Comparative study on the host-parasite-interactions of eel and swim bladder parasites of the genus *Anguillicola*

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A.a.	*Anguillicola australiensis*
A.c.	*Anguillicola crassus*
A.g.	*Anguillicola globiceps*
A.n.	*Anguillicola novaezelandiae*
A.p.	*Anguillicola papernai*
BCA	bicinchoninic acid
bp	base pairs
C	condition factor; the ratio of the fish somatic mass ∗ 100/fish total length³
cox1	mitochondrial cytochrome c oxidase subunit 1
dpi	days post infection
ELISA	enzyme-linked immunosorbent assay
F1	first filial generation; offspring of distinctly different parental types
F2	second filial generation; offspring between two F1 individuals
hsi	hepatosomatic index; ratio of fish liver mass/fish somatic mass ∗ 100
hsp70	heat shock protein with a molecular weight of 70 kDa
L2	second larval stage of nematode
L3	third larval stage of nematode
L4	forth larval stage of nematode
MI	mean intensity
n	number of samples
NIS	nonindigenous species
P	prevalence
PCR	polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sp.	species
spp.	species pluralis
ssi	spleen-somatic index; ratio of fish spleen mass/fish somatic mass ∗ 100
TBS	tris-buffered saline

Summaries

1.1 Summary

Biological invasions emerge worldwide and rise with increasing globalisation and world trade. While nonindigenous species (NIS) can affect endemic species up to whole ecosystems they are also hosts to parasites which may be introduced into the new area with them (Hatcher & Dunn 2011; McGeoch et al. 2010). One of the best examples for a parasitic NIS is the eel parasite *Anguillicola crassus* (Knopf 2006). Nematodes of the genus *Anguillicola* are eel-specific swim bladder parasites with copepods as intermediate hosts. In the eel final host larval stages are found in the swim bladder wall while adult stages migrate into the swim bladder lumen and feed on the host's blood. The original occurrence of *Anguillicola* spp. was restricted to eels of the Indo-Pacific region but since eels are traded alive worldwide, two species of the genus were introduced into new areas and were able to establish stable populations in new host species (Moravec 2006).

In 1975 *Anguillicola novaezelandiae* was introduced to Lake Bracciano (Italy) with Short-finned eels (*Anguilla australis*) from New Zealand. The parasite was able to establish a stable population in the indigenous European eel (*Anguilla anguilla*) but never spread to other locations (Moravec 2006; Moravec et al. 1994a; Paggi et al. 1982). While *A. novaezelandiae* remained noninvasive, the close relative *A. crassus* became a successful invader. Quickly after its introduction to Germany with eels from Taiwan in the early 1980s, the parasite spread throughout Europe. Today *A. crassus* can be found as an invasive species in Africa, Europe and North America and was recorded as an alien parasite in six eel species. After *A. crassus* was introduced into Lake Bracciano it apparently replaced *A. novaezelandiae* in the location (Moravec 2006).

The original host of *A. crassus*, the Japanese eel (*Anguilla japonica*), is well adapted to the parasite and almost no harmful effects are recorded (Nagasawa et al. 1994). The naïve European eel on the other hand had no coevolution with the parasite and was therefore never able to gain efficient defence mechanisms against *Anguillicola* sp. In the European eel, *A. crassus* reaches higher intensities and prevalences of infection than in the original host. The swim bladder of European eels is damaged by heavy infections and no effective immune reactions could be recorded (Kennedy 2007; Knopf & Lucius 2008; Knopf 2006).

The aim of the present thesis was to gain more information on host-parasite-interactions of eels and *Anguillicola* spp. Whereas *A. crassus* was studied well since its introduction to Europe, little is known on the other species of *Anguillicola*. Differences, which distinguish *A. crassus* and *A. novaezelandiae*, an effective invader around the world on the on hand and a species only able to establish in a single location but never spreading from the point of introduction on the other hand, should be revealed. Therefore, the indigenous occurrence of *A. novaezelandiae* in its eel final host was studied. From two different regions (South Island and North Island) in New Zealand Short-finned eels were sampled and examined for the presence of swim bladder parasites. All stages of *A. novaezelandiae* were recorded and the prevalence as well as the infrapopulation composition in eels of both regions were compared. Since only the life cycle of *A. crassus* was known so far, an infection experiment with *A. novaezelandiae* was performed in the laboratory. European eels were infected with 20 larvae of *A. novaezelandiae* (20x *A.n.*-group) and sampled after 30, 60, 90 and 120 days post infection (dpi). An additional group was infected with 40 larvae (40x *A.n.*-group) to study density dependent effects, which are known for *A. crassus*. To ascertain the comparability of the study with literature data, one group of eels was infected with *A. crassus* (20x *A.*c.-group). The 40x *A.n.*-group, 20x *A.c.*-group and the uninfected control group were dissected 120 dpi. During dissection blood samples as well as liver samples were taken from all eels, to study the stress response of the host against the infection with *Anguillicola* sp. Subsequently, plasma cortisol and hepatic hsp70 levels were analysed. By performing an additional infection experiment with both *Anguillicola* species in one eel, the hypothesis of hybridisation between the two species was tested. After 222 days all recovered parasites were identified morphologically and genetically. Eggs of the uteri of the recovered females were isolated and analysed genetically.

The present study is not only the first to cover all stages of *A. novaezelandiae* in the natural final host, the Short-finned eel, but also revealed crucial differences to other *Anguilla-Anguillicola* systems. *A. novaezelandiae* is a common parasite of the Short-finned eel in New Zealand and was present in both regions evaluated. Larval stages dominate infrapopulation composition but massive encapsulation of larvae, as in other original *Anguilla-Anguillicola* systems, did not occur. Eels from both localities show differences in their infection status with *A. novaezelandiae* in respect to prevalence, abundance and intensity. While eels of the South Island were only infected with larval stages, preadult and adult stages could be detected in eels of the North Island. The infection pattern indicated a possibly seasonal occurrence of *A. novaezelandiae* infections in New Zealand. This was so far unknown for *Anguillicola* species.

The experimental infections of European eels allowed to study the life cycle of *A. novaezelandiae*. First adult stages were found after 60 days, while eggs with second stage larvae (L2) appeared only at 120 dpi. Infection experiments with *A. crassus* in European eels recorded first larval stages as soon as 50 dpi (Weclawski et al. 2013). The experiment revealed a synchronised development of *A. novaezelandiae* under laboratory conditions. No more larval stages of *A. novaezelandiae* were

recorded after 120 dpi, whereas in eels infected with *A. crassus* over 16 % of the recovered parasites were in a larval stage. Furthermore, a density dependent development, as stated for *A. crassus*, could not be observed. Whereas a synchronised development may be beneficial for *A. novaezelandiae* in the indigenous eel host, where infections seem to occur in a seasonal pattern, these differences in life cycle could be one factor which favoured *A. crassus* in Lake Bracciano, where both species occurred in sympatry in the 1990th. The less harmonised growth of *A. crassus* specimens could allow the parasites to produce eggs over a longer period, than the synchronised egg production of *A. novaezelandiae*.

Stress levels induced by *A. novaezelandiae* are comparable to those of *A. crassus*. While the eels' cortisol response is induced by larval stages (probably L3), hepatic hsp70 levels were enhanced by adult stages. Both species induce stress in eel in similar ways and levels of stress markers differed most likely because of difference in infrapopulation composition. Stress induced in the final host seems not to be the key element of *A. crassus*' more successful invasiveness.

After dissection of an eel infected with both *Anguillicola* species, twelve living adult parasites were recovered. All specimens were identified morphologically as well as with PCR using *cox I*. Recovery rates of both species were alike. Two female parasites of each species were found. To characterise their offspring, ten eggs were dissected from each uterus. Subsequent microsatellite analyses revealed that one female could be fertilised by several males. Analyses of the eggs extracted from *A. crassus* females showed only alleles typical for *A. crassus*. In contrast to this, eggs of *A. novaezelandiae* females showed not only eggs with alleles typical for *A. novaezelandiae* but also nine eggs with *A. crassus* alleles. The results prove that both species can co-infect a single eel and mature together in the same swim bladder. Microsatellite analyses also gave first evidence for the possibility of hybrid offspring between female *A. novaezelandiae* and male *A. crassus* but not vice versa. We could not prove whether viable and fertile nematodes grow from hybrid eggs. But even if eggs are not viable, this would benefit *A. crassus* over *A. novaezelandiae* in an area of sympatric occurrence. Therefore, hybridisation might be an explanation for the disappearance of *A. novaezelandiae* from Lake Bracciano.

The results of the present thesis substantially increase the knowledge on the host-parasite interactions of freshwater eels (*Anguilla*) and nematode parasites of the genus *Anguillicola* in general and in particular of *A. novaezelandiae*. Differences between the natural occurrence of *A. crassus* and *A. novaezelandiae* were revealed and larval stages of the latter species were studied for the first time in New Zealand. By performing an experimental laboratory infection of European eels with *A. novaezelandiae* it was possible to compare the species to recent studies on *A. crassus* and reveal fundamental differences. The eels' hsp70 response related to *Anguillicola* sp. infections was studied for the first time. Levels of stress induced by *A. novaezelandiae* were comparable to those induced by *A. crassus*. A co-infection of eel with both species was performed and the hypotheses of hybridisation tested. In summary the results prove, that the knowledge on one species cannot simply be transferred

to other species of the same genus. Further research is needed to extend our understanding of *Anguillicola* and its eel host. The *Anguilla-Anguillicola* system is an excellent example of effects on indigenous species caused by world trade and the negative impact of invasive parasites. In the future eel trade, in particular with live eels, should be handled more cautiously to prevent the further spread of *Anguillicola* species.

1.2 Zusammenfassung

Die Invasion fremder Arten ist ein weltweites Phänomen und nimmt durch die zunehmende Globalisierung und den wachsenden Welthandel weiter zu. Das Vorkommen von Neobiota kann sich auf einzelne endemische Arten bis hin zu ganzen Ökosystemen auswirken. Da im besonderen Tiere auch gleichzeitig einen Lebensraum für Parasiten darstellen, kann mit Neobiota auch deren Parasitenfauna in das neue Ökosystem eingeschleppt werden (Hatcher & Dunn 2011; McGeoch et al. 2010). Eines der besten Beispiele für einen solchen neozoischen Parasiten ist der Aalparasit *Anguillicola crassus* (Knopf 2006). Nematoden der Gattung *Anguillicola* sind aalspezifische Schwimmblasenparasiten, denen Ruderfußkrebse (Copepoda) als Zwischenwirte dienen. Im Endwirt, dem Aal, findet man larvale Stadien in der Schwimmblasenwand, während adulte Stadien in das Lumen der Schwimmblase eindringen und sich dort von Wirtsblut ernähren. Das ursprüngliche Vorkommen von *Anguillicola* spp. war auf Aale des Indo-Pazifischen Raums beschränkt, doch da Aale weltweit lebend gehandelt werden, wurden zwei Arten der Gattung in neue Gebiete verschleppt und konnten dort stabile Populationen in neuen Wirtsarten bilden (Moravec 2006).

Im Jahr 1975 wurde *Anguillicola novaezelandiae* mit Kurzflossen-Aalen (*Anguilla australis*) aus Neuseeland in den Bracciano See (Italien) eingeschleppt. Der Parasit konnte zwar eine stabile Population im endemischen Europäischen Aal (*Anguilla anguilla*) etablieren, doch konnte er sich nie in andere Gebiete ausbreiten (Moravec 2006; Moravec et al. 1994a; Paggi et al. 1982). Während *A. novaezelandiae* sich nicht invasiv verbreitet hat, wurde die eng verwandte Art *A. crassus* zu einem erfolgreichen Invasor. Kurz nach seinem Eintreffen in den 1980ern in Deutschland (mit Aalen aus Taiwan) breitete sich der Parasit europaweit aus. Heute kommt *A. crassus* als Neozoe in Afrika, Europa und Nordamerika vor und wurde in sechs Aalarten als nicht endemischer Parasit nachgewiesen. Nachdem *A. crassus* auch in den Bracciano See eingeschleppt wurde, scheint er dort *A. novaezelandiae* verdrängt zu haben (Moravec 2006).

Der ursprüngliche Wirt von *A. crassus*, der Japanische Aal (*Anguilla japonica*), ist gut an den Parasiten angepasst und es wurden kaum Schäden durch *Anguillicola*-Befall nachgewiesen (Nagasawa et al. 1994). Der nicht angepasste Europäische Aal dagegen hatte keine gemeinsame Koevolution mit dem Parasiten und konnte somit keine wirksamen Abwehrmechanismen gegen *Anguillicola* sp. entwickeln. Dadurch treten *A. crassus*-Infektionen in Europäischen Aalen mit höheren Intensitäten und Prävalenzen auf als im ursprünglichen Wirt. Durch starken Befall wird die Schwimmblase des

Europäischen Aals geschädigt. Im neuen Wirt konnte keine effektive Immunreaktionen nachgewiesen werden (Kennedy 2007; Knopf & Lucius 2008; Knopf 2006).

Ziel der vorliegenden Dissertation war es, das Wirt-Parasit-Verhältnis von Aalen und Schwimmblasenparasiten der Gattung *Anguillicola* besser zu verstehen. Während *A. crassus* nach seinem Auftreten in Europa gut untersucht wurde, ist wenig über die anderen *Anguillicola*-Arten bekannt. Darum sollten Unterschiede zwischen *A. crassus,* einem weltweit erfolgreichen Invasor, und *A. novaezelandiae*, einer Art die sich nur in einem einzigen Habitat etablieren konnte, gefunden werden. Hierzu wurde das endemische Vorkommen von *A. novaezelandiae* im Endwirt Aal untersucht. In Neuseeland wurden Kurzflossen-Aale zweier Standorte (Südinsel und Nordinsel) beprobt und auf eine Infektion mit Schwimmblasenparasiten hin untersucht. Alle *A. novaezelandiae*-Stadien wurden erfasst und die Prävalenz sowie die Zusammensetzung der Infrapopulationen der beiden Regionen verglichen. Da bisher nur der Lebenszyklus von *A. crassus* bekannt war, wurde im Labor ein Infektionsversuch mit *A. novaezelandiae* durchgeführt. Hierzu wurden Europäische Aale mit je 20 Larven von *A. novaezelandiae* (20x *A.n.*-Gruppe) infiziert und 30, 60, 90 und 120 Tage nach Infektion beprobt. Eine weitere Gruppe wurde mit 40 Larven infiziert (40x *A.n.*-Gruppe) um eine mögliche, für *A. crassus* bekannte, Dichteregulation zu untersuchen. Um die Vergleichbarkeit der Studie mit Literaturdaten zu überprüfen, wurde eine Aalgruppe mit *A. crassus* infiziert (20x *A.c.*-Gruppe). Die Aale der 40x *A.n.*-Gruppe, der 20x *A.c.*-Gruppe sowie nicht infizierte Kontrollaale wurden 120 Tage nach Infektion seziert. Während der Sektion wurden allen Aalen Blut- sowie Leberproben entnommen, um die Stressreaktion des Aals auf eine Infektion mit *Anguillicola* zu untersuchen. Anschließend wurden Plasmacortisolgehalte sowie hsp70-Gehalte der Leber bestimmt. Durch einen zusätzlichen Infektionsversuch, bei dem ein Aal gleichzeitig mit beiden *Anguillicola*-Arten infiziert wurde, konnte die Hypothese einer Hybridisierung beider Arten überprüft werden. Nach 222 Tagen wurden alle vorhandenen Parasiten morphologisch und genetisch bestimmt. Eier aus den Uteri der vorgefundenen Weibchen wurden entnommen und genetisch untersucht.

Die vorliegende Studie ist nicht nur die erste, die alle Stadien von *A. novaezelandiae* in seinem natürlichen Endwirt, dem Kurzflossen-Aal umfasst, sondern hat zudem entscheidende Unterschiede zu anderen *Anguilla-Anguillicola*-Systemen offengelegt. *A. novaezelandiae* ist ein häufiger Parasit des Kurzflossen-Aals in Neuseeland und konnte an beiden untersuchten Standorten nachgewiesen werden. Larvale Stadien dominierten die Infrapopulationen, es trat jedoch, im Gegensatz zu anderen natürlichen *Anguilla-Anguillicola*-Systemen, keine massenhafte Einkapselung von Larven auf. Aale beider Standorte unterschieden sich in ihrem Infektionsstatus, hinsichtlich Prävalenz und Intensität. Während Aale der Südinsel nur mit larvalen Stadien infiziert waren, konnten präadulte sowie adulte Stadien in Aalen der Nordinsel nachgewiesen werden. Das Infektionsmuster spricht für ein mögliches saisonales Auftreten von *A. novaezelandiae* Infektionen in Neuseeland. Dies war bisher für keine *Anguillicola*-Art bekannt.

Durch die experimentelle Infektion des Europäischen Aals war es möglich, die Lebenszyklusdauer von *A. novaezelandiae* zu untersuchen. Erste adult Stadien wurden nach 60 Tagen gefunden, während Eier mit sogenannten zweiten Larvenstadien (L2) erst nach 120 Tagen vorhanden waren. Bei Infektionsversuchen mit *A. crassus* im Europäischen Aal traten erste adult Stadien bereits nach 50 Tagen auf (Weclawski et al. 2013). Im Experiment zeigte sich eine synchronisierte Entwicklung von *A. novaezelandiae* unter Laborbedingungen. Nach 120 Tagen konnten keine larvalen Stadien von *A. novaezelandiae* mehr nachgewiesen werden. In mit *A. crassus* infizierten Aalen befanden sich dagegen über 16 % der wiedergefundenen Parasiten in larval Stadien. Darüber hinaus konnte keine Dichteabhängigkeit in der Entwicklung beobachtet werden, wie sie für *A. crassus* postuliert wird. Eine synchronisierte Entwicklung für *A. novaezelandiae* mag im natürlichen Aalwirt, indem die Infektion ein saisonales Muster aufzuweisen scheint, vorteilhaft sein. Dieser Unterschied könnte allerdings ein Faktor sein, der sich vorteilhaft für *A. crassus* im Bracciano See ausgewirkt hat, in dem beide Arten in den 1990ern gemeinsam vorkamen. Das uneinheitliche Wachstum der *A. crassus* Individuen könnte es dem Parasiten ermöglichen, Eier über einen längeren Zeitraum zu produzieren, im Gegenteil zur synchronisierten Eiproduktion von *A. novaezelandiae*.

Durch *A. novaezelandiae* verursachte Stresslevel sind mit den durch *A. crassus* verursachten vergleichbar. Während die Cortisolreaktion des Aals durch larvale Stadien (vermutlich L3) hervorgerufen wird, verursachen adulte Stadien eine Erhöhung der Leber-hsp70-Werte. Da beide Arten Stress in ähnlichem Maße verursachen und Unterschiede im Niveau der Stressreaktion höchstwahrscheinlich auf unterschiedlich zusammengesetzte Infrapopulationen zurückzuführen sind, scheint der im Endwirt induzierte Stress nicht der entscheidende Faktor für die weitaus erfolgreichere Invasivität von *A. crassus* zu sein.

