Fitness of the marine parasitic nematode *Anisakis simplex* s. str. in temperate waters of the NE Atlantic

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ABSTRACT: In temperate waters of the NE Atlantic, third-stage larvae of *Anisakis simplex* sensu stricto collected from 3 paratenic host species were identified by restriction fragment length polymorphisms. The condition of wild larval infrapopulations was assessed by examining morphometric and growth characteristics. The differentiation patterns and the excretory/secretory products of larvae grown *in vitro* were also examined. An extensive morphometric, growth and differentiation variability was found between parasite larvae collected from different paratenic host sources. Nematode infrapopulation larvae from the squid comprise those smaller individuals with the lowest values of survival rates and moult success. It may be then concluded that the fitness of *A. simplex* s. str. larvae is not the best possible in the squid, which impaired the competitiveness of the parasite and its chances of developing into an adult. This suggests that the microenvironments impaired by the paratenic host may provide larval infrapopulations with unique ecological factors probably influencing its recruitment to the final host populations.

KEY WORDS: *Anisakis simplex* s. str. · Fitness · Recruitment

INTRODUCTION

The common whaleworm or herringworm includes an important and diverse array of parasitic organisms, with a certain degree of specificity in their geographic distribution and their host preference in major areas and fisheries of the marine ecosystem (Mattiucci et al. 1997). Recent studies on *Anisakis simplex*, the casual agent of human anisakiasis as larvae, show the existence of at least 3 reproductively isolated sibling species, differing in their genetic structure, life history and geographic distribution (Nascetti et al. 1983, 1986, Mattiucci et al. 1997).

Parasite recruitment, usually defined as the number of parasitic larvae from a single breeding that enter the host population, might be expected to relate to the total biomass of the spawners comprising a stock. The low predictive power of stock-recruitment relationships characterized by high variance, and the difficulties associated with predicting recruitment with accuracy,

has been a central problem in anisakid population models (Des Clers 1990), which are a key element for the epidemiological control of the so-called 'fish nematode problems' in nature (ICES-WGPDMOC 1995). Since few of the many eggs produced by anisakids eventually give rise to adults (Smith 1984), it is believed that small changes in the mortality rates of the larval stages can lead to the observed high variability in recruitment. Although there has been substantial controversy over the ontogenetic stage at which recruitment is determined, the third-larval stage is still considered important in this respect because it is the stage infective to the final host in which the adults are formed.

According to Mattiucci et al. (1997) all the samples of *Anisakis simplex* s. str. from the Atlantic Ocean were in Hardy-Weinberg equilibrium and no significant differences were found between samples collected in different years or in different paratenic hosts at the same locality. These authors stated that because of the homogenizing effects of gene flow, enhanced by the high vagility of intermediate/paratenic and definitive

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hosts, a remarkable genetic homogeneity is detected within *A. simplex* s. str. However, because of the 2-layer environment defined by Pavlovsky (1934) many biological and physical factors can interact to affect the condition and mortality of *Anisakis* larvae. Physical processes have often been suggested as the main sources of larval mortality in the ocean (Smith 1983a, Likely & Burt 1989, Burt et al. 1990, Pascual et al. 1996). On the other hand, differential growth within the microenvironment may also be important because it determines the time of moult and ecdysis of infective larvae and thus the length of time the larvae are susceptible to any mortality source mostly related to the host immunological responses (Abollo 1999).

The study of condition is believed to be a good indicator of the general 'well-being or fitness' of the population under consideration. The aim of this paper is to determine the role of the host species inducing intraspecific variability in some expressions of this fitness related to the success of establishing of *Anisakis simplex* s. str. larval infrapopulations in the final host.

MATERIALS AND METHODS

Collection of larvae. Post-recruit specimens of the blue whiting *Micromesistius poutassou* (n = 100), horse mackerel *Trachurus trachurus* (n = 100), and broad-tailed short-finned squid *Todaropsis eblane* (n = 100), were collected by fishermen in the Galician fishing grounds (42º05'–45º15'N, 07º00'–09º20'W). All specimens were placed on flake ice and transported to the laboratory where they were examined on arrival while still fresh.

