Mutual adaptation in differently evolved host-parasite systems

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Michelle Keppel

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That's life

That's what all the people say

You're riding high in April, shot down in May

But I know I'm gonna change that tune

When I'm back on top, back on top in June.

F. Sinatra

Angaben zur Prüfung

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

1. Gutachter: Prof. Dr. Bernd Sures

2. Gutachter: PD Dr. Klaus Knopf

3. Gutachter:

Vorsitzender des Prüfungsausschusses: Prof. Dr. Markus Kaiser

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Good things come to those who wait.

Table of contents

List of abbreviations

Aa	*Anguilla anguilla*
Ac	*Anguillicola crassus*
Aj	*Anguilla japonica*
An	*Anguillicola novaezelandiae*
Bc	Blood capillary
BCA	Bicinchoninic acid
dpi	Days post infection
E	European eel
f	Female
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hsp	Heat shock protein
hsp70	Heat shock protein with a molecular weight of 70 kDa
J	Japanese eel
L	Lumen
L2	Second stage larvae of nematodes
L3	Third stage larvae of nematodes
L4	Fourth stage larvae of nematodes
Lp	Lamina propria
m	Male
MI	Mean intensity
Mm	Muscularis mucosae
N	Number of samples
S	Submucosa
SBW	Swim bladder wall

SD	Standard deviation
SDI	Swimbladder Degenerative Index
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SK	Schadensklasse (damage level)
SOD	Superoxide dismutase
sp.	Species
spp.	Species pluralis
TBS	Tris-buffered saline

List of figures

List of tables

1 Summaries

1.1 Summary

Host-parasite systems are an ideal field of research for studying co-evolutionary processes since the two involved individuals are continuously interacting. In this respect, adaptation processes are directly targeted at coping with the antagonistic species. Invasive parasite species are of particular importance when comparing data on established and new host-parasite associations. The invasive swim bladder nematode *Anguillicola crassus*, originally parasitizing the Japanese eel (*Anguilla japonica*), has spread throughout populations of the European eel (*Anguilla anguilla*) since its introduction in the early 1980s. The present study is based on infection experiments of these two eel host species with *A. crassus* and its close relative *A. novaezelandiae*, which is endemic to the Short-finned eel (*Anguilla australis*) and thus non-native to both analyzed host species. Accordingly, four host-parasite associations differing in terms of mutual adaptation were investigated. With these it was possible to contrast systems featuring long-term, short-term, and no co-evolutionary relationship at all.

Infection success, swim bladder pathogenicity, morphological features, and stress response based on hsp70 levels of both *Anguillicola* species in each eel species were analyzed in order to reveal host-specific differences, which could be related to co-evolutionary processes. Recovery rates of both parasite species proved to be significantly lower in Japanese eels than in European eels indicating that Japanese eels developed a strong genus-specific immune response against nematode infections with the ability to encapsulate and kill invading larvae. Those encapsulation processes proved to implicate a distinct thickening of the swim bladder walls. Detailed morphological measurements showed that both nematode species attain a reduced maximum body size in Japanese eel hosts compared to European eels. Highly increased hsp70 responses could be detected in *A. crassus* individuals taken from Japanese eels, slightly increased levels were observed for *A. crassus* taken from European eels indicating that adapted hosts induce a higher stress response in the parasites. *Anguillicola novaezelandiae* showed neither elevated hsp levels in Japanese nor in European eel hosts. However, taken as a whole, the present results strongly hint at an impaired development of both *Anguillicola* species

when confronted with the strong immune response of a well-adapted eel host species.

1.2 Zusammenfassung

Wirt-Parasit-Systeme bieten ideale Voraussetzungen zur Erforschung von koevolutionären Prozessen, da die beteiligten Individuen in kontinuierlicher Interaktion stehen. Insofern sind die entsprechenden Adaptationsprozesse gezielt darauf ausgerichtet, die Wirkungen des Gegenspielers abzuschwächen. Invasive Parasitenarten sind in diesem Zusammenhang von großer Bedeutung, denn sie lassen Vergleiche zwischen etablierten und neu entstandenen Wirt-Parasit-Verbindungen zu. Die invasive, ursprünglich im Japanischen Aal (*Anguilla japonica*) parasitierende Schwimmblasennematoden-Art *Anguillicola crassus* hat sich seit ihrer Einführung in den frühen 1980er Jahren über Populationen des Europäischen Aals (*Anguilla anguilla*) in ganz Europa ausgebreitet. Die vorliegende Studie basiert auf Infektionsexperimenten mit diesen beiden Wirtsaalarten und *A. crassus* sowie der nahe verwandten Parasitenart *A. novaezelandiae*, die endemisch im Kurzflossenaal (*Anguilla australis*) vorkommt und dementsprechend in beiden untersuchten Aalarten nicht heimisch ist. Daraus resultieren vier unterschiedlich angepasste Wirt-Parasit-Systeme, die eine Gegenüberstellung von langfristig, kurzfristig und nicht-koevolvierten Beziehungen ermöglichen.

Die Untersuchungen beziehen sich auf den Infektionserfolg, die morphologischen Eigenschaften und die Stressreaktionen, gemessen an den Expressionsniveaus von Hitzeschockproteinen (hsp70), der beiden *Anguillicola*-Arten in den verschiedenen Wirtsarten sowie die jeweilige im Wirt hervorgerufene Schwimmblasenschädigung, um wirtsspezifische Unterschiede herauszustellen, die in Zusammenhang mit koevolutionären Prozessen stehen. Die Wiederfindungsraten beider Parasitenarten waren in Japanischen Aalen signifikant niedriger als in Europäischen Aalen, was auf die Etablierung einer effektiven Genus-spezifischen Immunantwort auf Infektionen mit *Anguillicola*-Arten im Japanischen Aal hindeutet, der in der Lage ist, eindringende Larven einzukapseln und zu töten. Diese Einkapselungen gingen mit einer deutlichen Verdickung der Schwimmblasenwände einher. Detaillierte morphometrische Analysen konnten zeigen, dass beide Parasitenarten in Japanischen Wirten auch eine deutlich geringere Körpergröße als in Europäischen Wirten erreichten. Stark

erhöhte Hitzeschockproteinwerte konnten in Individuen der Art *A. crassus* in Japanischen Aalen festgestellt werden, während *A. crassus* im Europäischen Aal leicht erhöhte Werte aufwies, was darauf hinweist, dass leicht bis stark an die jeweilige Infektion angepasste Wirtsarten eine höhere Stressreaktion in den Parasiten hervorrufen als unangepasste Arten. *Anguillicola novaezelandiae* zeigte weder im Japanischen noch im Europäischen Wirt gesteigerte hsp70-Niveaus. Dennoch deuten die Ergebnisse der vorliegenden Arbeit zusammen betrachtet deutlich auf eine beeinträchtigte Entwicklung beider *Anguillicola*-Arten in Reaktion auf die ausgeprägte Immunantwort eines stark angepassten Wirtes.

2 General introduction

2.1 Background

In the course of globalization and due to the rising demand of fresh foods from all over the world, the transportation of live animals has expanded sharply. When transferring animals into a new environment, this may have severe effects on the animal itself as well as on the newly captured ecosystem. When carrying a parasite species, this may also implicate a decisive change for the parasite itself and for potentially suitable new hosts. The invasion success of an unintentionally or consciously introduced parasite species and its infection success in a new host species are strongly dependent on many different factors, as illustrated in figure 2.1.

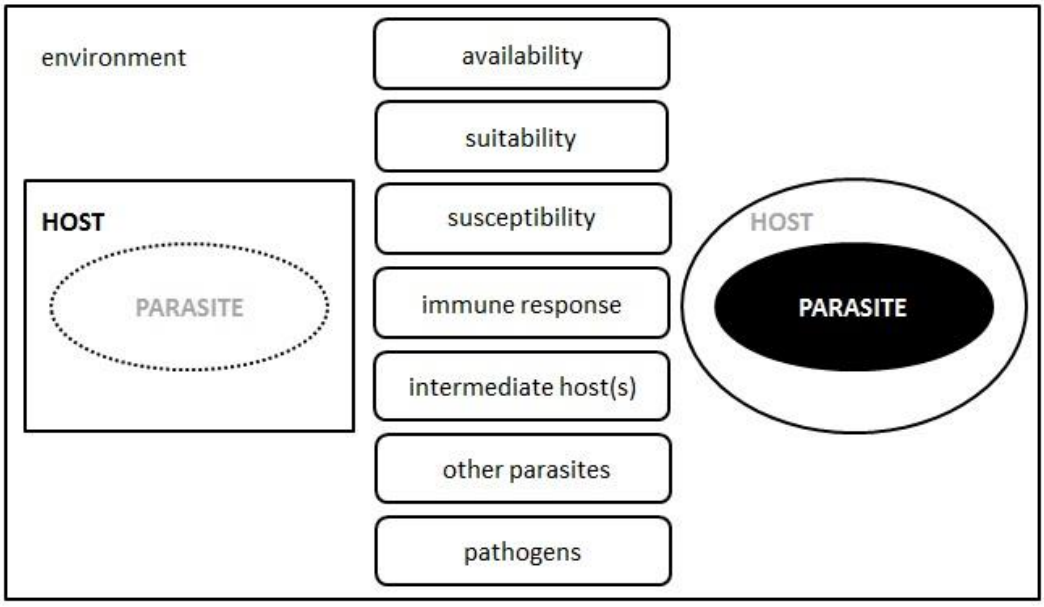

Fig. 2.1 Relations between hosts and parasites and factors of mutual dependence

The figure shows different factors affecting the relationship between a parasite and its host (centre). The original host (right) is represented by a round shape, the new host (left) features a rectangular shape.

When a parasite together with its host is transferred into a new environment, it is initially uncertain if it will be capable of parasitizing other host species. First of all, environmental conditions like the ambient temperature, in which the other host species lives, may be different. The availability of the host candidate has to be given as well, for example if the parasite is able to reach the host or – depending on the life cycle of the respective parasite – the intermediate host on its own or through ingestion. The requirements of the host's suitability and susceptibility might be highly dependent on its immune defence system, which is closely connected to co-evolutionary processes related to former infections (Poulin 2007). Other parasite species might confront the invading species with a competitive situation, as well as other pathogens either competing for the same resources or infecting and weakening the parasite itself.

If a parasite species becomes a successful invader, it is assumed that the coexistence of this newly established host-parasite system will be subject to co-evolutionary mechanisms, since both involved partners evolve in response to the other (Thompson 2005). Evolutionary processes are expected to happen quite rapidly in antagonistic interactions between hosts and parasites compared to other forms of ecological relationships, mainly due to the parasite's usually short reproduction times and to a very strong selection pressure in finding susceptible hosts (Buckling and Brockhurst 2012). A long-standing host-parasite association is therefore supposed to be consequentially complex and well-advanced, whereas newly acquired host populations often suffer from detrimental health impairments supposedly due to a lack of adaptation.

A well-studied example providing many insights about parasitic invasion over the past few decades are swim bladder nematodes of the genus *Anguillicola*, of which the most invasive species *A. crassus*, endemic to the Japanese eel (*Anguilla japonica*), has been accidentally introduced into populations of the European eel (*A. anguilla*) (Kennedy 2007). Just shortly after its first appearance in Europe, it has spread across the entire European continent through to North Africa and North America (Johnson et al. 1995; El Hilali et al. 1996). Additionally, the closely related species *A. novaezelandiae*, originating from New Zealand and Australia, respectively, and being native to the Short-finned eel (*A. australis*), is able to infect the European eel with a high prevalence and intensity as well (Moravec et al. 1994b). It was introduced into Lake Bracciano in Italy via transportation of Short-finned eels from

New Zealand in 1975 (Paggi et al., 1982). After its introduction, its prevalence reached up to 80% within the population of European eels in that lake (Moravec et al. 1994b), but it could not establish any populations outside of the lake. The subsequent introduction of *A. crassus* to Lake Bracciano was initially followed by a short phase of coexistence of the two *Anguillicola* species, but subsequent samplings did only identify *A. crassus* individuals (Moravec et al. 1994b; Münderle 2005). Table 2.1 provides an overview of the distribution of these two *Anguillicola* species and their host switches.

Tab. 2.1 Host-parasite associations between *Anguilla* and *Anguillicola* species

	Anguillicola crassus	***Anguillicola novaezelandiae***
Original (co-evolved) host	Japanese eel *(Anguilla japonica)*	Short-finned eel *(Anguilla australis)*
Introduced to (year)	Europe (1980) North Africa (1994) USA (1994)	Lake Bracciano, Italy (1975)
Invasiveness	spread throughout Europe, North Africa, USA	remained in Lake Bracciano
New host	European eel *(Anguilla anguilla)* American eel *(Anguilla rostrata)* *Anguilla bicolor* *Anguilla marmorata* *Anguilla mossambica*	European eel *(Anguilla anguilla)*

Due to their different grades of mutual adaptation, these host-parasite associations provide a perfect system for experimentally analyzing interactive processes. For conducting laboratory infections of selected host-parasite associations, the life cycle of the parasites (Fig. 2.2) has to be simulated. Under natural conditions, adult *Anguillicola* nematodes settle in the swim bladder of eels, where they nourish on eel blood. Following sexual reproduction, they release eggs containing second stage larvae (L2), which are subsequently excreted by the eel. Second stage larvae then hatch out of the eggs into the water and through ingestion, they reach the visceral cavity of copepods, which serve as intermediate hosts. In this phase, the larvae develop from the second to the third larval stage (L3). The infected copepods are eaten by the eels and so the L3 larvae enter their final host. At this stage, the nematode larvae penetrate the intestine of the eel, in order to cross the abdominal cavity and to reach the swim bladder wall, where they moult into fourth stage larvae (L4). The larvae finally enter the eel's swim bladder and develop to their adult stage, producing and emitting eggs again. Within this life cycle, an additional interposed (paratenic) host may ingest the intermediate host and may be eaten by the final host itself. If there is another host interposed, a further moult does not take place, but only an enrichment of the larvae (Kirk 2003).

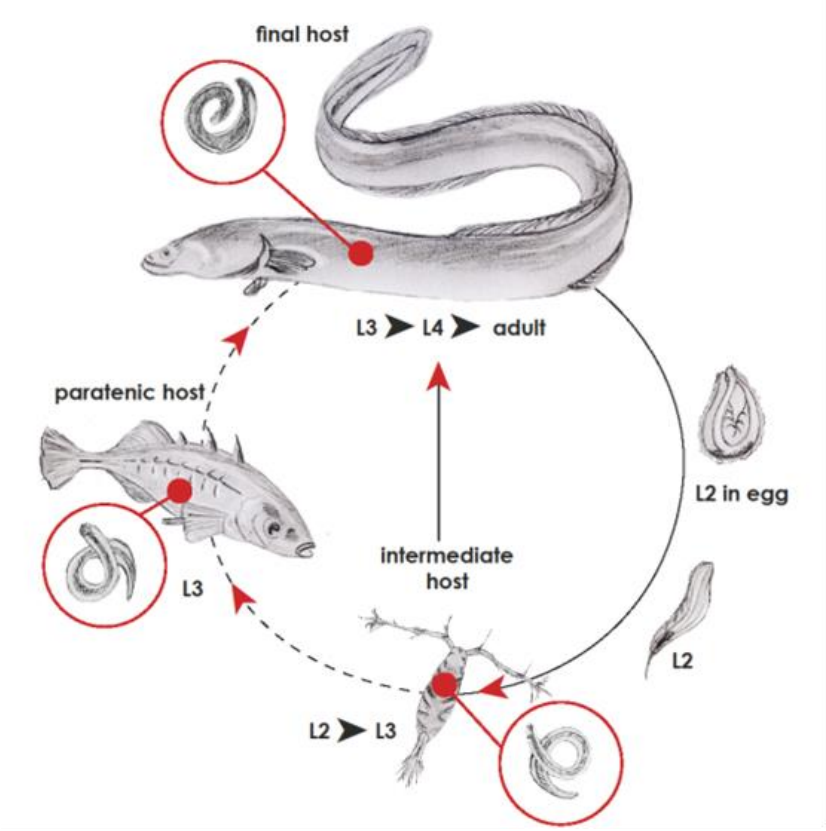

Fig. 2.2 Life cycle of *Anguillicola* sp.

The life cycle of the nematodes requires the availability of an intermediate and a final host. A paratenic host may be interposed without involving a further development but inducing an enrichment of the larva.

The present thesis focuses on experimental studies of interactions between eel hosts and *Anguillicola* species, because infection experiments provide exact parameters related to parasite dose entering the host, infection period, environmental conditions and host condition especially with regard to health status, age and former infections. *A. crassus* and *A. novaezelandiae* were chosen due to their close relationship and their competitive encounter in Lake Bracciano. This study is based on these two *Anguillicola* species infecting the two final host species *A. japonica* and *A. anguilla*, so there are cases of long-time adaptation in a well-adapted system (*A. crassus* and *A. japonica*), short-time adaptation since the 1980s (*A. crassus* and *A. anguilla*), and no adaptation at all (*A. novaezelandiae* and both eel host species), since *A. anguilla* has encountered *A. novaezelandiae* only for a limited period in Lake Bracciano excluding any further spread to other European host populations and *A. japonica* has

never encountered *A. novaezelandiae* under natural conditions. The experimental design of the present study is outlined in Fig. 2.3.