Nach Sektion eines mit beiden *Anguillicola* Arten infizierten Aals wurden zwölf lebende Parasiten als adulte Stadien gefunden. Alle Individuen wurden sowohl morphologisch als auch mittels *cox I* PCR bestimmt. Die Wiederfindungsrate beider Arten war gleich hoch und von beiden Arten waren je zwei Weibchen vorhanden. Um deren Nachkommen zu untersuchen, wurden jedem Weibchen zehn Eier aus dem Uterus entnommen. Eine anschießende Mikrosatellitenanalyse zeigte, dass ein Weibchen von mehreren Männchen befruchtet werden kann. Die Analyse der Eier aus den *A. crassus* Weibchen wies nur *A. crassus*-typische Allele auf. Im Gegensatz dazu wurden in Eiern der *A. novaezelandiae* Weibchen nicht nur Allele für *A. novaezelandiae*, sondern in neun Eiern auch *A. crassus* Allele nachgewiesen. Durch den Versuch konnte bewiesen werden, dass beide Arten einen einzelnen Aal gleichzeitig infizieren und gemeinsame in derselben Schwimmblase heranwachsen können. Durch Mikrosatellitenanalyse konnten erste Nachweise für die Möglichkeit zur Hybridisierung zwischen *A. novaezelandiae* Weibchen und *A. crassus* Männchen erbracht werden, jedoch nicht umgekehrt. Es konnte nicht getestet werden, ob aus Hybrideiern lebensfähige und fertile Nematoden heranwachsen können. Doch selbst wenn die Eier nicht lebensfähig sind, wäre dies für *A. crassus* gegenüber

A. novaezelandiae in Gebieten gemeinsamen Vorkommens von Vorteil. Darum könnte Hybridisierung eine Erklärung für das Verschwinden von *A. novaezelandiae* aus dem Bracciano See sein.
Die Ergebnisse der vorliegenden Dissertation erweitern das Wissen über das Wirt-Parasit-Verhältnis von Aalen (*Anguilla*) und parasitischen Nematoden der Gattung *Anguillicola* im Allgemeinen, als auch über *A. novaezelandiae* im Besonderen. Unterschiede im natürlichen Vorkommen von *A. crassus* und *A. novaezelandiae* wurden aufgezeigt und larvale Stadien der letztgenannten Art erstmals in Neuseeland untersucht. Durch die experimentelle Infektion des Europäischen Aals mit *A. novaezelandiae* konnte die Art mit Studien über *A. crassus* verglichen und grundlegende Unterschiede entdeckt werden. Die Effekte einer *Anguillicola* sp. Infektion auf die hsp70-Reaktion des Aals wurde erstmals untersucht. Durch *A. novaezelandiae* und *A. crassus* induzierte Stresslevel waren vergleichbar. Eine Infektion des Aals mit beiden Parasitenarten wurde durchgeführt und die Hypothese einer Hybridisierung getestet. Zusammengefasst zeigen die Ergebnisse, dass man das Wissen über eine einzelne Art nicht einfach auf andere Arten der gleichen Gattung übertragen kann. Weitere Forschungsarbeiten sind nötig, um *Anguillicola* und seinen Aalwirt besser verstehen zu können. Das *Anguilla*-*Anguillicola*-System stellt ein exzellentes Beispiel für die Auswirkungen des Welthandels auf endemische Arten und deren Schädigung durch invasiver Parasiten dar. Zukünftig sollte der Aalhandel, insbesondere mit lebenden Aalen, viel vorsichtiger gehandhabt werden, um eine weitere Ausbreitung von *Anguillicola* Arten zu vermeiden.

General introduction

2

2.1 Background

Globalisation of human activities and worldwide trade of goods, plants and animals can lead to intended and accidental introduction of organisms into new areas. Of those species that are introduced, only few become established in the new area and even less spread from the initial population to new territories and become invasive species (see Figure 2.1, Kolar & Lodge 2001).

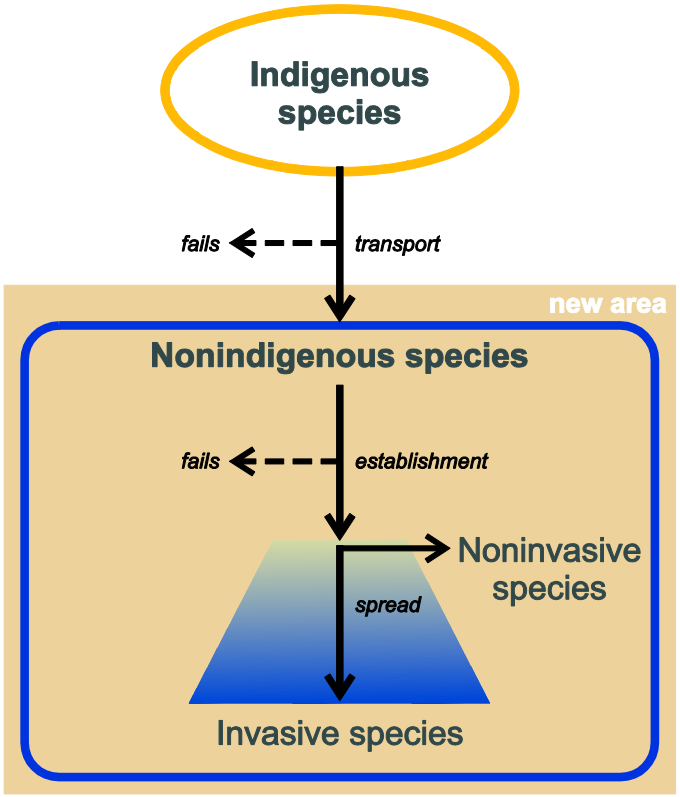

Figure 2.1 The invasion process (modified after Kolar & Lodge 2001).

In its native range a species is called an indigenous species. In the invasion process the species must be transported to a new area and survive transport and introduction to the new area. In the new area this species is then called a nonindigenous species (NIS). A NIS then starts interacting with the invaded ecosystem and might be able to establish a self-sustaining population. NIS that remain localised around the point of introduction are called noninvasive species, whereas invasive species spread widely in the new ecosystem (Kolar & Lodge 2001).

Table 2.1 Overview on the genus *Anguilla*.

Sources: Froese and Pauly 2014; Roskov et al. 2014

Species Subspecies	Author	English name
Anguilla anguilla	(Linnaeus, 1758)	European eel
Anguilla australis	Richardson, 1841	Short-finned eel
Anguilla australis australis	Richardson, 1841	Short-finned eel
Anguilla australis schmidtii	Phillipps, 1925	
Anguilla bengalensis	(Gray, 1831)	Indian mottled eel
Anguilla bengalensis bengalensis	(Gray, 1831)	Indian mottled eel
Anguilla bengalensis labiata	Peters, 1852	African mottled eel
Anguilla bicolor	McClelland, 1844	Indonesian shortfin eel
Anguilla bicolor bicolor	McClelland, 1844	Indonesian shortfin eel
Anguilla bicolor pacifica	Schmidt, 1928	Indian short-finned eel
Anguilla breviceps	Chu & Jin, 1984	
Anguilla celebesensis	Kaup, 1856	Celebes longfin eel
Anguilla dieffenbachii	Gray, 1842	New Zealand longfin eel
Anguilla interioris	Whitley, 1938	New Guinea eel
Anguilla japonica	Temminck & Schlegel, 1846	Japanese eel
Anguilla luzonensis	Watanabe, Aoyama & Tsukamoto, 2009	Luzon mottled eel
Anguilla malgumora	Kaup, 1856	Indonesian longfinned eel
Anguilla marmorata	Quoy & Gaimard, 1824	Giant mottled eel
Anguilla megastoma	Kaup, 1856	Polynesian longfinned eel
Anguilla mossambica	(Peters, 1852)	African longfin eel
Anguilla nebulosa	McClelland, 1844	Mottled eel
Anguilla nigricans	Chu & Wu, 1984	
Anguilla obscura	Günther, 1872	Pacific shortfinned eel
Anguilla reinhardtii	Steindachner, 1867	Speckled longfin eel
Anguilla rostrata	(Lesueur, 1817)	American eel

Biological invaders may not only harm endemic organisms but can even change biodiversity and community structure in the new ecosystem (Hatcher & Dunn 2011; McGeoch et al. 2010). The success of an invasion and its impact on the community can be modified by parasites, and host-parasite associations can be altered by invasions. Invaders may introduce new parasites to the recipient community (spillover), they can acquire parasites from the new area (spillback) or lose parasites during the invasion (enemy release) (Hatcher & Dunn 2011). New host-parasite associations can have negative effects on the affected populations due to missing host-parasite coevolution and subsequent coadaption.

The present study focused on the host-parasite interactions of freshwater eels (*Anguilla*) and nematode parasites of the genus *Anguillicola*. Eels are of tremendous worldwide importance for trade and food production. Several species of *Anguilla* are traded alive, are thereby introduced into new environments and bring with them new parasites into the new area (Egusa 1979; FAO 2012; Sures 2011; Wang et al. 2006).

Freshwater eels of the genus *Anguilla* comprise 19 species, three of which are further divided into two subspecies (see Table 2.1; Froese and Pauly 2014; Roskov et al. 2014). Eels are catadromous fish, with a juvenile continental life and spawning grounds in tropical oceans. So far spawning grounds of only four species are known with certainty (*A. anguilla*, *A. celebesensis*, *A. japonica* and *A. rostrata*) (Righton et al. 2012). After eels hatch from the eggs in the deep see, their larvae are shaped like leafs and called leptocephali. They travel over thousands of kilometres to the continental shelf, transfer into the round glass eel stage and enter coastal waters, estuaries, streams and rivers. When they inhabit coastal and continental waters the yellow eel stage begins. This continental life can last several decades (usually 5 to 20 years, but even 80 year old eels have been recorded) before eels start their downstream migration and mature to the so called silver eel stage. Silver eels migrate to their spawning grounds into the oceans where they mate, lay their eggs and die (Dekker 2008; Tesch 1999).

Since 2010 the European eel, *Anguilla anguilla*, is listed as 'Critically Endagered' on the IUCN Red List of Threatened Species™ and in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), which restricts international trade with the species (Freyhof & Kottelat 2010). Recruitment of juvenile eels has collapsed in the past decades with juvenile abundance dropping by 99 %. But juvenile abundance of the American eel, *Anguilla rostrata*, as well as of the Japanese eel, *Anguilla japonica*, have also declined dramatically (Anonymous 2003). Several factors are discussed as reasons for the decline, such as climate change, changes in ocean circulation, overfishing of glass eels and habitat loss (Freyhof & Kottelat 2010; Kettle et al. 2011). In their continental life eels are exposed to anthropogenic pollutants such as poly-chlorinated biphenyls (PCB), their migration routes are blocked by dams and pumping stations, they are overfished and exposed to diseases such as the eel virus European X (EVEX) or the swim bladder parasite *Anguillicola crassus* (ICES 2011; Righton et al. 2012; Sures & Knopf 2004a).

Nematodes of the genus *Anguillicola* are eel-specific swim bladder parasites with copepods as intermediate hosts. Moravec (2006) split the family Anguillicolidae into the two genera *Anguillicola* and *Anguillicoloides* due to morphological criteria. But since the phylogenetic study of Laetsch et al. (2012) on the family found no support for this separation, all species of the family will be referred to as *Anguillicola* in the following (see Table 2.2).

The genus *Anguillicola* comprises five species which are native parasites of Indo-Pacific eel species. The best studied species is *Anguillicola crassus* and the knowledge on this species is therefore often

Table 2.2 Taxonomy of the genus *Anguillicola.*

Sources: Laetsch et al. 2012; Moravec 2006; Roskov et al. 2014

Taxonomic rang	Author	Original eel host
Phylum: Nematoda	Rudolphi, 1808	
Class: Secernentea	Linstow, 1906	
Order: Spirurida	Chitwood, 1933	
Superfamily: Anguillicoloidea	Yamaguti, 1935	
Family: Anguillicolidae	Yamaguti, 1935	
Genus: *Anguillicola*	Yamaguti, 1935	
Species: *Anguillicola australiensis*	(Johnston & Mawson, 1940)	*Anguilla reinhardtii*
Species: *Anguillicola crassus*	(Kuwahara, Niimi & Itagaki, 1974)	*Anguilla japonica*
Species: *Anguillicola globiceps*	Yamaguti, 1935	*Anguilla japonica*
Species: *Anguillicola novaezelandiae*	Moravec & Taraschewski, 1988	*Anguilla australis*
Species: *Anguillicola papernai*	Moravec & Taraschewski, 1988	*Anguilla mossambica*

used exemplary for the whole genus. The basic life cycle of the species is rather simple (Figure 2.2). Adult parasites are found inside the swim bladder of the eel where they mate and produce eggs. The eggs contain so called second stage larvae (L2) which leave the swim bladder via the pneumatic duct and are then released into the water with the faeces. The larvae hatch in the water and start with typical undulating movements, which presumably attract the intermediate host. As soon as suitable intermediate hosts (mostly cyclopid copepods) ingest the L2, the larvae penetrate the hosts' digestive tract and enter the body cavity for further development. The larvae grow and moult into the third stage larvae (L3), which are infective to the final host, the eel. As soon as L3 are ingested by the final host the larvae penetrate the intestine and migrate into the wall of the swim bladder. Here, they moult again into fourth stage larvae (L4), grow further and molt into a preadult stage, which enters the lumen of the swim bladder. Inside the swim bladder the adult parasites bite blood vessels, suck blood, grow and mate (Kennedy 2007; Kirk 2003; Køie 1991; Moravec 2006). In Europe over 30 fish species but also tadpoles of frog and newt, as well as some aquatic invertebrates, have been recorded as additional paratenic host to *A. crassus* (De Charleroy et al. 1989, 1990; Haenen et al. 1994a; Moravec & Konecny 1994; Moravec & Skorikova 1998; Moravec 1996; Pazooki & Székely 1994; Székely 1994; Thomas & Ollevier 1992). Paratenic hosts are facultatively included in the life cycle. Parasites do not develop within this host but accumulate and remain infective to the eel host (see Figure 2.2). Whether other *Anguillicola* species are able to include paratenic hosts and if paratenic host are included in the life cycle of *A. crassus* outside of Europe, has so far not been recorded or studied experimentally.

A. crassus was brought to Europe in the early 1980s through the import of life eels from Asia (see Figure 2.3 and Table 2.3, Kennedy 2007; Kirk 2003; Koops & Hartmann 1989). The original host of this parasite, the Japanese eel (*Anguilla japonica*), is well coevolved with the parasite, while the

European eel is naïve to this nematode species. Soon after its first record from Germany in 1982 *A. crassus* spread throughout the European eel populations in Europe and Northern Africa (Koops & Hartmann 1989; Peters & Hartmann 1986, for a review see Kirk 2003). In 1995 *A. crassus* was first described in North America infecting the American eel (*Anguilla rostrata*) (Johnson et al. 1995). After transportation of living European eels to the Island of Reunion *A. crassus* was also introduced to Southern Africa and was found in three African eel species (*Anguilla bicolor*, *Anguilla marmorata*, *Anguilla mossambica*) in 2005 (Sasal et al. 2008). Even though *A. crassus* seems to be the most efficient invader of the genus, it was not the first to be successfully introduced to Europe. In 1975 Short-finned eels (*Anguilla australis*) from New Zealand infected with *Anguillicola novaezelandiae* were released into Lake Bracciano in Italy (Paggi et al. 1982; Welcomme 1981). The parasites were able to infect the European eel and build a stable population in the lake (Moravec et al. 1994a; Paggi et al. 1982). *A. novaezelandiae* is coevolved with Short-finned eels in Australia and New Zealand, but only little is known on its endemic life cycle, biology or pathogenicity (Lefebvre et al. 2004; Moravec & Taraschewski 1988; Moravec et al. 1994a). Lake Bracciano is a very unique location. It is so far the only known location worldwide, where one eel species is infected with two nonindigenous *Anguillicola* species (Moravec et al. 1994a).

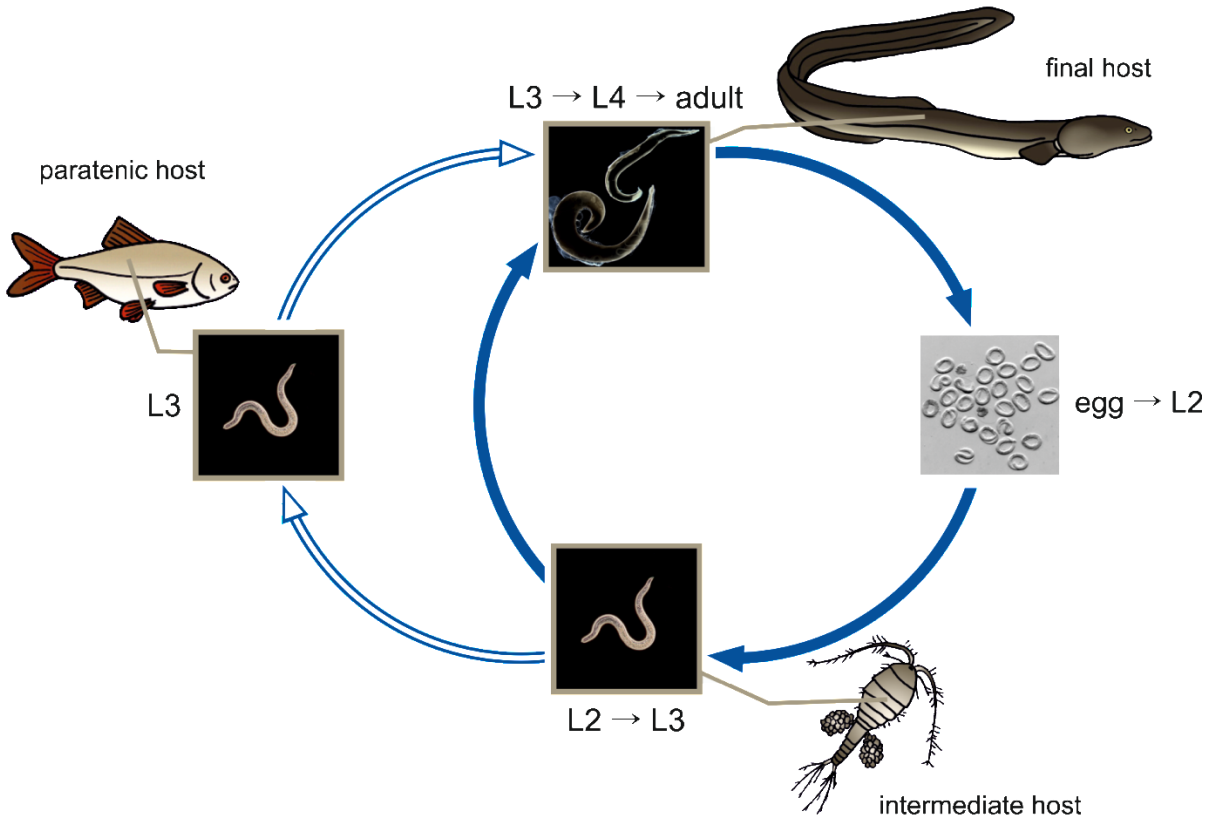

Figure 2.2 The life cycle of *Anguillicola crassus*. The basic life cycle (*blue darts*) includes eels as final hosts and copepods as intermediate hosts. By integrating additional paratenic hosts (e.g. fish) the life cycle can be broadened (*white darts*).

