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Insight into the role of cetaceans in the life cycle of the tetraphyllideans (Platyhelminthes: Cestoda) ☆

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Abstract

Four types of tetraphyllidean larvae infect cetaceans worldwide: two plerocercoids differing in size, 'small' (SP) and 'large' (LP), and two merocercoids referred to as *Phyllobothrium delphini* and *Monorygma grimaldii*. The latter merocercoid larvae parasitize marine mammals exclusively and exhibit a specialised cystic structure. Adult stages are unknown for any of the larvae and thus the role of cetaceans in the life cycle of these species has been a long-standing problem. The SP and LP forms are thought to be earlier stages of *P. delphini* and *M. grimaldii* that are presumed to infect large pelagic sharks that feed on cetaceans. A molecular analysis of the D2 variable region of the large subunit ribosomal DNA gene based on several individuals of each larval type collected from three Mediterranean species of cetaceans showed consistent and unique molecular signatures for each type regardless of host species or site of infection. The degree of divergence suggested that LP, *P. delphini* and *M. grimaldii* larvae may represent separate species, whereas SP may be conspecific with *M. grimaldii*. In all host species, individuals of SP accumulated in the gut areas in which the lymphoid tissue was especially developed. We suggest therefore that these larvae use the lymphatic system to migrate to the abdominal peritoneum and mesenteries where they develop into forms recognizable as *M. grimaldii*. The plerocercoid stage of *P. delphini* remains unknown. In a partial phylogenetic tree of the Tetraphyllidea, all larvae formed a clade that included a representative of the genus *Clistobothrium*, some species of which parasitize sharks such as the great white which is known to feed on cetaceans. A bibliographic examination of tetraphyllidean infections in marine mammals indicated that these larvae are acquired mostly offshore. In summary, the evidence suggests that cetaceans play a significant role in the life cycle of these larvae. In addition, it seems clear that cetaceans act as natural intermediate hosts for *P. delphini* and *M. grimaldii*, as within these hosts they undergo development from the plerocercoid stage to the merocercoid stage. Because tetraphyllidean species use fish, cephalopods and other marine invertebrates as intermediate hosts, the inclusion of cetaceans in the life cycle would have facilitated their transmission to apex predators such as the large, lamnid sharks. The biological significance of infections of LP in cetaceans is unclear, but infections do not seem to be accidental as such larvae show high prevalence and abundance as well as a high degree of site specificity, particularly in the anal crypts and bile ducts.

Keywords: Tetraphyllidea; Plerocercoid; *Phyllobothrium delphini*; *Monorygma grimaldii*; *Clistobothrium*; Cetacean; Molecular diagnostics; Life cycle

1. Introduction

Four types of tetraphyllidean metacestodes (terminology follows Chervy, 2002) have been recognized in marine mammals worldwide (Agustí et al., 2005a,b). Two of these are plerocercoids, collectively referred to as *Scolex pleuronectis* Müller, 1788, that occur throughout the gut and hepatopancreatic ducts. These forms exhibit the same morphology but can be readily distinguished by size differences, hence the names 'small' (SP) and 'large' (LP) used to differentiate them (Agustí et al., 2005a). Two other types are bladder-like merocercoids with clear morphological, molecular and ecological differences: *Phyllobothrium delphini*

☆ Nucleotide sequence data reported in this paper are available in the GenBank under the Accession No.: DQ839568–DQ839593.

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(Bosc, 1802) Van Beneden, 1868, occurring in the subcutaneous (s.c.) blubber, and *Monorygma grimaldii* (Moniez, 1889) Baylis, 1919 in the peritoneum and mesenteries of the abdominal cavity (Agustí et al., 2005b and references therein).