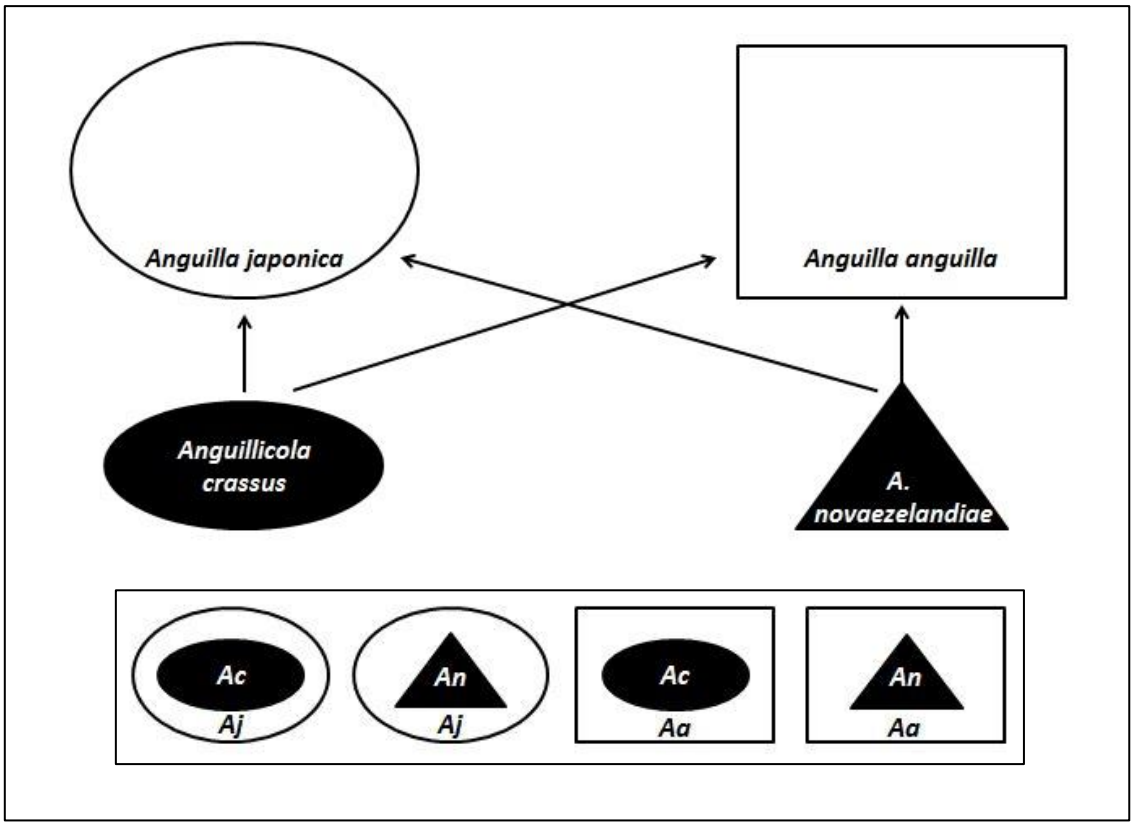

Fig. 2.3 Experimental design of infections with *Anguilla* and *Anguillicola* species

Figure shows final host species (blank) and parasite species (black) used for infection experiments. The four combined groups are presented below.

2.2 Aims

Based on previous findings and assumptions regarding adaptive processes between hosts and parasites in general, the central objective of this work was to find out if these four host-parasite associations (*A. crassus* x *A. japonica*; *A. crassus* x *A. anguilla*; *A. novaezelandiae* x *A. japonica*; *A. novaezelandiae* x *A. anguilla*) can provide any visible traces of adaptation, especially with regard to immune response and immune evasion, respectively and if those factors driving the infection success of *Anguillicola* species are specific to the respective host species.

Analyses on infection success based on recovery rates of the two parasite species in the two different host species and the related effects on the host's swim bladder presented in chapter 3, are supposed to give new insights into the performance of all involved species, whether adapted or not to the respective association. Chapter 4 considers the question whether size differences between the two *Anguillicola* species and selected morphological traits are ontogenetically determined or dependent on the particular host species. The main aim pursued in chapter 5 was to analyze the stress responses of the parasites, which were confronted with stressors related to the different host species.

2.3 Hypotheses

With regard to the different analyzed factors of recovery rate, swim bladder pathogenicity, morphology, and hsp response, the present study will focus on a number of hypotheses, which are based on previous studies and consequent assumptions.

A. japonica and *A. crassus* have passed through a long-term association and they both could have undergone some kind of adaptive mechanisms in order to fight against defence or evasion strategies by the antagonist species. This eel host might have developed a species-specific immune response to its endemic parasite species, whereas no specific selection has taken place in relation to the alien parasite species *A. novaezelandiae*. The response of the European host species has not yet been subject to selection concerning a successful defence against both parasite species, so infection rates, swim bladder pathogenicity, parasite body parameters and stress

responses are expected to considerably differ between the four analyzed host-parasite associations.

Chapter 3 will investigate the following hypotheses regarding infection success and swim bladder reactions:

1. The infection rate of *A. crassus* in the European eel will exceed the rate in the Japanese eel.
2. The infection rate of *A. novaezelandiae* in both eel species will be higher than the rate of *A. crassus*.
3. Swim bladder walls of European and Japanese eels will show a higher damage degree following infection with *A. novaezelandiae*.

In chapter 4, the conducted morphological analyses are supposed to test the following hypotheses:

4. The body size measurement values of both nematode species in the Japanese eel will be lower than those in the European eel.
5. Morphological parameters of *A. novaezelandiae* in the European and in the Japanese eel will be similar.

The underlying hypotheses with regard to the parasites' stress responses analyzed in chapter 5 are the following:

6. Both parasite species show a higher stress response in the Japanese eel than in the European eel.
7. The stress level of *A. crassus* in both eel species will be higher than the level of *A. novaezelandiae*.

3 Comparison of infection success, development and swim bladder pathogenicity of two congeneric *Anguillicola* species in experimentally infected *Anguilla anguilla* and *A. japonica*

Michelle Keppel, Kerstin Claudia Dangel & Bernd Sures

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3.1 Summary

Two closely related parasites, *Anguillicola crassus* and *Anguillicola novaezelandiae*, originally parasitizing swim bladders of the Japanese eel (*Anguilla japonica*) and the Short-finned eel (*Anguilla australis*), respectively, were used for analyzing the infection success of each parasite species on either long-known, recently acquired or new definitive host species and the associated effects on the eels' swim bladders. On that account, European eels (*Anguilla anguilla*) and Japanese eels were experimentally infected with both *Anguillicola* species in the laboratory. Susceptibility of the two eel species to both parasite species was determined by analyses of infection data. Subsequently, histopathological effects of the nematodes on the hosts' swim bladders were characterized according to already established indices.

The present study revealed significant differences between the four different host-parasite systems regarding recovery rates, infrapopulations, and damage levels. Both nematode species achieved significantly lower recovery rates in Japanese eels than in European eels, since the examined swim bladders of Japanese eels contained a high amount of dead encapsulated larvae, whereas those of European eels contained only living nematodes. Encapsulation of larvae in Japanese eels was associated with a distinct thickening of the swim bladder walls. The swim bladders of uninfected Japanese eels turned out to be generally thicker than those of European eels. Infection with both *Anguillicola* species resulted in a further thickening process of the swim bladder walls in Japanese eels, whereas those of European eels showed only minor changes. The two established classification systems turned out to be

inapplicable, since the measurements and the macroscopic evaluations of the swim bladders of the two infected eel species did not entirely correspond to the underlying criteria.

3.2 Introduction

Anguillicoloid swim bladder parasites that have accidentally been introduced into the population of European eels (*Anguilla anguilla*) belong to the best known examples of parasites that negatively affect newly acquired hosts. The nematode species *Anguillicola crassus* was introduced to Europe via import of infected eels from Taiwan in the 1980s (Neumann 1985), whereas *Anguillicola novaezelandiae*, the endemic parasite of the Short-finned eel (*Anguilla australis*) was transported from New Zealand to Lake Bracciano, Italy, in 1975 (Moravec and Taraschewski 1988). These two closely related species differ with regard to their natural hosts and thus to their infection potential and their pathogenicity towards other eel hosts. While various studies were focused on the susceptibility of the European or the Japanese eel towards *A. crassus* both in wild eel populations and in experimental studies (Knopf et al. 1998; Knopf and Mahnke 2004; Knopf 2006; Münderle et al. 2006; Han et al. 2008; Heitlinger et al. 2009; Weclawski et al. 2013), there is only one study dealing with experimental infections with *A. novaezelandiae* in intermediate hosts (Moravec et al. 1994) and two in European eels (Grabner et al. 2012; Dangel et al. 2013).

In order to comparatively analyze the infection success of the two nematode species *A. crassus* and *A. novaezelandiae* in the two eel species *A. anguilla* and *A. japonica*, experimental infections of all four possible host-parasite combinations were performed in a common garden experiment. Based on recent studies, it can be assumed that the infection success of *A. crassus* in the European eel will exceed the rate in the Japanese eel, as the immune system of the Japanese eel has already been adapted to *A. crassus*, while the European eel has only been exposed to *A. crassus* within the last three decades (Knopf 2006). With respect to *A. novaezelandiae*, the first hypothesis being tested is that its infection rate is higher than the rate of *A. crassus* in both eel species since the former parasite has never naturally encountered the Japanese host species and has only encountered the European host species in Lake Bracciano for a period assumed to be too short to allow for adaptation processes in the population of the European eel.

Infestation of swim bladders with *A. crassus* usually causes damage of the swim bladder wall including thickening, inflammation, fibrosis, and changes in the epithelial cells (Knopf et al. 2008; Würtz and Taraschewski 2000). In severe cases, pathologic alterations even lead to a complete loss of the swim bladder lumen, or the lumen becomes totally filled with worms. From these massive alterations of the swim bladder, one may expect a loss of its function which will be especially relevant when eels start their spawning migration through the Atlantic (Sures and Knopf 2004a), with diurnal vertical migrations ranging between 40 to 1,000 m (Aarestrup et al. 2009). In order to specify the degree of swim bladder damage, a number of analyses of histopathological effects on the swim bladder walls of the respective eel hosts have been performed (Hartmann 1994; Haenen et al. 1994; Molnár et al. 1993, 1995; Würtz and Taraschewski 2000; Abdelmonem et al. 2010; Neto et al. 2010), and the results were used in order to classify swim bladder damages into categories (Liewes and Schaminee-Main 1987; Csaba et al. 1993; Hartmann 1994; Molnár et al. 1994; Beregi et al. 1998; reviewed by Lefebvre et al. 2011). From these studies, it emerges that infections with *A. crassus* may cause severe swim bladder damages in European eels (Kirk 2003), whereas the impact on Japanese eels is usually rather low (Egusa 1992). Data on natural infections of the Short-finned eel with *A. novaezelandiae* (Lefebvre et al. 2004a, b) revealed only little – if any – damage. Accordingly, within the present study, degenerative changes of the swim bladder walls of two different eel species were directly compared following experimental infection with the two different nematode species and related to the already existing knowledge on histopathological changes and their related categorization. The underlying hypothesis is that a higher degree of swim bladder wall damage in European and Japanese eels is to be expected following infection with *A. novaezelandiae*, since both eel species are not adapted to this parasite.

3.3 Material and methods

3.3.1 Source and maintenance of final and intermediate hosts and parasites

This study includes two different eel species (*A. anguilla*, *A. japonica*) and two different nematode species (*A. crassus*, *A. novaezelandiae*). Uninfected European eels were purchased from a German eel farm known to be free of *A. crassus* infections (Albe Fischfarm, Haren/Ruetenbrock, Germany) and were kept at a constant temperature of 20 °C in aerated tap water. Eels were fed with pellet food (DAN-EX 2848, BioMar A/S, Brande, Denmark) twice a week. Japanese eels were imported from an eel farm in Japan (Omori-Tansui Co., Ltd., Miyazaki, Japan) and were kept in tanks separated from the other eel species but under the same conditions. In order to exclude already existing infections, a group of 10 eels of each species was dissected prior to experimental infections and their swim bladders were screened for the presence of nematodes. Cyclopoid copepods, serving as intermediate hosts, were caught from an urban pond, kept in small tanks, and fed twice weekly. Second stage larvae (L2) of *A. crassus* were extracted from swim bladders of European eels that were previously infected in the laboratory (see Grabner et al. 2012). Second stage larvae of *A. novaezelandiae* were collected from swim bladders of naturally infected Short-finned eels in New Zealand (see Dangel and Sures 2013).

3.3.2 Experimental infections

In order to generate third stage larvae (L3) of both nematode species, copepods were individually infected by placing two L2 per copepod into 24-well plates filled with tap water. In the following 3 weeks, the copepods were fed twice a week, and subsequently, the L3 were separated from the copepods by means of a tissue potter (55 ml tissue grinder, Wheaton, Millville, New Jersey, USA) (see Haenen et al. 1994). The remaining suspension of larvae in 0.8 % saline was poured through a common paper tea filter into a centrifuge falcon tube, allowing the larvae to migrate through the membrane of the filter. After at least 2 hours rest time, the larvae were collected from the bottom of the tube by means of a Pasteur pipette and then stored in medium

(Minimum Essential Medium Eagle, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) at 8 °C until use.

Infections of European eels were performed with 20 L3 of either parasite species. In order to achieve a comparable intensity, 40 larvae were used for infections of the Japanese eel, since previous studies suggest a lower susceptibility of Japanese eels towards *A. crassus* compared to European eels (Knopf and Mahnke 2004). The respective eels were selected according to comparable sizes of about 100-250 g per individual. Eels were gently wrapped in a well-soaked cloth, and subsequently, a small-sized volume of the suspension containing the larvae was administered by means of a stomach tube (1.5 mm diameter; B. Braun Melsungen AG, Melsungen, Germany) as described by Sures and Knopf (2004b). Each infection group was kept separated from the other groups in large tanks at a constant temperature of 20 °C. In order to prevent unintended release of *A. novaezelandiae* individuals to the environment, all occurring waste water was heated to at least 80 °C in order to kill the L2 potentially discharged by the hosts. Uninfected control groups of both eel species were kept separated from the infected groups but under the same conditions.

3.3.3 Examination and analysis of host-parasite systems

All groups (see Table 3.1) were kept for 90 and 120 days post infection (dpi), respectively. Prior to examination, the eels were beheaded, measured, and weighed. Then, the swim bladders were removed and cut open. Adult nematodes were collected in physiologic saline, divided according to genders, counted, and immediately stored in -80 °C for further analyses.

The swim bladders were macroscopically examined for any alterations or damages, which were categorized into degenerative levels on the basis of classifications by Hartmann (1994), modified by Knopf (1999) (Schadensklasse (SK)) and Lefebvre et al. (2011) (Swimbladder Degenerative Index (SDI)). The SK classification is based on a macroscopic diagnosis regarding thickness and opacity of the swim bladder wall, whereas the SDI classification uses three criteria: transparency/opacity, pigmentation/exudate, and thickness. After removal and macroscopic examination, the swim bladders were compressed between two

plexiglass plates and checked for the presence of dead or living L3 or L4 by means of a stereomicroscope with a magnification of x8-40. After recording the number of larvae, a sample of each swim bladder was stored for histological analyses in a tissue embedding cassette (Histosette I, M498, VWR International GmbH, Darmstadt, Germany), immersed in a formalin solution containing 10 % formaldehyde.

Recovery rates were determined in percent by dividing the total number of recovered nematodes by the total number of administered larvae. In order to describe the composition of infrapopulations, mean intensities were calculated by dividing the total number of nematodes found in one host by the total number of hosts that have been infected (see Bush et al. 1997). Statistical analyses of all data were performed by means of the t-test (GraphPad Prism 5, GraphPad Software, Inc., USA).

Tab. 3.1 Overview of experimental infections with eel and *Anguillicola* species

Table shows host-parasite systems that were used for experimental infections, the amount of administered L3 stage larvae and the respective system labels.

Host/parasite species	*Anguillicola crassus*	*Anguillicola novaezelandiae*
European eel	20 x L3 (E20Ac)	20 x L3 (E20An)
Japanese eel	40 x L3 (J40Ac)	40 x L3 (J40An)

3.3.4 Histological analyses

The fixed swim bladder samples were embedded in paraffin wax, sectioned into layers of 5 µm by means of a microtome (Leica Microsystems), and applied to microscope slides. These sections were dried and subsequently stained with hematoxylin and eosin. The histological samples were examined and photographed using a light microscope (Olympus). The thickness of the swim bladder walls was measured by means of an image-processing program (Image J, National Institutes of Health, USA). Histopathological data were checked for significance using the t-test (GraphPad Prism).

3.4 Results

3.4.1 Infection success and development of Anguillicola spp*. in Anguilla* spp*.*

Recovery rates of both *Anguillicola* species at 90 dpi are significantly higher in European eels compared to Japanese eels, with a slightly higher infection success for *A. novaezelandiae* compared to *A. crassus* (Fig. 3.1). In Japanese eels, recovery rates of both *Anguillicola* species ranged below 10 %. European eels infected with *A. crassus* contained 17 % L3, 6 % L4, and 76 % adults, whereas the infrapopulation of *A. novaezelandiae* consisted of 96 % adults and 4 % L4 (Fig. 3.2). Both nematodes in Japanese eels as hosts lack living larval stages but contain 46 % (*A. crassus*) and 59 % (*A. novaezelandiae*) dead larvae, located in the swim bladder walls.

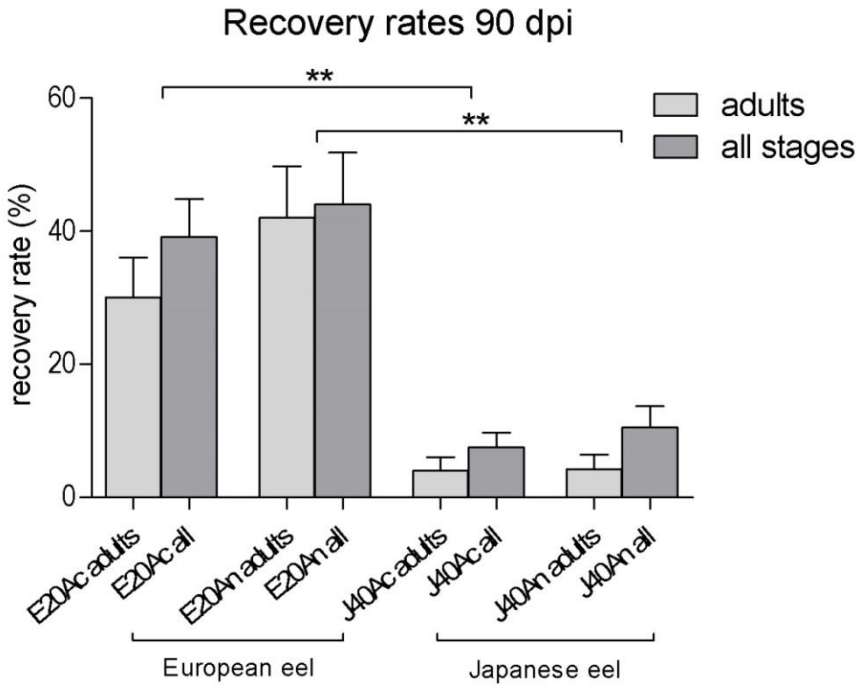

Fig. 3.1 Recovery rates of adults and all stages of *Anguillicola* spp.

