Parasitology Research (2019) 118:57–62
https://doi.org/10.1007/s00436-018-6114-1

ORIGINAL PAPER

Non-native parasitic copepod *Neoergasilus japonicus* (Harada, 1930) utilizes non-native fish host *Lepomis gibbosus* (L.) in the floodplain of the River Dyje (Danube basin)

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Received: 10 July 2018 / Accepted: 7 October 2018 / Published online: 23 October 2018

Abstract
The parasitic copepod *Neoergasilus japonicus* (Harada, 1930) (Ergasilidae), native to east Asia, is widely distributed in Asia, Europe, and North and Central America. Recently, this species appeared in lentic water bodies of the River Dyje floodplain (Danube basin, Czech Republic). It was first recorded in 2015 and in 2 years it reached a 100% prevalence in recently expanding non-native fish host, *Lepomis gibbosus* (Linnaeus, 1758) (Centrarchidae, native to North America) at two borrow pits. Abundance of *N. japonicus* increased with fish length, with maximum intensity of infection reaching 99 parasites per fish. The parasite was most frequently found attached to the dorsal and anal fins of fish, while preference for the dorsal fin was more evident with lower infection intensities. Utilization of expanding fish hosts in water bodies that are regularly interconnected via natural or managed flooding may support the rapid dispersal of this non-native parasite.

Keywords *Neoergasilus* · Copepod · *Lepomis* · Species introductions · Centrarchidae · 18S, 28S rDNA

Introduction

Intentional or unintentional alien species introductions resulting from anthropogenic activities are often a cause for a range of ecological and economic impacts. Non-native species introductions may also carry, among other consequences, the risk of novel disease introductions when local species are capable of hosting the new parasite/pathogen (termed as "spillover"), or risk of shift in local native or non-native parasite/pathogen population dynamics when introduced species are a suitable host (termed as "spillback") (Kelly et al. 2009). Increasing abundance of the parasite in the environment may then become a threat to native host species, especially when potential hosts are endemic or endangered (Suárez-Morales et al. 2010).

Neoergasilus japonicus (Harada, 1930) (Ergasilidae) is a widespread parasitic copepod that has been recorded from numerous fish species in eastern Asia, Europe, and North and Central America (Suárez-Morales and Mercado-Salas 2013). The parasite is suspected to be native to eastern Asia and expanding via transport of exotic fish hosts associated with the fish-culture industry (Hudson and Bowen 2002). The expected mode of distribution of *N. japonicus* involves aquaculture, the aquarium trade, bait releases, or ballast water introductions. In its non-native range, *N. japonicus* is often found on a variety of hosts families, including cyprinids and percids in Europe, supplemented by centrarchids and ictalurids from North America (Hudson and Bowen 2002) and Neotropical cichlids from Central America (Suárez-Morales et al. 2010). In its native range, this parasite successfully utilizes alien freshwater fishes in addition to a wide range of native host species (Nagasawa and Uyeno 2012). In Europe, *N. japonicus* was first reported in the 1960s in Slovakia (Hanek 1968) and Hungary (Pónyi and Molnár 1969), and later in other countries such as France (Lescher-Moutoué 1979), Britain (Mugridge et al. 1982), Finland

Section Editor: Shokoofeh Shamsi

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(Tuuha et al. 1992), and more recently Germany (Knopf and Holker 2005), Italy (Alfonso and Belmonte 2010), and Turkey (Soylu and Soylu 2012). Nevertheless, Tuuha et al. (1992) suggest that the actual European distribution is more abundant than expected as this species can be dislodged during the fish sampling and therefore absent from parasite surveys.

Lepomis gibbosus (Linnaeus, 1758) (Actinopterygii: Centrarchidae) is one of the most successful introduced fish species in Europe (Copp and Fox 2007). At the end of nineteenth century, the species was imported to Europe for recreational angling and as an aquarium and garden pond fish (Holčík 1991) from its native range covering freshwaters of eastern North America. The species is now established in most European countries as a result of subsequent introductions and natural spreading into adjacent waterbodies (Copp and Fox 2007). In the Czech Republic, populations of *L. gibbosus* exhibit patchy distribution in the River Elbe (North sea drainage) and the River Morava (Black sea drainage) basins (Musil et al. 2010), with sporadic occurrence in the main rivers and stable populations in adjacent lentic waterbodies. In Morava basin, the species occurs in sand lakes along the river, but reports on its presence in the lower Dyje (Morava tributary, confluence located over 100 km downstream the lakes) have been very rare the last few decades until 2010 (Lusk et al. 2011). After this period, *L. gibbosus* invaded several isolated borrow pits in the Dyje floodplain where it established large population sizes. During a parasitological survey on the newly established populations of *L. gibbosus* in the Dyje floodplain, we observed the presence of the non-native copepod parasite *N. japonicus*, a species that has not been previously documented in the Dyje basin and the Czech Republic (Moravec 2001). The aim of this study was to assess the susceptibility of *L. gibbosus* to *N. japonicus* infection.