Delicate capsules are present around some larvae in marine fish only 34 h after infection (Smith 1974). Following these authors, and according to Abollo (1999) and Pascual et al. (1999), only those 'long-term persistent' encapsulated *Anisakis simplex* s. str. larvae inhabiting the preferred site of infection (liver, *Micromesistius poutassou*; stomach, *Trachurus trachurus* and *Todaropsis eblanae*) were removed from surrounding host tissue. Furthermore, according to Smith (1984) the length distribution of younger herringworm larvae did not differ significantly from that of older larvae; thus, the size of the host was not considered in the analysis. *Anisakis* larvae were then randomly collected from many hosts individuals to avoid intraspecific variability related to infrapopulation subdivision in nature.

PCR-based Restriction Fragment Length Polymorphisms (PCR-RFLP) analysis. PCR-RFLP analysis of the rDNA has been identified as a useful approach for the accurate identification of sibling species of the complex *Anisakis simplex* (D'Amelio et al. 2000). The method was used to falsify the hypothesis that the dif-

ferences observed in the fitness of *Anisakis* from squid and fishes may be biased by taxonomic differences in the *Anisakis* taxa involved.

DNA extraction was performed according to the protocol proposed by Holmes & Bonner (1973). Two conserved primers, i.e.: A (forward), GTCGAATTCGT-AGGTGAACCTGCGGAAGGATCA; and B, GCCGG-ATCCGAATCCTGGTTAGTTTCTTTT-CCT (Bachellerie & Qu 1993), were used in PCR to amplify an rDNA region between the 3' end of the small subunit rRNA and the 5' end of the large subunit rRNA genes, spanning the internal transcribed spacer 1, the 5.8S subunit and the internal transcribed spacer 2. PCR amplifications were performed for each reaction, starting from 10 to 20 ng of DNA template, using 0.5 µl Amplitaq Gold Polymerase (5 U µl^{-1}, Perkin-Elmer), 10 µl Gene Amp 10× PCR Buffer II (Perkin- Elmer), 10 µl of MgCl$_2$, 25 mM (Perkin-Elmer), 8 µl dNTPs (dCTP, dGTP, dATP, dTTP) 2.5 mM each, 0.3 µl of each primer, 0.05 µM µl^{-1}. Sterile distilled water was added to a final volume of 100 µl. The PCR conditions were as follows: 10 min at 95°C, 30 cycles of 30 s at 95°C, 30 s at 55°C and 75 s at 72°C and followed by a final elongation of 7 min at 72°C. A negative control, containing distilled water, was included in each amplification in order to detect any contaminating DNA. Following amplification, samples were subjected to electrophoresis in 1% agarose gel. No specific bands were detected and therefore amplicons were directly subjected to RFLP analyses, using 6 individual restriction enzymes: *Alu*I, *Hha*I, *Hinf*I, *Mbo*I, *Rsa*I and *Taq*I. After digestion, fragments were separated by electrophoresis on a 2% agarose gel.

Morphometric analysis. As suggested by Berland (1961), Fagerholm (1979) and Valero et al. (1991), the sample size and nature, the fixation and clearing method and the measurement methodology were standardized in order to discriminate between morphometric and/or growth variations due to the parasite's intraspecific variability and those due to host (i.e. microenvironment) influences. Quantitative measurements of body structures by light microscopy (LM) were used to evaluate allometric growth parameters of *Anisakis* larvae from different host species. In order to limit manual operations, digital images of single worm structures, ranging from ×40 to ×400 magnification, were recorded by a CDD camera fitted onto a microscope, and then digitized and processed by a filtering operation on a PC. After separation of worms from the image background, the pixel positions of the worm outline were analysed by algorithms to describe size characteristics. To this end, morphometric data were collected on 75 *A. simplex* s. str. larvae collected from the 3 host source groups (25 per host) using the Image Analysis System IBAS 2000. Eight measurements and

