Interaction of non-indigenous endoparasites of the European eel *Anguilla anguilla*

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Et hätt noch immer jot jejange.

- Kölsches Grundgesetz

Angaben zur Prüfung

Die der vorliegenden Arbeit zugrunde liegenden Experimente wurden in der Abteilung für Aquatische Ökologie der Universität Duisburg-Essen durchgeführt.

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1.1 Abbreviations

AC = *A. crassus*

AN = *A. novaezelandiae*

Bp = base pairs

cox I = mitochondrial cytochrome c oxidase subunit 1

DNA = deoxyribonucleic acid

dpi = days post inoculation

dsDNA = double-stranded deoxyribonucleic acid

ELISA = enzyme linked immune sorbent assay

F0 = parental generation

F1 = first filial generation

F2 = second filial generation

IMH = Invasional meltdown hypothesis

L2 = second stage larvae

L3 = third stage larvae

L4 = forth stage larvae

m/M = male

n = total number

nD = not determinable

PCR = polymerase chain reaction

SD = standard deviation

sp. = species

spp. = species pluralis

w/W = female

a x b = crossbreeding between a and b

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2. Summaries

2.1 Summary

Biotic interaction is an essential feature of ecosystems, expressing their stable and dynamic structure. The present thesis is focusing on the interaction of different non-indigenous endoparasites of the European eel (*Anguilla anguilla*). One of them, *Anguillicola crassus*, is topic of research since decades, however very few is known about its interaction with other parasites. After this invasive parasite was found inside an acanthocephalan cyst (*Pomphorhynchus* spp.) – the second protagonist of the present thesis – it was indicated that there might be some beneficial interaction between both species. This work proves that hidden nematode larvae are still infectious to their final host, revealing previously unidentified but effective ways to complete their life cycle. It is already known that invasive parasites influence their new habitats to some extent. The here applied invasional meltdown hypothesis describes the beneficial influence of two non-indigenous species by accelerating the distribution of each other. Because this hypothesis was only used to describe interactions of free-living species, *Pomphorhynchus* spp. and *A. crassus* are the first parasites it is applied to.

Furthermore, the co-evolutionary adaptation of host-parasite systems is considered in this thesis, as both parasites are native to different eel species. By analyzing plasma cortisol levels after inoculation, the co-evolutionary well adapted system of *A. crassus* with Japanese eels (*A. japonica*) revealed the lowest stress response, whereas the same eel species displayed the highest response to *Pomphorhynchus* spp. as a naïve parasite. Cortisol levels of the European eels ranged between those of the Japanese eel, but likewise with lower levels to the familiar parasite. The results underline the clear dependence of the cortisol response on mutual adaptation occurring in host-parasite systems, from high cortisol levels in naïve systems and low cortisol levels in adapted systems.

When considering the invasional success of *A. crassus*, the focus of this thesis is not only on its interaction with the phylogenetically distinct parasites *Pomphorhynchus* spp. but equally with the phylogenetically close nematode *A. novaezelandiae*. This species, native to the short-finned eel *A. australis*, was established in Lago Bracciano, Italy, even before the introduction of *A. crassus* to Europe. Only couple of years later it got displaced by its Asian relative. Since then, it is unknown how *A. crassus* was able to outcompete *A. novaezelandiae* in such a

short period of time. Some determinants might be a more efficient life cycle, for instance by releasing eggs over a longer period of time; or consequences of unidirectional hybridization between these two species, meaning that only *A. novaezelandiae* females fertilized by *A. crassus* males are able to produce viable offspring, but not the other way around. As only rudimental information is known about this hybridization hypothesis, the here presented thesis is also researching the viability of hybrid offspring for both hybridization patterns. By microsatellite analysis, it is detected that both crossbreeding produce hybrid eggs, but only those of the already described pattern *A. novaezelandiae* female x *A. crassus* male develop further into adult nematodes. This discovery not only underlines the understanding of what has happened at Lago Bracciano in the early 1980s, but further conduces to a better understanding of the global invasion success of *A. crassus*.

The interaction of invasive parasites in newly conquered ecosystems is a critical component for understanding successful invasion processes. The phylogenetic relation of the parasites studied in this work highlights the extensive variance with which organisms such as *A. crassus* can establish and spread in new areas.

2.2 Zusammenfassung

Biotische Interaktionen sind ein wesentliches Merkmal von Ökosystemen, die deren stabile und dynamische Struktur zum Ausdruck bringen. Die vorliegende Dissertation befasst sich mit der Interaktion verschiedener nicht einheimischer Endoparasiten des Europäischen Aals (*Anguilla anguilla*). Obwohl der Nematode *A. crassus* seit Jahrzehnten Gegenstand der Forschung ist, ist nur sehr wenig über seine Interaktion mit anderen Parasiten bekannt. Erst nachdem dieser in einer Acanthocephalen-Zyste (*Pomphorhynchus* spp.) - dem zweiten Protagonisten der vorliegenden Dissertation - gefunden wurde, deutete sich an, dass es möglicherweise zwischen diesen beiden Arten eine mutualistische Interaktion gibt. Die Ergebnisse der vorliegenden Arbeit beweisen, dass versteckte Nematodenlarven aus diesen Zysten immer noch für ihren Endwirten infektiös sind, und zeigen so bisher unbekannte, aber effektive Wege auf, wie *A. crassus* seinen Lebenszyklus vollenden kann. Es ist bereits bekannt, dass invasive Parasiten ihre neuen Lebensräume zu einem gewissen Grad beeinflussen. Die hier angewandte Invasional-Meltdown-Hypothese beschreibt den gegenseitigen, positiven Einfluss zweier nicht-einheimischer Arten, indem sie die Verbreitung der jeweils anderen Art beschleunigen. Da diese Hypothese bisher nur zur Beschreibung von Interaktionen freilebender Arten verwendet wurde, sind *Pomphorhynchus* spp. und *A. crassus* die ersten Parasiten, auf die sie angewendet wird.

Des Weiteren wird in dieser Arbeit die koevolutionäre Anpassung von Wirts-Parasit-Systemen behandelt, da beide Parasitenarten in verschiedenen Aalarten endemisch sind. Bei der Analyse der Plasmacortisolwerte nach einer Infektion zeigte sich, dass das koevolutionär gut angepasste System von *A. crassus* mit Japanischen Aalen (*Anguilla japonica*) die geringste Stressreaktion aufwies, während dieselbe Aal-Art die höchste Reaktion auf *Pomphorhynchus* spp. als naiven Parasiten zeigte. Die Cortisolwerte der Europäischen Aale lagen zwischen denen des Japanischen Aals, aber ebenfalls mit niedrigeren Werten für den vertrauten Parasiten. Die Ergebnisse unterstreichen die deutliche Abhängigkeit der Cortisolreaktion von der gegenseitigen Anpassung in Wirt-Parasit-Systemen, von hohen Cortisolwerten in naiven Systemen und niedrigen Cortisolwerten in angepassten Systemen.

Bei der Betrachtung des Invasionserfolgs von *A. crassus* liegt der Schwerpunkt dieser Arbeit nicht nur auf der Interaktion mit dem phylogenetisch weit entfernten

Parasiten *Pomphorhynchus* spp. sondern auch mit dem phylogenetisch nah verwandten Nematoden *A. novaezelandiae*. Diese Art ist im Kurzflossen Aal *A. australis* endemisch und war bereits vor der Einführung von *A. crassus* in Europa im Lago Bracciano, Italien, etabliert. Nur wenige Jahre später wurde sie von ihrem asiatischen Verwandten vollständig verdrängt. Seitdem ist unklar, wie *A. crassus* in so kurzer Zeit *A. novaezelandiae* verdrängen konnte. Eine mögliche Erklärung dafür könnte ein effektiverer Lebenszyklus sein, wie zum Beispiel durch die Abgabe von Eiern über einen längeren Zeitraum, oder die Folgen einer unidirektionalen Hybridisierung zwischen diesen beiden Arten, was bedeutet, dass nur *A. novaezelandiae*-Weibchen und *A. crassus*-Männchen lebensfähige Nachkommen hervorbringen können, nicht aber umgekehrt.

Da über diese Hybridisierungshypothese nur rudimentäre Informationen vorliegen, wird in der hier vorgestellten Dissertation auch die Lebensfähigkeit der Hybridnachkommen für beide Hybridisierungsmuster untersucht. Mittels Mikrosatellitenanalyse wird festgestellt, dass Nachkommen beider Kreuzungsmuster Hybrideier produzieren, aber nur die des bereits beschriebenen Musters von *A. novaezelandiae*-Weibchen x *A. crassus*-Männchen sich weiter zu adulten Nematoden entwickeln. Diese Entdeckung untermauert nicht nur das Verständnis dessen, was am Lago Bracciano in den frühen 1980er Jahren geschah, sondern trägt auch zu einem besseren Verständnis des weltweiten Invasionserfolgs von *A. crassus* bei.

Die Interaktion von invasiven Parasiten in neu besiedelten Ökosystemen ist ein wichtiger Bestandteil für das Verständnis von erfolgreichen Invasionsprozessen. Die phylogenetische Zugehörigkeit der in dieser Arbeit untersuchten Interaktionspartner zeigt die breite Varianz auf, mit der sich Organismen wie *A. crassus* in neuen Gebieten etablieren und verbreiten können.

3. Introduction

Human life on earth would not be possible without functioning ecosystems. They do not only provide us with clean water, air and nutrients, but are also essential for protection against flooding, detoxification of pollutants and impoundment of water and soil resources. In recent decades, some ecosystems have become increasingly important as a source of relaxation and conviviality for people, like forests, or areas close to lakes and rivers (Boenigk, 2021). Human activities have changed the world and ecosystems to such an extent that the term "Anthropocene" has even come to be used for the current epoch (Tandon, 2021; Zalasiewicz et al., 2011). One characteristic of the Anthropocene is that mankind is increasingly travelling and trading around the globe and is thereby having a direct impact on various ecosystems by the introduction of new species, accidental or intended (Marcogliese, 2023).

The main causes for the introduction of new species into aquatic ecosystems are associated to trade of life animals (Jacoby and Gollock, 2014; Torchin et al., 2002), escapes of farmed fish into the marine environment (Atalah and Sanchez-Jerez, 2020; Bouwmeester et al., 2021), escaping or suspended pets (Dorcas et al., 2012), or unintended transport of organisms e.g. with ballast waters of ships (Carlton and Geller, 1993; Corkum et al., 2004). But not every newly introduced species will become an invasive threat as they first have to overcome the biotic resistance, which is the ability of the native community to limit invasions (Byun and Lee, 2017). Most introduced species struggle too much with it and are not able to cope with this resistance (Elton, 1958; Simberloff and Von Holle, 1999). Many organisms die already during transportation or soon after, because of transportation stress and/or the lack of a proper food source.

After the introduction to a new area, invaders need to establish themselves by coping with new habitat conditions, predators, or parasites. Only if they are able to cope with all of these factors and reproduce in large quantities, they might become invasive organisms that cause changes in local communities e.g. by outcompeting native species (Boenigk, 2021; Hatcher and Dunn, 2011; Kolar and Lodge, 2001). Thus, adaptation to the new ecosystem and displacement of native species are basically the common characteristics of all invasive species, regardless of the different mechanisms by which this is achieved (Boenigk, 2021; Torchin et al., 2002). Due to the lack of enemies and competitors from the native distribution range,

invasive species can reach even higher abundances and body sizes in their new environments compared to their native habitats (Torchin et al., 2002).

The River Rhine, one of the largest and most important waterbodies in Germany and western Europe (Tittizier and Krebs, 1996; Uehlinger et al., 2009), is described as a hotspot for biological invasions due to its economic importance and therefore an extensive anthropogenic influence (Leuven et al., 2009). Starting from degradation of the Rhine in the 19th century, the river was redirected with the aim of better and cheaper transportation of goods, resulting in a well-connected system of European rivers (Leuven et al., 2009). From this starting point, not only ships can move quickly between waterbodies that were previously isolated, but also the flora and fauna of the waterbodies can access new areas by themselves. Carlton and Geller (1993) estimated, that several thousand species are being transported daily in ballast waters around the globe. Like this, species are easily transferred between different water bodies (Ricciardi and MacIsaac, 2000; van der Velde et al., 2002) and even though only a small percentage of these transported species is able to establish themselves, it is a tremendous amount of species day by day.

The inauguration of the Rhine-Danube-Canal is one of the main causes for the increase of invasive species, most of them invertebrates, in the River Rhine (Leuven et al., 2009; Ricciardi and MacIsaac, 2000; Sures et al., 2019). Due to this inauguration, waterbodies of the Ponto-Caspis, which includes the Black Sea, Caspian Sea and Azov Sea, have a direct connection to European waterbodies, which led to a huge number of invasions within the last decades. Since then, species such as the Zebra Mussel (*Dreissena polymorpha*), the killer shrimp (*Dikerogammerus villosus*) and goby species (*Neogobius* spp. or *Ponticola kessleri*) established themselves in the River Rhine (IKSR, 2013). According to Nehring (2003), nearly 20% of invertebrate species in 2003 were non-native in the Rhine river system. Furthermore, the IKSR (International commission for the protection of the River Rhine) reported, that the fish community in the River Rhine in 2013 consisted of ten native and 54 non-native species (IKSR, 2015).

