found in the epilithic mosses of the study region. In the epilithic lichens, the lingulate amoebae were absent. At 0 to 1.5 m above soil, we found the monotactic, dactylopodial, fan-shaped, mayorellian, striate, acanthopodianl, eruptive morphotypes, making up 78% of all the recorded morphotypes. Amoebae of the lens-like and the lingulate morphotypes, 22% of all recorded morphotypes, were not collected at 1.5 m above soil.

Similarly, all nine morphotypes of amoebae were present in the epilithic mosses at soil level (0 m). Seven morphotypes of amoebae were observed in the epilithic lichens at that level, with the exception of the lens-like and lingulate morphotypes. In the epilithic mosses at soil level, the most common amoeba were of acanthopodial (98%), eruptive (95%), striate (67%), mayorellian (67%), dactylopodial (67%) morphotypes; the least common were amoebae of monotactic (5.5%) and lingulate (17%) morphotypes; for fan-shaped and lens-like amoebae, the frequency of occurrence was 33%. In the epilithic lichens at soil level, the most common were the amoebae of striate (67%) and eruptive (70%) morphotypes. Frequency of occurrence of amoebae of other morphotypes did not exceed 37% (Figure 3).

At 0.5 m above soil, the frequency of occurrence and the diversity of amoebae of certain morphotypes did not change essentially (Figure 3). In the epilithic mosses, seven of the morphotypes were represented (78% of all morphotypes). In the epilithic lichens, the amoebae of eight morphotypes (89%) were present.

At 1.0 m above soil the humidity levels and frequency of occurrence of certain amoebic morphotypes were lower. The amoebae of the epilithic mosses at that height were of dactylopodial (11%), mayorellian (33%), striate (33%) and eruptive (41%) morphotypes. The amoebae of epilithic lichens were of monotactic (5.5%), dactylopodial (11%), mayorellian (17%), fan-shaped (22%), acanthopodial (31%), eruptive (33%) and striate (50%) morphotypes (Figure 3).

The diversity of amoeba morphotypes was analyzed using Chekanovsky-Sørensen index. The resulting two groups are shown on Figure 4. The first group included the morphotypes of amoebae found in epilithic mosses and lichens (0–0.5 m), with 76% bootstrap support. The second group included the morphotypes of amoebae from the epilithic mosses at 1 m above soil. That complex was defined by the amoebae of dactylopodial, mayorellian, striate and eruptive morphotypes. The results of clustering analysis of the amoeba species diversity were in line with

these findings. *Vahlkampfia* sp. (1), *Thecamoeba* sp., *Mayorella* sp., *Korotnevella* sp. formed a separate complex of species, and their morphotypes were grouped similarly.

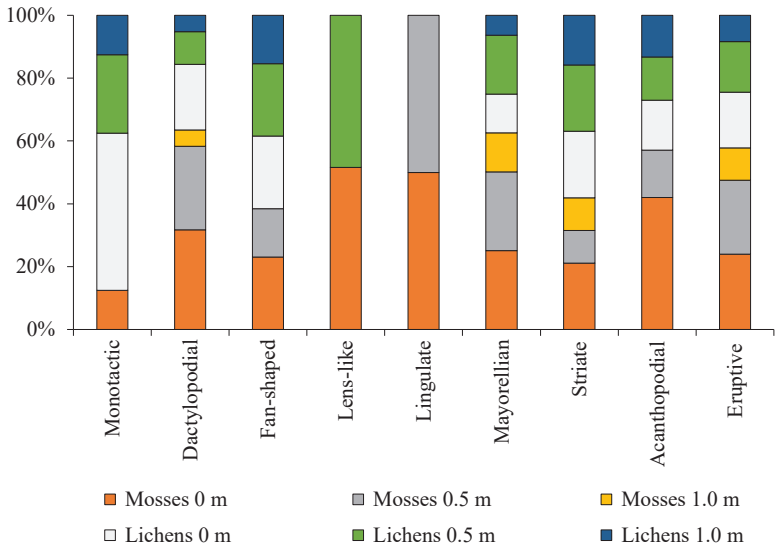


Figure 3. Frequency of occurrence of amoeba morphotypes in epilithic biotopes at different height above soil in Zhytomyr region (Ukraine)

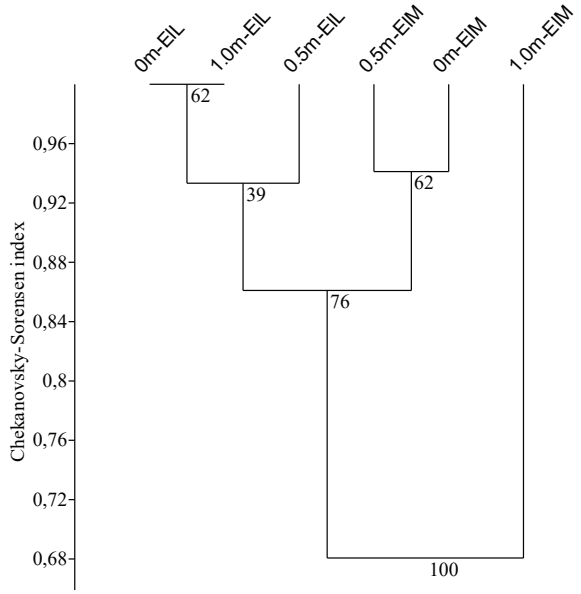


Figure 4. Similarity of morphotype diversity of naked amoeba in epilithic mosses and lichens at different height above soil in Zhytomyr region (ElL – epilithic lichens, ElM – epilithic mosses)

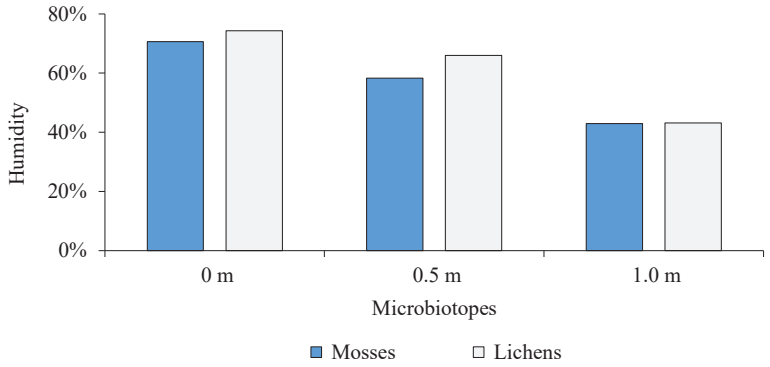


Figure 5. The mean values (in %) of the change of humidity in the studied microbiotopes with the increasing height above ground

Thus, 16 species of naked amoebae of the molecular clusters of Tubulinea, Discosea and Discoba were identified in present study of the species composition of naked amoebae in the epilithic mosses and lichens of Zhytomyr region, Ukraine. Most species grouped together in clusters are eurybionts found in the water bodies, soils, and epilithic biotopes. The changes in species composition of amoebae in the epilithic mosses and lichens occur at 0.5–1 m above soil. Factors that determine the species composition of amoebae in such biotopes are distance to soil and humidity levels (Figure 5). According to the cluster analysis, the species diversity of amoebae of the epilithic biotopes of Zhytomyr region consists of two complexes. One of them includes the amoebae of the epilithic lichens at varying height above soil and epilithic mosses at 0.5 m above soil (47% bootstrap support), the other is formed by of amoebae of the epilithic mosses at 1.0 m above soil (100% bootstrap support). In the epilithic mosses of the study region we found amoebae of monotactic, dactylopodial, fan-shaped, lens-like, lingulate, mayorellian, striate, acanthopodial and eruptive morphotypes. The amoebae of lingulate morphotype are not found in the epilithic lichens. At 1.5 m above soil, the amoebae of the lingulate and lens-like morphotypes are not present. The cluster analysis of the morphotype diversity results in two groups of the amoeba morphotypes similar to the clusters of amoeba species diversity. The species and morphotype compositions of the naked amoebae are similar in structure according to the results of cluster analysis, that is, the representatives of different morphotypes prefer similar ecological conditions.

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New sheath nematode, *Hemicycliophora paraconida* n. sp. (Rhabditida: Hemicycliophoridae) from Northern forest in Iran

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Keywords 28S D2/D3, Hemicycliophoroidea, morphology, morphometric, new species, taxonomy

Abstract *Hemicycliophora paraconida* n. sp. from the Tilekenar forest in northern Iran, is described and illustrated. The new species is characterized by having female body length of 907–1197 µm, 286–341 body annuli and a 96–115 µm long stylet. Lateral fields with three longitudinal lines forming two rows of blocks with breaks and anastomosis of transverse striae; each annulus in lateral field with four subcuticular ovate markings. Tail elongated uniformly conoid, with distinct annuli and narrowly rounded to acute terminus. Male possess robust and semi-circular spicules and a well-developed penial tube. Based on molecular and morphological comparisons, the new species comes close to seven species of the genus namely: *H. conida*, *H. iwia*, *H. ovata*, *H. hellenica*, *H. poranga*, *H. ornamenta* and *H. halophila*, but it differs from them by the body size, R related indices, stylet length, lateral field structure, vulva position and tail shape. Molecular analyses based on sequences of D2-D3 expansion segments of 28S rRNA gene supported its morphological status as a new species and revealed that the new species is closest to the *H. conida* clade in the dendrogram inferred using 28S D2-D3 gene.

Nowy gatunek nicienia, *Hemicycliophora paraconida* n. sp. (Rhabditida: Hemicycliophoridae) z lasu w Północnym Iranie

Słowa kluczowe 28S D2/D3, Hemicycliophoroidea, morfologia, morfometria, nowy gatunek, taksonomia

Streszczenie Wykonano opis wraz z ilustracjami nowego gatunku, Hemicycliophora paraconida n. sp. z lasu Tilekenar w północnym Iranie. Samica nowego gatunku charakteryzuje się długością ciała 907–1197 µm, segmentami pierścieniowymi w liczbie 286–341 oraz sztyletem o długości 96–115 µm. Pola boczne z trzema liniami podłużnymi tworzącymi dwa rzędy brodawek z przerwami i poprzecznych prążkami; każdy pierścień z boku ma cztery subkutikularne,

owalne znamiona. Ogon wydłużony, jednolicie stożkowaty, z wyraźnymi pierścieniami i wąsko zwężający się do samego końca. Samce z mocnymi, półokrągłymi spikulami mają oraz dobrze rozwiniętym trzonem penisa. Na podstawie porównań molekularnych i morfologicznych nowy gatunek jest spokrewniony z siedmioma gatunkami z tego samego rodzaju: *H. conida*, *H. iwia*, *H. ovata*, *H. hellenica*, *H. poranga*, *H. ornamenta* i *H. halophila*, od których różni się wielkością ciała, wskaźnikami R, długością sztyletu, strukturą pola bocznego, usytuowaniem otworu genitalnego i kształtem ogona. Analizy molekularne oparte na sekwencjach segmentów D2-D3 genu 28S rRNA potwierdziły jego odmienność morfologiczną jako nowego gatunku i usytuowały go najbliżej kladu *H. conida* w dendrogramie skonstruowanym na podstawie genu 28S D2-D3.

Introduction

The plant-parasitic nematodes of the superfamily Hemicycliophoroidea Skarbilovich, 1959 commonly known as 'sheath nematodes. These nematodes are a group of obligate ectoparasitic nematodes. Many species belonging to the superfamily have been found associated with agricultural crops, although pathogenicity has only been studied for a few species (van den Berg et al., 2018). Some species of *Hemicycliophora e.g., H. arenaria* Raski, 1958, *H. conida* Thorne, 1955, *H. parvana* Tarjan, 1952, *H. poranga* Monteiro & Lordello, 1978, *H. similis* Thorne 1955, *H. typica* de Man, 1921 have been reported as damaging agricultural crops such as carrot, celery, citrus, maple and tomato in several countries (Chitambar & Subbotin, 2014). Host symptoms produced in response to nematode feeding are not well-known for most species within the genus. Some species could induce root galls, while the others, such as *H. typica*, may induce only slight swellings or no symptoms. Stubby root symptoms on carrots was induced by *H. typica* in sandy soil and *H. conida* caused host stunt and aberrant root development in The Netherlands (Chitambar, 1993).

Considering synonymy by Raski and Luc (1987), the superfamily includes two families with three genera *i.e. Hemicycliophora* de Man 1921, *Caloosia* Siddiqi & Goodey, 1964 and *Hemicaloosia* Ray & Das, 1978 (Chitambar, Subbotin, 2014). The taxonomic position of the genus *Hemicycliophora* has been changed by some authors since 1921 when de Man recovered a single male nematode specimen in a compost heap in Netherland and described it as a new species and genus. For a long time, males and females of this genus were been considered as two different nematodes by nematologists until Loos (1948) discovered males and females together in a population collected in Sri Lanka. The genus *Hemicycliophora* now belongs to subfamily Hemicycliophorinae and is known to have a global distribution with 135 valid species (Maria et al., 2018; Subbotin et al., 2014; van den Berg et al., 2018). The subfamily Hemicycliophorinae including the monotypic genus, is characterized by the presence of an extra cuticular layer adpressed or loose from the inner cuticle along the body. The lip region has two or three lip annuli which are not modified or separated. Transverse vulval slit bearing lips which are mostly modified. Males have smooth and offset labial region and semi-circular, U- or hook-shaped spicule (Chitambar, Subbotin, 2014; Raski, Luc, 1987).

Loof (1984) described six new and one known species of *Hemicycliophora* based on material collected by Sturhan from different regions in Iran during 1970–1974. More recently, five other members of the genus have been reported from Iran (Ghaderi et al., 2019; Ghaderi et al., 2018). In a nematological survey conducted in a forest of Mazandaran province in northern Iran, a population of an unknown *Hemicycliophora* species was recovered from soil samples collected from the rhizosphere of grasses. It is herein described as *H. paraconida* n. sp. through morphological observation and molecular characterization by the partial 28S D2-D3 rRNA gene sequence.

Materials and Methods

Sampling, extracting, mounting and drawing. Specimens of *Hemicycliophora paraconida* n. sp. were obtained from soil samples collected around roots of grasses in Tilekenar Forest, Mazandaran province. To obtain a cleaner suspension of nematodes, the tray method was used (Whitehead, Hemming, 1965). Nematodes were handpicked under a stereomicroscope, heat-killed by adding boiling 4% formalin solution, transferred to anhydrous glycerin (De Grisse, 1969), mounted on permanent slides and examined under a Nikon E200 light microscope. Drawings and measurements were made using a drawing tube attached to the microscope. Photographs were taken using a camera (Dino-Lite digital microscope) attached to the same microscope.

DNA extraction, PCR and sequencing. Total DNA was extracted from single individual nematodes using worm lysis buffer containing proteinase K (Williams et al., 1992) and DNA extracts were stored at -20ºC. The forward D2A (5'–ACAAGTACCGTGAGGGAAAGTTG–3') and reverse D3B (5'–TCGGAAGGAACCAGCTACTA–3') primers were used for amplification and sequencing of the fragment of the 28S rRNA gene (Nunn, 1992). Fifty µL of PCR mixture contained 1× Taq DNA polymerase incubation buffer, 2.5 mM MgCl2, 0.2 mM of each dNTP, 0.5 µM of each primers and 2 µL of DNA. The PCR amplification profile consisted of 5 min at 95ºC; 35 cycles of 30 s at 94ºC, 45 s at 55ºC and 2 min at 72ºC, followed by a final step of 10 min at 72ºC. Two µL of the PCR product was run on a 1.2% TBE buffered agarose gel (100 V, 40 min). The band of the correct size was excised and purified using QIAquick gel Extraction Kit (Qiagen: www.qiagen.com) and sequenced directly for both strands using the amplification primers with an ABI 3730XL sequencer (Macrogen Corporation, South Korea).

DNA sequence alignment and phylogenetic inference. The obtained sequences of the partial 28S D2-D3 region of the nematode specimen were aligned using the online version of MAFFT version 7 (http://mafft.cbrc.jp/alignment/server/) (Katoh, Standley, 2013) with those of the other *Hemicycliophora* species and some related genera available in GenBank using the BLAST homology search program. After manually trimming the alignment, the Gblocks program (version 0.91b) using all three less stringent parameters, a server tool at the Castresana Lab (http://molevol.cmima.csic.es/castresana/Gblocks_server.html), was used to eliminate poorly aligned regions or divergent positions. The best-fitted model of DNA evolution was obtained using MrModeltest 2 (Nylander, 2004). Phylogenetic analyses was then performed using the Akaike-supported model, a general time reversible model, including among-site rate heterogeneity and estimates of invariant sites (GTR + G + I) together with the base frequency, the proportion of invariable sites, and the gamma distribution shape parameters and substitution rates. The tree topology was confirmed using MrBayes v3.2.3 (Ronquist & Huelsenbeck, 2003) with four chains (three heated and one cold). The number of generations for the total analysis was set to 2×10^6, with the chain sampled every 100 generations, and the burn-in value was 25%. The Markov chain MonteCarlo (MCMC) method within a Bayesian framework was used to estimate the posterior probabilities of the phylogenetic trees using 50% majority rule (Larget, Simon, 1999). A maximum likelihood (ML) tree was also reconstructed by using RaxmlGUI 1.3 (Silvestro, Michalak, 2012) software using the same alignment and nucleotide substitution model as in the Bayesian inference in 1000 bootstrap (BS) replicates. The consensus tree were selected to represent the phylogenetic relationships with branch length and support level and visualized using Dendroscope V.3.2.8 (Huson, Scornavacca, 2012) and redrawn in Adobe® Photoshop® 7.0 ME. *Paratylenchus hamatus* and *Gracilacus* sp. (Accession numbers: KF242218 and KM061782) were used as the outgroup taxa. The Bayesian

posterior probability (BPP) and ML BS values exceeding 0.50 and 50%, respectively, are given on appropriate clades in the format of BPP/ML BS.

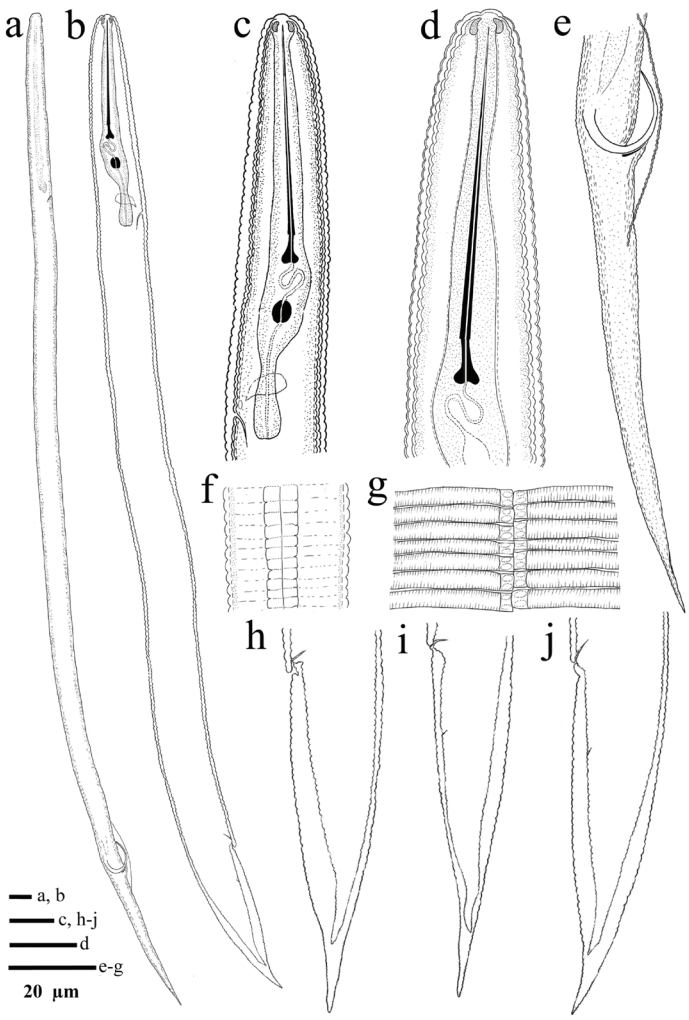
Results

Hemicycliophora paraconida n. sp.

Table 1. Morphometrics of *Hemicycliophora paraconida* n. sp. All measurements are in µm and in the form: mean ± s.d. (range).

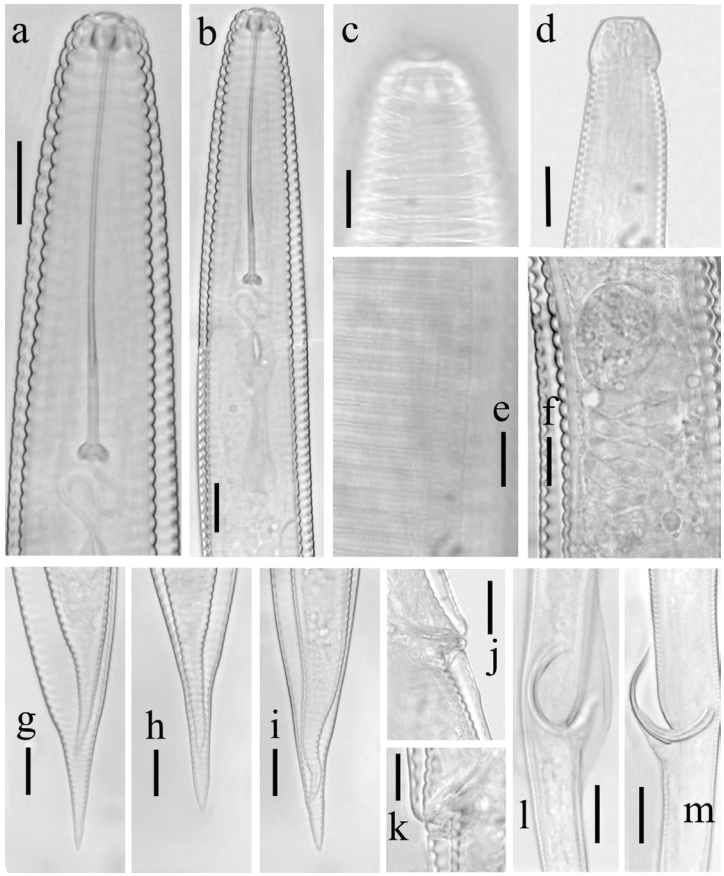
Characters	Female		Male
	holotype	paratypes	paratypes
n	–	12	10
L	933	1017 ±100 (907–1197)	973 ±21 (944–994)
L’	786	879 ±96 (766–1047)	801 ±21 (773–820)
a	21	21 ±1 (19.5–22.5)	37 ±3.3 (33–41)
b	4.6	5.3 ±0.5 (4.6–6.1)	7.5 ±0.8 (6.8–8.4)
c	6.3	7.4 ±0.7 (6.3–8.3)	5.6 ±0.2 (5.5–6)
c’	3.9	3.3 ±0.3 (2.8–3.9)	6.5 ±0.5 (6–7)
V or T	83.6	83.0 ±0.6 (82–84)	15 ±3 (12–17)
V’	99	96 ±2 (94–99)	–
Head height	10	9.5 ±0.8 (8.5–10.5)	8.3 ±0.5 (8–9)
Head width	20.5	21 ±0.8 (20–22)	12 ±0.0
Stylet	101	104 ±6 (96–115)	–
Conus	81	82.5 ±4 (78–91)	–
Shaft	20	21 ±3.5 (16–27)	–
m	80.2	80 ±2.5 (75–84)	–
DGO from base of knobs	17	13 ±2.2 (11–17)	–
Anterior end to centre of median bulb	137	134 ±6.5 (123–143)	94 ±13 (87–114)
Nerve ring from anterior end	170	159 ±8 (147–170)	131 ±4.2 (128–134)
Excretory pore from anterior end	195	188 ±17 (150–213)	165 ±8 (154–171)
Pharynx length	201	190 ±9 (176–201)	130 ±16 (113–145)
Head–vulva	780	844 ±84 (757–1002)	–
Max. body diam.	45	48.5 ±3 (44–53)	27 ±2.5 (24–30)
Anal/cloacal body diam.	38	43 ±4 (37–49)	27 ±2.5 (24–30)
Vulva – anus	43	45 ±4.5 (36–53)	–
Ovary or testis length	398	359 ±80 (259–507)	145 ±27 (114–163)
Tail length	147	138 ±11 (118–150)	172 ±5.5 (166–179)
Rs_t	32	30 ±3 (23–33)	–
R_{ex}	57	52 ±4 (46–57)	–
R_{pha}	59	53 ±5 (44–59)	–
RV(ant)	268	244 ±16 (230–272)	–
Ran	287	259 ±18 (243–290)	–
Rvan	19	15 ±2.5 (12–19)	–
R	338	304 ±21 (286–341)	–

VL/VB	4.3	3.8 ±0.3 (3.3–3.4)	–
Spicule (arc line)	–	–	55.5 ±2 (54–58)
Spicule width (straight distance between extremities)	–	–	28 ±0.6 (27–29.5)
Penial tube length	–	–	17 ±2 (15–19)
Gubernaculum length	–	–	12 ±0.5 (11.5–13)



a – male entire body; b – female entire body; c – female pharyngeal region; d – female anterior region; e – male posterior region; f, g –: lateral lines at mid body in male and female, respectively; h–j – female posterior region.

Figure 1. Line drawings of paratypes of *Hemicycliophora paraconida* n. sp.



a, b – female anterior region; c – surface view of male anterior region; d – male anterior region; e – details of lateral field in female; f – genital tract showing spermatheca and crustaformeria; g–i – female tail region; j, k – vulval region; l, m – cloacal region. Scale bars: a, b, g–i, l, m = 20 µm, c–f, j, k = 10 µm.

Figure 2. Photomicrographs of paratypes of *Hemicycliophora paraconida* n. sp.

Description. *Female.* Relatively large sized nematodes (Figures 1 and 2). Body straight or irregularly arcuate upon fixation, cuticular sheath fitting closely or loosely to body, more loosely on tail and vulval region. Cuticle with coarse annulation, annuli. Annuli outside lateral field marked by a row of short scratches at their anterior and posterior border. Lateral field bordered by two distinct longitudinal lines; within the lateral field the body annuli may continue as complete transverse striae or show breaks appearing as a third longitudinal line in the middle and resulting in two longitudinal rows of blocks, each block ornamented by two vague subcuticular ovate markings, hardly visible just in few specimens (Figure 2e). Labial region broad, rounded and anteriorly truncate, continuous with body contour, but slightly narrower than body at base, bearing two distinct annuli (three in two specimens) with elevated labial disc. Stylet slender, straight or slightly curved, conus extremely larger than shaft *i.e.* 75–84% of total stylet length. Stylet

knobs large and oblong to rectangular shape, posteriorly sloping creating large "cavity". Pharynx typically criconematoid with a fused procorpus and metacorpus with a large valve, a short isthmus connected to a reduced basal bulb. Secretory- Excretory pore varying in position from three annuli anterior to the base of pharynx to nine annuli posterior to it, but usually located opposite to base of basal bulb; hemizonid indistinct. Genital system mono-prodelphic occupying 26–42% of the body length, with outstretched ovary comprising oocytes in single line, spermatheca rounded to oblong in shape, offset and filled with tiny spheroid sperm cells. Crustaformeria more or less indistinct visible as a combination of small cells in few specimens; uterus thin-walled and less developed lacking a post uterus sac (PUS); vagina oblique. Vulva a wide transverse slit, not modified, vulval lips usually bearing two to three annuli appearing rounded protuberant. Tail elongate conoid with distinct annulation, tapering gradually and uniformly; approximately three times anal body diam. in length. Distal portion in some specimens slightly offset with a shallow depression at terminal one-third of tail region and tapering to a narrowly rounded to acute terminus. Intestine with indistinct lumen; rectum faintly distinguishable, most likely non-functional.

Male. Body almost cylindrical, slightly curved ventrally, marked by finer annuli than female. Labial region distinctly trapezoid, offset by distinct expansion, without annulation. Lateral fields marked by three distinct lines, two outers incisures strongly areolated. Pharynx consumptive, sometimes appearing as an open chamber. Hemizonid distinct, three to four annuli long, located three to five annuli anterior to excretory pore, marked externally by slight elevation of cuticle. Spicules robust and semi-circular; the tips slightly recurved; penial tube well-developed and almost completely covering spicule. Gubernaculum slightly curved, and slightly thickened proximally. Bursa well developed, crenate, folded over ventrally and covering spicule. Tail elongate conoid, tapering to an acute terminus.

Juvenile. Body straight or slightly ventrally arcuate. Resembling female except for lower values of body length, stylet length, and total annuli body.

Type host and locality. Specimens were recovered during July to September 2015, from the rhizosphere of undetermined grasses collected in Tilekenar Forest (coordinates: 36°37'04.9"N 51°18'43.6"E), Motelqo city, Mazandaran province, northern Iran.

Type material. Holotype female, five paratype females and three paratype males (Slides HPH001- HPH003) deposited in the nematode collection of the Department of Plant Protection, College of Agriculture and Natural Resources, University of Tehran, Iran. Three female paratypes and two paratype males deposited in the National Nematode Collection of the Department of Nematology, Iranian Research Institute of Plant protection, Tehran, Iran. Two paratype females and two paratype males deposited in Wageningen Nematode Collection, The Netherlands.

Polytomous key code. According to the polytomous key of Chitambar and Subbotin (2014) the new species has the following specific alphanumeric codes: A2, B7, C4, D1, E2, F1, G2, H1, I2, J2, K4, L3, M2, N1, O23, P1, Q2, R2, S3, T12, U2, V-, W2, X2, Y1

Diagnosis. *Hemicycliophora paraconida* n. sp. is characterized by female being 907–1,198 µm long with 286–341 body annuli. Broad labial region, anteriorly truncate, bearing two distinct annuli and an elevated labial disc. Stylet 96–115 µm long with three rounded, posteriorly sloped knobs creating a large "cavity". Lateral fields with by three longitudinal lines forming two rows of blocks with breaks and anastomosis of transverse striae; each annulus in lateral field with four hardly visible subcuticular ovate markings. Vulva not modified and vulval lips appearing rounded protuberant, bearing two to three annuli. Tail elongated uniformly conoid except for slightly marked narrower terminal third, with distinct annulation and narrowly rounded to acute

tail end. Male with robust and semi-circular spicules with slightly recurved tips, anteriorly inside of well-developed penial tube of 15–19 µm.

Relationships. After molecular comparisons, four species namely *H. conida*, *H. hellenica* Vovlas, 2000, *H. poranga* (syn. *H. ripa* Van den Berg, 1981) (van den Berg et al., 2018) and *H. halophila* Yeates, 1967 shows closest similarity to the new species. Furthermore, three other species, i.e. *H. iwia* Brzeski, 1974, *H. ovata* Colbran, 1962, *H. ornamenta* Bajaj, 1998 are selected because of the similarity in morphological characters such as Lateral field structure, cephalic region shape, body length and female tail shape.

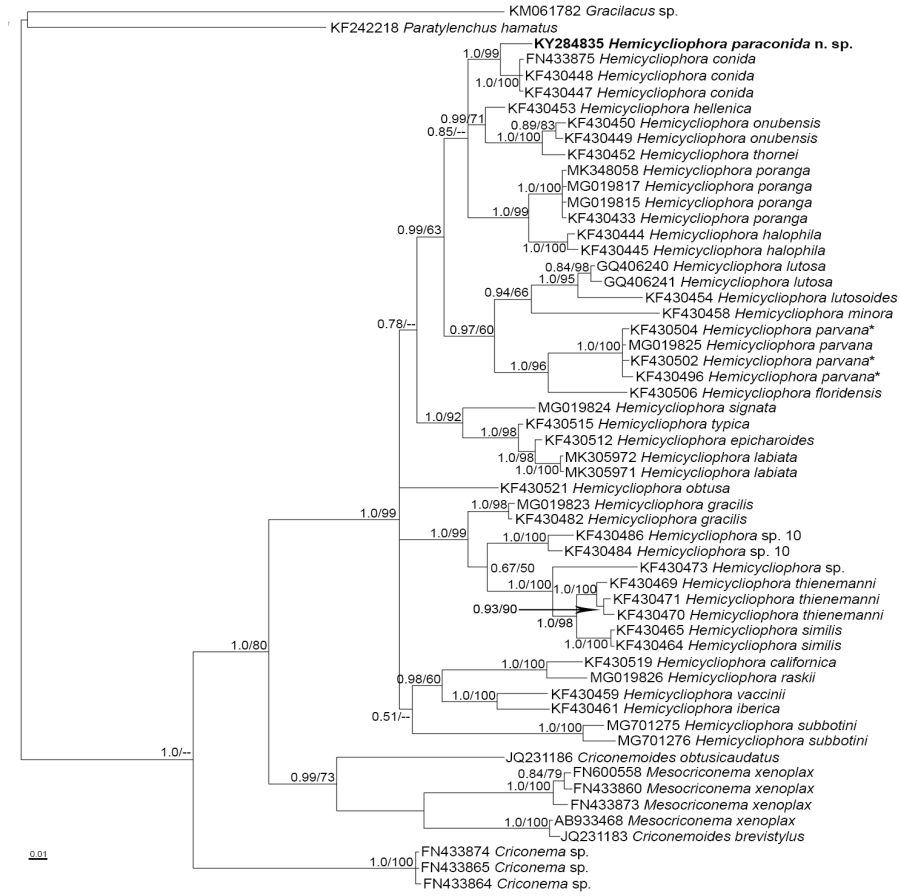
According to morphological, morphometrical and molecular comparisons, *Hemicycliophora conida* is the closest species to the new species. Table 2 represents differential morphometric characters of *H. paraconida* n. sp. and *H. conida*; the new species has a longer body length, longer stylet, more body annuli, longer tail, more posterior vulva and greater spicule compared to *H. conida.* The new species differs from *H. iwia* by having different type of lateral field (three faint lines *vs* without lateral line often with irregular longitudinal striae), anteriorly position of vulva (V = 82–84 *vs* 86–94), more body annuli (R = 286–341 *vs* 188–219), longer tail in female and male (c = 6.3–8.3 *vs* 13–16.6 and 5.5–6 *vs* 10.5, respectively) and sharper ending tail in both sexes. It differs from *H. ovata*, in having a slightly different lateral field (four subcuticular ovate markings *vs* two rows of ovate ornamentations), different vulval lips (vulval lips protuberant bearing two to three annuli *vs* not protuberant) and different tail shape (elongated uniformly conoid *vs* convex–conoid). The new species differs from *H. hellenica* by having shorter body length (907–1197 *vs* 1078–1634 µm and 944–994 *vs* 1013–1302 µm in female and male respectively), different cephalic region (anteriorly truncate labial region with slightly elevated labial disc *vs* greatly protruding offset labial disc), shorter stylet (96–115 *vs* 115–143µm), shorter spicule (arc line = 54–58 *vs* 58–72 µm in arc line). It's distinguishable from *H. poranga* by greater length of female tail (c index = 6.3–8.3 *vs* 8.9–12) and slightly greater stylet length (96–115 *vs* 82–100 µm) (Chaves, 1983; Costa Manso, 1996; Crozzoli, Lamberti, 2006; Monteiro, Lordello, 1978). It differs from *H. ornamenta* by having slightly posteriorly position of vulva (V = 82–84 *vs* 78–81 %), less annuli number of whole body (R = 286–341 *vs* 355–390), different tail terminus (narrowly rounded to acute *vs* filiform), and completely different form of cuticular ornamentation (four subcuticular ovate markings *vs* appearing as blocks inside annuli). It differs from *H. halophila* by having more annuli number of whole body (R = 286–341 *vs* 190–238), greater length of female and male tail (c = 6.3–8.3 *vs* 9.1–14.6 and mean = 5.6 *vs* 8.6 respectively), shorter spicule (arc line mean = 55.5 *vs* 68 µm in arc line) and penial tube (17 *vs* 22µm) and shape of tail terminus (narrowly rounded to acute terminus *vs* rounded) (Reay, 1984; van den Berg, 1977, 1987; Yeates, 1967).