No life cycle of a tetraphyllidean species has been demonstrated to date (Caira and Reyda, 2005). In the case of the species whose larvae infect marine mammals, the inability to positively identify the adult stage has severely hampered our knowledge of key aspects of their biology. First, the actual number of species infecting marine mammals is yet to be determined, as attempts to identify species based on larval morphology (Testa and Dailey, 1977 and references therein) were inconclusive (Agustí et al., 2005b). Second, the relationship among larval types is unclear, although it has been suggested that the SP and LP may represent earlier stages of *P. delphini* and *M. grimaldii* (Skrjabin, 1972; Fernández et al., 2003; Agustí et al., 2005a). This hypothesis was based on circumstantial evidence, i.e., the morphological resemblance of the scolex, the co-occurrence of the four larval types in the same individual hosts and the presence of plerocercoids close to the sites of infection where the merocercoids are found (Agustí et al., 2005a). Finally, whether or not the role of marine mammals in the life cycle of these larvae is obligatory is still debated (Williams, 1968; McColl and Obendorf, 1982; Walker, 2001; Raga et al., 2002). In a recent molecular analysis of *P. delphini* and *M. grimaldii* from Western Mediterranean striped dolphins, *Stenella coeruleoalba*, the merocercoids showed a high level of genetic similarity with *Clistobothrium montaukensis* Ruhnke, 1993 (Agustí et al., 2005b), suggestive of congeneric status. Given that some species of *Clistobothrium* (i.e., *Clistobothrium carcharodoni* Dailey and Vogelbein, 1990 and *Clistobothrium tumidum* Linton, 1922) infect sharks that include marine mammals in their diet (Dailey and Vogelbein, 1990; Cortés, 1999), it may be that these mammals play an obligatory role in the life cycle of some tetraphyllidean species.

In this paper, we carried out an extensive survey of three cetacean species in the Western Mediterranean, including a molecular analysis and a detailed description of the sites of infection of the four tetraphyllidean larval morphotypes that commonly infect these hosts. The results shed light on the identity and phylogenetic relationships among the larval types, their distribution within hosts and the role of cetaceans in their life cycles, as well as raising new questions.

2. Materials and methods

2.1. Samples

Fifty striped dolphins, *S. coeruleoalba* (27 males, range of total length 150–213 cm; 23 females, 106–211 cm), eight Risso's dolphins, *Grampus griseus* (four males, 172–308 cm; four females, 289–305 cm) and four bottlenose dolphins, *Tursiops truncatus* (three males, 238–322 cm; one female, 260 cm) stranded during 1990–2003 along the Mediterranean coast of Spain (between 42°20′N, 3°11′E and 37°34′N, 1°04′W) were examined for tetraphyllidean larvae. All carcasses were in good condition (codes 2–3 sensu Geraci and Lounsbury, 1993). Larval types were identified according to the morphological descriptions of Agustí et al. (2005a,b).

Organs were not always available for analysis of all larval types; the sample size in each case is given in Table 1. Hosts were examined fresh for the presence of *P. delphini* and *M. grimaldii*. The former was detected through transversal and longitudinal slices on s.c. blubber. Radial cuts were made every 0.5 cm from the anal-genital slits, the region where these larvae mostly occur (Walker, 2001 and references therein). Searching was stopped when no larva was found after 20 consecutive cuts. The peritoneum and mesenteries of the abdominal cavity were thoroughly examined for *M. grimaldii*.

The stomach, intestine, liver and pancreas were generally frozen for subsequent examination, although in some dolphins organs were analysed fresh. After thawing, organs were examined for SP and LP. The three stomach chambers (i.e., forestomach, main stomach and pyloric stomach) were examined separately. The intestine was divided into 20 sections of equal length (approximately 1 m). The contents of each chamber and intestinal section were flushed with physiological saline through a 0.2 mm mesh sieve,

Table 1
Number of individuals of striped dolphin, *Stenella coeruleoalba* (Sc), Risso's dolphins, *Grampus griseus* (Gg) and bottlenose dolphins, *Tursiops truncatus* (Tt) used for analysis of infection patterns of tetraphyllidean larvae

Host species	Larval type	No. of dolphin individuals analysed	
		Prevalence	Intensity
Sc	*Phyllobothrium delphini*	50	10
	Monorygma grimaldii	50	10
	LP[a]	50	20
	SP[b]	15 (stomach)	15 (stomach)
		50 (intestine, bile ducts)	20 (intestine, bile ducts)
Gg	All larval types	8	8
Tt	All larval types	4	4

[a] 'Large' plerocercoid.
[b] 'Small' plerocercoid.