Taking a closer look on invasive species, in many cases they carry parasites, which are therefore co-introduced with their hosts. Similarly to the host, the parasite has to cope with the new habitat and is additionally dependent on the availability of suitable hosts to establish in a new ecosystem. Goedknegt et al. (2016) describe different mechanisms how introduced parasites and their hosts interact with new habitats: e.g. “spillover”, “spillback” and “parasite release”. In the case of spillover

newly introduced parasites find suitable hosts in the conquered ecosystem, so that they can reach high abundances by occupying new microhabitats, especially when they are not yet occupied by other parasite species. An example for spillover is the eel swim bladder nematode *Anguillicola crassus*, which was introduced from Asia into Europe in the early 1980s (Jacoby and Gollock, 2014). This parasite is native to the Japanese eel (*Anguilla japonica*), where adult nematodes are living in the swim bladder. Here, they are mating and releasing the eggs, containing second stage larvae (L2), via the ductus pneumaticus and the feces into the freshwater, where the larvae hatch and are eaten by the intermediate host, a copepod, where they migrate into the haemocoel and molt into the infective third stage larvae (L3). If this copepod is eaten by an eel, the life cycle can be completed, as the larvae can migrate into the lumen of the swim bladder and mate. Alternatively, the infective copepod is eaten by another fish species (paratenic host), where the nematode larvae is not able to develop, but to survive and accumulate in the swim bladder wall. In this case, the life cycle is complete, if the paratenic host is eaten by an eel (De Charleroy et al., 1990; Szekely, 1994). Since *A. crassus* quickly found suitable intermediate hosts and was very successful in infesting the European eel (*Anguilla anguilla*) population, it is a prominent case of spillover. “Spillback” describes a mechanism of host-parasite interaction, where introduced free-living species are suitable hosts for native parasites, leading to an increase of the abundance of native parasites and a change of the local species communities. An example for spillback is the acanthocephalan *Acanthocephalus tumescens*, which is native in Argentina and showed an increased abundance in local hosts after the introduction of non-native rainbow trout (*Oncorhynchus mykiss*) (Kelly et al., 2009; Rauque et al., 2003). The scenario of “parasite release” occurs if a host species benefits from the introduction to a new ecosystem, because its parasites do not cope with the new situation, which often relays in not finding suitable hosts anymore, leading to a decrease and finally total loss of the parasites (Colautti et al., 2004). One example is the European green crab (*Carcinus maenas*), whose population growth in its native range is regulated partly by frequent infestation of the castrating rhizocephalan barnacle *Sacculina carcini*. This castrator is not coping with new environments, which leads to a complete loss of the parasite for invasive green crabs in new habitats. This is one reason, why population sizes in introduces ecosystems are larger, compared to native ones (Torchin et al., 2001).

Another explanation for the distribution of invasive species is the invasional meltdown hypothesis (IMH), which is based on the fact, that two invasive species benefit from each other's establishment (Green et al., 2011; Simberloff and Von Holle, 1999). For example, one nonindigenous species modifies the habitat in a way that another nonindigenous species benefits from the habitat transformation. Even though the IMH is a well-known phenomenon it was only described for free living species, not for parasites so far. However, Emde et al. (2014) described the invasive nematode *A. crassus* inside a cyst of the likewise invasive acanthocephalan *Pomphorhynchus* sp. The authors sampled *N. melanostomus* from the River Rhine, as it is the dominant invasive fish species (Borcherding et al., 2011) and the parasitological examination revealed *Pomphorhynchus* sp. in the mesenteries. The larval acanthocephalans were encysted and some of the cysts contained larvae of the swim bladder nematode *A. crassus* (Emde et al., 2014). *A. crassus* is usually using the swim bladder as a hide out from the immune system, for example in paratenic hosts. However, as gobies lack a swim bladder it seems, that *A. crassus* is using the cyst instead. This co-occurrence of two non-native parasites provided the possibility to examine, if the IMH also applies to these parasites, as the nematode seems to use the already established species *Pomphorhynchus* sp. to gain a better distribution. The interaction of these two parasite species from different phyla with different suitability for the European eel, is subject of chapters one and two of the present thesis.

As described earlier, not only free-living organisms but also their parasites can change local communities. Hohenadler et al. (2019) described the influence of the acanthocephalan *Pomphorhynchus laevis*, introduced as a hidden passenger of gobies from the Ponto-Caspis to the River Rhine system. Briefly, the history of *Pomphorhynchus* spp. in the River Rhine is characterized by many misidentifications between *P. tereticollis*, *P. laevis* and *P. bosnicaus* (Hohenadler et al., 2018; Reier et al., 2019). Whereas *P. tereticollis* is the native species in the River Rhine, *P. laevis* and *P. bosniacus* are invasive ones from the Ponto-Caspis (Nachev et al., 2022). Hohenadler et al. (2018) revealed that the invading species changed the parasite community drastically, by replacing the native species completely, as no *P. tereticollis* was identified in eel samples from German rivers from 2004 onwards. Despite the question which exact species are present in the German Rivers, all species have in common, that they parasitize gammarids as intermediate hosts (Kennedy, 2006). Even though they seem to prefer different species, e.g.

P. bosniacus prefers *Dikerogammerus villosus* and *P. tereticollis* and *P. laevis* prefers *Gammerus pulex* (Vogel and Taraschewski, 2023). As their final host all *Pomphorhynchus* spp. parasitize fish species (Sures et al., 2019), with a preference for barbel (*Barbus* spp.), chub (*Squalius cephalus*) and brown trout (*Salmo trutta*) (Hine and Kennedy, 1974; Kennedy et al., 1978; Nachev et al., 2022; Perrot-Minnot et al., 2019). In European eels, a prevalence of 80% is described, but as *Pomphorhynchus* spp. does not grow properly and does not reach maturity, this fish species is considered as a dead-end host for *Pomphorhynchus* spp. (Thielen et al., 2007).

By altering the community, it is apparent that invaders have an impact on native species. Parasites, in particular, impact their naïve hosts as they often have no defense mechanisms against the new species. Looking more closely at the consequences of parasite infections, it quickly becomes clear that there is no general answer. The range is wide, from nearly not affected at all, to lethal. Furthermore, the severity of the adverse effects on the host depends on its role for the parasite, i.e. if it functions as intermediate or final host, with intermediate hosts usually suffering more (Lucius and Loos-Frank, 2008). The consequences that most people have directly in mind when thinking of parasites are the loss of nutrients consumed by the parasite and the destruction of tissues as the parasite is migrates through different parts of the host body. There are plenty examples where parasite infestation reduces the fitness of their hosts, e.g. by reducing the number of offspring (Lucius and Loos-Frank, 2008). This may not have a huge impact on the individuals themselves, but for the host population it increases the risk of extinction. Furthermore, it is known, that infested individuals are avoided by sexual selection, which decreases the genetic variance of the population. Contrary, in many cases parasites depend on their hosts, so it is a disadvantage for them, if parasites harm their hosts in such a way, that the host dies – of course there are likewise some parasites that do not depend on the host survival, as they already reproduced sufficiently. Still, well-adapted host-parasite-systems, such as *A. crassus* and the Japanese eel, are normally characterized by a long history of coevolution, in which both species can survive in an acceptable way (Lucius and Loos-Frank, 2008). Several studies already showed that consequences of an infestation with *A. crassus* for the native hosts are comparatively minor, but not for the newly conquered host – the European eel. The consequences are a higher parasite load, a thickening of the swim bladder and thus a loss of function so that they cannot reach their

spawning grounds, an increase in stress and thus a suppression of the immune system and a higher susceptibility to other parasites, pathogens, and drugs (Barry et al., 2014; Dangel et al., 2014; Genc et al., 2008; Keppel et al., 2016, 2014; Knopf, 2006; Knopf et al., 2000; Sures and Knopf, 2004; Van Ginneken et al., 2009). Soon after its introduction to Europe, *A. crassus* spread rapidly in the European eel population with abundances of nearly 75% (FAO and ICES, 2007).

To better understand the impact of interactions between invasive parasites, Chapter I and II of the present thesis are both dealing with *A. crassus* larvae inside the acanthocephalan cyst. Whereas Chapter I is focusing on the question whether *A. crassus* is still infectious for the final host, after hiding in the acanthocephalan cyst, Chapter II is focusing on the adaptation in terms of stress response of hosts to infestation with both of these newly acquired parasites. Accordingly, the effect of infestation with *A. crassus* on the new fish host, the European eel, was investigated in comparison with effects on the native fish host, the Japanese eel. Additionally, *Pomphorhynchus* sp., a parasite native to the European eel, was used to infest naïve Japanese eels. To measure the stress response plasma cortisol, which is the main corticosteroid of fish (Mommsen et al., 1999) was used. It plays an important role in various pathways and is measurable with Enzym-Linked-Immuno-Sorbent-Assay (ELISA) with blood from the caudal vein. Plasma cortisol in European eels has already been establish as a viable biomarker by different studies that examined infestations with *Anguillicola* sp., heavy metals, drugs or temperature changes (Dangel et al., 2014; Gollock et al., 2005; Sures et al., 2001; Teles et al., 2004).

When taking a closer look into interactions of *A. crassus* with other species, one close relative appears in the history of *A. crassus* in Europe right at the beginning of its invasion history. Even before the introduction of the Asian swim bladder parasite, *A. novaezelandiae*, which originally parasitizes the swim bladder of the short-finned eel (*A. australis*) from New Zealand, was introduced in Lake Bracciano, Italy. This parasite was able to successfully build a stable but isolated population in the European eel population of the Italian lake. After its introduction to Europe, *A. crassus* also invaded Lake Bracciano and competed with *A. novaezelandiae* as they share the same final host and habitat – the Europeans eels swim bladder. After some years of co-existence, *A. novaezelandiae* was not found in Lake Bracciano anymore. Since today, the mechanism behind the replacement of *A. novaezelandiae* by *A. crassus* is not understood. Hence, different hypotheses were formulated that explain these observations, one of them linked to the more

efficient life cycle of *A. crassus* compared to the one of *A. novaezelandiae* described by Dangel et al. (2013), another hypothesis by Grabner et al. (2012) is focusing on the possibility of hybridization as the main mechanism of replacement. Accordingly, chapter III of the present thesis addresses the phenomenon of hybridization between the two closely related *Anguillicola* species and concentrates on the question of vitality and infectivity of the hybrid offspring and if the hybrid pattern is facing another benefit for *A. crassus* over *A. novaezelandiae*.

With the chosen examples, the present work provides fundamental insights into the behavior of invasive parasites. Current theories on invasion biology are specifically tested on parasites by confirming the invasional meltdown hypothesis for the first time and by investigating the significance of hybridization between closely related species of one genus. The host perspective was also taken into account by studying the stress response of eels experimentally infected with parasites exhibiting different degrees of adaptation to their hosts.

4. Publications

4.1 Chapter I: First evidence for a possible invasional meltdown among invasive fish parasites

Authors: M. A. A. Hohenadler*, **K. I. Honka***, S. Emde, S. Klimpel & B. Sures
*: shared first-authorship

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Contributions		
Conception	0%	Bernd Sures and Sven Klimpel developed the concept, Bernd Sures supervised the study.
Conduction of experimental work	50%	Sebastian Emde provided *N. melanostomus* from River Rhine. Michael Hohenadler conducted pre-experiments. Katrin Honka and Michael Hohenadler prepared the parasites and inoculated the eels. Katrin Honka took care of eels during the experiment and dissected the fish at the end of the experiment.
Data analysis	50%	Katrin Honka prepared all the data for statistical analysis.
Writing the manuscript	50%	Katrin Honka and Michael Hohenadler wrote the draft manuscript. Sebastian Emde provided the pictures.
Revision of the manuscript	30%	Bernd Sures, Sven Klimpel and Sebastian Emde revised the manuscript and Katrin Honka and Michael Hohenadler enhanced the manuscript accordingly. All authors approved the final manuscript.

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Signature of the Doctoral Candidate Signature of the Doctoral Supervisor

First evidence for a possible invasional meltdown among invasive fish parasites

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Biological invasions are frequently studied topics in ecological research. Unfortunately, within invasion ecology parasite-associated aspects such as parasite impacts on new environments and on local host populations are less well-studied. Round gobies migrating from the Ponto-Caspian region into the Rhine River system are heavily infested with the Ponto-Caspian acanthocephalan parasite *Pomphorhynchus laevis*. As shown by experimental infestations the acanthocephalans occur as pre-adults in host-encapsulated cysts within the internal organs of the migrating gobies, but remain infective for their definitive host chub. Recently, we described the occurrence of larvae of another parasite, the invasive eel swim bladder nematode *Anguillicola crassus*, in these *Pomphorhynchus* cysts. In the present study, we could prove the infectivity of the nematode larvae for European eels for the first time. After experimental inoculation of *Pomphorhynchus* cysts occasionally infested with *A. crassus* larvae, the nematodes grow to maturity and reproduce whereas all *P. laevis* were unviable. We therefore postulate that the nematode larvae behave like immunological hitchhikers that follow a "Trojan horse strategy" in order to avoid the paratenic host's immune response. Accordingly, the interaction between both invasive parasites gives first evidence that the invasional meltdown hypothesis may also apply to parasites.

Introduction

Invasion of free-living organisms and their effects on new habitats has emerged as a major threat for ecosystems around the globe, partly with irreversible consequences for the local biota. Invasive species might cause habitat modification, extinctions of endemic species, affect human health, and therefore engender enormous economic costs[1–3]. However, not every newly introduced species will be able to establish itself in a new habitat[4]. Success rates depend on different biotic and abiotic conditions such as absence/presence of enemies, competition with local species for resources, and climatic conditions[5-7]. Besides these aspects, the occurrence of other invasive species is one of the most substantial factors for invasion success. The so-called invasional meltdown hypothesis (IMH) states that if several new species invade the same habitat, they usually facilitate each other's establishment since one species might serve e.g. as food or energy resource for another, which initiate its invasion process[8,9]. This might result in an increased rate of invasion, leading to crucial impacts within the new habitat[10]. In this context it seems surprising that alien parasites, although generally co-introduced to new environments with invasive host species[11-14], are often not taken into account when evaluating the effects and mechanisms of invasion. This is even more surprising as parasites are considered an important response variable for ecosystem health[15-17]. Although the IMH did not show any significant differences among taxonomic groups that have been studied yet[18] it remains unclear if it also applies to nonindigenous parasites.