Molecular characterisation. Amplification of the D2-D3 expansion segment of 28S, sequence from *Hemicycliophora paraconida* n. sp. specimens yielded a single fragment of 698 bp. The 698-bp 28S D2-D3 sequence data was less than 97.9% homologous from any available DNA sequences from GenBank. For molecular analysis, the species with the highest identity matches in BlastN search were included. A majority consensus phylogenetic tree generated using both Bayesian and ML method of the D2-D3 of the 28S rRNA gene sequence alignment under the GTR + I + G model is presented in Figure 3. The dataset for phylogenetic tree composed of respectively 661 total characters from which 311 characters were variable after aligning with MAFFT and manually editing. Based on the D2-D3 28S gene sequence, *H. paraconida* n. sp. clustered with the phylogenetically most similar species *H. conida* (FN433875, KF430448 and KF430447) with maximal BPP and high ML BS values (0.1/99) (Fig. 3). This clade is a part of a greater clade with *H. hellenica*, *H. onubensis* van den Berg, Tiedt, Liebanas, Chitamber, Stanley, Inserra, Castillo,

Table 2. Comparison of morphometrics between *Hemicycliophora paraconida* n. sp. and *H. conida,* reported by different authors

	H. paraconida n. sp.	*H. conida* Thorne, 1955						
	Iran	Ireland		The Netherlands		Spain		USA
	Present syudy	Paratypes Loof (1968)	Paratypes Brezeski (1974)	Loof (1968)		Castillo et al. (1989)	Subbotin *et al.* (2014)	Subbotin *et al.* (2014)
Female (n)	12	7	4	100	83	9	10	3
L	1,017 (907–1197)	700–780	810 (750–850)	840 (660–990)	790 (640–870)	817 (683–912)	770 (683–912)	836 (810–873)
a	21 (19.5– 22.5)	18–23	24 (23–26)	23 (18–27)	23 (18–28)	21 (20–22.5)	28.6 (26–31)	25 (24–26.4)
b	5.3 (4.6–6.1)	4.9–5.2	5.5 (5.3–5.7)	5.2 (4.5–6)	5.1 (4.1–5.7)	5.7 (5.1–6.2)	5.4 (5.2–5.7)	5 (4.8–5.3)
c	7.4 (6.3–8.3)	9.5–11.4	10 (9.2–10.8)	11.1 (8.7–14.4)	11 (8.3–13.5)	10.3 (9–13.7)	11.4 (10.2–12.6)	10.1 (9.6–10.4)
c’	3.3 (2.8–3.9)	–	–	–	–	2.7 (1.9–3.2)	2.9 (1.3–3.6)	3.5 (3.5–3.6)
V	83.0 (82–84)	85–88	86 (85–87)	86 (84–88)	87 (83–90)_	84 (84–87)	85.6 (84–870	87.3 (86–88)
Stylet	104 (96–115)	73–82	82 (79–84)	87 (78–96)	80 (69–86)	85 (77–94)	74 (70–80)	83.6 (77–89)
Tail length	138 (118–150)	–	–	–	–	80 (60–93)	73 (66–79)	83 (78–91)
R	304 (286–341)	180–201	202 (190–211)	245 (227–274)	204 (180–220)	231 (219–240)	238 (209–278)	250 (246–254)
RV (ant)	244 (230–272)	–	165 (155–170)	–	–	–	194 (167–229)	206 (202–210)
RV (pos)	60 (53–70)	31–41	–	49 (38–57)	41 (35–46)	46 (41–51)	44 (42–49)	45 (41–49)
PV/ABD	4.3 (4.9–5)	–	–	–	–	4.6 (3.9–5.6)	4.7 (4–5)	5.2 (5–5.6)
Male (n)	10	–	–	51	5	–	–	–
L	973 (944–994)	–	–	660 (530–840)	620–680	–	–	–
a	37 (33–41)	–	–	30 (22–39)	29–33	–	–	
b	7.5 (6.8–8.4)	–	–	6.1 (5.1–7)	5–6	–	–	–
c	5.6 (5.5–6)	–	–	7.2 (6.4–9)	7–8	–	–	–
c’	6.5 (6–7)	–	–	5.6 (4.6–6.1)	4.9–5.2	–	–	–
Spicule width	28 (27–29.5)	–	–	23 (18–29)	18–20	–	–	–
Tail length	172 (166–179)	–	–	92 (79–109)	81–88	–	–	–

Subbotin 2018, *H. thornei*, *H. poranga* and *H. halophila*. The comparison of pairwise sequence revealed that the sequences of 28S domain for the new species with other similar species were largely different and the sequence divergences are marked in the tree (Figure 3). The branch length of the new species clade was fairly long compared with specimens of *Hemicycliophora conida,* its sister species.



* Identified as *H. wyei* by Zeng et al. (2015).

Figure 3. Phylogenetic relationships of *Hemicycliophora paraconida* n. sp. within related species and genera based on partial 28S D2/D3 under GTR+I+G model. Bayesian posterior probabilities (BPP) and maximum likelihood bootstrap (ML/BS) values >50% are given for appropriate clades in the form: BPP/ML BS

Alignment of the D2-D3 28S sequence obtained from the new species with the sequence from the molecularly similar species of *H. conida* isolates, yielded 692–661 total characters with 675–647 identical nucleotides (97.54–97.90% identity). The new species forms a well-supported molecular clade with *Hemicycliophora* spp. (BPP/ML, 0.1/99) which is in accordance with

Subbotin et al. (2014). However, according to the 28S tree topology, the new species clade is sister to all other *Hemicycliophora* as in Subbotin et al. (2014), whereas ancestral states in the D2-D3 28S gene sequence tree, it is sister to an assemblage of three paraphyletic genera, *Criconema* Hofmänner & Menzel, 1914, *Criconemoides* Taylor, 1936 and *Mesocriconema* Andrássy, 1965. In our phylogram, *Hemicycliophora* appeared to be monophyletic, a finding that agrees with previous studies (Subbotin et al., 2014; Subbotin et al., 2005).

Because of the unevenness of deposited sequences, *e.g.*, many species were sequenced with only one locus, either half or near-full-length of 18S or 28S domain and other markers, more detailed phylogenetic relationship of new species is not clear. However, both Bayesian and Maximum likelihood phylogenetic analysis suggested that the new species belongs to Clade I (Subbotin et al., 2014), which includes *H. conida*, *H. poranga*, *H. halophila*, *H. thornei* Goodey, 1963, *H. hellenica,* and many other undescribed/unidentified species.

Remarks. *H. conida* shows a wide range of morphometrical features in different populations (Brzeski, 1974; Castillo et al., 1989; Loof, 1968; Subbotin et al., 2014). The populations of this species were divided into two conspecific morphological forms only based on their morphometric features (Loof, 1968). The form I with longer female body size at least *ca* 230 annuli, Secretory-excretory pore located *ca* 50 annuli from the anterior end, stylet length with average of *ca* 90 µm and spicule chord (straight distance between extremities) *ca* 23 µm. The form II, has 180–220 annuli in female body, excretory pore *ca* 40 annuli from the anterior end, average stylet length of 80 µm and spicule width *ca* 19 µm. The new species shows highest morphological similarity to *H. conida* within the genus, although molecular study proved the uniqueness of the new species. The difference between these two close species become greater when they were morphometrically compared (Table 2). However, between these two morphometrical forms of *H. conida*, the new species came closer to form I, although *H. paraconida* n. sp. was distinct from Form I with having $R \geq 285$ annuli (vs. 230) in female body, $R_{ex} = 52$ annuli (vs. 40), average stylet length of *ca* 104 µm (vs. 90 µm) and spicule width *ca* 28 µm (*vs* 23 µm).

Discussion

This study described a new sheath nematode species with its molecular characterization on D2-D3 expansion segment of 28S rDNA. Molecular analysis was performed based on rDNA sequence and proved this sequence region efficiency in differentiation among morphologically closed populations of *Hemicycliophora*.

Although *Hemicycliophora* spp. have many distinct morphological characters, it becomes very hard, time-consuming and somehow impossible to identify the species using only their morphology, mainly because of the overlapping in morphological features of the species and intra- and inter-specific variations. It suggested that integrative taxonomy using both morphological and molecular study will increase accuracy and decrease the difficulty for identification in this species rich genus. Furthermore, approving novelty of *H. paraconida* n. sp. as a new species using molecular study, despite having morphological similarities with *H. conida* can be considered as an example of the existence of cryptic species among the species of genus, especially those with wide range conspecific features. Furthermore the current study suggests that two conspecific forms *i.e.*, form I and II in *H. conida* may represent two distinct species. In this regard, phylogenetic study on two conspecific forms of *H. conida* is highly recommended as small sequence divergences within the species-complexes may be interpreted as various stages in the speciation process, from recently diverged populations to distinct biological species.

The proposal of *H. wyei* Cordero López, Robbins and Szalanski, 2013 as a junior synonym for *H. parvana* by van den Berg et al. (2018) is also approved in our molecular analysis with maximal support (BPP/ML, 0.1/100). Moreover, the results of the present study also suggest that the observed genetic diversity of *Hemicycliophora* is significantly higher than that shown by morphological observations. Thus, species diversity in *Hemicycliophora* based on morphological characters needs a thorough re-examination of the type materials of the genus. Also, our data suggest that the biodiversity of sheath nematodes is still not fully clarified and other morphological data are needed to help understanding the taxonomy and molecular phylogeny of the genus in the future.

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Richness and diversity of odonates of the agricultural college and research institute, Vazhavachanur, Tamilnadu, India

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Keywords Vazhavachanur, Dragonfly, Damselfy, Libellulidae and Coenagrionidae

Abstract Investigations on the diversity of Odonata in and around the Agricultural College and Research Institute, Vazhavachanur, Tamil Nadu, India were studied. Eight locations were selected, of which sixteen Odonata species were recorded. In total, eleven dragonfly and five damselfly species were identified from Thiruvannamalai district, Tamil Nadu, India. *Pantala flavescens, Diplacodes trivialis*, *Brachythemis contaminata* and *Ischnura aurora* were recorded from all eight locations. *Trithemis pallidinervis* and *Agriocnemis pygmaea* were recorded from seven locations except from the farm pond and the open stretch area. *Rhyothemis variegata was* recorded only at the open stretch area. The results clearly show that, Odonates have specific habitat preferences for their growth and development. Four families Libellulidae, Gomphidae, Aeshnidae and Coenagrionidae were observed and collected during the study. Libellulidae were the most abundant family (56.25%) and comprised of 9 species, followed by Coenagrionidae (31.25%) with 5 species. Low species abundance was recorded within the Gomphidae and Aeshnidae families with 6.25% each. Simpson and Shannon Index showed maximum diversity in the open stretch area (0.8743 and 2.186, respectively) and minimum in location 8 (crop production area) and location 4 (Sathakuppam), respectively. Open stretch area showed maximum Richness Index (Margalef) (2.275) followed by farm pond (2.196) where recorded minimum in sathakuppam (1.276) location. Equitability J index, maximum species evenness (0.788) was recorded Location 6 i.e. farm pond (0.6727) followed by open stretch area 0.686.

Bogactwo i różnorodność ważek z terenu kolegium rolniczego i instytutu badawczego, Vazhavachanur, Tamilnadu, Indie

Słowa kluczowe Vazhavachanur, ważki różnoskrzydłe, ważki równoskrzydłe, Libellulidae i Coenagrionidae

Streszczenie Zbadano różnorodność Odonata w Kolegium Rolniczym i Instytucie Badawczym w Vazhavachanur, Tamil Nadu w Indiach i wokół niego. Wybrano osiem lokalizacji, gdzie

odnotowano 16 gatunków Odonata. Łącznie w okręgu Thiruvannamalai w stanie Tamil Nadu w Indiach zidentyfikowano 11 gatunków ważek różnoskrzydłe i 5 gatunków ważek równoskrzydłych. *Pantala flavescens*, *Diplacodes tryialis*, *Brachythemis contaminata* i *Ischnura aurora* odnotowano we wszystkich ośmiu lokalizacjach. *Trithemis pallidinervis* i *Agriocnemis pygmaea* odnotowano w siedmiu miejscach, z wyjątkiem stawu hodowlanego i otwartego obszaru. *Rhyothemis variegata* odnotowano tylko na otwartym terenie. Wyniki wyraźnie pokazują, że ważki mają określone preferencje siedliskowe dotyczące ich wzrostu i rozwoju. Podczas badań zaobserwowano i zebrano cztery rodziny: Libellulidae, Gomphidae, Aeshnidae i Coenagrionidae. Najliczniejszą rodziną były Libellulidae (56,25%), składające się z 9 gatunków, następnie Coenagrionidae (31,25%) z 5 gatunkami. Niską liczebność gatunków odnotowano w rodzinach Gomphidae i Aeshnidae po 6,25%. Indeks Simpsona i Shannona wykazał maksymalne zróżnicowanie na otwartym obszarze (odpowiednio 0,8743 i 2,186) i minimalne w lokalizacji 8 (obszar upraw) i lokalizacji 4 (Sathakuppam). Otwarty obszar wykazywał maksymalny wskaźnik bogactwa (Margalef) (2,275), następnie był staw hodowlany (2,196), minimalny wskaźnik bogactwa odnotowano w lokalizacji Sathakuppam (1,276). Wskaźnik równości J, maksymalna równość gatunkowa (0,788) odnotowano w lokalizacji 6, tj. Staw hodowlany (0,6727), a następnie na otwarty obszarze (0,686).

Introduction

Odonataare an important predatory order which includes Dragonflies (Anisoptera) and Damselflies (Zygoptera). The odonate larvae are in aquatic habitat and the adults are excellent aerial predators. The insect possess large compound eye which helps to capture the prey comfortably. Of which, dragonflies are performing strong flying activity under the suborder called Anizoptera and damselflies are weak flier under the suborder of Zygoptera (Tofilski, 2004). The insects distributed almost throughout the world (Tillyard, 1917). Selvarasu et al. (2019), reported that it is the second largest aquatic insect order in the animal kingdom. Chovanec and Waringer (2001) reported that Odonates are so sensitive and it will react immediately even small fraction of changes in the habitation.Rowe (2003) reported that, the odonates nymph indirectly helps to control the epidemic diseases of human beings *viz*., malaria, dengue, filaria, since, the mosquito larvae are aquatic and it will be of good source of food for odonates nymphs. It prefers live fresh water, non-polluted and oxygenated habitats (Manwar et al., 2014). Nesemann et al. (2011) reported that, odonates are playing an important role in climate change mainly due to evolutionary history and its adaptations.

In India, 474 species and 50 subspecies which belongs to 142 genera and 18 families were recorded. Seventeen Odonates species recorded from Howrah district, West Bengal (Dwari, Mondal, 2017) and 36 species from Meghalaya (Acharjee, Karzee, 2016) in India. The Odonates diversity have been studied in all the agro-ecosystems, wherein 31 species were recorded in Amravathi city, Maharashtra (Rathod et al., 2012)

Many studies were carried out on Odonates diversity different parts of Tamil Nadu, *viz*., 16 species in rice field from Coimbatore district (Gunathilagaraj et al., 1999), 21 species from Coimbatore and Salem district (Arulprakash, Gunathilagaraj, 2010), 29 species from Kanyakumari district (Elanchezhyan et al., 2017) and 8 species were recorded in Mangroove forest (Muthukumaravel et al., 2015). Odonata diversity is lacking in Thiruvannamalai district, Tamil Nadu and hence, the present study have been undertaken forthe taxonomic identification, richness and abundance.

Materials and methods

The present study was undertaken in and around Agricultural College and Research Institute, Vazhavachanur, Thiruvannamalai, Tamil Nadu. Annual rainfall of Thiruvannamalai district is 1074.70 mm, the average annual temperature is 28.3°C and it comes under the tropical climate. Thiruvannamalai gets more rainfall during the summer season than winter season. Nearly 45% of the rainfall is received during north east monsoon period (October–December). The sources of water is Sathanur Dam, wells and in some cases bore wells. Most commonly available tree species in our collection premises are Neem, Vaagai, Silk cotton, Tamarind tree, Banyan tree, Peepal tree, Prosopis, etc. and common weed species are *Cyperus*, *Lantana*, *Parthenium*, *Chaff flower*, swollen finger grass, wire weed, Crab grass, *Corchorus* etc.

Wet land ecosystem

It is distinct ecosystem which is inundated by water either permanently or seasonally. In the present study, the paddy crop was used for collection of odonates to study the abundance and species richness. Six different paddy field were selected to observe the abundance of odonates. Each paddy field showed different growth phases and the bunds also were smothered with vigorous weed growth.

Pond (Stagnated water)

To study the Odonata diversity the College farm pond was selected where human inferences is very minimum. The Farm pond gets filled with water during January to March whenever there is flow of water from the canal present near the college boundary. The water will be available for few months and gets dried up during summer.

River canal

It is located adjacent to Thenpennai river for irrigation of farmer field and it is near to campus.

Open land stretch

Barren land were selected to study the diversity.

Geographical information's

Table 1. Geographical information of the different locations

Location details	Latitude (n)	Longitude (e)	Altitude (m)	Crop	Aquatic status	Shade cover
Seed farm 1	12°4'37"	78°58'60"	153.98	Paddy	Seasonal aquatic	Partial
(Paddy field) Near college gate	12°4'18"	78°59'8"	158.98	Paddy	Seasonal aquatic	Partial
Seed farm 2	12°4'28"	78°59'59"	149.98	Paddy	Seasonal aquatic	Partial
Sathakuppam	12°4'18"	78°58'41"	148.98	Paddy	Seasonal aquatic	No
Open stretch area	12°4'33"	78°59'0"	156.98	–	Seasonal aquatic	Partial
Farm pond	12°4'33"	78°59'6"	165.98	–	Temporary	Partial
Seed farm 3	12°4'39"	78°58'83"	164.65	Paddy	Seasonal aquatic	Partial
Crop production area	12°4'22"	78°59'12"	141.98	Paddy	Seasonal aquatic	No

Survey and collection of Odonates

Odonata specimens were collected through direct survey from various locations in and around the Agricultural college campus. The areas surveyed are presented in Plate 1, 2 and 2a. Collections were done by different methods *viz*., zigzag collection, visual observation were also done. Zigzag method is used to collect odonates by using aerial net along with walk. Transect or zigzag walk conducted to cover the selected areas. This method has been widely used for quantitative sampling encountered while visual survey along transects is used for abundance of insect species. The total number of individuals in each species encountered during six months of sampling period (December 2018 to April 2019) is used for comparing their abundance and diversity.

The collection of insects were done by using a long-handled aerial net. A net opening of at least 30 cm diameter makes the job easier. Some collectors feel that a dark net bag (black or green) is less conspicuous to dragonflies and thus is more effective than a white one. Observing patrolling dragonflies before swinging at them often pays off; positioning yourself in the most advantageous location, especially if it is somewhat concealed, is usually fruitful. When attempting to a dragonfly, move deliberately. Refrain from waving the net around; keep it as inconspicuous as possible. Swing at fast-flying agile species from behind as they fly by; many will easily dodge a net swung head-on. Collected insects were anaesthetized and preserved properly for further studies.

The collected odonates were photographed for the species identification. Odonate richness, abundance and diversity (Ludwig, Reynolds, 1998) was calculated by using the software 'Biodiversity Calculator':http://www.alyoung.com/labs/biodiversity_calculator.html.

Results

A total of 16 odonates species representing 4 families *viz*., Libellulidae (9), Gomphidae (1), Aeshinidae (1) and Coenagrionidae (5) were observed and taxonomic studies were studied. The detailed taxonomic identification from genera, species, family level have been identified.

Diversity of Odonata

Out of 11 dragonfly species, *Pantala flavescens* were observed from paddy field and Open stretch area and recorded more population (303 ind.) followed by *Diplacodes trivialis* (195 ind.) observed from farm pond and least populations were recorded from *Anax* sp. (2 ind.) were observed from open stretch area (Table 2).

Table 2. Diversity of odonata in & around ac&ri, Vazhavachanur from December to April

No.	Common name	Scientific Name	Family	Habitat	Abundance (Nos.)
Dragonfly					
1.	Wandering glider	*Pantala flavascens* (Fabricius, 1798)	Libellulidae	Paddy field & Open stretch area	303
2.	Chalky percher	*Diplocodes trivialis* (Rambur, 1842)	Libellulidae	Farm pond	195
3.	Ditch jewel	*Brachythemis contaminata* (Fabricius,1793)	Libellulidae	Paddy field & Open stretch area	98
4.	Coral- tailed cloud wing	*Tholymis tillarga* (Fabricius,1793)	Libellulidae	Farm pond	43

Continuation of Table 2

5.	Long – legged marsh glider	*Trithemis pallidinervis* (Kirby, 1889)	Libellulidae	Paddy field	142
6.	Scarlet skimmer	*Crocothemis servilia* (Drury, 1770)	Libellulidae	Paddy field & Open stretch area	67
7.	Slender skimmer	*Orthetrum sabina* (Drury, 1770)	Libellulidae	Open stretch area	39
8.	Variegated flutterer	*Rhyothemis variegata* (Linnaeus, 1763)	Libellulidae	Openstretch area	5
9.	Common club tail	*Ictinogomphus rapax* (Rambur, 1842)	Gomphidae	Farm pond	9
10.	–	*Anox* sp.	Aeshnidae	Open stretch area	2
11.	Asian pintail	*Acisoma panorpoides* (Rambur, 1842)	Libellulidae	Open stretch area	8
Damselfly					
1.	Yellow wax tail	*Ceriagrion coromandelium* (Fabricius, 1798)	Coenagrionidae	Paddy field & Farm pond	41
2.	Blue river damsel	*Pseudagrion microcephalum* (Rambur, 1842)	Coenagrionidae	Farm pond	51
3.	Golden darlet	*Ischnura aurora* (Brauer, 1865)	Coenagrionidae	Paddy field	502
4.	Wandering midget	*Agriocnemis pygmaea* (Rambur, 1842)	Coenagrionidae	Paddy field	283
5.	Marsh blue tail	*Ischnura senegalensis* (Rambur, 1842)	Coenagrionidae	Farm pond	32
Total					1,820

Details	Loc 1	Loc 2	Loc 3	Loc 4	Loc 5	Loc 6	Loc 7	Loc 8
Dragonfly								
Pantala flavescens	✓	✓	✓	✓	✓	✓	✓	✓
Diplocodes trivialis	✓	✓	✓	✓	✓	✓	✓	✓
Brachythemis contaminata	✓	✓	✓	✓	✓	✓	✓	✓
Tholymis tillarga	–	–	–	–	–	✓	–	–
Trithemis pallidinervis	✓	✓	✓	✓	✓	–	✓	✓
Crocothemis servilia	✓	✓	✓	–	✓	✓	✓	–
Orthetrum sabina	✓	✓	✓	✓	✓	✓	✓	–
Rhyothemis variegata	–	–	–	–	✓	–	–	–
Ictinogomphus rapax	–	–	–	–	✓	✓	–	–
Anax sp.	–	–	–	–	✓	–	–	–
Acisoma panorpoides	–	–	–	–	✓	✓	–	–
Damselfly								
Ceriagrion coromandelium	–	–	✓	–	✓	✓	–	–
Pseudoagrion microcephalum	–	–	–	–	✓	✓	–	–
Ishnura aurora	✓	✓	✓	✓	✓	✓	✓	✓
Agriocnemis pygmaea	✓	✓	✓	✓	✓	✓	✓	✓
Ishnura senegalensis	–	–	–	–	✓	✓	–	–

Out of 5 damselfly species, *Ischnura aurora* (502 ind.) dominated in and around the AC&RI, Vazhavachanur campus which was recorded from paddy field followed by *Agriocnemis pygmaea* (283 ind.) also recorded from Paddy field and *Ischnura senegalensis* (32 ind.) recorded very less population from farm pond in our campus.

Diversity of Odonata in different locations

The dragonflies, *Pantala flavescens, Diplacodes trivialis* and *Brachythemis contaminata* and damselflyspecies, *Ischnura aurora* were recorded from all eight location (Table 3).

Table 3. Diversity of Odonata in different locations

Loc 1: Seed farm 1	Loc 5: Open stretch land
Loc 2: Paddy field (Near college gate)	Loc 6: Farm pond
Loc 3: Seed farm 2	Loc 7: Seed farm3
Loc 4: Sathakuppam	Loc 8: Crop production area

Trithemis pallidinervis and *Agriocnemis pygmaea* recorded seven locations except farm pond and open stretch area. Whereas, *Rhyothemis variegata* recorded only one location i.e., open stretch area. The results clearly shows that, Odonates have specific habitat for its growth and development.

Odonata diversity in different family level

Representatives of four families *viz*., Libellulidae, Gomphidae, Aeshnidae and Coenagrionidae were observed and collected during the study. Out of four families, Libellulidae occupies maximum abundance (56.25%) which comprises of 9 dragonfly species, followed by Coenagrionidae (31.25%) occupies 5 damselfly species and minimum of Gomphidae and Aeshnidae recorded 6.25% and 6.25%, respectively (Figure 1).

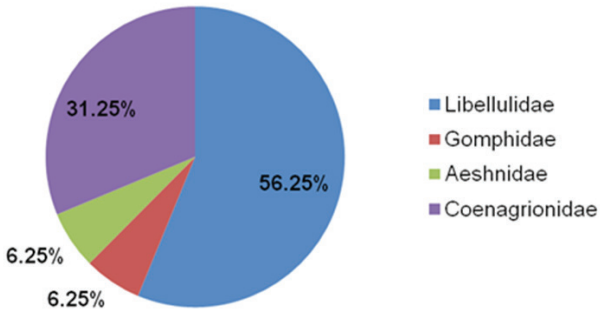


Figure 1. Abundance of Odonata in different families

Abundance and Diversity of Odonata

Simpsonand Shannon Index showed maximum diversity in the open stretch area (0.8743 and 2.186, respectively) and minimum in location 8 (crop production area) and location 4 (Sathakuppam), respectively (Table 4). Open stretch area showed maximum Richness Index

(Margalef) (2.275) followed by farm pond (2.196) where recorded minimum in Sathakuppam (1.276) location. Equitability J index, maximum species evenness (0.788) was recorded Location 6 i.e. farm pond (0.6727) followed by open stretch area 0.686.

Table 4. Abundance and diversity of Odonata

Location	Simpson dominance index	Shannon index	Margalef richness index	Evenness
Location 1	0.6777	1.391	1.427	0.502
Location 2	0.7996	1.691	1.665	0.609
Location 3	0.8176	1.766	1.831	0.637
Location 4	0.6015	1.242	1.276	0.448
Location 5	0.8103	1.904	2.275	0.686
Location 6	0.8743	2.186	2.196	0.788
Location 7	0.8165	1.752	1.659	0.632
Location 8	0.6727	1.281	1.385	0.462

Loc 1: Seed farm 1.
Loc 2: Paddy field (Near college gate).
Loc 3: Seed farm 2.
Loc 4: Sathakuppam.
Loc 5: Open stretch land.
Loc 6: Farm pond.
Loc 7: Seed farm 3.
Loc 8: Crop production area.

Discussion

Odonates are extremely susceptible to the insecticides and the number of doses will be increased in the rice filed, the abundance also reduced (Smith, Straton, 1986; Tang, Siegfried, 1995).

The odonata family Libellulidae was the widespread species and this was in accordance with the result of Shelton and Edwards (1983). In our study also, the Libellulidae family accounts 56.25% besides four families. It clearly shows that, this family is one of the most prevalent one all over the globe. Biotic and abiotic factors straightly affect the odonate insect inhabitants (Kittelson 2004; Goldsmith 2007; Bispo, Oliveira 2007).

Damselfly population was recorded minimum when compare to dragonfly in this study. This is in accordance with the results of Weir (1974) who stated that less abundance is mainly due to their limited dispersal ability and Clark and Samways (1996) reported that partial/ absence of shade cover. Arulprakash and Gunathilagaraj (2010) also reported that shade over the habitat which facilitates rich abundance of damselflies. Maximum Odonates were observed and recoded from the farm pond area. This was in accordance with the studies of Baruah and Saikia (2015) i.e., water bodies will attract the insects.

Krishnasamy et al. (1984), Asaithambi and Manikavasagam (2002) and Bhatacharya et al. (2006) reported that *Diplacodes trivialis*, *Pantala flavescens*, *Orthetrum sabina* (Anisoptera), *Ischnura aurora* and *Agriocnemispygmaea* (Zygoptera) were the dominant species and observed from transplanting to harvesting stage. This was also in accordance with the present study.

Open Stretch area recorded maximum richness (2.275) followed by farm pond (2.196) in our study, where as Baruah and Saikia (2015) also reported that species richness or Margalef's richness (DMg) index was found to be maximum in open tracts.

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By-catch mortality of beaked Sea Snake *Hydrophis schistosus* (Daudin, 1803) by entanglement in shore-seine operation in Goa-India

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Keywords Sea snake, *Hydrophis schistosus*, *Arius jella*, by-catch, Goa; India

Abstract *Hydrophis schistosus* (Daudin, 1803) commonly known as beaked sea snake occurs in shallow marine and estuarine habitats of India. The population of *H. schistosus* has been frequently noted in the shore-seine net as By-catch. Only a few studies are available about By-catch of sea snakes in Indian coasts. Being a top predator, their mortality and ecological consequences are largely unknown. The present study was conducted along the Caranzalem-Miramar coast of Caranzalem bay, Goa during monsoon (August-September, 2017 and 2018) to focus on the by-catch mortality of *H. schistosus* and their feeding preferences. Observations indicated that the fishing activities, particularly during every shore-seine operation, results in a significant by-catch of sea snakes where approximately 20–60 individuals get entangled in the fishing net. The study indicated a large number (90%) of juveniles being caught in the shore-seine. Gut content analysis of *H. schistosus* revealed that 80% of snakes preferred catfish *Arius jella* as the desired prey species. Sea snakes being top predator play a vital role in checking the local catfish population. Hence, in concern to save *H. schistosus* population and sea snakes, in general, the fishing methods, as well as awareness towards conservation of sea snakes among fishermen, should be raised.

Śmiertelność węża morskiego Hydrophis schistosus (Daudin, 1803) w sieciach typu niewód w Goa – Indie

Słowa kluczowe wąż morski, *Hydrophis schistosus*, *Arius jella*, sum morski, przyłów, Goa, Indie

Streszczenie Wąż *Hydrophis schistosus* (Daudin, 1803) występuje powszechnie w płytkich wodach morskich i estuariach na wybrzeżu Indii, gdzie często jest łapany jako przyłów w sieciach typu niewód.

Do dzisiaj niewiele wiadomo na temat wielkości przyłowu tego gatunku na wybrzeżu Indii. Ponieważ wąż ten jest drapieżnikiem znajdującym się wysoko w piramidzie troficznej ekosystemu wód przybrzeżnych Indii, jego dodatkowa śmiertelność może mieć znaczący wpływ na funkcjonowanie całego ekosystemu. W artykule zawierto dane dotyczące wielkości przyłowu I preferencji pokarmowych *H. schistosus* z wybrzeża Caranzalem-Miramar zatoki Caranzalem (Goa) w czasie monsunu (sierpień–wrzesień, 2017 i 2018 r.). Obserwacje wskazują, że aktywność rybacka, a szczególnie używanie sieci typu niewód, powodują znaczną śmiertelność węży (20–60 osobników zaplątanych w jednej sieci). Młode osobniki stanowią 90% przyłowu. Analiza żołądków *H. schistosus* wykazała, że 80% węży preferowała suma morskiego *Arius jella* jako zdobycz, co powodowało częste zaplątywanie się węży w sieci używane do połowu suma morskiego. Konieczne jest rozważenie alternatywnych metod połowowych, aby uniknąć przyłowu węży.

