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Mode of Infection with Larval Anisakid Nematodes in the Japanese Flying Squid, *Todarodes Pacificus* (Cephalopoda: Ommastrephidae)

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Abstract: *The Japanese flying squid, Todarodes pacificus, is generally infected by third-stage larvae of two species of anisakid nematodes. Anisakis sp. is typically observed encapsulated in the outer wall of the stomach and caecum of infected squids, whereas Lappetascaris sp. is found at the anterior end of the mantle musculature. To investigate the infection process, third-stage larvae of these two anisakid species were labelled by injecting a mixture of 5% fluorescein isothiocyanate-dextran and 5% biotin-dextran into their caudal end. The labelled larvae were placed inside the muscle tissue of dead prey fish which were then fed to freely swimming squids held within a large aquarium. Several days later, the squids were removed, killed and dissected to observe the characteristics of infection by fluorescing anisakids. Anisakis sp. larvae were found infecting the outer walls of the stomach and caecum, but no Lappetascaris infections were observed. Squids held inside custom-made plastic packs containing free anisakid larvae were not infected with Anisakis sp. but Lappetascaris sp. larvae were found attached to the gills. We conclude that Anisakis sp. penetrates the squid digestive tract after squids consume Anisakis-infested prey; while Lappetascaris sp. infects squids by direct entry through their gills following inhalation of ambient seawater.*

Keywords: *Anisakid nematodes, Todarodes pacificus, fluorescent labelling, FITC*

1. INTRODUCTION

The larval stages of anisakid nematodes are the most common parasites of the Japanese flying squid, *Todarodes pacificus*. These nematodes include *Anisakis simplex* sensu lato (cf. 1, 2), here referred to as *Anisakis* sp., and *Lappetascaris* sp., both at the third larval stage [3-5]. The larval stages of the life cycle of *Anisakis* sp. involve several hosts, including squid and fish. Adult parasites live in the guts of the definitive hosts, which typically are marine mammals, especially cetaceans [6-9]. The cycle begins when the definitive host releases the parasite eggs in their faeces. The eggs develop and hatch in the environment, releasing free-living third-stage larvae [10, 11]. Planktonic or semi-planktonic crustaceans are intermediate hosts, ingesting these larvae. Fish and squids act as paratenic hosts, acquiring larvae through the diet by preying upon infected crustaceans and/or other fish species. The definitive hosts of these parasites are cetaceans, in which two molts occur (third-stage to fourth-stage, then to adult) before sexual maturity and egg production [9, 12-15].

The life cycle of *Lappetascaris* sp. is largely unknown. The definitive host of this species is thought to be a predatory marine fish [3, 4], although the source of larvae found in these fish is unknown.

The larvae of *Anisakis* sp. commonly appear encapsulated in the outer walls of the stomach and caecum of squids, whereas those of *Lappetascaris* sp. are found in the anterior end of the mantle musculature [3-5].

The North Pacific krill species, *Euphausia pacifica* and *E. similis*, have been successfully infected experimentally by *A. simplex* larvae in the laboratory [16, 17]. However, the infection process in squids is unknown. The present study aimed to determine the route of infection of captive squid, *T. pacificus*,

with third-stage larvae of the two anisakids. For the purpose of observing the infection process in *T. pacificus* a new method of fluorescence labeling of anisakid larvae was devised.

2. MATERIALS AND METHODS

2.1. Collection of Larval Anisakid Nematodes

Japanese flying squids, *Todarodes pacificus* (Steenstrup, 1880), were caught in set nets off the Pacific coast of southeast Hokkaido, Japan, in November, 2009, and October, 2010 and inspected for parasite infestation. Third-stage larvae of the parasitic nematode *Anisakis simplex* sensu lato [1, 2], henceforth referred to as *Anisakis* sp., were obtained from the outer wall of the squid stomach and caecum. Third-stage larvae of *Lappetascaris* sp. were obtained from the anterior end of the squid mantle musculature. Both were found in freshly caught squid and in squid purchased at a fish market in Hakodate, southern Hokkaido, Japan, in October, 2010.