In order to be able to invade a new habitat parasites usually depend on free-living alien hosts[19,20]. Therefore, the presence of a sufficient number of free-living invasive species is an obligate prerequisite for the establishment of non-indigenous parasites. Nevertheless, the question whether a certain parasite species also benefits from the occurrence of other invasive parasites remains to be unanswered. The Rhine River, a Western European river is considered a hot spot for biological invasion, and thus might be an ideal system to study the relevance of the IMH for invasive parasites[21,22]. Although many nonindigenous species were able to establish in the Rhine River over the past decades, invaders from water bodies of the Ponto-Caspian steppe were among the most successful[22]. Species

such as the amphipod *Dikerogammarus villosus* or the fish species *Neogobius melanostomus* or *Ponticola kessleri* usually become dominant species in newly invaded areas due to their invasion strategy that provide them with competition advantages against local species[23,24]. Recent research has shown that both the amphipods as well as the fish species introduce the acanthocephalan *Pomphorhynchus laevis* to the river Rhine since the mid 1990's after the inauguration of the Main-Danube-Canal[25]. Subsequently, the parasite spread rapidly and successfully established itself along the river Rhine, showing a high prevalence in cyprinid fishes as well as in predators that feed on infected intermediate host species[26,27]. After a potential paratenic host ingests pre-adult individuals of *P. laevis*, a cyst will be formed by both the hosts' immune response and the parasite itself. Such parasite stages thus occur encapsulated in the hosts' internal organs as well as in its body cavity[28-30]. Infection experiments with chub, *Squalius cephalus*, have demonstrated that encapsulation does not have any apparent effect on the parasite since it remains infective for its definitive host (unpublished data). Recent research has also shown that cysts of *P. laevis* in *N. melanostomus* may contain larvae of another invasive parasite species, *Anguillicola crassus*[31]. This nematode causes severe health impacts for the native eel species in Europe[32,33]. Initially it was co-introduced with Japanese eels (*Anguilla japonica)* to European waterbodies in the early 1980's [34]. Shortly after its arrival, *A. crassus* adapted to local environmental conditions and accepted the European eel (*Anguilla anguilla*) as its suitable final host. Within a short period, the infestation rates of *A. crassus* in *A. anguilla* increased to more than 90 % in large parts of Western and Central Europe (e.g.[33,35-37]). The nematode parasitizes the swim bladder of its final host after undergoing different development stages by using a wide variety of species as intermediate and paratenic hosts[38]. Accordingly, the eel's swim bladder is frequently affected to a significant extent, leading to a reduced functionality, which might result in the host's death during its spawning migration from the European coast to the Sargasso Sea[39]. In fact, *A. crassus* is also held partly responsible for the massive decline of the overall stock of European eel that resulted in its occurrence on the list of critically endangered species by the International Union for Conservation of Nature[40,41].

The fact that individuals of *A. crassus* utilize cysts of encapsulated *P. laevis* individuals provides evidence that establishment of a parasite species might have been facilitated by the arrival of another invasive parasite within the Rhine River. Hyperparasitized cysts – what in detail describes acanthocephalan cysts that were simultaneously infested by *P. laevis* and *A. crassus* - which were gathered from *N. melanostomus* individuals from the Rhine River demonstrated that *A. crassus* frequently enters the cyst most likely to avoid immune responses of the paratenic host. Generally, third-stage larvae (L3) of *A. crassus* evoke an immune response of their paratenic hosts, with diversified intensities among the various host species, which might cause the parasites' death[31]. Recently, it was suggested that *A. crassus* might use the cyst as a "hideout" to evade the immune response of the round goby, which might serve as prey for *A. anguilla*, the parasites' main definitive host. Therefore, the nematode larvae are protected from host defenses while being in the goby. Theoretically, with such a "Trojan horse" strategy the parasite could be able to infest the hosts' swim bladder more readily. However, it is still unknown whether *A. crassus* is still infectious for the definitive host after entering the acanthocephalan cyst. If yes, this could be seen as support that the IMH also applies to nonindigenous parasites. In order to test the viability and infectivity of encapsulated *A. crassus* larvae, we therefore conducted an infection experiment where European eels were inoculated with cysts collected from Ponto-Caspian gobies.

Results

The initial screening of cysts removed from *N. melanostomus* (*cf.* figure 1) showed a prevalence of 12 % of *A. crassus* larvae within the cysts. In all 200 cysts 96 larvae of *A. crassus* were detected, with a mean intensity of four nematodes per cyst (ranging between one to twelve larvae per cyst). Individuals of *P. laevis* found in the cysts were alive and showed a normal activity level.

Eels administered with intact cysts showed a prevalence with *A. crassus* of 40 % 154 days post infection (dpi). While two eels were found to be infested by an individual *A. crassus* (either male or female) each,

two eels showed a double infestation. In one eel two females occurred, whereas a pair of both sexes containing eggs with L2 larvae was detected in the second eel. In sum, 164 cysts were administered to the eels, which corresponds to a total of 79 *A. crassus* when considering the results of the initial cyst screening. Based on these results, the recovery rate can be determined as 7.6 %. The size of the *A. crassus* individuals found in the eels corresponds with the developmental period of 154 days when compared with previous infection experiments[42,43]. Further parasitological examination of the eels did not show any infection with *P. laevis* in the experimental group. Eels of the uninfected control did not contain any individual of either parasite species.

Discussion

The present study demonstrates for the first time that larvae of *A. crassus*, enclosed in the cysts of encapsulated *P. laevis*, remain able to infest their definitive host, the European eel. The experiment showed that *A. crassus* is still able to complete its life-cycle and produce offspring after entering the cysts in a potential paratenic host. Moreover, as the invasive nematode larvae use the cyst of an invasive acanthocephalan parasite species, the invasional meltdown hypothesis is supported.

Parasitological examination of the eels revealed a prevalence of 40 % of *A. crassus* with a recovery rate of 7.6 %. Previous experiments with eels using isolated L3 of *A. crassus* under similar conditions showed generally higher recovery rates of up to 40 %[42,43]. Apart from the fact that the number of introduced *A. crassus* larvae in the present study can only be estimated as an average value and not using exact data, the relatively low infestation rate might also be related to this, so far unknown, way of transmission of *A. crassus*. It was demonstarted that encapsulation might be a barrier for some parasites in order to establish themselves after beeing transmitted to a new host[44]. As implied by the relatively low prevalence and recovery such a barrier effect might also apply for *A. crassus*. Nonetheless, the use of cysts containing encapsulated *P. laevis* in fish lacking a swim bladder represents an additional way of transmission to the preferred final host for *A. crassus*.

The results demonstrate the infectivity of *A. crassus* individuals from cysts co-infected with *P. laevis.* Thus, *A. crassus* was able to develop to mature adults whereas no individual of *P. laevis* was detected inside the eels at the end of the experiment although *P.laevis* is regularly found in eels from the Rhine River[25]. This is a striking result since encapsulated *P. laevis* that were ingested by their preferred definitive hosts such as *S. cephalus* and *Barbus barbus* are able to mature[28,44,45], which was also confirmed by additional infection experiments in which encapsulated *Pomphorhynchus* individuals developed to full maturity after beeing infested to individuals of *S. cephalus* (unpublished data). The lack of any *P. laevis* in the examined eels after 154 dpi might therefore be related to the following reasons. On the one hand, the European eel as a non preferred host was used for laboratory infestation experiments. Even if *P. laevis* can regularly be found in eels in the field[21] this might be a result of eels ingesting cystacanths from the first intermediate host, i.e. different species of amphipods and not by feeding on paratenic hosts. On the other hand, it is also conceivable that the lifetime of *P. laevis* in its non-preferred hosts is shorter than the time of seven to eight months estimated for this species in their preferred definitve hosts[46]. In the latter case, the acanthocephalans might have already been shed from the eels after 154 dpi. However, during daily inspections, no acanthocephalans were recovered in the tanks.

Both parasites have been described as succsessful invadors in European waterbodies and have been intensively studied during the past decades[47-49]. Nonetheless, a relation or possible interaction between the two invasive parasites was only discovered recently[31]. The reason might be that usually *P. laevis* is carefully removed from the cysts and then further examined while the tissue of the cyst is treated as waste material. Simultaneously, the larvae of *A. crassus* are not recognized since they are hardly seen by bare eye. Accordingly, the parasite has always been overlooked prior to the preliminary field study by Emde et al.[31]. Furthermore, we assume that if individuals of *A. crassus* have already been detected in gobies before, their exact localization (in the cysts) was not recognized. However, in the context of these findings and the results of the present study we assume that *P. laevis* might facilitate *A. crassus'* establishment and distribution in a new environment. This corresponds to the invasional

meltdown hypothesis (IMH), which has never been described for invasive parasites before, although interactions of free-living invasive species are already referred to as a major aspect of biological invasion[9,18,50]. The IMH states that the arrival of nonindigenous species in an environment facilitates the establishment of other invasive species[8]. The fact that both parasites were able to establish themselves successfully in environments that are recognized as hotspots for invasion, such as the river Rhine, and the fact that *A. crassus* seems to benefit from the presence of encapsulated invasive parasites supports the assumption that the IMH also applies to invasive parasites.

Although *A. crassus* larvae utilize cysts and thereby eventually avoid the paratenic host's immune response (of e.g. *N. melanostomus*) this could also be a side effect associated to the fact that gobies lack a swim bladder. It is already known that *A. crassus* larvae can be found in many different tissues of paratenic hosts[51-54]. The idea that the parasite uses a "Trojan horse strategy" was firstly mentioned in 2014[31]. Although the present results do not directly support a trojan horse strategy as no immunological responses were analysed, they show that *A. crassus* benefits from the presence of the cysts of encapsulated *P. laevis* individuals as it represents an additional way of infecting the definitive host. Obviously, the distribution and establishment of *A. crassus* is (at least partly) facilitated by another invasive parasite that consequently turned a possible dead-end host into a paratenic host in order to increase the nematodes' infestation success. As there are not many other fish species described in which *P. laevis* occurs in cysts[29,55], the particular type of co-occurrence of both parasites that is described here is only known for gobies.

The fact that both parasite species have been studied intensively over the past decades but their interaction was only discovered recently demonstrates the necessity of future research on possible interactions between (invasive) parasites in order to evaluate the effects of parasites invasion on local biota.

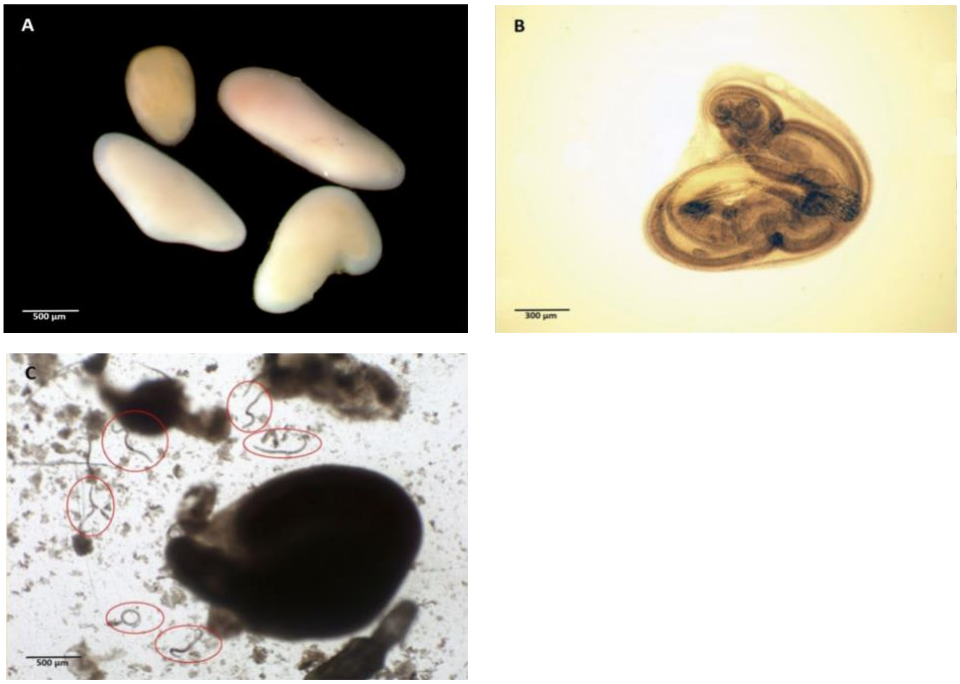
Methods

A total of 22 individuals of the invasive goby *Neogobius melanostomus* were collected by professional fishermen with bow nets in the River Rhine close to the city of Grieth at Rhine km 844 (North Rhine Westfalia, Germany). Within two days after sampling, all fish were sacrificed and examined for the presence of acanthocephalans of the genus *Pomphorhynchus*, which were discovered encapsulated in the abdominal cavities of the fishes. All encapsulated *P. laevis* individuals (n = 364) were stored in a 0.9 % sodium-chloride (saline) solution at 5 °C.