Introduction

Sea snakes are an integral part of coastal ecosystem in the tropical Indo-Pacific region and they comprise about 86% of living marine reptile species (Rasmussen, 2011). They inhabit the soft bottom shallow coastal seas to lagoons, estuaries and mangrove areas (Limpus, 1975; Sharma, 2007). Sea snakes are active migrants in the sea but they are less efficient on land and die usually if stranded on the beach for a longer duration (Whitaker et al., 2004; Duarte, 2009).

Sea snakes are protected in Australia under the Environment Protection Biodiversity and Conservation Act 1999, but they are listed as Least Concerned (LC) in the IUCN Red list of threatened species (IUCN, 2018). The beaked sea snake *Hydrophis schistosus* (=*Enhydrina schistosa*) (Daudin, 1803), is a well-distributed Indo-Pacific species and reported as most common species from the Indian coast (Lobo et al., 2004). This species is known for its highly potent neurotoxin as the venome more toxic than those of land snakes; nevertheless, they are found to be rarely aggressive (Chippaux, 1998; Senanayake et al., 2005) despite it, most of the fatalities due to sea snake's bite are associated with *H. schistosus* around the globe (Reid, 1975). The occurrence of this species has been well reported from the entire Indian coast (Lobo et al., 2004; Karthikeyan, Balasubramanian, 2007; Zacharia, 2008; Walmiki et al., 2012; Muthukumaran et al., 2015; Hatkar, Ramesh, 2016). Studies indicate that estuaries comprise a suitable habitat for this species during pre-monsoon and monsoon season to provide ample food or bearing young (Shantov, 1971; Wassenberg et al., 1994). *H. schistosus* is a piscivorous species preferentially feeding on Catfishes and Pufferfish (Voris et al., 1978; Garrett et al., 1982; Jeyabaskaran et al., 2015) but also forage on species of Clupeidae and Engraulidae and fish eggs (Lobo et al., 2005; Emilie et al., 2010).

In India, this species is also impacted by coastal pollution (Mote et al., 2015) and commonly encountered as a trawl by-catch and other fishing operation (Rao et al., 2017). The commercial fishing operation appears to be the foremost threat to this sea snake species due to high volume by-catch (Padate et al., 2009). Due to sporadic reporting of this species mainly as a product of by-catch associated mortality, a significant knowledge gap exists about the population ecology (Hatkar, Ramesh, 2016) and feeding preferences from Indian waters. The aim of the present study was to know the sea snakes by-catch fate and feeding preferences of these top predators. The present observation highlights the ecological importance of *Hydrophis schistosus* wherein we hypothesize that as mega-benthic predators they profoundly influence the local estuarine benthic food chain.

Materials and Methods

Study area

The present study was carried on the central west coast of India at Caranzalem-Miramar coast (3.5 km stretch, Lat. – 15º28'09.75"N; Long. – 73º47'40.01"E), located at the mouth of Mandovi estuary (Figure 1). This place is known for fishing by artisanal fishing by local community and also a popular tourist beach in Goa.

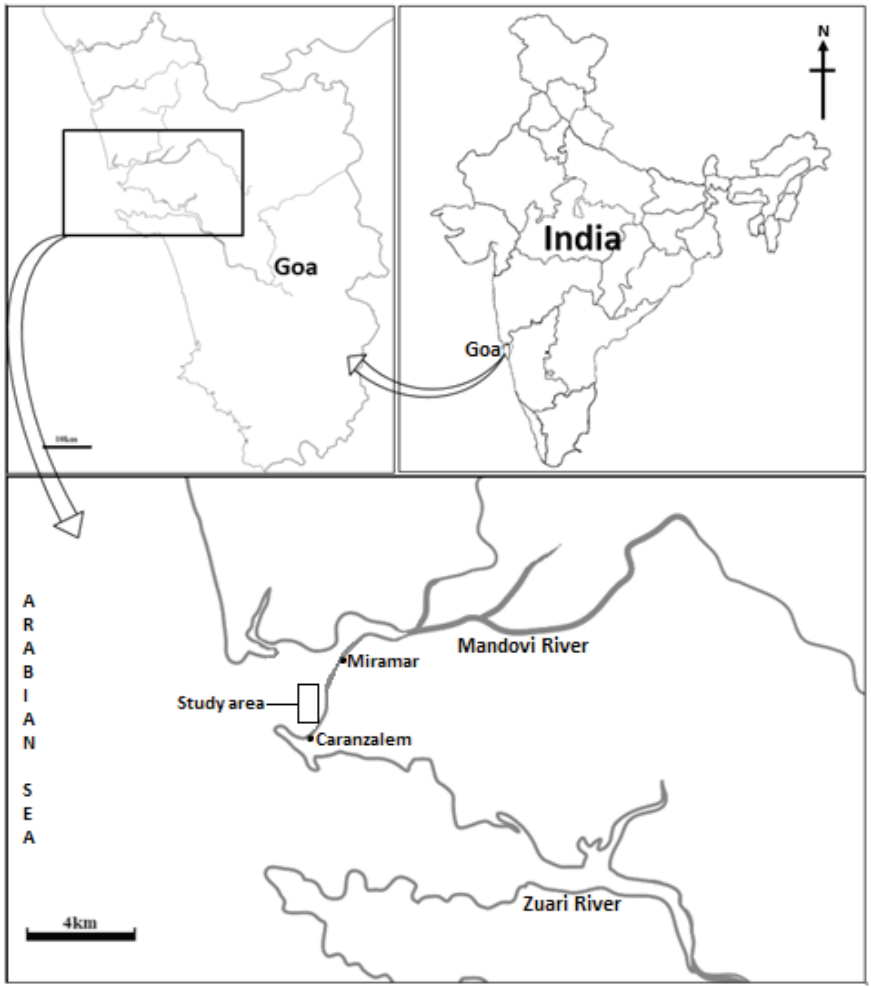


Figure 1. Location of the study area

Collection of sample

Sampling was carried out twice in a week during August–September 2017 and 2018 after the annual fishing ban period (June–July, a total 61 days during the Southwest monsoon). Counts were carried out of all the sea snakes entangled in the shore-seine net and landing on the beach, followed by a count of dead and live sea snakes. Immediately, after the sorting of landed fishes live snakes were released back into the sea while the dead snakes were brought to the laboratory for further analysis. Morphological identification confirmed that the sea snake species is *Hydrophis schistosus* (Rasmussen, 2001). Fishing gears operated in study area were noted, which revealed that traditional fishing activities such as shore-seine net (locally known as 'Rampani/Yandi'), operated at a depth of 4–5 m, covering an area of 1.5 km^2 at the mouth of the Mandovi estuary (Figure 2a). The mesh size varies from 10–20 mm and seine nets use sinkers at the bottom and float at the surface, and manually dragged. This traditional fishing method results in the considerable amount of by-catch along with the targeted fish species (Figure 2b). Significant by-catch of sea snakes *H. schistosus* during this fishing operation (Figure 2c) was noted. In the laboratory, measurements were conducted for length-weight of each dead snake specimen (n = 40) followed by gut content analysis (Figure 2d, e).



Figure 2. *In-s*itu and laboratory observations during the study (a) Seine net operation activity, (b) Discard of by-catch during sorting, (c) Sorted catch after hauling and By-catch removal (note the trails in the fore ground made by stranded snakes), (d) Sea snake species *Hydrophis schistosus*, (e) Dissected *H. schistosus*showing gut content , (f) Specimen from the gut (indigested *Arius jella*)

The species gut content confirmed as *Arius jella* using standard literature (Fischer and Bianchi, 1984).

Results

A total of 1,448 individuals (ind.) of *H. schistosus* were landed as by-catch during the study period. In 2017, 920 ind. were counted in by-catch (695 live and 225 dead), among those 89 were adults (9.67% of total catch; Mean 5.6 ±1.59 SD) and 831 juveniles (90.3% of total catch; Mean 51.9 ±11.82 SD). While in 2018, total 528 ind. of *H. schistosus* were recorded, among them 56 were adults (10.6% of total catch; Mean 3.5 ±1.90 SD) and 472 juveniles (89.4% of total catch Mean 29.5 ±12.69 SD), among those 407 were alive and 121 were dead (Table 1). Other by-catch species along with sea snake *H. schistosus* were Bamboo sharks, Stingrays, Guitarfishes, Puffers, Catfish and other pelagic juveniles of fishes.

Table 1. Account of *H. schistosus* by-catch during the study period

Year		Total no. of *H. schistosus*	Adults	Juveniles	Live	Dead
2017	total	920	89	831	695	225
	mean	57.5	5.6	51.9	43.4	14.1
	SD	12.61	1.59	11.82	11.74	4.55
	percentage	–	9.7	90.3	75.5	24.5
2018	total	528	56	472	407	121
	mean	33	3.5	29.5	25.4	7.6
	SD	14.13	1.90	12.69	10.09	4.47
	percentage	–	10.6	89.4	77.1	22.9

The length-weight ratio of *H. schistosus* indicated the presence of juveniles and sub-adults with an average length of 43 cm and weight 35 g (Figure 3). Among the dead specimen collected for laboratory analysis, only one adult was noted with length 97 cm and weighing 359 g. Generally, *H. schistosus* attain maturity at a length of 66–73 cm (Voris, Jayne, 1979; Rasmussen, 2001; Jeyabaskaran et al., 2015). The gut content analysis of *H. schistosus* revealed presence of catfish *A. Jella* in 80% of snake specimens (Figure 2f) and the remaining 20% guts were empty or with the semi-digested or completely digested material (identification of prey items was not possible). The length of *A. Jella* in snake gut varied between 4.32–11.68 cm (Mean 5.94 ±1.65 SD) and weighed between 0.11–11.79 g (Mean 1.42 ±1.89 SD).

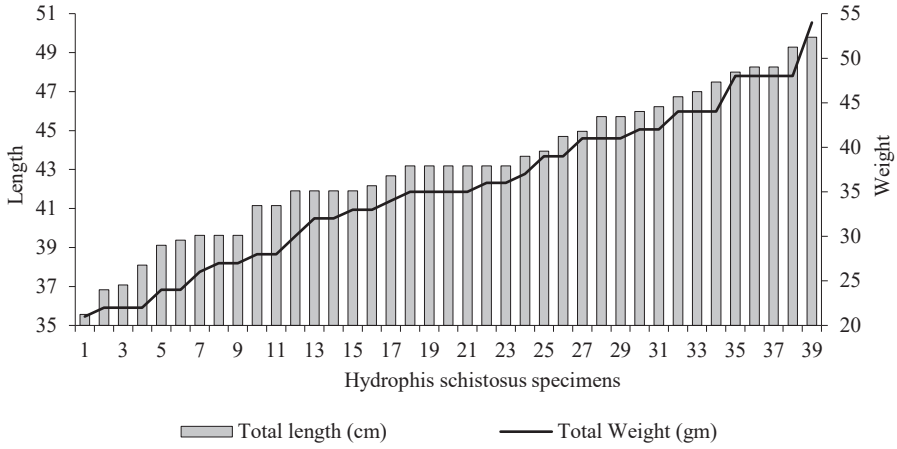


Figure 3. Length-weight ratio of *H. schistosus* sampled at the study location

Discussion

The mortality of sea snake *H. schistosus* was found to be high in this part of the Arabian Sea. Rao et al. (2017) recorded a total 379 species of sea snakes as by-catch at Vengurla, Devgarh and Sindhudurg districts of Maharashtra within 100 km of present study area. Whereas, Padate et al. (2009) encountered 43 individuals of *H. schistosus* in 70 trawl operations. While on the east coast of India, Muthukumaran et al. (2015) reported the occurrence of 234 entangled sea snakes in trawling nets and seine nets. Several studies revealed that most of the specimens were juveniles and sub-adults (Dell et al., 2009). Use of traditional shore-seine nets is widespread in the Indian coast and known for its by-catch discards where in sea snakes are usually discarded on the shore (Raj et al., 2017). Out of all the sea snakes, Wassenberg et al. (1994) revealed that about 88% of *H. schistosus* being caught as by-catch in the coastal region at the Gulf of Carpentaria, Australia.

During this study, approximately 20–60 live *H. schistosus* entangled were found after hauling of the shore-seine net on the beach (Table 1). Therefore, considering their high abundance in the by-catch indicates that estuarine mouth benthic environment serves as an important habitat for the juveniles by providing ample food. By-catch related mortality of juvenile of marine species arises across the world oceans, as well as in Indian seas is a serious concern (Bijukumar, Deepthi, 2006; Lobo, 2012). A large quantity of by-catch and discard due to trawling and unregulated use of small meshed net causes loss of marine life including endangered species like sea turtle, sea horses and elasmobranchs (Shankar et al., 2004; Salin et al., 2005; Tiralongo et al., 2018).

The present study site at the Mandovi estuary is known to be highly productive area (Kumari et al., 2002; Ansari et al., 2003) and attracts a rich diversity of fishes as it also serves as a nursery ground for most of the species. The high abundance of sea snakes during post-monsoon as compared to pre-monsoon season has been reported in estuarine and inshore waters of Goa and the abundance of small-sized fish attracts more sea snakes into this habitat (Padate et. al., 2009). Several mega-benthic predators including sea-snakes, mostly juveniles and sub-adults forage the

area and take refuge in the bay (Brown et al., 2013). Reportedly juveniles of *H. schistosus* may remain in estuarine and riverine habitats for protection and migrate into open water when nearing maturity (Voris, Jayne, 1979; Voris, 1985). The increased number of sea snake *H. schistosus* juveniles in the present study suggest that by-catch mortality is alarming in this area, compared to adjacent regions of Maharashtra, Pondicherry, Kerala and Tamilnadu states of India (Karthikeyan, 2007; Jeybasskaran et al., 2015; Muthukumaran, 2015; Rao et al., 2017). Sea snakes are also known for their philopatry and site fidelity (Shetty, Shine, 2002); therefore, continuous by-catch allied mortality of this species in the study area could wipe out the local population and could impose broad scale effect on the ecosystem as well as the artisanal fishery in the region.

The gut content analysis revealed the catfish *A. jella* as the only prey species of *H. schistosus* indicating species specificity. Such a high preference for Ariid catfishes was also noted by Voris et al. (1978) in a feeding experiment as *H. schistosus* prefers benthic organisms as their prey items (McCosker, 1975; Voris, Jayne, 1979). Moreover, the ray finned catfish *A. jella* is a known benthic predator (Ritukumari, Rajukumar, 2007) and regionally has low commercial value as compared to the other economically important fishes. This implies that as a top predator, this sea snake *H. schistosus* controls the population of *A. jella* in the coastal subtidal benthic habitats. As a megabenthic important predator, if *H. schistosus* a natural predator of *A. jella* is removed from the habitat, *A. jella* will dominate this habitat as it is voracious feeder and an omnivore. This dominance of *A. jella* will indirectly out compete other economically important species of Carangidae, Stromateidae, Sciaenidae, Sillaganidae and Cynoglossidae families which are wellnoted from the study area (Noted from shore-siene operation). Apparently, due to reduced predatory pressure, the catfish dominance could impact the population dynamics of other benthic species in the bay and result in population reduction of other commercially important species.

Although the exact population of the *H. schistosus* in the bay region is difficult to decipher, but based on the by-catch mortality it can be said that considerable loss of population may result in ecosystem-level repercussions. Because of habitat destruction and capture a large number of non-target species and juveniles, small meshed seine nets are considered to be detrimental practice (Divakarannair, 2007). However, by-catch reduction is achievable through mesh size regulation with fixed and regulatory bans (Boopendranath et al., 2007); hence, strict implementation of mesh size regulation should be followed. Additionally, development and use of excluder devices and speedy release of the entangled snakes could help to avoid human fatalities and save the sea snake population (Steve, 2007). In addition, worldwide decline of sea snake population is alarming and the high mortality rate of these diminishing populations could threaten the ecosystem (Gibbons, 2000). A long-term monitoring program and the implementation of proper conservation strategies are required to protect this species.

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Changes in innate and adaptive immune response to *Lagovirus europaeus* GI.1 (Rabbit Haemorrhagic Disease Virus -RHDV) infection

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Keywords neutrophil, lymphocytes, viral infection, rabbits, rabbit haemorrhagic disease virus

Abstract *Lagovirus europaeus* GI.1 (Rabbit Haemorrhagic Disease Virus – RHDV) is a virus that causes rabbit haemorrhagic disease (RHD) that affects domestic and wild rabbits worldwide. Due to the fact that the impact of RHDV on the host organism, and more precisely, its influence on the immune system, is crucial for the spread of this infection in rabbits, it is very important to understand the role of the immune system components in the course of infection with this virus. The aim of the study was to present innate and adaptive immune response to three strains (V411, 1092V, Wika) of the *Lagovirus europaeus* GI.1 (RHDV) identified at different times. Twelve indices were examined for the innate immune response and nine for the adaptive response. The results indicate that PMN cells (polymorphonuclear cells, neutrophils) play an important role in the response to this infection and give new insights into the function of $CD4^+$ Th and $CD8^+$ Tc lymphocytes. The most immunogenic was the Wika strain from 1996.

Zmiany we wrodzona i nabytej odpowiedź immunologicznej po zakażeniu *Lagovirus europaeus* GI.1 (Rabbit Haemorrhagic Disease Virus – RHDV)

Słowa kluczowe neutrofil, limfocyty, infekcja wirusowa, królik, wirus krwotocznej choroby królików

Streszczenie *Lagovirus europaeus* GI.1 (Rabbit Haemorrhagic Disease Virus – RHDV) infekuje króliki domowe i dzikie na całym świecie, wywołując u nich krwotoczną chorobę królików (RHD-rabbit haemorrhagic disese). Z uwagi na fakt, że wpływ RHDV na organizm żywiciela, a dokładniej jego wpływ na układ odpornościowy, ma kluczowe znaczenie dla rozprzestrzeniania się tej infekcji u królików, bardzo ważne jest poznanie roli elementów układu odpornościowego w przebiegu zakażenia tym wirusem. Dlatego też celem badań było przedstawienie zmian we wrodzonej i nabytej odpowiedzi immunologicznej po zakażeniu trzema szczepami (V411, 1092V, Wika) *Lagovirus europaeus* GI.1 (RHDV), zidentyfikowanymi w różnym czasie. W zakresie wrodzonej odpowiedzi immunologicznej zbadano dwanaście wskaźników, a w nabytej dziewięć. Uzyskane rezultaty wskazują na udział komórek PMN (polimorfonuklearnych, neutrofilii) jako ważnych elementów w odpowiedzi na to zakażenie oraz rzucają nowe spojrzenie

na udział limfocytów $CD4^+$ Th i $CD8^+$ Tc. Najbardziej immunogenny okazał się szczepem Wika z 1996 r.

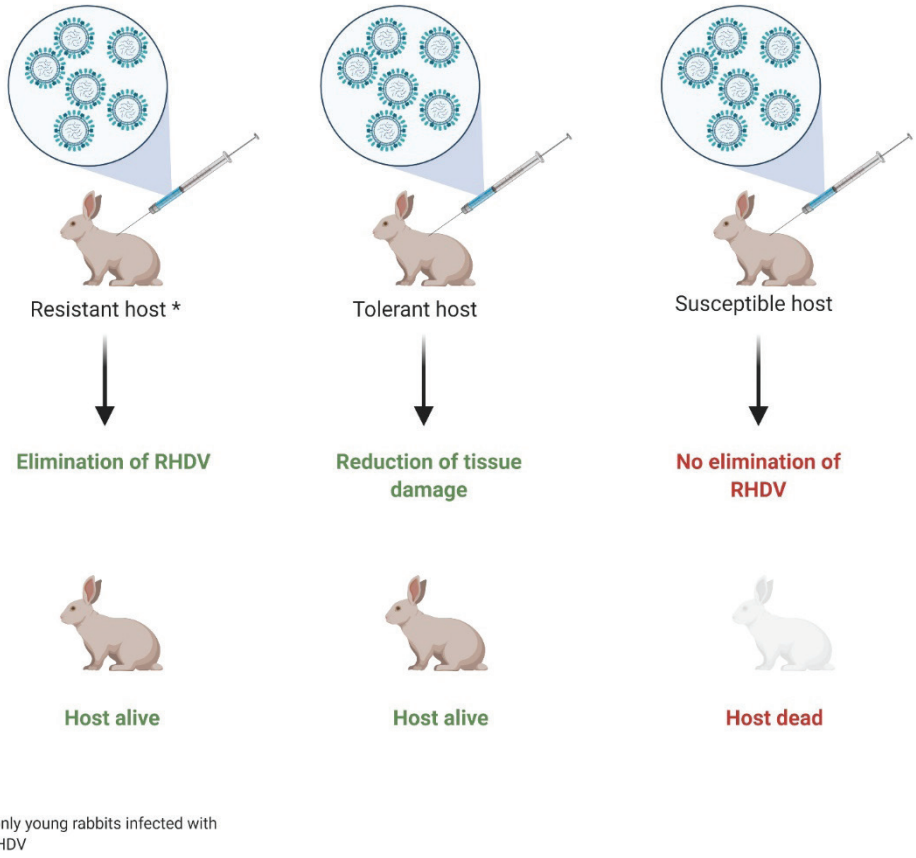
Introduction

The protection of the host against infection is ensured by well-functioning innate and adaptive immunity and increased host tolerance to infection (Iwasaki, Pillai, 2014). Recently, intensive research has been undertaken in Lagomorphs (rabbits, hares, etc.) to learn about the immune system in terms of its defensive reactions to pathogenic factors, especially viruses causing acute diseases (Esteves et al., 2018; Neves et al., 2015; Pinheiro et al., 2016). This is associated with the fact that the rabbit is one of the most frequently used animal laboratory models for immunological tests, including tests for viral and bacterial infections but also for other pathological conditions (Esteves et al., 2018; Neves et al., 2015).

Lagovirus europaeus GI.1 (Rabbit Haemorrhagic Disease Virus – RHDV) (Le Pendu et al., 2017) causing rabbit haemorrhagic disease (RHD) was first reported in 1984 in China in rabbits imported from Germany (Liu et al., 1984). Since the identification of RHDV more than 30 years ago, researchers have discovered its new variants GI.1a (G6/RHDVa) (in 1996) (Capucci et al., 1998) and new genotypes – GI.2 (RHDV2/b) (in 2010) (Le Gall-Recule, Zwingelstein, 2011). The impact of RHDV on the host organism, and more specifically, its effect on the immune system (Holmes, Grenfell, 2009), are key for the spread of this infection in rabbits (Figure 1).

That is why it is so important to understand the role of the immune system components in the course of infection with RHDV. Studies conducted in Poland (Hukowska-Szematowicz, 2013; Hukowska-Szematowicz, Deptuła, 2008; Hukowska-Szematowicz, Deptuła, 2011; Niedźwiedzka-Rystwej, 2013; Niedźwiedzka-Rystwej, Deptuła, 2010; Tokarz-Deptuła, 2009; Trzeciak-Ryczek, Tokarz-Deptuła, Deptuła, 2016; Trzeciak-Ryczek, Tokarz-Deptuła, Deptuła, 2017) and other centres (Huang, 1991; Marques et al., 2012; Semerjyan et al., 2019; Teixeira et al., 2012) have shown that numerous elements of the immune system, such as macrophages, T and B lymphocytes in the liver, spleen and lymph nodes as well as peripheral blood leukocytes and their products play a large role in the infection of *Lagovirus europaeus* GI.1 (RHDV) and course of RHD.

Therefore, the aim of this study was to present the changes in the host's innate and adaptive immune response to the infection with three strains of *Lagovirus europaeus* GI.1 (RHDV): Hungarian 1092V, Austrian V411 and German-Wika. The strains origin from the first years of diseases apperance in Europe 1988 (1092V), 1989 (V411) and 1996 (Wika). At this work has been analyzed twelve parameters of innate immune response (adherence capacity of polimorfonuclear cells-PMN cells, neutrophils); absorption capacity of PMN cells- absorption index and percent of absorbing cells; nitroblue tetrazolium (NBT) reduction tests – spectrophotometric, spontangeous, stimulated and index of stimulation (IS); metabolic activity coefficient of PMN cells (WAMG) – spontaneous and stimulated; myeloperoxidase (MPO) activity and lysozyme (LZM) concentration and activity) and seven parameters of adaptive immune response (percent of $CD5^+$ T lymphocytes, percent of $CD4^+$ Th lymphocytes, % of $CD8^+$ Tc lymphocytes, percent of $CD25^+$ lymphocytes, percent of CD19+ B lymphocytes, and IgG serum amount, total Ig concentration).



A resistant host regains physical fitness by recognizing and eliminating the pathogen (animals less than two months old do not show symptoms of RHD, infection does not lead to their death). A tolerant host recovers from infection through the effectiveness and integrity of the mechanisms of innate and adaptive immunity, which reduces tissue damage. A susceptible host recognizes the pathogen but is unable to eliminate the virus through its immune mechanisms, which leads to death.

Figure 1. Host strategies in RHDV infection

Materials and Methods

Animals

The studies were performer on 45 mixed-breed rabbits of both sex, about weight 2.5–4.5 kg. Marked as conventional animals (Annon, 1987), from a utility farm conducted under comlete zoo-technical and veterinary supervision. The animals did not present any symptoms pointing to a disease, were not subjected to any vaccination, and did not feature anti-RHDV antibodies in serum. During the study, the rabbits remained at the vivarium of the University of Szczecin,

where the conditions of the rooms in the aspect of temperature, humidity and lighting conformed to national standards (Annon, 2006). In the room, apart from natural lighting, there was also artificial lighting- glow discharge tubes. Animals were fed with full-portion rabbit feed LSK, manufactured in Miłosławiec near Poznań, in the quantity of 0.15–0.20 kg/day and had *at libitum* access to water.

Lagovirus europaeus GI.1 (RHDV)

Three European RHDV strains, differing in their identification times, were used to infect the animals. Strain 1092V from Hungary – 1988, V411 from Austria – 1989, and Wika from Germany – 1996. The presence and titre of the virus used for the experiment were defined with haemagglutination reaction (HA), using erythrocytes of human 0 group (Rh+), and immunoenzymatic test ELISA. The titer in the HA reaction for the studied strains RHDV was respectively: 1,280, 2,560, 10,240. The pathogenicity of all strains up to 48 hours of the study was 100%. RHDV strains were administered to the rabbits intramuscularly (leg muscles) in the form of suspension in 1 ml of glycerol. In turn, control animals were administered with placebo- 1 ml of glycerol. Each of the RHDV strains used for infecting rabbits came from the naturally dead animals. The strains in the form of liver homogenisate, were used for experimental infection of rabbits from whom after death liver was sampled, which was then used for infection of animals studied, by administering the liver tissue to them in the form of 20% homogenisate cleared by centrifugation at 3,000 rpm, 10% chloroforming for 60 minutes and centrifugation again, and then suspension in glycerol in the 1:1 proportion (Niedźwiedzka-Rystwej, Deptuła, 2010). All the antigens of RHDV prepared had the same number of molecules determined with floating density in caesium chloride of 1.31–1.34 g/dm^3.

The scheme of the experiment

The animals designed for infection were divided into three groups of ten animals, who were administered intramuscularly (lower limb muscles) RHDV suspended in 1 ml of glycerol (group 1 – 1092V strain, group 2 – V411, group 3 – Wika). Rabbits of control groups – three group of five animals each analogically received 1 ml of glycerol. Blood for immunological studies was drawn through a port from the marginal vein of the ear onto an anti-coagulant or without it, depending on the needs and requirements of the method applied in the study. For all groups of the studied experimental animals, blood was drawn at hour "0", namely before the administration of the RHDV in the group of infected rabbits and glycerol in the group of control animals, and then at hours 8, 12, 24, 36. All animals died to 48 hours of the experiment. In animals infected with the RHDV, at particular hours of the study, clinical symptoms were recorded by observation of animals and mortality was recorded (on the basis of which the mortality index was calculated). Before commencement of the experiment, all rabbits were subjected to the test for presence of anti-RHD antibodies using ELISA test. During the experiment, zoo-hygienic parameters were controlled every 24 hours, and the reading was always done at 8.00 AM.

Indices of innate and adaptive immune response

Adherence capacity of PMN cells in peripheral blood was determined with Lorente et al. (1973) method. The PMN cells' absorption capacity of the model strain of *Staphylococcus aureus* bacteria, strain 209P, was marked with Brzuchowska and Ładosz method, as modified by Deptuła

(1991), Zeman (1995) and Hukowska (2000). The capacity for reduction of the NBT was determined with cytochemical method in a spontaneous and stimulated test and with spectrophotometric method (Park, Fihring, Smithowich, 1968; Raman & Poland, 1975). Also, metabolic activity coefficient of neutrophilic granulocytes (WAMG) was marked acc. to Grządzielska (1976), as well as stimulation index (IS) acc. to Lechowski et al. (1991). MPO activity in PMN cells was assessed according to the Graham method described by Zawistowski (1976) and Afanasyev and Kolot (1971). In turn, the LZM concentration and activity in serum was determined by the method of plate diffusion according to Hankiewicz & Świerczek (1975) and Szmigielski (1972). Marking of the percentage of T, Th, Tc cells and lymphocytes with $CD25^+$ receptor was done according to the method described by Deptuła et al. (1998). It involved monoclonal antibodies (mouse anti-rabbit) (Serotec, USA) to identify $CD5^+$ (T cells), $CD4^+$ (Th cells), $CD8^+$ (Tc cells), lymphocytes with receptor $CD25^+$ and $CD19^+$ (B cells). The marked cells were measured at the flow cytometer FACScan by Becton Dickinson (USA) with FACSDiva software. Marking of total number of immunoglobulins in serum was done using McEwan (1970) method. Serum IgG immunoglobulin was marked with platelet method according to the IgG standards (ICN), according to the manufacturer's protocol.

Statistical Analysis

Data are presented as mean±standard deviation (SD). All statistical analyses were performed using StatView-5 Software (SAS Institute, Cary, NC, US). Between-group differences (infected rabbits and non-infected rabbits-control) evaluated using non-parametric tests (Mann-Whitney U or Kruskal-Wallis) or analysis of variance (ANOVA) with Fisher's PLSD. A p-value ≤ 0.05 was considered to indicate statisticallly significant.

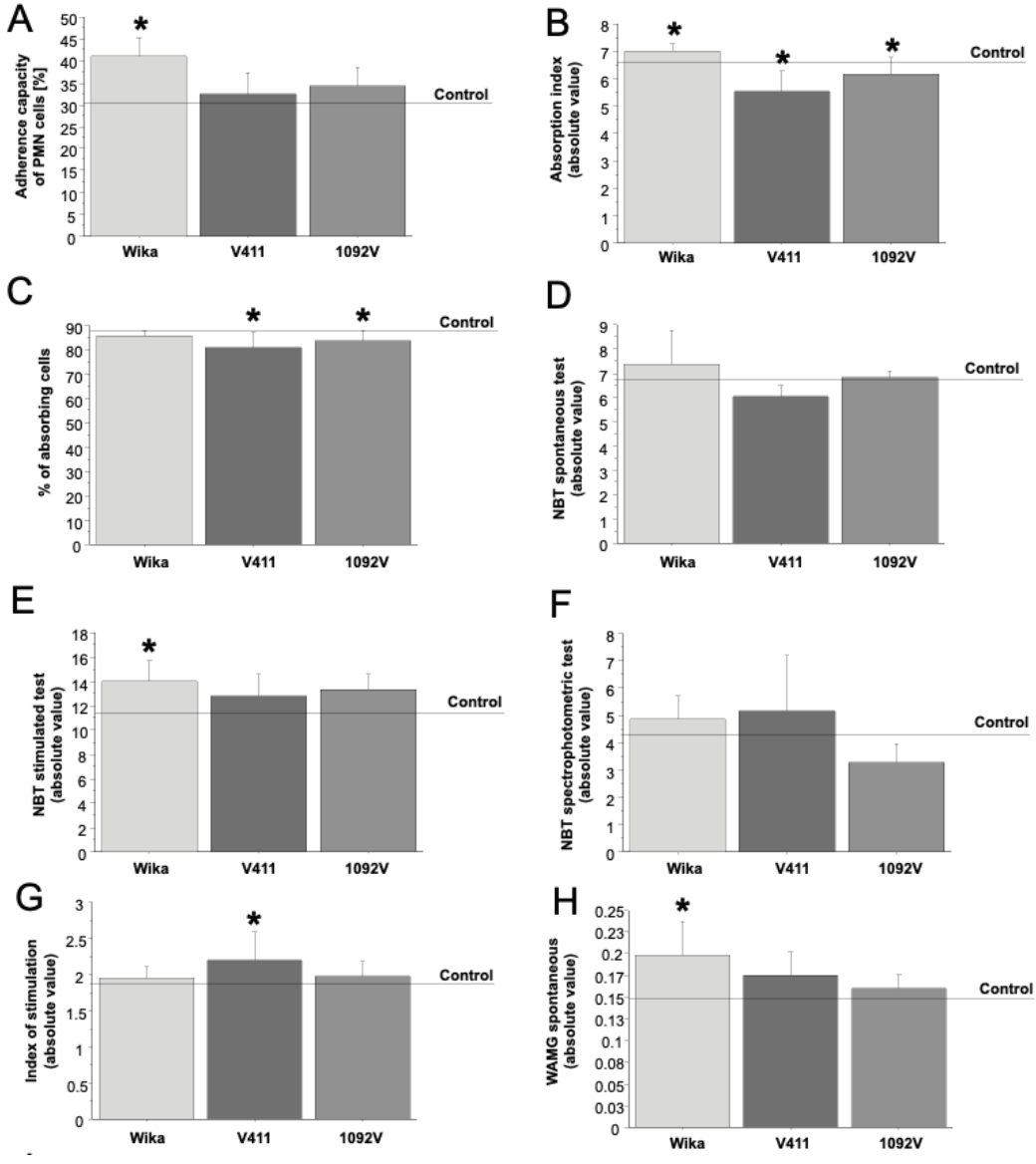
Results

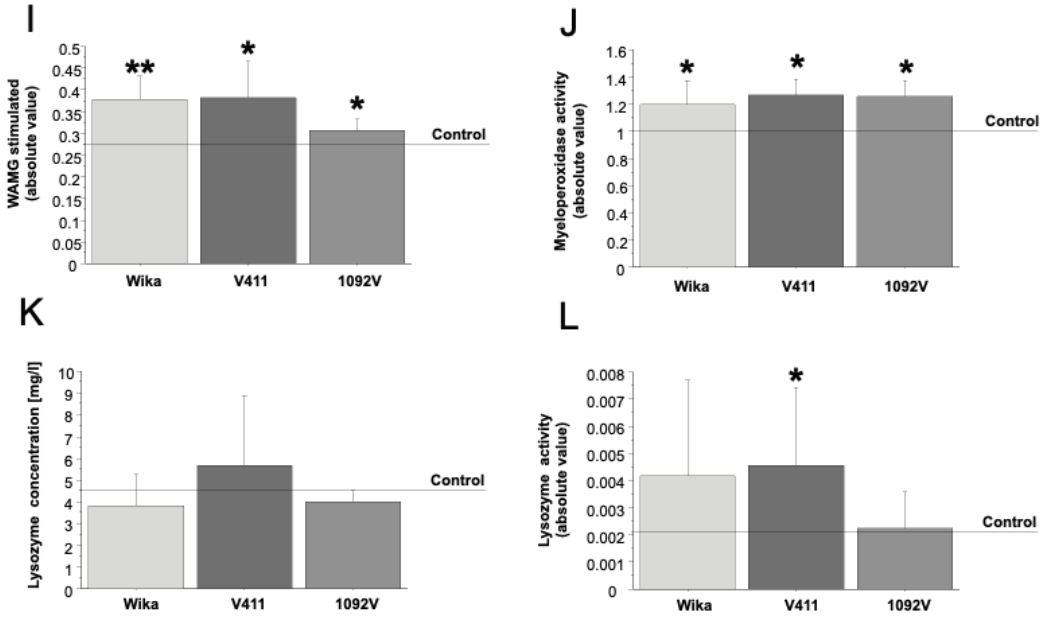
Changes in the innate immune response to *Lagovirus europaeus* GI.1 (RHDV) infection

At this work, analyzed the changes of innate and adaptive immune response, evoked by three strains – 1092V, V411 and Wika during infections and compared these findings with the levels obtained in non-infected rabbits (control). Despite the fact, that during the experimrnt, blood samples were collected from animals in a dynamic (temporal) system, the results of the researches were presented in a static form (not taking into account time), due to the fact that there are limited data on this subject. The research presented the next step in understanding the role of the immune system components in the course of RHDV infection.