Figure shows recovery rates of adults (light grey bars) and all developmental stages (L3, L4 and adults; dark grey bars) of *A. crassus* and *A. novaezelandiae* in European and Japanese eels at 90 days post infection (dpi).
E20Ac = European eel infected with 20x *A. crassus*, N=10; E20An = Europ. eel infected with 20x *A. novaezelandiae*, N=10; J40Ac = Japanese eel infected with 40x *A. crassus*, N=10; J40An = Jap. eel infected with 40x *A. novaezelandiae*, N=10

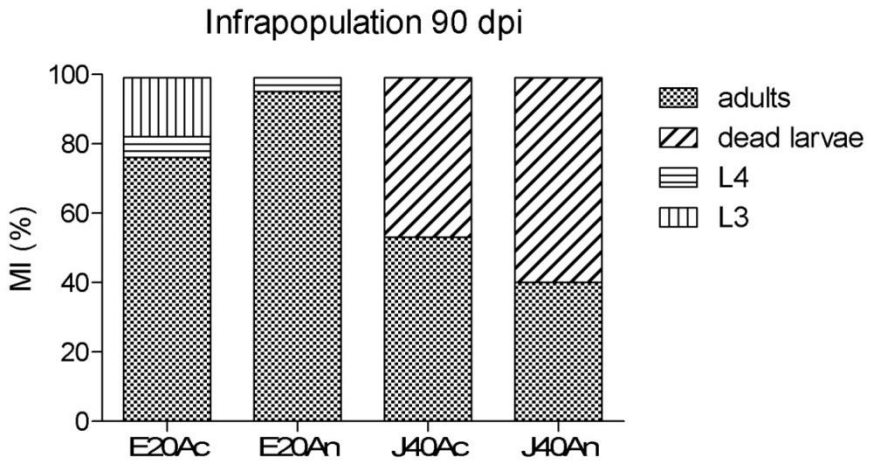

Fig. 3.2 Infrapopulations of *Anguillicola* spp. 90 dpi

Figure shows infrapopulations of *A. crassus* and *A. novaezelandiae* in European and Japanese eels at 90 dpi.
E20Ac = European eel infected with 20x *A. crassus*, N=10; E20An = Europ. eel infected with 20x *A. novaezelandiae*, N=10; J40Ac = Japanese eel infected with 40x *A. crassus*, N=10; J40An = Jap. eel infected with 40x *A. novaezelandiae*, N=10

At 120 dpi, recovery rates of adults (Fig. 3.3) of both nematode species in the European eel were similar with means of 41.4 % (*A. crassus*) and 39.5 % (*A. novaezelandiae*). In the Japanese eel, only a mean of 6.3 % (*A. crassus*) and 3.6 % (*A. novaezelandiae*) adults were found. When including larval stages, however, the highest recovery rate was found for European eels infected with *A. crassus* followed by European eels infected with *A. novaezelandiae*. In the Japanese eel, *A. novaezelandiae* showed a higher recovery rate compared to *A. crassus*. Values concerning adults as well as all stages of both *Anguillicola* spp. in the European and the Japanese eel featured a highly significant difference. Infrapopulations at 120 dpi (Fig. 3.4) showed a similar composition compared to the results at 90 dpi. The number of L3 found in European eels infected with *A. crassus* was slightly reduced, and at the same time, the number of L4 was increased. European eels infected with *A. novaezelandiae* were not found to contain larval stages. In the Japanese eel infected with *A. novaezelandiae*, the share of dead larvae rose towards 71 %.

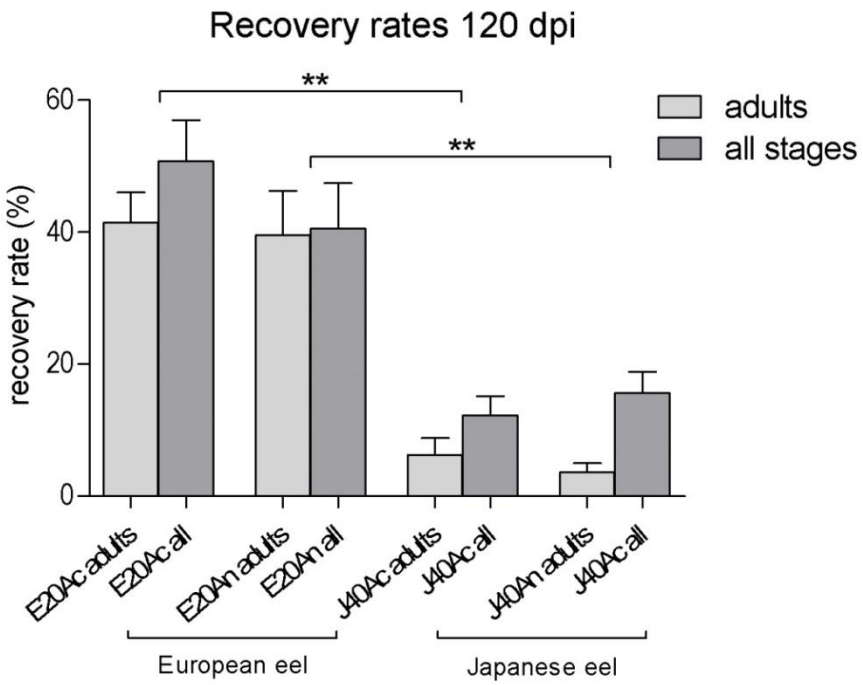

Fig. 3.3 Recovery rates of adults and all stages of *Anguillicola* spp. 120 dpi

Figure shows recovery rates of adults (light grey bars) and all developmental stages (L3, L4 and adults; dark grey bars) of *A. crassus* and *A. novaezelandiae* in European and Japanese eels at 120 days post infection (dpi).
E20Ac = European eel infected with 20x *A. crassus*, N=10; E20An = Europ. eel infected with 20x *A. novaezelandiae*, N=10; J40Ac = Japanese eel infected with 40x *A. crassus*, N=10; J40An = Jap. eel infected with 40x *A. novaezelandiae*, N=10

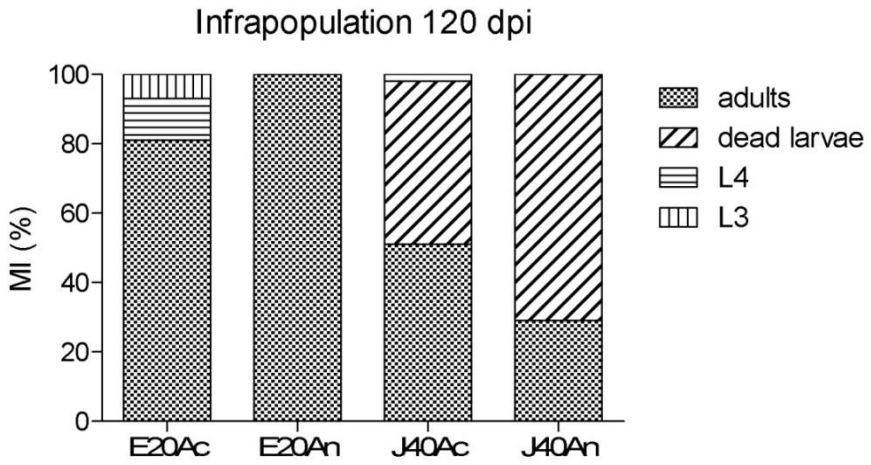

Fig. 3.4 Infrapopulations of *Anguillicola* spp. 120 dpi

Figure shows infrapopulations of *A. crassus* and *A. novaezelandiae* in European and Japanese eels at 120 dpi.
E20Ac = European eel infected with 20x *A. crassus*, N=14; E20An = Europ. eel infected with 20x *A. novaezelandiae*, N=10; J40Ac = Japanese eel infected with 40x *A. crassus*, N=10; J40An = Jap. eel infected with 40x *A. novaezelandiae*, N=11

3.4.2 Histological analyses

Representative samples of the analyzed swim bladder walls of each group of eels are shown in Figs. 3.5 and 3.6. All layers of the swim bladders of Japanese eels – including the control group – were thicker than those of the European groups (Fig. 3.7). Both the epithelium and the muscularis mucosae showed higher thicknesses compared to all groups of the European eel. While the layers of the muscularis mucosae in Japanese eels were slightly thickened in consequence of infection with both *Anguillicola* species, the thickness of the epithelia partially increased threefold. Japanese eels infected with *A. crassus* featured the greatest thickening of all groups. Statistical tests showed a significant difference ($p = 0.0308$) between both uninfected control groups as well as between Japanese eels infected with *A. crassus* and *A. novaezelandiae*. Differences between European and Japanese eels infected with *A. crassus*, between European and Japanese eels infected with *A. novaezelandiae* as well as between uninfected Japanese eels and those infected with *A. crassus* were found to be highly significant ($p < 0.008$) (see Fig. 3.7).

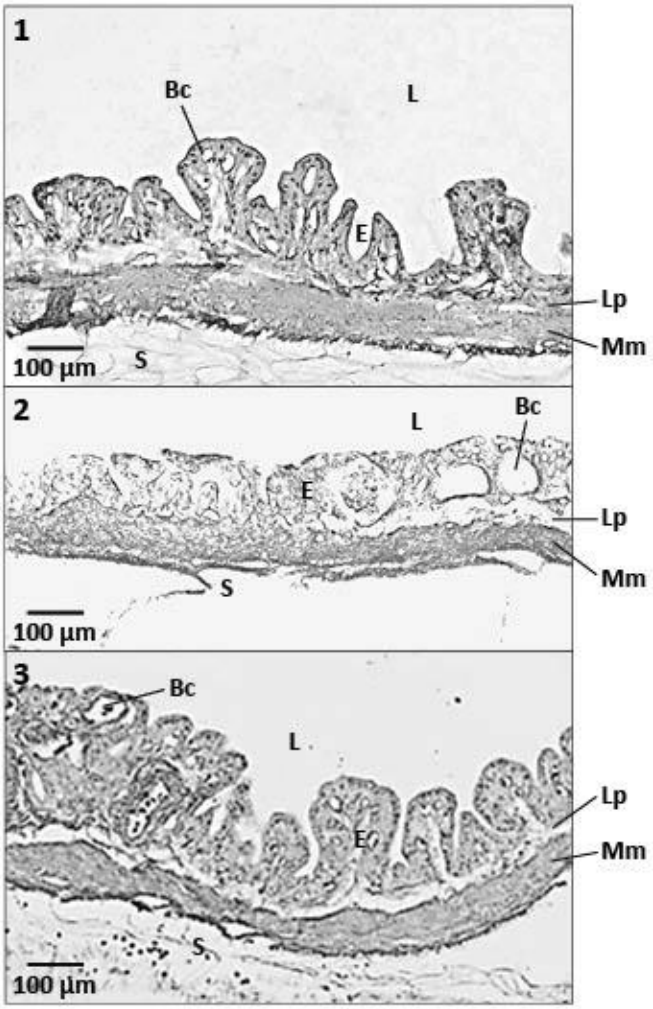

Fig. 3.5 Cross-sections of swim bladders of European eels at 120 dpi

Image 1 shows a cross-section of a swim bladder of an uninfected European eel, image 2 shows a swim bladder infected with *A. crassus*, image 3 shows a swim bladder infected with *A. novaezelandiae*.
Bc = Blood capillary, E = Epithelium, L = Lumen, Lp = Lamina propria, Mm = Muscularis mucosae, S = Submucosa

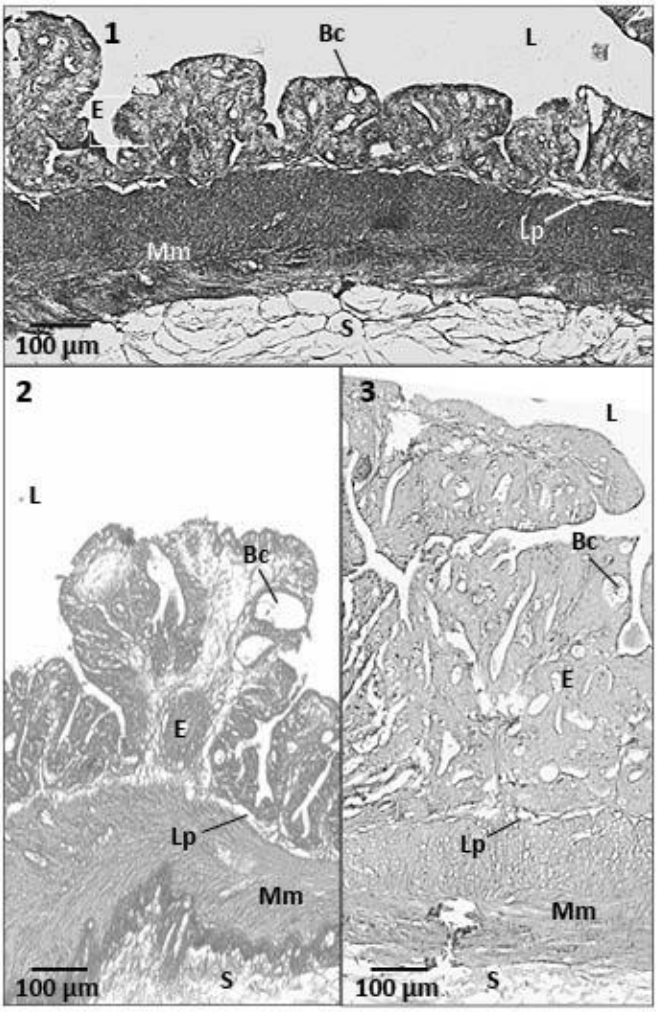

Fig. 3.6 Cross-sections of swim bladders of Japanese eels at 120 dpi

Image 1 shows a cross-section of a swim bladder of an uninfected Japanese eel, image 2 shows a swim bladder infected with *A. crassus*, image 3 shows a swim bladder infected with *A. novaezelandiae*.
Bc = Blood capillary, E = Epithelium, L = Lumen, Lp = Lamina propria, Mm = Muscularis mucosae, S = Submucosa

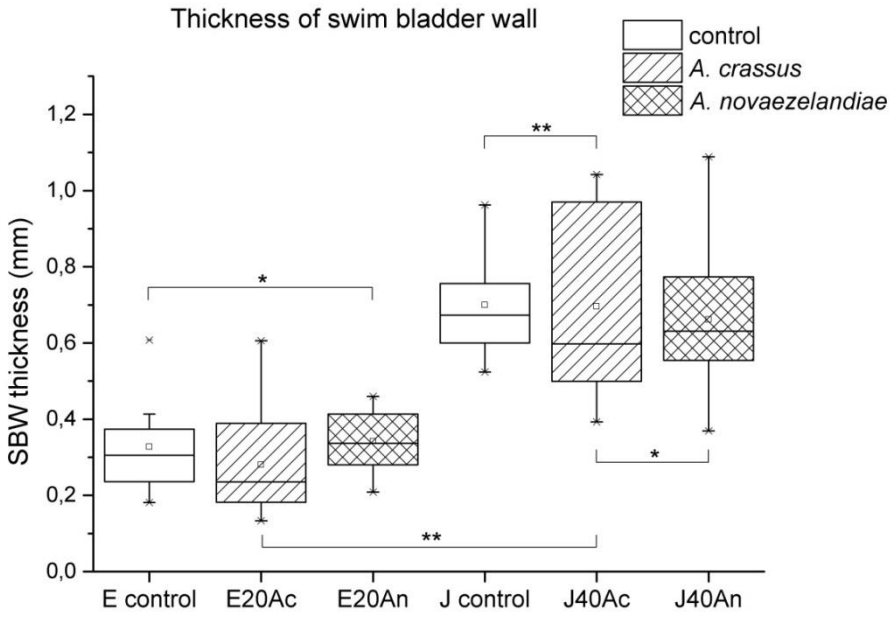

Fig. 3.7 Thickness of swim bladder walls of infected and uninfected European and Japanese eels at 120 dpi

Boxplots show ranges of thickness of swim bladder walls measured in mm. Data on *A. crassus* are shown in striped boxes, those on *A. novaezelandiae* in a check pattern.
E control = European eel uninfected, N=10; E20Ac = European eel infected with 20x *A. crassus*, N=14; E20An = Europ. eel infected with 20x *A. novaezelandiae*, N=10; J control = Japanese eel uninfected, N=10; J40Ac = Japanese eel infected with 40x *A. crassus*, N=10; J40An = Jap. eel infected with 40x *A. novaezelandiae*, N=11

3.4.3 Damage levels of swim bladders

The percentages of the assessed swim bladder damage levels according to the SDI are presented in Fig. 3.8; those of the SK are shown in Fig. 3.9. While 25 % of the European eels infected with *A. novaezelandiae* had no visible damages and were thus ranked with an SDI of 0, all other groups showed certain swim bladder alterations. SDI 1 was found in approximately 92 % of European eels infected with *A. crassus*, in 75 % of European eels infected with *A. novaezelandiae*, in 50 % of Japanese eels infected with *A. crassus*, and in 10 % of Japanese eels infected with *A. novaezelandiae*. SDI 2 was only determined for European eels infected with *A. crassus* (~8 %) and Japanese eels infected with *A. crassus* (50 %) and with

A. novaezelandiae (50 %). Solely swim bladders of Japanese eels infected with *A. novaezelandiae* correspond with SDI 3 (40 %).

Analyses of the swim bladders regarding the SK revealed a high percentage of SK 1 in European eels infected with *A. crassus* (~92 %) and with *A. novaezelandiae* (100 %). In Japanese eels infected with *A. novaezelandiae*, only 10 % of the swim bladders featured an SK 1. SK 2 was determined in approximately 8 % of the swim bladders of European eels infected with *A. crassus*; those infected with *A. novaezelandiae* showed no cases of SK 2. In Japanese eels infected with *A. novaezelandiae*, a share of 20 % was assigned to the SK 2 level. SK 3 was only identified in the Japanese eel infected with *A. novaezelandiae* with a share of 60 %. SKs for Japanese eels infected with *A. crassus* could not be determined due to mismatch of features with the underlying study.