200 isolated cysts were transferred one by one into a well-plate chamber to check whether cysts were infested by *Anguillicola crassus*. Wells were filled up with artificial stomach acid-solution, composed of 1 % hydrochloric acid and Pepsin (0.5 g per 100 ml)[56]. Filled-up well plates were incubated for 40 minutes at 37 °C to induce cysts to break open and allow parasites to be released and eventually found free in the solution (*cf.* figure 1C). After the incubation time, the content of each chamber was carefully examined in order to determine whether cysts were infested by *A. crassus* and if so to what extent. The mean infestation rate (number of *A. crassus* per cyst of *P. laevis*) was calculated as 0.48, which was then used as a basis for subsequent infection experiments with European eels. We infested ten European eels (mean size of 426 mm) that were provided by a commercial eel farm known to be free of any infestation with *A. crassus* and/or *Pomphorhynchus* sp. with the remaining cysts (n = 164). Apart from a longstanding cooperation with the eel farm, eels are regularly checked by parasitological examinations to verify absence of *A. crassus* as well as of any other endoparasites. A total of 16 to 18 cysts (resembling approximately 7.7 to 8.6 *A. crassus*) were manually administered to each eel by a stomach tube (diameter of 0.5 mm). Following infection, the eels were kept individually in a single water tank (30 l) at a water temperature between 10 and 13°C with permanent air supply. An uninfected control group of five eels (mean size of 464 mm) was kept under the same experimental conditions to verify that the eels were free of parasites. The eels were killed and examined for parasites 154 days post infection (dpi). Internal organs were removed and digestive tracts and swim bladders

were carefully examined under a stereomicroscope for the presence of *A. crassus* and *P. laevis*. Individuals of *A. crassus* were subsequently categorized according to their developmental stage and sex.

All experimental protocols were approved by the Ethics Council (Landesamt für Natur, Umwelt und Verbraucherschutz, Nordrhein-Westfalen, permit number: 84-02.04.2017.A245) and were carried out in accordance with the relevant guidelines and regulations.



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219 Figure 1: A) Cysts of encapsulated *P. laevis* individuals as detected and removed from the digestive
220 tracts of *N. melanostomus* B) Encapsulated *P. laevis* irradiated with high light intensity C) Digested cyst
221 with released *A. crassus* individuals

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Author Contributions Statement

BS and SK conceived the study and supervised the project. MAAH and KIH conducted the experiments and wrote the manuscript. SE collected infected gobies. BS, SK and SE oversaw the writing and reviewed the manuscript.

Additional Information

The authors declare no competing interests.

Data availability statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

All experimental protocols were approved by the Ethics Council (Landesamt für Natur, Umwelt und Verbraucherschutz, Nordrhein-Westfalen, permit number: 84-02.04.2017.A245) and were carried out in accordance with the relevant guidelines and regulations.

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377

4.2 Chapter II: Mutual adaptations between hosts and parasites determine stress levels in eels

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Contributions		
Conception	50%	Bernd Sures and Katrin Honka developed the concept.
Conduction of experimental work	90%	Katrin Honka and Michael Hohenadler inoculated the eels. Katrin Honka draw blood at all time points and dissected the eels at the end of the experiment
Data analysis	100%	Katrin Honka prepared and conduced all the data for statistical analysis.
Writing the manuscript	100%	Katrin Honka wrote the draft manuscript including the preparation of pictures and tables.
Revision of the manuscript	50%	Bernd Sures revised the manuscript and Katrin Honka amended the manuscript accordingly. Both authors approved the final manuscript.

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Signature of the Doctoral Candidate Signature of the Doctoral Supervisor

Mutual adaptations between hosts and parasites determine stress levels in eels

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Abstract

Invasive parasites may severely affect their new hosts. Two invasive parasites occurring in the European eel (*Anguilla anguilla*) are the Asian swim bladder nematode *Anguillicola crassus* and the Ponto-caspian acanthocephalan *Pomphorhynchus* sp., which were introduced to the river Rhine in the early 1980/90s. The Japanese eel (*Anguilla japonica*), as the native host of *A. crassus*, developed mutual adaptations to the swim bladder parasite, which are lacking in the European eel. Therefore, after its spread to Europe, infestations of European eels with *A. crassus* were found to be more severe and caused massive swim bladder wall damages mainly due to the feeding activity of the adult nematodes. A suppression of the immune system also appears to be likely, which allows secondary infections e.g. by bacteria or other parasites in European eels. Acanthocephalans of the genus *Pomphorhynchus* have not been described so far in Japanese eels, in contrast to European eels, which regularly show infestations with *Pomphorhynchus* sp.. By using these differentially adapted host-parasite associations for experimental studies, host stress responses were examined in the present study in relation to the degree of mutual adaptations between eel hosts and parasites.

Under laboratory conditions, Japanese and European eels were each inoculated with *A. crassus* and *Pomphorhynchus* sp., respectively, to investigate their stress responses against differently adapted parasites. The stress response was determined by analyzing plasma levels of cortisol, which is the main corticosteroid hormone during stress response of fish. The results show a strong cortisol release in European eels after infestation with *A. crassus* whereas Japanese eels only react against *Pomphorhynchus* sp. infestations. These results are consistent with the initial hypothesis that a low degree of host-parasite adaptations lead to stronger host stress responses against the parasite.

Keywords: cortisol, stress response, *Anguilla anguilla*, *Anguilla japonica*, *Anguillicola crassus*, *Pomphorhynchus* sp.

1. Introduction

Ecosystems are dynamic habitats with a variety of abiotic and biotic interactions. Among biotic components, invasive species often negatively affect native communities and change interactions within ecosystems (Simberloff and Rejmanek 2011). Although such invasive species are attaining increasing interest, their parasites are often neglected, even if co-introduced parasites affect new habitats occasionally to a greater extent than their hosts (Ballari et al., 2016; Keppel et al., 2014; Lymbery et al., 2014). However, invasive parasites do not only affect native hosts, but parasite communities can also be changed (Hatcher and Dunn, 2011; Hohenadler et al., 2019). Recently, two invasive parasites, *Anguillicola crassus* (Kuwahara, Niimi and Hagaki, 1974) and *Pomphorhynchus* sp. Monticelli, 1905, were shown to influence each other's establishment, supporting the invasion meltdown hypothesis that also applies to parasites (Hohenadler et al., 2018a). Specifically, the nematode *A. crassus* utilizes encapsulated cysts of the acanthocephalan *Pomphorhynchus* sp. as a hideout in a paratenic host (*Neogobius melanostomus* (Pallas, 1914)) to escape the fish' immune response (Emde et al., 2014). Thereby *A. crassus* exploits an alternative way of infesting the European eel (*Anguilla anguilla* (Linnaeus, 1758)) as the definitive host (Hohenadler et al., 2018a).

The swim bladder nematode *A. crassus* was introduced to Europe in the early 1980s and ever since spread rapidly over the European eel population (Hartmann, 1993; Koops and Hartmann, 1989) and a few years later through American and African eel populations likewise (Barse et al., 2001; Sasal et al., 2008). This parasite is characterized by a long-lasting history of mutual adaptations with its native definitive host, the Japanese eel (*Anguilla japonica* Temmick and Schlegel 1846), while all the other *Anguilla* spp., such as the European eel can be considered as non-adapted final hosts (Taraschewski, 2006). The long-lasting co-adaptations between Japanese eels and *A. crassus* lead to an effective immune response keeping the numbers of successfully establishing nematodes low. Moreover, almost no physiological consequences following an infestation in Japanese eels are known (Dangel et al., 2015; Keppel et al., 2014). Nevertheless, mutual adaptations allow the nematode to successfully infest its original host (Keppel et al., 2016). As these co-adaptations are missing in the European eel, the

pathogenicity of *A. crassus* infestations in the new host is more severe and accompanied by a less effective immune defense of the host (Keppel et al., 2014; Knopf, 2006). One indication of the physiological imbalance within Japanese and European eels with *A. crassus* might be their stress response, which can be determined by analyzing their plasma cortisol concentrations after infestations with *A. crassus*. According to the current state of knowledge, infestations with *A. crassus* induce acute stress in European eels, throughout larval and young adult stages, in the form of increased plasma cortisol levels (Dangel et al., 2014; Sures et al., 2001). In contrast to laboratory infestations (Keppel et al., 2014; Sures et al., 2001), no cortisol increase for wild infested eels have been detected, which might be due to the fact that the duration of the infestation is unknown (Kelly et al., 2000). Consequences of increased cortisol levels are diverse: on short term it up-regulates the energy mobilization such as gluconeogenesis, on long term it down-regulates pathways, which cost a lot of energy, such as growth, reproduction and immune functions (Faught and Vijayan, 2016). These processes in turn might lead to higher susceptibility for secondary infections such as viruses, bacteria and other parasites (Sures, 2001; Sures et al., 2006).

Even though it is known that eels are unsuitable definitive hosts for species of the genus *Pomphorhynchus*, a high prevalence of immature acanthocephalans is usually found in eels (Thielen et al., 2007). *Pomphorhynchus* spp. prefer barbel (*Barbus barbus* (Linnaeus, 1758)) or chub (*Squalius cephalus* (Linnaeus, 1758)) as final hosts and do not mature in the European eel, which is therefore considered a dead-end host for these parasites (Sures et al., 2019). However, cystacanths of *Pomphorhynchus* sp. occur in the round goby (*Neogobius melanostomus*), which might be used as a paratenic host for the acanthocephalan. In addition, part of these *Pomphorhynchus* sp. cystacanths were found to harbor *A. crassus* larvae (Emde et al., 2014) allowing for a successful transmission of *A. crassus* to the European eel (Hohenadler et al., 2018a). The exact species of *Pomphorhynchus* sp. appearing in eels in the River Rhine remains unclear due to morphological similarities between *P. tereticollis* (Rudolphi, 1809), *P. laevis* (Zoega in Müller, 1776) and *P. bosniacus* Kiskároly and Čanković, 1967 which have frequently been misidentified (Emde et al., 2012; Hohenadler et al., 2018b; Reier et al., 2019). Nevertheless, species of the genus *Pomphorhynchus*, most likely *P. tereticollis* (Hohenadler

et al., 2018b), are known for a long period to be present in eel populations (Sures et al., 1999; Thielen et al., 2007) but have most likely been replaced during the last 20 to 30 years (Sures et al., 2019). Hence, it seems reasonable that European eels might have developed adaptations to cope with infestations with this acanthocephalan genus to a greater extent than to infestations with *A. crassus*. Compared to European eels, no records of *Pomphorhynchus* sp. in Japanese eels are known (Amin et al., 2007; Katahira and Nagasawa, 2014; Leidy and Van Cleave, 1924; Van Cleave, 1925), which can therefore be considered as a presumably naïve system.

Since it appears that mutual adaptations of hosts and their parasites can be reflected in the stress response, we aimed to test the hypothesis that the stress response of eels against non-adapted parasites is higher than against adapted ones. We tested this hypothesis using the following differently adapted host-parasite systems: 1. Japanese eel and *A. crassus* (well-balanced mutual adaptations due to a long lasting history); 2. European eel and *Pomphorhynchus* sp. (presumably established mutual adaptations based on a rather long period of co-occurrence); 3. European eel and *A. crassus* (presumably weak mutual adaptations due to the recent invasion of *A. crassus* 40 years ago); and 4. Japanese eel and *Pomphorhynchus* sp. (no mutual adaptations due to missing co-occurrence).

2. Materials and Methods

2.1 Experimental design

To characterize the stress response of eels experimentally inoculated with *A. crassus* and *Pomphorhynchus* sp., fish were divided into six groups of ten individuals each (Figure 1). One group of each eel species remained un-inoculated and served as a control, one group of each eel species was inoculated with 15 third stage larvae (L3) of *A. crassus* per eel, and the last group of each eel species was inoculated with 16-18 acanthocephalan cysts, partly containing *A. crassus* (see Hohenadler et al., 2018a). Both, the isolated L3 as well as the cysts were administered by a stomach tube to each eel. Therefore, eels were gently wrapped in a well-soaked cloth, and the respective number of larvae or

cysts was administered by a stomach tube (1.5 mm diameter; B. Braun Melsungen AG, Melsungen, Germany) as described by Sures and Knopf (2004). Following inoculation, eels were maintained for 154 days in individual, aerated 30 l tanks using a flow through system and fed twice a week with eel pellets (DAN-EX 2848, BioMar A/S, Brande, Denmark) until parasitological examination. Plastic tubes served as hiding places in every tank.

2.2 Animal Source

European and Japanese eels were obtained from eel farms known to be free of *A. crassus* (Albe Fischfarm, Haren/Rütenbrock, Germany and Omori-Tansui Co., Ltd., Miyazaki, Japan). To verify the absence of any endoparasites, initially ten eels of each species were randomly chosen, killed, dissected and screened with light microscopy for parasites. For experimental infestation of eels, eggs containing second stage larvae (L2) of *A. crassus* were collected from European eels caught by fishermen from the River Rhine. Development to the L3 stage was performed by offering copepods (*Macrocyclops albidus*) freshly hatched L2, cultured in the lab. Cysts of the acanthocephalan *Pomphorhynchus* sp. were collected from naturally infested invasive gobies (*Neogobius melanostomus*) provided by a professional fishermen as described in Hohenadler et al. (2018a). All experimental protocols were approved by the Ethics Council (Landesamt für Natur, Umwelt und Verbraucherschutz, Nordrhein-Westfalen, Germany, permit number: 84-02.04.2017.A245) and were carried out in accordance with the relevant guidelines and regulations.

2.3 Cortisol analyses

In order to measure plasma cortisol levels, blood samples of 150 µl were drawn from the caudal vein of each eel at 0, 28, 42, 70, 98 and 154 days post infestation (dpi). Eels were not sedated, considering that it took a maximum of 40 seconds between netting, drawing blood and transferring them back to

the tank. If blood drawing was impossible within this time frame, eels were transferred back and no sample was taken at this occasion. Blood samples were allowed to clot for two hours at room temperature and then centrifuged for 10 min at 5.000 g to separate serum from other blood parts. Only serum samples were frozen at -80°C until further examination. Analyses of cortisol in eel sera were performed according to the manufacturer's instructions by an enzyme linked immunosorbent assay (Cortisol ELISA RE 52611, IBL International GmbH, Germany). Samples were transferred to microtiter plates coated with rabbit anti-cortisol antibodies. After the coloring reaction, optical density was measured at λ=450 nm on a microplate reader (Tecan, infinite M200). Samples were measured in triplicates.