Source: Moravec 2006

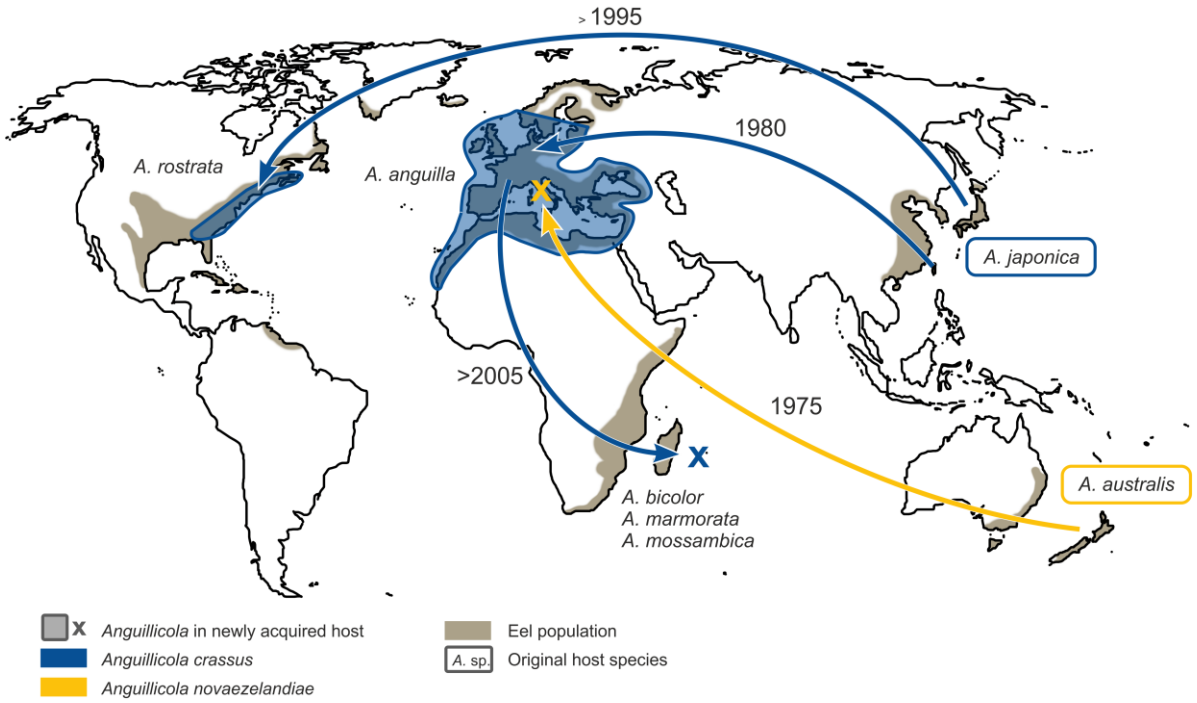

Figure 2.3 Expansion of invasive *Anguillicola* species. The world-wide translocation of *A. crassus* (*blue*) and *A. novaezelandiae* (*orange*) into new areas and subsequent establishment of the parasites in the populations of eels in Europe, America and Africa is shown.

Sources: Aieta & Oliveira 2009; Kirk 2003; Paggi et al. 1982; Sasal et al. 2008; Tesch 1999

In Lake Bracciano *A. novaezelandiae* showed high prevalence and intensity of infection in European eels but it never established populations outside the lake. There are no records of any damage to the swim bladder of eels. After *A. crassus* had also been introduced into the lake, both species were found in 1993, but no mixed infections were recorded. *A. crassus* quickly became the dominant species in Lake Bracciano and so far it is unclear if *A. novaezelandiae* is still present or not (Moravec et al. 1994a).

In the naïve European eel *A. crassus* did not only establish rapidly, but also reached significantly higher infection rates (intensities and prevalence) than in the well adapted Japanese eel (Egusa 1979; Kennedy 2007). These high rates of infection can cause severe damages of the swim bladder, suggesting that heavy and chronically infected European eels are unable to reach their spawning grounds in the Sargasso Sea (5,500 km transoceanic migration) (Palstra et al. 2007; Sures & Knopf 2004a).

In contrast to the disease pattern as an alien parasite, infrapopulations of *A. crassus* in the Japanese eel are small and cause low pathogenicity towards the eel (Han et al. 2008; Heitlinger et al. 2009; Münderle et al. 2006; Nagasawa et al. 1994). Experiments of Knopf & Mahnke (2004) confirmed that

Table 2.3 Occurrence of invasive *Anguillicola* species.

Sources: Johnson et al. 1995; Koops & Hartmann 1989; Moravec & Taraschewski 1988; Nagasawa et al. 1994; Neumann 1985; Paggi et al. 1982; Sasal et al. 2008

Parasite species	*Anguillicola crassus*	*Anguillicola novaezelandiae*
Endemic occurence		
host	*Anguilla japonica*	*Anguilla australis*
site	China, Japan, Korea, Taiwan	Australia, New Zealand
New occurence		
first record	1982 (introduced 1980)	1982 (introduced 1975)
host	European eel (*Anguilla anguilla*)	European eel (*Anguilla anguilla*)
site	Europe (Germany)	Europe (Italy)
invasiveness	spread throughout Europe and North Africa	remained in Lake Bracciano (Italy) (last confirmed record 1994)
	1995	
	American eel (*Anguilla rostrata*)	
	North America (USA)	
	still spreading throughout North America	
	2005	
	three African eel species (*Anguilla bicolor* , *Anguilla marmorata* , *Anguilla mossambica*)	
	Southern Africa (Island of Reunion)	
	recorded in eel populations, invasive potential in pacific eel species still unclear	

recoveries of *A. crassus* are higher in European eels (33.2 %) than in Japanese eels (13.8 %), in which the development is slower, the size of adult parasites is smaller and a greater proportion of larvae are encapsulated in the swim bladder wall and die. The original host is able to mount efficient protective immune responses against *A. crassus*, whereas the newly acquired host seems to lack this ability (Knopf et al. 2000; Nielsen 1999). It was also shown in an immunisation experiment that resistance can be induced only in Japanese eels (Knopf & Lucius 2008). Furthermore parasite infection does not only lead to immune reactions but also causes stress for the host (Marcogliese & Pietrock 2011). In experimental studies with European eels Sures et al. (2001, 2006) showed an increase in cortisol and glucose level in the blood serum of eels infected with *A. crassus*.

Due to its pathogenicity towards European as well as American eels, which are also a naïve host to *Anguillicola* spp., *A. crassus* is in focus of science since the 1960s (Egusa 1979; Egusa et al. 1969; Kennedy 2007; Nagasawa et al. 1994). It was studied in wild and cultured Japanese, European and American eels as well as in Japanese and European eels infected in the laboratory and also in copepod intermediate and numerous paratenic hosts (overview see Moravec 2006). In contrast to this, very little is known on the other four *Anguillicola* species. While literature on eels infected with *A. australiensis*

and *A. papernai* comprises only one published article each (*A. a.*: Kennedy 1994; *A. p.*: Taraschewski et al. 2005) four focus on *A. globiceps* (Nagasawa et al. 1994; Suyehiro 1957; Wang & Zhao 1980; Wu 1956) and six on *A. novaezelandiae* (Hine 1978; Lefebvre et al. 2004; Moravec & Rohde 1992; Moravec et al. 1994a; Paggi et al. 1982; Rid 1973).

2.2 Aims of the present study

Even though *A. crassus* proved to be an extremely successful invasive species on three continents (Africa, North America and Europe) and in two eel species (*A. anguilla* and *A. rostrata*) with crucial effects on the new eel hosts, our knowledge on the whole genus of *Anguillicola* is based on only one species. There is an urgent need to figure out which characteristics make *A. crassus* such an effective invader. Therefore, it was the aim of the present thesis to gain more information on the host-parasite-interactions of eels and *Anguillicola* spp. By comparing indigenous eel infections of *A. novaezelandiae*, a species which infected the European eel successfully and established a stable population in one European lake, with those of *A. crassus* it was possible to show differences in the native infection patterns of the two species. Experimental infections of European eel with both species were performed to identify differences in the infection success, as well as the development in the new final host. The host's reaction against both species was investigated by analysing different stress markers, to figure out different effects on the eel. Furthermore, a hybridisation experiment between both *Anguillicola* species in the European eel was performed, to detect possible reasons for the disappearance of *A. novaezelandiae* in Lake Bracciano.

The results of the present study provide first evidence that the original infection patterns of *Anguillicola* species may differ essentially. The life cycle of *A. novaezelandiae* was studied for the first time experimentally in the lab. The finding of hybrids could be a first step to explain the competitive success of *A. crassus* over *A. novaezelandiae* in Lake Bacciano. The results increase the knowledge on the biology and the host-parasite-interactions of eels and parasites of the genus *Anguillicola* in general.

Natural *Anguillicola novaezelandiae* infection – Is there seasonality in New Zealand?

3

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Knowledge of natural *Anguillicola* infections of Short-finned eels (*Anguilla australis*) in New Zealand is very limited. So far, no study contains data on all life cycle stages of *Anguillicola novaezelandiae* in naturally infected eels. In order to study the frequency of *A. novaezelandiae* in New Zealand Short-finned eels, we examined eels of the North and the South Island for the presence of the swim bladder parasite. The results show that *A. novaezelandiae* is a common parasite of the Short-finned eel. The parasite was present in both regions. Eels from both localities show differences in their infection status with respect to prevalence, abundance and intensity. While eels of the South Island were only infected with larval stages, adult and preadult stages could be detected in eels of the North Island. Nevertheless, infrapopulations at both sites were dominated by larval parasite stages. This unique composition of infrapopulations has never been described for any *Anguillicola* species before and suggests a seasonal occurrence as a possible reason. Export of live eels should be handled cautiously to prevent the spread of *A. novaezelandiae* throughout other eel populations.

3.1 Introduction

Within the genus of freshwater eels (*Anguilla*), four species are of major importance for worldwide trade and food production (FAO 2012): The European eel (*Anguilla anguilla*), the Japanese eel (*Anguilla japonica*), the American eel (*Anguilla rostrata*) and the Short-finned eel (*Anguilla australis*). Adult eels are traded as processed dish, smoked, frozen and also alive. With this export and import of live eels, not only alien fish species travel around the world but with them a variety of parasites. As the spread of the gill parasites *Pseudodactylogyrus bini* and *Pseudodactylogyrus anguilla* and the swim bladder nematode *Anguillicola crassus* have shown, this trade can lead to the introduction of alien parasite species. *A. crassus* has spread quickly throughout the European eel population and can cause damage to the eels' swim bladder (Jakob et al. 2009; Kirk 2003; Køie 1991). This swim bladder damage could disable the eel in reaching its spawning ground in the Sargasso Sea (Palstra et al. 2007) and could thus be one factor contributing to the decline of the European eel population (Sures & Knopf 2004a).

The family Anguillicolidae consists of five species, which are all adapted to different Pacific eel species. In 2006, Moravec split the genus *Anguillicola* into the two genera *Anguillicola* and *Anguillicoloides* due to morphologic differences. The phylogenetic study of Laetsch et al. (2012) on Anguillicolidae found no support for the maintenance of these two genera; therefore, all species of the family are referred to as *Anguillicola* in the following.

Whilst the European eel is not an original host to any *Anguillicola* species, the Japanese eel is the natural host of the two species *A. crassus* and *Anguillicola globiceps*, *Anguillicola novaezelandiae* is a parasite of the Short-finned eel. *Anguillicola australiensis* is parasitic in the Long-finned eel (*Anguilla reinhardtii*) and *Anguillicola papernai* is found in *Anguilla mossambica* (Moravec & Taraschewski 1988). Two of these *Anguillicola* species have shown their invasive potential and were able to infect alien eel species. Besides *A. crassus*, which has been in focus of science since live eel trade started in the 1960s (Egusa 1979; Kennedy 2007), *A. novaezelandiae* has also been able to infect European eels and built a stable population in an Italian lake (Moravec et al. 1994a; Paggi et al. 1982). However, the latter species disappeared from the lake after the arrival of *A. crassu*s, probably due to hybridisation between *A. crassus* and *A. novaezelandiae* (Grabner et al. 2012). Apart from eels which are used as definitive hosts, all *Anguillicola* species use mainly copepods as obligate intermediate hosts. In the eel host, larval stages are found in the swim bladder wall as third stage (L3) and fourth stage larvae (L4) while preadult stages migrate into the swim bladder lumen, feed on the host's blood and moult to the adult stage Moravec (2006).

Anguillicola novaezelandiae is native to Australia and New Zealand, where its host, the Short-finned eel (*A. australis*) occurs. So far, there is only limited data on the distribution of the parasite in Short-finned eel populations (Hine 1978; Lefebvre et al. 2004; Rid 1973). The latest work of Lefebvre et al.

(2004) concentrates solely on adult parasites in the lumen of the swim bladder. Older studies mainly gave prevalence data or only report if eels were infected or not. So far no study has presented data on all stages of the parasite. In order to evaluate the potential risk of spreading *A. novaezelandiae* with the trade of live Short-finned eels, its occurrence in the original host (including all larval stages within the swim bladder wall) has to be analysed. Therefore, eels from two sampling sites, located on each of the New Zealand main islands, have been checked for the presence of *A. novaezelandiae* in the present study. In addition, we compared the demographic structure of *A. novaezelandiae* in the present study with that reported in other *Anguillicola-Anguilla* systems, in order to infer potential differences in seasonal patterns and/or population dynamics.

3.2 Material and Methods

3.2.1 Eel sampling

Wild Short-finned eels (*A. australis*) were obtained from professional fishermen in November and December 2007 and transported alive to the lab. Sampling sites are located on each of the New Zealand islands. The Hydro Lakes of the Waikato River were sampled on the North Island. On the South Island, eels were sampled from Lake Ellesmere (Table 3.1).

The eels were kept in large oxygenated tanks, killed by decapitation within two weeks after capture and examined immediately. The length and weight of the eels were measured, whereupon the swim bladder was removed and examined for the presence of *A. novaezelandiae*. The condition factor (C factor) was calculated as described by Schäperclaus (1990). With C as the ratio of the fish somatic mass $*$ 100 $*$ total length^{-3}.

Table 3.1 Sampling sites of *Anguilla australis*.

Locality	Date	n	Length N.S.	Weight **	C factor *
North Island (Waikato River Hydro Lakes, Waikato)	03-07 Dec 2007	105	62.4 ± 3.5	517.3 ± 79.3	0.21 ± 0.02
South Island (Lake Ellesmere, Canterbury)	24-25 Nov 2007	101	58.0 ± 3.5	339.2 ± 51.3	0.17 ± 0.02

Length, weight and C factor of all eels as mean ± SD; length in centimetre; weight in gram
n: number of eels; C factor: condition factor; *N.S.*: not significant,
*: $p < 0.05$, **: $p < 0.001$ significance tested with unpaired Student's t-test

3.2.2 Parasitological examination of eel swim bladders

All parasites were removed from the swim bladder lumen. They were identified and their number and sex was recorded. Species identification was performed morphologically according to the key of Moravec (2006) and subsequently verified by molecular analyses. Preadult and adult parasites were stored in 70 % alcohol. After removal of adult worms, the swim bladder was examined between two plexiglass plates with a stereomicroscope (magnification x8 to x50) for larval stages (L3 and L4). Since the differentiation between L3 and L4 stage is not always possible, all larvae longer than 1.5 mm were considered as L4, as described in Blanc et al. (1992). Length and width of preadult and adult parasites were measured using a stereomicroscope (magnification x8 to x50). Prevalence (P) and mean intensity (MI) of parasites in the eels were calculated as described in Bush et al. (1997). For the description of the host-parasite relation, a frequency distribution was used. In order to check for an aggregated pattern of the parasite's population, the variance-to-mean ratio was calculated as described in Shaw and Dobson (1995; $k = s2/MA$; where MA is mean abundance). Overdispersion (negative binominal distribution) is characterised by $k > 1$, while a random or Poisson distribution is characterised by $k \approx 1$ (Shaw & Dobson 1995).

3.2.3 Molecular identification of *A. novaezelandiae*

For genetic verification of the morphologically identified species, a subsample of eleven nematodes was analysed genetically. DNA was extracted from six randomly chosen parasites and the five largest specimens using the JETQUICK Genomic DNA Purification Kit (Genomed) based on the method of Bowtell (1987). A part of the *coxI* gene was amplified using the primers HCO2198 and LCO1490 (Folmer et al. 1994) and PCR conditions as described in Wielgoss et al. (2008). PCR products were purified with a JETQUICK PCR Product Purification Kit (Genomed) and sent for sequencing (GATC) with HCO2198 and LCO1490 primers. A BLAST search, implemented in the homepage of the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi), was conducted with the sequences obtained to check for homologies in the database.

3.2.4 Statistical treatment of data

For comparisons of eel length, weight and C factor, the unpaired Student's t-test was used with a significance level of $p < 0.05$ and $p < 0.001$, respectively. Dependence of infection status or number of parasites on the C factor was examined by one-way analysis of variance (ANOVA). All statistical tests were conducted with STATISTICA 10.

3.3 Results

3.3.1 Eel data

Data on the Short-finned eels, their origin, length, weight and condition factor are summarised in Table 3.1. Eels from the North Island were longer and heavier than eels from the South Island. The weight of both eel samples shows a highly significant difference, while the length shows no significant difference. Infection status or number of parasites had no influence on the C factor ($p < 0.05$).

3.3.2 Infection patterns of *A. novaezelandiae*

The 105 eels from the North Island were infected with 145 *Anguillicola* individuals, whereas the 101 South Island eels harboured 675 nematodes. All nematodes detected in the swim bladder were identified as *A. novaezelandiae* according to the key of Moravec (2006). The nematode infrapopulation in eels from South Island was dominated by L3 (79.7 %), while the infrapopulation in eels from North Island was dominated by L4 (53.1 %) (Figure 3.1). Eels of the South Island harboured only larval stages with a prevalence of 89.1 %. The total prevalence in North Island eels was 51.4 % of which 21.0 % of the eels were found to be infected with preadult and adult stages (Table 3.2).

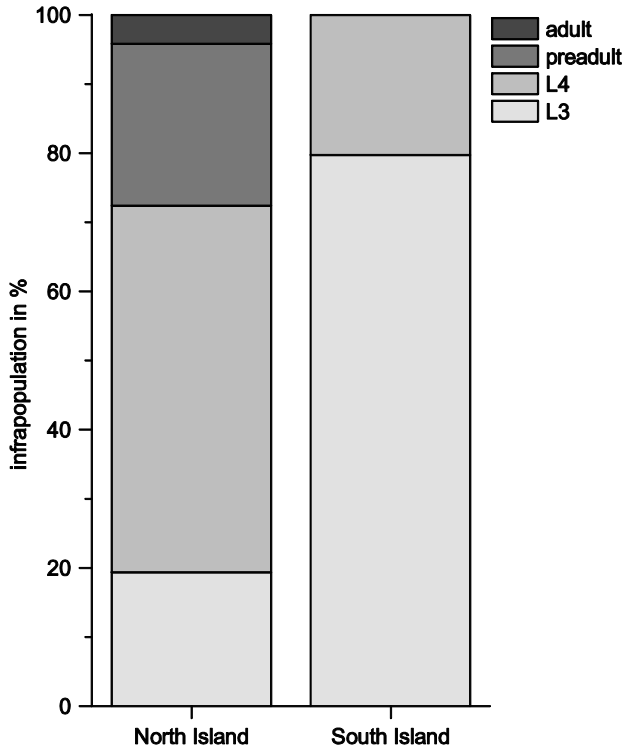

Figure 3.1 Infrapopulation of *A. novaezelandiae* developmental stages. Eels from the North Island were infected with all stages of *A. novaezelandiae*, while eels originating from the South Island were only infected with larval parasite stages in the swim bladder wall.

Table 3.2 Infection parameters of *Anguillicola novaezelandiae*.