3 indices (Koyama et al. 1969) were analysed on cleared specimens: total body length (BL), maximum body width (BW), oesophagus length (OL), oesophagus width (OW), ventriculus length (VL), ventriculus width (VW), tail length (from anus to tip of tail; TL), the distance of the mouth to nerve ring (MN), alfa (α = BL BW^{-1}), beta (β = BL OL^{-1}), beta 3 (β_3 = BL VL^{-1}), gamma (γ = BL TL^{-1}). Non-parametric tests were used to discriminate host-induced variability in morphometric variables. In addition, a comparison of regression lines describing allometric relationships of larvae from different host source was assessed by means of analyses of covariance (ANCOVA). Furthermore, data were subjected to a stepwise forward discriminant analysis to select a subset of variables. The variables selected were then subjected to a canonical discriminant analysis (CDA). Mahalanobis distances between individual observations were calculated. Individual observations were subsequently allocated to the group for which they had the minimal Mahalanobis distance. These analyses were carried out with the raw data matrix.

In vitro culture. *In vitro* cultures were performed to determine the role of the microenvironment as a factor inducing the observed morphometric variability of wild larval infrapopulations. Worm larvae collected from the 3 host source groups were cultured in the same media and then subjected to the same environmental selection pressure.

Only healthy larvae randomly collected from several infrapopulations were carefully selected under a stereomicroscope and placed in a defined, complex specific culture medium follow Perteguer et al. (1996). After 7 d culture, the larvae were removed and fixed in hot 70% ethanol. Worms were examined at intervals of 24 h, and the number that had completed ecdysis was counted. The ecdysis was used as a measure of development (Sommerville & Davey 1976).

In vitro cultures were also performed to test the influence of the host source on excretory/secretory (ES) larval products. After 3 and 7 d culture, the supernatants were collected: ES1, 1 to 3 d and ES2, 4 to 7 d. After dialysing against PBS and concentration in an Amicon YM-10, the protein content was estimated (Bradford 1976) and the supernatant was finally frozen at –80ºC until use. Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with ES products of third stage (L3) worm larvae was carried out (Laemmli 1970, Hames 1986) using a Mini Protean® cell (BioRad). The gel consisted of a 4% stacking gel and a 5 to 20% lineal gradient separating gel, under reducing conditions (García-Palacios et al. 1996). Broad range molecular weight markers (150 to 15 kDa, BioRad) were incorporated into each electrophor-

etic run. The gels were stained with a silver staining kit (BioRad), following the manufacturer's instructions.

RESULTS

PCR-RFLP analysis

The amplification of the rDNA region spanning the ITS-1, ITS-2 and the 5.8 subunit produced a fragment of ~1 kb. Fig. 1 shows the length of rDNA fragments of *Anisakis simplex* L3 digested with 6 different enzymes. Restriction with *Alu*I produced 4 fragments of approximately 450, 240, 180 and 100 bp. Restriction with *Hha*I produced 2 fragments of approximately 550 and 430 bp. Restriction with *Hinf*I produced 2 fragments of approximately 620 and 250 bp plus a fragment shorter than 100 bp. Restriction with *Rsa*I produced 4 bands (550, 300, 125 bp, plus a fragment shorter than 100 bp). Restriction with *Taq*I produced 3 bands (430, 400 and 100 bp) and *Mbo*I produced 3 bands (430, 430 and 100 bp). Few fragments of small size were also visible on the gel, but their resolution on the gel was not sufficient to determine exact sizing, thus not affecting the reliability of the assay. Restriction patterns allowed the specific identification of all the specimens analysed as corresponding to the sibling *A. simplex* s. str. (Table 1). Moreover, no variation in restriction patterns were observed among individual larvae of wild *A. simplex* s. str. collected from *Micromesistius poutassou*, *Trachurus trachurus* and *Todaropsis eblanae*. However, some differences were observed due to partial digestion of the amount of DNA, for example see Fig. 1D, lanes 2, 4 and 6.

Morphometric analysis

Data on larval morphometrics are given in Table 2. Larvae collected from *Todaropsis eblanae* were smaller in overall length than those collected from *Micromesistius poutassou* and *Trachurus trachurus*.