Material and methods

Fish were collected at two separated borrow pits, the artificial pools created during dyke construction in the River Dyje floodplain (Rohlik, coordinates 48° 38′ 53,478 N, 16° 55′ 39,129 E and Hvezda, 48° 38′ 36,405 N, 16° 55′ 56,703 E; Czech Republic, Danube basin), with possible migration of fish between the two pits during the flooding in spring. A total of 97 *L. gibbosus* were collected during spring 2013 ($N = 21$; Rohlik), 2015 ($N = 20$; Hvezda), and 2017 ($N = 24$; Rohlik and $N = 32$; Hvezda; Table 1). Fish were caught using backpack electrofishing equipment (LENA portable-pulsed direct-current electrofishing device, Bednář, Czech Republic) and transported live in aerated tanks with local water to the laboratory where they were euthanized and dissected within 1 day (Kvach et al. 2016). All fish were measured to the nearest 1 mm (standard length [SL]) and weighed to the nearest 1 g (total weight [w]) prior to dissection. Fins, gills, and body surface were examined for the presence of ergasilid copepods. Parasites were removed from the host tissue, preserved in 4% formaldehyde or 96% ethanol. The morphological identification of copepod specimens was performed according to Gusev et al. (1985) using an Olympus BX50 light microscope equipped with phase contrast, differential interference contrast, and Olympus MicroImage™ Digital Image Analysis software (Olympus Optical Co.).

Ethanol-preserved specimens were processed by molecular identification techniques to obtain nuclear ribosomal DNA sequence data (28S and 18S rDNA). Genomic DNA was extracted from 14 specimens of *N. japonicus* (7 specimens per each locality) by using a DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The 18S rDNA fragment was amplified using primers 18SF (5′-AAG GTG TGM CCT ATC AAC T- 3′) and 18SR (5′-TTA CTT CCT CTA AAC GCT C-3′) (Song et al. 2008); and the 28S rDNA fragment with primers 28SF (5′-ACA ACT GTG ATG CCC TTA G-3′) and 28SR (5′-TGG TCC GTG TTT CAA GAC G-3′) (Song et al. 2008). The amplification reactions were performed in a total reaction volume of 20 μl containing 3 μl DNA, 1.5 mM MgCl2, 1× PCR buffer with NH4(SO4)2 (Fermentas), 0.2 mM dNTPs, 0.2 μM of each primer, and 1 unit of Taq polymerase (Fermentas). PCR was carried out in the Mastercycler ep gradient S (Eppendorf) using the following steps: an initial denaturation at 94 °C for 5 min, followed by 39 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s and extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min. PCR products were checked on 1.5% agarose gels purified using an ExoSAP-IT™ (Affymetrix Inc., Santa Clara, USA) and sequenced directly from both strands using the PCR primers. DNA sequencing was carried out using BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystem by Thermo Fisher Scientific, Praha, Czech Republic) and an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems). DNA sequences obtained from the 18S and 28S regions were assembled and edited using Sequencher software (Gene Codes Corp., Ann Arbor, MI, USA). The newly obtained sequences were compared to the NCBI database using the nucleotide basic local alignment search tool (BLASTn) and were deposited in GenBank under the following accession numbers: MH167969 and MH167970 for 18S, and MH167967 and MH167968 for 28S.

Following Bush et al. (1997), parasite abundance was expressed as the number of individuals of a particular parasite species (N_P) on a single host regardless of whether or not the host is infected; mean abundance (A) was calculated as the mean number of parasites per all hosts examined: $A = \Sigma N_P/N_H$; intensity of infection was expressed as the number of individuals of a particular parasite on a single infected host; mean intensity of infection (II) was calculated as the mean number of individuals of a particular parasite species per all infected hosts: $II = \Sigma N_P/N_{Hinf}$. and prevalence (P) as a

Table 1 Number of *L. gibbosus* examined (N) with fish standard length (SL, in cm), total weight (w, in g), prevalence (in %), mean abundance, and intensity of *N. japonicus* infection during two sampling years at Hvezda and Rohlik borrow pits in the River Dyje floodplain (Danube basin)