Swim bladders of European eels infected with both *Anguillicola* species featured no macroscopically visible thickening and no encapsulated larvae in their swim bladder walls. In contrast, 50 % of swim bladders in Japanese eels infected with *A. crassus* had a thickened wall and 40 % contained encapsulated larvae. When infected with *A. novaezelandiae*, 90 % of the swim bladder walls in Japanese eels were thickened and 100 % showed encapsulated larvae.

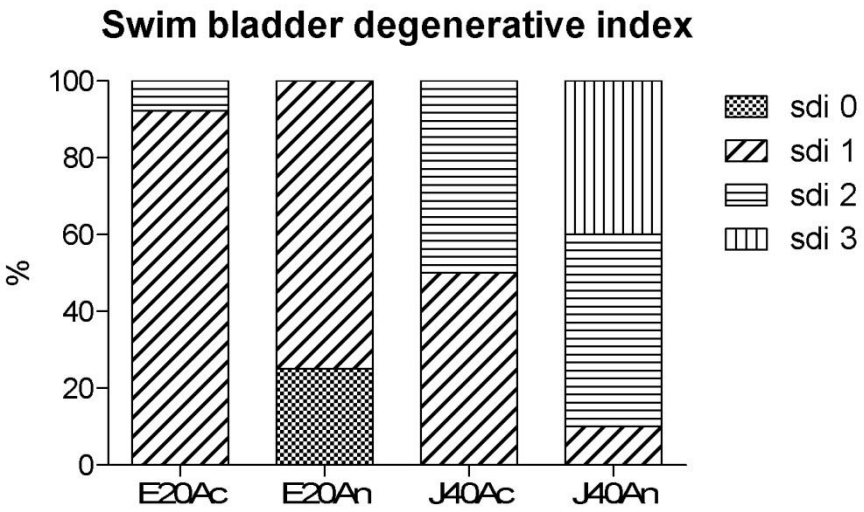

Fig. 3.8 Swim bladder degenerative index (SDI) at 120 dpi

Bars show distributions of SDI classifications according to Lefebvre et al. (2002).
E20Ac = European eel infected with 20x *A. crassus*, N=14; E20An = Europ. eel infected with 20x *A. novaezelandiae*, N=10; J40Ac = Japanese eel infected with 40x *A. crassus*, N=10; J40An = Jap. eel infected with 40x *A. novaezelandiae*, N=11

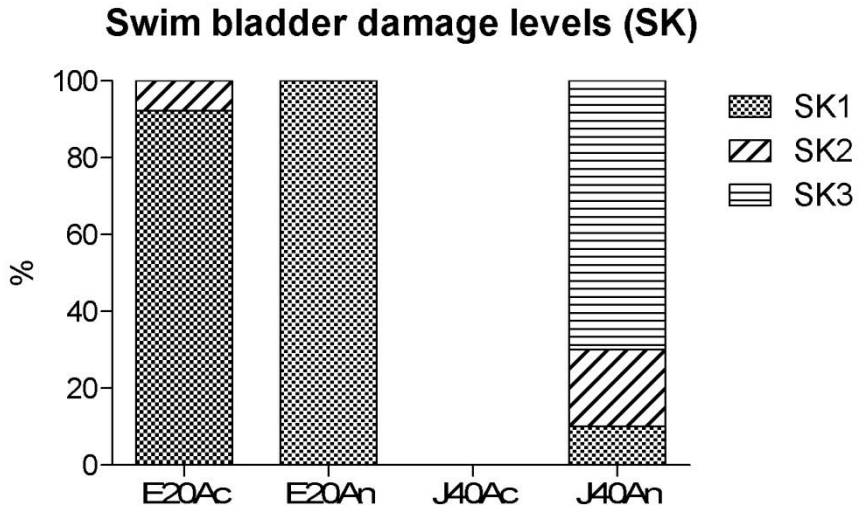

Fig. 3.9 Swim bladder damage levels (SK) at 120 dpi

Bars show distributions of swim bladder damage classifications according to Knopf (1999).
E20Ac = European eel infected with 20x *A. crassus*, N=14; E20An = Europ. eel infected with 20x *A. novaezelandiae*, N=10; J40Ac = Japanese eel infected with 40x *A. crassus*, N=10; J40An = Jap. eel infected with 40x *A. novaezelandiae*, N=11

3.5 Discussion

The present study showed distinct differences in the recovery rate and the development of fully adapted, newly joined up, and completely unfamiliar host-parasite systems. Recovery rates were generally found to be higher in the European eel than in the Japanese eel irrespective of the particular nematode species they were infected with. Especially in the European eel, *A. novaezelandiae* featured a higher infection success and a faster development compared to *A. crassus*, which is in correspondence to the results of Dangel et al. (2013). These findings may lead to the conclusion that the development of *A. crassus* in the European eel is retarded due to the adapted immune response of the host, which has been living together with this parasite for more than 30 years now. On the contrary, *A. novaezelandiae* is able to develop more unopposedly in this virtually unadapted host. As already discussed by Dangel et al. (2013), *A. novaezelandiae* shows a much more synchronized development than *A. crassus*. While the former parasite completed its development in the European eel at 120 dpi and no more larval stages could be detected, the latter parasite species featured an irregular development with both L3 and L4 stages at 90 and 120 dpi. The results on *A. crassus* in both eel species also correspond to a susceptibility study of Knopf and Mahnke (2004), in which similar recovery rates and infrapopulations for the Japanese and the European eel were determined, each infected with 30 larvae of *A. crassus* at 98 dpi. These findings imply that *A. crassus* in the European eel shows the least synchronized development of all four analyzed parasite-host systems. This might indicate that *A. crassus* in the European eel has an advantage over other parasite-host systems since it is able to produce more eggs over a longer period of time.

Interestingly, both parasite species performed quite similar in the Japanese eel. There were no L3 or L4 stages present, except for a single L4 finding of *A. crassus* at 120 dpi. The individuals found in the swim bladders of Japanese eels were either dead or fully developed. So the present study gives new insights into defence mechanisms of the Japanese eel towards a long-known parasite on the one hand and a new parasite species on the other hand. The results on the analyses of swim bladders in experimentally infected Japanese eels suggest that the revealed responses are not species-specific. Both systems involving Japanese eels featured thickening as well as encapsulation processes. From an evolutionary point of view, this reaction might be explained by the long-standing adaptation of the Japanese eel

to *A. crassus*, which may have led to a similar response to *A. novaezelandiae*, although the host has never naturally encountered this parasite. The rather low recovery rates and the high amounts of dead encapsulated larvae in both Japanese eel-nematode systems point to the assumption that this eel species has developed a strong immune response, which is unspecific to the parasite species. Studies by Nielsen (1999) and Knopf and Lucius (2008) have proved that *A. anguilla* lacks a specific humoral immune response against antigens of *A. crassus*, which can yet be found in *A. japonica.* This ability of generating a high immune competence against this long-known nematode may have led to a comparable reaction to the closely related species *A. novaezelandiae*. The effective response of Japanese eels to the particular antigens emerging from both parasite species is likely to contribute to a successful elimination of larvae penetrating the swim bladder wall. This reaction is also mirrored in the high percentage of encapsulated larvae found in Japanese eels which, in turn, corresponds to the findings on the thickening of their swim bladder walls. Although – indicated by the results of the control group – the swim bladders of Japanese eels inherently feature a higher thickness than those of European eels, the present study shows that the thickening of the swim bladder walls still correlates with the encapsulation of larvae. Since European eels had no encapsulated larvae at all, no thickening of their swim bladder walls occurred irrespectively of infection with either parasite species. As soon as encapsulated larvae were present in the swim bladder walls of Japanese eels, a further thickening occurred. While all layers of the swim bladder walls of Japanese eels were affected by thickening, those of European eels showed no sign of thickening. Except for the studies by Molnár (1994), Molnár et al. (1995), and Audenaert et al. (2003), in which an encapsulation of *A. crassus* larvae in European eels taken from Lake Balaton and from different sites in Belgium was observed, there has been no evidence for such a response of the European eel.

The present study generally revealed only minor damages of the swim bladders of European eels. A study by Würtz and Taraschewski (2000) pointed out that natural *A. crassus* infections of European eels caused a heavy folding of the epithelium, whereas laboratory infections were mainly characterized by inflammatory processes that were not associated with thickening. The described characteristic folding of the epithelium can yet be observed in Japanese eels experimentally infected with either parasite species (Fig. 3.5). It remains unclear if there are strong differences between naturally or experimentally infected eels. Studies by Würtz et al.

(1998) and Abdelmonem et al. (2010) also disclosed a possible thickening of swim bladder walls in wild European eels. Würtz and Taraschewski (2000) concluded that a thickening of the swim bladder wall is much more likely in naturally infected European eels, whereas experimentally infected individuals normally show no signs of thickening unless other impairing factors are involved. This indicates that, apart from a nonrecurring *Anguillicola* infection, other factors possibly occurring under natural conditions may lead to a thickening of the swim bladder wall. The present study shows those swim bladder damages that are solely associated with the respective *Anguillicola* infection and excludes all other factors that could have previously affected the swim bladders.

When regarding the results of the thickness measurements, there is a clear association between the respective thickening process and the corresponding swim bladder damage level as one main criterion for both classification systems is the thickness of the wall or the volume of the lumen, respectively. In some cases, however, it was difficult to clearly define the particular damage class using the established indices. While the SDI by Lefebvre et al. (2002) takes all grades of damage into account by adding up all observed alterations, the SK by Hartmann (1994) and Knopf (1999), respectively, is limited to clearly defined grades, which cannot be combined or graded any further. This fixed classification made it impossible to conclusively assign the swim bladder damages of the Japanese eel to a particular SK (Fig. 3.8). In some cases, there was a variance of three grades within the SK classification, as there was an overlap of visible features belonging to three different grades. One might assume that this index is only applicable to the well-investigated host-parasite system of the European eel infected with *A. crassus*. It also seems to be questionable whether those damage classes may be equally employed on both wild and laboratory eels. The present study shows those swim bladder damages that are solely associated with the respective *Anguillicola* infection and excludes all other factors that could have previously affected the swim bladders. Other impairing factors or repeatedly occurring infections influencing the condition of swim bladders in natural eel populations might have led to more severe histopathological damages like extreme thickening, inflammation, and fibrosis that were previously described in other studies (Würtz et al. 1998; Würtz and Taraschewski 2000; Abdelmonem et al. 2010). Moreover, the results on the definite measurements of the swim bladder walls imply that the existing classification

systems cannot be equally applied to both eel species. Hartmann has assigned a benchmark of up to 1.00 mm defining a normal swim bladder thickness; a value of up to 5.00 mm is classed with the most severe damage class of SK 5. The SDI by Lefebvre also takes a value of less than 1.00 mm as a basis for an unaffected swim bladder, whereas the highest damage degree (value 2) in this category is based upon a thickness value of more than 3.00 mm. Taking these specified values into account, the measurement results in this study are contradictory to the scales determined by the two mentioned classification systems. In this regard, almost all analyzed swim bladder walls would be regarded as unimpaired since most of them featured a thickness value lower than 1.00 mm.

A macroscopic evaluation of the thickness of swim bladders seems to be quite vague and arbitrary, so an objective classification based on visible alterations is difficult. Lefebvre et al. (2011) proposed involving two independent observers in order to ensure objectivity, but even with two observers, there was a high coefficient of variation with regard to the SDI. The present study suggests that it might be useful to include histological measurements into a damage classification, since thickness can be precisely measured. In this case, the criterion of easiness, which should generally pertain to an index, is subordinated. A similar difficulty applies to the evaluation of opacity which is another underlying criterion of SDI and SK, since opacity cannot be precisely measured and a macroscopic evaluation might be biased as well.

The findings of this study suggest that the two considered damage classification systems proved to be unsuitable for comparing naturally and experimentally infected eels on the one hand and European and Japanese eels on the other hand. Since there has been no study focusing on swim bladder damages and a possible classification system for either naturally or experimentally infected Japanese eels so far, further research should be conducted on this host species.

4 Size does matter – a closer look on *Anguillicola* morphology

Michelle Keppel, Kerstin Claudia Dangel & Bernd Sures

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4.1 Summary

The present study deals with morphological differences between two closely related parasitic nematode species (*Anguillicola crassus* Kuwahara et al., 1974 and *Anguillicola novaezelandiae* Moravec & Taraschewski, 1988) in two different experimentally infected eel species (*Anguilla anguilla* Linnaeus, 1758 and *Anguilla japonica* Temminck & Schlegel, 1847). Furthermore, it considers the question whether size differences between those two species are ontogenetically determined or host species-dependent. In order to analyze these questions, experimental infections with the four possible host-parasite systems have been performed, followed by precise morphometric measurements related to body size and head structures of all resulting nematodes 120 days post infection.

Body size measurements (length and width) of *A. crassus* generally exceeded those of *A. novaezelandiae*, while both *Anguillicola* species turned out to be smaller in Japanese eels than in European eels. Comparative measurements of neck width, maximum oesophagus width, and posterior head end width were found to be highly significant with regard to the different host-parasite systems. Shape and width of neck have been identified as reliable discriminating factors for species distinction. Generally, the relation of anterior head end width and neck width proved to be distinctly species-specific and can thus serve as a decisive and easily measureable distinguishing feature.

4.2 Introduction

The family Anguillicolidae represents five morphospecies of *Anguillicola* nematodes parasitic to freshwater eels (*Anguilla* sp.), which were originally divided into the two subgenera *Anguillicola* with one species (*A. globiceps* Yamaguti, 1935) and *Anguillicoloides*, consisting of four species (*A. australiensis* Johnston & Mawson, 1940; *A. crassus* Kuwahara et al., 1974; *A. novaezelandiae* Moravec & Taraschewski, 1988; *A. papernai* Moravec & Taraschewski, 1988) (Moravec and Taraschewski 1988). Although there are common properties among the species of *Anguillicoloides*, *A. globiceps* features some morphological distinctions. However, a study by Laetsch et al. (2012) applying DNA taxonomy revealed that there is no molecular evidence for a division of Anguillicolidae into two different genera. So on that account, as suggested by Laetsch et al. (2012), the present study refers to the two species *A. crassus* and *A. novaezelandiae* as members of the genus *Anguillicola*. For distinguishing the different species on a morphological basis, there are several studies describing anatomical features and detail measurements of body characteristics of adult (Taraschewski et al. 1987; Moravec and Taraschewski 1988; Moravec and Rohde 1992; Lefebvre et al. 2004; Münderle 2005; Moravec 2006; Münderle et al. 2006; Brunanska et al. 2007, 2010; Rolbiecki 2008) and larval anguillicoloid species (Blanc et al. 1992) taken from wild eel populations and from experimentally infected eels (Moravec et al. 1994a). In order to analyze potential evolutionary divergence, one study has focused on measuring nematode populations of one species from different sites in the world in two experimentally infected eel species (Weclawski et al. 2014). These studies show that there is quite a wide morphometric range among individuals of one single species and overlapping size ranges of body characteristics between different *Anguillicola* species, which sometimes causes problems with morphological species identification. Assuming that parasites require a much higher effort to resist the original host's immune defence, one can hypothesize that time of co-evolution may also affect size characteristics of the nematodes. For example, individuals of *A. crassus* are generally smaller in their original host, the Japanese eel (*Anguilla japonica* Temminck and Schlegel, 1847), than in the newly adapted European eel (*Anguilla anguilla* Linnaeus, 1758) (Weclawski et al. 2014). Referring to findings on recovery rates by Keppel et al. (2014), Japanese eels presumably feature a similarly strong immune response to different *Anguillicola* species such as *A. crassus* and *A. novaezelandiae*. Accordingly,

one may expect that the morphological parameters of both nematode species in the Japanese eel will also strongly deviate from the data that will be assessed in the European eel.

While *A. crassus* and the Japanese eel constitute a long-standing original parasite-host association (Kuwahara et al. 1974), this nematode species was recorded from European eels in European open waters for the first time in the mid-1980s (Neumann 1985) and has even spread to populations in North Africa ever since (Maamouri et al. 1999; Loukili and Belghyti 2007; Abdelmonem et al. 2010). *A. novaezelandiae* is endemic to the Short-finned eel (*Anguilla australis* Richardson, 1841) from New Zealand and Australia (Moravec 2006) and has been introduced to Europe in 1975 (Paggi et al. 1982). To the present day, this nematode species has only been found in a single European spot, in Lake Bracciano, Italy, and seems to have disappeared after its last record in 1993 (Moravec et al. 1994b; Dangel et al. 2015), which suggests that no adaptation could have occurred.

As there has not been any study dealing with comprehensive morphometric comparisons of different *Anguillicola* species in different host species under laboratory conditions, the aim of the present study was to use differently adapted *Anguillicola*-host combinations to test for host-dependent size differences versus species-specific morphological characteristics. Accordingly, the present study includes parasite-host systems that have undergone long-term (*A. crassus* and *A. japonica*), short-term (*A. crassus* and *A. anguilla*), and no mutual adaptation at all (*A. novaezelandiae* and *A. japonica*/*A. anguilla*). This study was also aimed at revealing precise and easily detectable morphometric distinctions between the two *Anguillicola* species and to check both species for any specific morphological features that are ontogenetically determined in contrast to those which are host-dependent.

4.3 Material and methods

4.3.1 Experimental infections of eel hosts

European and Japanese eels were experimentally infected with either 20 or 40 larvae of each *Anguillicola* species, were kept constantly at 21 °C, and were examined at 120 days post infection (dpi). All parasites used in the present study have been taken from infection experiments as described in Dangel et al. (2013) and Keppel et al. (2014). An overview of the experimental setup is presented in Table 4.1. Based on previous studies, which revealed different recovery rates for the two *Anguillicola* species, European eels were generally infected with 20 larvae and Japanese eels with 40 larvae. This proportion was supposed to ensure comparable infrapopulation sizes. In order to check for any density-dependent effects, a group of two European eels was additionally infected with 40 larvae of *A. novaezelandiae*.

Tab. 4.1 Overview of experimental infections with different eel and *Anguillicola* species

Table shows host-parasite systems that were used for experimental infections including eel sample sizes, eel body size ranges, the respective amounts of administered L3 stage larvae, mean intensities for each system, and the nematode sample sizes for each sex.