Table 2
Hosts and sites of infection of specimens used for molecular analysis

Larval type:	*Phyllobothrium delphini*	*Monorygma grimaldii*	LP[a]		SP[b]
Site of infection:	Blubber	Peritoneum-mesentery	Anal canal	Liver	Terminal colon
Host					
Stenella coeruleoalba	8[c] AY741599–AY741606 BMNH 2004.8.18.6-13	8[c] AY741591–AY741598 BMNH 2004.8.18.14-21	8[d] Sc1-PGA (DQ839574/ BMNH.2004.10.13.14) Sc1-PGB (DQ839575/ BMNH.2004.10.13.15) Sc1-PGC (DQ839576/ BMNH.2004.10.13.16) Sc2-PG2 (DQ839577/ BMNH.2004.10.13.17) Sc2-PG4 (DQ839570/ BMNH.2004.10.13.18) Sc2-PG6 (DQ839571/ BMNH.2004.10.13.19) Sc3-PGA (DQ839578/ BMNH.2004.10.13.20) Sc3-PGB (DQ839579/ BMNH.2004.10.13.21)	3 Sc4-PGD (DQ839569/ BMNH.2006.8.17.1) Sc25-PGA (DQ839568/ BMNH.2004.10.13.22) Sc25-PGB (DQ839572/ BMNH.2004.10.13.23)	1 Sc3-PPA (DQ839588/ BMNH.2004.10.13.25)
Grampus griseus	2 Gg1-PdA (DQ839593/ BMNH.2004.10.13.4) Gg1-PdB (DQ839589/ BMNH.2004.10.13.5)	2 Gg1-MgC (DQ839585/ BMNH.2004.10.13.9) Gg1-MgD (DQ839586/ BMNH.2004.10.13.10)	1 Gg1-PG2 (DQ839573/ BMNH.2004.10.13.13)		1 Gg1-PP2 (DQ839587/ BMNH.2004.10.13.24)
Tursiops truncatus	3 Tt1-PdA (DQ839590/ BMNH.2004.10.13.1) Tt1-PdB (DQ839591/ BMNH.2004.10.13.2) Tt1-PdC (DQ839592/ BMNH.2004.10.13.3)	3 Tt1-MgA (DQ839582/ BMNH.2004.10.13.6) Tt1-MgB (DQ839583/ BMNH.2004.10.13.7) Tt1-MgC (DQ839584/ BMNH.2004.10.13.8)	2 Tt1-PGA (DQ839580/ BMNH.2004.10.13.11) Tt1-PGC (DQ839581/ BMNH.2004.10.13.12)		
Total	13	13		14	2

[a] 'Large' plerocercoid.

[b] 'Small' plerocercoid.

[c] Previously published samples: see Agustí et al. (2005b) for additional information.

solid remains being collected in a Petri dish. The wall of each chamber/section was examined under a stereomicroscope to detect larvae in situ. The hepatic and pancreatic ducts were opened and examined under a stereomicroscope, and the liver and pancreas were subsequently sliced and washed on a sieve to collect plerocercoids that may have been overlooked in situ. Plerocercoids were fixed in 70% (v/v) ethanol.

Although we attempted to collect and count all larvae infecting each individual host, there were obvious difficulties to make accurate calculations when dolphins were not fresh, particularly in the case of plerocercoids. Therefore, we adopted a gross intensity index (INI) for all larval types: 1 (1–10 larvae); 2 (11–100); 3 (101–1000), and 4 (>1000). The index was always assigned by the same observer (C.A.).

2.2. Molecular analysis

Samples of the four larval types were collected from the three dolphin species and fixed in 95% ethanol for molecular diagnostic analysis. The D2 variable region (~650 bp) of the nuclear large subunit ribosomal DNA (lsrDNA) gene was characterized from a total of 26 specimens and combined with eight D2 sequences (AY741591–AY741606) each of *M. grimaldii* and *P. delphini* from the work of Agustí et al. (2005b), see Table 2 for the hosts and sites of infection sampled. The D1–D3 regions (~1400) were characterized from one LP (Sc25-PGA; DQ839568) in order to test the variability of the more conserved D1 and D3 regions, which were found to be too conserved for comparisons among the larval types with adult reference sequences. No specimen from the pyloric stomach of any host species was available for molecular analysis.