	Hvezda 2015	Hvezda 2017	Rohlik 2013	Rohlik 2017
N	20	32	21	24
SL mean (range, cm)	6.6 (5.5–7.3)	5.9 (4.2–8.0)	8.4 (7.0–9.7)	6.3 (3.8–7.4)
w mean (range, g)	8.1 (4.9–11.0)	6.2 (2.0–15.3)	20.1 (11.6–29.9)	7.8 (1.6–11.3)
Prevalence (%)	50	100	0	100
Mean abundance ± SD	1.5 ± 2.2	15.0 ± 18.1	0	4.4 ± 3.7
Intensity of infection				
Mean (range)	2.9 (1–9)	15.0 (1–99)	0	4.4 (1–17)

proportion of infected hosts: $P = N_{Hinf} \times 100/N_H$, where N_H represents number of all hosts examined and N_{Hinf} number of infected hosts. Abundance of *N. japonicus* was compared using a Man-Whitney U test between samples, while prevalence was compared using Fisher's exact test. Spearman rank correlation was used to test the association between fish SL and parasite abundance.

Results

The copepods obtained from all samples were morphologically identified as *N. japonicus*. The sequences of the 28S and 18S rDNA generated in this study were 623 bp and 1355 bp long, respectively. No sequence variation was observed within each locality, while single base differences were observed between localities. The sequence of 28S rDNA generated from the parasites from Rohlik (accession no. MH167968) showed complete identity to the sequence of *N. japonicus* already deposited in GenBank (accession no. KR048823). The sequence of 28S obtained from specimens from the Hvezda borrow pit (accession no. MH167967) had one nucleotide substitution (A–G) at position 525 of the reference sequence KR048823. For 18S, sequence variation between localities was also observed: two nucleotide substitutions were detected (A–T at position 385 and T–C at position 723). The 18S sequences from both localities (accession no. MH167969—locality Hvezda, MH167970—locality Rohlik) showed 99% identity to 18S sequence of *N. japonicus* deposited in GenBank (accession no. KR048752).

Presence of adult females of *N. japonicus* was detected at Hvezda in 2015 and at both localities in 2017, while no ergasilid copepods were observed in Rohlik in 2013 (Table 1). Prevalence significantly increased from 50 to 100% ($p < 0.001$) and mean abundance from 1.5 to 15.0 ($Z = 4.9$, $p < 0.001$) in Hvezda between 2015 and 2017. In 2017, mean abundance was lower in Rohlik (4.4) compared to Hvezda ($Z = 2.9$, $p = 0.004$), despite a 100% prevalence at both sites (Table 1). Parasite abundance increased with fish SL only in Hvezda in 2017 ($r_s = 0.61$, $p < 0.001$), while no association between abundance and SL was found in samples with relatively low-parasite load (i.e., Hvezda in 2015 and Rohlik in 2017).

Neoergasilus japonicus was located mainly on fins (99.5% of all copepods collected), and occasionally on gill cartilage, body surface, and nostrils (Fig. 1). Parasites exhibited preference for dorsal fins in samples with lower parasite load (79% in Hvezda 2015 and 62% in Rohlik 2017), and preference for both dorsal and anal fins in a sample with high-parasite load (Hvezda 2017, 48 and 37%, respectively).

Discussion

Though *N. japonicus* has been previously reported in several European countries, our findings represent, to our knowledge, its first occurrence in Czech Republic and the River Dyje basin. Previous records of this parasite in the nearby regions of central and western Europe have been reported from localities in Slovakia (Hanek 1968), Hungary (Pónyi and Molnár 1969), and Germany (Knopf and Hölker 2005), which are farther than 200 km from our sites not naturally connected through the river network. Additionally, intensive surveys of parasite fauna of the three potentially susceptible local hosts (cyprinid and percid fish) were conducted in the late 1990s throughout a range of waterbodies of the lower Dyje floodplain, including sites used in the present study (Kadlec et al. 2003). Their results based on investigation of over 1000 fish individuals showed absence of *N. japonicus* in the hosts' parasite communities (Kadlec et al. 2003). We, therefore conclude that the recent occurrence of *N. japonicus* in the lower Dyje is linked rather to unintentional releases from the global live fish trade (of unknown source, possibly including trade with *L. gibbosus*) than its natural spread or failed detection of the parasite in parasitological surveys as suggested by Hanek (1968) or Hudson and Bowen (2002). Therefore, although the source of parasite release remains unknown, the co-introduction with *L. gibbosus* offers one possible explanation as the parasite's recent spread corresponds to the spread of this non-native fish host. However, to resolve this question requires further investigation, including for example molecular genetics of both the host and parasite, which is out of the scope of the present study. While non-native parasites are usually introduced into a new locality with their natural non-native hosts, there are examples of parasite introduction regardless of the host (e.g., as free swimming stages) or co-introduction with other host species (summarized in Lymbery et al. 2014). In the case of *N. japonicus*, original source of introduction is believed to be associated with commercial fish introductions from Asia to Europe/North America (Pónyi and Molnár 1969; Hudson and Bowen 2002). Consequently, its further spread may then be related to the fish introduction for aquaculture and sport fishing within particular continents (Alfonso and Belmonte 2010), utilizing either natural (Asian) or new (native or non-native) fish species as a vector.