Table 1. Taxonomic key based on 3 diagnostic restriction enzymes (*Hha*I, *Taq*I and *Hinf*I) for the identification of sibling species of the complex *Anisakis simplex* (adapted from D'Amelio et al. 2000)

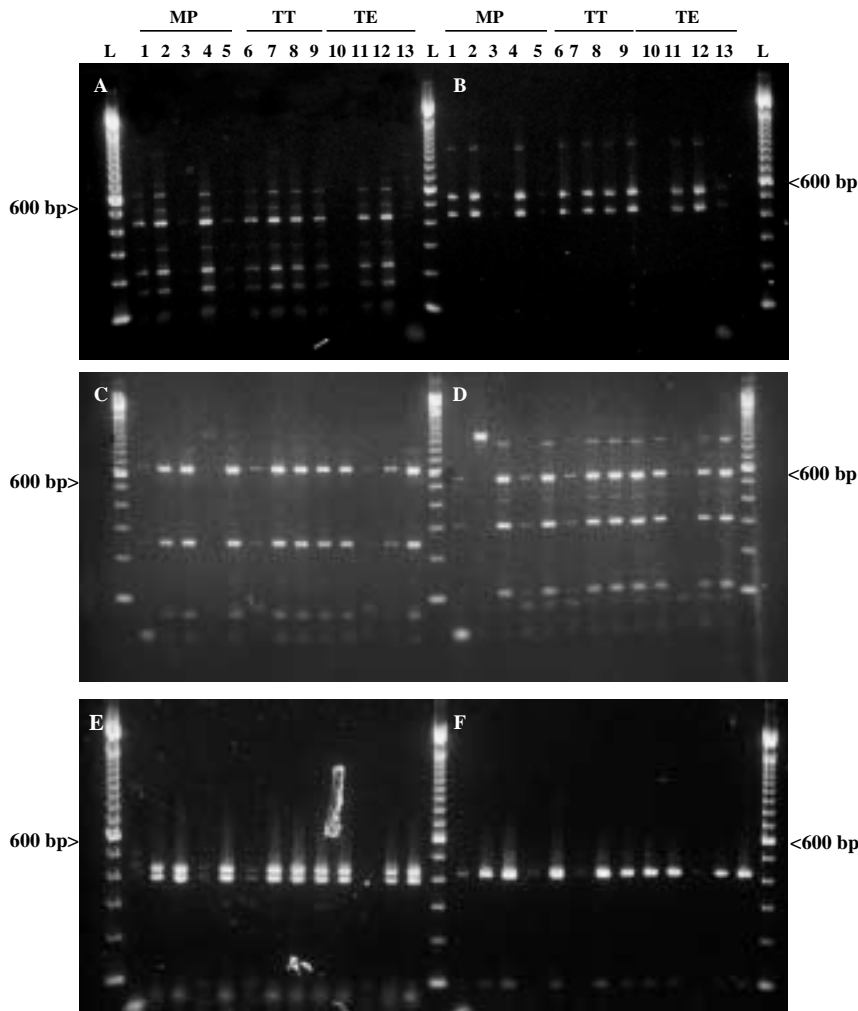| Enzyme	Fragments	Species
*Hinf*I	370-300-250	*A. pegreffii*
	620-250-80	*A. simplex* sensu stricto and *A. simplex* C
*Taq*I	400-320-150	*A. pegreffii*
	430-400-100	*A. simplex* sensu stricto and *A. simplex* C
*Hha*I	550-430	*A. simplex* sensu stricto
	550-300-130	*A. simplex* C

Fig. 1. *Anisakis simplex* s. str. Restriction fragment polymorphisms of larvae from 3 different paratenic hosts. rDNA digested with (A) *Alu*I, (B) *Hha*I, (C) *Hinf*I, (D) *Rsa*I, (E) *Taq*I, (F) *Mbo*I. Lanes 1–5: larvae from *Micromesistius poutassou*. Lanes 6–9: larvae from *Trachurus trachurus*. Lanes 10–13: larvae from *Todaropsis eblanae*. MP, *M. poutassou*; TT, *T. trachurus*; TE, *T. eblanae*; L, 100 bp ladder

the 2 discriminant functions were 1.40 and 0.63. The 2 roots accounted for 69 and 31% of the variance respectively. Calculation of Mahalanobis distance for individual cases revealed 81.33% discrimination between wild *Anisakis simplex* s. str. larvae from the 3 host sources as seen by the classification matrix (Table 5).