Host species (N)	Host length (range in cm)	infected with	N parasites recovered (mean)	N parasites measured	
				male	female
A. anguilla (7)	32.0 – 39.5	20x *A. crassus*	8.3	44	16
A. anguilla (4)	37.5 – 46.5	20x *A. novaezelandiae*	7.9	29	25
A. anguilla (2)	52.0 – 54.0	40x *A. novaezelandiae*	18.5	25	7
A. japonica (5)	39.4 – 47.5	40x *A. crassus*	2.5	11	13
A. japonica (4)	44.0 – 59.0	40x *A. novaezelandiae*	1.5	12	7

4.3.2 Examination of parasites

Morphometric analyses included measurements of the parasites' overall length and width at the broadest part of the body as well as other six detail measurements of heads and oesophagi, respectively (Fig. 4.1). Measurement 1 refers to the width of the anterior head end, which is characterized by a slightly expanded head structure. Measurement 2 was taken at the neck of the nematodes' heads; number 3 represents the width of the upper part of the oesophagus. The maximum oesophagus width, i.e. its width at the broadest part, is represented by measurement 4, while its total length is represented by measurement 5. The posterior head end width (6) was measured at the end of the oesophagus. Although previous studies suggest measurements of the buccal capsule for distinguishing between the two nematode species, this was not taken into account in this study as one of the aims was to define easily measurable features without extensive preparations. All morphometric measurements were conducted by means of a stereomicroscope (magnification x8 to

x50) with a built-in camera (Olympus) and an image-processing program (ImageJ, National Institutes of Health, USA).

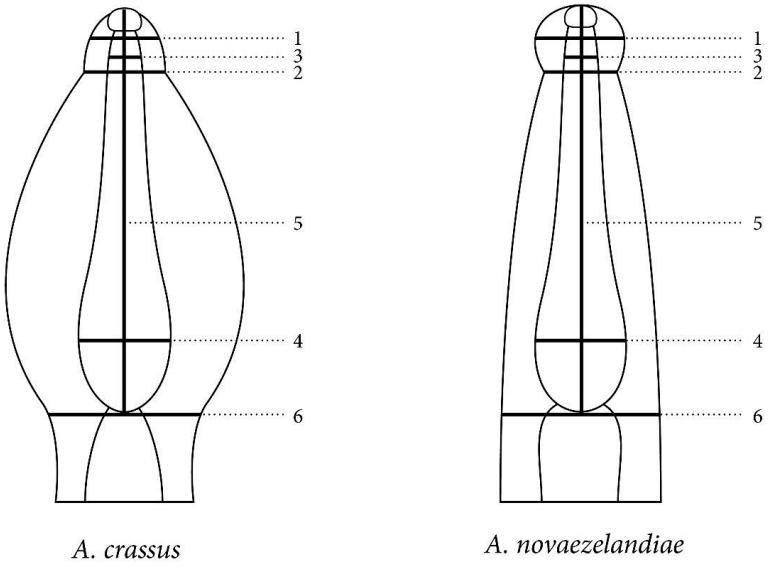

Fig. 4.1 Morphometric measurements of *Anguillicola* species

All conducted measurements (1-6) on *A. crassus* (left) and *A. novaezelandiae* (right): 1 anterior head end width, 2 neck width, 3 upper oesophagus width, 4 max. oesophagus width, 5 oesophagus length, 6 posterior head end width

4.3.3 Statistical treatment of data

Differences between the mean values of the infection groups were tested for significance by means of the t-test (SigmaPlot 12, Systat Software Inc.) and are displayed using Origin Pro 9.0G (OriginLab Corp.). Significance was accepted when $p < 0.05$ (*) or $p < 0.001$ (**), respectively. All measurement results were checked for significant ratios and quotients. In order to ascertain distinctive morphometric factors for the two nematode species, a discriminant analysis has been performed (Origin Pro 9.0G).

4.4 Results

4.4.1 Morphometric analyses of parasites

All recovered parasites were analyzed according to Fig. 4.1. Ranges of all measured data are summarized in Table 4.2, and selected characteristics are presented in Figs. 4.2, 4.3, 4.4, 4.5, 4.6, and 4.7. Females of both species were generally found to be larger than the respective males, both in length and in width. Compared to *A. novaezelandiae*, *A. crassus* individuals of both sexes were longer and wider in each eel species. Both nematode species had a reduced length in the Japanese eel in relation to those in the European eel (Fig. 4.2). Individuals taken from European eels infected with 40x *A. novaezelandiae* tended to be even larger than those infected with only 20 larvae. Comparisons between the mean values of width in all infection groups (Fig. 4.3) showed highly significant differences between *A. novaezelandiae* in the European eel on one side and in the Japanese eel on the other side. Additionally, highly significant width differences were found for *A. crassus* compared to *A. novaezelandiae* in the Japanese eel. Width values of *A. crassus* and *A. novaezelandiae* in the European eel differed significantly ($p < 0.05$).

Tab. 4.2 Data on morphometric measurements of all *Anguillicola* spp. infection groups 120 dpi

Table shows all considered morphometric measurements of two *Anguillicola* species in two different host species 120 days post infection. All measurements are given in mm. N = number of individuals

Host	**European eel**						**Japanese eel**			
Parasite species	**20x *A. crassus***		**20x *A. novaezelandiae***		**40x *A. novaezelandiae***		**40x *A. crassus***		**40x *A. novaezelandiae***	
Sex	**male**	**female**	**male**	**female**	**male**	**female**	**male**	**female**	**male**	**female**
N	44	16	29	25	25	7	11	13	12	7
length	4.776 - 27.728	21.072 - 40.426	7.650 - 19.055	12.386 - 27.417	12.206 - 23.571	19.634 - 28.004	8.856 - 16.912	22.732 - 31.980	2.041 - 11.750	10.808 - 16.616
max. width	0.235 - 1.898	1.821 - 3.803	0.498 - 1.527	1.064 - 2.571	0.796 - 1.756	1.519 - 2.926	0.806 - 1.519	2.464 - 3.639	0.141 - 0.748	0.612 - 1.364
length/width	8.849 - 20.323	7.171 - 15.603	10.734 - 18.174	8.172 - 14.725	10.017 - 19.104	8.911 - 13.594	8.422 - 14.848	6.458 - 10.162	10.816 - 21.610	11.591 - 15.077
anterior head end width (1)	0.057 - 0.264	0.179 - 0.430	0.065 - 0.260	0.110 - 0.412	0.039 - 0.244	0.195 - 0.212	0.068 - 0.221	0.103 - 0.224	0.060 - 0.149	0.108 - 0.261
neck width (2)	0.074 - 0.289	0.194 - 0.495	0.048 - 0.222	0.078 - 0.249	0.034 - 0.179	0.110 - 0.194	0.088 - 0.274	0.129 - 0.283	0.049 - 0.105	0.098 - 0.157
upper oesophagus width (3)	0.031 - 0.139	0.069 - 0.175	0.028 - 0.101	0.046 - 0.230	0.017 - 0.140	0.078 - 0.140	0.036 - 0.153	0.062 - 0.182	0.021 - 0.061	0.045 - 0.113
max. oesophagus width (4)	0.108 - 0.261	0.183 - 0.308	0.062 - 0.237	0.075 - 0.358	0.075 - 0.263	0.136 - 0.301	0.098 - 0.238	0.155 - 0.256	0.040 - 0.139	0.089 - 0.213
oesophagus length (5)	0.414 - 0.972	0.459 - 1.108	0.344 - 0.803	0.456 - 1.404	0.327 - 0.695	0.549 - 0.676	0.509 - 0.762	0.564 - 0.832	0.249 - 0.675	0.500 - 0.774
oesophagus length/width	2.187 - 6.288	1.246 - 5.235	2.433 - 9.586	1.865 - 6.587	1.901 - 6.704	2.010 - 4.779	2.139 - 6.908	2.431 - 4.819	4.656 - 8.408	3.028 - 7.517
ratio of oesophagus/ body length	1 : 8.724 - 49.500	1 : 26.618 - 54.612	1 : 14.023 - 44.081	1 : 16.726 - 46.588	1 : 22.479 - 52.245	1 : 30.206 - 49.142	1 : 14.330 - 33.226	1 : 30.095 - 47.168	1 : 4.830 - 24.763	1 : 16.155 - 30.444
posterior head end width (6)	0.142 - 0.638	0.359 - 0.787	0.185 - 0.774	0.253 - 0.700	0.226 - 0.473	0.321 - 0.478	0.182 - 0.382	0.292 - 0.832	0.081 - 0.300	0.138 - 0.462

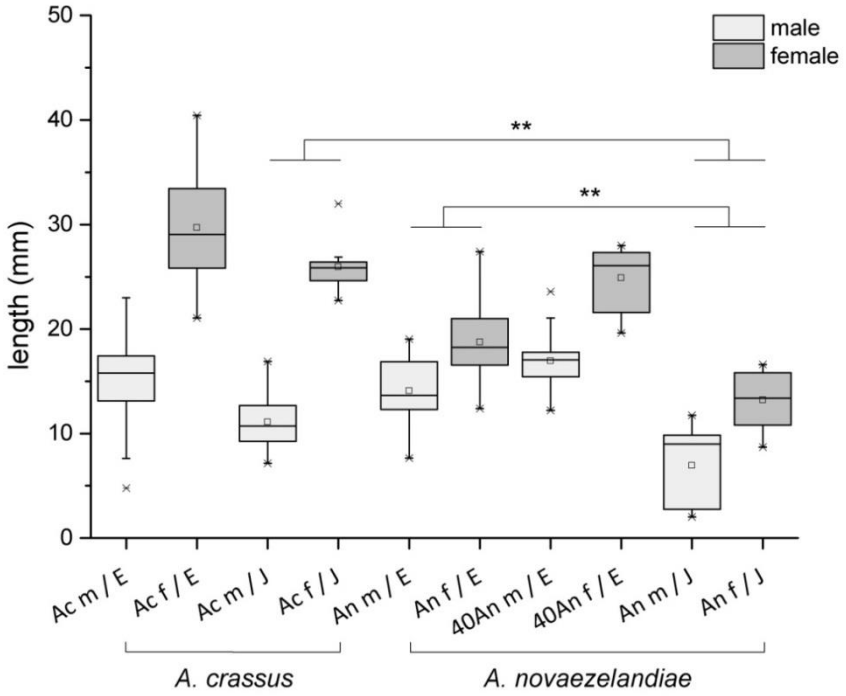

Fig. 4.2 Length measurements of all *Anguillicola* spp. infection groups 120 dpi

Box plots show ranges of length measurements of both *Anguillicola* species in five infection groups. Male nematodes are presented in light grey, and female nematodes in dark grey.
Ac = *A. crassus*, An = *A. novaezelandiae*, m = male, f = female, E = European eel, J = Japanese eel, 40 = infected with 40 larvae; significance levels: $p < 0.05$ (*), $p < 0.001$ (**)

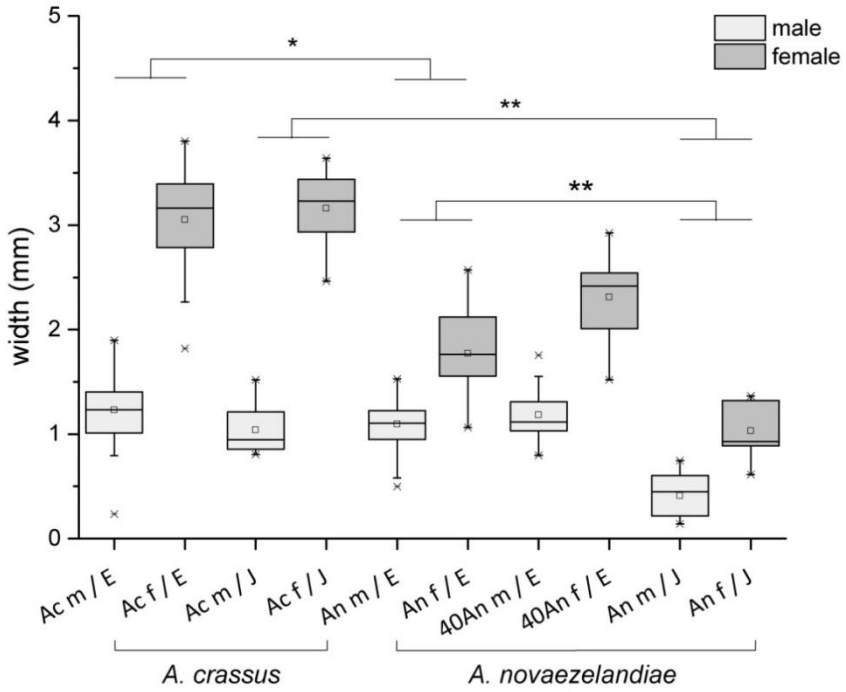

Fig. 4.3 Width measurements of all *Anguillicola* spp. infection groups 120 dpi

Boxplots show ranges of width measurements of both *Anguillicola* species in five infection groups. Male nematodes are presented in light grey, and female nematodes in dark grey.
Ac = *A. crassus*, An = *A. novaezelandiae*, m = male, f = female, E = European eel, J = Japanese eel, 40 = infected with 40 larvae; significance levels: $p < 0.05$ (*), $p < 0.001$ (**)

Data on neck width (Fig. 4.5), maximum oesophagus width (Fig. 4.6), and posterior head end width (Fig. 4.7) turned out to be statistically relevant. In those three measurement categories, *A. crassus* and *A. novaezelandiae* in the European eel and *A. novaezelandiae* in the European eel compared to *A. novaezelandiae* in the Japanese eel featured highly significant differences. In terms of neck width, all pooled values on *A. crassus* differed highly significantly from those on *A. novaezelandiae.* In both eel species, *A. novaezelandiae* individuals of both sexes featured lower neck width values compared to *A. crassus*.

By means of the discriminant analysis, the variable of neck width proved significantly suitable for generally discriminating between *A. crassus* and *A. novaezelandiae*, irrespective of the particular host species. The factor of maximum oesophagus width turned out to be the second best suitable factor. When comparing values of anterior head end width (Fig. 4.4) and neck width measurements, the two nematode species can be distinctly distinguished (Fig. 4.8). Basically, all neck width values of *A. crassus*

were found to be higher than anterior head width values, whereas neck width measurements of *A. novaezelandiae* were generally lower than anterior head end width measurements.

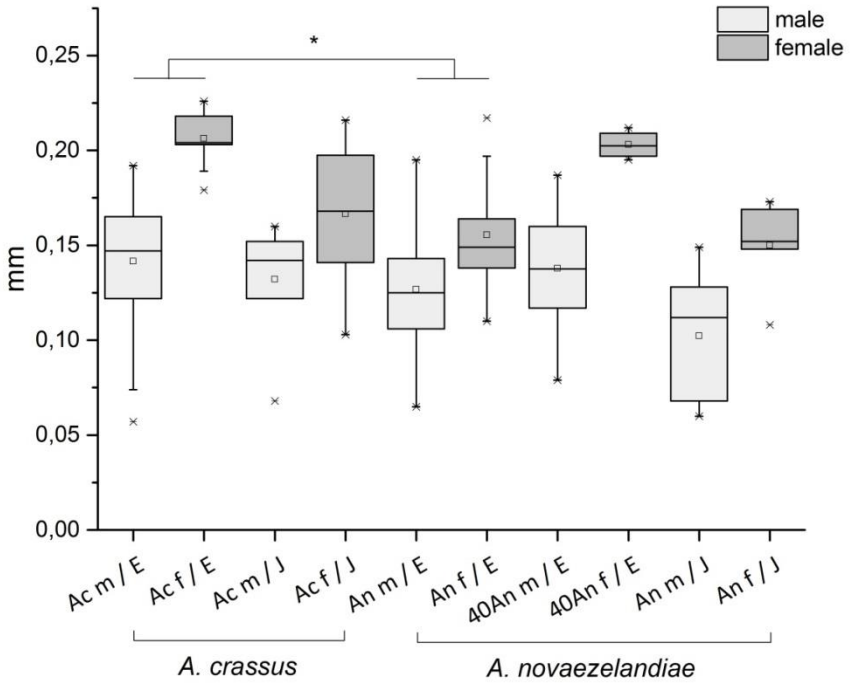

Fig. 4.4 Measurements of anterior head end width of all *Anguillicola* spp. infection groups 120 dpi

Boxplots show ranges of anterior head end width (1) measurements of both *Anguillicola* species in five infection groups. Male nematodes are presented in light grey, and female nematodes in dark grey.
Ac = *A. crassus*, An = *A. novaezelandiae*, m = male, f = female, E = European eel, J = Japanese eel, 40 = infection with 40 larvae; significance levels: $p < 0.05$ (*), $p < 0.001$ (**)

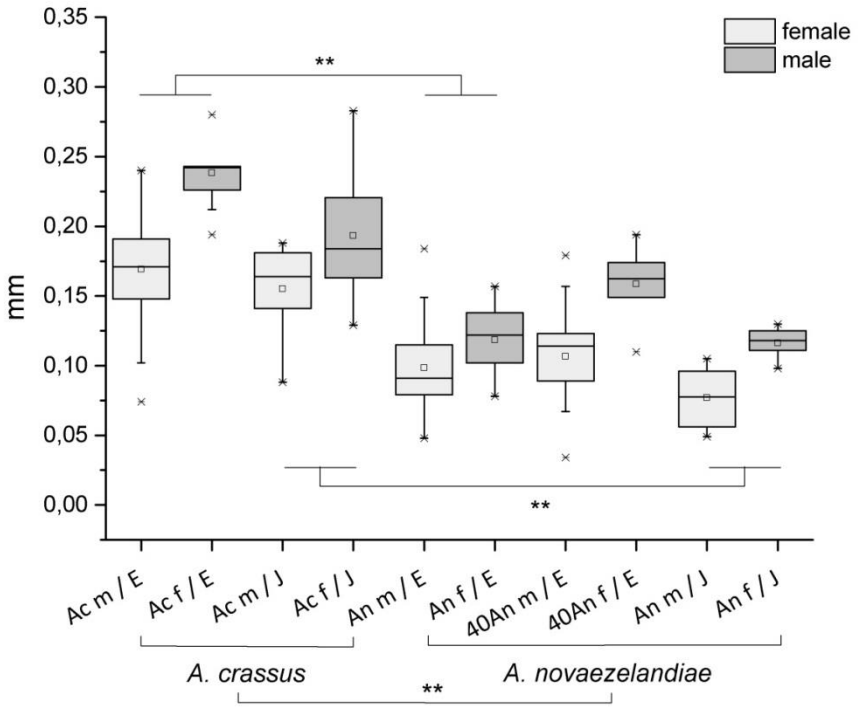

Fig. 4.5 Measurements of neck width of all *Anguillicola* spp. infection groups 120 dpi