2.4 Statistical Analysis

Graphs were plotted with Graphpad prism Version 8.4.1. Outliers have been removed by performing the ROUT test with Q=1%. One individual of the control group of European eel had more than 50% of time points removed, so the complete individual was removed. In the groups of inoculation with L3-larvae only eels with *A. crassus* infestation were considered. In the groups of inoculation with *Pomphorhynchus* sp. cysts, all eels were considered regardless the underlying infestation with *A. crassus*.

3. Results

At the end of the experiment, direct administration of L3 of *A. crassus* resulted in a higher infestation rate in European eels than in Japanese eels (Table 1). Individuals of *Pomphorhynchus* sp. were not found in either eel species inoculated with the acanthocephalan cysts. However, individuals of *A.*

crassus were identified in four eels of each species following administration of acanthocephalan cysts. Control groups of both eel species were free of *Pomphorhynchus* sp. and *A. crassus*. Details of the parasitological examination are shown in table 1.

To check the stress response, plasma cortisol levels of inoculated and untreated control eels were measured and the results are shown in figure 2. The mean of serum cortisol concentration of European eels infested with L3 of *A. crassus* increased from 3.5 ± 1.6 ng/ml plasma cortisol at 0 dpi to 11.2 ± 5.4 ng/ml at 28 dpi. European eels inoculated with acanthocephalan cysts showed an increase of serum cortisol concentration from 3.5 ± 1.5 ng/ml at 0 dpi to 7.2 ± 7.1 ng/ml at 14 dpi. The group of Japanese eels infested with *A. crassus* shows an initial plasma cortisol level of 3.3 ± 0.9 ng/ml and an increase to 5.3 ± 1.2 ng/ml at 28 dpi. In the group inoculated with *Pomphorhynchus* sp. the plasma cortisol concentration was initially at 2.7 ± 0.9 ng/ml and increased to 9.5 ± 10.3 ng/ml at 14 dpi. The cortisol levels of un-inoculated control eels stayed constant during the whole experiment with a mean of 3.9 ± 2.5 ng/ml for European eels and 3.2 ± 1.9 ng/ml for Japanese eels. From 70 dpi onwards, cortisol levels of all groups of both eel species ranged in a similar range. Plasma cortisol concentrations for both eel species after inoculation with *Pomphorhynchus* sp. cysts were independent of a subsequent successful establishment of *A. crassus*.

4. Discussion

The present results clearly support our hypothesis that mutual adapted host-parasite systems are characterized by a lower stress level of the hosts compared to rather new host-parasite associations. The response of both eel species to infestations with *Pomphorhynchus* sp. cysts have not been investigated so far, whereas the cortisol values of both, the Japanese and the European eel, infested with *A. crassus* determined during the present study confirm previous results (Dangel et al., 2014; Sures et al., 2001). Taking a closer look on the results, all groups of the Japanese eel, including the un-

192 inoculated control group, had a low starting value of plasma cortisol. The well adapted system of
193 Japanese eels with *A. crassus,* showed only slightly increased plasma cortisol concentration after
194 infestations with this parasite. In comparison to that, the group inoculated with *Pomphorhynchus* sp.,
195 which represents the group with presumably no mutual adaptations, showed the strongest cortisol
196 increase by more than threefold compared to the initial plasma cortisol concentrations at 14 dpi.
197 Comparing this to the results of European eels, an opposite pattern is evident. European eels started
198 with a slightly higher mean cortisol concentration compared to Japanese eels. In the group of
199 presumably weak adaptations with *A. crassus*, an almost three times higher cortisol release was
200 detected at 28 dpi, whereas in the presumably established system with *Pomphorhynchus* sp. the
201 cortisol concentration doubled at 14 dpi but then decreased again before remaining approximately at
202 the cortisol level of the un-inoculated control eels. No effects of the *A. crassus* larvae encapsulated in
203 the cysts of *Pomphorhynchus* sp. were detected on cortisol concentrations in any of the eel species.
204 Accordingly, the idea "that the nematode larvae behave like immunological hitchhikers that follow a
205 Trojan horse strategy in order to avoid the paratenic host's immune response" (Hohenadler et al.,
206 2018a) obviously also applies to other physiological processes, i.e. in avoiding a stress response by the
207 host. The high standard deviations obtained at some days can most likely be attributed to individual
208 differences, which were also described earlier (Dangel et al., 2014; Silva et al., 2018; Sures et al., 2006).
209 However, despite a comparably high SD, clear patterns emerge, suggesting a stress response for less
210 mutually adapted host-parasite systems.

211 Consequences of increased cortisol levels as shown here are diverse. On short term cortisol up-
212 regulates the energy mobilization such as gluconeogenesis, on long term it down-regulates pathways
213 which cost a lot of energy, such as growth, reproduction and immune functions (Faught and Vijayan,
214 2016). Consequences of *A. crassus* infestations on eels in general are well known; various data reveal
215 an influence on the swim bladder function, an increase of stress parameters and therefore a decrease
216 of the eel's immune system, which makes the eels more susceptible to secondary infections with
217 viruses, bacteria or other parasites (Barry et al., 2014; Kennedy, 2007; Kirk, 2003; Schneebauer et al.,

2017; Würtz et al., 1996; Würtz and Taraschewski, 2000). Therefore, some of the known consequences of *A. crassus* infestations in European eels might be related to increased cortisol expression (Sures et al., 2006, 2001).

Whereas *A. crassus* is a highly specific eel parasite, the eel is considered a dead-end host for *Pomphorhynchus* sp. - even though the acanthocephalan is able to establish and start growing in this fish species (Hohenadler et al., 2018b). To the best of our knowledge, consequences of *Pomphorhynchus* sp. infestations to either of the eel species were never investigated. Furthermore, the appearance of *Pomphorhynchus* sp. was only described in European eels, but never for Japanese eels (Katahira and Nagasawa, 2014; Nagasawa and Katahira, 2017; Thielen et al., 2004). Van Cleave described a single appearance of *Acanthocephalus gotoi* sp. Van Cleave, 1925 in Japanese eels from fish markets, nearly 100 years ago (Van Cleave, 1925). More recent studies described acanthocephalan infestations of giant mottled eels (*Anguilla marmorata* Quoy and Gaimard, 1824) from Japan (Katahira and Nagasawa, 2014) and infestations of the Japanese eel with a single individual of *Echinorhynchus cotti* Yamaguti, 1939 and *Pseudorhadinorhynchus samegaiensis* Nakajima and Egusa, 1975 (Amin et al., 2007). In European eels, some acanthocephalan species are known to be host-specific such as *Acanthocephalus anguillae* (Müller, 1780) and *Paratenuisentis ambiguus* (Van Cleave, 1921), as they also mature in European eels (Kennedy, 2006; Taraschewski et al., 1987). Species of the genus *Pomphorhynchus* can survive in European eels, where they are commonly found, but as they never reach maturity, eels are not a suitable definitive host for this parasite (Bates and Kennedy, 1991; Thielen et al., 2004).

The cortisol release of Japanese eels to infestations with the acanthocephalan cysts and the European eel to infestations with *A. crassus* indicates that both eel species showed similar stress responses to unknown parasites. In contrast, slightly or fully adapted parasites do not influence the cortisol response of their hosts. The pattern of a lower cortisol release for better adapted host-parasite-systems has also been observed for other species. For example, the ectoparasite *Caligus rogercresseyi* Boxshall and Bravo, 2000 affects the Chilean salmon industry as it infests primarily Atlantic salmon

(*Salmo salar* Linnaeus, 1758), but not Coho salmon (*Oncorhynchus kisutch* (Walbaum, 1792)), which appears to be immune to infestation by this crustacean (Valenzuela-Muñoz et al., 2016). Comparative studies of these host-parasite systems also demonstrated that the better adapted *O. kisutch* has a considerably lower cortisol release than the less well adapted *S. salar* following infestation with *C. rogercresseyi* (Vargas-Chacoff et al., 2019).

The results of the present study as well as of previous investigations on salmon parasites suggests that the stress response of the host can be used to indicate differently adapted host-parasite systems. The well-balanced mutual adaptations between the Japanese eel and *A. crassus* do not lead to a measureable stress response of the host following inoculation with the parasite. In contrast, the cortisol response of the Japanese eel to inoculation with *Pomphorhynchus* sp. was the highest cortisol release during the experiment, which might indicate a complete lack of adaptation due to missing co-occurrence under natural conditions. The cortisol response of the European eel - as well as their degree of adaptation with the parasites chosen - ranges between that of the Japanese eel. Since nearly 40 years, *A. crassus* and the European eel co-occur in the River Rhine. This period might not be enough to lead to a mutual adaptation as can be seen by a rather strong cortisol release. Even if European eels are not suitable final host for *Pomphorhynchus* sp. they do have a long history of co-occurrence with some species such as *P. tereticollis* (Hohenadler et al., 2018b; Sures et al., 2019) what might provide them with some adaptations, which is also reflected in a relatively weak cortisol release compared to *A. crassus* infestations.

Conclusions

Invasive species must adapt to their newly conquered ecosystem - the same is valid for parasites and their new hosts. Mutual adaptations determine the success of the invasion process. Following inoculation of eel hosts with parasite larvae, the cortisol release relates negatively to the degree of

adaptation. Specifically, highly adapted systems such as the Japanese eel with *A. crassus* showed no cortisol response in contrast to systems with no adaptation at all, such as the Japanese eel following inoculation with cystacanths of *Pomphorhynchus* sp., which showed by far the strongest cortisol response of all investigated host-parasite systems investigated within this study.

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395

396

Figures and Legends

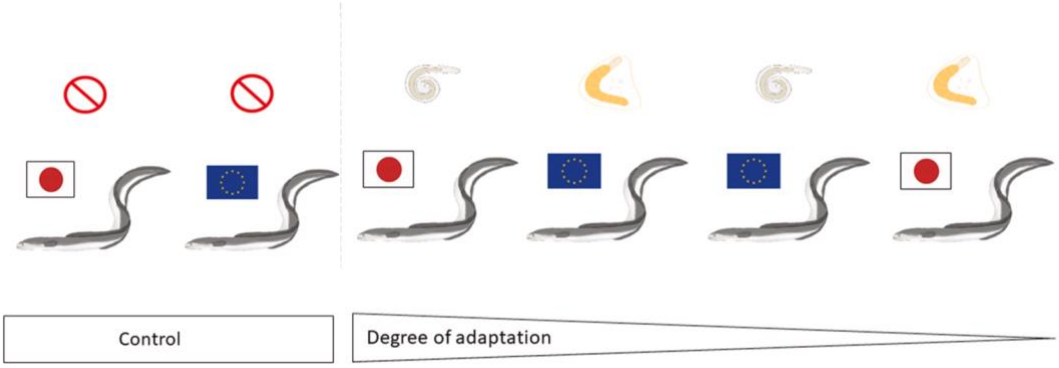
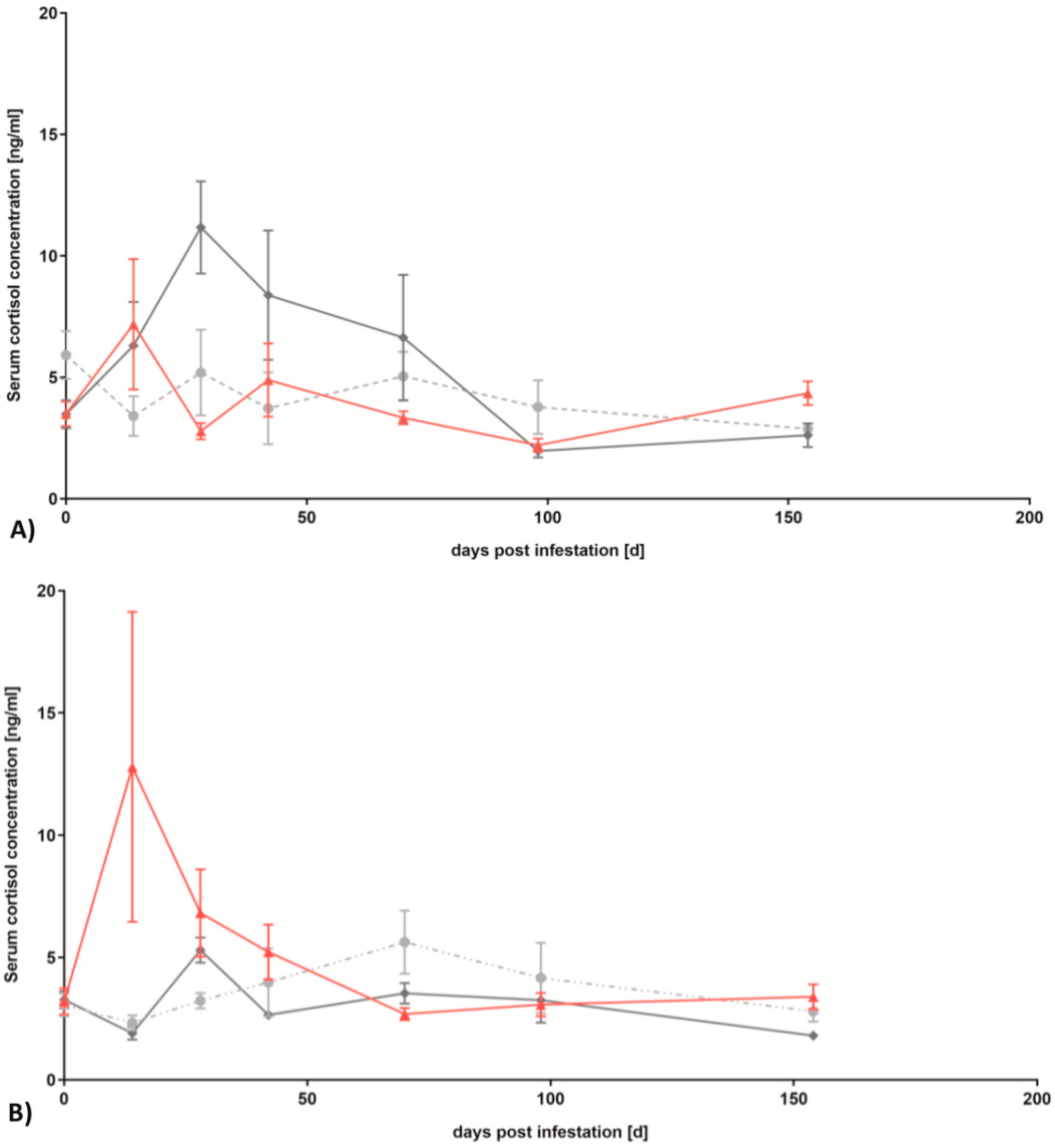