'In vitro' culture

After 7 d culture, no statistically significant differences (p > 0.005) were observed in any of the morphometric characters in larvae from the 3 host species (Table 3). Nevertheless, unequal mortality was observed. Thus, over 20 to 25% of the larvae collected from *Micromesistius poutassou* and *Trachurus trachurus* died earlier, up to the Day 7 of the experiment, whereas in the case of squid nematodes, the mortality increased to 80%. Larvae from *Todaropsis eblanae* died 3 d before in culture and in the remaining larvae, the third ecdysis lagged until 10 d; even then, the moult was not completed in most of these larvae.

SDS-PAGE analysis

The banding patterns of ES1 of L3 collected from different host species were similar. ES1 were separated into major bands in the 75 to 15 kDa range and an additional 4 bands within the ≥150 kDa range (Fig. 2). Nevertheless, the banding patterns of the ES2 products differed considerably between the larvae collected from both fish hosts and the squid host. The ES2 antigen lanes of larvae collected from *Micromesistius poutassou* and *Trachurus trachurus* revealed 8 different bands that were not observed in the banding pattern of the ES2 larval products from *Todaropsis eblanae*. Furthermore, the banding pattern of ES2 products of larvae from *T. eblanae* was very similar to the observed ES1 products, except for those bands within the ≥150 kDa range.

DISCUSSION

In temperate waters of the NE Atlantic the *Anisakis simplex* complex is considered a highly successful parasite, as it is suggested to be the most prevalent (an

Morphometric data showed that larval infrapopulation from *T. eblanae* are more homogenous that those from *M. poutassou* and *T. trachurus*. Thus 41.7% of the morphometric variables of *Anisakis simplex* s. str. larvae collected from *T. eblanae* are conservative (CV < 10%); 25% and 33.3% of the morphometric variables were conservative for larvae collected from *M. poutassou* and *T. trachurus* respectively.

The values of the non-parametric and ANCOVA tests showed the influence of the host source in various morphometric characters (especially for MN and α index, which are conservative in larvae collected at any of the 3 host species) (Table 3) and growth patterns (Table 4). An *a priori* stepwise forward discriminant analysis showed 7 variables that were used in the canonical discriminant analysis. The eigenvalues for

Table 2. *Anisakis simplex* s. str. Comparative morphometric data; mean ± SD (range). Larvae collected from different paratenic hosts in temperate waters off the NE Atlantic

Variable	*Micromesistius poutassou*	*Trachurus trachurus*	*Todaropsis eblanae*
BL	24.66 ± 3.27 (19.40–30.57)	24.21 ± 3.50 (19.48–30.27)	23.15 ± 2.33 (18.51–28.56)
BW	0.44 ± 0.06 (0.30–0.53)	0.42 ± 0.07 (0.30–0.56)	0.43 ± 0.05 (0.30–0.53)
OL	2.29 ± 0.21 (1.71–2.62)	2.25 ± 0.26 (1.86–2.95)	2.05 ± 0.16 (1.68–2.29)
OW	0.11 ± 0.01 (0.08–0.13)	0.13 ± 0.02 (0.09–0.16)	0.12 ± 0.01 (0.10–0.13)
VL	1.06 ± 0.14 (0.76–1.33)	1.02 ± 0.14 (0.79–1.27)	1.03 ± 0.11 (0.79–1.25)
VW	0.17 ± 0.05 (0.09–0.25)	0.21 ± 0.03 (0.15–0.27)	0.21 ± 0.03 (0.13–0.24)
TL	0.12 ± 0.02 (0.08–0.16)	0.14 ± 0.02 (0.10–0.17)	0.12 ± 0.10 (0.07–0.14)
MN	0.30 ± 0.02 (0.26–0.34)	0.30 ± 0.02 (0.25–0.33)	0.28 ± 0.01 (0.26–0.32)
α	57.00 ± 5.48 (42.73–64.38)	58.55 ± 5.56 (46.57–70.44)	54.36 ± 3.90 (48.77–62.70)
β	10.80 ± 1.28 (7.84–14.04)	10.87 ± 0.89 (9.79–12.40)	11.33 ± 0.98 (9.14–13.00)
β_3	23.32 ± 2.56 (19.08–27.10)	23.69 ± 2.32 (19.98–30.27)	22.58 ± 2.46 (19.14–27.86)
γ	218.11 ± 41.17 (137.19–305.19)	179.35 ± 34.65 (122.71–269.05)	202.38 ± 39.50 (148.10–335.92)