Locality	L3			L4			Preadult			Adult			All		
(n eels)	n	P %	MI	n	P %	MI	n	P %	MI	n	P %	MI	n	P %	MI
North Island (105)	28	14.3	1.9 ± 1.8	77	41.0	1.8 ± 1.3	34	14.3	1.7 ± 1.1	6	5.7	3.0 ± 2.8	145	51.4	2.7 ± 2.7
South Island (101)	538	85.1	6.3 ± 6.0	137	54.5	2.5 ± 2.0	0	0.0	0.0	0	0.0	0.0	675	89.1	7.5 ± 6.9

Prevalence and mean intensity for all eels examined ± SD
n: number of specimens; P: prevalence in percent; MI: mean intensity

Only two eels of the North Island were infected with mature parasites. While one eel was infected with a single male parasite, the other eel contained three male and two female parasites. The swim bladder of the latter eel also contained second stage larvae (L2). Only one encapsulated larva could be observed in the swim bladder wall. No swim bladder showed signs of pathological changes due to the infection with *Anguillicola*. Frequency scale of infection showed negative binominal distribution for parasites from North Island eels. Of the eels from the South Island, 33.6 % were infected with more than seven parasites (Figure 3.2). The variance-to-mean ratio for these data was computed for both sampling sites (s^2/MA). Both were > 1, indicating overdispersion (Table 3.3).

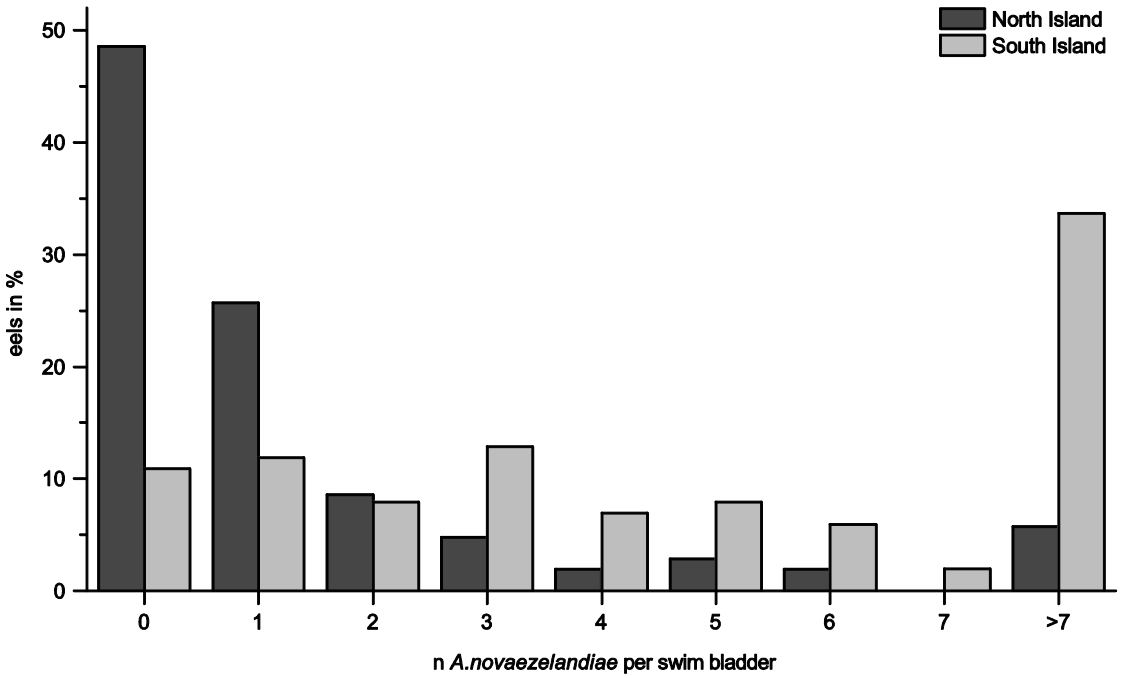

Figure 3.2 Frequency distribution of *A. novaezelandiae*. Data on the North Island (*dark*) show a negative binominal distribution. On the South Island (*light*) 33.6 % of the eels were infected with more than seven parasites.

Table 3.3 Variance to mean ratio of *A. novaezelandiae*. Dispersion of larval stages in the swim bladder wall (wall), preadult and adult stages inside the swim bladder lumen (lumen) and all stages joint (all) are shown.

	Variance-to-mean ratio (k)					
Locality	**Wall**	**Dispersion**	**Lumen**	**Dispersion**	**All**	**Dispersion**
North Island	3.3	o.d.	2.3	o.d.	3.8	o.d.
South Island	7.1	o.d.	-	-	7.1	o.d.

o.d.: overdispersed

Morphometric measurements of preadult and adult stages show two size classes (Figure 3.3). The first group (n = 25) had a mean length of 4.58 ± 0.85 mm, while the second group (n = 4) was 28.69 ± 12.63 mm long. The longest parasite was a 40.16 mm-long male with a width of 1.992 mm. The two female parasites of the same eel could not be measured as they were opened immediately after removal from the swim bladder to obtain eggs for experimental infections described elsewhere (Dangel et al. 2013; Grabner et al. 2012 / Chapter 4 and Chapter 6).

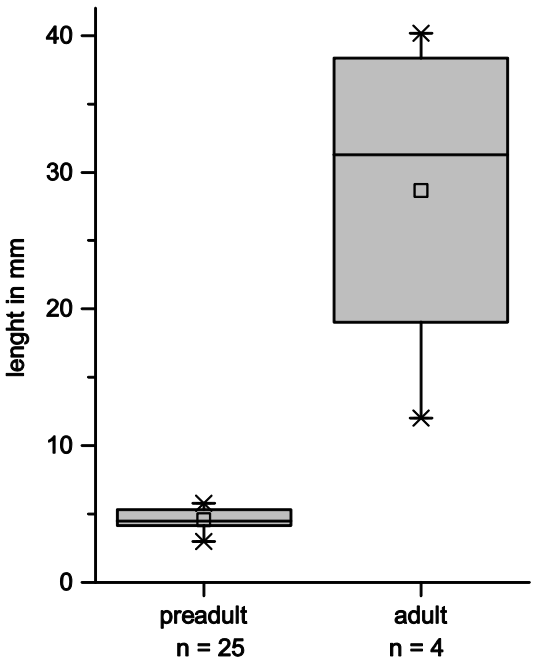

Figure 3.3 Size classes of parasites in swim bladder lumen. Preadult parasites show a very small size range (4.58 ± 0.85 mm), while length of adult parasites varied between 12.0 and 40.2 mm (mean length 28.69 ± 12.63 mm).

3.3.3 Molecular species identification

The sequences obtained from the eleven specimens used for molecular analysis (length 597 bp) were identical except for one polymorphic position at 119 bp. New sequence information was deposited in GenBank under accession no. JX868555 and JX868556. A comparison to sequences of

A. novaezelandiae from Laetsch et al. (2012) (originating from Tasmania, Australia) using BLAST shows only 89.1 % identity. Greatest consensus showed the sequence of *A. australiensis* with 90.2 % identity. *A. crassus* showed an identity of 89.3 %.

3.4 Discussion

According to morphological identification, all parasites found in the swim bladder belong to the species *A. novaezelandiae*. The *cox1* sequences identified showed genetic difference to all other *Anguillicola cox1* genes. As Laetsch et al. (2012) mentioned, the genetic difference of the *cox1* gene between *A. novaezelandiae* from New Zealand and Tasmania suggests the hypothesis of the existence of two sibling species. The geographic distance between New Zealand and Australia could have led to isolation of both *A. novaezelandiae* populations and thus to the genesis of sibling species. This seems plausible since the taxonomic status of the host species *A. australis* is still discussed and it is currently uncertain if the species may consist of the two subspecies *A. australis schmidtii* (New Zealand) and *A. australis australis* (Australia and Tasmania). The latest studies found both morphological (Watanabe et al. 2006) and genetic differences (Shen & Tzeng 2007) comparing *A. australis* from New Zealand and Australia. Therefore, it is possible that both eel subspecies are infected by different *A. novaezelandiae* sibling species. Further studies combining morphological and genetic approaches should be performed to verify the taxonomic status of the two parasite populations.

The finding of only one *Anguillicola* species is in accordance with several findings of *A. novaezelandiae* in the 1970s (Hine 1978; Moravec & Taraschewski 1988; Rid 1973) as well as with the latest study of Lefebvre et al. (2004). So far *A. novaezelandiae* is the only known species of *Anguillicola* in New Zealand as well as in *A. australis*. However, three of the four male adult specimens in our study were larger than all *A. novaezelandiae* specimens described so far (see Figure 3.4). This might reflect the lack of data on *A. novaezelandiae* in literature (Lefebvre et al. 2004; Moravec & Rohde 1992; Moravec & Taraschewski 1988; Paggi et al. 1982). Further studies are needed to gain more knowledge of the species in terms of their morphology, life cycle and distribution.

This study is the first to cover all stages of *A. novaezelandiae* in eel. By including larval stages in the swim bladder wall, the parasite could also be found in eels of the South Island. A study only covering stages in the swim bladder lumen would have detected no parasites, even though 89 % of the eels were infected. Having this in mind, a lack of *A. novaezelandiae* findings in the literature (as described for some locations by Hine (1978) and Lefebvre et al. (2004)) must be viewed with caution. A possible explanation for the differences in infrapopulation composition of the two sampling sites could be

Table 3.4 Overview on morphometric measurements of adult male *Anguillicola novaezelandiae*. The data give an overview on all known measurements of male *A. novaezelandiae* described in literature.

		Length		Width		
Land of Origin	**n**	**Min**	**Max**	**Min**	**Max**	**Source**
New Zealand	4	12.00 -	40.16	0.650 -	1.992	own data
New Zealand	4	5.34 -	22.29	0 476 -	0.680	Lefebvre et al. 2004
New Zealand	4	5.54 -	8.57	0.476 -	0.680	Moravec & Taraschewski 1988
Italy	3	11.63 -	14.92	1.470 -	1.560	Moravec & Taraschewski 1988
Italy	10	10.25 -	25.44	0.500 -	1.800	Paggi et al. 1982
Australia	3	20.60 -	24.40	1.100 -	1.300	Moravec & Rohde 1992

n: number of parasites measured; length and width in millimetre

seasonality. While eels from South Island harboured only larval stages, 14 % of the eels from the North Island harboured preadults. The preadult stages were all about the same size (4.6 ± 0.9 mm). Only two eels from the North Island were infected with adult stages, one with a single worm and the other eel with adult stages exceeding all so far known size data for *A. novaezelandiae*. A possible explanation is that these adults overwintered in the eel, while all other eels were freshly infected in spring 2007. The hypothesis of seasonality is supported by the fact that 20 % of South Island *A. novaezelandiae* are in L4 stage and 23 % of North Island parasites are in the preadult stage. Parasites of the North Island seem to be a step ahead in their life cycle. An explanation is the about 2°C warmer temperature on the northern sampling site compared to the South Island sampling site (NIWA 2012). Prevalence data of preadult and adult stages of *A. novaezelandiae* by Lefebvre et al. (2004) also support this hypothesis. Prevalence in October was lower than in December and eels from the North Island were infected with a higher prevalence at the same sampling date than those from the South Island. So far, indications for seasonality have not been described for any other *Anguillicola-Anguilla* system. Compared to data from Australia (*A. australiensis* from *A. reinhardtii*; Kennedy 1994) and Taiwan (*A. crassus* from *A. japonica*; Münderle et al. 2006) clear differences become obvious for *A. novaezelandiae* from New Zealand. While only one dead larva was found during our study, a large number of dead larvae was described in Kennedy (1994) and Münderle et al. (2006). Furthermore, the dominance of larval stages in the eels *A. novaezelandiae* infrapopulation in both locations of the present study is a unique finding in an *Anguillicola-Anguilla* system (see Figure 3.4), as in Australia and Taiwan, the infrapopulations were all dominated by worms in the swim bladder lumen (Kennedy 1994; Münderle et al. 2006). For *A. crassus* lower prevalences in wild and cultured Japanese eel populations were described in winter (Egusa et al. 1969; Han et al. 2008; Kim et al. 1989; Münderle et al. 2006; Nagasawa et al. 1994) but a lack of adult stages in spring was never observed in

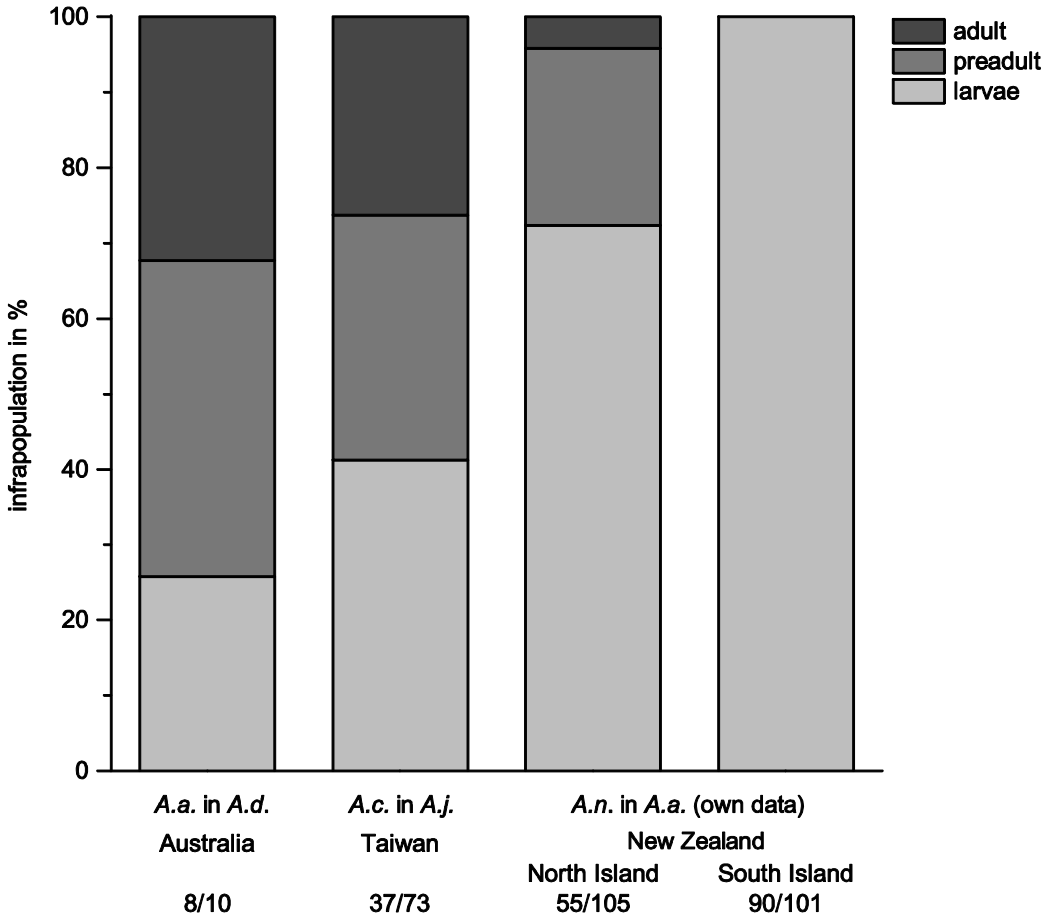

Figure 3.4 Comparison of *Anguillicola* infrapopulations in native hosts. While about 30 % of the *Anguillicola* infrapopulations in Australia and Taiwan consist of adult parasites, only eels of one location in New Zealand were infected with adult parasites (4 %), whereas the second sampling site lacks any adult *A. novaezelandiae*. Only living stages were taken into account to allow a comparison between the different localities; all data are of spring samples.

Australia: data from the sampling on the 1 December 1993 at Bundaberg were used as an example (details see Kennedy 1994; Kennedy personal communication 2012); Taiwan: data from Kao-Ping River sampled in March 2003 were used as an example (worms < 10 mm were counted as preadult; details see Münderle 2005 and Münderle et al. 2006).

numbers indicate the number of infected eels per sampled eels

neither the natural nor the newly invaded distribution areas. All these data support the hypothesis of seasonal occurrence of *A. novaezelandiae* in New Zealand, nevertheless, more eel sampling should be carried out to verify this theory as other factors like different water temperature regimes of the sampling sites may also be important.

The frequency distribution of all stages and both sampling sites exhibited an aggregated dispersal. This is in accordance with other natural *Anguillicola-Anguilla* systems (Han et al. 2008; Kennedy 1994; Kim et al. 1989; Münderle et al. 2006). The observed overdispersion is a characteristic of macroparasites in wildlife populations and arises from heterogeneities in exposure of host populations or under infection pressure (Shaw & Dobson 1995). Eels of the South Island showed a more

aggregated infection pattern than eels of the North Island. The rate of infection with parasites in the swim bladder lumen in this study is comparable to literature data of Hine (1978), Lefebvre et al. (2004) and Rid (1973). Prevalence of *A. novaezelandiae* in the swim bladder lumen in the natural habitat never exceeded 12 % (Boustead (1982) mentioned that eels in Hine (1978) originating from Rangitaiki River, Bay of Plenty, with P of 72.7 % were of an eel farm; also Long-finned and Short-finned eel infection is combined in the paper). The infection of Short-finned eels with *A. novaezelandiae* is similar to infections of other Anguillicolidae in their natural eel host in wild habitats. Prevalence of adult *A. papernai* in *A. mossambica* varies between 8 and 63 % (Taraschewski et al. 2005). Kennedy (1994) found 27 – 78 % of *A. reinhardtii* infected with adult and preadult *A. australiensis*. Japanese eel populations showed a maximum prevalence of around 60 % for *A. crassus* (all stages; Han et al. 2008; Münderle et al. 2006), while *A. globiceps* infected the same eel species with a prevalence of 6 to 60 % (all stages; Nagasawa et al. 1994; Suyehiro 1957; Wang & Zhao 1980; Wu 1956). Infection of non-native eel species is more severe. *A. crassus* was introduced to Europe in the 1980s and to North America in the 1990s. The alien parasite infects its new host, the European eel with a prevalence of up to 100 % (overview in Jakob et al. 2009). In Germany, a well-studied country, prevalence is around 80 % and mean intensity varies between 1 and 14 parasites per eel (Jakob et al. 2009; Sures & Streit 2001; Sures et al. 1999; Würtz et al. 1998).

The findings in the present study seem to be comparable with other natural *Anguillicola-Anguilla* systems. No swim bladder damage could be observed. The invasive potential, shown by the introduction of the species to Europe in the 1970s, should be reason enough for further studies of the species in its natural habitat. As the populations of the most important eel species have fallen to catastrophic lows (Stone 2003), Short-finned eels may be of growing importance in eel production worldwide. There is no glass eel fishery for this species, so live eels are exported from New Zealand as yellow eels with their parasite load. Wang et al. (2006) showed the import of a number of parasite species via live trade of Short-finned eels to Taiwan. In future, more attention should be paid to the risk of this trade. In order to give a risk assessment, the distribution of *A. novaezelandiae* throughout the Short-finned eel population should be studied including larval stages. In order to study the seasonal occurrence, some eel populations should be monitored regularly throughout a year. The knowledge of a distribution pattern and the seasonal occurrence of the species are essential to give advice to eel traders and prevent the parasite from spreading throughout alien eel species.

