博士論文（要約）

Ecological and reproductive implications on the distributions of *Anisakis simplex* sensu stricto and *Anisakis pegreffii* (Nematoda: Anisakidae) in Japan

（アニサキス科線虫 *Anisakis simplex* sensu stricto と *Anisakis pegreffii* の

生態・生殖と両種の日本における地理的分布との関係）

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Chapter 1. General introduction

The *Anisakis* genus is a cosmopolitan group of marine parasitic nematodes. Adult worms are frequently found inside the gastric compartments of cetaceans, their definitive hosts. Crustaceans serve as intermediate hosts and third-stage (L3) larvae are subsequently transferred to fish and cephalopods, which are paratenic hosts. Humans can become accidental hosts when they consume raw or undercooked infected seafood. Japan alone accounts for more than 90% of the global reported cases of anisakiasis but the introduction of exotic eating habits have increased the frequency of human cases in other locations.

Two sibling species *Anisakis simplex* sensu stricto (s.s.) and *A. pegreffii* are the most common. Even though their specificity in paratenic hosts is low, it has previously been suggested that their distributions are quite distinct and delimited. In Japan *A. simplex* (s.s.) is predominantly located in the Pacific coast and northern part of the archipelago while *A. pegreffii* is more commonly found around the East China Sea and Sea of Japan.

The reasons contributing to this differentiated distribution have not been elucidated. Two hypotheses can be raised: one is that the two sibling species may have different final host specificity and the distribution of these hosts may vary locally; and the other is that abiotic factors, especially water temperature, may distinctively affect tge two sibling species. These two hypotheses are tested in this study.

Chapter 2. Analysis of host factors

We examined the infections with *Anisakis* spp. in intermediate and paratenic hosts to confirm their distribution patterns in Japan (Section 1) and in definitive hosts to elucidate their host specificity (Section 2). Collected worms were identified to the sibling species level molecularly (PCR-RFLP).

Section 1. Infection in intermediate and paratenic hosts

Anisakis spp. in fishes, cephalopods and euphausiids from Japanese waters

Chum salmons (*Oncorhynchus keta*), Alaska pollocks (*Gadus chalcogrammus*), blue mackerels (*Scomber australasicus*), Pacific cods (*Gadus macrocephalus*), chub mackerels (*Scomber japonicus*), Japanese flying squids (*Todarodes pacificus*), Japanese pilchards (*Sardinops sagax melanostictus*) and Pacific krill (*Euphausia pacifica*) from different areas were examined. In general, the different distributions of the two nematodes species were confirmed. However, *A. simplex* (s.s.) was surprisingly the most abundant species even in chub mackerel from the East China Sea (95.3%) and in Japanese flying squid from the Sea of Japan (100%), areas where *A. pegreffii* has been regarded as predominant. These findings suggest that the distributions of the two sibling species might be more fluctuant and less rigid than previously thought, possibly depending on the time of the year.

Section 2. Infection in final hosts

Anisakis spp. in baleen whales from coastal and offshore areas in the Northwestern Pacific Ocean

Anisakis samples of common minke whales (*Balaenoptera acutorostrata*) from two coastal locations (Abashiri and Kushiro) in Hokkaido and one offshore area in the North Pacific as well as samples of sei whales (*B. borealis*) from the latter locality were examined. This study also analyzed the histopathological changes associated with the attachment of *Anisakis* worms in the stomachs of both cetacean species.

Worms were separated into mature (macroscopically visible gonads) and immature (macroscopically not visible gonads). Overall, most common minke whales and sei whales were infected with *A. simplex* (s.s.) regardless of the maturity of the parasites. While some of the common minke whales from the two coastal areas were mostly infected with mature and immature forms of *A. simplex* (s.s.), others were mainly parasitized with mature forms of *A. pegreffii* and with immature forms of *A. simplex* (s.s.). Two stocks of common minke whales are known in Japanese waters: the O stock is prevalent in the Pacific side, migrating from offshore areas towards the coast and then north; and the J stock, which is located mainly in the Sea of Japan, moving northwards from the Yellow Sea and East China Sea to the coast of Hokkaido, where both stocks meet. Interestingly, it was observed that most of the common minke whales predominantly infected with mature *A. pegreffii* adults belonged to the J stock (percentage of animals mostly infected with *A. pegreffii* mature forms belonging to the J stock: 71.43%) whereas most of those mainly parasitized by mature *A. simplex* (s.s.) adults were from the O stock (61.54%). It is possible that the whales co-infected with mature *A. pegreffii* and immature *A. simplex* (s.s.) had migrated from the Sea of Japan to northern and eastern Hokkaido coasts. Tthe examined *Anisakis*-associated lesions in both whales were usually very superficial in depth, localized in extension and mild in intensity.