A few larvae were fixed in 70% ethanol or 5% formaldehyde and cleared (rendered transparent) using glycerol, enabling inspection of the internal organs of the larvae for identification. *Anisakis* sp. larvae can be identified based on the presence of a long ventriculus with an obliquely shaped posterior end, a short rectum, and a rounded tail; while *Lappetascaris* sp. larvae are distinguished by the presence of an elongate-oval ventriculus, a very long ventricular appendix, a conspicuous oesophageal gland, and a conical tail [3].

Living larvae of both species were removed and placed in Petri dishes filled with seawater, maintained at 8°C under a 12:12 circadian light regime. The water was changed every day. These larvae were checked daily for viability by reaction to gentle agitation with a needle and dead larvae were removed.

2.2. Labeling of Larvae

Living larvae were incubated at 8°C for three days then labelled [18] by injecting a mixture of 5% fluorescein isothiocyanate-dextran and 5% biotin-dextran (FITC-biotin) in 0.2M KCl into the caudal end of each larva and viewed using fluorescence microscopy (LEICA MZ16F, GFP2 filter set: excitation 480/40 nm; barrier 510 nm). An Eppendorf microinjector was used to introduce the dye, with the larvae placed in a glass dish coated with 1% agar and filled with seawater. The quantity injected was just sufficient to coat the inside of the anisakid cuticle with FITC-biotin. Injected larvae of both species were returned to a new Petri dish filled with seawater at 8°C.

2.3. Feeding Experiments

Live *T. pacificus* were caught using set nets in November 2009 and October 2010 in the waters off southern Hokkaido. The squid were maintained together in a 10,000-L aquarium, in which the water temperatures variation was 15-16°C. In November 2009, live *T. pacificus* were tagged on a fin using a plastic ribbon tag for individual identification. Tagging was performed under cold-water anesthesia (0-1°C seawater) [19]. Three to five FITC-labelled larvae of *Anisakis* sp. were placed in pieces of frozen fillets of Pacific saury (*Cololabis saira*), which were then fed to each of three squids for two days. In the same way, labelled larvae of *Lappetascaris* sp. were fed to eight different squids. Each squid received two or three infested fillets per day.

Any food that was not consumed was collected and the number of larvae remaining was counted. Experimentally infected squid were observed for 1 hour to ensure that larvae were not regurgitated. The aquarium was cleaned daily by siphon and inspected for expelled larvae until the end of the experiment. After 4-5 days of feeding on infested feed, all squid were netted, killed and examined for larval infection using fluorescent microscopy. All obtained larvae were fixed in 70% ethanol or 5% formaldehyde and cleared using glycerol for microscopic observation.

In October, 2010, about six labelled larvae of *Lappetascaris* sp. were placed in each of a number of pieces of frozen saury fillet. Two or three pieces were fed to each of four tagged squids and 2 or 11 hours after feeding, the squids were collected and examined for larval infection.

2.4. Exposure of Squid to Free-Swimming Larvae

In November 2009, three individual live *T. pacificus* were placed individually with 13-20 labelled larvae of *Anisakis* sp. in separate 5-L transparent plastic transporter bag containing 1.5-2.5-L of seawater at 0-1°C with approximately 2.5-3.5 l of oxygen gas (custom-made plastic packs used for transporting live

squid [19]). Similarly, five live *T. pacificus* individuals and 20-30 labelled larvae of *Lappetascaris* sp. were placed in identical plastic transporter bags.

The bags were placed in an incubator (8°C) for 1 d (12 h dark, 12 h light). The squids were then removed and examined for larval infection.