The adherence capacity of PMN cells (Figure 2A) has been elevated during Wika strain infections (1.4-fold, $p < 0.05$). After V411 and 1092V strains infection, the adherence of capacity was comparable to controls. Moreover, infection with all three strains has been different in absorption capacity of PMN cells (Figure 2B,C). The analysis showed elevated values of absorption index (Figure 2B) after Wika strain infection (1.1-fold, $p < 0.05$), and decreased after V411 (16% reduction, $p < 0.05$) and 1092V (6% reduction, $p < 0.05$). However, the percentage (%) of absorbing cells (Figure 2C) after Wika antigen infection has been comparable to controls ($p = 0.3$). In regard to this analysis, a reduction has been observed after V411 (7% reduction, $p < 0.05$) and 1092V

(4% reduction, $p < 0.05$) infection strains. From all performed NBT reduction tests (Figure 2D-F), only the stimulated NBT analysis showed increased values for Wika strains (1.2-fold, $p < 0.05$). For all other analyzes strains, do not indicated any statistical changes. A change was also recorded in the case of the stimulation index (Figure 2G), but only in the case of V411 strain infection (1.0-fold, $p < 0.05$).





* $p < 0.05$; ** $p \leq 0.0001$. A. Adherence capacity of PMN cells; B. Absorbsion index; C. Percent of absorbing cells; D. NBT spontaeeous test; E. NBT stimulated test; F. NBT spectrophotometric test; G. Index of stimulation; H. WAMG spontaneous; I. WAMG stimulated; J. Myeloperoxidase activity; K. Lysosyme concentration; L. Lysosyme activity.

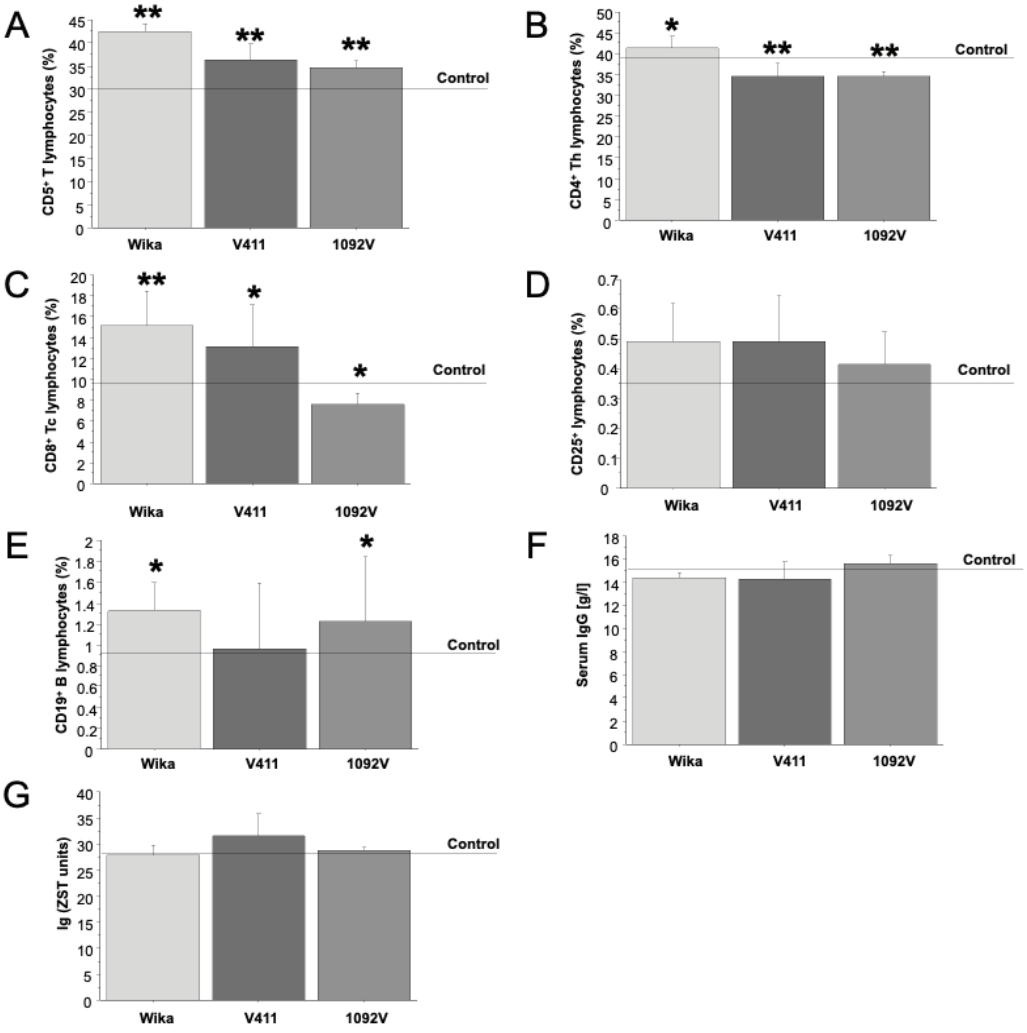
Figure 2. Indices of innate immune response after infection with three strains of *Lagovirus europaeus* GI.1 (RHDV)

Analysis of spontaneous and stimulus WAMG (Figure 2H–I) showed changes depending on the strains applied. In regards to this analysis, only WAMG spontaneous showed increased during Wika strains infection (1.3-fold, $p < 0.05$). Moreover, all three analyzed strains has have elevated parameters of WAMG stimulated (Wika 1.4-fold, $p \leq 0.0001$; V411 1.4-fold, $p < 0.05$; 1092V 1.1-fold, $p < 0.05$) (Figure 2I). Similarly, the activity of MPO (Figure 2J) has been increased at all analyzed strains (Wika 1.1-fold, $p < 0.05$; V411 1.2-fold, $p < 0.05$; 1092V 1.2-fold, $p < 0.05$). The analysis did not show any changes in the parameter of LZM concentration (Figure 2K); however, LZM activity has been elevated only during V411 antigen infection (2.4-fold, $p < 0.05$) (Figure 2L).

Changes in the adaptive immune response to *Lagovirus europaeus* GI.1 (RHDV) infection

In order to assess changes in the adaptive immune response, individual subpopulations of lymphocytes were analysed on the basis of CD receptor ($CD5^+$, $CD4^+$, $CD8^+$, $CD25^+$, $CD19^+$) using flow cytometry (Figure 3A–E).

In terms of the adaptive immune response, Ig (ZST units) and IgG immunoglobulins were determined in the serum of infected rabbits (Figure 3F–G).



* $p < 0.05$; ** $p \leq 0.0001$. A. $CD5^+$ T lymphocytes; B. $CD4^+$ Th lymphocytes; C. $CD8^+$ Tc lymphocytes; D. $CD25^+$ lymphocytes; E. $CD19^+$ B lymphocytes; F. Serum IgG; G. Ig (ZST units).

Figure 3. Indices of adaptive immune response after infection with three strains of *Lagovirus europaeus* GI.1 (RHDV)

Analysis of $CD5^+$ T cells (Figure 3A) showed elevated overall frequency for all three antigens (Wika 1.4-fold, $p \leq 0.0001$; V411 1.2-fold, $p \leq 0.0001$; 1092V 1.1-fold, $p \leq 0.0001$). Analysis of $CD4^+$ Th cells (Figure 3B) showed elevated frequency only for Wika antigen (1.1-fold, $p < 0.05$). After infection with V411 and 1092V strains, observed a 12% reduction of $CD4^+$ Th cells abundance ($p \leq 0.0001$). Frequency of $CD8^+$ Tc cells (Figure 3C) has been increased after infection

with Wika (1.6-fold, $p \leq 0.0001$) and V411 (1.4-fold, $p < 0.05$) antigens. For 1092V strain, observed a 20% reduction of $CD8^+$ Tc cells count ($p < 0.05$). The amount of $CD25^+$ T cells (Figure 3D) did not show any changes after infection with three strains of RHDV. The analysis showed an elevated amount of B cells (Figure 3E) after Wika (1.5-fold, $p < 0.05$) and 1092V (1.4-fold, $p < 0.05$) strains infection. Infection with V411 antigen did not change the numbers of B cells in comparison to controls group ($p = 0.7$). Lack of changes in the total amount of serum Ig (ZST units) and IgG (Figure 3F–G) has been observed in all analyzed RHDV strains.

Clinical Signs of RHD and mortality

All rabbits infected with three analyzed strains RHDV died with symptoms of RHD within 24–48 h of the infections (Figure 4).

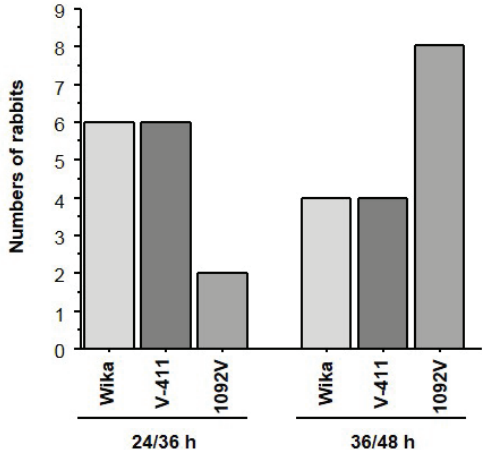


Figure 4. Mortality in the course of *Lagovirus europaeus* GI.1 (RHDV) infection

A few hours before death, rabbits showed listlesness and acute respiratory distress, characteristic for RHD. There was a marked difference in the survival rates and survival times depending on the strain of RHDV infection. The highest mortality for Wika and V411 strain has been noticed between 24/36 hours post infection with the number of 6 rabbits death. In contrast, mortality after 1092V antigen infection has been the highest between 36/48 hours post infection (8 rabbits death). Serological studies performer with ELISA test before the commencement of the experiment did not reveal presence of anti-RHDV antibodies in blood serum of rabbits in infected and control groups. Zoo-hygienic conditions in which the animals remained during the experiments, as regards the temperature, humidity and lighting, corresponded to standards applicable to rabbits in Poland.

Discussion

The response of the host immune system to the viral pathogen requires the activation of mechanisms related to the recognition of the virus with appropriate precision and in due time, followed by mechanisms of virus elimination (Chaplin, 2010; Chapel et al., 2009; Iwasaki, Pillai, 2014). An immense role is played by factors affecting the extent and severity of the infection pathogen-dependent factors including dose, virulence, and the route of entry, and host-dependent factors: integrity of immune mechanisms, efficiency of host immune system, previous exposure to the pathogen, and the occurrence of co-infections (Chapel et al., 2009). RHDV belongs to the group of viruses inducing acute infections associated with massive and rapid virus expansion, so the host immune response (both cellular and humoral) must be sufficient to block the penetration of viruses into cells and eliminate infected cells in order to limit the spread of the virus in the body.

The study showed that the infection of rabbits with three different RHDV strains triggered changes in both innate and adaptive immune response. In the innate immune response changes were recorded in nine (adherence of capacity, absorption capacity of PMN cells, % of absorbing cells, NBT reduction test-stimulated, index of stimulation, WAMG spontaneous and stimulated, MPO activity and LZM activity) out of twelve examined indices. All the above mentioned indices (except for LZM activity, whose activity was determined in serum) reflected the biological activity of PMN cells (neutrophils) (Berridge, Herst, Tan, 2005; Niedźwiedzka-Rystwej, Deptuła, 2008; Odobasic, Kitching, Holdsworth, 2016; Wang, Arase, 2014), which is an outstanding indication of phagocytosis activation during this infection. LZM activity has been elevated only during V411 antigen infection. A recent study by Hrynkiewicz, Bębnowska, Niedźwiedzka-Rystwej (2020) suggest that LZM and MPO may serve as a prognostic marker of the state of the immune system of rabbits infected with RHDV. Which may indicate antiviral effect and stimulating phagocytic activity of neutrophils and monocyte proliferation (Ibrahim, Matsuzaki, Aoki, 2001). This confirms the results of tests other RHDV strains (Hukowska-Szematowicz, 2013; Hukowska-Szematowicz, Deptuła, 2008; Hukowska-Szematowicz, Deptuła, 2011; Niedźwiedzka-Rystwej, 2013; Niedźwiedzka-Rystwej, Deptuła, 2010; Tokarz-Deptuła, 2009; Trzeciak-Ryczek, Tokarz-Deptuła, Deptuła, 2016; Trzeciak-Ryczek, Tokarz-Deptuła, Deptuła, 2017). The data presented in previous studies were collected in a dynamic system (several time points), while in this study they were presented in a static system. It should also be emphasized that PMN cells are not only first-line defence cells in this viral infections; they also modulate elements of adaptive immune response and can regulate T and B cells (Borregaard, 2010; Liew, Kubes, 2019). It should be remembered that there is a complex interaction between neutrophils and adaptive immunity to provide a smooth immune response to pathogens (Kołaczkowska, Kubes, 2013; Liew, Kubes, 2019; Mantovani et al., 2011). Moreover, it should be emphasized that out of three tested strains (1092V, V411, Wika) with very similar virulence, the youngest Wika strain from 1996 turned out to be the most immunogenic, which was manifested by the increase of innate immunity indices. The two phylogenetically oldest strains – Hungarian 1092V from 1988 and Austrian V411 from 1989 caused similar immune responses.

RHDV-induced adaptive immune response was observed in four (percent of $CD5^+$ T lymphocytes, percent of $CD4^+$ Th lymphocytes, percent of $CD8^+$ Tc lymphocytes, percent of $CD19^+$ B lymphocytes) out of the seven examined indices (Figure 3A-C, E). It should be noted that only the Wika strain infection caused an increase in lymphocytes $CD5^+$ T, $CD4^+$ Th, $CD8^+$ Tc, and $CD19^+$

B. This fact may indicate, high immunogenicity of the strain and efficient antiviral defense of the host, but not sufficient for the elimination of the virus and survival of animals. $CD5^+$ T, $CD4^+$ Th and $CD8^+$ Tc lymphocytes play a particularly important antiviral role by balancing the fight against the pathogen and the risk of chronic inflammation (Chaplin, 2010; Li et al., 2020; Sissons, Oldstone, 1980; Withmire, 2011). The biological role of $CD5^+$ T lymphocytes is to regulate the intensity of intracellular signaling induced by the antigen receptor, which is crucial for maintaining lymphocyte homeostasis (Chaplin, 2010, Chapel et al., 2009). This research confirms the increase in $CD5^+$ T lymphocytes during infection with each of the three tested strains. In turn, $CD4^+$ Th lymphocytes promote the production of virus-specific antibodies by activating T-dependent B cells (Whitmire, 2011). $CD8^+$ Tc cells are cytotoxic and may kill the virus-infected cells, which may reduce the disease by eliminating the infectious agent (Sissons, Oldstone, 1980). It should be emphasized that in viral infections $CD4^+$ T cells sustain antiviral $CD8^+$ T cell responses during infections, which is confirmed by the results after Wika infection (Whitmire, 2011). In the absence of $CD4^+$ T cells, there is minimal or no expression of $CD8^+$ T cells and deficiencies in cellular immunity (Whitmire, 2011), which is confirmed by research after infection with 1092V strain. It should be noted that after infection with Wika and 1092V strain, an increase in $CD19^+$ B lymphocytes was observed, which, as indicated by research, are the driving force of immunity in response to pathogens, during both primary and repeat infections (Lam, Baumgarth, 2019; Szikora et al., 2017). Infection of any of the tested strains did not affect the change of $CD25^+$ lymphocytes, whose biological role is to inhibit proliferation of effector lymphocytes and their secretion of proinflammatory cytokines (Lewkowicz, Lewkowicz, Tchórzewki, 2005). There were no changes in IgG and Ig (ZST units), whose role is to neutralize the virus, which prevents its binding to target cell receptors (Chapel et al., 2009). The above test results are partly consistent with earlier findings after infection with other RHDV strains (Hukowska-Szematowicz, 2013; Hukowska-Szematowicz, Deptuła, 2008; Hukowska-Szematowicz, Deptuła, 2011; Niedźwiedzka-Rystwej, 2013; Niedźwiedzka-Rystwej, Deptuła, 2010; Tokarz-Deptuła, 2009; Trzeciak-Ryczek, Tokarz-Deptuła, Deptuła, 2016; Trzeciak-Ryczek, Tokarz-Deptuła, Deptuła, 2017) however, as already highlighted, they were presented in the dynamic system. The mortality rate was 100%, and was similarity to the mortality reported for other RHDV strains (Fitzner, Niedbalski, 2017; Hukowska-Szematowicz, 2013, Niedźwiedzka-Rystwej, 2013; Niedźwiedzka-Rystwej, Deptuła, 2010; Tokarz-Deptuła, 2009).

Conclusion

Infection of rabbits with three different strains of *Lagovirus europaeus* GI.1 (RHDV) was manifested by changes in innate and adaptive immune response. The innate immunity indices and previous studies carried in Poland indicate a large role of PMN cells and their products as very important components in immune response in the corse of RHDV infection. However, the changes in adaptive immunity rates give new insights into the role of T lymphocytes and their subpopulations in this infection, with particular emphasis on $CD4^+$ Th and $CD8^+$ Tc lymphocytes. At the same time, they suggest that in this infection PMN cells may modulate the elements of adaptive immunity, which constitutes complex interaction ensuring fluent immune response to this pathogen. Of all the strains studied, the youngest evolutionary Wika strain from 1996 turned out to be the most immunogenic, followed by V411 and 1092V.

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MicroRNAs as new immunity regulators in viral and bacterial infections

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Abstract MicroRNAs (miRNAs) – small, conserved RNA molecules, containing 22 to 25 nucleotides and occurring in the cells of living organisms. As regulatory molecules, they have enormous biological potential and can influence a number of cellular processes. In the context of immunology, the role of miRNAs as novel immunity regulators is invaluable. The miRNAs regulate immune phenomena at many levels - starting from the impact on the processes of maturation, proliferation and differentiation of the immune system cells, through the regulation of the secretion of their products, to the regulation of intracellular signalling pathways. In all these areas, the miRNAs can play the role of both an inducer and an inhibitor by appropriately increasing the intensity of or suppressing the immune processes they regulate. In the future, it will be possible to regulate the host's immune response to the pathogen thanks to the properly controlled expression of miRNAs in the immune system cells.

Cząsteczki mikroRNA jako nowe regulatory odporności w infekcjach wirusowych i bakteryjnych

Słowa kluczowe mikroRNA, odporność, wirusy, bakterie

Streszczenie MikroRNA (miRNA), małe, konserwatywne 22–25 nukleotydowe cząsteczki RNA występujące powszechnie w komórkach żywych organizmów. Jako cząsteczki regulatorowe mają ogromny potencjał biologiczny i mogą wypływać na wiele procesów komórkowych. W kontekście immunologii nieoceniona jest rola miRNA jako nowych regulatorów odporności. MiRNA regulują zjawiska odpornościowe na wielu poziomach. Począwszy od wpływu na procesy dojrzewania, proliferacji oraz różnicowania komórek układu odpornościowego, przez regulację wydzielania ich produktów, po regulację wewnątrzkomórkowych szlaków sygnalizacyjnych. Na wszystkich tych polach miRNA może odgrywać rolę zarówno induktora, jak i inhibitora, odpowiednio zwiększając nasilenie lub wygaszając regulowane przez siebie procesy odpornościowe. W przyszłości dzięki właściwie pokierowanej ekspresji miRNA w komórkach układu odpornościowego możliwe będzie regulowanie przebiegu odpowiedzi immunologicznej gospodarza w odpowiedzi na patogen.

Introduction

MicroRNAs (miRNAs) are small, conserved and non-coding sequences containing 22–25 nucleotides (Bartel, 2004). They are assigned a role in controlling the expression of genetic information in many physiological and pathological processes such as cell proliferation and differentiation, embryogenesis, DNA repair, inflammation, immunity, viral and bacterial infections, apoptosis or neoplastic processes (Ardekani, Naeini, 2010; Bartel, 2004; Das et al., 2016; Hukowska-Szematowicz, Deptuła, 2010; Mahesh, Biswas, 2019; Olejniczak et al., 2018; Poczęta et al., 2018; Świetlik, Szemraj, 2017). Moreover, these molecules play a part in intercellular signalling (Barbu et al., 2020). So far, more than 38.000 various miRNAs have been identified, the activities of which were distinguished in individual biological processes (Annon, 2020a). Nevertheless, a single miRNA molecule can influence the expression of several or several dozen different genes (Bartel, 2004). MiRNA influences the reading of the target mRNA by identifying complementary fragments at the 3' end of the UTR of the mRNA strand (Bartel, 2004). The mRNA reading is regulated by reducing the level of its transcription or by post-transcriptional mechanisms such as mRNA cleavage or its dissociation from ribosome subunits (Pong et al., 2018). MiRNA distinguishes between target mRNAs by using complementary sequences containing 2–8 nucleotides, located at the 5' end of the miRNA. These sequences are called seed sequences/ seed regions (Das et al., 2016; Jeker, Marone, 2015; Olejniczak et al., 2018; Pong et al., 2018). Those miRNAs that target the same mRNAs have the same seed regions (sequences) (Jeker, Marone, 2015). MiRNAs are presumed to control the expression of 30% to 60% of protein-coding genes in humans (Acuña et al., 2020; Das et al., 2016; Ojha et al., 2016). It was also found that a part of miRNAs can bind to the 5'-UTR fragment and to the ORF region, which increases the number of genes they regulate.

The expression of miRNAs can be regulated genetically or epigenetically (DNA methylation, modification of histone proteins) (Barbu et al., 2020; Das et al., 2016). MiRNA biogenesis takes place in several stages (Bartel, 2004; Olejniczak et al., 2018). The initial stages occur in the cell nucleus with the next stages taking place in the cytoplasm. MiRNA biogenesis begins with the transcription of genes encoding miRNAs via RNA polymerase II (RNA Pol II). As a result, the primary miRNA transcript (pri-miRNA) is produced. The resulting transcript is recognized by the Microprocessor complex consisting of the Drosha protein and DGCR8. After trimming by the Microprocessor, a miRNA transcript called precursor miRNA (pre-miRNA) is produced. Pre-miRNAs are transported from the cell nucleus to the cytoplasm by exportin-5 (XPO5). This process is catalyzed by RAN GTP hydrolysis. Then, the pre-miRNA binds to the Dicer protein and its partner protein TRBP and undergoes the process of converting into double-stranded miRNAs. So created molecule is loaded onto the Argonaute (Ago) and TNRC6A/B/C proteins to form the RNA-induced silencing complex (RISC) (Bartel, 2004). When all the above mentioned items are combined, one of the miRNA strands is discarded and degraded; the other becomes the mature miRNA molecule. RISCs and mature miRNAs combine complementarily with the target mRNA, resulting in gene silencing by degrading the mRNA or inhibiting its translation (Barbu et al., 2020; Dickey et al., 2017; Michlewski, Caceres, 2019; Olejniczak et al., 2018; Pong et al., 2018). There are also several alternative miRNA biogenesis pathways. Some of the miRNAs can be collected in the so-called clusters and transcribed together, others can be synthesized without the Dicer enzyme via the Ago2 protein (Jeker, Marone, 2015; Michlewski, Caceres, 2019; Olejniczak et al., 2018). However, these are not all of the possible alternative pathways for miRNA biogenesis.

MiRNAs control gene expression, but miRNAs themselves can also be regulated. The biogenesis of miRNAs may be affected by homeostasis abnormalities, which may interfere with its

production at every stage (Olejniczak et al., 2018). Stress or viral and bacterial infections are an example of factors causing the body imbalance (Drury et al., 2017; Olejniczak et al., 2018).

Key miRNAs involved in immune regulation

As previously mentioned, miRNAs can affect many various processes. MiRNAs can regulate the mechanisms of both innate and adaptive immunity (Acuña et al., 2020). In the context of immunity, they, *inter alia*, influence the survival of the immune system cells, their differentiation and proliferation (Jeker, Marone, 2015).

MiRNAs affect the activity of many cells of the immune system, which can be observed, for example, in macrophages (Nazimek et al., 2015). MiRNA-17-5p, miRNA-20a and miRNA-106a regulate the biological activity of macrophages as early as in their differentiation and maturation phases. Also, miRNA-146a and miRNA-155 increase their expression during the differentiation of monocytes into macrophages, which shows they are necessary in this process. (Ahmed et al., 2016; Hukowska-Szematowicz, Deptuła, 2010; Nazimek et al., 2015). Interestingly, for example, the miRNA-146 molecule, the expression of which in macrophages is stimulated by the activation of TRL2, TRL4 and TRL5 receptors, inhibits the activity of macrophages M1, and thus also reduces an inflammatory response (Nazimek et al., 2015). The same miRNA molecule contributes to the immune tolerance of the body by inhibiting the production of pro-inflammatory cytokines in macrophages. MiRNA-155 is considered to be the key miRNA molecule involved in the regulation of immunity. This molecule, by targets and inhibits specific genes, affects a number of immune cells such as macrophages, B lymphocytes, T lymphocytes and their subpopulations and DC, NK cells. In these cells, it controls the secretion of inflammatory mediators (Dickey et al., 2017). It should be pointed out that the dysregulation of the above-mentioned processes can be affected by the development of inflammation caused by viral or bacterial infection present in the host organism (Das et al., 2016). In order to increase their pathogenicity and the chance of survival in the affected cells, pathogens, using the miRNAs they encode, cause changes in the mRNA expression of the infected cell (Das et al., 2016). This shows how pathogens themselves can influence the immune regulation and differentiation of immune cells in an infected organism. In the future, thanks to properly controlled miRNA expression in the cells of the immune system, it will be possible to regulate the host's time course of immune response to a pathogen (Barbu et al., 2020; Nazimek et. al., 2015).

Several miRNAs are involved in regulating the maturation and differentiation processes of immunocompetent cells. A few types of miRNAs are involved in the development and activation processes of a single type of immune cells.

MiRNA-146. The production of miRNA-146 in macrophages is associated with the activation of TLR2, TLR4 and TLR5 receptors (Nazimek et al., 2015). MiRNA-146 inhibits the activity in of macrophages with M1 phenotype, which has also been proven in relation to other cells of innate immunity (Acuña et al., 2020; Nazimek et al., 2015). Moreover, in macrophages, miRNA-146 reduces the production of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6, thereby exhibiting a tolerogenic effect. Moreover, miRNA-146 was found to negatively affect IRAK1 and TRAF6, through which it affects the NF-κB and MyD88 signalling pathways (Ahmed et al., 2016; Barbu et al., 2020; Zhao et al., 2015).

MiRNA-146a. The expression of miRNA-146a has been shown to increase during the differentiation of monocytes into macrophages (Li et al., 2016; Nazimek et al., 2015). Increased production of this type of miRNA is induced during bacterial infection, in response to bacterial

lipopolysaccharide (LPS), and during interaction with IL-1β and TNF-α. Such overexpression may result in macrophages becoming tolerant to LPS, rendering them unable to participate in the immune response. The expression of miRNA-146a is also enhanced by contact with the *Candidia albicans* and *Listeria monocytogenes*, *Mycobacterium bovis* (Li et al., 2016; Nazimek et al., 2015). In infected cells, miRNA-146a, under the influence of *Mycobacterium tuberculosis*, suppresses the expression of inducible nitric oxide synthase (iNOS), which is responsible for inducing the production of nitric oxide (NO) in macrophages (Das et al., 2016; Li et al., 2016). This results in a reduction of NO production thereby favouring the survival of mycobacteria inside these cells (Li et al., 2016). The suppression of iNOS expression occurs by the suppression of NF-κB and mitogen-activated protein kinases (MAPK) pathways and reduction of the expression of TNF receptor-associated factor 6 (TRAF6) (Li et al., 2016; Zhang et al., 2019). However, the level of miRNA-146a in peripheral blood cells in patients infected with *M. tuberculosis* is reduced in mononuclear cells, which, *inter alia*, include B and T lymphocytes, NK cells and monocytes. It is worth emphasizing that within the same type of bacteria, the same miRNA molecule may activate different functions. Research also shows that miRNA-146a influences the host's immune response in viral infections (Zhang et al., 2019).

MiRNA-150. The production of this type of miRNAs in monocytes was reported, which resulted in a lower susceptibility of these cells to human immunodeficiency virus-1 (HIV-1) infection. It is one of the miRNAs that binds directly to HIV RNA (Ojha et al., 2016). MiRNA-150 influences the regulation of T lymphocyte metabolism and the cytokines produced by T lymphocytes (King et al., 2016). In Th1 lymphocytes stimulated by CD46, miRNA-150 is necessary for their regulation and activation and for the initiation of the IL-10 secretion process (King et al., 2016). MiRNA-150 regulates the c-Myb transcription factor (cellular Myb), which influences the development of B lymphocytes. An increase in the activation and proliferation of $CD8^+$ T cells is another process regulated by this molecule (Badalzadeh et al., 2019).

MiRNA-155. This molecule is referred to as the master regulator of inflammation (Mahesh, Biswas, 2019). The high expression of miRNA-155 was reported in monocytes, macrophages, T and B lymphocytes, NK cells and DC (Dickey et al., 2017; Herrera-Uribe et al., 2018; King et al., 2016). In the above-mentioned cells, miRNA-155 is responsible for regulating the secretion of chemokines, cytokines and transcription factors in such a way that the immune response is optimal (Dickey et al., 2017). An increased expression of miRNA-155 has been observed in response to contact with *Candidia albicans*, as well as in macrophages derived from mice after contact with LPS (*Listeria monocytogenes*, *M. tuberculosis)* (Das et al., 2016; Nazimek et al., 2015). In macrophages, miRNA-155 is one of several miRNAs that are responsible for modulating TNF-α secretion. In the same cells, the secretion of this miRNA induces the development of specific M1 phenotype, and additionally prevents the development of the M2 phenotype (Dickey et al., 2017; Nazimek et al., 2015). Blocking the M2 phenotype formation is associated with a reduction in the IL-13 receptor expression. In T cells, miRNA-155 causes an increase in IFN-γ production and a decrease IL-2 production (Dickey et al., 2017). Moreover, it enhances the response of $CD4^+$ and $CD8^+$ T cells, which is dependent on IFN-γ and is involved in the maturation of Treg lymphocytes. It was shown that in the absence of miRNA-155, T cell responses were reduced during infections caused by certain neurotrophic viruses (Dickey et al., 2017). Moreover, miRNA-155 affects the maturation of NK cells (Dickey et al., 2017). Its deficiency results in the production of less NK cells and has a negative impact on their survival (Dickey et al., 2017). Moreover, variable expression of this miRNA was reported during infection with HIV and IAV (Barbu et al., 2020; Chen et al., 2017; Zuo et al., 2017).

MiRNA-223. This molecule is responsible for inducing monocyte maturation (Ahmed et al., 2016). In addition, studies have shown that the expression of the *NLRP3*, which is also found in macrophages, is inhibited under the influence of miRNA-223. The protein encoded by this gene is responsible for maintaining the balance inside the cell. Together with the miRNA let-7i, it is responsible for regulating the TLR4 signalling pathway (Nazimek et al., 2015). Moreover, the production of miRNA-223 blocks the IL-1β production in macrophages (Nazimek et al., 2015). In $CD8^+$T cells it increases the intensity of their proliferation and biological activity (Badalzadeh et al., 2019).