Can differences in life cycle explain differences in invasiveness? – A study on *Anguillicola novaezelandiae* in the European eel

4

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Anguillicola crassus is the most invasive species of its genus and it is a successful colonizer of different eel species worldwide. It is so far the only species of the genus *Anguillicola* whose life cycle has been studied completely. To analyse whether differences in life cycle may explain differences in invasiveness, we infected European eels with *Anguillicola novaezelandiae* under laboratory conditions. *Anguillicola novaezelandiae* shows a synchronised development in the European eel. Eggs with second-stage larvae appeared 120 days after infection. No density-dependent effect in parasite development could be found for *A. novaezelandiae*. The present study shows that the life cycle of *A. novaezelandiae* differs on final host level compared with *A. crassus* in ways which result in a less successful invasion of new host species.

4.1 Introduction

The current knowledge on the life cycle of all five species of the family Anguillicolidae is mainly based on one species. Moravec (2006) split the genus *Anguillicola* into the two genera *Anguillicola* and *Anguillicoloides* due to morphological differences. The phylogenetic study of Laetsch et al. (2012) on Anguillicolidae, however, found no support for the maintenance of these two genera, therefore all species of the family are referred to as *Anguillicola* in the following. *Anguillicola crassus* is the only species that was intensively studied under laboratory conditions. After its spread throughout the European eel population in the 1980s, this species was studied intensively in wild eel populations (reviewed in Jakob et al. 2009) as well as in experimental infection studies of copepod intermediate hosts (Ashworth et al. 1996; Bonneau et al. 1991; Kennedy & Fitch 1990; Moravec & Konecny 1994; Moravec et al. 1993; Petter et al. 1990; Thomas & Ollevier 1993) and eel final hosts (Ashworth & Kennedy 1999; De Charleroy et al. 1990; Fazio et al. 2008; Haenen et al. 1989, 1991, 1996; Knopf & Mahnke 2004; Knopf et al. 1998; Moravec et al. 1994b; Weclawski et al. 2013).

However, only few studies on the other species have been performed. One study of Taraschewski et al. (2005) proved that *Anguillicola papernai* can infect the European eel. But as only two eels were successfully infected, only limited information on the duration of the life cycle or on infrapopulation composition is available. Wang & Zhao (1980) studied the life cycle of *Anguillicola globiceps* in experimentally infected Japanese eels. Until now the life cycle of *Anguillicola australiensis* has not been studied. Moravec et al. (1994b) studied the life cycle of *Anguillicola novaezelandiae* by infecting the copepod intermediate host experimentally. Studies on the life cycle in eel have not yet been performed apart from a co-infection study of *A. novaezelandiae* and *A. crassus* in European eels focusing on possible hybridisation between these species (Grabner et al. 2012).

Thus, in the present study European eels were infected with *A. novaezelandiae* under controlled laboratory conditions in order to study the life cycle of this parasite for the first time at the final host level. This will help to unravel possible differences to its close relative *A. crassus*, which in turn may help to explain differences in invasion success of both species. As a final host, the European eel was used for several reasons: (i) the main interest in research on *Anguillicola* is the invasive potential of the different parasite species in the European eel; (ii) as most laboratory studies on *A. crassus* have been performed with the European eel, comparability is given; (iii) this eel species is easily available in Europe.

4.2 Material and Methods

4.2.1 Source and maintenance of parasites and hosts

Anguillicola novaezelandiae was collected from *Anguilla australis* originating from New Zealand in December 2007 as second stage larvae (L2, for details see Dangel & Sures 2013 / Chapter 3). The larvae were stored in tap water at ~ 8°C until use. The second stage larvae of *A. crassus* were collected from the swim bladder of naturally infected *Anguilla anguilla* from lake Müggelsee in Berlin, Germany. Copepoda of the order Cyclopoida were collected as intermediate hosts from an eel-free pond. They were kept at 20°C and fed 3 times a week with ground fish flakes (TetraMin, Tetra).

European eels (*Anguilla anguilla*) were obtained from a commercial fish farm (Albe Fischfarm, Haren/Rütenbrock, Germany) known to be free of *A. crassus*. The eels were placed in 300 l tanks and maintained in aerated tap water at 20°C. Eels were fed twice a week *ad libitum* with eel pellets (Dan-Ex 2848, BioMar A/S, Brande, Denmark). Polypropylene tubes were provided as a hide-out. In order to confirm the absence of parasites, ten eels were killed, dissected and examined for the presence of parasites prior to infection experiments. In order to prevent *A. novaezelandiae* from escaping to the environment, all wastewater from tanks with infected eels was heated to 80 °C to kill all potentially occurring L2 stage larvae.

4.2.2 Infection of hosts

Infective third stage larvae (L3) were produced based on the method of Haenen et al. (1994). Copepods and L2 were put together in 24-well plates containing tap water in a ratio of 1 : 3. The plates were kept at 20°C with a 12 h light cycle. Copepods were fed three times a week. After 24 days, *A. novaezelandiae* were collected as L3 by using a tissue potter (55 ml Tissue Grinder, Wheaton) as described by Haenen et al. (1994). *A. crassus* L3 were collected 20 days post infection (dpi). The pottered suspension was poured into a paper tea filter (Tee-Filter standard, Profissimo, dm-drogerie markt) and put into a 50 ml centrifuge falcon tube. The tube was filled with 50 ml Minimum Essential Medium Eagle (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and the larvae were allowed to migrate through the filter for at least 2 h at 20°C. The larvae could then be collected with a Pasteur pipette from the bottom of the tube and were stored at 8°C in fresh medium until use.

Eels were infected by administering the third stage larvae as a suspension in medium with a stomach tube (1.5 mm diameter; B. Braun Melsungen AG, Melsungen, Germany) as described in Sures & Knopf (2004). Thereto the eels were gently wrapped in a wet towel and their eyes covered. The calm eels could then be infected easily without the use of anaesthesia. This method usually allows eel infection within one minute. All infection experiments were conducted in compliance with national and institutional guidelines for the care and use of animals.

4.2.3 Experimental design

An overview on the experimental design and the number of eels for each group is given in Table 4.1. The composition of the parasites' infrapopulation and the duration of the life cycle were studied by infecting eels with 20 L3 of *A. novaezelandiae* (20x-*A.n.* group). About ten eels of this group were dissected every 30 dpi.

In order to study density-depending effects on the infrapopulation, a group of eels was infected with 40 L3 of *A. novaezelandiae* (40x-*A.n.* group). In order to confirm the comparability of this study with literature data, a group of eels was infected with 20 L3 of *A. crassus* (20x-*A.c.* group). As a control group of the eels' condition, ten eels were shame-infected with medium only (control group). All groups were kept in different tanks and fed twice a week.

Eels of the 20x-*A.n.* group were killed by decapitation and examined immediately 30, 60, 90 and 120 dpi each. All other eels (control, 40x-*A.n*. and 20x-*A.c*. groups) were killed after 120 days and examined immediately. The length and weight of the eels were measured, whereupon the swim bladder was removed and examined for the presence of *Anguillicola*. The condition factor (C factor) was calculated as described by Schäperclaus (1990) with C as the ratio of the fish somatic mass ∗ 100 ∗ total length^{-3}.

All parasites were removed from the swim bladder lumen, identified and their number and sex was recorded. About half of the parasites were stored in 70 % alcohol for subsequent morphometric studies whereas the other parasites were each stored in 2 ml microtubes (Sarstedt) at −80 °C for future molecular studies. The swim bladder was then examined between two plexiglass plates with a stereomicroscope (magnification x8 to x50) for all larval stages (L3 and L4). Since the differentiation between L3 and L4 stage is not always possible, all larvae longer than 1.5 mm were considered as L4 stage, as described in Blanc et al. (1992).

Prevalence (P) and mean intensity (MI) of parasites in the eels were calculated as described in Bush et al. (1997). The recovery rate was calculated as the ratio of all recovered parasites divided by the number of parasites administered, in per cent. Reproduction of the nematodes was recorded by the presence of eggs and L2 in the swim bladder lumen.

4.3 Results

4.3.1 Data on eel infection

Mersitic data on eels and parasite recovery are presented in Table 4.1. Condition factors varied between 0.15 and 0.18. All eels had inconspicuous swim bladders without any signs of pathological damages due to the infection with either of the *Anguillicola* species.

Table 4.1 Data on all groups of *A. anguilla*. Length, weight, C factor and recovery rate of all eels as mean ± SD.

Group	Sampling date	n	Length	Weight	C factor	Recovery rate
20x *A.n.*	30 dpi	8	42.3 ± 3.9	112.9 ± 30.5	0.15 ± 0.03	28.1 ± 17.9
20x *A.n.*	60 dpi	8	42.9 ± 4.7	132.6 ± 61.6	0.16 ± 0.03	37.1 ± 26.0
20x *A.n.*	90 dpi	10	44.4 ± 5.0	114.6 ± 59.2	0.16 ± 0.03	44.0 ± 24.7
20x *A.n.*	120 dpi	10	41.9 ± 3.2	112.6 ± 38.9	0.15 ± 0.03	39.5 ± 21.4
40x *A.n.*	120 dpi	4	53.3 ± 8.2	290.9 ± 126.0	0.18 ± 0.02	45.6 ± 9.0
20x *A.c.*	120 dpi	13	36.4 ± 3.0	75.3 ± 15.9	0.16 ± 0.02	50.0 ± 24.2
control	120 dpi	10	43.4 ± 3.2	130.0 ± 36.0	0.16 ± 0.02	-

n: number of eels; C factor: condition factor; length in cm; weight in g; recovery rate in per cent
A.n.: *A. novaezelandiae*; *A.c.*: *A. crassus*

Recovery rate of *A. novaezelandiae* ranged between 28 and 44 % for eels of the 20x-*A.n.* group. The highest recovery rate of 50 % was found in eels of the 20x-*A.c.* groups, while eels of the 40x-*A.n.* group showed a rate of 46 % (Table 4.1).

4.3.2 Development of *A. novaezelandiae*

Figure 4.1 shows the relative composition of the infrapopulations of all *A. novaezelandiae* stages in the 20x-*A.n.* group at different dpi. After 30 dpi, 46 % of the parasites were still in L3 stage, while after 90 dpi no more L3 were found. First adult stages were found at 60 dpi. As soon as adult stages occurred, they dominated the infrapopulation composition. After 120 days, first dead parasites were found in the swim bladder lumen (2 %). Eels had mean intensities of 5.6 - 8.8 parasites (Table 4.2). Only eels dissected at 120 dpi harboured L2 (Table 4.2).

Differences in relative infrapopulation composition between all eel groups dissected after 120 days are shown in Figure 4.1. Eels of the 20x-*A.n.* group showed exclusively adult and dead stages. Only 84 % of the parasites found were adult in eels of the 20x-*A.c.* group, compared with 98 % (20x-*A.n.* group) and 95 % (40x-*A.n.* group). The 20x-*A.c.* group still harboured L3 (5 %) and 11 % L4. No dead parasites were found in this group.

Table 4.2 Infrapopulation of eels infected with *Anguillicola.* Number of parasites, prevalence and mean intensity for all eels examined ± SD

	L3		L4		Preadult		Adult		Dead			
Group	**P %**	**MI**	**P %**	**MI**	**P %**	**MI**	**P %**	**MI**	**P %**	**MI**	**MI all**	**L2 %**
20x-*A.n.*												
30 dpi	45.8	2.3 ± 2.0	54.2	4.8 ± 0.8	0.0	0.0	0.0	0.0	0.0	0.0	5.6 ± 3.6	0
60 dpi	7.5	3.0 ± 0.0	18.5	1.3 ± 0.6	14.6	1.0 ± 0.0	59.4	10.2 ± 3.8	0.0	0.0	7.5 ± 5.2	0
90 dpi	0.0	0.0	6.3	2.0 ± 0.0	0.0	0.0	93.8	8.4 ± 4.9	0.0	0.0	8.8 ± 4.9	0
120 dpi	0.0	0.0	0.0	0.0	0.0	0.0	97.8	7.7 ± 4.3	2.2	2.0 ± 0.0	7.9 ± 4.3	80.0
40x-*A.n.*												
120 dpi	0.0	0.0	1.3	1.0 ± 0.0	0.0	0.0	95.4	18.0 ± 3.6	3.3	2.0 ± 0.0	18.8 ± 2.6	100.0
20x-*A.c.*												
120 dpi	4.9	1.6 ± 0.5	11.3	2.3 ± 1.7	0.0	0.0	83.8	8.2 ± 3.6	0.0	0.0	10.0 ± 4.8	69.2

n: number of parasites; P: prevalence in percent; MI: mean intensity; L2: presence of eggs/L2 in swim bladder lumen of eels in per cent

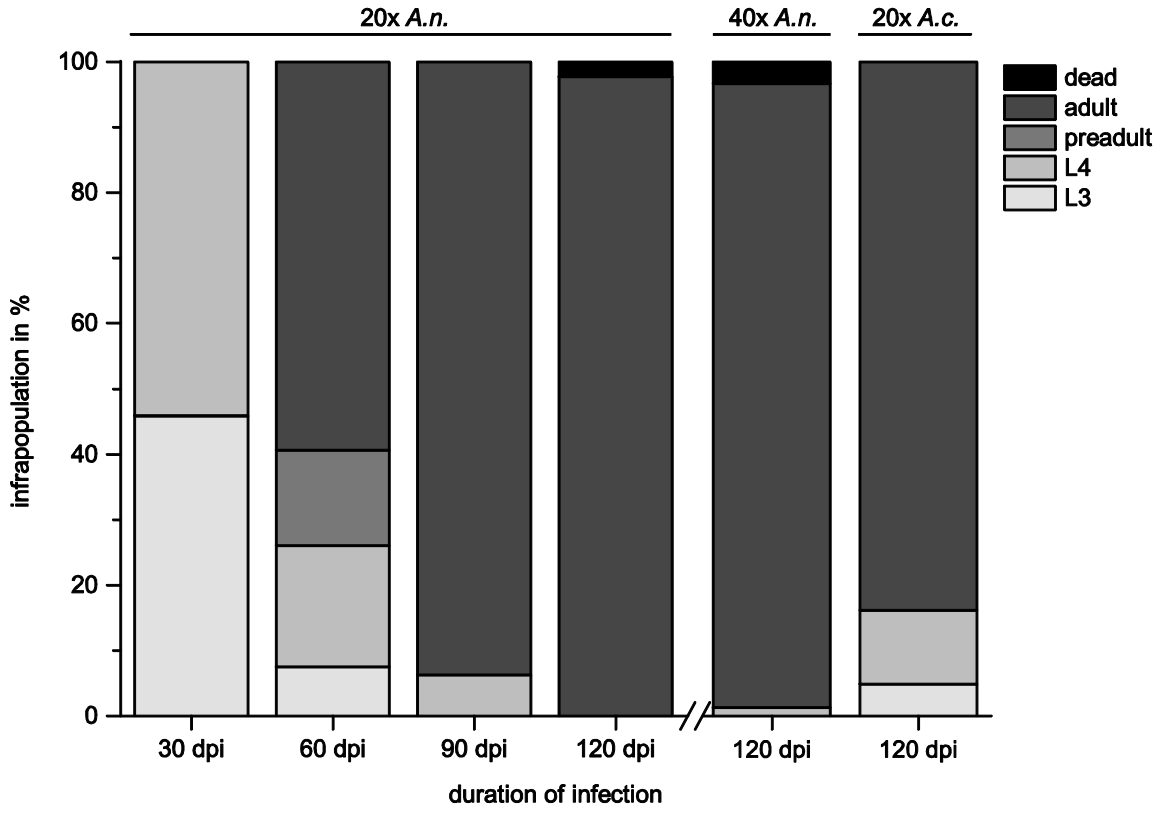

Figure 4.1 *Anguillicola* sp. infrapopulation of eels. 20x *A.n.* group: After 30 days all parasites were still in larval stages; first adult stages are visible after 60 days; 90 days after infection all parasites were in L4- and adult stage; the swim bladder of eels sampled at 120 dpi contains mainly adult stages and some dead parasites.
120 dpi: Eels infected with 20x *A. novaezelandiae* showed no larval stages, while L4 were present in both other groups. In the swim bladder of eels infected with 20x *A. crassus* over 16 % of the recovered parasites were still in larval stages.

4.4 Discussion

The number of recovered worms in the swim bladder of infected eels in the 20x-*A.n.* group varied between 1-16 parasites (MI 6-9), while eels of the 20x-*A.c.* group were infected with 3-18 parasites (MI 10). These data are comparable to natural infections of the European eel with other *Anguillicola* parasites. Mean intensity of *A. novaezelandiae* from Lake Bracciano eels varied between 2-11 (Moravec et al. 1994a), while records of *A. crassus* infection of the European eel showed mean intensities between 1-17 (overview in Jakob et al. 2009). Recovery rate of all groups is similar to laboratory infections of European eel with *A. crassus* (Fazio et al. 2008; Knopf & Mahnke 2004; Knopf et al. 1998; Weclawski et al. 2013).

The data presented here give first information on the life cycle duration of *A. novaezelandiae* in the eel final host and the infrapopulation composition during its maturation. Development to L4 was first recorded at 30 dpi and was completed at 90 dpi. First adult stages were found in the lumen of the eels' swim bladders at 60 dpi. The life cycle was completed at 120 dpi when no more larval parasites were found and eggs with L2 were recovered from the swim bladder. This infrapopulation composition of *A. novaezelandiae* differs strongly from the pattern of *A. crassus* infections. Whilst all studies performed with comparable infection doses and duration on the latter species show L3 and L4 (30x *A.c*. at 98 dpi at 23°C, Knopf & Mahnke 2004; 20x *A.c*. at 119 dpi at 19°C, Knopf et al. 1998) like eels of the 20x-*A.c*. group in this experiment, no larval stages were found at 120 dpi in eels of the 20x-*A.n.* group. *A. novaezelandiae* specimens seem to grow equally fast, in contrast to the non-uniform growth of *A. crassus*.

A. novaezelandiae L2 were first found at 120 dpi, while L2 of *A. crassus* were detected at 84 dpi at 20-25°C by Fazio et al. (2008) and at 50 dpi at 22°C by Weclawski et al. (2013). *A. novaezelandiae* needs thus more time to complete its life cycle and to produce second stage larvae infective to the copepod intermediate host. By comparing our findings with literature data, we found that in Taraschewski et al. (2005) the European eel infected with an unknown number of L3 of *A. papernai*, only harboured adult parasite with eggs when dissected 131 days after infection. In an unpublished study performed in our laboratory, we infected European eels with 13 or 20 L3 of *A. papernai* (the experiment was performed at 20°C). One eel infected with 13 parasites was dissected after 56 days. Only larval stages (1x L3, 4x L4) were found. Three other eels were dissected 140 and 168 dpi. While we found four adult parasites in two eels (both infected with 20x *A.p*.) and three adult parasites in the other eel (infected with 13x *A.p*.), no larval stages were recovered in the swim ladder wall of these eels. These findings suggest that parasites of *A. papernai* might also develop rather equally fast, but for reliable data more experimental infections should be performed to prove this hypothesis. In contrast to these findings, Knopf et al. (1998, 25x *A.c*. at 18°C) still recovered L3 and L4 of *A. crassus* after 195 days, while first adult stages were recorded after 50 days.

Only one eel infected with 40x *A. novaezelandiae* harboured a single fourth stage larva, which seems more likely due to a simple coincidence than a true density-dependent effect. While in the literature the development of *A. crassus* is stated to be density dependent (Ashworth & Kennedy 1999; Fazio et al. 2008; Weclawski et al. 2013), no such effect could be found in this study. Moreover our study shows a highly synchronised development pattern, which is the first finding of such a pattern in *Anguillicola* parasites.