Table 3. *Anisakis simplex* s. str. Values of the Kruskall-Wallis test to estimate the influence of the host source on the morphometric characters of wild and cultured larvae. ns: not significant

Variable		χ^2	df	p
BL	Wild	1.8723	2	ns
	Cultured	1.9171	2	ns
BW	Wild	1.1684	2	ns
	Cultured	3.0659	2	ns
OL	Wild	13.8072	2	<0.001
	Cultured	4.8969	2	ns
OW	Wild	14.7897	2	<0.001
	Cultured	0.5482	2	ns
VL	Wild	1.2545	2	ns
	Cultured	2.0390	2	ns
VW	Wild	8.0936	2	<0.05
	Cultured	0.4613	2	ns
TL	Wild	14.6755	2	<0.001
	Cultured	0.4105	2	ns
MN	Wild	8.9397	2	<0.05
	Cultured	3.5531	2	ns
α	Wild	8.7877	2	<0.05
	Cultured	1.2121	2	ns
β	Wild	4.3303	2	ns
	Cultured	0.2407	2	ns
β_3	Wild	2.9822	2	ns
	Cultured	0.8752	2	ns
γ	Wild	13.7182	2	<0.001
	Cultured	0.2359	2	ns

average of 60% mean occurrence in all host species examined) and numerically dominant macroparasite in commercial fishes. Worm counts of 7 (up to 100) larvae per host individual have been recorded. In fact, life-cycle traits of *A. simplex* s. str. in this geographic area revealed a well-adapted marine parasite, conspicuous at the free-living stage, cryptic at L3 in intermediate and paratenic hosts, eurixenous, moderately pathogenic and aggregately distributed within host populations (Abollo 1999). Such a scheme is of a parasite-host system in which parasite fitness will increase and selective pressures are primarily on the host (Combes

Table 4. *Anisakis simplex* s. str. ANCOVA values to compare allometric growth patterns of larvae collected from different host source. ns: not significant

Variable	*F*	df	p
BW	4.626	2	<0.05
OL	8.515	2	<0.001
OW	15.181	2	<0.001
VL	1.009	2	ns
VW	13.480	2	<0.001
TL	9.019	2	<0.001
MN	3.680	2	<0.05

Table 5. *Anisakis simplex* s. str. Classification matrix displaying the number out of 75 larvae allocated to groups for which they have the minimum Mahalanobis distance

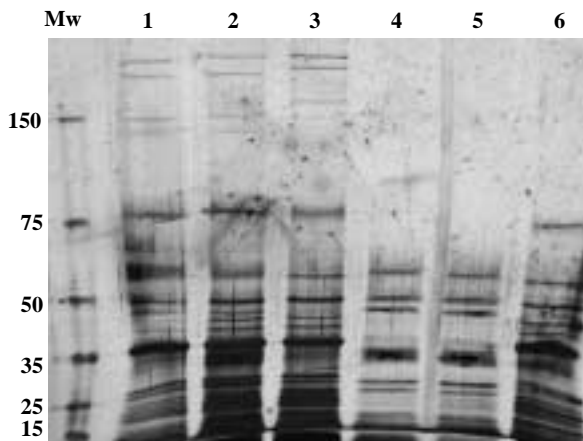| Source group	% correctly classified	Number allocated to group			Total
		T. eblanae	*T. trachurus*	*M. poutassou*	
Todaropsis eblanae	84	21	2	2	25
Trachurus trachurus	80	3	20	2	25
Micromesistius poutassou	80	3	2	20	25
Sum	81.33	27	24	24	75