Figure 1: Overview of experimental eel groups

406



407

408 Figure 2: Course of mean cortisol concentrations (± SD) of A) European eels and B) Japanese eels. (red

409 triangle = acanthocephalan cysts; dark grey square = L3 *A. crassus*; light grey circle = un-inoculated

410 control)

411

412

413 # Tables and Legends

414 Table 1: Mean (± SD) intensities of *A. crassus* following experimental inoculation in European and

415 Japanese eels

Eel species	Type of inoculation	Stage of *A. crassus*			
		adult	preadult	L4	L3
European eel	isolated *A. crassus* (L3)	2.0 ± 0.8	5.0 ± 0.0	2.1 ± 1.0	1.6 ± 0.8
	encysted *Pomphorhynchus* sp.	1.5 ± 0.5	-	-	-
	uninfested control	-	-	-	-
Japanese eel	isolated *A. crassus* (L3)	2.0 ± 1.0	1.0 ± 1.0	2.5 ± 0.5	1.0[a]
	encysted *Pomphorhynchus* sp.	1.5 ± 0.5	-	-	-
	uninfested control	-	-	-	-

416 [a] : only one individual was found.

417

4.3 Chapter III: Hybridization between *Anguillicola crassus* and *A. novaezelandiae*, and viability of the F1 generation

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Contributions		
Conception	50%	Bernd Sures, Daniel Grabner and Katrin Honka developed the concept.
Conduction of experimental work	100%	Katrin Honka inoculated the eels, prepared and conduced the laboratory work.
Data analysis	70%	Katrin Honka and Daniel Grabner prepared and conduced the data for statistical analysis.
Writing the manuscript	100%	Katrin Honka wrote the draft manuscript including the preparation of pictures and tables.
Revision of the manuscript	30%	Bernd Sures and Daniel Grabner revised the manuscript and Katrin Honka amended the manuscript accordingly. All authors approved the final manuscript.

_____ _____

Signature of the Doctoral Candidate Signature of the Doctoral Supervisor

Hybridization between *Anguillicola crassus* and *A. novaezelandiae* and viability of the F1 generation

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Abstract

For decades, it has remained unclear how the Asian swim bladder nematode *Anguillicola crassus* was able to supplant the previously stable population of its relative from New Zealand *Anguillicola novaezelandiae* in the Lake Bracciano, Italy. Previously, researchers have hypothesized that *A. crassus* possesses an ecological advantage due to a more efficient life cycle in combination with a pattern of unidirectional hybridization between *A. novaezelandiae* females and *A. crassus* males. The present study is focusing on the viability of hybrid offspring and their allelic pattern, particularly in developed adult stages of the hybrid F1-generation. While the percentages of hybrid individuals from *A. novaezelandiae* mothers and *A. crassus* fathers increased from egg to adult stages, it was more distinct in egg stages of *A. crassus* females and *A. novaezelandiae* males but did not occur in adult F1-individuals at all. Therefore, we corroborate the hypothesis of unidirectional hybridization by differentiating between egg and adult stages and suggest this as another explanatory factor for the extinction of *A. novaezelandiae* in Lake Bracciano in Italy and the predominance of *A. crassus*.

Keywords: invasive parasite, *Anguilla anguilla,* F1 generation

Introduction

Anthropogenic activities such as worldwide trade or travel led to an increase of invasive species around the world, which is a major driver for shifting species compositions (Colautti *et al.* 2006; Pejchar & Mooney 2009) and loss of ecosystem functions (Cardinale *et al.* 2012). Even though ecosystems are well organized and aligned structures, they are still dynamic and reorganize themselves continuously. If a non-indigenous species enters a habitat, it might face competition with native or other invasive species for space and food. In general, it is difficult to measure the impact of an invasive species on ecosystem functions in a new habitat due to the complexity of interacting factors in an ecosystem (Kumschick *et al.* 2015). Even though the magnitude of the impact is hard to measure, there are several studies attributing changes to the presence of introduced species (Atalah & Sanchez-Jerez 2020; Doody *et al.* 2017; Livingstone *et al.* 2020). Albins (2013) for example, compared the influence of an invasive predator fish (Lionfish, *Pterois volitans*) on the local diversity of a prey community at Bahamian coral-reef, to the influence of a native predator fish (coney grouper, *Cephalopholis fulva*). He illustrated that the presence of the invasive species had an impact on prey communities, regardless of whether the native piscivore was present or not. Nevertheless, it remains difficult to measure the long-term impact of the invader on the conquered ecosystem, as changes in prey community, including herbivores which are keeping seaweeds under control, or cleaner fish that control ectoparasite density on other fish species, may lead to a complete reconstruction of the coral-reef with unpredictable consequences (Albins 2013).

Comparing the number of introduced species with established invasive ones, it seems by far more common that new species are not able to establish themselves in a new environment. Local species are often more resilient, the amount of introduced individuals and the adaptability of new species to habitats have an decisive impact and lead only in few cases to stable, invasive populations (Carlton & Geller 1993; Kolar & Lodge 2001). In rare cases, introduced species are able to reproduce quickly with tremendous consequences for local ecosystems. For example, the introduction of brown snakes (*Boiga irregularis*) to Guam – where they are foraging on local birds and rodents, but not facing any predator – led to the extinction of many native bird species (Wiles *et al.* 2003).

Among the multitude of ecological factors that are covered in invasion research, a neglected but important topic are neozoan parasites (Poulin 2017). On the host-parasite level, the new host can be seen as the newly conquered ecosystem, with the same scenarios being possible as described above for free-living species. Accordingly, if a new parasite species conquers a new region, it is in need for suitable hosts. If such a host is already occupied by other parasites, it either needs to find other suitable hosts, outcompete the existing parasite or needs to co-occur within the same host; otherwise, it is not able to survive and establish a population.

However, if parasites do co-exist in one host and are closely related, it is also possible that they produce hybrid offspring (King *et al.* 2015). Several studies have demonstrated that hybridization is not only possible, but that that hybrid parasites might have a better host exploitation, faster maturation time and a better resistance against the host's immune system (Oey *et al.* 2019).

Natural hybridization is a mechanism which is commonly examined in evolutionary science and considered as one of the major drivers and sources for genetic variance (Arnold 2004; Barton 2008; Harrison *et al.* 2017). In some instances, hybrid offspring can develop in both host species of their parental generation, which provides them with a better host range. This was shown e.g. for hybrid offspring of *Schistosoma bovis*, a parasite of cattle, and *S. haematobium* a common human parasite, collected from children in Senegal (Webster *et al.* 2013). Schelkle *et al.* (2012) suggest that hybridized monogeneans may exhibit a higher capability to escape the host immune system. In contrast to that, it is also possible that hybridization between parasites can limit the adaptations that one species developed to a host and is therefore decreasing their infectivity (Dybdahl *et al.* 2008). However, hybrids may face subsequent reproductive challenges, as some may be unable to produce fertile offspring (Al-Ahmad *et al.* 2006; Thomsen *et al.* 2011).

The well-studied swim bladder nematode *Anguillicola crassus* is one example that managed to outcompete an already established invasive parasite, *A. novaezelandiae*, with the same habitat requirements: the swim bladder of European eels (*Anguilla anguilla*). In the 1970s the nematode *A. novaezelandiae*, originating from the shortfin eel *A. australis*, was introduced to Lake Bracciano, Italy, where the parasite was able to establish a stable population in the native European eel (*A. anguilla*) population (Paggi *et al.* 1982). However, because the lake is not connected to other waterbodies, the parasite population remained in this particular lake and did not spread further. After the introduction of the closely related invasive species *A. crassus*, originating from the Japanese eel (*A. japonica*) in the early 1980s, both species were reported to co-occur in Lake Bracciano, even though mixed infections in eels have never been reported (Moravec *et al.* 1994). Nevertheless, a few years later *A. novaezelandiae* seems to have gone extinct, and *A. crassus* is the only species reported from eels from Lake Bracciano in Italy (Münderle 2005). Later on, Grabner et al. (2012) could demonstrate that mixed infestations of both nematode species in one eel produce hybrid offspring under laboratory conditions.

The aim of this study is to build on the previous results from Grabner et al. (2012), which are based on a single infested eel. We combine infestation hybridization experiments with molecular validation of hybridization to more explicitly investigate hybridization events in the F1 generation of *A. crassus* and *A. novaezelandiae* in European eels. However, in order to validate if the genetic advantage of *A. crassus* might be an explanation for the disappearance

of *A. novaezelandiae* from Lake Bracciano a multi generation study with several eels have to be performed.

Materials and methods

Animal source:

European eels (*Anguilla anguilla*) were purchased from an eel farm (Albe Fischfarm, Haren/Rütenbrock, Germany), where *A. crassus* infections were not recorded in the past (Dangel *et al.* 2013; Hohenadler *et al.* 2018). The general absence of the parasite was verified by dissection of ten randomly chosen eels, which were checked by light microscopy for infestation of the swim bladder.

Larvae of *A. crassus* (L2) were collected from European eels from the River Rhine caught by fishermen. L2 of *A. novaezelandiae* were obtained from a lab culture, which originated from *Anguilla australis* from New Zealand (see also Dangel and Sures, 2013 & Grabner et al., 2012). Life-cycles were established according to Haenen et al. (1994).

To prevent the accidental release of *A. novaezelandiae* or potential hybrid larvae into the waste water system, all used tank water was collected and boiled before being discharged into the sewage system.

Infestation experiments

F0 generation

For the hybridization experiment, four eels were inoculated with 10 L3 of *A. crassus* and *A. novaezelandiae* each by a stomach tube (1.5 mm diameter; B. Braun Melsungen AG, Germany) according to Sures and Knopf (2004). After inoculation, eels were kept for 150 days in aerated 80 l tanks with a PVC tube as environmental enrichment. Twice a week, they were fed ad libitum with eel pellets (DAN-EX 2848, BioMar A/S, Brande, Denmark) and 1/3 of the water was changed the day after feeding. After 150 days post inoculation (dpi), eels were dissected, adult nematodes were counted, and sexes were distinguished by light microscopy.

F1 generation

Each gravid female from the previous hybridization experiment was carefully washed to remove eggs attached to the outer cuticle. Developed eggs containing F1 L2 were removed from the uterus. One batch of eggs was stored in 70% ethanol for further molecular analysis and another batch was transferred to tap water to initiate hatching of L2 that were fed to copepods (*Macrocyclops albidus*). Developed F1 L3 stages were removed from copepods after 14 days and 20 eels were inoculated with these as described above. Each eel was

134 inoculated with 11 –27 L3 individuals originating from a single female nematode. The further
135 procedure was performed as described above for the F0 generation, including checking gravid
136 females for embryonated eggs.

137 All experimental protocols were approved by the Ethics Council (Landesamt für Natur, Umwelt
138 und Verbraucherschutz, Nordrhein-Westfalen, Germany, permit number: 84–02.05.40.16.017)
139 and were carried out in accordance with the relevant guidelines and regulations.

140

141 Molecular analysis

142 Small pieces of the pharynx or cuticle were cut out of adult individuals, and washed multiple
143 times in Milli-Q water, to remove contaminations of the host tissue. DNA was extracted with a
144 salt precipitation protocol as described in Grabner et al. (2015). To verify species identity of
145 the parental generation, molecular barcoding was performed using species-specific primer
146 targeting ***cox I*** according to Grabner ***et al.*** (2012). Primer sets for each species were run
147 separately for every individual sample. The PCR reaction mix contained 10 µl OneTaq® 2X
148 Master Mix (New England Biolabs), 0.5 µM of each primer, 1 µl of sample DNA and was made
149 up to 20 µl with PCR grade water. The PCR was run on a peqStar Labcycler at 95 °C for 5
150 min, 35 cycles of 95 °C, 58 °C and 72 °C each for 45s and a final elongation at 72 °C for 5 min.
151 PCR products were checked by standard agarose gel electrophoresis (1.5% agarose, 85 volt,
152 100-1000 bp ladder). Bands for ***A. crassus*** are expected at 303 bp and for ***A. novaezelandiae***
153 at 404 bp.

154 Analysis of microsatellite markers was used to identify a possible hybrid origin of the F1
155 generation. The markers AcrCT04 and AcrCA102 (Wielgoss ***et al.*** 2007) were used as
156 described in Grabner et al. (2012). PCR was conducted as described above with the following
157 conditions: 94 °C for 5 min, 35 cycles of 94 °C, 55.9 °C and 72 °C each for 45 s and a final
158 elongation at 72 °C for 10 min.