Neoergasilus japonicus appeared to successfully utilize *L. gibbosus* as a suitable host, confirmed by the maximum prevalence (100%) observed at both localities in 2017 and intensity of infection reaching almost 100 parasite individuals per host in the Hvezda borrow pit (see Table 1). This is an exceptionally high infection rate found outside the natural range of *N. japonicus* as compared with recent observations from Europe (Beyer et al. 2005; Knopf and Hölker 2005; Soylu and Soylu 2012), North America (Hudson and Bowen 2002), and Central America (Suárez-Morales et al. 2010). Earlier studies note severe infections of *N. japonicus* in Hungary, but without providing details on infection intensities (Pónyi and Molnár 1969), and in Britain with intensities up to 250 parasites per *Rutilus rutilus* L., 1758 host (Mugridge et al. 1982). A high infection rate was found in the native range of *N. japonicus* in Japan (Nagasawa and Uyeno 2012), where introduced bluegill, *Lepomis macrochirus* Rafinesque, 1810, appeared to serve as competent host with maximum prevalence and intensity reaching over 200 (Nagasawa and Sato 2015) or even 700 (Urawa et al. 1991) parasites per host. In southern Japan, Nagasawa and Uyeno (2012) documented utilization of alien host species by this parasite, especially in reservoirs where native freshwater fishes decreased their abundance, confirming its low-host specificity. In addition, an increase over time in parasite infection rate at both localities supports the assumption for the competence of non-native *L. gibbosus* to *N. japonicus*. In Rohlik borrow pit, *L. gibbosus* was first recorded in 2012 (personal observation), while this locality was the only lentic water body in the Dyje floodplain out of the 18 surveyed sites where this fish was detected. In the following year (2013), the parasitological examination did not confirm presence of ergasilid copepods parasitizing *L. gibbosus*, but 4 years later all fish collected were infected, although with relatively low intensity of infection. Occurrence of *L. gibbosus* in Hvezda was first documented in 2013–2014 (personal observation). The infection rate shortly after the host's introduction into this borrow pit (after 1–2 years) was comparable to other European records, but during the 2 years the prevalence doubled and reached 100%, while mean abundance increased tenfold. The results therefore indicate that the presence of non-native host species (*L. gibbosus*) in the Dyje floodplain facilitates the population dynamics of the non-native parasite (*N. japonicus*). This potentially represents an example of Simberloff—Von Holle "invasion meltdown" model, demonstrating that facilitative interactions between non-native species can exacerbate the impact of individual invaders (Simberloff and Von Holle 1999).

The majority of parasites was attached to the fish fins, which is typical for *N. japonicus* and differing from other ergasilids (Pónyi and Molnár 1969). In concordance with other studies (Hudson and Bowen 2002; Soylu and Soylu 2012; Nagasawa and Obe 2013; Mirzaei et al. 2016), *N. japonicus* showed a preference for dorsal fins, especially at low-infection levels, and anal fins as the second preference with increasing intensity of infection (Fig. 1). As noted by Nagasawa and Obe (2013), dorsal and anal fins are preferentially infected with other sites such as other fins, body surface, or gills being parasitized when the preferred sites are fully occupied. The authors suggested that infective females of *N. japonicus* can easily attach to the posterior basal part of the dorsal and anal fins due to surrounding conditions of low turbulence. In our study, parasite abundance increased with fish size, but only in a sample with a higher infection rate, corresponding to the results of Hudson and Bowen (2002) who found that parasite infection of *N. japonicus* was strongly biased towards large fish. Moreover, the authors also observed a relatively high infection rate in 0+ juvenile *L. gibbosus* in comparison to the parasite's absence in other centrarchids *L. macrochirus* and *Ambloplites rupestris* (Rafinesque, 1817), indicating high susceptibility of *L. gibbosus* to *N. japonicus* among centrarchids in the North America (Hudson and Bowen 2002). However, the intensity of infection in 1+ fish (the youngest fish at the localities, as 0+ fish were not yet present in the spring sample) in our study was generally very low.