Boxplots show ranges of neck width (2) measurements of both *Anguillicola* species in five infection groups. Male nematodes are presented in light grey, and female nematodes in dark grey.
Ac = *A. crassus*, An = *A. novaezelandiae*, m = male, f = female, E = European eel, J = Japanese eel, 40 = infection with 40 larvae; significance levels: $p < 0.05$ (*), $p < 0.001$ (**)

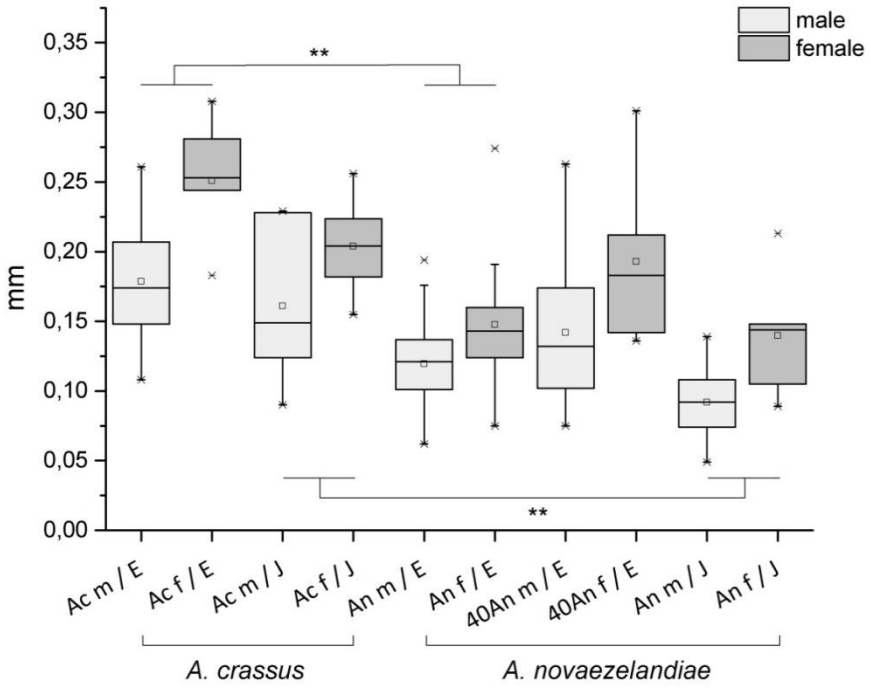

Fig. 4.6 Measurements of maximum oesophagus width of all *Anguillicola* spp. infection groups 120 dpi

Boxplots show ranges of maximum oesophagus width (4) measurements of both *Anguillicola* species in five infection groups. Male nematodes are presented in light grey, and female nematodes in dark grey.
Ac = *A. crassus*, An = *A. novaezelandiae*, m = male, f = female, E = European eel, J = Japanese eel, 40 = infected with 40 larvae; significance levels: $p < 0.05$ (*), $p < 0.001$ (**)

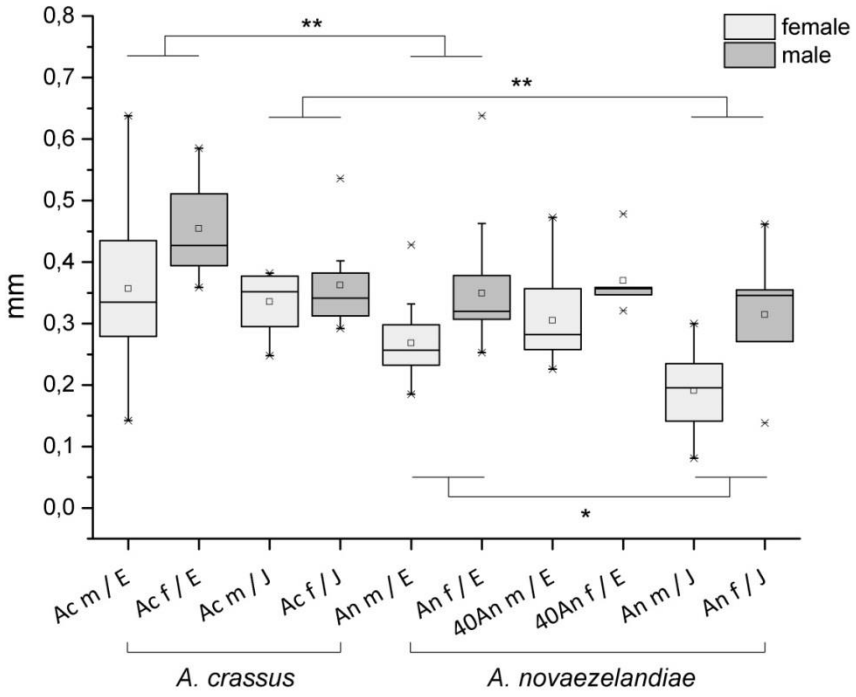

Fig. 4.7 Measurements of posterior head end width of all *Anguillicola* spp. infection groups 120 dpi

Boxplots show ranges of posterior head end width (6) measurements of both *Anguillicola* species in five infection groups. Male nematodes are presented in light grey, and female nematodes in dark grey.
Ac = *A. crassus*, An = *A. novaezelandiae*, m = male, f = female, E = European eel, J = Japanese eel, 40 = infected with 40 larvae; significance levels: $p < 0.05$ (*), $p < 0.001$ (**)

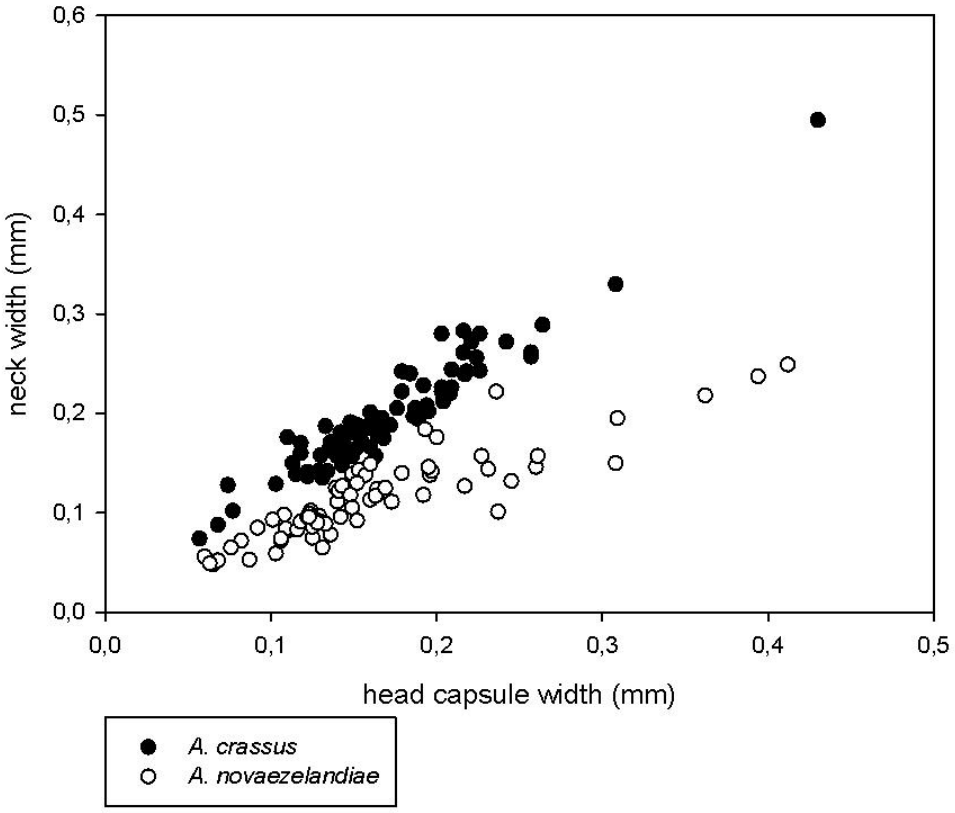

Fig. 4.8 Relation between anterior head end width and neck width of all *Anguillicola* spp. individuals 120 dpi

Circles show values of anterior head end width (1) and neck width (2) for each measured parasite. Dark circles represent values for *A. crassus*; light circles represent values for *A. novaezelandiae*

4.5 Discussion

The results of this study confirmed existing data but also revealed new findings on the morphology of the two analyzed *Anguillicola* species.

The remarkable sexual dimorphism according to Moravec (1994) could be substantiated by strong differences not only in body length and width but also in all measured head morphology values. Data on length could prove that both male and female individuals of *A. crassus* feature a reduced maximum size in the Japanese eel compared to the European eel, though with a much broader range.

A. novaezelandiae individuals were also found to be significantly smaller in the Japanese eel than in the European eel. Both findings support the assumption that Japanese eels are able to generate a strong immune defence, no matter which of the two considered *Anguillicola* species they are confronted with. These differences in size correspond to the study of Keppel et al. (2014), in which recovery rates for both nematode species in Japanese eels were significantly lower and a high number of dead larvae were recorded in the swim bladder walls. The marked size differences in the present study may be traced back to the nematodes' striving against the immune response of the host. General size data on *A. novaezelandiae* in the Japanese eel strongly deviate from findings in its original host *A. australis*. Existing data by Moravec and Taraschewski (1988), Lefebvre et al. (2004), and Dangel and Sures (2013) on the original wild parasite-host system show that there is usually a very wide size range, so host-specific regulation of size could not be observed, whereas present data on *A. novaezelandiae* in the Japanese eel indicate that the parasite's size is regulated by this particular host immune response. So far, there has been no study dealing with the immune response of *A. australis* to any *Anguillicola* infection, while *A. japonica* presumably features a strong immune defence to both nematode species and *A. anguilla* seems to have a comparably weaker immune response (Nielsen 1999; Knopf and Lucius 2008; Keppel et al. 2014).

When comparing the current results on *A. crassus* in the European eel to those by Moravec et al. (1994a), who collected measurements on *A. crassus* in experimentally infected European eels at 98 dpi and at a temperature of 20-22 °C, it is striking that their results on length and width are both far below those presented in this study. This could either be due to the small sample size (N=6) or to the shorter period of growth until the worms were examined. In comparison to the study by Weclawski et al. (2014), in which European eels were experimentally infected with *A. crassus* and analyzed at 100 dpi, the respective current data sets are all within the same range and are showing a similar maximum body length. With regard to the results on *A. crassus* in the Japanese eel, Weclawski et al. (2014) found strong variations between parasite populations originating from three different countries (Germany, Poland, and Taiwan). *A. crassus* individuals of the present study were taken from a German parasite population and are on average slightly bigger in terms of length and width than individuals of the German parasite population in Weclawski et al. (2014). Especially the female nematodes strongly differ in their minimum size.

This could possibly be explained by the 20 days longer growth period in the present study, so that the female individuals could turn gravid and thus feature a higher body size for harbouring mature eggs. On the other hand, the parasites originating from Poland and Taiwan showed a similar or even higher maximum body length, while the minimum length was comparatively low. These wide ranges might be based on the generally unsynchronized development of *A. crassus*, as stated by Dangel et al. (2013). Larvae of *A. crassus* mature within different time periods, so that rather young and small adults could occur simultaneously with older and larger ones.

The present study also dealt with the question whether particular morphological features of the two *Anguillicola* species are ontogenetically determined or dependent on other features. Moravec et al. (1994a) stated in their study on European eels experimentally infected with *A. crassus* that the body size of adults is related to the size of the host. Fazio et al. (2008) described a correlation between the host's swim bladder size and the body weight of nematodes, i.e. the overall size of nematodes. Van Banning and Haenen (1990) also stated that the nematode size is adapted to the restricted lumen of the host's swim bladder. In the present study, all eels were selected according to comparable body sizes, so that a relation to parasite size can be excluded (see Table 4.1). The length of the infected swim bladders was recorded as well, but this data could not be linked to the size of the nematodes. So one might assume that the body size of the two *Anguillicola* species is directly dependent on the particular host species, in fact on the immune response of the respective host, as already stated in other publications on oxyurid nematodes (Sorci et al. 2003) and on *Strongyloides ratti* Sandground, 1925 (Wilkes et al. 2004).

According to original species descriptions by Moravec and Taraschewski (1988), individuals of *A. novaezelandiae* were expected to feature a slight neck constriction in contrast to a tapering anterior head end of *A. crassus* (see also Fig. 4.1). They also provided measurement data, but those on *A. novaezelandiae* were based on a small sample size. This distinct attribute could be proved in this study, based on microscopic measurements and on statistical assessment. Consequently, this feature could be adduced as a distinct morphological trait for distinguishing those two closely related *Anguillicola* species. The present morphological measurements show plainly that there is an underlying general rule. Where *A. crassus* is concerned, the neck width value is higher than the anterior head width value, whereas *A. novaezelandiae* categorically features a lower neck width compared to the anterior

head end width. While Münderle (2005) suggested a regression analysis of body length in relation to head capsule width, the present method is applicable in an easier and quicker way. Cutting the nematodes' heads and measuring the head capsules is a much more complex procedure than the one presented here, and additionally, it is possible to perform these two measurements on living parasites.

In order to prove the validity of this species-specific morphological feature for wild parasite populations, further studies on both *Anguillicola* species taken from wild eels should be performed. Moreover, other *Anguillicola* species, for instance *A. australiensis* with its marked spherical-shaped head, could be checked for distinctive, statistically supported morphological traits, which could serve as distinguishing characteristics as well. The discovery of an easily measureable and quickly applicable body feature analysis like the one presented in this study may facilitate any species identification task apart from molecular analyses which may also allow precise species identification (Grabner et al. 2012).

5 The hsp70 response of *Anguillicola* species to host-specific stressors

Michelle Keppel, Kerstin Claudia Dangel & Bernd Sures

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5.1 Summary

The present study is based on infection experiments of two different swim bladder parasite species, *Anguillicola crassus* Kuwahara et al., 1974 and *A. novaezelandiae* Moravec and Taraschewski, 1988, which were experimentally transferred to the two eel species *Anguilla anguilla* Linnaeus, 1758 and *A. japonica* Temmink and Schlegel, 1846, respectively. The host-parasite groups were selected due to their different grades of mutual adaptation. The main aim of this study was to analyze the stress responses within the parasites, which were confronted with different hosts, i.e. different stressors related to the respective host. For this purpose, mean intensities, recovery rates, larvae output, and levels of synthesized heat shock proteins (hsp70) were determined in nematodes of each infection group. Increased stress responses were detected in the endemic system of *A. crassus* parasitizing *A. japonica* and *A. crassus* in its recently acquired host *A. anguilla*, which seems to be associated with the immune response of the particular host species and the expenditure of energy on producing larvae. *Anguillicola novaezelandiae* showed overall weak activities in its unknown host species *A. japonica*, with the lowest recovery rate of all examined groups neither featuring elevated hsp responses, nor a high mean intensity, nor any reproductive output. On the contrary, in *A. anguilla*, the parasite reached higher recovery rates, mean intensities, and reproductive output, but no increased hsp70 levels could be detected. The four considered factors proved partially interdependent, whereas few results did not follow a clear pattern.

5.2 Introduction

One of the best investigated non-indigenous aquatic parasite species is the hematophagous swim bladder nematode *Anguillicola crassus* Kuwahara et al., 1974 (synonym *Anguillicoloides crassus*, see Laetsch et al. 2012), which is endemic to the Japanese eel (*Anguilla japonica* Temmink and Schlegel, 1846) and which has been accidentally introduced into populations of the European eel (*Anguilla anguilla* Linnaeus, 1758) in the early 1980s (Neumann 1985). Since this newly acquired host has undergone only short-term adaptation compared to the original host, *A. crassus* attains remarkably higher infection intensities both in naturally and in experimentally infected eels (Knopf and Mahnke 2004; Moravec 2006; Keppel et al. 2014) and causes partially severe swim bladder damages (Würtz and Taraschewski 2000), which could result in impairment of the swim bladder function (Barry et al. 2014). Numerous studies have already focused on different effects of *Anguillicola* infections on original and new eel host species (Würtz et al. 1995; Kelly et al. 2000; Lefebvre et al. 2004; Knopf 2006; Han et al. 2008; Fazio et al. 2009; Fazio et al. 2012; Dangel et al. 2014), whereas there are only few studies dealing with the parasite's perspective of infecting different final host species (Münderle 2005; Fazio et al. 2008; Weclawski et al. 2014; Keppel et al. 2015). For example, it can be assumed that infecting a final host with a strong immune response is a stressful situation for the parasite. In order to unravel stress levels in organisms, different physiological reactions, commonly referred to as biomarkers, can be analyzed. In free-living organisms such as fish, nematodes, or amphipods, biomarker responses may give indication of stress responses on various abiotic and biotic stressors (Basu et al. 2001; Arts et al. 2004; Sures and Radszuweit 2007; Frank et al. 2013; Dangel et al. 2014; Grabner et al. 2014; Teigen et al. 2015). One of those established biomarkers is the group of highly conserved heat shock proteins (hsps), which can be detected in all organisms (Köhler 2009). Given the fact that hsp levels shed light on any kind of environmentally induced stress in potential hosts (Basu et al. 2002; Roberts et al. 2010; Sung and MacRae 2011), parasites may also show a stress response to environmental conditions within their hosts. Several studies have already revealed the mutual dependence of heat shock proteins in hosts and parasites as they feature a protective function against each other's responses during their relation and that hsps belong to the most abundant proteins expressed by many parasite species (Newport et al. 1988; Kaufmann 1990; Mazier and Mattei 1991; Maresca and Kobayashi 1994;

Feder and Hofmann 1999). Except for studies focusing on heat stress in parasites (Sures and Radszuweit 2007; Chen et al. 2014), there is no study available that analyzes the response of parasites on other forms of stress related to their environments in specific hosts. Therefore, the present study focuses on the stress response of the parasite exposed to host-specific stressors assessed by the expression of hsps with a molecular weight of 70kDa (hsp70) in the parasite's tissue. The aim was to elucidate how the parasite copes with responses of the host, intraspecific competition related to mean intensities and with energetically costly reproduction. For comparing different reactions, the two closely related nematode species *Anguillicola crassus* and *A. novaezelandiae* Moravec and Taraschewski, 1988 were used for experimental infections of the two eel host species *Anguilla anguilla* and *A. japonica.* It can be assumed that *A. crassus* and *A. japonica* have been subject to long-term mutual adaptation processes and that this parasite species has to struggle against the host's strong immune response, which was shown by the comparably low recovery rates and body sizes of *A. crassus* in its original host (Knopf and Mahnke 2004; Knopf 2006; Keppel et al. 2014) and the host's ability to restrict the worm burden by its own immune response (Knopf and Lucius 2008). Since *A. crassus* is known to parasitize *A. anguilla* for about 30 years now, there might have occurred short-term adaptation processes as well. *Anguillicola novaezelandiae*, an endemic parasite to the Short-finned eel (*Anguilla australis* Richardson, 1841) from New Zealand, has only been recorded from *A. anguilla* populations in one single lake in Europe (Lake Bracciano, Italy) for a limited period of 11 years, so it is unlikely that this parasite-host system has developed any mutual adaptation at all. Although *A. novaezelandiae* and *A. japonica* have never encountered under natural conditions, a recent study by Keppel et al. (2014) has revealed that this host species is able to successfully oppose both parasite species, presumably due to the development of highly effective defence mechanisms. In this way, it can be expected that both parasite species used for the present study are confronted with immune responses of the different host species on the one hand and to intraspecific competition in relation to mean intensities on the other hand. This study also considers the reproductive success of each *Anguillicola* species in different hosts, which is another important factor associated with stress protein expression.