3. RESULTS

All FITC-positive larvae were observed to be within the pseudocoelom, where they were easily recognized under the fluorescence microscope (Fig. 1). *Lappetascaris* sp. larvae showed active behavior even on an agar-coated dish, where they were observed to promptly burrow into the medium soon after being placed in the dish.

In feeding experiments, all squids consumed the fillets they were fed, and no larvae were regurgitated. Table 1 shows the results. *Anisakis* sp. larvae were found in each of the three squids tested. The larvae were encapsulated in the outer wall of the stomach or caecum, but no larvae were found in the mantle musculature or other internal organs. Fig. 2 shows a squid infected by *Anisakis* sp., where two larvae can be seen, one in the caecum and the other in the stomach. However, only the larva in the caecum was fluorescent, indicating that it was an experimental specimen; the one in the stomach was presumably from a natural infection when the squid was still in the wild. Interestingly, while no *Lappetascaris* sp. larvae were found in any of the squid 4-5 days after they had been fed with feed containing labelled larvae, another experiment revealed freely moving larvae in the squids 2 or 11 hours after feeding (Table 2), indicating that the squid did ingest these larvae in their food but did not suffer digestive tract infection by this species.

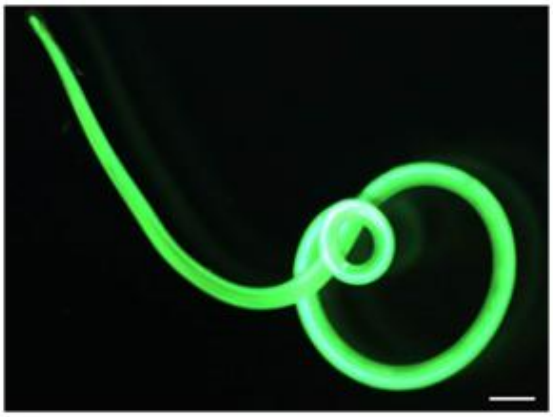

Fig1. *Third-stage larva of Anisakis sp. labelled by injecting a 5%FITC-biotin. Scale bar 1.0 mm.*

Table1. *Infection of Todarodes pacificus by third-stage larvae of two species of anisakid nematodes labelled with 5% FITC-biotin and included in the diet*

	Mantle length of squid (mm)	Body weight of squid (g)	No. of larvae given	Day of collection	Location of infection			
					Stomach	Caecum	Mantle anterior end	Mantle posterior end
Anisakis sp.	234	256	8	4	1	0	0	0
	250	334	11	5	0	1	0	0
	236	238	16	5	0	1	0	0
Lappetascaris sp.	245	269	5	4	0	0	0	0
	258	318	5	4	0	0	0	0
	240	293	5	4	0	0	0	0
	243	304	5	4	0	0	0	0
	225	221	7	4	0	0	0	0
	230	223	12	4	0	0	0	0
	251	276	14	4	0	0	0	0
	241	270	10	5	0	0	0	0