Other miRNAs involved in immune regulation. These include the miRNAs encoded by the Epstein-Barr virus (EBV) (Hartung et al., 2019; Wang et al., 2018; Zuo et al., 2017). HIV replication is inhibited by miRNA-133b, miRNA-138-5p, miRNA-326, miRNA-149-5p and miRNA-92a-3p (Balasubramaniam et al., 2018; Bernier, Sagan, 2018; Ojha et al., 2016). MiRNA-323, miRNA-491, miRNA-654, miRNA-584-5p and miRNA-1249 molecules reduce influenza A virus replication (Chen et al., 2017; Kumar et al., 2018; Zhao et al., 2015). MiRNAs belonging to the miRNA let-7 family are involved in the regulation of immunity in viral and bacterial infections (Ahmed et al., 2016; Rivera et al., 2016; Zhang et al., 2019). MiRNA-136 is responsible for the increase in the production of IL-6, IFN-α, IFN-β and TNF-α (Zhao et al., 2015). MiRNA-301b reduces the influx of phagocytes into the inflammatory focus (Li et al., 2016). MiRNA-21, miRNA-146a and miRNA-155 affect the NF-κB transcription factor during infection with *Salmonella sp.* (Ahmed et al., 2016).

The role of miRNAs in selected viral infections

Epstein-Barr Virus (EBV)

Epstein-Barr virus belongs to the *Herpesviridae* family, and contain dsDNA genome (Annon, 2020b). EBV belongs to the group of oncogenic viruses. Studies on the presence of antibodies against EBV indicate that this pathogen is present in approximately 90% of the worldwide adult population (Hartung et al., 2019; Lewandowisz-Uszyńska et al., 2018; Wang et al., 2018). After entering the body, particles of this virus may not be completely destroyed, and as a result, remain latent in the body even for the host's entire life (Barbu et al., 2020; Wang et al., 2018; Zuo et al., 2017). As numerous studies have shown, the presence of latent EBV infection can result in the development of various malignant neoplasms, including Burkitt lymphoma, Hodgkin lymphoma, nasopharyngeal cancer and stomach cancer (Barbu et al., 2020; Wang et al., 2018; Zuo et al., 2017).

In 2004, EBV was the first virus found to encode miRNAs (Pfeffer et al., 2004). So far, EBV has been shown to encode 25 miRNA precursors and 44 mature miRNAs that can affect mRNAs of both host and virus cells (Bernier, Sagan, 2018; Wang et al., 2018; Zuo et al., 2017). Viral miRNAs are expressed from two regions of the viral genome, i.e. BART and BHRF1 (Hartung et al., 2019; Wang et al., 2018; Zuo et al., 2017). During a latent infection, the virus shows a reduced level of its own protein synthesis, but a high level of expression of non-coding RNA molecules, including miRNAs (Zuo et al., 2017). Studies show that EBV miRNAs affect the host's immunity-related genes (Wang et al., 2018). They are also responsible for maintaining latent viral infection. In addition, it has been observed that EBV miRNA biosynthesis is dependent on the host's transcriptional mechanisms and it does not show significant differences as compared to cellular miRNA synthesis (Wang et al., 2018; Zuo et al., 2017). The key mechanism which allows the virus to avoid the host's immune response is to prevent cells from presenting antigen, inhibit

T cell responses, and disrupt cytokine and chemokine signalling pathways (Wang et al., 2018). Another function of the miRNAs produced by EBV is to reduce the synthesis of viral membrane proteins. These proteins, i.e. latent membrane protein 1 (LMP1) and latent membrane protein 2A (LMP2A), are responsible for activating specific signalling pathways of the immune cells of the infected organism (Wang et al., 2018; Zuo et al., 2017). They are referred to as viral antigens that stimulate NF-κB signalling and the cytotoxic effect of $CD4^+$ and $CD8^+$ T cells (Bernier, Sagan, 2018). The LMP1 protein increases the proliferation and transformation of viral cells (Wang et al., 2018). The LMP2A protein exhibits the effect of antigen to which T cells respond (Wang et al., 2018).

EBV miRNA also regulates the host's innate immune response (Wang et al., 2018). One of the ways to control it is the effect of miRNA-BHRF1-3 on the inhibition of the activity of the C-X-C motif chemokine 11 (CXCL-11 chemokine) (Bernier, Sagan, 2018; Wang et al., 2018; Zuo et al., 2017). Under the influence of IFN, this chemokine engages and activates NK cells (Wang et al., 2018) and regulates the influx of T cells (Bernier, Sagan, 2018). Additionally, the interaction between CXCL-11 and C-X-C motif chemokine receptor 3 (CXCR3 receptor) (for which the chemokine is a ligand) is important from the viewpoint of Th1 effector cells. MiRNA-BART6-3p suppresses RIG-I protein signalling and thus also type I IFN reactions. The RIG-I protein belongs to the pattern recognition receptors (PRRs) (Wang et al., 2018). RIG-I also stimulates the type I IFN response, which affects the time course of viral infections, including EBV infections (Gołąb et al., 2018; Wang et al., 2018). Another miRNA, miRNA-BART2-5p, suppresses the expression of MHC class I chain-related molecule B (MICB) on the surface of cells containing EBV (Bernier, Sagan, 2018; Wang et al., 2018; Zuo et al., 2017). When the amount of MICBs on the cell surface is reduced it somehow protects the infected cells from the reaction of NK cells and T lymphocytes (Wang et al., 2018; Zuo et al., 2017). Moreover, miRNA-BART3-3p reduces the cytotoxic effect by suppressing the expression of importin 7 (IPO7) (Zuo et al., 2017). This protein influences the stimulation of T lymphocytes and immune tolerance. In addition, it has been observed in macrophages that after the decrease in the amount of IPO7 due to the effect of miRNA-BART3, the secretion of IL-6 is reduced (Zuo et al., 2017). EBV miRNAs can also control the expression of many cytokines (Wang et al., 2018). MiRNA-BHRF1-2-5p directly affects the IL-1 receptor 1 (IL1R1) and prevents the activation of IL-1β stimulated NF-κB. MiRNA-BART6-3p can regulate the expression of the receptor for the IL-6, which is involved in the regulation of the anti-infective response (Gołąb et al., 2018; Wang et al., 2018; Zuo et al., 2017). Moreover, IL-6 is a growth factor for B lymphocytes and has a stimulating effect on T lymphocytes. Furthermore, miRNA-BART1, miRNA-BART2, miRNA-BART22 and miRNA-BHRF1-2 inhibit the secretion of IL-12 (Wang et al., 2018; Zuo et al., 2017). This cytokine increases the secretion of IFN-γ and TNF-α and is responsible for the activation of Th1 lymphocytes. The reduced activity of IL-12 caused by the above-mentioned miRNAs results in a decreased T cell response, differentiation and activation, thus increasing the survival of EBV-infected cells (Wang et al., 2018; Zuo et al., 2017). In EBV-infected cells, miRNA-BART20-5p and miRNA-BART8 affect the secretion of IFN-γ (Zuo et al., 2017). It has also been shown that miRNAs produced by EBV regulate part of the genes responsible for antigen presentation to immune cells (Wang et al., 2018). The lysosomal enzymes of class II MHC cells, responsible for epitope modification, are often their targets. Their regulation is influenced by such miRNAs as: miRNA-BART1, miRNA-BART2 or miRNA-BHRF1-2. In turn, miRNA-BART1-5p affects lymphocyte antigen 75 (LY75) which is responsible for the presentation of the antigen on the surface of MHC molecules and for the stimulation of $CD4^+$ and

$CD8^+$ T cells. This may result in the avoidance of T lymphocyte responses by EBV-infected cells (Wang et al., 2018).

Influenza Virus (IV)

Influenza virus belongs to the family *Orthomyxoviridae*, contain a single-stranded negative-sense RNA genome (Annon, 2020b). It causes respiratory symptoms, but also causes systemic symptoms by negatively affecting the respiratory system (Zhang et al., 2019). Due to the accumulation of numerous mutations in the viral genome during its replication, adaptive immunity is often insufficient to fight off infection (Brogaard et al., 2018). For this reason, IV present a new challenge to the immune system in each flu season. In humans, influenza A virus (IAV) is the most common cause of pneumonia-related death (Zhang et al., 2019). This virus is recognized by TLR-3, TLR-7, TLR-8 and RIG-I receptors (Brogaard et al., 2018; Kumar et al., 2018). In order to prevent an excessive immune response, the host organism produces factors that suppress signalling pathways such as suppressor of cytokine signalling 1–7 (SOCS1-7) and CUE domain-containing 2 (CUEDC2) (Kumar et al., 2018).

Comparative studies of miRNA expression in cells infected with IAV and control cells have shown an increase in the expression of microRNA- miRNA-21, miRNA-663b, miRNA-146a, whereas a decrease in the expression was reported for miRNA-211, miRNA-508-5p and miRNA-4298 (Zhang et al., 2019). These studies, indicated that, miRNA-146a has the greatest impact on IAV replication process. The said miRNA molecule contributes to an increase in viral replication in infected cells by suppressing the type I IFN (IFNs) response. Studies have shown that the decrease miRNA-146a expression in cells promotes the production of IFN-α and IFN-β, the synthesis of which is stimulated by IAV (Zhang et al., 2019). MiRNA-146a and miRNA-146 modulate IFN synthesis by targeting the *TRAF6* and suppressing its expression (Chen et al., 2017; Zhang et al., 2019). Thus, miRNA-146a weakens the host's antiviral defence. In addition, miRNA let-7c, miRNA-33a and miRNA-302c have been shown to be involved in IV replication (Zhang et al., 2019). Furthermore, miRNA-485-5p (Kumar et al., 2018), miRNA-323, miRNA-491 and miRNA-654 (Chen et al., 2017; Zhao et al., 2015) inhibit IAV replication by interacting with the gene encoding PB1 (Chen et al., 2017; Kumar et al., 2018). Moreover, miRNA-485-5p stimulates the antiviral response by increasing the stimulation of the signalling pathway mediated by the RIG-I receptor (Kumar et al., 2018). Studies have shown that during IAV infection, the miRNA-155 molecule acts as an immune regulator. The high expression of this molecule has been observed in macrophages (Chen et al., 2017). In these cells, miRNA-155, by affecting SOCS1 (on a feedback basis), increases IFNs signalling, which leads to the development of antiviral immunity. Studies have shown that during IAV infection, a reduction in miRNA-302a expression and an increase in the synthesis of interferon regulatory factor 5 (IRF-5) are observed (Chen et al., 2017). The latter factor is activated by the stimulation of TLR-7 or TLR-8 receptors. IRF-5 and plays an important role in the expression of type I IFN and pro-inflammatory cytokines IL-6, IL-8, IL-12 and TNF-α. Moreover, it is involved in the polarization of M1 macrophages. MiRNA-302a directly targets IRF-5 by suppressing its expression (Chen et al., 2017). It has also been observed that an increase in the amount of IRF-5 has a positive effect on the IAV replication process. During IAV infection, the reduction of miRNA-302a expression results in increased cytokine and chemokine synthesis and viral replication. The increased cytokine production leads to a "cytokine storm". During IAV infection, a decrease in the expression of let-7f miRNA has been observed, resulting in an increase in the production of IL-6 and IL-10 (Rivera et al., 2016). During infection with avian influenza

virus (H5N1), it was observed that this virus inhibited miRNA-324-5p expression (Kumar et al., 2018). This miRNA reduces replication of the influenza virus in infected cells by affecting the PB1 transcript. PB1 encodes one of the viral RNA polymerase subunits (Kumar et al., 2018). The host's miRNA molecules, i.e. miRNA-584-5p and miRNA-1249, target the gene encoding PB2 (the second subunit of viral RNA polymerase) thereby also negatively affecting its replication. According to studies, miRNA-324-5p also stimulates the host's innate antiviral immunity during H5N1 infection. This is because miRNA-324-5p targets the CUEDC2 transcript. CUEDC2 is the host's protein that affects the JAK-STAT signalling pathway. This pathway is responsible for the induction of type I and III IFN. Thus, CUEDC2 negatively influences the regulation of the antiviral response (Kumar et al., 2018). MiRNA-324-5p directly binds to the CUEDC2 inhibitor, suppressing its expression in the cell and increasing the body's immune response. Thus, this type of miRNA mediates in the increased synthesis of type I and III IFN during H5N1 infection (Kumar et al., 2018). In addition, miRNA-136 has also been shown to increase the production of IL-6, IFN-α, IFN-β and TNF-α (Zhao et al., 2015).

Human Immunodeficiency Virus (HIV)

The human immunodeficiency virus belongs to the *Retroviridae* family (Annon, 2020b). It is estimated that over 36 million people in the world are infected with HIV. The presence of HIV in the human body can lead to acquired immune deficiency syndrome (AIDS) (Balasubramaniam et al., 2018). The mechanism of viral pathogenesis is based on the attack and destruction of $CD4^+$ Th cells as well as monocytes, macrophages, DC (Balasubramaniam et al., 2018). So far, two types of virus have been distinguished: HIV-1 and HIV-2 (Barbu et al., 2020; Devadas et al., 2016; Flór, Blom, 2016). The HIV-2 type is less aggressive and is transmitted less frequently than the HIV-1 type (Devadas et al., 2016). In addition, a longer latency has been observed for HIV-2 type as compared to HIV-1 type (Devadas et al., 2016). In this study, most of the data collected relates to the HIV-1 type virus (hereinafter referred to as HIV) unless stated otherwise.

According to studies, viral replication in $CD4^+$ T cells does not start until their activation (Balasubramaniam et al., 2018). It is worth stressing that after activating infected cells, the amount of cellular miRNA negatively affecting viral RNA decreases in them. In studies on model cells, the effect of miRNA-132 on an increase in HIV replication in infected T cells has been observed (Balasubramaniam et al., 2018; Bernier, Sagan, 2018; Ojha et al., 2016). The increased expression of this miRNA also takes place in activated $CD4^+$ T cells (Bernier, Sagan, 2018). By directly affecting the viral RNA, MiRNA-133b, miRNA-138-5p, miRNA-326, miRNA-149-5p and miRNA-92a-3p reduce the virus replication by more than 40% (Balasubramaniam et al., 2018). In $CD4^+$ T cells, an increase in the expression of five cellular miRNAs, i.e. miRNA-28, miRNA-125b, miRNA-150, miRNA-223 and miRNA-382, that targets and inhibits viral mRNAs, has been observed (Balasubramaniam et al., 2018; Bernier, Sagan, 2018; Ojha et al., 2016). Upon activation of $CD4^+$ T cells, the expression of these miRNAs decreases, leading to reduced immunity of these cells to viral infection (Ojha et al., 2016). By affecting the mRNA of the virus, these particles contribute to reducing the amount of synthesized viral protein and progeny virions. The part of the miRNAs expressed in $CD4^+$ T cells targets mRNA encoding viral genes (*nef, vpr, vif* or *vpu*) and cellular factors (cyclin T protein and receptors necessary for virus to enter into the cell) (Ojha et al., 2016; Ortega et al., 2018). Cellular miRNA-29a, which affects the mRNA of the virus by suppressing its expression thus reducing its replication and infectivity, is also highly expressed in T lymphocytes infected with the virus (Balasubramaniam et al., 2018; Modai

et al., 2019; Ortega et al., 2018). Additionally, miRNA-146a/b increases during viral infection and reduces the NF-κB pathway signalling by targeting TNF receptor-associated factor 6 (TRAF6) (Barbu et al., 2020). Four of the five miRNAs mentioned above (excluding miRNA-223) were also detected in macrophages and monocytes (Nazimek et al., 2015). It was found that these molecules, as compared to macrophages, are more expressed in monocytes, which means that the latter are more resistant to HIV infections (Nazimek et al., 2015). The presence of two other miRNAs, i.e. miRNA-221 and miRNA-22, has been confirmed in macrophages. These miRNAs limit the possibility for HIV to enter the cell (Balasubramaniam et al., 2018). Research shows that miRNA-155 is also involved in controlling the spread of the virus, *inter alia*, by influencing the polarization of macrophages (Dickey et al., 2017). MiRNA-155 promotes macrophage differentiation towards the M1 phenotype, which is more resistant to HIV infection (Dickey et al., 2017). Moreover, miRNA-155 reduces the possibility of HIV binding to dendritic cells (Dickey et al., 2017; Ojha et al., 2016). The same miRNA inhibits the NF-κB pathway thus contributing to the maintenance of latent infection (Barbu et al., 2020). MiRNA-155 can influence HIV-1 replication by acting on factors involved in the development of infection in both the host and virus itself (Dickey et al., 2017). Increased expression of miRNA-155 has also been demonstrated after the stimulation of TLR3 receptors in macrophages (Dickey et al., 2017). Other studies have shown that this type of miRNA is involved in the transcriptional silencing of HIV-1 in T cells by acting on the host factor - tripartite motif-containing protein 32 (TRIM32) and E3 ubiquitin ligase. Through the agency of them, the factor NF-κB is stimulated, which affects the virus transcription. Moreover, the increased expression of miRNA-155 leads to a reduction of dendritic cell-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN) receptors on the surface of dendritic cells, which prevents the virus from binding to these cells (Dickey et al., 2017). MiRNA-155 may also bind to the HIV-1 genome and reduce the expression of viral genes (Dickey et al., 2017). HIV is presumed to encode its miRNAs (Balasubramaniam et al., 2018; Barbu et al., 2020). Five regions that can encode viral miRNAs have been identified in the viral genome. The miRNAs encoded from one of these regions are miRNA-TAR-5β and miRNA-TAR-3β. They are expressed in infected $CD4^+$ T cells. The function of these miRNAs is to prevent cell apoptosis and stimulate viral replication (Balasubramaniam et al., 2018; Barbu et al., 2020; Bernier, Sagan, 2018). However, the existence of these two types of miRNAs is debatable. Currently, researchers assume that HIV neither encodes any miRNAs nor strongly influences cellular miRNA expression, at least at the beginning of infection.

The role of miRNAs in selected bacterial infections

Salmonella Typhimurium

Salmonella is an intestinal gram-negative bacteria belonging to the *Enterobacteriaceae* family (Busse et al., 2012). It is classified as an absolute intracellular pathogen (Szewczyk et al., 2013; Zhou et al., 2018). Its presence in the body may lead to the development of typhoid fever or gastroenteritis (Naveed et al., 2017; Zhou et al., 2018). It is usually transmitted through food (mainly on products of animal origin contaminated with faeces) (Herrera-Uribe et al., 2018; Szewczyk et al., 2013). There are three main serotypes of *Salmonella*: Typhi, Typhimurium and Enteritidis (Zhou et al., 2018). This study deals with the serotype of *Salmonella* Typhimurium. This species of bacteria is most often isolated on products that are potential vectors of *Salmonella* infection (Herrera-Uribe et al., 2018). The bacterium under consideration is one of the causes

of salmonellosis in humans (Szewczyk et al., 2013). Studies show that abnormalities in miRNA function influence the pathogenesis of infection with this bacterium (Zhou et al., 2018).

According to research, bacteria of the genus *Salmonella* can regulate miRNAs in epithelial cells and in macrophages (Ahmed et al., 2016). In the above-mentioned cells, *Salmonella* Typhimurium changes the expression of miRNA-15, miRNA-21, miRNA-146a, miRNA-155 and let-7. In that case the expression of miRNA-146a is upregulated and the expression of let-7 is downregulated (Herrera-Uribe et al., 2018; Naveed et al., 2017). Mentioned molecules affect the genes that regulate the cell cycle, the proliferation of B and T lymphocytes, and the inflammatory response of the body (Herrera-Uribe et al., 2018). After detection, by TLR4 receptors, of LPS from *Salmonella* the expression of miRNAs from the miRNA let-7 family is reduced, *inter alia*, in macrophages (Ahmed et al., 2016; Huang et al., 2019; Naveed et al., 2017; Yao et al., 2015; Yao et al., 2016; Zhou et al., 2018). The reduced expression of the above miRNAs increases the production of IL-6 and IL-10, which are involved in immunity regulation (Ahmed et al., 2016; Naveed et al., 2017; Yao et al., 2015; Yao et al., 2016; Zhou et al., 2018). After infection with this bacteria, an increase in the expression of miRNA-155 in macrophages has been observed (Herrera-Uribe et al., 2018). On the other hand, after *Salmonella* Typhimurium infection the expression of miRNA-155 is reduced in macrophages, it's reduces the host's immune response through the influence of this molecule on lymphocytes and dendritic cells (Zhou et al., 2018). Studies have confirmed that in the absence of miRNA-155, the specific immune response is significantly reduced (Ahmed et al., 2016). This is due to aberrant antigen presentation by dendritic cells to B and T lymphocytes. The reduction of miRNA-155 synthesis in B cells also affects the levels of immunoglobulin G class (IgG1) antibodies produced by these cells. After *Salmonella* infection, three miRNAs: miRNA-21, miRNA-146a and miRNA-155 strongly affect the NF-κB transcription factor, thus reducing the regulation of B and T lymphocyte proliferation (Ahmed et al., 2016). It has been observed that miRNA-214 and miRNA-331-3p are involved in *Salmonella* Thypimurium infection, and they play a part in the regulation of the host immune response (Herrera-Uribe et al., 2018; Zhou et al., 2018). A decrease in miRNA-214 expression and an increase in miRNA-331-3p expression were demonstrated (Zhou et al., 2018). Moreover, it has been proven that during infection with *Salmonella* bacteria in human monocytes there is an increase in the expression of miRNA molecules such as: miRNA-23b, miRNA-24, miRNA-27a, miRNA-29 and miRNA-222 (Ahmed et al., 2016). In the case of *Salmonella* Typhimurium infection, it has been observed that the time course of the inflammatory response is regulated by TNF-α mediated miRNA-125a/b (Herrera-Uribe et al., 2018). These miRNAs have been shown to negatively affect TNF-α production (Herrera-Uribe et al., 2018; Yao et al., 2016). It has also been proved that miRNA125a/b affects proteins involved in the MHC class I antigen presentation. Proteins involved in regulation include proteasome subunit beta type-8 (PSMB8), protein disulfide isomerase family A member 3 (PDIA3) and heat shock protein 90 beta family member 1 (HSP90B1) (Herrera-Uribe et al., 2018). The PSMB8 protein is involved in the inflammatory response and cell death. MiRNA-125a/b regulates the secretion, by macrophages, of effector molecules such as nitrogen intermediates, reactive oxygen species, and cytokines - IL1β, IL6 or TNF-α. Moreover, the effects of miRNA-148a/b on the HSP90B1 protein and miRNA-1 on the PDIA3 protein have been observed (Herrera-Uribe et al., 2018).

Pseudomonas aeruginosa

Pseudomonas aeruginosa belongs to the *Pseudomonadaceae* family of opportunistic gram-negative bacteria (Busse et al., 2012). This bacterium is the aerobic, which is most often isolated

from the genus of *Pseudomonas* (Zhou et al., 2018). This bcteria is one of the leading causes of contact lens-associated bacterial keratitis (Muraleedharan et al., 2019; Xu, Hazlett, 2019). Moreover, in immunocompromised persons or those suffered from cystic fibrosis, it also causes chronic and acute respiratory infections (Shin et al., 2017).

Studies have shown that miRNA-155 expression increases during *P. aeruginosa* corneal infection (Xu, Hazlett, 2019). Its presence increases the tissue susceptibility to the development of inflammation. MiRNA-155 reduces the ability of macrophages to phagocytose and inhibits intracellular killing processes (Xu, Hazlett, 2019). The effect of the miRNA-183/96/182 cluster on the time course of infection caused by *P. aeruginosa* has also been observed (Xu, Hazlett, 2019). During infection, lowering the activity of miRNA-183/96/182 leads to a reduction in the inflammatory response in the cornea (Muraleedharan et al., 2019; Xu, Hazlett, 2019). Whereas, a decrease in the activity of miRNA-183/96/182 or its absence results in the stimulation of the phagocytosis process and intracellular killing of bacteria in macrophages and neutrophils (Muraleedharan et al., 2019; Xu, Hazlett, 2019; Zhou et al., 2018). An increase in the activity of these mechanisms is caused by the influence of miRNA-183/96/182 on the increase of the efficiency of synthesis process of reactive oxygen and nitrogen species. It has also been observed that the miRNA-183/96/82 cluster increases the level of proinflammatory cytokines in macrophages in response to *P. aeruginosa* corneal infection (Muraleedharan et al., 2019; Xu, Hazlett, 2019). It has also been shown that this cluster is expressed in innate immunity cells and during the differentiation of Th17 lymphocytes (Xu, Hazlett, 2019). This cluster promotes Th17 lymphocyte response and stimulates the production of pro-inflammatory cytokines. The miRNA-183/96/182 cluster targets the *DAP12* (which is synonym for the transmembrane immune signalling adapter (*TYROBP*) gene) whose macrophage expression contributes to the reduction of cytokine production in response to *P. aeruginosa* (Muraleedharan et al., 2019). The DAP12 is also found on the surface of NK cells. By lowering the cluster expression that occurs during the infection with the bacterium in question, the expression of the DAP12 increases thus resulting in extinguishing the immune response by reducing the production of cytokines by macrophages. Another gene that the miRNA-183/96/182 cluster targets is NADPH oxidase 2 (*Nox2*). The Nox2 is an enzyme necessary for the production of reactive oxygen and nitrogen species (Muraleedharan et al., 2019). Pulmonary *P. aeruginosa* infection also leads to the lung tissue inflammation development (Li et al., 2016). Studies in mice have shown that during infection, the expression of miRNA-301b is increased under the influence of LPS (Huang et al., 2019; Li et al., 2016). The same studies have shown that after infection with the bacterium in question, miRNA-301b-m (chemically synthesized miRNA-301b) reduces the recruitment of neutrophils. Therefore, miRNA-301b is also presumed to inhibit the early recruitment of phagocytes to the focus of inflammation (Li et al., 2016). The appearance of neutrophils at the site of inflammation is associated with an increase in the immune response in this area. The same miRNA-301b-m decreased the secretion of pro-inflammatory cytokines, i.e. IL-4 and TGF-β1, by macrophage cells (Li et al., 2016). In the same study, miRNA-301b was found to target the c-Myb transcription factor (Li et al., 2016; Zhou et al., 2018). The c-Myb expression decreases after bacterial infection and is further decreased by the miRNA-301b activity. According to research, c-Myb influences the production of IL-4 and TGF-β1, and its lack suppresses their release (Li et al., 2016). Moreover, the same studies have shown that influx of $CD4^+$ T cells into the lungs under the influence of *P. aeruginosa* infection was inhibited by miRNA-301b-m. miRNA-301a is also presumed to produce similar effect as miRNA-301b on the release of IL-4 in macrophages (Li et al., 2016).

Mycobacterium tuberculosis

Mycobacterium tuberculosis belongs to the *Mycobacteriaceae* family (Busse et al., 2012) and is one of the best studied intracellular mycobacteria. Approximately one third of the human population is infected with this bacteria (Kumar et al., 2015; Szewczyk et al., 2013; von Both et al., 2018). Tuberculosis caused by *M. tuberculosis* most often affects the lungs, although its extrapulmonary forms also occur (Szewczyk et al., 2013). Infection with these microorganisms occurs through droplets or dust particles (Szewczyk et al., 2013). Mycobacteria multiply in macrophage cells, which are subsequently destroyed and phagocytosed by subsequent macrophages and other phagocytic cells (Szewczyk et al., 2013). *M. tuberculosis* may remain latent in the host's organism (Kumar et al., 2015; Szewczyk et al., 2013). In order to increase survivability in infected cells, mycobacteria suppress the processes leading to phagolysosome formation in the cell (Szewczyk et al., 2013; von Both et al., 2018). In addition, *M. tuberculosis* developed other mechanisms for intracellular survival: suppression of antigen presentation and deregulation of class II MHC system (von Both et al., 2018).

Research shows that the regulation of immunologic factors is influenced by miRNA-223 (Zhou et al., 2018). This molecule modifies the time course of *M. tuberculosis* infection by regulating the influx of neutrophils to the site of inflammation. Its level in the blood of infected patients is significantly elevated (Das et al., 2016). It is also presumed that the increased expression of miRNA-223 lowers the level of chemokine (C-C motif) ligand 3 (CCL3), chemokine (C-X-C motif) ligand 2 (CXCL2) and IL-6 (Das et al., 2016). After infection with *M. tuberculosis*, miRNA-155 expression increases in macrophage cells and other affected cells (Ahmed et al., 2016; Das et al., 2016; Zhou et al., 2018). This miRNA increases the influx of macrophages to the site of infection and the production of reactive oxygen species, thereby reducing the intracellular survival of mycobacteria (Zhou et al., 2018). In addition, miRNA-155 stimulates apoptosis in infected cells through the agency of TLR2 and NF-κB signalling pathways. The same miRNA molecule may also have a favourable effect on the survival of mycobacteria in macrophages (Das et al., 2016; Zhou et al., 2018). This is, *inter alia*, achieved by inhibiting the expression of one of the zinc finger domains BTB or SHIP1 (SH2-domain containing inositol polyphosphate 5-phosphatase 1), which shows phosphatase activity (Zhou et al., 2018). Moreover, miRNA-155 increases survival and enhances the functions of T cells specific to *M. tuberculosis* infection (Zhou et al., 2018). It has also been observed that reducing the level of miRNA-155 in human macrophages inhibits the production of TNF-α (Ahmed et al., 2016; Das et al., 2016). Additionally, studies have shown that *M. tuberculosis* inhibits TNF-α production by upregulating miRNA-125b expression (Das et al., 2016). Studies have shown that *M. tuberculosis* inhibits miRNA let-7f expression (Ahmed et al., 2016; Das et al., 2016; Kumar et al., 2015; Zhou et al., 2018). The let-7f miRNA regulates the mRNA of the factor inhibitor NF-κB - A20. The A20 protein is a feedback inhibitor of the NF-κB pathway (Kumar et al., 2015). In this way, the tubercle bacilli increase their survivability in the affected cells (Das et al., 2016). The role of miRNA-144 in the immune response to mycobacterial infection was also demonstrated experimentally (Ahmed et al., 2016). MiRNA-144 reduces the synthesis of TNF-α and IFN-γ and inhibits the proliferation of T lymphocytes. The MiRNA-146a expression is also increased during *M. tuberculosis* infection. This molecule, through the signalling pathways TLR/NF-κB and interleukin-1 receptor-associated kinase-1 (IRAK-1), reduces the secretion of pro-inflammatory cytokines: TNF-α, IL-1β, IL-6 and monocyte chemoattractant protein 1 (MPC-1) chemokine (Das et al., 2016). During infection with this bacterium, the levels of miRNA-26a and miRNA-132 are increased, which indirectly contribute to the reduction of IFN-γ

in macrophages. Additionally, an increase in the miRNA-206 expression has been observed, which increases the secretion of pro-inflammatory cytokines- IL-1β, IL-6, IFN-γ, TNF-α and matrix metallopeptidase 9 (MMP9) (Das et al., 2016). It is presumed that *M. tuberculosis* increases the miRNA-99b expression in DC and macrophages, the presence of which in these cells suppresses the release of pro-inflammatory cytokines (IL-1β, IL-6, IL-12 and TNF-α) and increases the severity of infection (Ahmed et al., 2016; Das et al., 2016; Zhou et al., 2018). In addition to the TNF-α receptor genes, MiRNA-99b also targets the tumour necrosis factor receptor superfamily member 4 (TNFRSF-4) genes (Das et al., 2016). Research confirms that *M. tuberculosis* avoids immune response by increasing the miRNA-381-3p expression (Das et al., 2016). MiRNA-381-3p reduces the amount of CD1 antigen on the surface of infected dendritic cells (Das et al., 2016). This antigen is responsible for the presentation of *M. tuberculosis* antigens to T lymphocytes (Das et al., 2016; Gołąb et al., 2018).

Conclusion and perspectives

This article shows that miRNAs affect a number of processes related to immune regulation during viral and bacterial infections. This affect cannot be clearly defined as negative or positive. As we showed in our work depending on the type of infected cell or the infectious agent, the same miRNA can have different effects. In addition, miRNAs represent an exceptionally promising research object due to the diversity of their biological potential. They can be used not only as diagnostic material, but also as a therapeutic objective in the treatment of various diseases. Research on the influence of miRNAs on the course of the immune response during viral and bacterial infections provides more and more new evidence that miRNAs can be used in the future in immunological therapies. In the future, these molecules may provide an alternative to the traditional treatment of bacterial infections with antibiotics. More and more studies report the possibility of inhibiting or even combating, by means of miRNA regulation, infections with bacterial strains resistant to antibiotics.

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Report of *Trematosoma rotunda* (Ciliophora, Suctorea) as epibiont on harpacticoid copepod from western Indian coast

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Keywords epibiont ciliate, copepod, India, Arabian Sea

Abstract Suctorian ciliate *Trematosoma rotunda* (Allgén, 1952) is reported here for the first time as epibiont on a harpacticoid copepod collected from Mandovi river estuarine mouth, Goa, west coast of India, Arabian Sea. The description of the species, synonymy, data on species distribution and host taxa are also presented in this study.

Notatka o Trematosoma rotunda (Ciliophora, Suctorea) jako epibioncie występ[ującym na Herpacticoida z zachodniego wybrzeża Indii

Słowa kluczowe epibiontyczne orzęski, oczliki, Indie, Morze Arabskie

Streszczenie Po raz pierwszy odnotowano gatunek orzęska *Trematosoma rotunda* (Allgén, 1952) jako epibionta na Herpacticoida zebranym z ujścia rzeki Mandovi, Goa, zachodnie wybrzeże Indii, Morze Arabskie. W pracy przedstawiono opis gatunku, synonimizację oraz dane dotyczące rozmieszczenia gatunków i taksonów żywicieli.