5.3 Material and methods

5.3.1 Experimental infections of eel hosts

All analyzed parasites resulted from experimental infections described in detail by Keppel et al. (2014) and Dangel et al. (2013), respectively. Briefly, Japanese and European eels were infected with previously generated third-stage larvae (L3) of *A. crassus* and *A. novaezelandiae*, respectively, and were allowed to grow in the eels for 120 days. All host-parasite systems involved, the amounts of administered L3, the codes used for each group and the sample sizes of both hosts and parasites are presented in Tab. 5.1. The respective amounts of larvae determined for infections were based on preceding experiments.

Tab. 5.1 Design of experimental infections with eel and *Anguillicola* species

Overview of host-parasite systems used for experimental infections, the number of administered L3 stage larvae, the respective system labels, and all sample sizes of eels as well as male and female parasites

Host species (N)	infected with	code	N parasites analyzed	
			male	female
A. japonica (5)	40x *A. crassus*	J40Ac	11	13
A. anguilla (7)	20x *A. crassus*	E20Ac	41	20
A. japonica (4)	40x *A. novaezelandiae*	J40An	7	4
A. anguilla (4)	20x *A. novaezelandiae*	E20An	14	17
A. anguilla (3)	40x *A. novaezelandiae*	E40An	35	18

5.3.2 Examination of parasites

After 120 days post infection (dpi), all infected eels were dissected and examined for the presence of swim bladder nematodes, which were removed from the swim bladders, counted for determining mean intensities and recovery rates, individually frozen in liquid nitrogen and kept at -80 °C for further processing. Swim bladder lumina were checked for the presence of second stage larvae (L2) emitted by gravid females and swim bladder walls were checked under a binocular for the presence of remaining L3.

In order to exclude distortion of data, L2 were removed from gravid female nematodes by puncturing the uterus prior to protein analyses and all nematodes' intestines were drained. Hsp analyses of intestinal contents of the nematodes were performed as well to test for possible contamination of the nematode tissue material.
The frozen nematodes were mixed with extraction liquid at pH 7.5 consisting of 80 mM potassium acetate, 5 mM magnesium acetate, 20 mM HEPES (Carl Roth, Germany) mixed with 2 % protease inhibitor (Sigma-Aldrich, Germany) and subsequently homogenized using an ultrasonic homogenizer (Sonopuls HD, Bandelin, Germany). The added volume of extraction buffer was adjusted to the nematodes' body weights. Tissue homogenates were centrifuged for 10 minutes at 16,000g and 4 °C. Total protein quantification of supernatants was performed using a standardized protein analysis kit (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific Inc., USA) and was photometrically determined according to Bradford (1976). Following this, equal protein concentrations were adjusted for all samples in order to ensure a comparability of hsp levels.

A volume of 20 µl of each supernatant was diluted with 20 µl Laemmli buffer (see Laemmli 1970), heated at 95 °C for 5 minutes and used for separating proteins by means of SDS-PAGE (15 min at 80 V and 90 min at 120 V) in a Mini-PROTEAN Tetra Cell (Bio-Rad Laboratories, Germany). Constant protein weights of 10 µg per sample were added to the electrophoresis gels, which additionally contained a protein marker (Precision Plus Protein Dual Color, Bio-Rad Laboratories, Germany) for visualization and a reference protein standard extracted from eel liver for scaling the data. The proteins were subsequently transferred to nitrocellulose by Western Blot at 90 mA for 120 minutes per gel and then blocked for 90 minutes in a 1:1 solution of horse serum and Tris-buffered saline (TBS) (both Sigma-Aldrich,

Germany). Following this, the nitrocellulose filters were washed in TBS for 5 minutes and were incubated overnight in the refrigerator with a mouse anti-HSP70 monoclonal antibody solution (1:5,000 in horse serum and TBS; IgG1, antibodies-online GmbH, Germany). The blots were again washed for 5 minutes in TBS and incubated in a peroxidase-conjugated secondary antibody solution (1:1,000) for 90 minutes (polyclonal goat anti-mouse immunoglobulins, Dako Deutschland GmbH, Germany). After subsequent TBS washing, the hsp70 blot bands were visualized by a staining solution containing 4-chloro(1)naphtol. After 10 minutes, the staining process was terminated in distilled water. The filters were subsequently dried and scanned at 600 dpi for densitometric quantification using the image-processing software Image J (National Institutes of Health, USA).

5.3.3 Statistical treatment of data

Mean intensities were determined as the sum of the total numbers of individuals of one nematode species in one host species divided by the number of infected individuals of the respective host species. The total number of recovered nematodes was divided by the total number of administered larvae in order to assess recovery rates. The presence of L2 was calculated as percentages of occurrence in each host-parasite group sample.

Relative grey values of hsp70 concentrations (mean ± SD) were calculated with mean densities of the samples divided by mean densities of the standard sample. Statistical analyses of the different sample groups were performed by means of the Mann-Whitney test (GraphPad Prism 5, GraphPad Software Inc., USA). Significance of differences was accepted when $p < 0.05$ (*). All presented graphs were created using GraphPad Prism.

5.4 Results

5.4.1 Mean intensities and recovery rates

Mean intensities and recovery rates of adult nematodes recovered in eels of all groups are presented in Fig. 5.1. The highest intensities were found in the 40x *A. novaezelandiae* group in European eels, followed by 20x *A. crassus* and 20x *A. novaezelandiae* in the same eel host species. Mean intensities of the two other groups ranged below 5, with *A. novaezelandiae* showing the lowest intensity in Japanese eels. Recovery rates of *A. crassus* and *A. novaezelandiae* in European eels showed mean values between 38 and 44 %, whereas both *Anguillicola* species in the Japanese eel featured lower recovery rates ranging between 7 and 14 %.

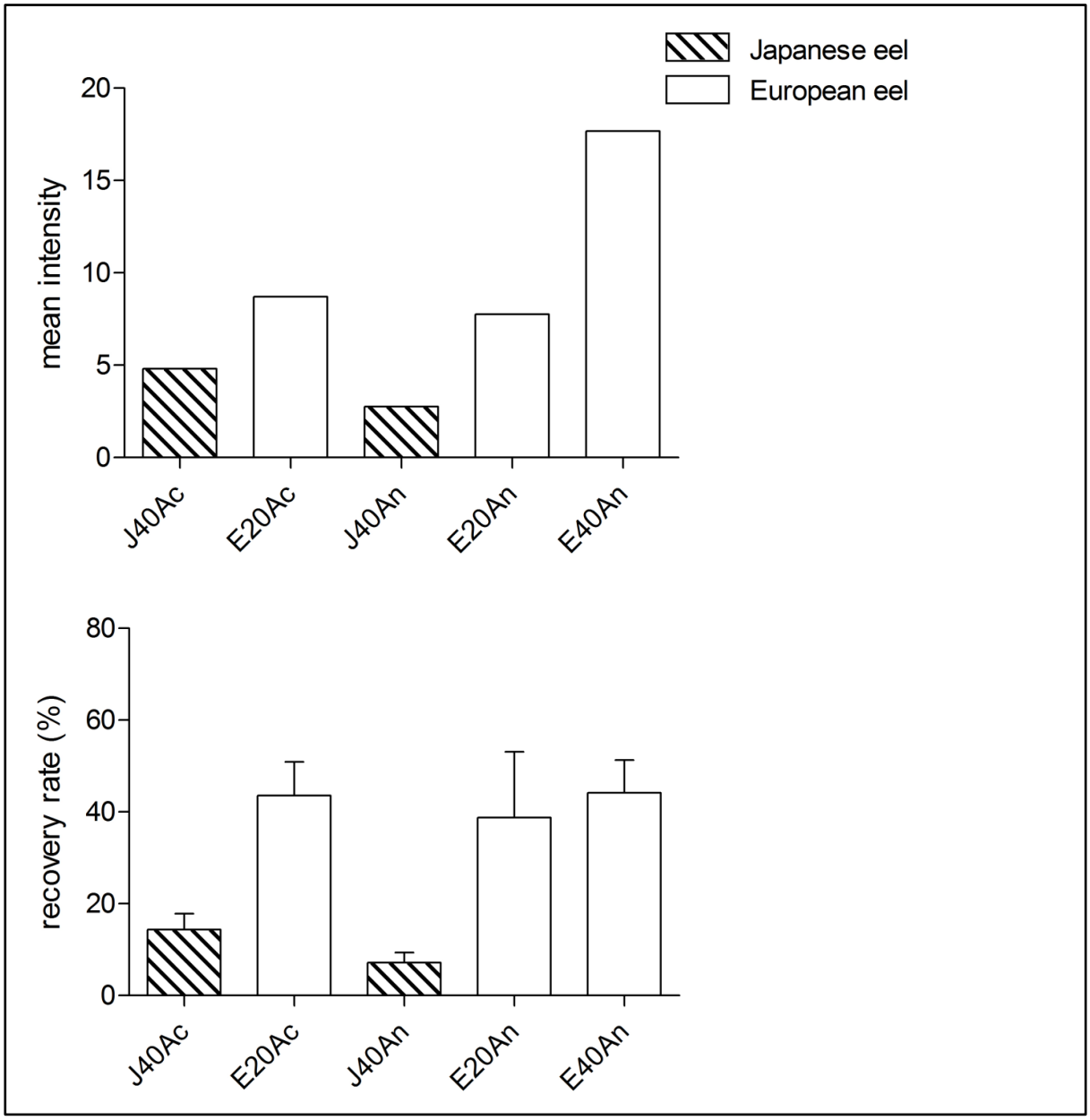

Fig. 5.1 Mean intensities and recovery rates of *Anguillicola* species in eel hosts 120 dpi

Figure shows mean intensities and recovery rates of *A. crassus* and *A. novaezelandiae* in the two eel host species *A. japonica* (Japanese eel – striped bars) and *A. anguilla* (European eel – white bars).
J = Japanese eel, E = European eel, 20 = infected with 20 larvae, 40 = infected with 40 larvae, Ac = *A. crassus*, An = *A. novaezelandiae*

5.4.2 Presence of L2 stage larvae

Second stage larvae could be detected in all examined swim bladders of European eels infected with 40x *A. novaezelandiae* (Fig. 5.2). In about 57 % of European eels infected with 20x *A. crassus*, in 50 % of European eels infected with 20x *A. novaezelandiae*, and in 30 % of all Japanese eels infected with 40x *A. crassus*, L2 were found in the swim bladders (Fig. 5.2). *Anguillicola novaezelandiae* females in the Japanese eel did not release any L2 and no fertilized eggs could be detected in their uteri.

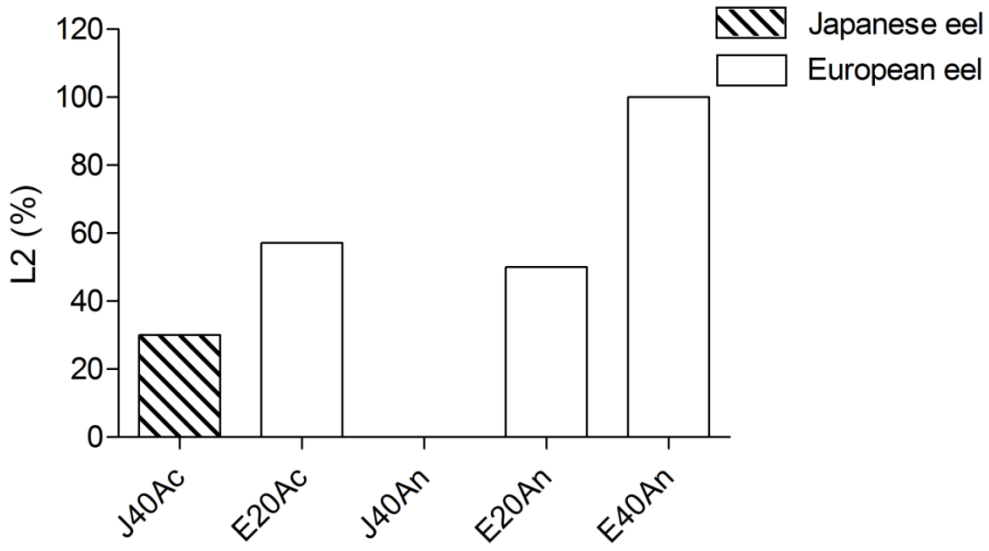

Fig. 5.2 Presence of L2 in all eel-*Anguillicola* groups 120 dpi

Figure shows the percentage of swim bladder samples in the two eel host species *A. japonica* (Japanese eel – striped bars) and *A. anguilla* (European eel – white bars) containing L2 of *A. crassus* and *A. novaezelandiae*.
J = Japanese eel, E = European eel, 20 = infected with 20 larvae, 40 = infected with 40 larvae, Ac = *A. crassus*, An = *A. novaezelandiae*

5.4.3 Hsp70 levels

Highest stress levels determined as relative hsp70 concentrations were found in *A. crassus* from experimental infection of Japanese eels (Fig. 5.3). These levels differed significantly from all other analyzed groups. The second highest values were determined for *A. crassus* in the European eel, while *A. novaezelandiae* (20x *A.n.*) in European eels featured the lowest levels. Values of 40x *A. novaezelandiae* in the Japanese and in the European eel showed no considerable differences. Hsp70 could not be detected in the samples removed from the parasites' intestines.

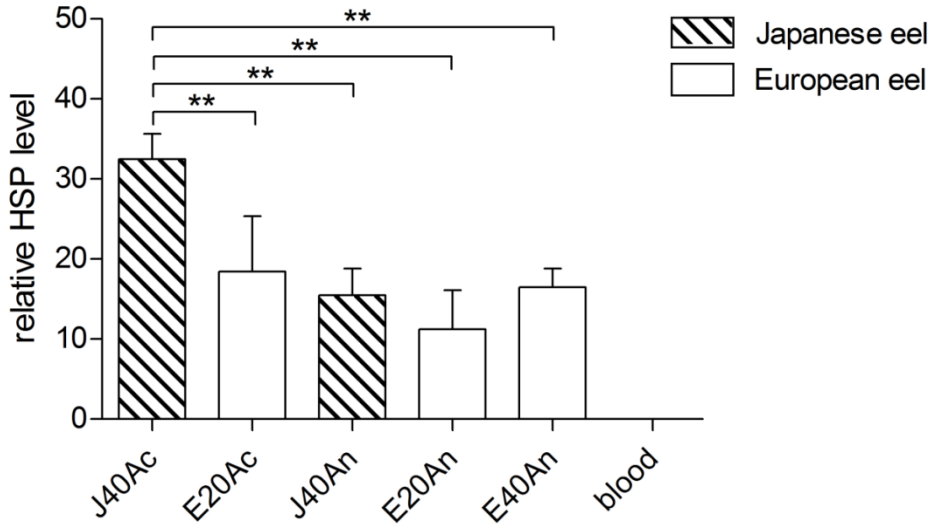

Fig. 5.3 Relative levels of hsp70 for all eel-*Anguillicola* groups 120 dpi

Levels of hsp70 in *A. crassus* and *A. novaezelandiae* in the two eel host species *A. japonica* (Japanese eel – striped bars) and *A. anguilla* (European eel – white bars) are displayed relative to the protein standard (mean ± SD); significance is represented by asterisks.
J = Japanese eel, E = European eel, 20 = infected with 20 larvae, 40 = infected with 40 larvae, Ac = *A. crassus*, An = *A. novaezelandiae*, ** significance level $p < 0.001$

5.5 Discussion

This study is the first attempt to analyze stress responses of anguillicoloid parasites. Contrary to previous studies focussing on the stress response of parasites exposed to either heat (Siamba et al. 2012, Chen et al. 2014), chemicals (van Leeuwen 1995), metals (Sures and Radszuweit 2007), or combined shifts of environmental conditions (Neumann et al. 1993), the two *Anguillicola* species in the present study were exposed to different hosts, which can be considered as different stressors due to their respective immune response. Since the parasites are inevitably bound to a specific host, there has been no possibility to create an unstressed control group as a basis. However, the results of the five different infection groups allow a direct comparison in terms of stress levels.

Anguillicola crassus in the Japanese eel showed comparably high hsp70 levels, which can be considered to be in accordance with its low mean intensity, recovery rate and L2 output. One may hypothesize that the immune response of its original host is harmful to the parasites leading either to failure of infestation or to a high level of stress in the successful parasite individuals. Since there are only few conspecifics as competitors in the same swim bladder (mean intensity of 4.8 nematodes), the parasite seems to spend most of its energy on striving against the host's immune system and on generating stress proteins and thus less energy on reproduction. Since hsps play a specific role in protection against killing mechanisms by the host cell (Mazier and Mattei 1991) and *A. crassus* attains only minor recovery rates (mean 14 %) and a high amount of encapsulated larvae in the Japanese eel (Keppel et al. 2014), this leads to the assumption that the main objective of the parasite was to survive in this well-adapted host.