The synchronised development of *A. novaezelandiae* may be beneficial for parasite populations with seasonal occurrence, where all host specimens are infected at the same time. As discussed in Dangel & Sures (2013 / Chapter 3), this could be possible for *A. novaezelandiae* in its original distribution area in New Zealand. As an alien species in Europe, the synchronised development could have been a disadvantage in competition with the related species *A. crassus* in Lake Bracciano. Assuming that both parasite species can produce the same amount of eggs with L2, the non-uniform growth of *A. crassus* will lead to L2 over a longer period, while the synchronised development of *A. novaezelandiae* will lead to production of L2 over a shorter period. This duration of L2 release could be an advantage, even though the density during the longer period may be lower. Accordingly, the non-uniform growth of *A. crassus* could be one important factor for its more successful invasion. However, other factors such as the involvement of paratenic hosts or the longevity of adult worms may also be important but are unknown for *A. novaezelandiae*.

These results show that there are differences in the life cycles of *A. novaezelandiae* and *A. crassus*. Not only infrapopulation composition, but also the periods until the first egg release vary strongly. *Anguillicola crassus* is often used as a model species for the whole genus, but this study proves that there are great differences between different *Anguillicola* species. Further studies should be performed with Short-finned eels (*Anguilla australis*) infected with *A. novaezelandiae* to determine how the native host influences the development of the parasite in terms of the infrapopulation composition and the duration until the first egg release. In order to study the temperature range of the parasites in Europe, infection studies of European eels with different water temperatures should be performed.

Effects of *Anguillicola novaezelandiae* on the levels of cortisol and hsp70 in the European eel

5

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The nematodes *Anguillicola novaezelandiae* and *Anguillicola crassus* are both alien parasites of the European eel with severe adverse effects on their new host. Both species differ in terms of their invasiveness and their severity of harmful effects on the European eel. The purpose of this study was to determine under laboratory conditions whether stages of *A. novaezelandiae* induce stress in European eels (*Anguilla anguilla*) and if these levels differ from stress levels induced by *A. crassus*. We analysed levels of plasma cortisol and hepatic hsp70 of eels experimentally infected with *A. novaezelandiae* and compared them to uninfected eels as well as to eels experimentally infected with *A. crassus*. Larval stages of *A. novaezelandiae* induced higher levels of plasma cortisol compared to uninfected controls, while adult parasites increased the levels of hepatic hsp70 above that of uninfected controls. The eels' cortisol response is induced by larval stages of *A. novaezelandiae*, while adult stages elevate levels of hepatic hsp70. Levels of stress induced by *A. novaezelandiae* are comparable to those induced by *A. crassus*.

5.1 Introduction

Swim bladder parasites of the genus *Anguillicola* are eel-specific parasites endemic to the Indo-Pacific region. Two species, *Anguillicola crassus* and *Anguillicola novaezelandiae*, are alien parasites of the European eel. After *A. novaezelandiae* had been introduced in 1975 to Italy, the species established in a single lake, but was never found in other locations in Europe and as of today it is unclear if the species is still present in European freshwater ecosystems (Moravec et al. 1994a; Paggi et al. 1982). The second species, *A. crassus*, was introduced in 1980 to Europe. It has quickly spread throughout the population of the European eel and is a much more invasive parasite than its congener *A. novaezelandiae*. It is currently known to be an alien parasite in five eel species (*Anguilla anguilla*, *A. rostrata*, *A. bicolor*, *A. marmorata*, *A. mossambica*; Johnson et al. 1995; Moravec 2006; Neumann 1985; Sasal et al. 2008). In Europe, *A. crassus* has a higher virulence and reaches significantly higher infection rates in terms of intensities and prevalence in its new host, the European eel, compared to the original host, the Japanese eel (Egusa 1979; Kennedy 2007). In the European eel, high infection rates cause severe damage of the swim bladder wall (Würtz & Taraschewski 2000), suggesting that these eels are unable to reach their spawning grounds in the Sargasso Sea (Palstra et al. 2007; Sures & Knopf 2004a). On the other hand, there are no records of any damage of the swim bladders of eels caused by *A. novaezelandiae* (Moravec et al. 1994a).

In addition, a parasite infection does not only lead to more or less visible damages of the host tissue caused by feeding activity, attachment or migration of larvae through host tissue, but can also have an impact on the host's homeostasis (Lucius & Loos-Frank 2008; Sures 2006). A disturbed homeostasis is commonly defined as stress (Wendelaar Bonga 2011). Fish, like other vertebrates, respond to stress on organismic as well as on cellular level. The organismic stress response is classified into three categories. The primary response results in an increase of stress hormones and cortisol blood levels. The second response is characterised by physiological effects of these hormones, while the tertiary response includes physiological and behavioural changes on animal level. Cortisol is the main corticosteroid hormone in regulation of the glucose metabolism of teleosts (Kiilerich & Prunet 2011). Besides this main function, cortisol is involved in the regulation of many physiological roles, such as growth, immune function, reproduction and osmoregulation (Kiilerich & Prunet 2011; McCormick 2011). It is rated to be an essential indicator of the stress status of fish and can easily be quantified (Moon 2011).

The cellular stress response includes changes of protein expression of e.g. heat shock proteins, metallothioneins and antioxidant enzymes (Wendelaar Bonga 2011). Heat shock proteins (hsps) function as molecular chaperones, which interact with other proteins by helping to prevent protein aggregation, promote with appropriate protein refolding and the repair of damaged proteins (Currie 2011; Roberts et al. 2010). Under stressful conditions hsps are up-regulated in all cells to protect or facilitate the repair of damaged proteins (Roberts et al. 2010).

Only few studies have analysed the stress response of eels to *Anguillicola* sp. infections. Sures et al. (2001, 2006) studied the effects of *A. crassus* infections on European eels with experimental infections. These studies showed a significant increase in cortisol levels of infected eels. The stress response was basically directed against larval and young adult stages, but not against older adult stages. In wild European eels, Kelly et al. (2000) found no effects of natural *A. crassus* infections on the plasma cortisol levels. Gollock et al. (2004, 2005a) found no difference in cortisol responses between eels that were uninfected or infected with *A. crassus*, when stress was imposed by netting and aerial exposure or acute temperature increase. However, eels infected with *A. crassus* showed a significantly greater corticosteroid stress response to hypoxia than uninfected eels (Gollock et al. 2005b.)

Here, we provide the first study on the effects of infections with *A. novaezelandiae* on the stress response of European eels in comparison to *A. crassus*. We used levels of plasma cortisol as a key messenger of the organismic stress response as well as hepatic hsp70 levels as part of the cellular stress response to characterise the eels' stress reaction against both parasite species.

5.2 Methods

The overall experimental design is displayed in Table 5.1 and described in detail in Dangel et al. (2013 / Chapter 4). Altogether, seven groups of eels were examined. Eels were either experimentally infected with parasites of *Anguillicola novaezelandiae* or *Anguillicola crassus* or remained uninfected and served as control. Four groups of eels were infected with 20 third stage larvae (L3) of *A. novaezelandiae* (20x-*A.n.* group) and dissected after 30, 60, 90 and 120 days post infection (dpi). One group was infected with 20 L3 of *A. crassus* (20x-*A.c.* group) to confirm the comparability of this study with literature data. The sixth group of eels was infected with 40 L3 of *A. novaezelandiae* (40x-*A.n.* group) to study density-depending effects of *A. novaezelandiae* infections. Control, 40x-*A.n*. and 20x-*A.c*. groups were killed after 120 dpi and examined immediately. All seven eel groups were handled alike. After decapitation of the eels, blood samples were immediately taken to analyse the plasma cortisol levels. After allowing the blood sample to clot for 1 h at 20 °C, the samples were centrifuged for 5 min at 2,000 g. Sera were collected and stored at -80 °C for cortisol analyses. During dissection, the liver was removed immediately, weighed and stored in 2 ml micro tubes (Sarstedt) at -80°C for subsequent analyses of hsp70 levels. Each spleen was also removed and weighed. The hepatosomatic index (hsi) and spleen somatic index (ssi) were calculated as fish liver mass/fish somatic mass ∗ 100 and fish spleen mass/fish somatic mass ∗ 100, respectively. The condition factor (C factor) was calculated as described by (Schäperclaus 1990). With C as the ratio of the fish somatic mass ∗ 100/total length^3. The whole infection experiment was conducted in compliance with national and institutional guidelines for the care and use of animals.

5.2.1 Cortisol analysis

Plasma cortisol levels (ng/ml) were measured by an enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (Cortisol ELISA RE 52611, IBL International GmbH, Germany) according to manufacturer's instructions. Cortisol was measured on a microplate reader (λ = 450 nm; Tecan, infinite M200).

5.2.2 Heat shock protein analysis

Hepatic hsp70 levels were measured using the discontinuous SDS-PAGE method of Laemmli (1970) followed by Western Blot with monoclonal antibodies. Liver tissue (10-20 mg) was mixed on ice with 200 µl of extraction buffer (containing 80 mM potassium acetate, 5 mM magnesium acetate, 20 mM HEPES, Roth, Germany; at pH 7.5; 2 % protease inhibitor, Sigma-Aldrich, Germany) and homogenized using a micropestle (Eppendorf, Germany). The tissue lysates were centrifuged at 16,000 g for 10 min at 4°C. 20 µl of the supernatant were taken for protein determination using the Pierce BCA Protein Assay Kit (Pierce Biotechnology, USA). 20 µl of the supernatant were mixed with 20 µl of Laemmli buffer (Laemmli 1970), heated to 95°C for 5 min and then frozen to -20°C until hsp70 quantification.

Proteins were separated on a Mini-PROTEAN Tetra Cell (Bio-Rad, Germany) for ~ 35 min at 180 V. On each gel, a protein marker (Precision Plus Protein Dual Color Standards, Bio-Rad, Germany) and a protein standard mix were added to normalise the data. Following the electrophoretic separation, the proteins were transferred onto nitrocellulose filters for 120 min at 120 mA. The filters were then blocked in a 1:1 solution of horse serum (Sigma-Aldrich, Germany) and TBS (tris-buffered saline, Sigma-Aldrich, Germany) for 60 min at room temperature, washed 5 min in TBS and subsequently incubated with a primary antibody against anti-heat shock 70kDa (hsp70) (antibodies-online GmbH, Germany) overnight in the refrigerator. The filters were washed for 5 min in TBS and incubated in a second antibody solution (peroxidase-conjugated goat anti-mouse IgG; P0447, Dako A/S, Denmark) for 120 min at room temperature and washed again.

The antibody cross reaction was visualised by a mix of 2 ml chloronaphthol (3 mg/ml in 99 % ethanol; Sigma-Aldrich, Germany), 20 ml TBS and 30 % hydrogen peroxide. After 3-10 min the reaction was stopped in aqua dest., filters were dried, scanned and the grey values of hsp70 bands were quantified by densitometric image analyses using the image processing program Image J.

5.2.3 Data analyses and statistical treatment

Results are shown as mean ± SD. Concentrations of all groups were tested using the Kruskal-Wallis test with the post-hoc Dunn's Test. Significance was accepted when $p < 0.05$. All statistical tests were conducted with GraphPad Prism 5. Spearman correlation analysis was performed with OriginPro 9G. Associations between the cortisol level, hsp70 level, condition factor, hsi, ssi, the number of larval *Anguillicola* spp. and the number of *Anguillicola* spp. in the swim bladder lumen were tested for significance.

5.3 Results

All results of condition factors, hepatosomatic indices, spleen somatic indices, plasma cortisol and hepatic hsp70 levels, are listed in Table 5.1. Condition factor, hepatosomatic index and spleen somatic index did not differ significantly between the groups and indicate that eels had normal body conditions.

Table 5.1 Measured parameters of all eel groups. Condition factor (C factor), hepatosomatic index (hsi) and spleen somatic index (ssi), cortisol and hsp70 levels in all eel groups (mean ± SD).

Group	Sampling date	n	MI	C factor	hsi	ssi	cortisol in ng/ml	hsp70
control	120 dpi	10	0.0 ± 0.0	0.16 ± 0.02	1.19 ± 0.18	0.092 ± 0.035	2.84 ± 2.45	65.4 ± 17.1
20x-*A.n.*	30 dpi	8	5.6 ± 3.6	0.15 ± 0.03	1.14 ± 0.27	0.082 ± 0.028	3.34 ± 2.83	73.9 ± 25.8
20x-*A.n.*	60 dpi	8	7.5 ± 5.2	0.16 ± 0.03	1.24 ± 0.27	0.079 ± 0.018	2.02 ± 1.01 *	66.0 ± 26.0
20x-*A.n.*	90 dpi	10	8.8 ± 4.9	0.16 ± 0.03	1.11 ± 0.20	0.078 ± 0.024	2.74 ± 1.76	92.4 ± 27.8 *
20x-*A.n.*	120 dpi	10	7.9 ± 4.3	0.15 ± 0.03	1.04 ± 0.21	0.103 ± 0.027 *	2.90 ± 2.66	90.0 ± 21.8
40x-*A.n.*	120 dpi	4	18.8 ± 2.6	0.18 ± 0.02	1.28 ± 0.21	0.087 ± 0.019	3.92 ± 1.89 *	111.4 ± 20.9
20x-*A.c.*	120 dpi	13	10.0 ± 4.8	0.16 ± 0.02	0.91 ± 0.17	0.090 ± 0.059	3.47 ± 3.50 *	73.7 ± 28.1 *

n: number of eels; MI: mean intensity of *Anguillicola* sp. infection; cortisol in ng/ml; hsp70 as relative grey value
A.n.: *A. novaezelandiae*; *A.c.*: *A. crassus*
*mean level/index was calculated with one value less than n

5.3.1 Stress on individual level

Plasma cortisol levels of eels infected with 20x *A. novaezelandiae* did not differ significantly from those of the control group (Table 5.1, Figure 5.1). Only at 30 dpi cortisol levels are elevated compared to control. Mean cortisol levels in eels infected with 40x *A. novaezelandiae* or 20x *A. crassus* were higher compared to eels infected with 20x *A. novaezelandiae*, but no significant difference could be found (Table 5.1, Figure 5.2).

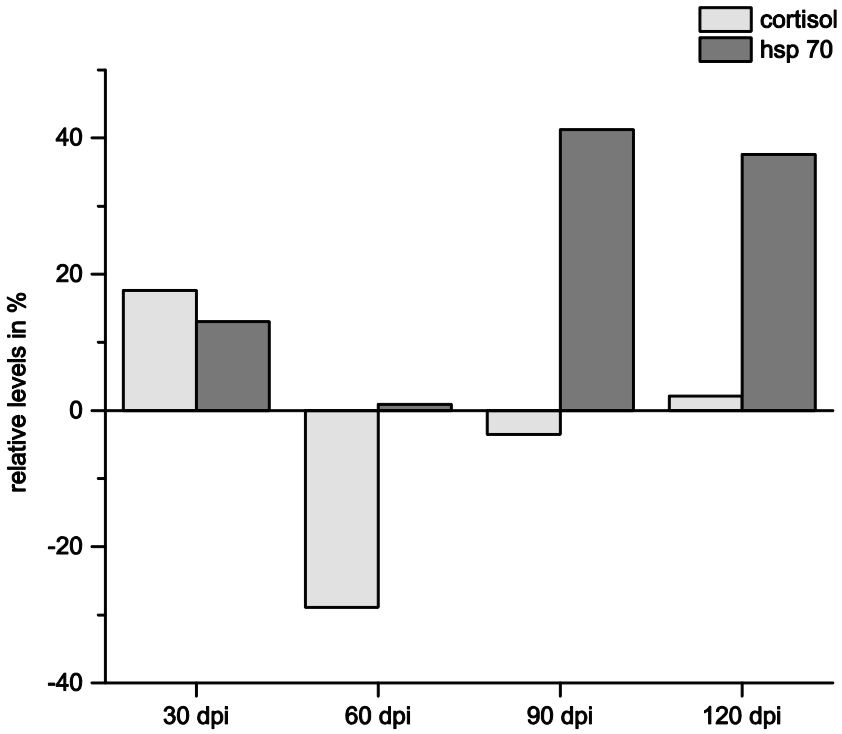

Figure 5.1 Relative cortisol and hsp70 levels of eels infected with 20x *A. novaezelandiae* in comparison to control eels. Eels dissected at 30 dpi show slightly elevated cortisol and hsp70 levels. All other groups show lower or similar levels of cortisol as control eels. Levels of hsp70 are elevated in eels dissected at 90 and 120 dpi.

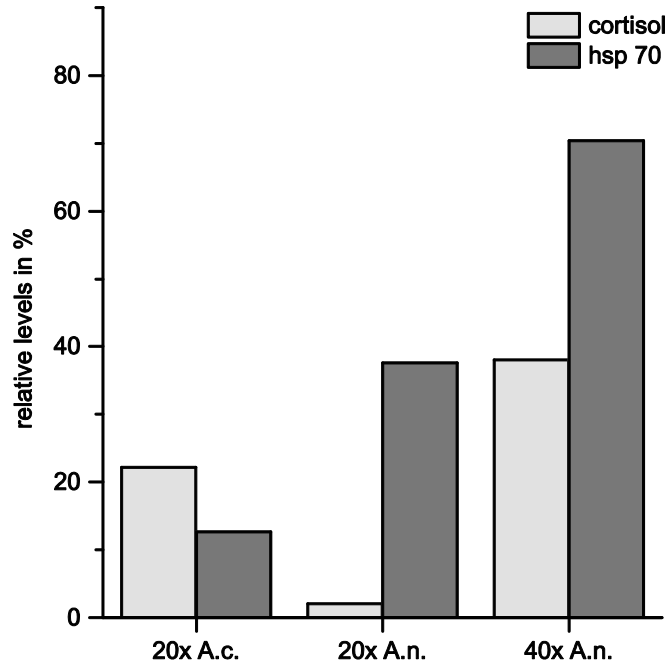

Figure 5.2 Relative cortisol and hsp70 levels of eels dissected 120 dpi in comparison to control eels. Eels infected with *A. crassus* show elevated cortisol levels compared to control and 20x *A. novaezelandiae* eels. Highest mean cortisol levels were measured in eels infected with 40x *A. novaezelandiae*. Highest levels of hsp70 were measured in eels infected with 40x *A. novaezelandiae*.

Hepatic hsp70 levels in eels infected with 20x *A. novaezelandiae* did not show significant differences to uninfected control eels (Table 5.1, Figure 5.1). However, at 90 and 120 dpi hsp70 levels were elevated compared to control. Eels infected with *A. crassus* showed lower hsp70 levels than eels infected with *A. novaezelandiae*. Eels infected with 40x *A. novaezelandiae* showed the highest hsp70 levels (Table 5.1, Figure 5.2).

The levels of hsp70 were significantly correlated ($p < 0.001$) with the number of *Anguillicola* spp. in the swim bladder lumen ($r = 0.44$). No other relationship showed significant correlations.