Introduction

Suctorian ciliates are common epibionts of marine meiobenthic invertebrates such as nematodes, halacarid mites, harpacticoid copepods, kinorhynchs, ostracods, bryozoans etc. (eg., Precht, 1935; Jankowski, 1981; 2007; Dovgal, 2002; 2013; Dovgal et al., 2008; 2009; Fernandez-Leborans, Tato-Porto, 2000a, b; Ingole et al., 2010; Fernandez-Leborans et al., 2012; Chatterjee et al., 1996; 2012; 2013a, b, c; 2014a, b; 2018; 2019a, b, c; 2020a, b, c, d, e, f; Durucan, 2019).

Trematosoma rotunda (Allgén, 1952) a marine loricate suctorian ciliate described by Allgén (1952) as epibiont on marine nematodes *Desmodorella tenuispiculum* (Allgén, 1928) and

Croconema stateni Allgén, 1928 collected in the Antarctic (Graham Land) and the Falkland Islands, was named as *Acineta rotunda*. In the same paper another suctorian species (*Acineta ovoidea*) inhabiting on nematodes was described. However, Curds (1985) synonymized *A. ovoidea* with *A. rotunda* showing that *A. ovoidea* was nothing but lateral view of *A. rotunda*. Dovgal (2002) transferred *A. rotunda* into genus *Trematosoma* Batisse, 1972 based on arrangement of its tentacles in two rows.

Dovgal et al. (2009) reported this species on the nematode *Pseudochromadora* sp. collected near Ratnagiri, Maharastra State, central west coast of India (Arabian Sea), and provide detailed remarks on the systematics and nomenclature of this species.

Fisher (2003) found epibiont ciliate on nematode *Pseudochromadora cazca* Gerlach, 1956, from the intertidal zone of North-Eastern Queensland, Australia; Dovgal et al. (2009a) identified this species based on Fisher's photo 'probably as *Trematosoma rotunda* (Allgén, 1952)'.

Bhattacharjee (2014), Ansari and Bhaduri (2016), Ghosh and Mandal (2019) reported the same species on the nematode host from Bay of Bengal, Indian Ocean. Baldrighi et al. (2020) found this species on nematode from NW Madagascar margin-deep sea pockmark and Gulf of Naples-shallow vent area.

We report here *T. rotunda* from Goa, west coast of India, Arabian Sea as an epibiont on harpacticoid copepod.

Material and Methods

Samples were collected from Mandovi river estuarine mouth (15°28'43.68"N and Long 73°41'41.28"E), Goa, west coast of India, Arabian Sea (Figure 1) at 20m depth. Samples were sieved in the laboratory using 45 µm mesh size sieve. Infested meiofauna were sorted, mounted on a 50% glycerine slide and sealed with DPX. The systematic position of suctorian ciliates follows Dovgal (2002; 2013). Measurements were carried out on four specimens using the program Toup View 3.7 for digital camera. Specimens are kept in the collection of the fourth author (MN) in CSIR—National Institute of Oceanography, Dona Paula, Goa.

Result and Discussion

Class Suctorea Claparéde and Lachmann, 1859
Subclass Endogenia Collin, 1012
Order Acinetida Raabe, 1964
Family Acinetidae Ehrenberg, 1834
Genus *Trematosoma* Batisse, 1972

***Trematosoma rotunda* (Allgén, 1952)** (Figure 2A–C)
=*Acineta rotunda* Allgén, 1952
=*Nemataclneta rotunda* (Allgén, 1952)
=*Conchacineta rotunda* (Allgén, 1952)
=*Acineta ovoidea* Allgén, 1952

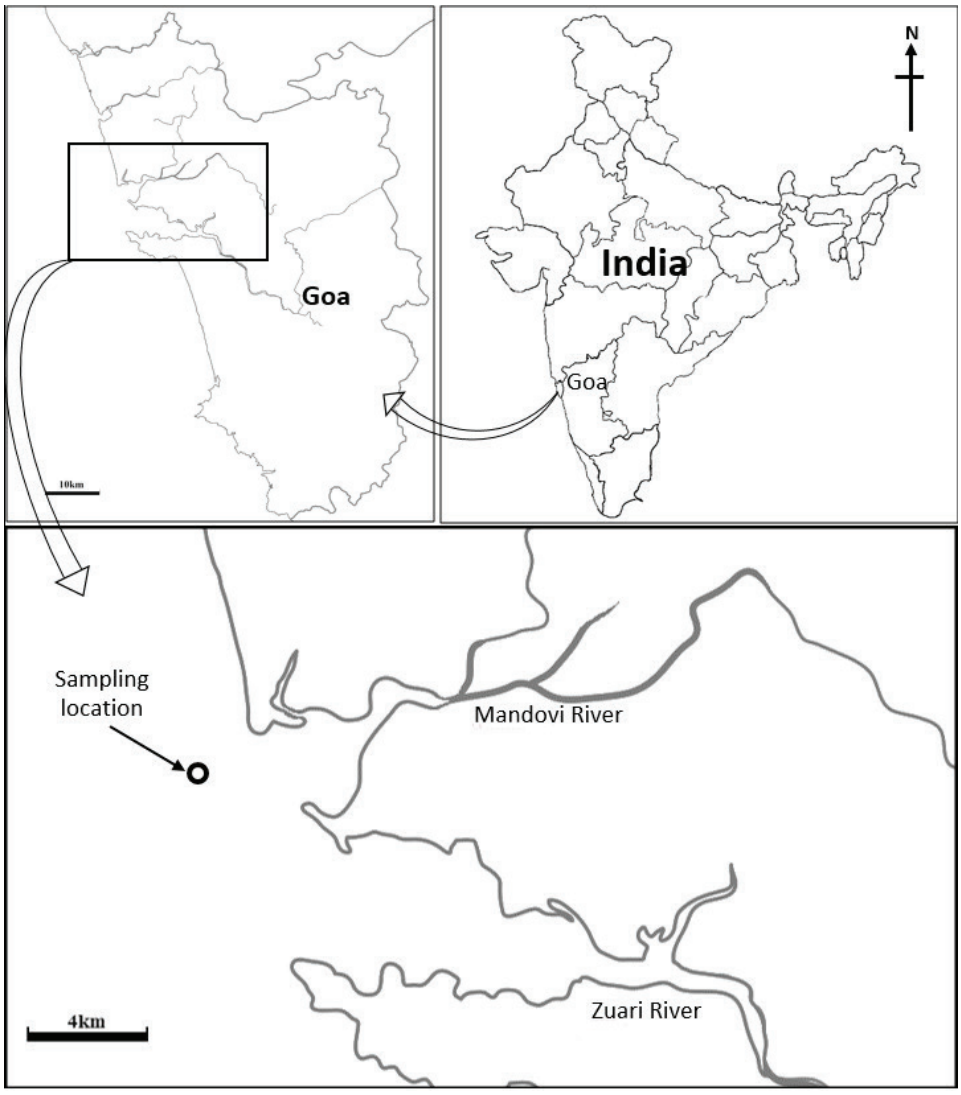


Figure 1. Map of collection site

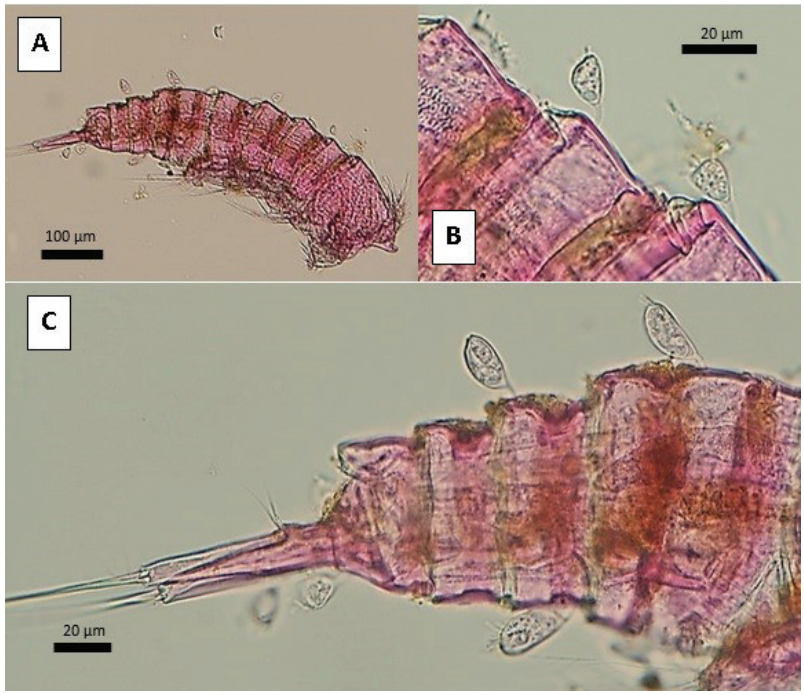


Figure 2. *Trematosoma rotunda* on harpacticoid host body

Material examined: Several individuals distributed throughout the harpacticoid copepod body.

Diagnosis

Marine loricate suctorian. Cell body short, rounded, laterally flattened, entirely fills up the lorica and clamped to their aperture border. Lorica delicate gently striated. Tentacles clavate, short, arranged in two groups of rows at the upper body surface. Macronucleus spherical or oval, centrally or slightly posteriorly located. Stalk short, thin, slightly ribbed.

Measurements

Morphometric measurements of specimens of *Trematosoma rotunda* observed in the present study (based on 4 individuals, in µm): Lorica length 12–27, lorica width 8–9, body length 11–17, body thickness 8, macronucleus diameter 3–6, stalk length 3–8, stalk diameter 1–3, length of tentacles 2–5; protomite length 7, width 3.

Distribution and host specificity

Table 1 shows both the basibiont species and known localities of *T. rotunda*, while Figure 3 illustrated the map of the species distribution.

Table 1. *Trematosoma rotunda* (Allgén, 1952) localities and basibiont species

Basibiont (Host)	Locality	Reference	Remarks
Nematode – *Desmodora tenuispiculum* Allgén, 1928	Falkland Islands, near the southern tip of Argentina (Fig. 2, location 1)	Allgén (1952)	Epibiont reported as *Acineta rotunda* and *A. ovoidea*. Curds (1985) synonymized *A. ovoidea* with *A. rotunda* showing that *A. ovoidea* was nothing but lateral view of *A. otunda*
Nematode – *Desmodora stateni* Allgén, 1928	Graham Land, Antarctica (Fig. 2, location 2)	Allgén (1952)	
Nematode - *Pseudochromadora* sp.	Ratnagiri, Maharastra, India, Arabian Sea (Fig. 2, location 3)	Dovgal et al. (2009)	
–	North-Eastern Queensland, Australia (Fig. 2, location 4)	Dovgal et al. (2009)	Dovgal et al. (2009) identified ciliates based on Figure 1 of Fisher (2003)
Nematode – *Chromaspirina* sp.	Rushikulya, Odisha India, Bay of Bengal (Fig. 2, location 5)	Bhattacharjee (2014)	
Nematode – *Dorylaimposis punctata*	Sagar Island, Sundarbans, Bay of Bengal – mangrove ecosystem (Fig. 2, location 6)	Ansari and Bhadury (2016)	
Nematode – *Desmodora scaldensis* de Man 1889	Matla estuary, Sundarbans, Bay of Bengal – subtidal (Fig. 2, location 7)	Ghosh and Mandal (2019)	
Nematode - *Tricoma* sp.	Karwar, Karnataka, India, Arabian Sea (Fig. 2, location 8)	Chatterjee et al. (2020d)	
Nematode – *Desmodora* sp.	NW Madagascar margin-deep sea pockmark (Fig. 2, location 9)	Baldrighi et al. (2020)	
Nematode – *Desmodora* sp.	Gulf of Naples-shallow vent area (Fig. 2, location 10)	Baldrighi et al. (2020)	
Harpacticoid copepod	Mandovi river estuarine mouth, Goa, India, Arabian Sea (Fig. 2, location 11)	Present Report	

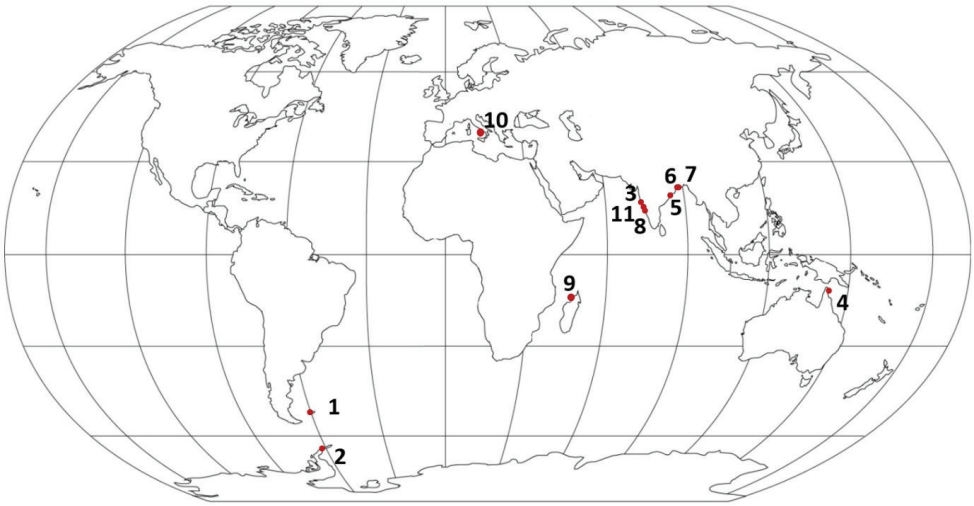


Figure 3. The map of world distribution of *Trematosoma rotunda* (the numbers of localities correspond to Table 1)

It should be presumed that the species is widely distributed, whose spread may be limited possibly by salinity. The relatively small number of finds of the species is apparently associated with a lack of research interest to ciliate epibiosis. This species was predominantly found on nematodes. Chatterjee et al. (2019b) have previously mentioned about *T. rotunda*, which most likely epibiont on nematodes only. However, our new finding is not only the first report of this species from Goa, but the first its registration on representative of new host taxon – Harpacticoida Sars, 1903. The last finding permits to conclude that *T. rotunda* may inhabit on a wide range of meiobenthic marine invertebrates.

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Preliminary investigations of the chironomid larvae fauna (Chironomidae, Diptera) from the Mavrovo reservoir – Republic of Macedonia

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Keywords reservoir, chironomids, community structure, dominance, seasonal density

Abstract Larval stages of the Chironomidae (Diptera) can be found in almost any aquatic habitat, including artificial lakes. They form an important fraction of the macrozoobenthos of most freshwater ecosystems. The Mavrovo reservoir as an artificial lake in the Republic of Macedonia, to date, has never been an object of hydrobiological research of this type. For that reason, the purpose of this study was to conduct research on the composition and structure of the chironomid larvae fauna that inhabits the Mavrovo reservoir. The quantitative samples for this research were collected during one year (2017/18), seasonally from five different localities (T1–T5) – T1-Ulazna, T2-Hotel Radika, T3-Middle of the reservoir, T4-Bunec, and T5-Old church (village Mavrovo). According to the results, Chironomidae was represented with five subfamilies, two tribes, and 25 taxa. The subfamilies are Chironominae with the two tribes Chironomini and Tanytarsini, Tanypodinae, Orthocladiinae, Diamesinae, and Prodiamesinae. The dominant taxon is *Tanytarsus* sp., and besides it, the following taxa: *Procladius* sp., *Chironomus plumosus, Harnischia* sp., *Polypedilum nubeculosum,* and *Orthocladius saxicola* are characterized by greater community participation. The highest density of chironomid larvae fauna was recorded during the summer, a total of 1013.69 ind/m^2, compared to the lowest registered in the spring season which is only 9.78 ind/m^2.

Badania wstępne nad fauną larw ochotkowatych (Chironomidae, Diptera) w zbiorniku Marvrovo – Republika Macedonii

Słowa kluczowe zbiornik, ochotkowate, struktura zgrupowań, dominacja, zagęszczenie sezonowe

Streszczenie Stadia larwalne Chironomidae (Diptera) są znajdowane w niemal wszystkich środowiskach wodnych, łącznie z jeziorami zaporowymi. Stanowią one bardzo ważną frakcję makrozoobentosu w większości słodkowodnych ekosystemów. Zbiornik Marvoro jest jeziorem zaporowym w Republice Macedonii i do tej pory nie było obiektem tego typu badań hydrobiologicznych. Z tego powodu celem było przeprowadzenie badań składu i struktury fauny larw ochotkowatych zamieszkujących zbiornik Mavrovo. Badania prowadzono za pomocą

prób ilościowych, pobieranych w ciągu jednego roku (2017/2018), w poszczególnych sezonach wegetacyjnych w następujących lokalizacjach (T1–T5) – T1 –Ulazna, T2 – Hotel Radika, T3 – środek zbiornika, T4 – Bunec i T5 – stary kościół (wieś Mavrovo). Fauna Chironomidae była reprezentowana przez pięć podrodzin, dwa plemiona i 25 taksonów. Podrodziny to Chironominae z dwoma plemionami Chironomini i Tanytarsini, Tanypodinae, Orthocladiinae, Diamesinae i Prodiamesinae. Dominującym taksonem był *Tanytarsus* sp., wysokimi udziałami w zbiorowisku charakteryzowały się również taksony: *Procladius* sp., *Chironomus plumosus*, *Harnischia* sp., *Polypedilum nubeculosum* i *Orthocladius saxicola*. Najwyższe zagęszczenie larw ochotkowatych odnotowano latem, łącznie 1013,69 ind/m^2, w porównaniu do najniższego zarejestrowanego w sezonie wiosennym, który wynosi zaledwie 9,78 ind/m^2.

Introduction

The reservoirs are artificial ecosystems and their ecological functioning has characteristics falling between a river and a lake (Callisto et al., 2005). They are built to provide water reserves for a variety of purposes, including electricity, households, industrial materials, transportation, irrigation, and recreation (Prat et al., 1992; Petridis, Sinis, 1993; Holdren et al., 2001; Tundisi et al., 2008; Tskhai, 2015). The construction of a dam causes significant changes in the natural conditions of the river ecosystem and neighboring territories (Armitage, 1984; 1987; Petts, 1984; Penczak et al., 1998, Kornijów, 2009; Tskhai, 2015). This has a great impact on the entire aquatic life in that area. Chironomidae are a family of Diptera (Culicomorpha) with a wide distribution in every type of aquatic ecosystems (Giłka, 2009). In Macedonia, to date, it has been widely researched, especially in natural lakes (Ohrid, Prespa, and Dojran Lake) (Angelovski, 1980; Angelovski, Shapkarev, 1983;1991;1994), while in the reservoirs it's been poorly explored (Sidorovska, 1988, Smiljkov, 1996; Miljanovič et al., 2004; Slavevska-Stamenkovic, 2007; Rimceska, 2014). Mavrovo Lake as an artificial reservoir over the years has almost never been the subject of research of this type. In the context of hydrobiological data for this reservoir, Stojkovski (1960) published a paper on the composition of the benthic community several years after the construction of the dam, ie after the creation of the lake. Shortly afterward, Popovska-Stankovic (1963) conducted another study of the Mavrovo reservoir on zooplankton from the first years of the lake's existence. Regarding the study of the physicochemical characteristics and the ecological status of Mavrovo Lake, a master thesis was prepared by Latifi, Lj. (2011). Recently, there is almost no hydrobiological research of the Mavrovo reservoir as an artificial lake. Starting from that fact, and taking into account the specific characteristics of the selected group of animals – chironomids, originated the need, and also the goal to conduct research on the composition and structure of the chironomid larvae fauna that inhabits the Mavrovo reservoir.

Study area

One of the biggest artificial reservoirs on the territory of the Republic of Macedonia is Mavrovo Lake. It is located in the northwest region of the municipality of Mavrovo at the base of the mountains Bistra and Korab. In the south, it is limited by forested and grassland Vlainica and in the north by Shar Planina, at an altitude of 1223 m a.s.l. The lake started to form in 1953 when a high dam was built in the western part of the valley between Mavrovi Anovi and the village of Mavrovo. The Mavrovo reservoir belongs to the category of mountain lakes of alpine character, so it freezes during the winter. It is the highest mountain lake in Macedonia. The total length of the lakeshore is 24.25 km. The depth of the lake is relatively variable, but it is considered

that the maximum depth is 40 m. Water temperature has significant seasonal and altitude changes. The average monthly temperature of the lake water in July and August is around 20°C, and the highest limit can exceed that limit.

The average monthly temperatures in the investigated area are negative in the three winter months and in January they are –2.2°C. The highest average temperatures are in July, 16.3°C, and August 16°C, so that the average annual temperature variation is 18.7°C. The samples for this research were collected seasonally in the period from July 2017 to June 2018 from 5 different points – Ulazna (T1), Hotel Radika (T2), Middle point of the Lake (T3), Bunec (T4), Old church (T5) (Figure 1). In continuation follows a brief description of the investigated localities:

T1 (41°42'27.5"N 20°46'34.5"E) – this point is located on the northern part of the lake, before the entrance to the settlement of Mavrovi Anovi. The shore of the lake is partly overgrown with grass, and the substrate of the littoral part is composed of coarser and finer stone. The substrate at the bottom is mainly represented by fine sand. Due to the variable level of the reservoir as well as the substrate of this locality, in the months of September, March, and June no samples were collected for quantitative analysis. The depth of this point is 3 m.

T2 (41°41'53.9"N 20°47'06.6"E) – this site is located between the two villages on the east shore of the lake, Nikiforovo, and Leunovo. The bottom of the lake is composed of sandy substrate and sporadic with plant material residues, due to drifts. The depth of this point is 4.5 m.

T3 (41°41'31.0"N 20°45'34.1"E) – the middle point of the lake is located in the middle of the widest part of the lake, from where on one side you can see the Mavrovo dam, and on the other side is the village Nikiforovo. At the time the samples were collected, the depth was 20 m.

T4 (41°42'03.7"N 20°47'31.3"E) – this site is located on the north side of the lake, between T1 and T3. The lake bottom in this locality is represented by finer and coarser sand. The depth of this locality is 7.4 m.

T5 (41°39'40.9"N 20°44'09.7"E) – this locality is in the southern part of the lake, near the old church which in some periods of the year is submerged in the lake. It is important to note that due to large seasonal changes in the water level, the depth in this locality is not constant. The base of this locality is composed of fine sand and finer and coarser stone, mainly near the Mavrovska River, before the inflow into the lake, while around the lake the ground is muddy. The depth of this locality in the period of material collection was 5 m.

Materials and methods

The materials used for this research were collected from five points during one year (2017/18), seasonally. Three samples were taken from each point, and the results are shown as mean values. The collection of the samples was carried out with the Ekman dredge with a volume of 225 cm^2 which is used for collecting from a substrate with small particle diameter (fine sand, silt) from the lake. Immediately after taking the samples, they were preserved with 70% ethanol. After the preservation they were transported to the laboratory were permanent microscopic slides were made using a mounting medium of Canada-balsam and then determined to the species level. The identification was done using proper taxonomic keys: D.R. Oliver et al. (1978), B. Rossaro (1982), J.H. Epler (2001), Orendt et al., (2012), Andersen et al., (2013), B. Rossaro and V. Lencioni

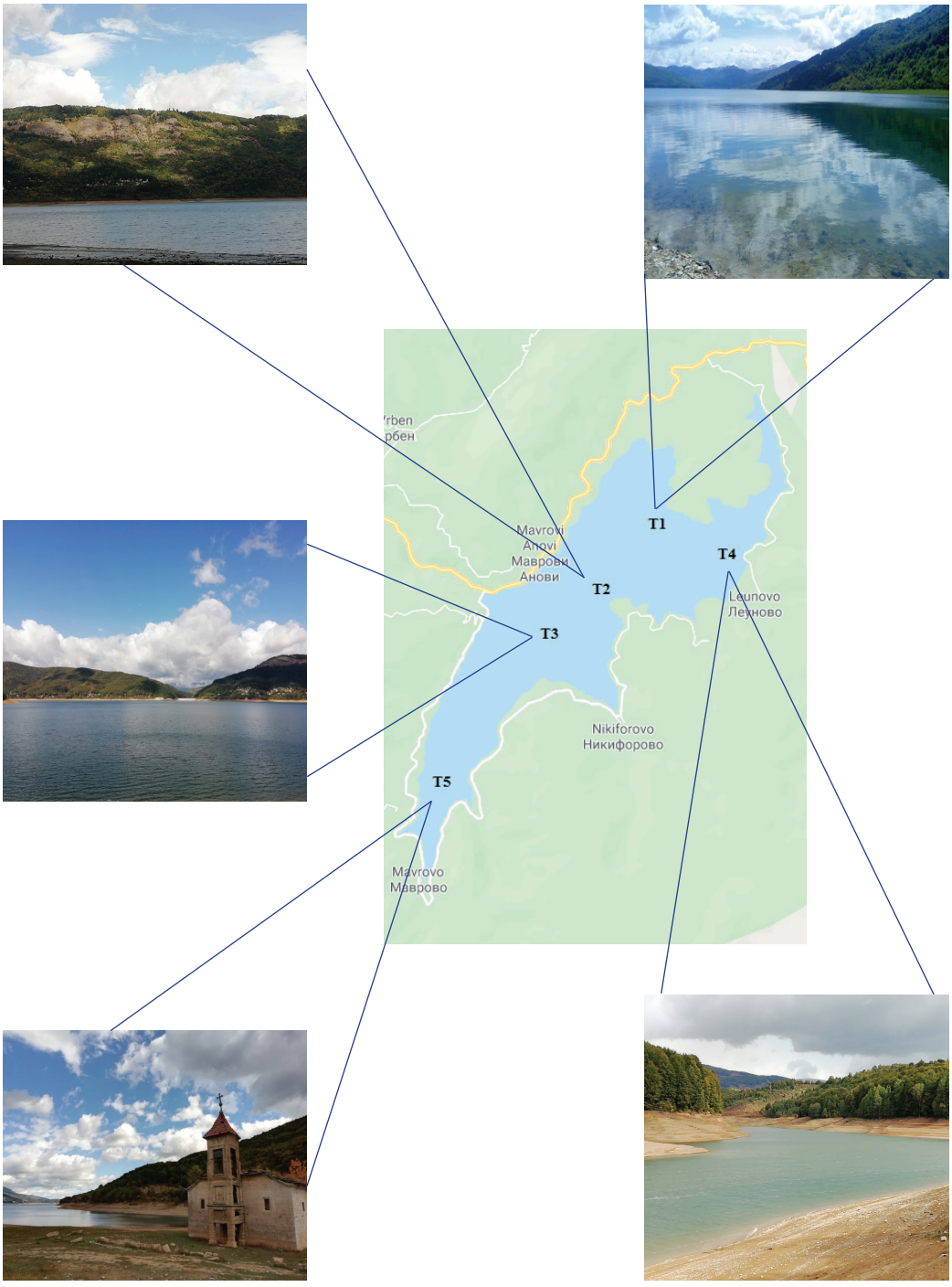


Figure 1. Map of the investigated points

(2015). All identified taxa are in a larval stage, and for their identification were used keys from authors that identified Chironomidae larvae, not pupae or adults. The abundance is shown as the number of individuals of unit meter square (ind/m^2) and dominance is calculated with the formula of Balogh (1958). According to this author the values for dominance are divided into four groups: for dominance greater than 10% are considered dominant (D), between 5–10% subdominant (SD), between 1–5% recendent (R), and lower than 1% subrecendent (SR).

Results

During the research conducted on the chironomid larvae fauna of the Mavrovo reservoir in the period 2017/18, the presence od 25 species was established. They belong to five subfamilies and two tribes: subfamilies Tanypodinae, Orthocladiinae, Diamesinae, Prodiamesinae, and Chironominae with the two tribes Chironomini and Tanytarsini (Table 1). As can be seen from the table, most species belong to the subfamily Chironominae, 13 in total.

Table 1. List of taxa of Chironomidae larvae fauna from the Mavrovo reserovir

Taxa
Subfam. Tanypodinae
Procladius choreus Meigen, 1804
Procladius sp. Skuse, 1889
Thiennemannymia sp. Fittkau, 1957
Subfam. Chironominae – tribe Chironomini
Harnischia sp. Kieffer, 1921
H. fuscimania Kieffer, 1921
H. curtilamelata Malloch, 1915
Cryptochironomus deffectus Kieffer, 1913
Polypedilum nubeculosum Meigen, 1804
P. scalaneum Schrank, 1803
Chironomus plumosus Linnaeus, 1758
C. thummi (riparius) Kieffer, 1911
Microtendipes pedellus De Geer, 1776
Cladopelma viridula Linnaeus, 1767
Subfam. Chironominae – tribe Tanytarsini
Cladotanytarsus mancus Walker, 1856
Tanytarsus sp. Van der Wulp, 1874
Micropsectra sp. Kieffer, 1908
Subfam. Orthocladiinae
Orthocladius saxicola Kieffer, 1911
Thienemanniella clavicornis Kieffer, 1911
Brilia bifida (modesta) Kieffer, 1990
Cricotopus sylvestris Fabricius, 1794
Parakiefferiela batophila Kieffer, 1912
Paracricotopus niger Kieffer, 1913

Continuation of Table 1

Psectrocladius sordidelus Zetterstedt, 1838	
Subfam. Prodiamesinae	
Prodiamesa olivacea Meigen, 1818	
Subfam. Diamesinae	
Diamesa insignipes Kieffer, 1908	
Total	**25**

According to the results shown in Table 2, the average annual density of the chironomid larvae fauna of the Mavrovo reservoir is 539.13 ind/m^2. The average annual density ranges from 6.67 ind/m^2 in the spring, which is the lowest value, to 2225.9 ind/m^2, which is the highest density in the summer season.

The Tanytarsini tribe from the subfamily Chironominae has by far the largest representation in this reservoir, 2484.39 ind/m^2, or 73.18% of the total chironomid larvae (Figure 2). Next is the subfamily Tanypodinae with a presence of 457.29 ind/m^2 (13.47%), then the tribe Chironomini from the subfamily Chironominae with 330.78 ind/m^2 (9.74%). Diamesinae and Orthocladiinae with 64.45 ind/m^2 (1.90%) and 51.28 ind/m^2 (1.51%) respectively have a smaller share in relation to the overall chironomid larvae fauna. Finally, the subfamily Prodiamesinae with a presence of 6.67 ind/m^2 or only 0.2% accounts for the smallest and almost insignificant share of the chironomid community in this reservoir.

Table 2. Annual dynamics of the density (ind/m^2) of the Chironomidae larvae broken down by seasons and points

Seasons/Points	T1	T2	T3	T4	T5
Summer	**2,225.9**	93.35	909.01	1,840.30	/
Autumn	/	1,251.30	215.58	291.15	1,397.95
Winter	/	688.98	526.73	/	1,293.50
Spring	/	42.23	/	/	**6.67**
Average annual density (ind/m^2)			**539.13**		

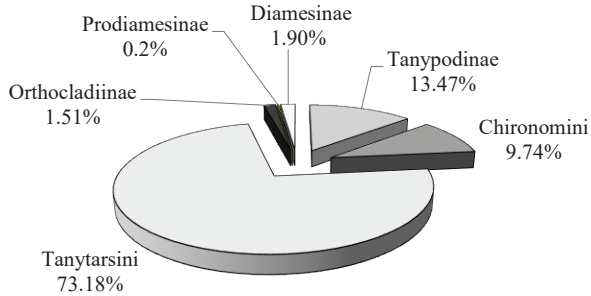


Figure 2. Percentage representation (%) of the subfamilies/tribes in the investgated period

In terms of the individual species of the benthos of the reservoir, the dominant taxon is *Tanytarsus* sp., with a share of 67.78% (7318.7 ind/m^2) in this community (Table 3). The taxa *Procladius* sp*., Chironomus plumosus,* and *Harnischia* sp. are subdominant with a share of 8,32% (896,8 ind/m^2), 8, 03% (865,66 ind/m^2) and 5,68% (612,31 ind/m^2), respectively.

Table 3. Average annual density (ind/m^2) and percentage representation (%) of the species from the Mavrovo reservoir in the investigated period

Species	ind/m^2	%	Dominance	Species	ind/m^2	%	Dominance
Tanytarsus sp. Van der Wulp, 1874	7318.70	67.88	D	*Micropsectra* sp. Kieffer, 1908	50.01	0.46	SR
Procladius sp. Skuse 1889	896.8	8.32	SD	*Cryptochironomus deffectus* Kieffer, 1913	45.56	0.42	SR
Chironomus plumosus Linnaeus, 1758	865.66	8.03	SD	*Parakiefferiela batophila* Kieffer, 1912	20.00	0.19	SR
Harnischia sp. Kieffer. 1921	612.31	5.68	SD	*Thiennemannymia* sp. Fittkau, 1957	17.78	0.16	SR
Polypedilum nubeculosum Meige, 1804	398.94	3.70	R	*Cladopelma viridula* Linnaeus, 1767	11.11	0.10	SR
Brilia bifida (modesta) Kieffer, 1990	195.58	1.81	R	*Prodiamesa olivacea* Meigen, 1818	6.67	0.06	SR
Orthocladius saxicola Kieffer, 1911	131.13	1.22	R	*Cricotopus sylvestris* Fabricius, 1794	4.45	0.04	SR
Cladotanytarsus mancus Walker, 1856	84.46	0.78	SR	*Thienemanniella clavicornis* Kieffer, 1911	3.34	0.03	SR
Diamesa insignipes Kieffer, 1908	64.45	0.60	SR	*Paracricotopus niger* Kieffer, 1913	2.22	0.02	SR
Microtendipes pedellus De Geer, 1776	51.12	0.47	SR	*Psectrocladius sordidelus* Zetterstedt, 1838	2.22	0.02	SR

At all points (depths) they occur with different density (Figure 3). It ranges from a minimum of 412.83 ind/m^2 at point T3, which is also the greatest depth at which the samples were collected, up to a maximum of 674. 53 ind/m^2 at T5. At the other three points, the density is 556.46 ind/m^2 at T1, 518.95 ind/m^2 at T2, and 532.85 ind/m^2 at T4.