Fig. 2. *Anisakis simplex* s. str. SDS-PAGE of excretion/secretion antigens at L3 collected from 3 different paratenic hosts. Mw, molecular weight. Lane 1: ES1 of larvae from *Micromesistius poutassou*. Lane 2: ES1 of larvae from *Trachurus trachurus*. Lane 3: ES1 of larvae from *Todaropsis eblanae*. Lane 4: ES2 of larvae from *M. poutassou*. Lane 5: ES2 of larvae from *T. trachurus*. Lane 6: ES2 of larvae from *T. eblanae*

1997). In addition to the specific identity of intermediate and definitive host (Nadler 1987), the main factors considered to induce intraspecific variability in anisakid nematodes are the geographical origin of host populations (George-Nascimento & Llanos 1995) and the complexity of the life-cycle (Bullini et al. 1986). As a consequence of different selection pressures, the identification of morphological, biochemical and genetic markers has revealed substantial intraspecific variation within anisakids among different microenvironment types. This has been well documented in *Contracaecum osculatum* s. l. (Fagerholm 1989, Nascetti et al. 1993, Orecchia et al. 1994), *Anisakis simplex* s. l. (Threlfall 1982, Smith 1983b, Mattiucci et al. 1997, Siles et al. 1997, Pascual et al. 1999), and *Pseudoterranova decipiens* s. l. (Paggi et al. 1991, Bristow & Berland 1992, George-Nascimento & Llanos 1995).

Morphometric and growth variations among wild larvae collected from the 3 host sampled species agreed with the electrophoretic variations of ES2 products of larvae grown *in vitro*, which are known to be produced during invasion of the host's tissues (Raybourne et al. 1986). Since enzymes involved in tissue-penetration (ES1 in the present study) were similar in wild third-stage larvae from a squid or a fish host, these differences must arise from some other as yet unknown environmental pressure against those larvae inhabiting the invertebrate paratenic hosts. Data on ES2 patterns of larvae from a squid or fish host suggest different morphoanatomical developments and development timings on cultured larvae depending on host source. Consequently, *Anisakis* infrapopulations will have ontogenetic trajectories which will be different in

larvae collected from each host species. Furthermore, the 3 host species sampled share the same sources of infection via diet (Abollo 1999) and the accumulation of infrapopulation worm larvae in the squid is determined by the combination of genotypes arriving from blue whiting and other different fish hosts ingested; these are shared between sympatric paratenic hosts in the sampling area, as noted by Pascual et al. (1996) and Rasero et al. (1996). Sugane et al. (1989) found that the patterns of 2 different fish paratenic host-derived DNA of *Anisakis* larvae type I were exactly the same in hybridised fragments generated by 6 endonucleases which reinforces the idea that *Anisakis* larva (I) genomic DNA from different paratenic host is not affected by environmental conditions.

The reasons for the differences in morphometric and growth pattern and also those in survival rates, and time of moult and ecdysis of larvae from squid and fish host, are not clear. Current views on the control of differentiation and growth of parasitic nematodes (Rogers & Sommerville 1968, Davey 1972) show that external stimuli activate internal regulatory mechanisms, including neurosecretions, which in turn generally regulate the various aspects of growth and differentiation, including moult and ecdysis behaviour *in vitro*. The external stimuli can be simple combinations of various components likely to be found in the alimentary tract of the host. Thus, the moult of exsheated third-stage larvae of *A. simplex* to an established parasite is probably initiated by temperature and carbon dioxide in the gas phase which reproduces some features of the stomach of the host (Sommerville & Davey 1976). Perhaps freshly hatched encapsulated larvae are less able to grow in the physicochemical environment impaired by the squid gastrointestinal tract, possibly due to the inhibitory effect of larval activity. Nevertheless, this is not likely because of the similar physicochemical environment that larvae might encounter on passage through a squid or a fish host (Packard 1972). Moreover, even significant differences should not be expected in relation to host immunity. The tissue response to the presence of *Anisakis* in an ommastrephid squid or a teleost fish is typical of a chronic granulamatous inflammatory reaction, as described by Abollo et al. (1998) and Ramakrishna & Burt (1991) respectively. The paratenic host encapsulates the parasite, preventing its continual migration and consequent destruction of tissues. It has been found that the capsules of 'later' and 'old' stages of infections differ in quantitative aspects, such as intensity of response and thickness of capsule (Ramakrishna & Burt 1991). However, all larvae sampled here were collected from 'later' capsules showing a healthy aspect (shining and transparent cuticle) and vigorous movements.