Parasite species identity was confirmed by combined morphological and molecular approaches. Based on the available genetic data of *N. japonicus* and other representatives of Ergasilidae in Genbank, nuclear 18S and 28S rDNA fragments were selected for molecular characterization of parasites studied. Within the family Ergasilidae, both rDNA markers have proven to be highly effective for species identification (Song et al. 2008) and for evaluating phylogenetic relationships (Kim and Kim

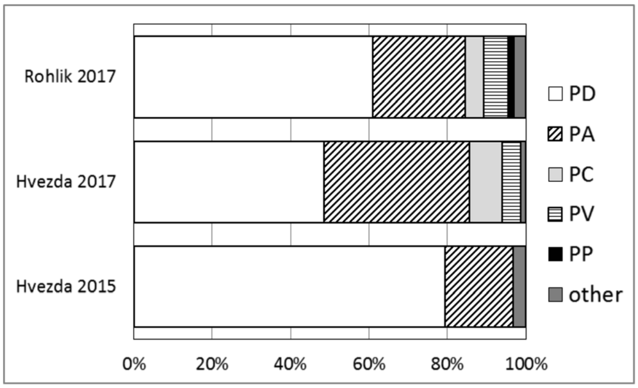

Fig. 1 Proportion of *N. japonicus* attached to the particular fish organs. PD dorsal fins, PA anal fin, PC caudal fin, PV ventral fins, PP pectoral fins, other (body surface, gill cartilage, nostrils)

2000; Song et al. 2008). Comparison of newly obtained sequence data revealed a 100% identity of the 28S rDNA sequence from Rohlik, the locality where *L. gibbosus* appeared first in the Dyje floodplain (see above) with the reference sequence from Korea (KR048823, Beak and Hwang, unpublished), the native range of *N. japonicus*. The sequence of 28S from Hvezda and 18S sequences showed a 99% identity to reference sequences of *N. japonicus*. For both rDNA markers, a low-intraspecific variation between populations was found (0.14% for 18S and 0.16% for 28S). Considering that all specimens analyzed in this study are morphologically very similar, we are currently unable to ascertain if the observed genetic distances simply represent a higher level of intraspecific variation or reflect recent speciation events or cryptic species diversity. Moreover, low-intraspecific divergence in 18S and 28S was also recorded in other representatives of ergasilid copepods (Song et al. 2008). Using more sensitive mitochondrial genes, such as cytochrome c oxidase subunit I sequence (COI mtDNA), and eventually additional morphological studies performed on a larger sample size will be necessary to resolve this question.

Due to its good swimming capability (Pónyi and Molnár 1969), high fecundity, and fast life cycle (Urawa et al. 1991), *N. japonicus* is presumed to possess high infectivity. In habitats altered by non-natives such as *L. gibbosus* in the River Dyje floodplain—a species that appears highly susceptible to this parasite—*N. japonicus* may rapidly impact the local freshwater fish species, for example via reduced growth rate (Mugridge et al. 1982). In the Dyje floodplain, natural reproduction of mainly phytophilic and phytolithophilic species is supported by natural and/or managed flooding during spring (Jurajda et al. 2004), enabling fish migration among the localities and the adjacent river. We can therefore expect further dispersal of this non-native parasite along with its non-native fish host in the region.

Acknowledgements We would like to thank Dr. Seth White for proofreading the English text.

Funding information This study received financial support through the European Centre of Ichthyoparasitology under the Grant Agency of the Czech Republic—Centre of Excellence Grant No. P505/12/G112.

Compliance with ethical standards

Ethical note statement The research was undertaken in line with the ethical requirements of the Czech Republic, and has been approved by an appropriate ethics committee. The sampling, transportation, maintenance, and care of experimental fish, as well as method of fish killing complied with legal requirements in the Czech Republic (§7, Law No. 114/1992 about the protection of nature and landscape and § 6, 7, 9, and 10 regulation No. 419/2012 about the care, breeding, and using experimental animals). Researchers involved in this study (MO and PJ) are certified according to Czech legal requirements (§15, Law No. 246/1992 on Animal Welfare) to work with experimental animals.

Conflict of interest The authors declare that they have no conflict of interest.

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