As for the percentage representation of subfamilies and tribes, the dominance of the Tanytarsini tribe above all others, along with the vertical distribution, is due to the large representation of the *Tanytarsus* sp. (Figure 4). The highest percentage of this taxon (82.78%) is represented at point T1 and the lowest percentage (53.79%) is represented at T5 regarding the overall chironomid community. On the other hand, the point at which all subfamilies/tribes are represented is T5 (5 m). Here alongside *Tanytarsus* sp., *Chironomus plumosus* is also characterized by great representation, i.e. the 23.97% share in the communitycomposition.

The seasonal dynamics of the chironomid larvae fauna in the investigated period 2017/18, ranges from 1013,69 ind/m^2, which is the highest density recorded in the summer period, to 9,78 ind/m^2, which is the lowest density recorded in the spring period (Figure 5).

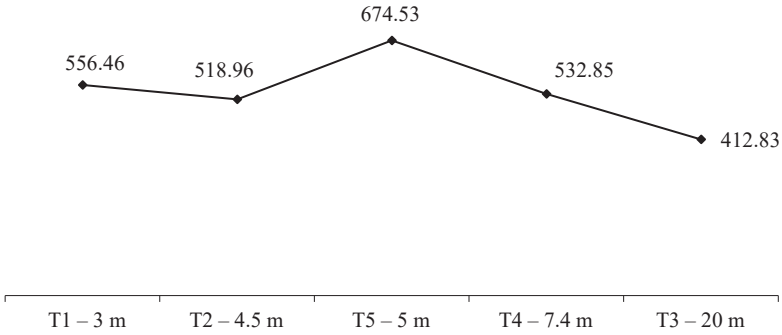


Figure 3. Dynamics of the average density (ind/m^2) of the chironomid community from the Mavrovo reservoir

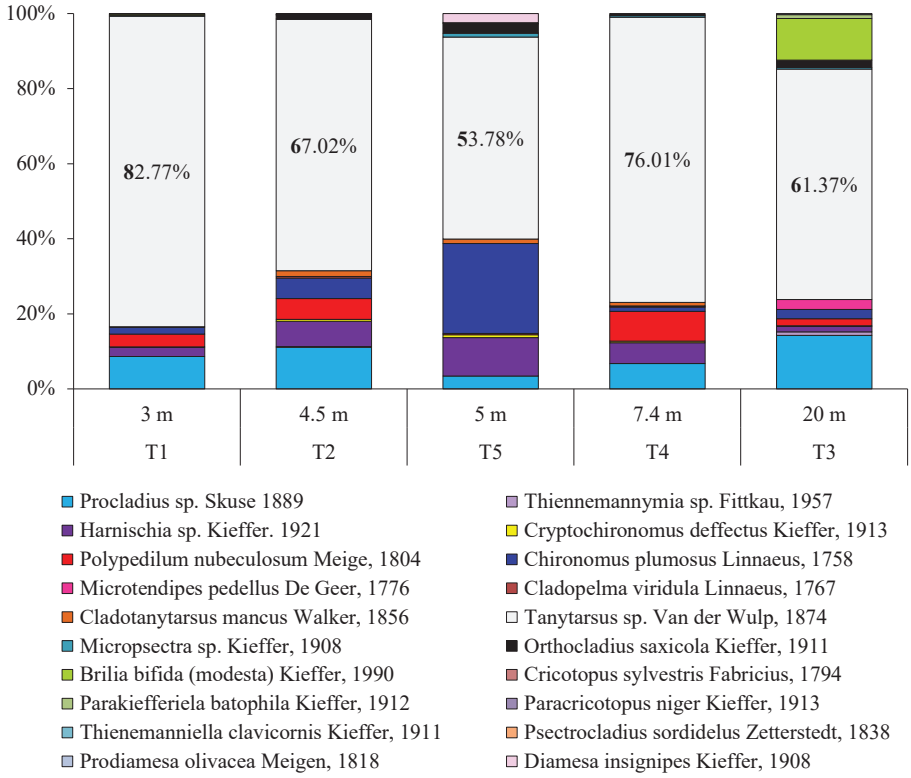


Figure 4. Average percentage annual representation of the chironomid species broken down by points

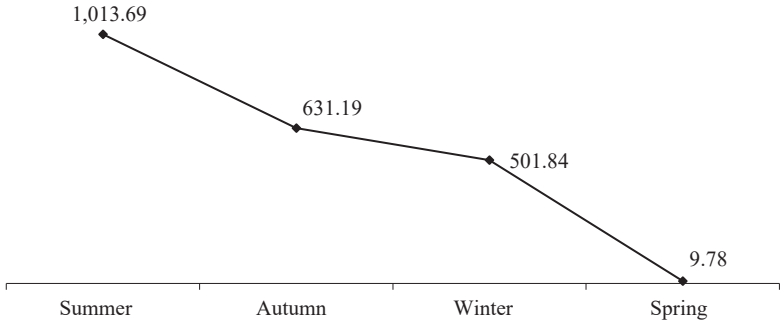


Figure 5. Seasonal dynamics of the average density (ind/m^2) of the chironomid larvae fauna in the investigated period

As we can see from Figure 6, in all four seasons *Tanytarsus* sp. is the dominant taxon. Beside it, *Chironomus plumosus* is also characterized by a high percentage in autumn (13.52%), winter (14.70%) and spring (22.72%).

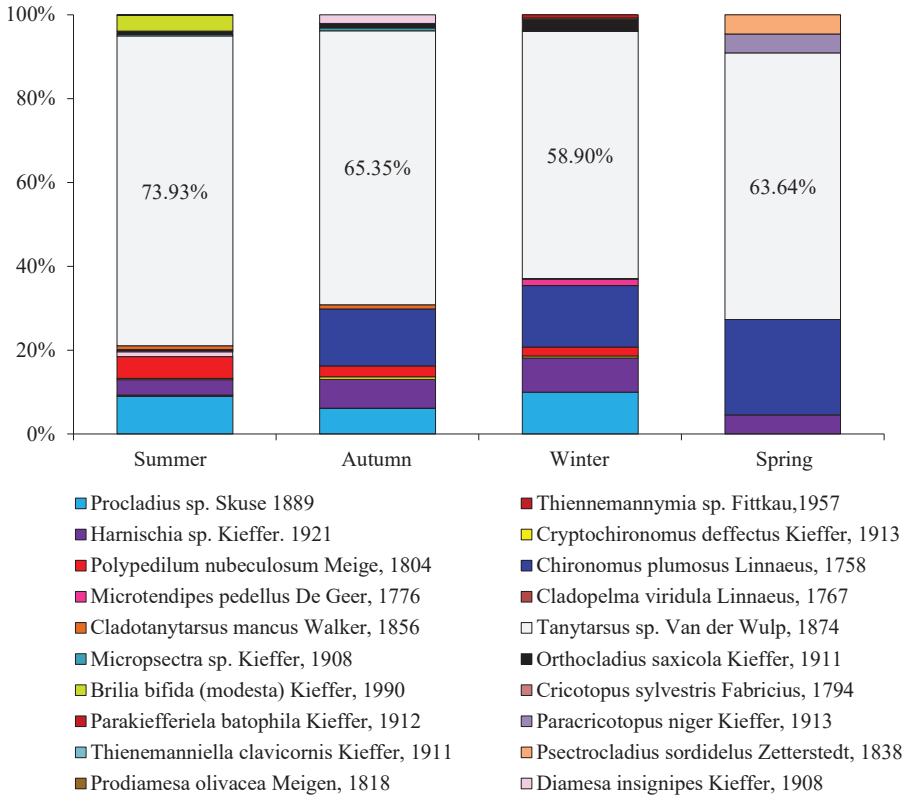


Figure 6. Average percentage annual representation of the chironomid species followed by seasons

Discussion

Mavrovo Lake is an artificial reservoir in the western part of Macedonia. This research is the first study of the species composition of the chironomidae larvae in this reservoir. The results showed the presence of a total of 25 species in the lake. Most species (13) belong to the subfamily Chironominae with the two tribes Chironomini and Tanytarsini, followed by the subfamily Orthocladiinae (6) and Tanypodinae (4). Of the subfamilies Diamesinae and Prodiamesinae, 1 species was found. Each subfamily has its own predispositions to certain environmental conditions. The subfamily Chironominae and certain species of the subfamily Tanypodinae prefer finer sand and silt sediments (Pinder, 1977; Carew, 2007). The general conclusion is that there is a strong correlation between the organic composition of the sediment and the density of most species of these two subfamilies (Moore, 1979; Vos et al., 2004; Carew, 2007). However, interactions between environmental factors can greatly contribute to hide this correlation (Vodopich, Cowell, 1984). Also, many species belonging to the subfamily Chironominae can tolerate conditions with low oxygen concentrations, as well as heavy metal contamination with certain exceptions (Pinder, 1986; Tomilina et al., 2016; Zelalem, Prokin, 2017). Given that in some of the points from which the materials were collected (T2, T5), the conditions coincide with the above mentioned, more precisely it has an anthropogenic impact, the number of taxa with which the subfamily Chironominae is represented is not unexpected.

The dominant taxon in this study is *Tanytarsus* sp. It was present in all seasons during our research. According to Ekrem (2003) and Epler (2014), their adaptability allows them to survive in waters with low quality and clean waters as well. This finding was confirmed by Brundin (1958), who indicates that the dominant representation of the tribe Tanytarsini is associated with a moderate-oligotrophic character of water. According to H.R. Ingvason et al. (2004), in terms of nutrition, Tanytarsini are considered larvae that feed non-selectively on detritus and debris from the surface parts of the sediment, which is another proof and confirmation that they can survive in different types of habitats and sediments. Apart from *Tanytarsus* sp*., Chironomus plumosus* is another taxon that covers most of the chironomid larvae fauna of the Mavrovo reservoir. It is important to note that the highest density of this species at point T5 is consistent with the fact that in that part, i.e. in the vicinity of this point, there are a number of catering facilities that discharge their wastewater directly into the lake. The fact that this species inhabits the littoral zone of mesotrophic and eutrophic lakes is confirmed by a number of authors, including Saether (1979). It is characteristic that the density trend of this species shows somewhat similar values in the autumn and winter season at T5. As we said before, along *Tanytarsus* sp. and *Chironomus plumosus*, also *Procladius* sp*., Harnischia* sp*., Polypedilum nubeculosum,* and *Orthocladius saxicola* are characterized by greater community participation in the Mavrovo reservoir. *Procladius choreus* and *Ch. plumosus* are also dominant in the reservoirs Matka (Smiljkov, 1996), Mantovo reservoir (Slavevska-Stamenkovic, 2006), and Prilepsko Lake (Rimcheska, 2014). Regarding *Tanytarsus* sp., it is dominant in Prilepsko Lake, subrecendent in the Mantovo reservoir and it's not found in the Matka reservoir.

The vertical distribution of the chironomids shows oscillations at different points, with the lowest density established at T3, which is also the highest depth (20 m) at which the samples were collected, while the highest density is at T5 (5 m). According to Salmoiraghi et al. (2001), for most lakes and reservoirs, density, diversity, and the number of species have the highest values in the littoral zone (less than 10 m), and as the depth increases, the number of species decreases. Since point T3 has the greatest depth, it is not an exception to the rule that the more the conditions

deviate from the normal, or rather, the normal optimum of the most species, the smaller the number of inhabited species (Odum, 1959). Also, according to Smiljkov and Slavevska-Stamenkovic (2006), low oxygen concentration, in the deepest parts of the lake, acts as a limiting factor in the development of benthic organisms.

Regarding the seasonal density of the chironomid larvae fauna, in this study, the lowest density was recorded in the spring season. According to various authors, spring is the period when the lowest density of the chironomid larvae fauna is recorded, which coincides with the finding of this research, because in this season there is an emergence of adults. Considering the altitude at which the reservoir is located, as well as the lower temperatures that are characteristic of this mountainous area, the presence of only one generation per year of these larvae can be ascertained. This coincides with the research of certain authors (Tokeshi, 1995, Lencioni, 2004), according to which, voltinism of less than 1 generation is a characteristic of species that inhabit predominantly cold regions. This is confirmed by the research of T. Stojkoski (1960), where the composition of the benthos in Mavrovo Lake was quantitatively examined immediately after the dam was constructed and where it was established that in the spring season (May) there is the lowest density of the chironomids while in the summer-autumn season (September-October) the density of this dipterian family reached its maximum.

Conclusions

Based on the above data, the following conclusions can be drawn:

1. Generally, the dominant taxon in this study is *Tanytarsus* sp., which indicates that the water of this reservoir most likely is of moderate-oligotrophic character.
2. The dominance of *Chironomus plumosus* at T5 is consistent with the fact that at this locality we have a larger amount of waste material which is discharged directly in the lake by the nearby catering facilities.
3. In terms of vertical distribution, the highest density is recorded at point T5 (5 m), while the lowest density at T3 (20 m). It is evident that the depth is a limiting factor that affects the density, ie the deepest parts of the reservoir, which are also the poorest in oxygen, are characterized by lower community density.
4. The seasonal dynamics of the chironomid larval fauna showed the highest community density in the summer season and the lowest density in the spring season. This indicates that most likely the chironomids are univoltine, ie they have one generation per year in the Mavrovo reservoir.

However, this research has a preliminary character. In order to get a complete picture of the structure and composition of the Chironomidae larvae, it is necessary to collect more samples and to do longer, wider, comprehensive studies, including physicochemical analysis of the water.

Acknowledgment

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Conservation genetics – science in the service of nature

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Abstract Conservation genetics is a subdicipline of conservation biology which deals with the extinction risk and many other problems of nature conservation by using genetic tools and techniques. Conservation genetics is a very good example of the practical use of scientific achievements in nature protection. Although its name seems to be self-defining, its specific area of interest, conceptual apparatus and methodological workshop are not widely known and recognizable. The purpose of this review is to clarify any ambiguities and inconsistencies in this respect. It explore what is conservation genetics, what research and practical issues does it deal with and how they can be solved.

Genetyka konserwatorska – nauka w służbie przyrody

Słowa kluczowe depresja wsobna, ekogenomika, genomika, jednostki zarządzania, obciążenie genetyczne, ryzyko wyginięcia, wir wymierania

Streszczenie Genetyka konserwatorska jest subdyscypliną biologii konserwatorskiej, która zajmuje się ryzykiem wyginięcia gatunków i wieloma innymi problemami ochrony przyrody, przy użyciu narzędzi i technik genetycznych. Genetyka konserwatorska jest bardzo dobrym przykładem praktycznego wykorzystania osiągnięć nauki w ochronie przyrody. Choć jej nazwa wydaje się samodefiniująca, to właściwy jej obszar zainteresowań, aparat pojęciowy i warsztat metodyczny nie są powszechnie znane i rozpoznawalne. Celem artykułu przeglądowego jest wyjaśnienie wszelkich niejasności i niespójności w tym zakresie. Artykuł wyjaśnia czym jest genetyka konserwatorska, jakie problemy badawcze i praktyczne podejmuje oraz w jaki sposób mogą być one rozwiązane.

Introduction

Recognition of the importance of conserving diversity and wildlife populations goes back to Darwin (1896), but the modern field of practice did not really emerge until the latter half of the 20th century. The birth of conservation genetics is associated with the publication of Frankel's

(1974) article *Genetic Conservation: our evolutionary responsibility* and Frankel and Soulè's (1981) book *Conservation and evolution*.

Conservation genetics is an interdisciplinary science which deals with the genetic factors that affect extinction risk of species and aims to preserve endangered species and biodiversity in general, through genetic methods (Frankham, 2003). This one of the most frequently cited definitions outlines the general area of interest of conservation genetics, but does not include some important details. The following definition shows greater information load and greater precision: conservation genetics is the science of gene pool conservation, to preserve biodiversity and to ensure the continuity of evolutionary and ecological processes responsible for its formation and maintenance (Frankham et al., 2004; Allendorf, Luikart, 2007; Avise, 2008; Skorupski, 2015). Similarly, the definition provided by Frankham, Briscoe and Ballou (2002) – the theory and practice of genetics in the preservation of species as dynamic entities capable of evolving to cope with environmental change to minimize their risk of extinction.

An important consequence of the last two cited definitions is the recognition of the fact that the overriding goal of conservation genetics is to suppress the populations and species gene pools depletion, due to their extinction by non-natural (anthropogenic) causes and restoration of optimal conditions for gene pools natural ability to evolve in order to adapt to environmental changes and minimizing the risk of extinction (Skorupski, 2015). What's more, the conservation genetics strives to stop the decline of biotic diversity driven by destructive human activity, without interfering in natural speciation and processes leading to species disappearance (Skorupski, 2015).

Conservation genetics is a theoretical and applied science within conservation biology, constituting the scientific foundations of nature conservation, and combines and benefits from many disciplines of genetics (molecular, population, evolutionary, ecological, landscape and breeding genetics, as well as genomics) and other biological sciences, such as ecology, systematics, evolutionary biology, bioinformatics and biomathematics (Skorupski, 2015). This combination of different fields of biology makes conservation genetics multidisciplinary science that explore and describe the possibilities of utilizing the theoretical achievements and practical tools of genetics (Soulé, 1985; Frankham et al., 2004; Allendorf, Luikart, 2007; Meine, 2010; Skorupski, 2015).

Scope of conservation genetics interest

The conservation of nature requires a combination of strategies, including the protection of endangered species, ecological reserves, control of human actions that harm ecosystems, ecosystem restoration, captive breeding, control of non-native species, and conservation biology education (Trombulak et al., 2004).

There are several issues that conservation genetics studies are conducted. Frankham (2003) categorized these main issues into five categories:

- management and reintroduction of captive populations, and the restoration of biological communities,
- description and identification of individuals, genetic population structure, kin relationships, and taxonomic relationships,
- detection and prediction of the effects of habitat loss, fragmentation, and isolation,
- detection and prediction of the effects of hybridization and introgression,
- understanding the relationships between adaptation or fitness and the genetic characters of individuals or populations.

In genere the conservation genetics focuses on genetic diversity and phylogenetic diversity (Skorupski, 2015). Both terms refer to the gene pool, and are source of other levels of biotic diversity (species and biocenotic diversity), and the resulting diversity at ecosystem, landscape and biogeographical levels (Frankham et al., 2004; Gaston, 2010; Skorupski, 2015). Genetic diversity is the degree of diversity within a species, determined by the differentiation of intra- and interpopulation genetic resources, while phylogenetic diversity is understood as a resource of evolutionary history of a species or population (Allendorf, Luikart, 2007; Skorupski, 2015).

According to Skorupski (2015) and Skorupski et al. (2017), and supported by Woodruff (2000), Frankham et al. (2004), Allendorf and Luikart (2007), Avise (2008), the following areas of conservation genetics can be listed:

1. Molecular genetics issues: use of molecular genetic methods in species biological research, identification of molecular markers best describing inter- and intrapopulation genetic variation, application of genetic analysis in counteracting poaching of protected species, determining kinship and genealogy, molecular diagnostics of pathogens associated with invasive alien species, developing non-invasive sampling for genetic analysis.
2. Population genetics issues: inbred depression, outbred depression, preservation of genetic variation of small populations, determination of genetic structure of populations, identification of populations and species at risk of extinction, detection and identification of genetic pollution (GMOs, genetically modified organisms), hybridization and introgression, identification of population size and gender identification, monitoring of invasive alien species populations, accumulation and loss of deleterious mutations, determination of effective population size.
3. Evolutionary genetics issues: explanation of taxonomic uncertainties, definition of management units and units of evolutionary significance within the species, phylogenetic analysis, detection of genetic variation, loss of genetic diversity and evolutionary capacity in response to changes in the natural environment, adaptive genetic variation.
4. Ecological genetics issues: characterization of genomic-environment interactions in transcriptional and epigenetic studies, genotoxicity studies.
5. Landscape genetics issues: genetic isolation and gene flow detection, population fragmentation and gene flow restriction, genetic drift leaving the importance of natural selection.
6. Breeding genetic issues: genetic adaptation to breeding in captivity and its negative effects on the success of reintroduction, enhancement of reproductive potential of individuals keept in captivity, management of captive populations.
7. Genomics issues: cognitive genomic research, multilocus genotyping.

Conservation genetics' methodology

Methods and techniques used by conservation genetics widely varies due to the broad range of raised issues. Conservation genetics uses a whole range of genetic methods, such as DNA sequencing, real-time PCR, gene editing technologies, analysis of SNPs, microsatellites, mtDNA and nuclear markers, DNA barcoding or reduced representation methods (GBS, RAD-tag, ddRAD sequencing), but also coalescent modelling and Bayesian statistical methods (Skorupski et al., 2017; Moro et al., 2018; Pertoldi, Randi, 2018).

Particular trend is currently the ongoing transition from conservation genetics to conservation genomics (Romanov et al., 2006; Pertoldi et al., 2010; Pertoldi, Randi, 2018). Genomic developments have spawned the assays of micro amounts of DNA in pooled environmental samples, such

as soil, and environmental DNA (eDNA) methods are becoming integral in a wide range of areas, including conservation, biosecurity and forensics, as well as in monitoring and tracking cryptic species (Thomsen, Willerslev, 2015; Bissett et al., 2016).

Molecular genetic methods are also used widely in forensic evidence for litigation. These include the detection of illegal hunting and collection. To understand these factors, coalescene and gene tree analyses provide useful tools. Also PCR based genetic markers can be used to detect illegal hunting or collecting (Frankham et al., 2004).

Genetics

Throughout the history scientists had a better understanding of the importance of genetics in conservation. The importance of genetics in conservation biology is first recognized by Darwin (1896). Steadiness of biodiversity depends upon the protection of the environment and maintenance of habitat, but it is the genetics that has played a significant and diverse role in conservation biology in the last few years (Allendorf, Luikart, 2007).

It should be assumed that in the future genetics will play an even more important role in nature conservation than at present. The use of research results on the genome of humans and other organisms will provide not only theoretical knowledge, but also practical solutions that can be implemented in the protection of endangered species. Analyzes of large amounts of data may be of particular importance, the statistical analysis of which, supported by an evolutionary perspective, will allow for a proper assessment of their biological significance and correct inference and forecasting based on these data. In addition, those data will permit an understanding of evolutionary events and processes (nonselective, including bottlenecks or gene flow, or selective, like detrimental or adaptive mutations) that shaped gene pools of endangered species in the past (Trombulak et al., 2004).

Genetic diversity has a major role in conservation. All the phenotypic plasticity is dependent on the genetic variability which helps the individuals to survive and adapt in harsh environments and abiotic stress, thus protect from endangering risk (Soumen, Tanmay, 2016). Genetic diversity is important because it helps to maintain the health of a population, by including alleles that may be valuable in resisting diseases, pests and other environmental stresses. Maintaining diversity gives the population a buffer against change, providing the flexibility to adapt these harsh conditions (Frankham et al., 2004). If the environment changes, a population that has a higher variability of alleles will be better able to evolve and adapt to the new environment. In extreme situations, such as drought or epidemics, diversity could even condition the survival of the population. Thus, if the genetic diversity of a population increases, the risk of extinction decreases (Frankham et al., 2004; Weeks et al., 2016).

The abundant genetic diversity found in large populations contrasts with that found in many small or bottlenecked populations (Garner et al., 2005). Most threatened species have lower genetic diversity than related non-endangered species with large population sizes. Of 170 threatened species examined, 77% had lower genetic diversity than related non-endangered species. Overall, threatened species have about 60% of the genetic diversity of non-endangered species. the most likely explanation is that threatened species have suffered reductions in population size that directly result in loss of genetic diversity (Frankham et al., 2004).

Inbreeding depression and mutation meltdown

Of the various possible genetic problems which face a declining population, loss of genetic variability and inbreeding depression have historically received most attention (O'Brien, 1994). Inbreeding occurs when related individuals of a species mate with one another. Inbreeding reduces the genetic variability and cause loss of genetic diversity which enables species to be prone to extinction. Inbred individuals generally have reduced fitness in comparison to noninbred individuals from the same population because of their increased homozygosity. Inbreeding depression is the reduction in fitness (or phenotype value) of progeny from matings between related individuals relative to the fitness of progeny between unrelated individuals. Inbreeding depression in natural populations will contribute to the extinction of populations under some circumstances (Keller, Waller, 2002).

The size of a population is important here. It is generally inversely related with inbreeding, the loss of genetic information due to chance events connected with survival and reproduction, and susceptibility to extinction. Therefore, small populations are, in general, at greater risk of extinction than large populations (Trombulak et al., 2004). Nevertheless, assuming that a species has lost useful variability, what are the probable results? One possibility is that fitness will be reduced as a direct consequence of a reduction in the number of heterozygous loci. This proposition stems from the fact that some, and maybe much, selectively important variability is maintained by balanced polymorphisms in which the heterozygote is better than either homozygote. Although such locus-specific effects could operate entirely separately from the more general aspects of inbreeding depression, in practice the two processes are virtually impossible to disentangle (Amos, Balmford, 2001).

For inbreeding depression to affect population viability it must affect traits which influences the population viability. Although inbreeding results in demonstrable costs in captive and wild situations, it has yet to be shown that inbreeding depression has caused any wild population to decline. Similarly, although loss of heterozygosity has baneful impact on individual fitness, no population has gone extinct as a result (Allendorf, Luikart, 2007). According to Amos and Balmford (2001), an alternative solution for the problem of inbreeding depression is based on introduction of new genetic diversity from other populations.

On the other hand, if the genetic load increases in a population, the population might decrease in size, such that destructive mutants of larger effect become efficiently neutral and, subsequently, are more likely to become fixed. This feedback process has been named "mutation meltdown" and, in theory, could result in the extinction of small populations (Lynch et al., 1995; Higgins, Lynch, 2001). However, it is not clear how significant mutation meltdown actually is because the extinction probability owing to other causes might be high in such small populations and other factors could also counter its effects (Hedrick, 2001).

The accumulation of new deleterious mutations is predicted to constitute a significant threat to the survival of finite sexually reproducing populations (Gilligan et al., 1997). Genetic loads, measured as fitness differentials between inbred and non-inbred lines, derive from each population under productivity of single pairs and competitive conditions, while smaller populations are proven to not exhibit greater genetic loads than larger populations under either benign or competitive conditions (Gilligan et al., 1997).

Natural selection is able to remove deleterious alleles from a population, but under natural conditions it takes so long that in the meantime new mutations emerge before the previous ones are removed from the gene pool, especially in the case of recessive alleles. As a result, a specific

balance is created between the appearance of deleterious alleles by mutation and their removal by selection (mutation-selection equilibrium). Consequently, low frequencies of deleterious alleles (mutation load) occur in all naturally intersecting populations (Gilligan et al., 1997; Frankham et al., 2004).

Extinction vortex

Inbreeding depression can potentially contribute to a so-called extinction vortex, in which decline reduces fitness which in turn hastens the decline, increasing both inbreeding depression and vulnerability to stochastic events in a destructive feedback loop (Lacy, Lindenmayer, 1995; Amos, Balmford, 2001). The term "extinction vortex" covers the biotic and abiotic forces affecting small populations that draw them into the chain of events leading to progressive decline in size and endanger their long-term survival which make possible the population size downward to extinction (Allendorf, Luikart, 2007).

Fagan and Holmes (2006) compiled a small time-series database of ten vertebrate species (two mammals, five birds, two reptiles and a fish) whose final extinction was witnessed via monitoring. They confirmed that the time to extinction scales to the logarithm of population size. In other words, as populations decline, the time elapsing before extinction occurs becomes rapidly (exponentially) smaller and smaller. They also found greater rates of population decline nearer to the time of extinction than earlier in the population's history, confirming the expectation that genetic deterioration contributes to a general corrosion of individual performance (fitness). Finally, they found that the variability in abundance was also highest as populations approached extinction, irrespective of population size, thus demonstrating indirectly that random environmental fluctuations take over to cause the final extinction regardless of what caused the population to decline in the first place. It was fundamentally the first empirical demonstration that the theory of accelerating extinction proneness occurs as populations decline, meaning that all attempts must be made to ensure large population sizes if there is any chance of maintaining long-term persistence. This relates to the minimum viable population size concept that should underscore each and every recovery and target set or desired for any population in trouble or under conservation scrutiny (Bradshaw, 2008).

Conservation genetics gaps

Despite the fact that the knowledge about genetic diversity is increased, the insufficiency of applying this information to the conservation has created a gap between genetic knowledge and conservation (Allendorf et al., 2010). Although its recognised importance for species' persistence, integrating genetics into conservation management has proved problematic, creating a so called conservation genetics gap, which could widen with the advent of advanced genomic techniques. Bridging this gap requires a clear understanding of the barriers to use of genetics by conservation practitioners (Taylor et al., 2017).

Management Unit & Evolutionary Significance Unit

It is a common belief, that in biological conservation, what should be protected are species (Mace, 2004). As, due to the limited resources, it is not possible to protect all populations

of a given species, it is necessary to focus on restricted number of local populations, prioritized for conservation purposes in accordance with the concept of the Management Unit (MU), defined as demographically independent populations (Moritz, 1994; Allendorf, Luikart, 2007).

Evolutionarily Significant Unit (ESU), conceptualised by Ryder (1986), is considered as a conservation unit, providing a theoretical background for prioritising taxa for conservation purposes (Moritz, 1994). The ESU may be seen as an entity identical to a species (encompassing one evolutionary lineage), or a species may encompass multiple ESUs. Evolutionarily Significant Units may also constitute a metapopulation or separate populations showing adequate gene flow and sharing a common evolutionary history (Fraser, Bernatchez, 2001). Several, sometimes contrasting, definitions have been proposed for ESU, however all of these definitions refers to "segments of species whose divergence can be measured or evaluated by putting differential emphasis on the role of evolutionary forces at varied temporal scales" (Casacci et al., 2014).

Studies regarding Management Unit (MU) for non-endangered species are much less common. Meanwhile, intraspecific diversity can provide important information on how to identify biogeographic areas and regions for conservation planning. According to Araújo (2002), the study of a large number of different species can help target appropriate areas for conservation purposes by identifying the amount and distribution of species diversity, especially important in case of species distributed in biodiversity hotspots and areas of endemism.

The designation of Management Units and Evolutionarily Significant Units is critical to the planning and achievement of short- and long-term nature conservation and management goals. MUs, as populations that are demographically independent, while maintaining a genetic link with other populations, are subject to short-term nature management objectives, expressed as maintaining stable reproduction of Management Units and excluding excessive pressure on small populations. ESUs have an independent conservation status defined by significant reproductive isolation and unique adaptations, and thus are targeted by the long-term management goals, i.e. preserving the structure, evolutionarily significant differences, maximum genetic diversity and evolutionary potential of native populations (Efremov, 2007).

Genomics

With the recent developments in genetic tools and technologies, another new research field has emerged – genomics, targeting whole genomes or their great segments. Genomics offer lots of promises, and the ongoing transition from conservation genetics to conservation genomics is currently taking place (Allendorf et al., 2010; Avise, 2010; Ouburg et al., 2010; Funk et al., 2012; Pertoldi, Randi, 2018).

The growing importance of genomics in conservation biology concerns especially identifying the segments of genomes responsible for local adaptation and therefore important for preservation, but also ecological issues, such as population size estimation using non-invasive methods (Puechmaille, Petit, 2007). Genomic methods can therefore be an alternative to costly and laborious radio and satellite tracking tracking in estimating movements and dispersal (Fabbri et al., 2007; Sahlsten et al., 2008).

One area where genomic data is particularly helpful is in attempts to trace past demographic processes, such as population size fluctuations, where traditional genetic techniques, using a limited number of markers, are insufficient. Meanwhile, genomic data, even from a single individual, can provide useful insights in an endangered species past population dynamics (Zhan et al., 2013). Genomic analysis of the past demographics can explain whether given species have

been endangered and bottlenecked also during its past evolutionary history or whether its present threat status is a consequence of current events and situation of the species (McMahon et al., 2014). The knowledge about the past demographics could be used in planning and implementation of conservation actions (McMahon et al., 2014).

The techniques, approaches and data of functional genomics are currently often adopted and applied to native populations, since gene function can only be defined in close relation to the environment in which it is measured (Feder, Mitchell-Olds, 2003; Ouborg, Vriezen, 2007). The problem is the transposition of the functional genetics achievements for model organisms (*Arabidopsis thaliana*, *Caenorhabditis elegans*, *Mus musculus* and *Drosophila melanogaster*), in a limited set of well-defined genotypes and under strictly controlled environmental conditions, into real natural conditions and situations. There are several challenges to developing an ecological and evolutionary approach to genomics. These involve changing from genomic model species, that have enough genomic resources and tools available, to ecological model species, for which these resources and tools are generally absent. Adopting an ecological and evolutionary functional genomic approach for conservation purposes referees to as ecogenomics, often utilizing techniques and approaches originally designed for detailed studies at the individual level for the population-wide studies (Feder, Mitchell-Olds, 2003).

Conclusions

To sum up, conservation genetics, as a subdicipline of conservation biology, plays an important role in conservation of biodiversity. Using genetic tools and implementing genomics into conservation enables better understanding of how to preserve the natural environment. The importance and application of this interdiciplinary science is increasing day by day.

The role of conservation genetics is to provide a scientific basis for nature conservation activities, and conservator geneticists role is to transfer knowledge and skills, i.e. scientific research results into practice, to carry out appropriate scientifically validated conservation practices (Skorupski, 2015; Skorupski et al., 2017).

Conservation genetics should focus not only on endangered and extinct species, but efforts taken should strive to protect the biotic diversity (gene pools), including anticipatory and preventive actions that protect species or populations from extinction threat (Skorupski, 2015).