5.4 Discussion

The plasma cortisol levels in this study were consistent with other studies of non-stressed eels (Gilham & Baker 1985; Sures et al. 2001, 2006). Nevertheless, slightly higher stress levels were obvious in selected groups of eels. Plasma cortisol levels of eels infected with 20x *A. novaezelandiae* were elevated only at 30 dpi compared to uninfected control eels. Longer periods since initial infection did not lead to elevated cortisol levels which might be due to the synchronised development of the nematodes within the eel. It is already known, that the *A. novaezelandiae* infrapopulation of experimentally infected European eels at 30 dpi consists only of larval stages (see Dangel et al. 2013 / Chapter 4). As L3 of *Anguillicola* spp. are the mobile stages which migrate into the swim bladder wall before molting to the less mobile L4 (De Charleroy et al. 1990) this migration could be a possible reason for the slightly elevated levels of cortisol measured in this study. Similarly, in their studies with *A. crassus*, Sures et al. (2001, 2006) stated that elevated cortisol levels were mainly caused by larval and probably young adult stages which is in accordance to the findings of the present study. Compared to the previous studies of Sures et al. (2001, 2006), in which plasma cortisol levels in the same eels were repeatedly analysed by drawing blood from the caudal vein of living eels, fish in the present study had no additional handling stress. Accordingly, the stress levels were mainly induced by infection with *Anguillicola* spp. individuals.

Regarding cortisol levels measured at 120 dpi, eels of the 20x *A. crassus* and the 40x *A. novaezelandiae* group showed elevated levels of plasma cortisol compared to the control group. The elevated level of the 20x *A. crassus* group could be explained by the fact that the development of *A. crassus* is less uniform than that of *A. novaezelandiae* (Dangel et al. 2013 / Chapter 4; Fazio et al. 2008; Knopf et al. 1998; Weclawski et al. 2013). At 120 dpi, still 16.2 % of *A. crassus* were in a larval stage (see Dangel et al. 2013 / Chapter 4) which, in accordance with the fact that mainly larvae and probably young adults cause cortisol release (Sures et al. 2001, 2006), would explain the elevated cortisol levels. The infection with a higher number of adult *A. novaezelandiae* could cause stress in eels due to a highly increased feeding activity of adults, which damages the swim bladder mechanically and leads to blood loss.

Elevated cortisol levels could be beneficial for *Anguillicola* sp. during the early infection of eels as (Sures et al. 2006) found a highly significant negative correlation between levels of cortisol and anti-*A. crassus* antibodies. However, the suppression of the host's antibody reaction is only beneficial for the parasite in its original eel host (i.e. *A. crassus* in *Anguilla japonica*, see Knopf & Lucius 2008) as no effect of the humoral immune response in European eels could be found (Knopf & Lucius 2008; Knopf et al. 2000; Sures & Knopf 2004b).

In contrast to the levels of plasma cortisol, the levels of hepatic hsp70 measured in this study showed a different pattern. Eels of the 20x *A. novaezelandiae* group had elevated hsp70 levels at 90 and 120 dpi compared to uninfected control eels. This could result from the blood feeding of adult parasites. At 90 dpi, almost all parasites migrated into the swim bladder lumen and started feeding on blood by biting veins in the swim bladder (De Charleroy et al. 1990). This tissue damage could result in the elevation of hsp70 levels. Hepatic hsp70 levels of eels infected with 40x *A. novaezelandiae* were even more elevated at 120 dpi compared to control. If more parasites inside the swim bladder lumen fed on blood the hsp70 levels were elevated. Spearman correlation analysis showed a highly positive correlation between hepatic hsp70 levels and the number of parasites inside the swim bladder lumen. In the 20x *A. crassus* group, hsp70 was elevated to a lower degree compared to *A. novaezelandiae*. A principal elevation is in line with data on *A. novaezelandiae* and shows that the presence of adult parasites increases the hsp70 level of eels. A possible reason why the hsp70 level in eels infected with *A. crassus* is lower compared to that of eels infected with a similar number of *A. novaezelandiae* could be the different infrapopulation composition. While eels infected with *A. novaezelandiae* harbored only adult stages at 120 dpi, the infrapopulation of eels infected with *A. crassus* consisted of larval as well as adult stages (Dangel et al. 2013 / Chapter 4). On the other hand almost 35 years of infections with *A. crassus* could have been enough time for the European eel population to start an adaption process to this alien species, provided that eels infected with low infection rates are capable of migrating back to the Sargasso Sea for spawning.

In conclusion it is clear, that different life stages of *A. novaezelandiae* induce different stress responses in the eel host, even though significant differences between groups were not given, probably due to the small sample size of the single groups. While larval stages induce a cortisol response in the European eel, the hsp70 response seems to be associated to the number of adult stages inside the swim bladder. Levels of stress induced by both *Anguillicola* species vary slightly as a result of differences in the infrapopulation composition.

Merging species? Evidence for hybridisation between the eel parasites *Anguillicola crassus* and *A. novaezelandiae* (Nematoda, Anguillicoloidea)

6

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Background: The eel parasitic nematodes *Anguillicola crassus* (originating from Asia) and *Anguillicola novaezelandiae* (originating from New Zealand) were both introduced to Europe, but occurred in sympatry only in Lake Bracciano in Italy, where they both infected the European eel (*Anguilla anguilla*). *A. novaezelandiae* was introduced to the lake in 1975 and disappeared soon after *A. crassus* was also found there in 1993. We tested the hypothesis if hybridisation of the two species might be an explanation for the findings at Lake Bracciano.

Findings: After laboratory infection of one European eel with ten third stage larvae of each parasite, two living female and four male adults of each species were found to co-occur in the swim bladder after 222 days post exposure. In 9 out of 17 eggs, isolated in total from uteri of the two *A. novaezelandiae* females, alleles were detected by microsatellite analysis that are characteristic for *A. crassus*, suggesting the hybrid origin of these eggs. In contrast, none of the eggs isolated from *A. crassus* females possessed alleles different from those found in *A. crassus* adults, but it was revealed that one female can be inseminated by several males.

Conclusion: Our results show that *A. crassus* and *A. novaezelandiae* can co-infect a single eel and can mature together in the same swim bladder. We also provide evidence for the possibility of hybridisation of *A. crassus* males with *A. novaezelandiae* females. Therefore, hybridisation might be an explanation for the disappearance of *A. novaezelandiae* from Lake Bracciano.

6.1 Background

Nematodes of the genus *Anguillicola* are eel-specific swim bladder parasites using copepods as intermediate hosts (some *Anguillicola* spp. were moved to the genus *Anguillicoloides* according to Moravec (2006), but recent molecular findings support the original taxonomy (Laetsch et al. 2012)). In eel species as final hosts, larval stages are found in the swim bladder wall while adult stages migrate into the swim bladder lumen and feed on the host's blood (De Charleroy et al. 1990). The best studied and most abundant species is *Anguillicola crassus* (Kuwahara et al. 1974), which was introduced to Europe in the early 1980s through the importation of living eels from Asia (Kennedy 2007; Kirk 2003; Koops & Hartmann 1989; Taraschewski et al. 1987). The original host of *A. crassus*, the Japanese eel (*Anguilla japonica*), is well adapted to the parasite and can limit parasite load, for example by massive encapsulation of larval stages (Heitlinger et al. 2009; Knopf 2006). In contrast, high infection intensities and prevalences were found in European eels (*Anguilla anguilla*), in which the nematode causes severe damage of the swim bladder wall, a general stress response and mortality of the host (Egusa 1979; Kennedy 2007; Sures & Knopf 2004a; Sures et al. 2001). Therefore, the parasite is expected to threaten European eel populations, especially as the loss of swim bladder function might impair the spawning migration of eels (Palstra et al. 2007; Sures & Knopf 2004a). *A. crassus* seems to be the most efficient invader of the genus, but it was not the first to be introduced to Europe. As early as 1975, Short-finned eels (*Anguilla australis*) from New Zealand infected with *Anguillicola novaezelandiae* were released into Lake Bracciano in Italy (Moravec & Taraschewski 1988; Welcomme 1981), where the parasite was able to infect European eels and built a stable population in the lake (Moravec et al. 1994a; Paggi et al. 1982). *A. novaezelandiae* showed high prevalence and intensity of infection in European eels, but there are no records of any damage to the swim bladder. To the best of our knowledge, there is no record of *A. novaezelandiae* being established in waters outside of Lake Bracciano, as this lake is not connected to other water bodies. In 1993, *A. crassus* was described for the first time in Lake Bracciano, but no mixed infections of eels with both *Anguillicola* species were recorded (Moravec et al. 1994a). Subsequently, *A. crassus* became the dominant species in the lake and so far *A. novaezelandiae* was not found any more (Moravec et al. 1994a; Münderle 2005). One possible explanation is that *A. crassus* outcompeted and replaced *A. novaezelandiae*, but the two species might have also formed viable hybrids that resemble *A. crassus* morphologically. To assess the latter hypothesis we performed a laboratory infection experiment with subsequent microsatellite analysis, to test i) if *A. crassus* and *A. novaezelandiae* can co-infect the same eel and ii) if they are able to mate and produce hybrid offspring.

6.2 Material and Methods

6.2.1 Infection experiment

European eels (*Anguilla anguilla*) were obtained from a commercial fish farm (Albe Fischfarm, Haren/Rütenbrock, Germany), where no cases of *Anguillicola crassus* infections were ever reported. To confirm the absence of parasites before the experiment, ten eels from the stock were killed, dissected and examined for the presence of parasites.

Anguillicola novaezelandiae collected from *Anguilla australis* originating from New Zealand and *A. crassus* collected from *A. anguilla* from lake Müggelsee in Berlin, Germany, were used for the laboratory life cycles. Infective third stage larvae (L3) of *A. crassus* and *A. novaezelandiae* were produced by the method of Haenen et al. (1994).

For the experiment, one European eel (40 cm, 106 g) was infected with 10 L3 of *A. crassus* and 10 L3 of *A. novaezelandiae* by administering L3 with a stomach tube (1.5 mm diameter; B. Braun Melsungen AG) as described in (Sures & Knopf 2004b). The eel was kept in a 80 l fish tank at 20°C and was fed twice a week for a period of 222 days in order to guarantee the presence of second stage larvae for molecular analyses (for details on the life cycle see Knopf et al. 1998). After 222 days post infection (dpi), the eel was killed by decapitation and examined immediately for infection of the swim bladder. Adult parasites were counted and sex was determined. For storage, worms were fixed separately in 70 % ethanol. *Anguillicola* species were identified morphologically according to (Moravec 2006). The identification was done without knowledge about the PCR results to avoid bias of the investigator. The infection experiment was conducted in compliance with national and institutional guidelines for the care and use of animals.

6.2.2 Molecular species identification

Small pieces of the cuticle or the pharynx were cut off from each adult nematode recovered from the eel using sterile technique. Care was taken not to carry-over intestinal content of the worms. DNA was extracted from each sample using a JETQUICK DNA Clean-Up Spin Kit (Genomed) according to manufacturer's instructions. Species-specific primers targeting *cox I* were designed for both nematode species according to a multiple alignment including *cox I* sequences from *A. crassus* (GenBank accession no.: EU376921), *Anguillicola globiceps* (JF805673), *Anguillicola papernai* (JF805697), *Anguillicola australiensis* (JF805640), *A. novaezelandiae* isolate from Tasmania (JF805629), *A. novaezelandiae* New Zealand isolate (JX868555) from the present study and European eel (HQ600683). Sequences of *A. crassus* specific primers were: crasscox for 5′-CCT TTT GTT AGG TGA TGG GCA A-3′, crasscox rev 5′-TAG CGA GAT CAA CAC TTA TAC CAG-3′, amplifying a product of 303 bp and for *A. novaezelandiae* (New Zealand isolate): novcox for 5′-ATT GGG TGA CGG CCA GTT ATA-3′, novcox rev 5′-ACT TAT ATG CTC CAG AGT AAT AGA ACT A-3′,

amplifying a product of 404 bp. PCR conditions were optimized and specificity of primers was tested. One 20 µl PCR reaction mix contained 4 µl of 5x Crimson Taq buffer (New England Biolabs), 0.2 mM dNTP mix (New England Biolabs), 0.5 µM of each primer 0.5 U Crimson Taq (New England Biolabs) and 1 µl template DNA. The mix was topped up to 20 µl with PCR grade water. PCR was conducted on a TGradient thermocycler (Biometra) with the same program for both primer pairs: 95°C for 5 min, 35 cycles of 95°C, 58°C and 72°C each for 45 s and a final elongation at 72°C for 5 min. PCR products were analysed by standard agarose gel electrophoresis.

6.2.3 Microsatellite analysis

Microsatellite markers for *A. crassus* were previously developed by Wielgoss et al. (2007). We tested those seven markers also for *A. novaezelandiae* and selected two markers (AcrCT04 and AcrCA102) that produced a clearly distinguishable pattern of PCR products for the two species. Each forward primer was labelled at the 5′-end with a fluorescent dye (FAM for AcrCT04 and HEX for AcrCA102).
To analyse the allelic pattern of the adult worms, their DNA was amplified with AcrCT04 and AcrCA102 according to Wielgoss et al. (2007). For characterization of the offspring, ten eggs were dissected from each uterus of female nematodes. Eggs were washed by repeated transfers to drops of clean distilled water on a sterile petri dish. Subsequently, single eggs were placed in reaction tubes, DNA was extracted as described above and amplified with AcrCT04 and AcrCA102. PCR products were purified with a JETQUICK PCR Product Purification Spin Kit (Genomed). Products of AcrCT04 and AcrCA102 were pooled and sent for analysis on an Applied Biosystems DNA Analyzer ABI3730 (GATC). Fluorescent peaks were analysed with the Peak Scanner™ Software v1.0 (Applied Biosystems).
To check for contamination of egg DNA from one nematode species with DNA from the other, all eggs were tested with the *cox I*-PCR. As *cox I* is a mitochondrial marker, it should show the identity of the female irrespective of a possible hybrid origin.

6.3 Results and discussion

6.3.1 Infection experiment

Both primer pairs designed according to the *cox I* sequence of *A. crassus* and *A. novaezelandiae* were species specific and did not cross react with eel DNA.
In total, 13 adult nematodes were isolated from the swim bladder of the eel, five of which were female and eight male. One of the females was found dead and partly decomposed, therefore this specimen was not used for further analyses. No larval stages (L3 or L4) were detected in the swim bladder wall. The morphological species identification was in accordance with the *cox I* PCR results. Only the DNA

from one female produced a strong band for *A. novaezelandiae* and an additional faint band with the crasscox-primers, indicating contamination of the sample. This problem remained even though new DNA was extracted and tested again twice from this specimen (Table 6.1). As the *A. crassus*-band was faint, we assumed that this specimen was *A. novaezelandiae*, in compliance with the result of the morphological identification. Two female and four male individuals were identified for each species, which equates to a recovery rate of 60 % (ten third stage larvae from each species were used for infection) for each species with an overall recovery rate of 65 %, considering also the dead female. This shows that co-infection of the final host with both species is possible, and might have also occurred in Lake Bracciano.

Table 6.1 Results of morphological and molecular examination of adult nematodes

No.	***A. crassus***						***A. novaezelandiae***						**dead**
	AC1	**AC2**	**AC3**	**AC4**	**AC5**	**AC6**	**AN1**	**AN2**	**AN3**	**AN4**	**AN5**	**AN6**	**-**
Sex	♀	♀	♂	♂	♂	♂	♀	♀	♂	♂	♂	♂	♀
Morphology	AC	AC	AC	AC	AC	AC	AN	AN	AN	AN	AN	AN	ND
cox I PCR	AC	AC	AC	AC	AC	AC	AN	AN	AN AC*	AN	AN	AN	ND
Alleles AcrCT04	123	135	123	135	135	135	109	109	109	109	109	109	ND
	198	163	146	159	-	198	-	-	-	-	-	-	
Alleles AcrCA102	325	305	305	305	305	305	-	-	-	-	-	-	ND
	-	325	325	-	325	325	-	-	-	-	-	-	

AC: *A. crassus*, AN: *A. novaezelandiae*; numbers indicate allele size in bp; ND: not determined; *faint band

6.3.2 Microsatellite analysis

According to the analysis of adult nematodes, allele sizes for marker AcrCT04 ranged from 123 bp - 198 bp for *A. crassus*, while a single 109 bp allele was characteristic for *A. novaezelandiae* specimens. AcrCA102 amplified two alleles, one 305 bp and one 325 bp. These were detected only in *A. crassus* samples (Table 6.1). Analysis of microsatellite patterns of the eggs revealed information on parental combinations. Only "*A. crassus*-alleles" were found in the eggs analysed from the females AC1 and AC2, showing that species boundaries were not crossed. Characteristic alleles of *A. crassus* males, allowed the identification of the potential father in some cases (Table 6.2). Apparently, one female was inseminated by several males, giving rise to eggs fertilised by different sperm (e.g. AC3 and AC6 for *A. crassus* female 1; see also Table 6.2).

Table 6.2 Results of microsatellite analysis for eggs

AC1	**egg 1**	**egg 2**	**egg 3**	**egg 4**	**egg 5**	**egg 6**	**egg 7**	**egg 8**	**egg 9**	**egg 10**
cox I PCR	AC	AC	AC	AC	AC	AC	AC	AC	AC	AC
Alleles AcrCT04	123	146	123	ND	123	123	123	135	198	123
	135	198	198		198	198	198	198	-	135
Alleles AcrCA102	325	305	305	ND	325	305	305	325	305	305
	-	325	325		-	325	325	-	325	325
potential fathers	AC4 AC5 AC6	AC3	AC3 AC6		AC3 AC6	AC3 AC6	AC3 AC6	AC4 AC5 AC6	AC6	AC4 AC5 AC6
AC2	**egg 1**	**egg 2**	**egg 3**	**egg 4**	**egg 5**	**egg 6**	**egg 7**	**egg 8**	**egg 9**	**egg 10**
cox I PCR	AC	AC	AC	AC	AC/**AN**	AC	AC	AC	AC/**AN**	AC
Alleles AcrCT04	163	135	C	146	123	135	C	135	159	ND
	198	-		163	163	146		159	163	
Alleles AcrCA102	325	305		325	305	325		305	305	ND
	-	325		-	325	-		325	-	
potential fathers	AC6	AC4 AC5 AC6		AC3	AC3	AC3		AC4	AC4	
AN1	**egg 1**	**egg 2**	**egg 3**	**egg 4**	**egg 5**	**egg 6**	**egg 7**	**egg 8**	**egg 9**	**egg 10**
cox I PCR	AN	AN	AN	AN	AN	AN	AN	AN	AN/**AC**	AN
Alleles AcrCT04	109	109	109	109	109	109	109	109	ND	109
	123	-	**146**	**123**	-	-	**135**	--		**146**
Alleles AcrCA102	**325**	-	**325**	**325**	-	-	**305**	--	ND	**305**
	-		-	-			--			--
potential fathers	AC3		AC3	AC3			AC4 AC5 AC6			AC3
AN2	**egg 1**	**egg 2**	**egg 3**	**egg 4**	**egg 5**	**egg 6**	**egg 7**	**egg 8**	**egg 9**	**egg 10**
cox I PCR	AN	AN	AN	AN	AN	AN	AN/**AC**	AN	AN	AN/**AC**
Alleles AcrCT04	109	109	109	109	109	109	ND	109	109	ND
	-	-	**198**	**159**	-	**198**		**198**	-	
Alleles AcrCA102	-	-	**305**	**305**	-	**325**	ND	**305**	-	ND
			-	-		-		-		
potential fathers			AC6	AC4		AC6		AC6		

AC: *A. crassus*, AN: *A. novaezelandiae*; numbers indicate allele size in bp
C: more than 2 alleles were present, indicating contamination with DNA of 2nd individual
alleles indicating hybrids or PCR results showing contaminations are highlighted in bold.