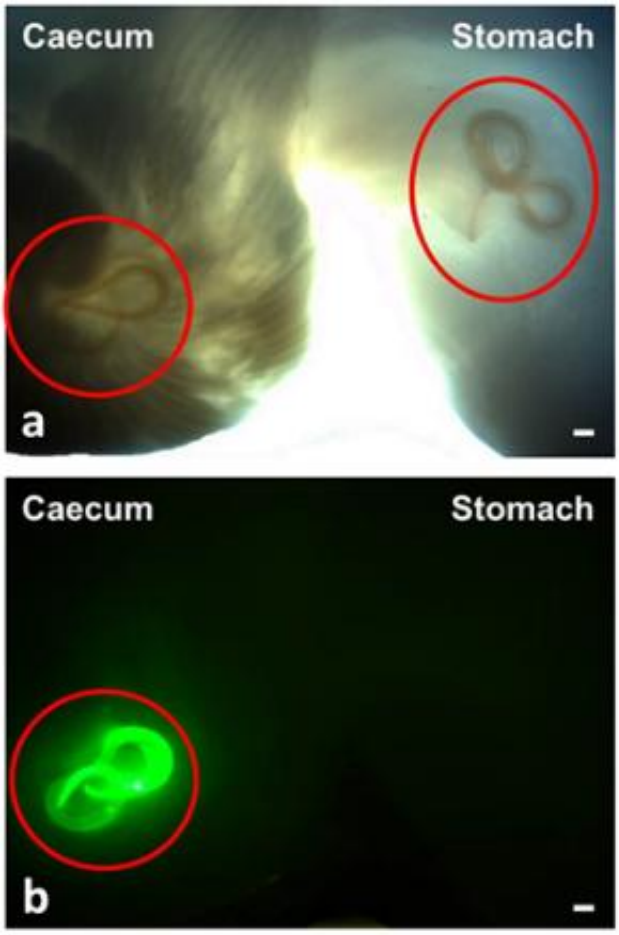

Fig2. *Infection of squid by Anisakis sp. larvae after the feeding experiment.*

(a) Viewed by conventional microscopy and normal light source. Two larvae can be seen encapsulated in the outer wall of the stomach and the caecum.

(b) Viewed by fluorescence microscopy. Note that only the larva in the caecum shows FITC-fluorescence, so it was introduced experimentally, while that in the stomach wall was presumably introduced naturally before the squid was captured. Scale bar 1.0 mm.

Table2. *Infection of T. pacificus by third-stage larvae of Lappetascaris sp. that were labelled with 5% FITC-biotin and included in the diet*

Mantle length of squid (mm)	Body weight of squid (g)	No. of larvae given	Hour of collection	Location of larvae in host			
				Stomach*	Caecum	Mantle anterior end	Mantle posterior end
209	174	5	2	1	0	0	0
184	122	5	2	2	0	0	0
192	133	6	11	6	0	0	0
211	191	7	11	7	0	0	0

Table 3 gives the results of the exposure experiment. No *Anisakis* sp. larvae were found in the internal organs or mantle musculature, although two larvae were found freely moving in the mantle cavity of two squids. In contrast, *Lappetascaris* sp. larvae were found attached to the gills of two of the five squids. These larvae remained active (Fig. 3).

Table3. *Infection of T. pacificus by third-stage larvae of two species of anisakid nematodes that were labelled with 5% FITC-biotin and introduced into the tank containing the squid.*

	Mantle length of squid (mm)	body weight of squid (g)	No. of larvae introduced	Hour of collection	Location of larvae in host	
					Mantle cavity	Attached to gill
Anisakis sp.	252	311	15	24	0	0
	237	298	13	27	2	0
	246	305	20	27	2	0
Lappetascaris sp.	233	264	20	22	2	2
	251	311	20	22	0	6
	265	397	30	24	4	0
	258	343	24	27	2	0
	245	259	24	27	3	0

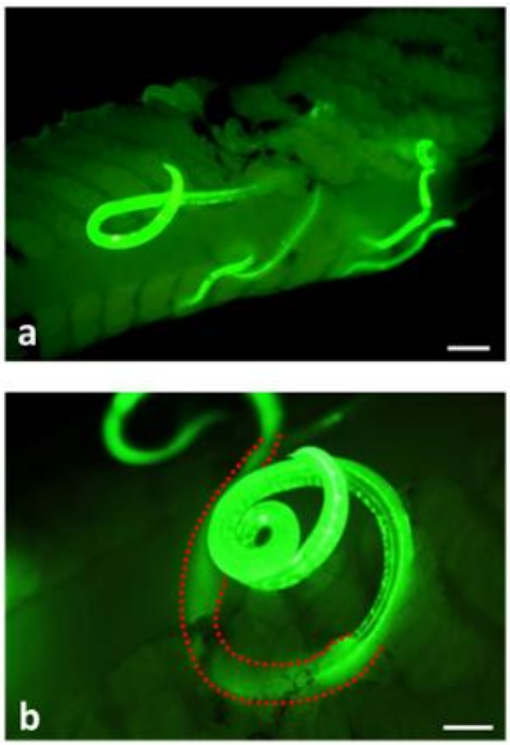

Fig3. *Infection of squid by Lappetascaris sp. larvae after the exposure experiment.*

(a) Fluorescent view of the gill of the infected squid. (b) Magnified image of (a). These infecting larvae have burrowed into the tissue. The dotted line represents the body of the larva within the gill. Scale bars 100 μm in (a) and 50 μm in (b).