On the other hand, it was stated that those larvae that have been passed from fish to fish (i.e., from one paratenic host to another) may have insufficient energy stores or enzymes to establish in a new micro-environment, as was demonstrated experimentally by Wootten & Smith (1975) and Burt et al. (1990). Worm larvae from the squid had naturally passes through at least one cephalopod (by cannibalism) and/or fish host (mainly *Micromesistius poutassou* but also *Trachurus trachurus*) used as prey to infect this host; the short-finned squid are largely voracious piscivorous at mantle length ≥120 cm (Rasero et al. 1996). In the sampling area these larger, mature squids in which the whole-parasite recruitment takes place (Pascual et al. 1995, 1996, 1999) are mainly feeding (up to 60 % of its diet) on heavily infected 20 to 26 cm blue whiting (Rasero et al. 1996). According to Wootten & Smith (1975) and Burt et al. (1990), this may explain the low survival rate and moult success of squid larvae grown *in vitro* compared with those from a fish host. Schiemer (1987) stated that even small differences in energy transfer efficiencies and energy allocation patterns to perform adaptive functions can have profound effects on nematode fitness under certain habitat conditions. Besides the energetic cost through metabolic adaptations for host tissue invasion (i.e., the invasion of a new micro-environment), larvae inhabiting the squid may also have suffered a larger sequence of severe host responses than those from the 2 other fish hosts, which had probably acquired the infection directly by entering an invertebrate intermediate host.

We stated that in the sampling area, cephalopods (especially large ommastrephid squids) seem to be less favourable paratenic hosts for *Anisakis simplex* s. str. larvae than those fish species with a similar ecological niche. However, it should also be noted that cephalopods are a key trophic bridge in order for the parasitic life-cycle to be completed in the long-finned pilot whale (Abollo et al. 1998). Thus, the narrow trophic relationship which has been demonstrated between cephalopods and long-finned pilot whales should also be considered as an important element for understanding the widespread dissemination of *Anisakis* in all major seas and oceans, especially in those areas where no other potential small-cetacean final hosts are abundantly distributed.

Fitness is defined as 'the average contribution of one allele or genotype to the next generation or to succeeding generations, compared with that of other alleles or genotypes' (Futuyma 1986). We might assume that a higher rate of 'maladaptation' of *Anisakis simplex* s. str. larvae to the squid host in relation to the fish host (i.e. the observable morphometric, growth, biochemical and behavioural characteristics which impair the ability of worm larvae to survive and differentiate in a particular environment, thereby reducing their fitness) is not likely to be due to insufficient time for evolution by natural selection to take place. The high level of gene flow (Paggi & Bullini 1994) and a heterogeneous environment may prevent larvae from being equally efficient in all paratenic host species. Furthermore, the first causes of variation may be changes in that part of the genome of the larvae that is expressed, or in that part that regulates expression. Thus the expression of proteinases that play an important role in the invasion of host tissues (Hotez et al. 1994), parasite nutrition (McKerrow 1989), secretion of pharmacological mediators (Rhoads 1984) and suppression of host immune responses (Leid et al. 1987) is often developmentally regulated (Newport et al. 1988).

Our experiments show that the squid host reduces growth and differentiation times of *Anisakis simplex* s. str. larvae. But perhaps of greater interest is the demonstration that parasite recruitment to the long-finned pilot whale, the final host in the sampling area, is expected to be reduced in those L3 from this invertebrate paratenic host that has obviously negative effects on parasite fitness.

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