The fact that the second highest stress response could be detected for *A. crassus* in the European eel – a host which could have developed a certain degree of immunological adaptation by now – might indicate that the immune reaction of this host is strong enough to cause a slightly elevated stress response in the parasite as well, although a quite high percentage of recovery was recorded. The respective mean intensity was not high enough to assume a high pressure through intraspecific competition in this group, but on the other hand, the percentage of gravid females in this host was much higher than in the Japanese eel, so that one can conclude that the parasite's energy was spent on both stress response and reproduction.

Surprisingly, relative hsp levels of *A. novaezelandiae* in the Japanese eel were not elevated, since this parasite species was expected to be strongly opposed by this host as well. A low intensity and the absence of L2 also suggest that there might have been a high stress level for the parasite. But when considering the extremely low recovery rates (mean 6.88 %), the high number of dead larvae detected by Keppel et al. (2014) and the parasites' small body sizes determined by Keppel et al. (2015) for these nematodes, it appears that the parasite could have been heavily weakened by its struggle for survival. The fact that the female individuals did not feature fertilized eggs in their uteri shows that their development is strongly retarded. Both stress protein synthesis and reproduction might have been too costly in terms of general energy demand.

Interestingly, both groups of European eels infected with *A. novaezelandiae* showed no notably high hsp70 levels. The mean intensity of the 20x *A. novaezelandiae* group was at 8.25, which slightly exceeded the values of the other host-parasite groups. L2 output (50 %) in turn was lower than expected. Parasites of the 40x *A. novaezelandiae* group showed a slightly higher hsp level, but considering the fact that they featured the highest L2 output (100 %), one might assume that most of their energy was spent on reproduction.

Since heat shock proteins are usually produced as rapidly as possible following a sudden environmental change, it remains uncertain at what stage in the nematode's life cycle increased hsp levels may be induced. Analyses on anisakid nematodes taken from infected mackerels by Chen et al. (2014) proved that hsp90 showed gradually increasing transcript levels within the first couple of hours after exposure to heat, i.e. after a decisive change of environmental conditions, but decreasing levels soon afterwards. Results on the hsp expression of the parasitic nematode *Nippostrongylus brasiliensis* Travassos, 1914 in rats showed that in critical phases of its life cycle, in L3 and adult stages, it featured upregulated levels (Arizono et al. 2011). Therefore, stress markers in larval stages of *Anguillicola* sp. could be analyzed soon after entering the final host as a significant environmental change or subsequent to the penetration of the swim bladder wall, where encapsulation as a process of the host's immune response especially in Japanese eels might occur.

In conclusion, most of the results presented here could be basically explained by the relations between stress response, recovery rate, mean intensity, and

reproductive output, especially with regard to energetic costs. A few other results, however, leave unanswered questions.

6 General discussion

Most of the findings in this study revealed significant differences between the four analyzed host-parasite associations, generally supporting the assumption that these differences can be traced back to adaptation as part of a co-evolutionary process with regard to the host's immune response. With reference to the underlying hypotheses, the infection rate of *A. crassus* proved to be more successful in the European eel than in the Japanese species. As hypothesized, both nematode species performed better in the European host due to its lack of adaptation compared to the Japanese host. On the contrary, *A. novaezelandiae* featured even a lower infection success than *A. crassus* in the Japanese eel host species, which could be associated with the generally strong genus-specific immune system of the host and at the same time, the unadapted immune response of the parasite. The swim bladder walls of Japanese eels turned out to be generally thicker compared to European eels and infections with both *Anguillicola* species caused even a further thickening, which could be linked to the host's general ability to encapsulate invading larvae and to its innate defence system.

The present study could reveal a simple tool for distinguishing both *Anguillicola* species by identifying a distinct and easily measurable morphological difference. Both nematode species attained a lower maximum body size in *A. japonica* also consequentially supporting the fact that the host's immune system is targeted at any *Anguillicola* infection. Elevated stress responses could be assessed for *A. crassus* in both host species suggesting that it requires some efforts to fight against well-adapted as well as against slightly adapted hosts, while *A. novaezelandiae* showed no significant responses to both unadapted host species.

In conclusion, contrary to initial assumptions that the Japanese host is not adapted to fighting against the alien species *A. novaezelandiae* at all, the presented results in total proved that *A. japonica* shows strong defence mechanisms against both *Anguillicola* species presumably as a consequence of continuous co-evolutionary processes during coexistence with *A. crassus*, although no natural encounter with *A. novaezelandiae* ever occurred.

Low rates of recovery in *A. japonica* could be explained by the host's ability to encapsulate and thus kill invading larvae of both nematode species. Encapsulation has frequently been recorded as an important cellular immune reaction and is well-

studied especially in insects encapsulating invading nematode larvae in their hemocoels (Götz 1986; Peters and Ehlers 1997). A couple of studies have described histopathological effects of helminth infections in fish tissues (Ramakrishna and Burt 1991; Ramakrishna et al 1993; Dezfuli et al. 2000) reporting on encapsulation and inflammatory reactions by macrophages, giant cells, mast cells, and neutrophils. Immunohistological approaches to eel host's reactions to *Anguillicola* infections have been conducted by Molnár et al. (1995) outlining alterations of the connective tissue of the subserosa in European eel swim bladder walls, where the parasites were found to be surrounded by melanomacrophages. Knopf et al. (2008) revealed increased migration activity of phagocytes in European eels following experimental infection with *A. crassus*. Abdelmonem et al. (2010) reported on thickened swim bladder walls in wild European eel samples. Hyperplasia could also be observed in the swim bladder tissue of American eels *Anguilla rostrata* infected with *A. crassus* (Sokolowski and Dove 2006). The present study could not verify any visible histopathological effects on the swim bladders of European eels following infections with both *Anguillicola* species, but it showed distinct histological effects in the Japanese eel to any *Anguillicola* infection identifying encapsulation as an effective defence mechanism, which is apparently associated with a swim bladder thickening requiring some of the host's energy. A couple of antibody reactions have been identified in eel hosts in response to the presence of either larval or adult *Anguillicola* stages (Höglund and Pilström 1994; Nielsen and Buchmann 1997; Knopf et al. 2000). Buchmann (2012) investigated immune responses of fish to endoparasitic helminth infections and the parasites' immune evasion strategies and argues that severe inflammatory reactions are rather related to dead worms than to living worms. Given the fact that the combating of adult nematodes as well as the removal of dead parasite material is a physiologically costly process and that there might be an increased emission of parasitic antigens after the parasite's death, which also requires high immunological efforts, encapsulation of invading larvae appears to be the best strategy for coping with the burden caused by helminth infections. According to findings by Molnár (1994), Molnár et al. (1995), and Audenaert et al. (2003) stating that European eels are still able to encapsulate *A. crassus* larvae under natural conditions, it remains uncertain if co-evolutionary processes will lead to a similarly powerful immunocompetence of European eels like the one that Japanese eels

evidently feature on a genus-specific level and perhaps on an even higher taxonomic level.

These defence mechanisms of the Japanese eel are also reflected in the generally smaller body size of both *Anguillicola* species in this host species and could be verified in this thesis. Many parasitological studies have revealed host-derived factors influencing the parasite's body size. There might be a strong correlation between host body size and parasite body size in some cases indicating that a small host provides a small habitat and vice versa, so that the parasite's environment is restricted to the available space in or on the respective host (Poulin 2007). Sorci et al. (2003) identified a negative correlation between the nematode's body size and the concentration of eosinophils in the host's blood stating that nematodes in hosts with a strong eosinophil response are generally smaller compared to those featuring a weaker response, since white blood cells are essentially involved in the host's immune defence against nematode infections. With regard to the model organisms of the present work, a study by Weclawski et al. (2014) supports the relation of parasite size to co-evolutionary developments, since they found out that in Japanese eel hosts, the Taiwanese population of *A. crassus* featured a significantly smaller body size than *A. crassus* individuals taken from European eels. So when including this fact as well, the host's defence system does not work similarly intense against all *Anguillicola* infections; it is also dependent on previous adaptations of the parasite. Taken together, these aspects hint at a strong interdependence of the defence mechanisms applied by host and parasite.

When considering immune evasion strategies of the parasite, the present study could show elevated hsp70 levels of *A. crassus* in its endemic Japanese eel host indicating a reciprocal struggle against the antagonist defence system. Both host and parasite have developed mechanisms adjusting immune regulation and immune evasion, respectively. For instance, Nielsen and Buchmann (1997) discovered the anti-oxidant function of Glutathione S-transferase in *A. crassus* as an agent promoting immune evasion. Other anti-oxidant enzymes like superoxide dismutase (SOD) may be involved in quenching the host's protective release of reactive oxidants. The presence of these enzymes has been detected in several nematode species (Callahan et al. 1988; Selkirk et al. 1998; Lattemann et al. 1999; Behm 2002) and should be analyzed in *Anguillicola* species regarding their function and their interrelation with the eel hosts' immunoregulatory molecules in future works.

Some studies have analyzed the immunosuppressive effects of different parasitic helminth species and interestingly, there are several hints that an infection with one particular species does not only diminish the host's immune response in favour of the successful establishment of this particular species, but also suppresses the host's resistance to other subsequently invading helminth species (Alghali et al. 1985; Christensen et al. 1987; Haukisalmi and Henttonen 1998). These heterologous synergistic interactions may be examined for anguillicoloid nematodes as well for further investigating their immune evasion functions, especially with reference to multiple infections in wild eel populations. Regarding the host's immune response, it might be a challenging subject to study the eel host's ability to develop a concomitant immunity, which allows the first parasites to survive and establish, but following infections by the same parasite are strongly opposed, which highly impedes the infection success of following invaders (Buchmann 2012). This could be examined through recurring experimental infections in order to transfer those findings to natural infections. Hence, this might lead to new interpretations of former results on the parasites' development in wild eel populations.

Quite a few papers have studied the detrimental impact of the successful invader *A. crassus* on newly acquired host species, whereas no considerable effects could ever be detected in the well-adapted Japanese eel. This might pose the question if the European eel or other non-adapted eel host species are able or will be able to generate an immune response similarly strong like the Japanese eel over time. With reference to the evolutionary arms race model (Dawkins and Krebs 1979), one might suggest that there is a selective pressure related to the currently occurring health risk emanating from such an invasive parasite like *A. crassus* and that this pressure will lead to adaptive processes setting up a more effective defence system.

Studies on other eel species (*Anguilla australis*, *A. mossambica*, *A. reinhardtii*) with their endemic nematodes (*Anguillicola novaezelandiae*, *A. papernai*, *A. australiensis*) should shed light on the question whether *A. japonica* has developed an unparalleled immune system and whether another *Anguillicola* species has a similar potential to successfully capture new habitats and hosts such as *A. crassus*, but never had any opportunities to encounter new host species, considering that there are numerous examples of suitable host-parasite associations under laboratory conditions without any contact under natural conditions (Perlman and Jaenike 2003).

All these presented findings contribute to extending the current knowledge about interactions between *Anguilla* and *Anguillicola* species, particularly about the host-specific factors that may play an important role in determining the infection success of the different parasite species. Referring to the introductory figure listing several influential factors affecting host-parasite relationships, we may conclude that based on the present results, the immune response of the host species represents the key factor for the analyzed model systems (Fig. 6.1).

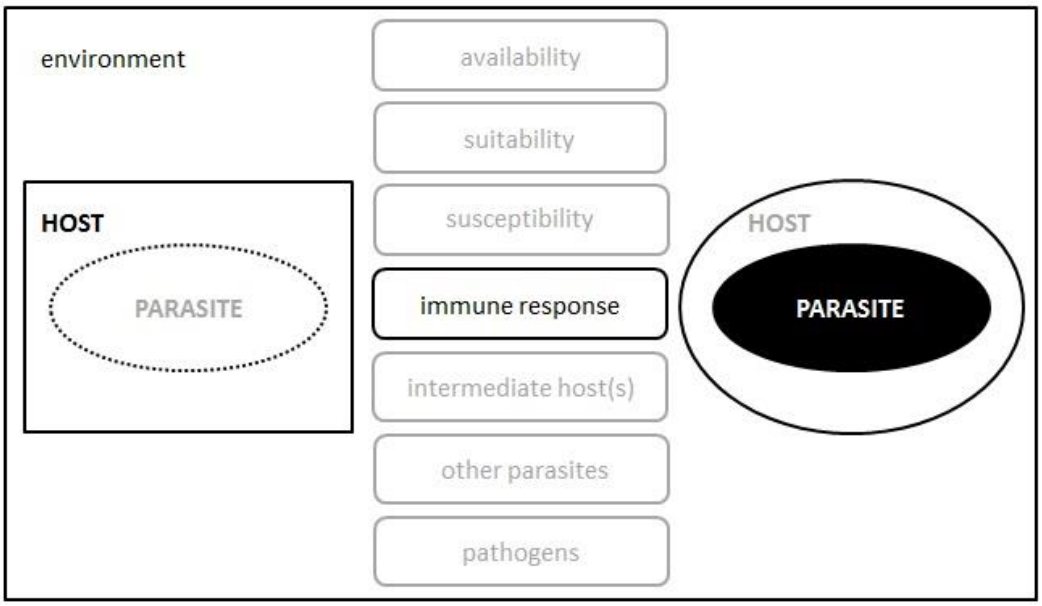

Fig. 6.1 Relations between hosts and parasites and factors of mutual dependence

The image shows different factors affecting the relationship between parasite and host (centre) highlighting immune response as a decisive factor in host-parasite relationships.

Especially when excluding environmental conditions and external factors like in the conducted infection experiments, in which all infected individuals were exposed to the same conditions and no side effects caused by other competitors or previous infections occurred, the central aspect of this study is that the host's immune response is decisive for both infection and invasion success of the parasite.

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Appendix

The Appendix is attached to the last page of the thesis as a data file on CD.

Appendix I: Data on recovery rates and mean intensities of all analyzed eel-*Anguillicola* groups (chapter 3)

Appendix II: Data on assessment of swim bladder damage levels and thickness measurements of swim bladder walls in all infected eels (chapter 3)

Appendix III: Morphometric data of all analyzed *Anguillicola* individuals in both eel host species (chapter 4)

Appendix IV: Collected morphometric data of all *Anguillicola* spp. including previous studies (chapter 4)

Appendix V: Levels of hsp70 in all analyzed *Anguillicola* individuals (chapter 5)

Eigenabgrenzung

Die vorliegende Dissertation wurde gemeinsam mit Prof. Dr. Bernd Sures konzipiert. Soweit im Folgenden nicht anders aufgeführt, wurden alle Analysen und Ergebnisse durch mich persönlich erbracht. Die Beiträge meiner Kollegin Dr. Kerstin Claudia Dangel zu den einzelnen Kapiteln/Artikeln sowie mein jeweiliger eigener Anteil stellen sich wie folgt dar:

3 Comparison of infection success, development and swim bladder pathogenicity of two congeneric *Anguillicola* species in experimentally infected *Anguilla anguilla* and *A. japonica*

Die zugrundeliegenden Infektionsversuche, die Sektion der Aale sowie die Entnahme der Parasiten wurden größtenteils gemeinsam mit Dr. Kerstin Dangel durchgeführt. Die Schwimmblasenuntersuchungen sowie die Auswertung und Interpretation der Daten zu Infektionsraten, Infrapopulationen und Schwimmblasenschädigungen erfolgte durch mich. Die Konzeption des Kapitels/Artikels wurde mit Prof. Bernd Sures abgesprochen. Das Kapitel/der Artikel wurde vollständig und eigenständig von mir verfasst und gemeinsam mit Prof. Dr. Bernd Sures vor Einreichung geringfügig überarbeitet.

4 Size does matter – a closer look on *Anguillicola* morphology

Die zugrundeliegenden Infektionsversuche, die Sektion der Aale sowie die Entnahme der Parasiten wurden größtenteils gemeinsam mit Dr. Kerstin Dangel durchgeführt. Die morphologischen Untersuchungen sowie die Auswertung und Interpretation der erfassten Daten erfolgte durch mich. Die Konzeption des Kapitels/Artikels wurde mit Prof. Bernd Sures abgesprochen. Das Kapitel/der Artikel wurde vollständig und eigenständig von mir verfasst und gemeinsam mit Prof. Dr. Bernd Sures vor Einreichung geringfügig überarbeitet.

5 The hsp70 response of *Anguillicola* species to host-specific stressors

Die zugrundeliegenden Infektionsversuche, die Sektion der Aale sowie die Entnahme der Parasiten wurden größtenteils gemeinsam mit Dr. Kerstin Dangel durchgeführt. Die Analysen der Hitzeschockproteine sowie die Auswertung und Interpretation der erfassten Daten erfolgte durch mich. Die Konzeption des Kapitels/Artikels wurde mit Prof. Bernd Sures abgesprochen. Das Kapitel/der Artikel wurde vollständig und eigenständig von mir verfasst und gemeinsam mit Prof. Dr. Bernd Sures vor Einreichung geringfügig überarbeitet.

Die Aufnahme der Veröffentlichungen in die Dissertation verletzt keine Urheberrechte.

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Hiermit bestätige ich die oben gemachten Angaben.

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Lebenslauf

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

Erklärungen

Erklärung I:

Hiermit erkläre ich, gem. § 7 Abs. (2) d) + f) der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbstständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient, bei der Abfassung der Dissertation nur die angegebenen Hilfsmittel benutzt und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

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Hiermit erkläre ich, gem. § 7 Abs. (2) e) + g) der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe und dass diese Arbeit von keiner anderen Fakultät/Fachbereich abgelehnt worden ist.

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Erklärung III:

Hiermit erkläre ich, gem. § 6 Abs. (2) g) der Promotionsordnung der Fakultät für Biologie zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „Mutual adaptation in differently evolved host-parasite systems“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Frau Michelle Keppel befürworte und die Betreuung auch im Falle eines Weggangs, wenn nicht wichtige Gründe dem entgegenstehen, weiterführen werde.

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