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New record of *Thecacineta calix* (Ciliophora, Suctorea) from Florida coast, Gulf of Mexico

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Keywords Ciliate, *Thecacineta calix*, distribution, harpacticoid copepod, Florida, USA

Summary The suctorian ciliate *Thecacineta calix* is reported off the coast of Panama, Florida (USA) epibiont on meiobenthic harpacticoid copepod. This is the first report of *T. calix* from Florida coast. The length of stalk in the present specimens, epibiont on harpacticoids, ranged from one seventh to one eighth of the lorica length.

Nowe stwierdzenie *Thecacineta calix* (Ciliophora,Suctorea) z wybrzeża Florydy, Zatoka Meksykańska

Słowa kluczowe orzęski, *Thecacineta calix*, rozmieszczenie, widłonóg z rzędu Harpacticoida, Floryda, USA

Streszczenie Sysydlaczek *Thecacineta calix* został stwierdzony na wybrzeżu Florydy (USA) jako epibiont na widłonogu z rzędu Harpacticoida. Jest to pierwsze stwierdzenie tego gatunku na wybrzeżu Florydy. Długość nóżki u okazów epibiontów występujących na Herpacticoida, wahała się od jednej siódmej do jednej ósmej długości loriki.

Introduction

Suctorian ciliate *Thecacineta calix* (Schröder, 1907), the type species of genus *Thecacineta* Collin, 1909, was firstly described as epibiont on marine nematodes from Kerguelen Islands, Antarctica (Schröder, 1907). After first description, the species was not observed during a long time. Matthes (1956) was the first after Schröder who found and reinvestigated the *T. calix* in Rovinj, Croatia, the Adriatic Sea. He described the peculiarities of morphology and reproduction of *T. calix* and some its congeners in details and erected the new suctorian family Thecacinetidae Matthes, 1956 for loricate suctorians with apical bundle of tentacles and vermigemmic budding.

Detailed remarks on the systematics and nomenclature of this species are provided in Dovgal et al. (2008).

Several recent investigations of suctorian ciliates distribution demonstrated that *T. calix* is wide spread in marine and brackish waters. The species has been reported as an epibiont on nematodes, copepods, and halacarid mites from the Atlantic, Pacific, Antarctic and Indian Oceans, from the intertidal region to the deep sea. Details of distribution and host specificity of this species is given in Chatterjee et al. (2019). However, further findings of thecacinetid suctorians are important to clarify the ecology and distribution of sessile ciliates.

This paper reports the epibiont *Thecacineta calix* collected off Panama city, Florida, USA (Gulf of Mexico).

Material and Methods

Specimens of suctorian ciliate *Thecacineta calix*, epibiont on meiobenthic harpacticoid copepods were collected off Panama City, Florida (USA), in Gulf of Mexico (Latitude: 30.063N, Longitude: 85.695W) (Figure 1), at 22 m water depth. Sediment samples were collected by 1st and 2nd authors (Baban Ingole and Biswajit Chakraborty), using Vibro Corer operated on a Research Vessel: MV Sharp (USA) in a joint expedition as a part of "Multi-Institutional shallow water experiment (GulfEx11) off Panama City, Florida (USA), in Gulf of Mexico" on 11 April 2011.



Figure 1. Map of sampling area

Meiobenthic samples were initially fixed in formalin Rosebengal and latter sieved in the laboratory using a 45 µm mesh size sieve. Infested meiofauna were hand sorted, mounted on a 50% glycerine slide and sealed with DPX.

Measurements were carried out on six specimens using the program Toup View 3.7 for digital camera.

Specimens are kept in the collection of the first author (BI).

Results

Class Suctorea Claparéde & Lachmann, 1859
Subclass Exogenia Collin, 1912
Order Vermigemmida Jankowski, 1973
Family Thecacinetidae Matthes, 1956
Genus *Thecacineta* Collin, 1909

***Thecacineta calix* (Schröder, 1907)** (Figures 2 and 3)
(for synonyms see Chatterjee et al., 2019)

Diagnosis: Marine loricate suctorian. Cell body entirely fills the lorica and attach to its base. Loricais ribbed with a series of transverse annular ridges that become progressively closer toward the base. Apical part of body protrudes beyond lorica aperture. Clavate tentacles (up to 30) arise from apical surface of body. Curved stalk with apical widening. The macronucleus ovoid and located in the basal region of the cell body. Large contractile vacuole located usually near macronucleus in the basal region of body.



Figure 2. Epibiont *Thecacineta calix* at host (harpacticoid copepod) body

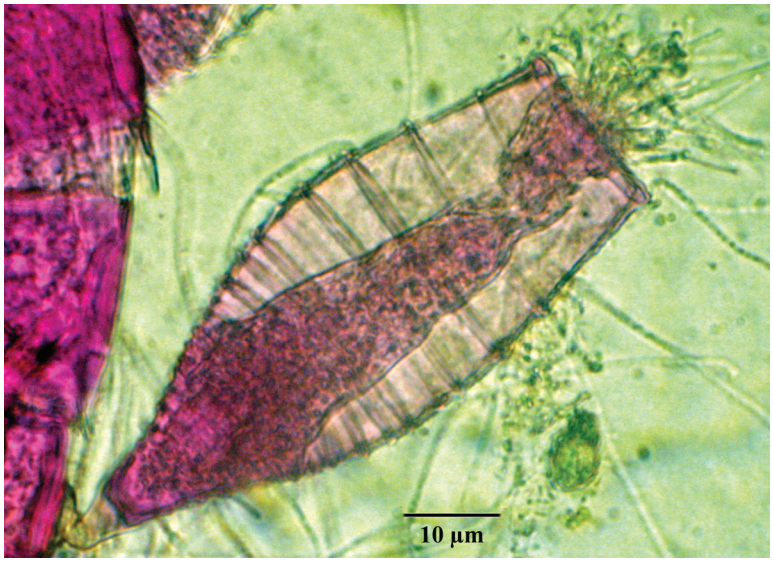


Figure 3. The *Thecacineta calix* from Florida coast, Gulf of Mexico

Morphometric measurements observed in the present study (Six individuals were measured)**:** Length of lorica: 56–63 µm; Maximal width oflorica: 16–26 µm; Width of lorica aperture: 12–18 µm; Length of body: 63–64 µm; Maximal width of body: 12–19 µm; Stalk length: 8 µm; Stalk diameter: 4µm; Macronucleus diameter: 6–7 µm; Length of tentacles: 6–19 µm.

Discussion

Thecacineta calix has been reported as an epibiont on nematodes, copepods, and halacarid mites from the Atlantic, Pacific, Antarctic and Indian Oceans, from the intertidal region to the deep sea (Chatterjee et al., 2019).

This ciliate species was recorded on harpacticoid copepod from East St. John, U.S. Virgin Islands, 53 m depth; Caja de Muertos Island near Ponce, Puerto Rico, 52 m depth; Buoy, La Parguera, Puerto Rico, 46 m depth, Caribbean Sea (Chatterjee et al., 2014); on harpacticoid copepod from Bajo de Sico, a mesophotic reef formation located in Mona Passage of Puerto Rico, 70 m depth, Caribbean Sea (Chatterjee et al., 2020). It was also reported among algae *Enteromorpha flexuosa* from Tamiahua lagoon (near Tampache), Veracruz, Mexico (Martínez-Murillo, 1997). This is the first record of this species from the Florida coast.

It should be mentioned that the stalk of *T. calix* from Florida, epibiont on harpacticoids in present observation is short, the stalk length ranged from one seventh to one eighth of the lorica length.

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Endophytic colonization of tomato by *Beauveria bassiana* for control of the greenhouse whitefly, *Trialeurodes vaporariorum* (Hemiptera: Aleyrodidae)

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Keywords colonization, endophyte, entomopathogenic fungus, leaf spray, Whitefly

Abstract We evaluated the efficacy of four different methods for endophytic inoculation of entomopathogenic fungus *Beauveria bassiana* in tomato plants. Fourteen days after inoculation, root dipping and leaf spraying allowed recovery of *B. bassiana* from leaves, while the fungus was recovered from roots in all inoculation methods, except soil drenching. Significant increases in mortalities of nymphs and adults of the greenhouse whitefly, 36 to 52%, were recorded on *B. bassiana*-endophyt plants. The total phenolic and protein contents of tomato plants were increased by endophyte colonization. Results of this study confirm the efficiency of foliar spraying of *B. bassiana* for colonization in tomato plants and its insecticidal activity against whitefly. The persistence of *B. bassiana* as endophyte was confirmed up to 56 days after inoculation. Given the insecticidal activity and beneficial effects of the endophyte on plant growth, *B. bassiana* could be considered as a suitable element in integrated pest management.

Endofityczna kolonizacja pomidora przez Beauveria bassiana w celu zwalczania mączlika szklarniowego, *Trialeurodes vaporariorum* (Hemiptera: Aleyrodidae)

Słowa kluczowe kolonizacja; endofit; grzyb entomopatogenny; dolistny aerosol; mączlik

Streszczenie Oceniliśmy skuteczność czterech różnych metod endofitycznej inokulacji entomopatogennego grzyba *Beauveria bassiana* do pomidorów. 14 dni po zaszczepieniu stwierdzono obecność *B. bassiana* w liściach w przypadku inokulacji poprzez zanurzanie korzeni i oprysk liści, natomiast obecność grzyba w korzeniach stwierdzono przy wszystkich metodach inokulacji, z wyjątkiem zwilżania gleby. Na pomidorach zainfekowanych *B. bassiana* odnotowano znaczny wzrost śmiertelności nimf i osobników dorosłych mączlika szklarniowego, wynoszący od 36 do 52%. Całkowita zawartość fenoli i białka w tkankach pomidora wzrosła w wyniku kolonizacji endofitów. Wyniki badań potwierdzają skuteczność oprysku dolistnego *B. bassiana*

w kolonizacji pomidora i jego owadobójcze działanie na mączlika szklarniowego. Trwałość *B. bassiana* jako endofitu została potwierdzona do 56 dni po inokulacji. Biorąc pod uwagę aktywność owadobójczą i korzystny wpływ endofitu na wzrost roślin, *B. bassiana* można uznać za odpowiedni element zintegrowanej ochrony roślin przed szkodnikami.

Introduction

Fungal endophytes inhabit the internal tissues of a plant for at least part of their life cycle without causing any apparent symptoms in the host (Wilson, 1995). These endophytes have been reported to benefit plant health and performance by enhancing growth, protecting plant tissues against pathogens, parasitic nematodes, and herbivorous pests, and direct translocation of nitrogen from insect cadavers to the host plant (Saikkonen et al., 2004; Behie, Bidochka, 2014; Vega, 2018). The endophytic association of fungi has been reported in many plants, including some important agricultural crops such as wheat, soybeans, maize, and tomatoes (Vega et al., 2008; McKinnon et al., 2018).

Beauveria bassiana (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae) is a soil borne fungus that attacks an extensive host range of insects at all stages of development (Ownley et al., 2004). In addition to insecticidal activity, *B. bassiana* has been found to protect plants against certain plant pathogens (Ownley et al., 2004; 2008) as well as to function as a biofertilizer (Vega, 2018; Afandhi et al., 2019). Different isolates of *B. bassiana* have been reported as naturally occurring endophytes in a variety of plant species such as tomato, potato, maize, cotton, cucumber, melon, and rice (Vega, 2008; Brownbridge et al. 2012; Vidal, Jaber, 2015). The insecticidal and antimicrobial activity of endophytic *B. bassiana* has been well documented in some previous studies (Cherry et al., 2004; Ownley et al., 2008; Gurulingappa et al., 2010; Akello, Sikora, 2012). *B. bassiana* has been successfully inoculated into tomato plants using different application methods and their insecticidal activity has been confirmed against two chewing-tunneling lepidopteran pests (*i.e.* the tomato fruitworm, *Helicoverpa armigera* (Hübner) (Lep: Noctuidae) and the tomato leafminer, *Tuta absoluta* (Meyrick) (Lep: Gelechiidae) (Qayyum et al., 2015; Allegrucci et al., 2017).

In this study, we evaluated the efficacy of different methods for establishment of *B. bassiana* as endophyte in tomato plants. Then, the *in planta* efficiency of fungal endophyte against a sucking insect, greenhouse whitefly *Trialeurides vaporariorum*, Westwood (Hem: Aleyrodidae), was investigated under greenhouse conditions. As whitefly management programs are currently dependent on the use of chemical insecticides (Schlaeger et al., 2018) on the freshly consumed tomato in most countries, our results could be important in considering alternative pest control methods.

Materials and methods

Plant

Seeds of tomato plants, *Solanum lycopersicum* L. cv. Falat, were cultivated in plastic pots (15 cm height, 20 cm diameter) filled with a mixture of soil, peat and perlite. Plants were grown in a research greenhouse (26 ±3°C, 60 ±10% RH, and 16L:8D photoperiod) and a nutrient solution (NPK) was applied according to manufacturer recommendations.

Fungus

Beauveria bassiana TV isolate, was obtained from the Biological Control Laboratory at Department of Plant Protection, University of Tehran (Karaj, Iran). The virulence of this isolate has been previously reported against insect species in the laboratory (SeyedTalebi et al., 2016; Kosari et al., 2016). The fungus was cultured on Sabouraud dextrose agar (SDA) medium and kept in a growth chamber at 25 ±1°C, 70 ±10% RH, and 16L:8D photoperiod. For preparation of fungal suspension, the conidia of *B. bassiana* were collected from 14-day-old cultures, suspended in distilled water, and filtered through sterile filter paper in a Buchner funnel. The conidia were quantified using a hemocytometer and a light microscope. The required fungal suspension (10^8 conidia ml^{-1}) was adjusted with regards to conidial viability according to the method described by Goettel and Inglis (1997).

Insect

Trialeurodes vaporariorum was collected from tomato greenhouses in Karaj County (Alborz Province, Iran) and maintained on tomato plants. The whiteflies were kept into wooden cages (60 × 40 × 40 cm) covered by net in a growth chamber at 26 ±1°C, 70 ±10% RH, and 16L:8D photoperiod.

Plant inoculation

Tomato plants were treated with *B. bassiana* concentration 10^8 conidia ml^{-1} using four inoculation methods; seed dressing with fungal suspension, rhizosphere inoculation, foliar application of conidial suspension, and soil drenching. For all inoculation methods, tomato seeds were immersed in 0.5% sodium hypochlorite for 1 min and washed in sterile distilled water for one min. The seeds were then surface sterilized by placing them in 70% ethanol for 2 min, and rinsed two times in sterile distilled water for one min. Treated seeds were placed on sterile paper towels to dry for 30 min. For seed inoculation, the treated seeds were immersed in 10 ml of the conidial suspension of *B. bassiana*. After 48 hours of incubation, the seeds were sown in pots in the greenhouse with above-mentioned condition. For rhizosphere and foliar application methods, tomato seeds were individually sown in plastic pots and the 14-day old seedlings were used for inoculation. For rhizosphere inoculation, 10 ml of the conidial suspension was added to the soil around tomato stems. For foliar application, tomato leaves were sprayed with 10 ml of the fungal suspension using a glass hand sprayer. To avoid conidial runoff to the soil, the top of each pot was covered with aluminium foil. In soil drenching method, 50 ml of the conidial suspension was added to one kg of soil in pots and one tomato seed was sown in each pot after 24 hours of inoculation. In control, tomato leaves were sprayed with 10 ml of distilled water.

Evaluation of endophytic establishment

For all application methods, the colonization of tomato plants by *B. bassiana* was evaluated 7 and 14 days after inoculation. For this purpose, the plants were removed from the soil and their root was washed with distilled water. For each plant, one gr of roots and one gr of leaves were cut and surface-disinfected by immersion in 0.5% sodium hypochlorite for 2 min, followed by one min in distilled water, and 2 min in 70% ethanol and rinsed with sterile distilled water three times in a laminar flow cabinet. The samples were then dried on sterile paper towels for 30 min.

Subsamples of the surface sterilized leaves and roots were placed on SDA media to ensure that residual conidia have not retained their germination potential. The samples were finely crushed in 10 ml distilled water and one ml of the resultant extract was spread over dishes containing SDA media. The dishes were daily checked and the number of fungus colonies was determined after 14 days of incubation at 25°C. A total of 8 plants were used for each application method. After determination of the most efficient technique, this experiment was repeated to explore the changes in frequency of fungal colonies at days 7, 14, 21, 28, and 56 following inoculation. This experiment was also performed with four replicates.

Insecticidal effect

After selection of the most efficient inoculation method based on the number of colonies on foliar and root samples, the insecticidal activity of endophytic *B. bassiana* against *T. vaporariorum* adults was evaluated using laboratory bioassays. To explore the capability of *B. bassiana* to systemically colonize the entire plant and protect the plant parts other than the treated parts, two spraying procedures (*i.e.* spraying of the fungal suspension on either a single leaf or the whole plant) were applied. For this purpose, tomato plants were sprayed with 10 ml of the fungal suspension using hand sprayer either thoroughly or on one randomly selected leaf. In control, the plants were treated with the same volume of distilled water. After 7 days of plant inoculation, treated plants were placed in cages covered by insect net and 15 pairs of adult *T. vaporariorum* were released on each plant. After 48 hours of oviposition, adult whiteflies were removed and an equal number of eggs was retained on each plant. The plants were then maintained under controlled conditions in a greenhouse and the mortality of different life stages of was recorded until adult emergence. The percentage of egg mortality was determined by dividing the number of unhatched eggs by the total egg number. The early (first and second) and late (red-eye) nymphal stage mortality was estimated by counting the number of dead nymphs in each life stage. The percentage of adult mortality was estimated by knowing the number of emerged pupa and the total number of pupae. The dead whiteflies were incubated at room temperature and monitored for presence of mycosis. All bioassays were performed with 4 replicates.

Plant biochemical changes

The effect of endophytic colonization of tomato plants by *B. bassiana* on total phenols and total protein content of leaves was evaluated in either absence or presence of whitefly infestation. Tomato plants were sprayed with 10 ml of the fungal suspension using glass hand sprayer. In control, the plants were treated with the same volume of distilled water. On the seventh day after fungal inoculation, half of the plants in both control and treated groups were exposed to 15 pair of adult *T. vaporariorum* for 48 hours to provide a total of four treatments including i) endophyte-free plants without whitefly infestation, ii) endophyte-free plants with whitefly infestation, iii) plants with endophyte, but without whitefly infestation, and iv) plants with both endophytic fungi and whitefly infestation. Twenty-four days after whitefly infestation, the leaves were removed and freeze-dried at –80°C for further analyses. Assessment of total phenolic content was performed according to the Folin-Ciocalteu method (Slinkard, Singleton, 1977) with slight modifications. Briefly, 0.1 gr of the freeze-dried samples were homogenized in 5 mL of 95% EtOH and incubated at darkness for 24 hours. The mixture was then centrifuged at 4,000 rpm for 10 min and the clear supernatant was collected as crude extract. One hundred µl of the crude extract were mixed with 0.2 ml of Folin-Ciocalteu reagent and 2 ml of distilled water. After 3 min, the reaction

was neutralized by addition of 1 ml of sodium carbonate (Na_2CO_3) (15%) and incubating at room temperature for 2 hours. The absorption was read at 765 nm in a spectrometer. Gallic acid was used as a standard and the total phenolic content was expressed as gallic acid equivalents (GAE) in $mg \cdot g^{-1}$ of fresh weight.

To measure the amount of protein, proteins were extracted from the foliar part of the plant at 0 to 4°C on ice. For this purpose, one gram of plant freeze dried tissue in a mortar containing three milliliters of buffer phosphate of (50 milimolar, pH = 7.2), containing ethylene diamine tetra-acetic acid (EDTA), phenyl methane sulfonyl fluoride (PMSF), and polyvinyl pyrrolidone (PVP) 1% were shed. The extract was placed in refrigerated centrifuge for 4 minutes at 4,000 g and 4°C for 15 minutes. A supernatant solution was used to study the protein assay (a solution of each sample was stored in several microtubes and stored at –80°C). The total protein content was measured according to the procedure described by Bradford (1976). To test tubes, 0.1 ml of protein extract, 5 ml of biuret was added and rapidly vortexed. After two minutes, the absorption was read at 595 nm in a spectrometer. The protein concentration was calculated using bovine serum albumin. Protein content was expressed as $mg.g^{-1}$ of fresh weight.

Data analysis

Statistical analyses for data of assays were performed using ANOVA in SAS software version 9.1 (SAS Institute, 2002). For mean comparisons, F-LSD test was used as post-ANOVA. T-test was used for two-sapmle comparisons. Percentages data were normalized by arcsine square root transformation before being analyzed.

Results and discussion

Endophytic establishment

Seven days after inoculation by all techniques, no colonization was obtained in roots, while the establishment of *B. bassiana* in leaf tissues was confirmed only in plants treated with foliar spraying, but not other inoculation techniques (Table 1).

Table 1. The frequency of *Beauveria bassiana* colonies (±SE) recovered from roots and leaves of tomato plants 7 and 14 days after inoculation with four different techniques

Inoculation method	Leaf tissue		Root tissue	
	day 7	day 14	day 7	day 14
Control	00.00 ±00.00^{b}	00.00 ±00.00^{c}	00.00 ±00.00^{a}	00.00 ±00.00^{c}
Soil drenching	00.00 ±00.00^{b}	00.00 ±00.00^{c}	00.00 ±00.00^{a}	00.00 ±00.00^{c}
Seed inoculation	00.00 ±00.00^{b}	00.00 ±00.00^{c}	00.00 ±00.00^{a}	8.50 ±0.25^{b}**
Root inoculation	00.00 ±00.00^{b}	15.00 ±3.48^{b}**	00.00 ±00.00^{a}	37.75 ±5.76^{a}**
Leaf spraying	2.25 ±0.63^{a}	133.75 ±25.93^{a}*	00.00 ±00.00^{a}	30.50 ±5.36^{a}**

Different letters within each column show significant differences at $P < 0.05$ level (LSD test); * and ** show significant difference between day 7 and 14 of each inoculation technique and each tissue sample at $P < 0.05$ and $P < 0.01$ levels, respectively (*t*-test).

Two weeks after inoculation, endophytic colonization of the fungus was detected in plant roots when they were inoculated using foliar spraying, seed treatment and rhizosphere inoculation. Similarly, foliar spraying and rhizosphere inoculation resulted in successful establishment of *B. bassiana* in leaf tissues 14 days after inoculation. These results are in consistent with previous studies indicating that inoculated fungi have the potential to move across the vascular tissues and systemically colonize the entire plant (Wagner, Lewis, 2000; Mantzoukas et al., 2015). The highest frequency of fungal colonies (133 CFU/ml) was obtained using foliar spraying at day 14 after inoculation, followed by rhizosphere inoculation (37 CFU/ml) and foliar spraying (30 CFU/ml) at day 7 after inoculation, respectively (Table 1). No evidence of fungal establishment was detected in soil drenching method and control (Table 1). Consistent with these results, Allegrucci et al. (2017) reported significant difference in endophytic colonization of *B. bassiana* in tomato plants when they used different techniques (leaf spraying, root dipping, and seed inoculation), with the highest efficiency was obtained from leaf spraying. However, these authors detected the highest percentage of colonization at day 7 post inoculation that was different from our current study. In another study, Afandhi et al. (2019) reported that the endophytic colonization of *B. bassiana* in common bean plants varies significantly depending on inoculation technique, parts of plant from which samples are taken (root, stem, or leaves), and the time elapsed after inoculation. Similarly, it was shown in coffee plants that foliar sprays favors leaf colonization whereas soil drenches favors root colonization (Parsa et al., 2013). A variety of factors, such as plant species/cultivar, fungal species/isolate, plant age at the time of inoculation, inoculation method, concentration of the inoculum, tissue specificity of endophytic fungus, and plant growing condition, have been argued to affect the capability of a fungus to establish as an endophyte (Parsa et al., 2013; Qayyum et al., 2015; Afandhi et al., 2019).

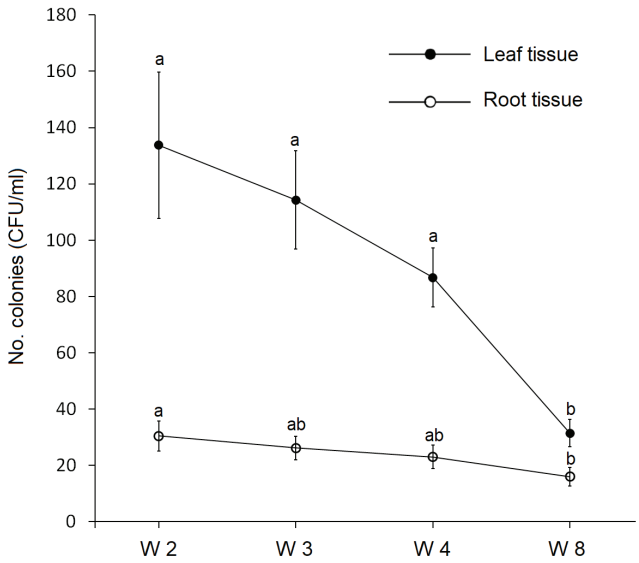


Figure 1. The frequency of *Beauveria bassiana* colonies (±SE) recovered from roots and leaves of tomato plants 2, 3, 4, and 8 weeks after foliar spraying; different letters on each curve show significant differences among weeks at $P < 0.05$ level (F-LSD test)

We used the leaf spraying technique to study the changes in frequency of fungal colonization in leaf and root tissues at days 7, 14, 21, 28, and 56. As expected from the previous experiment, the root and leaf tissues hosted 00.00 and 3.11 CFU/ml of fungal colony at day 7. The frequency of fungal colonies in both root and leaf samples maximized at week 2 and exhibited a descending pattern towards week 8 (Figure 1).

Consistent with these findings, the results of previous studies indicate that fungal colonization following foliar spraying maximizes between days 7 and 14 and decreases over time (Russo et al., 2015; Allegrucci et al., 2017; Afandhi et al., 2019). In different studies, *B. bassiana* has been shown to retain its endophytic association for up to three months with citrus lemon (Bamisile et al., 2019), jute plants (Biswas et al., 2012) and maize (Renuka et al., 2016), up to nine months with pine trees (Brownbridge et al., 2012), and up to eight months with coffee (Posada and Vega, 2006). A possible explanation for declines in fungal colonization over time can be the competition with other fungi and bacteria in the system, resulting in inhibition of *B. bassiana* growth (Russo et al., 2015).

Insecticidal effect

Significant differences were found in mortality percentages of whiteflies among different life stages and treatments. Although, the mortality rates of different life stages were not statistically different in control, an ascending pattern in mortality was observed by increase in whitefly age, with the highest mortality rate for the adult stage (Figure 2).

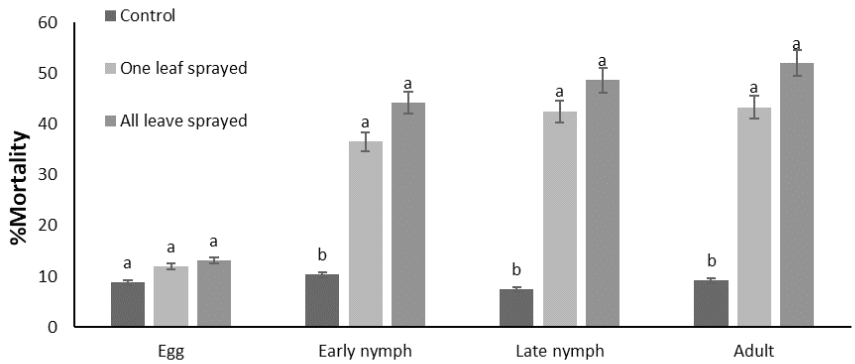


Figure 2. Mortality percentage of different life stages of *Trialeurodes vaporariorum* when reared on endophyte-free tomato plants (control) or plants sprayed with *B. bassiana* suspension on either a single leaf or the entire plant; different letters show significant difference among treatments in the same life stage at $P < 0.05$ level (F-LSD test)

The egg mortality was not affected by fungal inoculation. However, a significant increase in mortality of nymphal and adult stages was recorded on plants inoculated with *B. bassiana* (Figure 2). Inoculation of the entire plant with fungal suspension caused a higher mortality in all developmental stages of *T. vaporariorum* in comparison with inoculation of a single leaf, though the difference was not statistically significant (Figure 2). Garrido-Jurado et al. (2017) showed that the nymphal mortality of the sweet potato whitefly *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) ranges between 84–100% in melon leaves that are directly sprayed with *B. bassiana* suspension, but decreases (66–88%) in leaves of the same plant that are not directly sprayed. The mortality of nymphal and adult stages ranged from 36 to 52% in plants inoculated with *B. bassiana*. No mycosis was detected on any of dead whiteflies. These results are in line with some previous studies, indicating that endophytic fungi can cause mortality in herbivorous insects not only by direct contact of the conidia with the target insect, but also by expressing a variety of fungal metabolites either *in planta* or on plant surfaces (Arnold, 2008; Gurulingappa et al., 2011; Garrido-Jurado et al., 2017; Bamisile et al., 2019).

Plant biochemical changes

We measured the total phenolic and protein contents of tomato leaves following endophytic colonization by *B. bassiana* and/or infestation with *T. vaporariorum*. The results revealed significant increase in both phenolic and protein content of leaves following endophyte colonization regardless of whitefly infestation (Figure 3). The highest contents of both phenols and proteins were detected in plants simultaneously inoculated with fungal endophyte and infested with whiteflies. In absence of fungal endophyte, whitefly infestation had no significant effect on phenolic content; however, a significant increase in the total protein content was recorded following whitefly infestation (Figure 3). These results may highlight the promoting effect of fungal endophytes on protein and phenolic content of plants, which may be intensified following herbivore attack. In agreement with this results, previous studies also reported increased accumulation of compounds such as reducing sugar, total flavonoids, total phenolic compounds, trans-resveratrol, as well as increased activity of enzymes such as phenylalanine ammonia-lyase following endophyte colonization (Shoresh, Harman, 2008; Gomez-Vidal et al., 2009; Yang et al., 2018).

Compounds such as alkaloids, terpenoids, proteins, flavonoids, and phenols may be produced *in planta* either directly by endophytes themselves (Gunatilaka, 2006; Ownley et al., 2010) or indirectly by influencing on host plant (Shoresh, Harman, 2008; Gomez-Vidal et al., 2009; Gasoni, Gurfinkel, 2009). For example, colonization of date palm trees (*Phoenix dactylifera* L.) by *B. bassiana* and some other endophytic fungi resulted in increased production of proteins involved in plant defense, stress response, energy metabolism and photosynthesis (Gomez-Vidal et al., 2009). Different proteins were expressed following colonization by the fungal endophyte *Trichoderma harzianum* Rifai in maize (Shoresh, Harman, 2008) which were involved in carbohydrate metabolism, defense, and photosynthesis. In another study, the total phenlic content of 15-day-old cotton seedlings was reported to be about two times higher than that of 5-day-old seedlings following colonization by the endophytic fungus, *Cladorrhinum foecundissimum* Sacc. & Marchal (Gasoni, Gurfinkel, 2009).

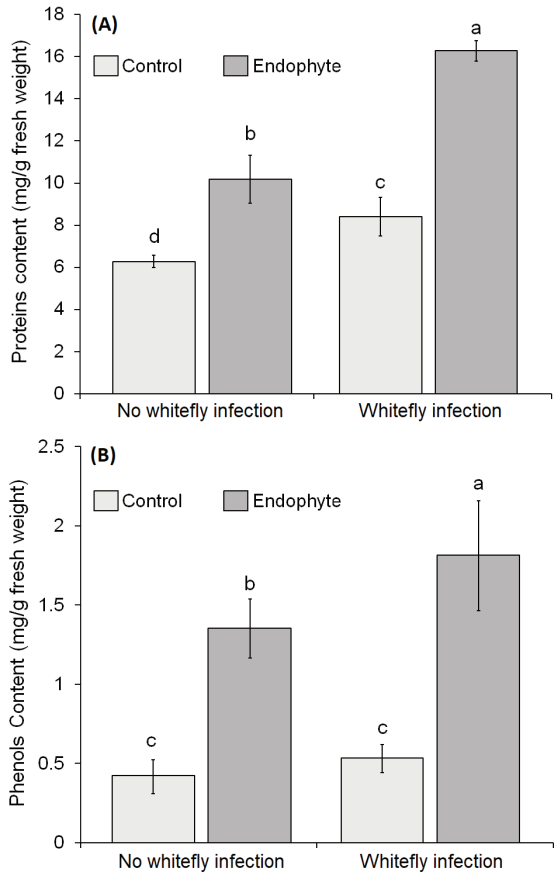


Figure 3. Effect of endophytic colonization by *Beauveria bassiana* or/and infestation by *Trialeurodes vaporariorum* on the total protein (A) and total phenol (B) content of tomato leaves; different letters show significant differences at $P < 0.05$ level (F-LSD test)

Conclusions

The successful establishment of *B. bassiana* (isolate TV) as an endophyte in tomato plant (cv. Falat) and its negative effects on different developmental stages of *T. vaporariorum* were reported for the first time in this study. Additionally, the fungal endophyte was found to promote the total phenolic and protein contents of tomato plants, especially when the plants were infested by *T. vaporariorum*. The leaf spraying was considered as the most efficient inoculation technique and fungal colonization was confirmed up to 56 days after inoculation. The persistence of entomopathogenic fungus as endophyte in plant tissues provides an opportunity to include these beneficial organisms in pest management programs (Lewis et al., 1996; Jaber et al., 2018; Gonzalez-Mas et al., 2019) and can be also used in integrated management programs of both insect pests and plant diseases (Ownley et al., 2008; Bamisile et al., 2019). Future studies may

focus on exploring the mechanisms underlying the systemic effect of endophytic *B. bassiana* on *T. vaporariorum* as well as assessing the potential sub-lethal effects of the endophyte on life parameters of the whitefly.

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