4. DISCUSSION

The methodology proposed in the present study is clearly effective for investigating infection with anisakid larvae. It was found possible to culture the injected larvae outside of the hosts for up to three weeks, after which they died, most likely due to starvation. Furthermore, fluorescence microscopy demonstrated that all the larvae were successfully labelled, thus validating injection of 5% FITC-biotin into the caudal end of larval anisakid nematodes as a good method for labelling nematodes.

Experimental infection of squids with *Anisakis* sp. larvae embedded within feed items resulted in encapsulation of larvae in the outer wall of the squid stomach or caecum. Takahara and Sakurai [5] reported that *T. pacificus* are infected with *Anisakis* sp. in the Sea of Okhotsk and the Oyashio region off eastern Hokkaido, suggesting that *T. pacificus* is infected with third-stage larvae of *Anisakis* sp. indirectly through preying on infected crustaceans or fish (e.g., walleye pollock) in the Sea of Okhotsk and the Oyashio region. Although the larvae were directly fed to the squid in the present study, it is very likely that these larvae will go on to infest larger predators that prey upon infected crustaceans, squid and fish. Thus large predators may accumulate enormous numbers of larvae. These findings suggest that the life cycle of *Anisakis* sp. relies on the predator-prey relationships in the food web.

Although a few *Lappetascaris* sp. larvae were found inside the stomach of some squid, they did not infect the host in the feeding experiments reported here. This result strongly suggests that infection by *Lappetascaris* sp. larvae does not occur through the food web. Rather, as demonstrated in the exposure experiment, some larvae of *Lappetascaris* sp. present in the seawater are taken into the mantle cavity during swimming and ventillation of the gills, into which they apparently burrow. In contrast, although *Anisakis* sp. larvae were found freely moving within the mantle cavity, they were not observed to attach themselves to any tissues unless ingested. The larvae of *Anisakis* sp. develop to the third stage while still in the egg and are free-living upon hatching [10, 11]. The life cycle of *Lappetascaris* sp. is largely unknown, although there appears to be a free-living period in its life cycle. In wild squid, the larvae are apparently found only in the anterior end of the mantle musculature [3-5]. *Lappetascaris* sp. larvae were observed to penetrate into the 1% agar plate during dye injection, while *Anisakis* sp. larvae did not show such behavior. From this result, we speculate that *Lappetascaris* sp. larvae may attach first to the gill, then burrow into the gill tissues before subsequently moving to the anterior end of the mantle musculature. Takahara and Sakurai [5] reported that *Lappetascaris* sp. in *T. pacificus* occurred on the coast off Hokkaido, suggesting that *T. pacificus* may become infected with third-stage larvae of *Lappetascaris* sp. directly by taking them into the mantle cavity with inhaled seawater during the migration of these squid around Hokkaido.

5. Conclusion

Recent studies have indicated that *T. pacificus* is infected by two anisakid nematodes, *Anisakis* sp. and *Lappetascaris* sp. We examined the route of infection for these two species in *T. pacificus* by means of experiments on captive squid, making use of a novel method of fluorescence labelling of the larvae. The results indicate that the third-stage larvae of *Anisakis* sp. infection happens indirectly following predation on infected euphausiids or fish; while infection with third-stage larvae of *Lappetascaris* sp. happens by direct exposure to larvae living free within the ambient seawater.

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