In the offspring of the two *A. novaezelandiae* females AN1 and AN2, alleles characteristic for *A. crassus* were detected. In five out of nine eggs from AN1 and in four out of eight eggs from AN2, alleles different from the 109 bp allele were amplified by AcrCT04. In all these individuals, either the 305 bp or the 325 bp "*A. crassus*-allele" amplified by AcrCA102 was found as well, strongly indicating hybrid origin of these eggs, attributable to at least three different *A. crassus* males. Contamination was excluded in these cases by *cox I*–PCR that detected only *A. novaezelandiae* (maternal) mitochondrial DNA in the hybrids (Table 6.2).

In some cases, no PCR product was obtained from single eggs (Table 6.2). Most likely, DNA-extraction was unsuccessful, because eggs were lost during collection and washing as they easily stick in pipette tips and other plastics. For egg no. 3 and egg no. 7 of *A. crassus* female no. 2, more than two alleles were detected by marker AcrCT04, which revealed contamination of the sample with DNA of another *A. crassus* individual. The *cox I* PCR also indicated a weak *A. novaezelandiae* contamination (faint bands) in DNA from eggs no. 5 and no. 9 isolated from AC female no. 2, although no AN alleles were detected in these samples (Table 6.2). Also, contamination of some DNA samples from *A. novaezelandiae* eggs with *A. crassus* DNA was detected by *cox I*-PCR (AN1: egg no. 9, AN2: egg no. 7 and no. 10; see Table 6.2). These eggs were not taken into account for evaluation of the microsatellite data. Reasons for the contaminations found in one adult nematode (AN3) and several eggs might have been carry over of tissue or eggs from other individuals during sampling or DNA extraction, remainders of sperm in the uteri, or errors during PCR setup. Nevertheless, most individuals were contamination free and allowed reliable interpretation of the results.

These results provide evidence that *A. crassus* and *A. novaezelandiae* can form hybrids, at least in laboratory infections. As *A. crassus* originated from Asia and *A. novaezelandiae* from New Zealand, these species would not have met under natural conditions without human influence. Therefore, evolving a morphological or behavioural reproductive barrier between the two species was not necessary. It is not clear, whether there is some pre- or postzygotic barrier for *A. novaezelandiae* males to fertilise *A. crassus* females, or if the lack of hybrids from *A. crassus* females was a coincidental result of our study. Further experimental double infections of eels with both *Anguillicola* spp. will help to answer this question.

Hybridisation within different groups of helminth parasites is a well-known phenomenon. In laboratory experiments, hybridisation was proven for different *Schistosoma* spp. (Fan & Lin 2005; LeRoux 1954; Taylor 1970; Theron 1989) and two other trematodes, *Fasciola hepatica* and *Fasciola gigantica* (Itagaki et al. 2011). In most of these laboratory studies, hybrids showed reduced survival and impaired fertility already in the F1 or F2 generation. However, hybridisation among parasites also occurs under natural conditions and was observed in monogeneans (Barson et al. 2010), schistosomes (Morgan et al. 2003) and nematodes (*Anisakis*) (Martín-Sánchez et al. 2005). The eggs isolated from the females from the present study were fixed in ethanol and therefore it could not be tested, if the

hybrid larvae produced are viable, infective, and if they develop into fertile adults, but these issues will be investigated in further studies. Therefore, it can only be speculated if hybridisation between *A. crassus* and *A. novaezelandiae* is an explanation for what happened in Lake Bracciano.

A case of replacement of one species by another through introgressive hybridisation among helminth parasites was described for *Schistosoma haematobium* and *Schistosoma intercalatum* in a part of Cameroon where *S. intercalatum* was completely replaced by the introduced *S. haematobium* and the hybrids of both species within about 30 years (Southgate & Rollinson 1981; Tchuem Tchuenté et al. 1997). Laboratory experiments proved that hybrids of *S. haematobium* and *S. intercalatum* were more successful in mating competition than both *S. intercalatum* and, to a lesser extent, *S. haematobium* (Webster & Southgate 2003). A similar scenario might be possible for Lake Bracciano, though the replacement of *A. novaezelandiae* by *A. crassus* occurred much faster. But incompatibility of *A. novaezelandiae* males with *A. crassus* females might quickly lead to a dominance of *A. crassus* genes in the population. The hybrid offspring would also be a selective disadvantage for *A. novaezelandiae* females, even if these hybrids are not viable. Of course alternative explanations for the disappearance of *A. novaezelandiae* from Lake Bracciano are likely such as a faster development of larval stages in the copepod intermediate hosts or a broader spectrum of suitable intermediate hosts for *A. crassus*. These and other life cycle parameters have to be studied in detail before introgressive hybridisation can be figured out as the main reason for the disappearance of *A. novaezelandiae*.

6.4 Conclusions

Results of the present study show that *A. crassus* and *A. novaezelandiae* might occur in the same host in areas of sympatry like Lake Bracciano. According to our results, hybridisation is possible between *A. novaezelandiae* females and *A. crassus* males, but not vice versa. This finding would fit well to the scenario of Lake Bracciano, where *A. novaezelandiae* seemed to have disappeared. Further laboratory hybridisation-experiments are planned to test if this hybrid offspring is viable and fertile, and to analyse their morphology if adult nematodes develop.

General discussion

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In New Zealand all parasites found in the Short-finned eel were identified as *Anguillicola novaezelandiae* according to morphological as well as *cox1* sequence identification. The finding of only one *Anguillicola* species is in accordance with all known data on the parasite fauna of Short-finned eel in New Zealand (Hine 1978; Lefebvre et al. 2004; Moravec & Taraschewski 1988; Rid 1973). The study covered all stages of *Anguillicola* in Short-finned eels and is therefore the first which includes larval stages. The inclusion of larval stages allowed the record of parasites in eels from the South Island, were 89 % of the eels were infected with larval stages but no adult stage was found. Differences in infrapopulation composition of the two sampling sites could be explained by seasonality. While eels from the South Island harboured only larval stages, 14 % of the eels from the North Island harboured preadults. The preadult stages were all about the same size and only two eels from the North Island were infected with adult stages. One eel was infected with a single worm and the other eel with adult stages exceeding all size data for *A. novaezelandiae* known so far. A possible explanation is that these adults overwintered in the eel, while all other eels were freshly infected in spring 2007. The hypothesis of seasonality is supported by the fact that 20 % of the South Island *A. novaezelandiae* are L4 and 23 % of the North Island parasites are in the preadult stage. Parasites of the North Island seem to be a step ahead in their life cycle. An explanation is the about 2°C warmer temperature on the northern sampling site compared to the South Island sampling site (NIWA 2012). The study of Lefebvre et al. (2004) also supports this hypothesis. Prevalence in October was lower than in December and eels from the North Island were infected with a higher prevalence at the same sampling date than those from the South Island.

The findings in this study differ clearly from other *Anguillicola-Anguilla* systems studied in their native range/distribution area. So far, indications for seasonality have not been described. Furthermore, only one dead larva was found during the study, whereas a large number of dead larvae was described in Kennedy (1994) and Münderle et al. (2006). The dominance of larval stages in the eel's *A. novaezelandiae* infrapopulation in both locations of the present study is a unique finding in an *Anguillicola-Anguilla* system. Nevertheless, the prevalence of *A. novaezelandiae* in Short-finned eels is similar to infections of other Anguillicolidae in their natural eel host in wild habitats.

The experimental infection of European eels with *A. novaezelandiae* is giving first information on the life cycle duration of *A. novaezelandiae* in the eel final host and the infrapopulation composition during its maturation. Recovery rates were comparable to laboratory infections of European eel with *A. crassus* (Fazio et al. 2008; Knopf & Mahnke 2004; Knopf et al. 1998; Weclawski et al. 2013) and mean intensities are comparable to natural infections of European eels with *Anguillicola* parasites (Jakob et al. 2009; Moravec et al. 1994a). After 120 days *A. novaezelandiae* completed its life cycle, produced eggs and no more larval stages were found inside the swim bladder wall. The infrapopulation compositions of *A. novaezelandiae* differ strongly from the pattern of *A. crassus* infections. *A. novaezelandiae* specimens seem to grow equally fast, in contrast to the non-uniform growth of *A. crassus*. *A. novaezelandiae* eggs and L2 were first found at 120 days post infection (dpi), while L2 of *A. crassus* were detected at 84 dpi at 20-25°C by Fazio et al. (2008) and at 50 dpi at 22°C by Weclawski et al. (2013). Thus, *A. novaezelandiae* needs more time to complete its life cycle and to produce L2 infective to the copepod intermediate host. While development of *A. crassus* is stated to be density dependent (Ashworth & Kennedy 1999; Fazio et al. 2008; Weclawski et al. 2013), no such effect could be found for *A. novaezelandiae*. Instead, the present study shows a highly synchronised developmental pattern, which is the first finding of such a pattern in *Anguillicola* parasites. The non-uniform growth of *A. crassus* could be one important factor for its enormous invasive success compared with *A. novaezelandiae*. The results show that there are differences in the life cycles of *A. novaezelandiae* and *A. crassus* and proves that there are great variations between different *Anguillicola* species.

In the eel host, different life stages of *A. novaezelandiae* induced different stress responses. While plasma cortisol levels were comparable to other studies of non-stressed eels (Gilham & Baker 1985; Sures et al. 2001, 2006), at 30 dpi cortisol levels of eels infected with 20x *A. novaezelandiae* were slightly elevated compared to the control group. Since the infrapopulation at 30 dpi consisted only of larval stages, while all other groups were dominated by adult stages, L3 could be the possible reason for the slightly elevated levels of cortisol measured in this study. L3 are the mobile stage of *Anguillicola* which migrate into the swim bladder wall (De Charleroy et al. 1990) and this migration could result in the elevation of cortisol levels. Elevated cortisol levels could be beneficial for *Anguillicola* sp. during the early infection of eels as Sures et al. (2006) found a highly significant negative correlation between levels of cortisol and anti-*A. crassus* antibodies. However, the suppression of the host's antibody reaction is only beneficial for the parasite in its original eel host as no effect of the humoral immune response in European eels could be found (Knopf & Lucius 2008; Knopf et al. 2000; Sures & Knopf 2004b). At 120 dpi only eels infected with *A. crassus* as well as eels of the 40x *A. novaezelandiae* group showed elevated levels of plasma cortisol compared to the control group. Reasons could be the less uniform development of *A. crassus* compared to *A. novaezelandiae* and the higher number of adult *A. novaezelandiae* in the 40x *A. novaezelandiae* group. The highly increased feeding activity of adults could cause stress in the latter eel group.

The blood feeding of adult parasites could also be the reason for elevated hepatic hsp70 levels of eels infected with 20x *A. novaezelandiae* at 90 and 120 dpi compared to the uninfected control group. Consistent to this, hsp70 levels of eels infected with 40x *A. novaezelandiae* were even more elevated at 120 dpi, while the 20x *A. crassus* group showed only slightly elevated levels. The reason for the difference between *A. novaezelandiae* and *A. crassus* could be the different infrapopulation composition. Spearman correlation analysis showed a highly positive correlation between hepatic hsp70 levels and the number of parasites inside the swim bladder lumen.

An experimental mixed infection of European eel with L3 of *A. crassus* and *A. novaezelandiae* was performed successfully in the lab and both *cox I* primer pairs designed were species specific. This shows that co-infection of the final host with both species is possible, and might have also occurred in Lake Bracciano. In nine of twenty eggs of the two female *A. novaezelandiae*, alleles characteristic for *A. crassus* were detected, strongly indicating hybrid origin of these eggs. Only "*A. crassus*-alleles" were found in the offspring analysed from both *A. crassus* females, showing that species boundaries were not crossed. All females were inseminated by several males. Nevertheless, it is not clear, whether there is some pre- or postzygotic barrier for *A. novaezelandiae* males to fertilise *A. crassus* females, or if the lack of hybrids from *A. crassus* females was a coincidental result of the study. Hybridisation within different groups of helminth parasites is a well-known phenomenon. In most of the studies, hybrids showed reduced survival and impaired fertility already in the F1 or F2 generation (Fan & Lin 2005; LeRoux 1954; Taylor 1970; Theron 1989). Whether hybrid larvae in the present study are viable and infective, and if they develop into fertile adults could not be tested. Results of the present study show that *A. crassus* and *A. novaezelandiae* might occur in the same host in areas of sympatry like Lake Bracciano. According to our results, hybridisation is possible between *A. novaezelandiae* females and *A. crassus* males, but not vice versa. This finding would fit well to the scenario of Lake Bracciano, where *A. novaezelandiae* seemed to have disappeared.

Overall, the results of the present thesis reveal fundamental differences in the biology of the two *Anguillicola* species compared. While the well-studied species *A. crassus* is infecting its final host during the whole year and parasites inside the swim bladder grow with uneven speed, *A. novaezelandiae* on the other hand seems to infect its original final host with a seasonal pattern and showed synchronised development in the laboratory. The synchronised development may be beneficial to the species in its endemic eel host with a seasonal occurrence, but could be a great disadvantage during the invasion process. By growing unequally fast, *A. crassus* is able to release eggs over a longer time period thus more suitable intermediate hosts could be infected. The evidence for hybrid offspring between *A. novaezelandiae* female and *A. crassus* males but not vice versa could be an additional advantage for *A. crassus* in locations of sympatric occurrence. If hybrid eggs are not viable, the egg output of *A. novaezelandiae* females would be reduced in mix-infected eels and in case hybrid offspring is viable *A. crassus* genes would exceed *A. novaezelandiae* genes after a while. Nevertheless,

other factors like selection on intermediate host level or simply different frequencies of access to human transport pathways may have favoured *A. crassus* as a successful invasive species compared to the noninvasive species *A. novaezelandiae*.

Even though this study increased the knowledge on *Anguillicola* in many ways it also raised several questions, which could be the starting point for future studies. To verify seasonality and to determine the natural occurrence of the species, sampling of natural infected Short-finned eels should be continued in New Zealand. The sampling of Short-finned eels in Australia and a subsequent comparison of the morphology and genetic analyses between *A. novaezelandiae* of the two origins could reveal whether both belong to the same species.

The present study proved that the knowledge on *A. crassus* cannot be transferred to other *Anguillicola* species one-on-one. Therefor the natural occurrence of all species should be studied further to reveal differences in their infection patterns. The findings on the South Island of New Zealand strongly suggest that larval stages should be included in ever study to obtain a complete overview on the occurrence of *Anguillicola* parasites. To further study the life cycle of *A. novaezelandiae* experimental infections of intermediate, potential paratenic and the natural final host should be performed. For stress induced in the final host seams not to be a key element of *A. crassus*' successful invasiveness, future research should focus on stress levels of the parasites. This could reveal whether the host induces stress in the parasite and if both species cope with host induced stress in similar ways. Even though hybrid offspring was detected, it is still unclear whether hybrid L2 are viable and can develop into fertile adults. If possible, F1 and F2 generations should be produced in the lab to study the hybrids' survival and fertility. Mix infections of eels should be repeated to verify, if *A. crassus* females can indeed not be fertilised by *A. novaezelandiae* males or whether this was a coincidence in the present study. Even though *A. crassus* was studied quite extensively in Europe, data on its indigenous occurrence is rare. In Asia sampling of eels could complement our knowledge on the distribution of *A. crassus* and additional sampling of potential paratenic hosts could verify whether these are involved in the life cycle naturally or only in Europe.

Two species of the genus *Anguillicola* have already proven their ability as biological invaders with crucial effects on the new eel hosts in case of *A. crassus*, the inter- and transcontinental trade with life eels should be handled more cautiously to prevent the further spread of *Anguillicola* species.

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Appendix

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

Appendix I: meristic data on *A. australis* and infection with *A. novaezelandiae* from New Zealand (Chapter 3)

Appendix II: morphometric measurements of adult and preadult *A. novaezelandiae* originating from New Zealand (Chapter 3)

Appendix III: data of experimental laboratory infections:
meristic data on *A. anguilla* and infection with *Anguillicola* sp. (Chapter 4)
as well as cortisol and hsp70 values (Chapter 5)

Appendix IV: data of experimental laboratory infection of mix infected eel:
meristic data on *A. anguilla* and infection with *Anguillicola* sp. (Chapter 6)

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

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. Hynek Burda

Tag der mündlichen Prüfung: 21.11.2014

Erklärungen

Erklärung:

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 selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient, bei der Abfassung der Dissertation nur die angegeben Hilfsmittel benutzt und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

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Erklärung:

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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Eigenabgrenzung

Soweit im Folgenden nicht anders aufgeführt, wurden alle Untersuchungen und Ergebnisse der vorliegenden Dissertation durch mich erbracht. Die Dissertation wurde gemeinsam mit meinem Doktorvater Prof. Bernd Sures konzipiert. Die Beiträge von Kollegen zu den einzelnen Kapiteln/Artikeln sowie eine genaue Aufschlüsselung meines eigenen Anteils an den einzelnen Kapiteln/Artikeln stellen sich wie folgt dar:

3 Natural *Anguillicola novaezelandiae* infection – Is there seasonality in New Zealand?

Alle praktischen Arbeiten wie auch die Probenahme in Neuseeland wurden von mir durchgeführt. Die Konzeption des Kapitels/Artikels wurde mit Prof. Bernd Sures abgesprochen. Das Kapitel/der Artikel wurde vollständig von mir verfasst und von Prof. Bernd Sures vor Einreichung beim Journal geringfügig überarbeitet.

4 Can differences in life cycle explain differences in invasiveness? – A study on *Anguillicola novaezelandiae* in the European eel

Der Infektionsversuch, die Sektion der Aale sowie die Bestimmung der Parasiten wurden gemeinsam mit Michelle Keppel durchgeführt. Die Auswertung und Interpretation der Daten erfolgte durch mich. Die Konzeption des Kapitels/Artikels wurde mit Prof. Bernd Sures abgesprochen. Das Kapitel/der Artikel wurde vollständig von mir verfasst und von Prof. Bernd Sures vor Einreichung beim Journal geringfügig überarbeitet.

5 Effects of *Anguillicola novaezelandiae* on the levels of cortisol and hsp70 in the European eel

Die Entnahme der Blut- und Leberproben erfolgte gemeinsam mit Michelle Keppel. Die Messung der hsp70-Level erfolgte unter meiner Betreuung durch Katharina Tabujew. Die Auswertung und Interpretation der Daten erfolgte durch mich. Die Konzeption des Kapitels/Artikels wurde mit Prof. Bernd Sures abgesprochen. Das Kapitel/der Artikel wurde vollständig von mir verfasst und von Prof. Bernd Sures vor Einreichung beim Journal geringfügig überarbeitet.

6 Merging species? Evidence for hybridisation between the eel parasites *Anguillicola crassus* and *A. novaezelandiae* (Nematoda, Anguillicoloidea)

Die Sektion der Nematoden, sowie die Eientnahme erfolgten gemeinsam mit Daniel Grabner. Das designen der Primer, molekulare Arbeiten sowie die Analyse der Mikrosatelliten erfolgten durch

Daniel Grabner. Die Konzeption des Kapitels/Artikels wurde mit Daniel Grabner und Prof. Bernd Sures abgesprochen. Das Kapitel/der Artikel wurde gemeinsam mit Daniel Grabner verfasst (mein Anteil 60%) und von Prof. Bernd Sures vor Einreichung beim Journal geringfügig überarbeitet.

Die Aufnahme der Veröffentlichungen in die Dissertation verletzt keine Urheberrechte.

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Hiermit bestätige ich die oben gemachten Angaben.

Essen, den _____ _____

Unterschrift des betreuenden Hochschullehrers