Anisakis spp. in toothed whales from Wakayama Prefecture

The *Anisakis* species infecting common bottlenose dolphin (*Tursiops truncatus*), pantropical spotted dolphin (*Stenella attenuata*) and striped dolphin (*Stenella coeruleoalba*) captured in Wakayama Prefecture were investigated to explore their potential role as final hosts of *A. simplex* (s.s.) and *A. pegreffii* in Japanese waters. An *Anisakis*-associated lesion from the stomach of a common bottlenose dolphin was also histologically examined.

Overall, *A. simplex* (s.s.), *A. pegreffii* and *A. typica* were collected from these hosts. *Anisakis typica* was the only species found in pantropical spotted dolphin and striped dolphin whereas in common bottlenose dolphin all three species were detected (*A. simplex* s.s. 78.05%; *A. typica* 15.85%; *A. pegreffii* 6.10%). The most prevalent parasitic species in this host was *A. typica* (present in 61.54% of the dolphins examined), followed by *A. simplex* (s.s.) (53.85%) and *A. pegreffii* (15.38%). The lesion analysis revealed a localized and deep ulceration with extensive tissue loss and moderate to severe histological changes.

The results obtained in this chapter indicate that various cetaceans including common minke whales, sei whales and common bottlenose dolphins can be definitive hosts for both *A. simplex* (s.s.) and *A. pegreffii* in Japanese waters because adult stages of both parasites were obtained from them. However, the different characteristics of the *Anisakis*-associated stomach lesions observed suggested distinct levels of host-parasite adaptation in baleen and toothed whales.

Chapter 3. Analysis of environmental factors

The main purpose of this chapter was to investigate the effects of temperature on eggs and recently hatched larvae in the water column and L3 larvae inside ectotherm intermediate and paratenic hosts.

Section 1. Eggs and recently hatched larvae

Preliminary studies on the effects of abiotic factors on adults' survival and fecundity

In order to optimize the *in vitro* conditions for the transportation and husbandry of adult *Anisakis* worms for the obtainment of eggs, 3 different experiments were conducted. In the first experiment, mature and immature actively moving *Anisakis* adults were incubated at 3–37ºC in PBS (pH 7.2) or RPMI-1640 (pH 4.5) solutions for 13 days and were monitored daily. The second experiment evaluated the fecundity of *A. pegreffii* females incubated in RPMI-1640 solutions with adjusted pH (3.01, 5.28, 6.5 and 7.3) at 20, 30 or 37ºC. Lastly, in the third experiment, the fecundity of *A. simplex* (s.s.) was assessed in RPMI-1640 (pH 4.5) at 3–37ºC.

Anisakis adults could generally survive longer in RPMI-1640 than in PBS. The mean survival times in RPMI-1640 ranged from 8.3 to 8.8 days at 21–37ºC, while it was considerably lower at lower temperatures, ranging from 1.7 to 3.3 days at 3–15ºC.

The mean number of eggs produced by *A. pegreffii* was highest at 37°C and lowest at 20°C, but little difference in the fecundity was detected among different pH solutions. On the other hand, the mean number of eggs produced by *A. simplex* (s.s.) increased with temperature until 33ºC and then decreased at 37ºC.

These observations indicate that the survival of *Anisakis* adults is optimized at 21–37ºC and their egg production is stimulated at 33ºC (*A. simplex* s.s.) and 37ºC (*A. pegreffii*).