159 PCR products were further analyzed with a Fragment Analyzer™ (Agilent Technologies) using
160 a 33 cm capillary and the dsDNA 905 Reagent Kit (Agilent Technologies, Inc). DNA
161 concentrations were quantified by Fragment Analyzer™ Automated CE System PROSize® 3.0.
162 Marker fragment sizes were evaluated based on PCR products amplified with the ArcCT04
163 and ArcCA102 of all F0 adult worms. The resulting fragment sizes were assigned to the
164 respective ***Anguillicola*** species. Because the microsatellite markers yield fragments of 100-
165 260 bp (ArcCT04) and 297-332 bp (ArcCA102) (Wielgoss ***et al.*** 2007), signals < 100 bp and >
166 400 bp, as well as signals with a relative intensity of < 5 % were not considered further. Further,
167 fragments between 136-139 bp are excluded, as they appear for both species.

To account for the uncertainty of 3-5 bp of the microsatellite measurement in the resulting fragments closely spaced bands were merged as follows resulting in fragment sizes that were exclusively found in one or the other species and named accordingly (AC: *A. crassus*, AN: *A. novaezelandiae*): AC1 = 115-117 bp; AC2 = 136-139 bp; AC3 = 146-149 bp; AN1 = 119-124 bp; AN2 = 129-133 bp; AN3 = 141-142 bp.

From F1 generation, in total 30 eggs and 48 adult individuals originating from *A. crassus* mothers and 50 eggs and 88 adult individuals originating from *A. novaezelandiae* mothers were individually examined.

Results

F0-Generation

All four inoculated eels were infested with various numbers of *Anguillicola* spp. individuals with recovery rates between 15-60%. Initial screening of the nematodes revealed that eel No I was infested with females only. Since no offspring is possible without male individuals, these nematodes were not considered for further investigation. The infracommunities of the other eels were composed as follows: eel No II: 6 ♀, 6 ♂; eel No III: 6 ♀, 4 ♂; eel No IV: 4 ♀, 4 ♂. All females were gravid, apart from two individuals - one in eel No II and one in eel No IV.

Species were determined by species-specific *cox I* primers and microsatellite analysis of the parental generation revealed three distinguishable alleles for both *A. crassus* (AC1-3) and *A. novaezelandiae* (AN1-3) (Table 1). Results of species identification by *cox I* primers matched results of species-specific microsatellite alleles consistently. Only one individual (IIIW6) showed unambiguous alleles of *A. novaezelandiae* but could not be clearly distinguished by *cox I* primers as bands for both *A. novaezelandiae* and for *A. crassus* were visible. Besides two individuals (IVW2 & IVW3) showed no band in the *cox I* PCR, but as their microsatellite alleles showed a clear *A. novaezelandiae* pattern, they were considered as such.

For six individuals (IIM4, IIIW1, IIIM3, IIIW5, IVM3, IVM4) no distinct microsatellite pattern was visible. The detected fragments were lying outside the range of the microsatellite alleles located for the two species, which are therefore considered as unspecific fragments. This remained the case even after repeating the measurement.

Table 1: Molecular analysis of microsatellite (AcrCT04) of adult *A. crassus* and *A. novaezelandiae* of all examined eels (I - IV). Fragments were merged: AC1 = 115-117 bp; AC2 = 136-139 bp; AC3 = 146-149 bp; AN1 = 120-124 bp; AN2 = 129-133 bp; AN3 = 141-142 bp. *species determination targeting *cox I*

	*A. crassus**					*A. novaezelandiae**						
Eel No I												
nematode	IW1	IW2	IW3									
sex	♀	♀	♀									
AcrCT04												
Eel No II												
nematode	IIW1	IIW4	IIM2	IIM4	IIM5	IIW2	IIW3	IIW5	IIW6	IIM1	IIM3	IIM6
sex	♀	♀	♂	♂	♂	♀	♀	♀	♀	♂	♂	♂
AcrCT04			AC1				AN1				AN1	AN1
	AC2	AC2	AC2		AC2	AN2	AN2	AN2	AN2	AN2	AN2	AN2
								AN3	AN3	AN3		
Eel No III												
nematode	IIIW1	IIIW2	IIIM2	IIIM3		IIIW3	IIIW4	IIIW5	IIIW6	IIIM1	IIIM4	
sex	♀	♀	♂	♂		♀	♀	♀	♀	♂	♂	
AcrCT04												
		AC2	AC2			AN2	AN2		AN2	AN2	AN2	
Eel No IV												
nematode	IVM1	IVM2	IVM3	IVM4	IVW1	IVW2	IVW3	IVW4				
sex	♂	♂	♂	♂	♀	♀	♀	♀				
AcrCT04					AC1	AN1	AN1	AN1				
	AC2	AC2				AN2	AN2	AN2				

F1-Generation

Recovery rates of nematodes originating from *A. crassus* mothers ranged between 4-67% with 3.0±2.2 ♀ and 3.1±3.2 ♂. Recovery rates of nematodes originating from *A. novaezelandiae* mothers ranged between 11-65% with 3.6±2.8 ♀ and 1.7± 1.3♂. Most of the females had either no eggs, or poorly developed/unembryonated eggs, except for three females originating from an *A. crassus* mother, which showed normally developed and embryonated eggs.

Ratio of hybrids and non-hybrids differ between eggs (containing L2) and developed adults of F1 generation (figure 1). Eggs that originated from *A. crassus* females revealed 13 % offspring with alleles of both species and 43 % offspring with *A. crassus* alleles only, whereas eggs that originated from *A. novaezelandiae* females revealed 4 % offspring with alleles of both species and 76 % offspring with only *A. novaezelandiae* alleles. Hybridization was not detected in any of the adult offspring originating from *A. crassus* females. Adult offspring of *A. novaezelandiae* females revealed a percentage of 25 % with alleles of both species, whereas 63 % showed only alleles of *A. novaezelandiae*. The number of individuals without a distinct pattern (nD) for offspring originating from an *A. crassus* mother differs between 43% (eggs) and 35% (adults)

and for offspring originating from an *A. novaezelandiae* mother between 20% (eggs) and 13% (adults).

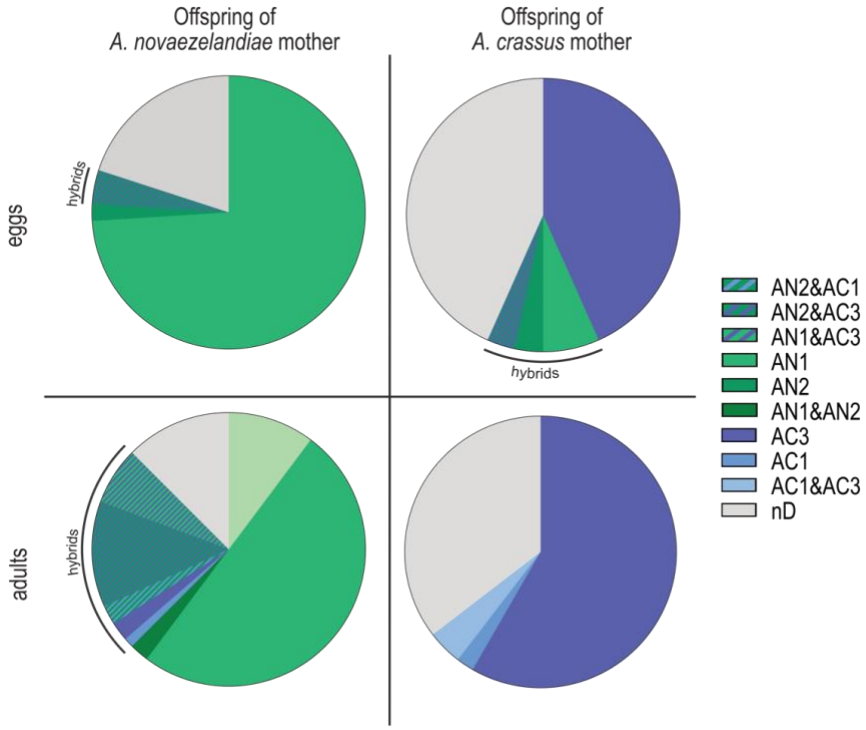

Figure 1: Ratio of alleles of F1 generation. Blue colors represent *A. crassus* alleles, green colors represent *A. novaezelandiae* alleles. a) eggs (with L2) from *A. novaezelandiae* mother (n = 50) b) eggs (with L2) from *A. crassus* mother (n = 30) c) adults from *A. novaezelandiae* mother (n = 88) d) adults from *A. crassus* mother (n = 48). AC1 = 115 – 117 bp; AC3 = 144 – 150 bp; AN1 = 119 – 126 bp; AN2 = 128 – 133 bp; nD = no distinct pattern of fragments; therefore, no species allocation possible.

Discussion:

In the present study, we provide additional details on the relevance of hybridization between the two eel swim bladder nematodes *A. crassus* and *A. novaezelandiae*. Previously, Grabner et al. (2012) suggested that *A. crassus* may have genetic advantages over *A. novaezelandiae*, as their findings indicated that hybridization appears to be possible only between *A. novaezelandiae* females and *A. crassus* males. Since they described this pattern based on nematodes obtained from one single eel only, it had remained uncertain whether this finding is reproducible. To verify the hypotheses of genetic advantages, the present study provides evidence for the viability of hybrid offspring and indicates that only hybrid offspring of *A. novaezelandiae* females can develop to F1 adults. We distinguish between hybrid larvae, which were released by the mother nematode, but not developed further and those that developed to the adult stage after passage through the copepod and experimental infection of an eel. Our results confirm the finding by Grabner et al. (2012) with respect to possible hybridization between both species of *Anguillicola*, and give further information about hybrid development.

In the present study, the length of the fragments amplified with the AcrCT04 primers varied between 120 and 142 bp, while previous data showed a uniform pattern for *A. novaezelandiae* of a single allele of 109 bp obtained by the AcrCT04 primers (Grabner *et al.* 2012). This is due to the fact that a new field isolate of *A. novaezelandiae* was used for the laboratory cycle in the present study, showing a different allelic pattern. Interestingly, alleles of *A. novaezelandiae* were found only in the egg stages obtained from *A. crassus* mothers. Those eggs obtained from *A. crassus* mothers that were passed through a copepod and were used to infest an eel showed exclusively *A. crassus* alleles in the developing F1-adults. This indicates that hybrid eggs and larvae originating from the *A. crassus* female/*A. novaezelandiae* male crossing only develop to the larval stages. Even though the length difference between AC1 (115-117bp) and AN1 (120-124bp) may be at the limit of measuring accuracy of the method, it was consistently the case in all measurements performed that samples of both species never exceeded this limit. Therefore, we consider the size assignment of the alleles as valid.

Nevertheless, we cannot exclude the possibility that those hybrid nematodes develop to adult stages, as the examined sample size is still too small to give complete poof. Among individuals that derived from an *A. novaezelandiae* mother, the number of hybrids increased from 4 % in the egg stage, to 25 % in adult nematodes, which is a strong indication, that the development proceeds with greater success in this hybrid crossing. Recovery rates of hybrid offspring originating from both species are not known, as we cannot detect if an infectious larva is a hybrid beforehand without dissecting it, still the further development into adult stages can be seen as a good predictor. In addition, the number of offspring without any distinct allelic pattern

266 is higher in individuals originating from *A. crassus* mothers (35-43%), compared to those from
267 *A. novaezelandiae* mothers (13-20%). This could be due to a different binding efficiency of the
268 AcrCT04 primers for the two species resulting in a lower density of bands for *A. crassus*.

269 The finding of the present study that *A. novaezelandiae* females/ *A. crassus* males crossings
270 exist and can even develop to the adult stage adds to the information provided by Grabner et
271 al. (2012) who analyzed only egg stages and could just speculate about the further
272 development. Applying our results to the situation on Lake Bracciano in the late 1980s, the
273 interpretation depends on the fate of the F1 adults, which we still cannot predict with certainty.
274 Basically, there are two possibilities – either hybrid offspring is viable and fertile, or
275 hybridization leads to a dead end of reproduction. If hybrids are fertile, it might be even a
276 disadvantage for *A. crassus* to produce hybrid offspring with *A. novaezelandiae*, as the former
277 has by far the better adaptation to the eels immune response (Keppel *et al.* 2014; Knopf *et al.*
278 2000) and the possibility is given, that hybrid offspring will lack some of this adaptations. The
279 life cycle of *A. crassus* is also more efficient compared to *A. novaezelandiae*, as the larvae are
280 released over a longer period of time. Accordingly, these larvae are capable of infesting the
281 intermediate host for a longer period of time as well (Dangel *et al.* 2013). Therefore, it may be
282 worse for hybrid offspring of *A. crassus* to lose this efficiency - although this is only speculation
283 according to current knowledge, since no valid data on hybrid offspring performance is
284 available. The effect of the hybrids on the populations of the two *Anguillicola* species would
285 also depend on the potential differential reproductive success of each of the two species with
286 the hybrids. However, we can only speculate about the further development and fertility of the
287 F1 generation. In other species, especially male hybrids are often facing sterility, which was
288 found for *Drosophila*, mice and other animals (Haldane 1922; Kagawa & Takimoto 2018; Price
289 & Bouvier 2002; Sun *et al.* 2004; Thomsen *et al.* 2011; Widmayer *et al.* 2020) , but on the same
290 side there is a variety of studies known, which show that hybrids can indeed be fertile (Close
291 & Bell 1997; Volf *et al.* 2007; Wallis & Beardmore 1980). If hybrid offspring is not fertile, it is to
292 some extent a disadvantage for both species, as some of their reproduction effort leads to a
293 dead end. Yet, it seems reasonable that *A. crassus* was able to combine its ecological
294 advantage of a more efficient life cycle (Dangel *et al.* 2013), underlined by theoretical modeling
295 of the population growth rate of the two species (Dangel *et al.* 2015), with some genetic
296 advantage to contribute to the extinction of *A. novaezelandiae* in Lake Bracciano. The latter
297 had to face an additional fitness impairment as it lost some reproductive output to non-viable
298 or non-fertile hybrids, which it was not able to cope with. Nevertheless, existing in constant
299 competition with another species is an energy-consuming process, so that in the long run it
300 was more beneficial to eliminate a competitor and accept possible minor disadvantages, for
301 example a slightly worse adaptation to the immune response of the host.