Influence of temperature and salinity on egg hatching and survival of recently hatched larvae

Naturally spawned eggs and those collected from dissected *A. simplex* (s.s.) and *A. pegreffii* females were incubated at 3, 9, 15, 21, 23, 25, 27, 29 and 37ºC in PBS and seawater (SW). The survival of the hatched larvae originated was subsequently monitored in the same conditions without changing the solutions.

Eggs of *A. simplex* (s.s.) hatched at 25ºC and lower temperatures while those of *A. pegreffii* hatched at 27ºC and below. Generally, hatching times increased as temperature decreased. In newly hatched larvae, mean survival times generally increased inversely to temperature. In naturally spawned *A. simplex* (s.s.) larvae mean survival times were 65–105 days at 3ºC and 4–7 days at 25ºC in sea water. In contrast, the mean survival times for *A. pegreffii* were 15–73 days at 3ºC and 13–22 days at 25ºC in the same conditions.

These results suggest that both the eggs and recently hatched larvae of *A. simplex* (s.s.) are more tolerant to lower temperatures than those of *A. pegreffii*.

Section 2. Third-stage (L3) larvae

Effects of temperature and salinity on the survival and activity of L3 larvae in vitro

The tolerance to temperature and salinity of the larvae of *A. simplex* (s.s.) and *A. pegreffii* infecting marine fishes was tested *in vitro*. Specimens were incubated at 10, 20 and 25°C for 1 or 3 weeks using PBS solutions of 280 and 970 mOsm/L, which respectively correspond to the approximate osmotic pressures of fish and crustaceans in the sea, and their survival was assessed. Then, their penetration capability was monitored during a 24-hour period on a gel column made of 1.75% agar with 0.8% nutrient broth and covered with a supernatant of 3% acetic acid in 0.85% NaCl solution at 37°C to infer about their activity. *Anisakis pegreffii* showed a higher survival rate and penetration capability than *A. simplex* (s.s.) in all experimental groups, particularly at 20–25°C, and both species generally performed better at the lower salinity solution. The present results suggest that *A. pegreffii* is more adapted than *A. simplex* (s.s.) to warmer temperatures also in the third larval stage *in vitro*.

Effects of temperature on the survival and activity of L3 larvae in vivo

Anisaksi simplex (s.s.) larvae were surgically inserted in the body cavities of rainbow trouts (*Oncorhynchus mykiss*) and Mozambique tilapias (*Oreochromis mossambicus*). The fishes were then reared for 6 or 12 weeks at 6 different temperatures (3, 9 or 15°C using rainbow trouts and 21, 27 or 33°C using Mozambique tilapias), after which fish were sacrificed and larval survival was assessed. Additionally, survival and gel penetration capability of *A. simplex* (s.s.) larvae from the same batch were tested separately in two parallel *in vitro* experiments at the same temperatures for 10 days and 12 weeks, respectively.
The survival rates of *A. simplex* (s.s.) larvae *in vivo* and *in vitro* were lower at higher temperatures than at lower ones, particularly at 27 and 33°C. Also, larvae were less active in migrating to the gel at 3 and 33ºC.

In this chapter, eggs, hatched larvae and L3 larvae of *A. pegreffii* showed higher tolerance to higher temperatures than those of *A. simplex* (s.s.), which suggested that *A. pegreffii* is more adapted to higher sea temperatures, when compared to *A. simplex* (s.s.).

Chapter 4. General discussion

Based on the present results from Japanese waters we could confirm that the distributions of *A. simplex* (s.s.) and *A. pegreffii* in paratenic hosts does not directly depend on final host specificity, since both sibling species infect the same cetacean species, but rather influenced by the region they inhabit and the predominant existent larvae. This effect was very clear in common minke whales, which seem to be a very important final host for both parasitic species in Japan. The identification of *Anisakis* spp. may even enable the distinction of stocks/populations of these cetaceans. On the other hand, eggs, hatched larvae and L3 larvae of *A. pegreffii* were observed to be more adapted to higher temperatures than those of *A. simplex* (s.s.), which means that distributions of these two sibling species are influenced by adaptation to different temperature zones.