Conclusively, this research contributes to a better understanding of what happened in the Lake Bracciano in the late 1980s and early 1990s - hybridization between the two species might have decreased the reproductive fitness of both, but due a more efficient life cycle and population growth rate, *A. crassus* could eventually make up for this disadvantage, while *A. novaezelandiae* has gone extinct.

Future experimental studies should focus on the viability and fertility of the F2 (2nd filial) generation to further clarify the fate of hybrid individuals in a population. Furthermore, the gene flow between the two Anguillicola species should be measured using a high number of genomic markers, using double- digest restriction-site-associated DNA, which has been shown to be efficient in detecting hybridization in previous studies (e.g. Xu & Hausdorf, 2021; Paulus et al., 2022).

Conflict of interest:

The authors declare none.

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5. General discussion

Anguillicola crassus, the protagonist of the present thesis is in the focus of research since decades. Nentwig et al. (2018) compared and published a list of the top 100 alien parasites from different phyla in Europe, based on the socioeconomic and environmental impact in their new habitats. Revealing that *A. crassus* takes place 81 of all species listed, and the 2nd place within the category of nematodes, after the plant parasite *Bursaphelenchus xylophilus*. This underlines that *A. crassus* is a successful invader with impact on its host populations. Research of the last 40 years generated a huge amount of knowledge about this parasite e.g. the description of the life cycle, stress influence on the host or changes of the gas composition in the host's swim bladder (Dangel et al., 2014, 2013; De Charleroy et al., 1990; Keppel et al., 2016, 2014; Knopf et al., 2000; Lefebvre et al., 2012; Newbold et al., 2015; Sures et al., 2001; Thomas and Ollevier, 1992; Würtz et al., 1996). This thesis contributes to what is known about *A. crassus* by adding its interactions with other parasites as further explanation for its successful invasion.

Even though *A. crassus* was already in focus of research for such a long period of time, the interaction of it with other parasites was a rather new finding of Emde et al. (2014). Results of chapter I of the present thesis underline the invasion success of *A. crassus* and emphasize the importance of a holistic approach to understand the ecology and lifestyle of this parasite.

In general, it is a key success of parasites, if they are able to establish their lifecycles in paratenic hosts, as this is an effective way to escape a dead-end host situation. In the case of *A. crassus*, it uses the swim bladder as the final habitat in both - final and paratenic hosts (Sures et al., 1999) - , in order to escape the fishes immune response, which could potentially kill it. Infestation results presented in chapter I clearly underline that this strategy to evade the immune system is so effective, that the nematode is generally searching for similar hiding places, if no swim bladder is present. As gobies are lacking a swim bladder, the acanthocephalan cyst appeared to be the next best suitable "organ". This way, *A. crassus* can extent its invasion success simply by using other parasite infestations and is transforming a dead-end host into a paratenic host.

Emde et al. (2014) who first described the finding of *A. crassus* hiding in the acanthocephalan cyst, opened the question if it may be hyperparasitism. At first glance, this appears to be reasonable, but with increasing knowledge, more and

more reasons emerge that speak against it. To follow the definition of a hyperparasite, *A. crassus* needs to benefit from *Pomphorhynchus* sp. itself and needs to harm it in some way (Boenigk, 2021; Lucius and Loos-Frank, 2008). Based on current knowledge, it seems more likely that the nematode is not parasitizing the acanthocephalan but is only coexisting in the cyst (personal observation and communication with Hohenadler), which is technically built up by the acanthocephalan and the host, why it is not part of the parasite itself. The co-existence is based on the observation, that all recognized nematodes appeared directly in the lumen of the cyst after opening it without damaging the acanthocephalan. Furthermore, *Pomphorhynchus* sp. is – probably – not harmed by the nematode, as the cyst is already fulfilling the need of a hiding place, which makes it unnecessary to invest more energy by infesting the acanthocephalan itself. The fact, that no adult of the acanthocephalan was found inside examined eels is more likely referable to the fact that eels are not a suitable host (Thielen et al., 2007), than the interaction with the nematode. Still, it is not deniable that there is some benefit from the interaction. It rather gives the impression that the acanthocephalan cyst serves as a kind of “paratenic host tissue”.

Based on that, the term “invasional meltdown hypothesis” mentioned in chapter I suits in a more accurate way, as it states the beneficial outcome for *A. crassus* without direct parasitizing the acanthocephalan. The implementation of another species for better survival in a new habitat is applying to the IMH by Simberloff & Von Holle (1999), which was so far only used to describe interactions of free-living species.

In the performed experiment, all cyst-inoculated eels were infested by individuals of the nematode, which is a clear statement, that *A. crassus* is using the other parasite to expand its transmission possibilities. Especially by considering that the exact number of applicated nematodes could only be estimated, the detected individuals proof that it is a realistic way for transmission of *A. crassus* in the wild. Simberloff (2006) notes that examples with unidirectional IMH, where only one participant is benefiting, provide weaker evidence, but are still worth taking into account. Results of the experiments of the present thesis did not confirm any benefits for the acanthocephalan. It might be possible, that there is some beneficial output for the acanthocephalan which could not be displayed in this thesis, e.g. a longer survival. To proof this, more experiments need to be performed with samples taken on different timepoints, especially in the first days in which the development and

interaction with the eels' immune system takes place. However, it is questionable if it is worth it, because the here presented results indicate that potential benefits are not substantial enough to allow acanthocephalan survival in eel hosts. No information about other, preferred definitive hosts of *Pomphorhynchus* spp. are known so far.

Even though infection rates seem low, it is impossible to estimate the exact recovery rate, as it cannot be calculated how many larvae were administered in the first place. Still, this thesis gives proof that the nematode is using another parasite for its benefit. This emphasizes the superior life cycle of *A. crassus* and its invasive characteristics. Regardless, laboratory inoculations were performed in the present thesis. Cysts from wild caught gobies were used, therefore it is most likely that it is a realistic infestation process of wild eels in the River Rhine. Albeit, this interaction was described recently, all species involved, namely *A. crassus*, *N. melanostomus* and *Pomphorhynchus* spp., are present in the river Rhine for at least 30 years. It is difficult to distinguish how intense *A. crassus* was benefitting from this way of interaction with the acanthocephalan, or if it was a neglectable way of distribution. Overall, it seems to be another way that *A. crassus* fulfills its reputation as a successful invader and illustrates the adaptability of the parasite to its environment.

Nevertheless, there are as well studies which rather promote the idea of a co-tolerance of two invaders instead of co-support between them, which was stated by Jackson (2015) in a meta-analysis of invasive animals. Still, the studies used in that meta-analysis only considered free-living animals and not parasites, which might have another impact. It is important to keep in mind, that the data of the current thesis is no proof of the IMH, but can be explained by it. Both, the IMH and the occurrence of *A. crassus* in Europe, are not new findings, but nonetheless, the possible connection between them was never taking into account as a reasonable explanation of the one with the other.

Another important key characteristic of *A. crassus* invasion success is the coevolutionary adaptation with its native final host, the Japanese eel (*Anguilla japonica)*. In general, those mutual adaptations are a good example for how species interact with each other in a well-balanced ecosystem. Both parties figured out a way how they can survive side by side, which only gets imbalanced, if those perfectly adapted systems are facing new counterparts. Invasive species can impact the new ecosystems e.g. by unknown hunting strategies, dominant habitat concurrence

behavior (Saul et al., 2013) or - in case of parasites - by higher infestation rates of new hosts, which in turn impact their immune system and suitability for other pathogens itself. The population of the European eel was facing this impact in the early 1980s, when *A. crassus* was introduced to Europe (Jacoby and Gollock, 2014). The native European eel species was not able to cope with this highly adapted eel parasite.

As can be seen in chapter II of this thesis, the degree of adaptation shows an important influence on the cortisol increase for the hosts. By linking the degree of adaptation with the stress response of the eels, it highlights the fact, that it is unprecise to say, that one parasite is more harmful than the other, as you always need to focus on the system in which the infestation is happening. The presented results confirm already published differences in the stress response between Japanese and European eels to its native (*A. japonica*) or invasive (*A. Anguilla*) parasite (Dangel et al., 2014). Furthermore, they underline that those adaptations are a unique system between the parasite and its host, which is not transferable in any kind. The systems used in chapter II vary in relatedness and adaptation degree, from coevolutionary adaptation to naïve systems, which have never occurred in the wild. The relation between the European and Japanese eel was close enough for *A. crassus* to accept it as a new final host and therefore infested it with all adapted effectiveness like it is used to in the native host. The unadapted European eel is overwhelmed by its new parasite and is therefore affected more severely. More broadly, the here presented results show, that the Japanese eel is not able to transfer its strategies to another parasite species, as highest cortisol responses were measured in the naïve system of *Pomphorhynchus* sp. and the Japanese eel. Consequence of this high cortisol distribution is a suppressed immune system of the eel, which is therefore easier accessible for the following parasites (Sures et al., 2001). Long-term cortisol increase is known to lead to inflammatory issues and an increase of apoptosis (Schreck and Tort, 2016; Tort, 2011), which is lowering the overall fitness of the individual. This underlines the importance of careful human actions in ecosystems, as they may have tremendous effects on further interactions.

Even though the Japanese eel is nearly not affected by its own native parasite, it showed an even higher cortisol response to the European parasite *Pomphorhynchus* sp., than the European eel to *A. crassus*. But not only phylogenetically divergent species influenced *A. crassus* in its distribution in

Europe. Even before its introduction in the early 1980s, the phylogenetically closely related species *A. novaezelandiae* was introduced into an isolated lake in Italy and managed to establish a stable population within the native eel population there. Shortly after the introduction of *A. crassus*, both species were reported from Lake Bracciano, but no coinfections within the same eel were described. Interestingly, a few years later *A. novaezelandiae* seemed to be extinct from the lake completely (Moravec et al., 1994; Münderle, 2005). Even though it is difficult to predict what happened 40 years ago, different hypotheses have been published during the last years. Whereas Dangel et al. (2013) provide an ecological explanation by a more sufficient life cycle, Grabner et al. (2012) gave genetic proof, that the two species not only co-exist, but can co-infest the same eel and furthermore can mate and produce hybrid eggs. As this genetic proof opened many questions concerning the viability of the offspring, chapter III of the present thesis is focusing on them. The here presented results proof, that hybrids are not only possible in the unidirectional pattern as proposed by Grabner et al. (2012), but vice versa, even though there are decisive differences in the viability. This thesis gives proof, that hybrid eggs of both crossing pattern are built, but only those of *A. crassus* males x *A. novaezelandiae* females develop further into adult stages, which confirms the unidirectional pattern of Grabner et al. (2012). On long-term, this might be an explanation for the extinction of *A. novaezelandiae* as males can fertilize numerous females of both species, but only produce offspring with females of the same species. Basically, this is cutting the reproducing rate in half, comparing to *A. crassus* males, which produce offspring with females of both species.

So, it leaves the question how this genetic appearance was interfering in the distinction of *A. novaezelandiae* in Europe. Considering that the life cycle of *A. novaezelandiae* is more compromised and the parasite therefore relies on finding the correct hosts in a shorter period of time, it is more depended on a sufficient genetic output, but exactly this output is minimized by the genetic interference of *A. crassus*.

To summarize the knowledge acquired in this thesis, results confirm the position of *A. crassus* as a successful invader. Not only that it uses native or already established species like the acanthocephalan *Pomphorhynchus* sp. as trojan horses to improve its distribution, it is also able to dominate the closely related species *A. novaezelandiae* on genetic level. Still, it leaves us with unanswered questions: How is *A. crassus* able to dominate its relative? What are the exact genetic

differences to *A. novaezelandiae* that make this species far more invasive? Maybe differences in some specific proteins might be the crucial distinction, as they are normally extremely sensitive to genetic changes/mutations, and have an essential role in organisms appearing (Boenigk, 2021). But from current knowledge we can only conjecture.

In general, invasive species are in most cases a threat to native ecosystems and can leave their conquered areas with tremendous ecological and likewise economic consequences. Once a specie is introduced, it is nearly impossible to remove it, that is why mankind needs to be more careful with all activities that have a potential to introduce new species.

To summarize the history of *Anguillicola* spp. in Europe, *A. novaezelandiae* was removed by its related species *A. crassus*, but with even worse consequences for the local eel population and therefore for the local ecosystem. The Asian swim bladder parasite improved its invasion success not only by using other native fish species as paratenic hosts, but also other invasive fish like gobies and furthermore other parasites (Emde et al., 2014; Li et al., 2015; Sures et al., 1999; Thomas and Ollevier, 1992). Once they infect the final host, they provoke a major stress response by increasing cortisol levels, which suppresses further immune responses, leading to easier secondary infestation. The superior genetic standing, nonetheless by hybridization other closely related species is completing its invasion success.

Therefore, the present thesis is providing new understanding of invasion mechanisms of parasites, which are often similar to better studied free-living species, but still need a special point of view.

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8. Curriculum vitae

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

9. Declarations

Declaration:

In accordance with § 6 (para. 2, clause g) of the Regulations Governing the Doctoral Proceedings of the Faculty of Biology for awarding the doctoral degree Dr. rer. nat., I hereby declare that I represent the field to which the topic “Interaction of non-indigenous endoparasites of the European eel *Anguilla anguilla*” is assigned in research and teaching and that I support the application of Katrin Isabel Honka.

Essen, date _____ Bernd Sures _____ _____

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

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

Declaration:

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

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Signature of the doctoral candidate

Declaration:

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

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Signature of the doctoral candidate