Scoleces of each specimen analysed genetically were retained for vouchers prior to genomic DNA (gDNA) extraction and deposited in ethanol in the helminth collection of the Natural History Museum, London (Accession Nos.: BMNH 2004.8.18.6-21; BMNH 2004.10.13.1-25; BMNH 2006.8.17.1; see also Table 2). Genomic DNA was extracted from the specimens using a Qiagen DNeasy™ tissue kit and used for PCR as described by Olson et al. (2003). A fragment (~1400 bp) of the lsrDNA gene spanning domains D1–D3 was amplified using primers LSU5 (5′-TAG GTC GAC CCG CTG AAY TTA AGC-3′) and 1200R (5′-GCA TAG TTC ACC ATC TTT CGG-3′) and the middle portion spanning the variable D2 region sequenced bi-directionally using internal primers 300F (5′-CAA GTA CCG TGA GGG AAA GTT-3′) and ECD2 (5′-CTT GGT CCG TGT TTC AAG ACG GG-3′). This region of the lsrDNA has been found to be informative for both diagnostic and phylogenetic work in tetraphyllidean and related taxa (e.g., Brickle et al., 2001; Reyda and Olson, 2003; Agustí et al., 2005b). Contiguous sequences were assembled and edited using Sequencher™ (GeneCodes Corp., ver. 4.6) and leading and trailing regions of the sequences without overlap were removed prior to analysis. Sequences are available from GenBank under Accession Nos. DQ839568–DQ839593 (see also Fig. 1).

Sequences were screened using BLASTn (McGinnis and Madden, 2004) to confirm their orthology with the lsrDNA genes of cestodes and aligned by eye using MacClade ver. 4.06 (Maddison, D.R., Maddison, W.P., 2000. MacClade 4: Analysis of phylogeny and character evolution. Ver. 4.06. Sinauer Associates, Inc., Sunderland, Massachusetts). A single representative sequence of each larval type from cetaceans was analysed together with 20 lsrDNA available tetraphyllidean sequences (see Agustí et al., 2005b). Phylogenetic affinities were estimated by Bayesian analysis using MrBayes ver. 3.1.2 (Ronquist and Huelsenbeck, 2003). Based on the results of MrModeltest ver. 1.1b (Nylander, J., 2002. MrModeltest Version 1.1b. Department of Systematic Zoology, EBC, Uppsala, Sweden; a simplified version of ModelTest by Posada and Crandall, 1998), a general time reversible model of nucleotide substitution incorporating among site rate variation and invariant sites was specified and the analysis run over 1 million generations, sampling topologies every 100th generation. Other program parameters were as specified in Olson et al. (2003). A consensus tree was constructed using the 'sumt' command with a 'burnin' value of 100 and the 'contype = allcompat' option. Trees were rooted using *Echeneibothrium maculatum* based on prior analysis of tetraphyllidean and related lsrDNA sequences (see Reyda and Olson, 2003). Comparisons of uncorrected genetic distances (shown parenthetically as the percent similarity; i.e., number of identical bases/number of sites compared * 100) were calculated using PAUP* ver. 4.0b10 (Swofford, D.L., 2002. Phylogenetic Analysis using Parsimony (* and other Methods). Vers. 4.0b10 Sinauer Associates, Sunderland, Massachusetts) as were bootstrap values based on 1000 replicates with 10 random-addition full heuristic searches/replicates.

2.3. Site selection analysis

Based on the results of the molecular analysis, we investigated patterns of site selection by each larval type. The following were considered as different sites of infection for comparison (Fernández et al., 2003; Agustí et al., 2005a,b and references therein): s.c. blubber; peritoneum + mesenteries of the abdominal cavity; forestomach; main stomach; pyloric stomach; intestinal sections 1–19; intestinal section 20; bile ducts (hepatic + pancreatic ducts). Due to sample size limitations, statistical comparisons of sites of infection for each larval type were performed only in the striped dolphin (Table 1). Differences in the frequency of occurrence per site were compared using Cochran's Q tests with MacNemar post hoc comparisons, and differences in INI were tested with Friedman tests with post hoc comparisons (Zar, 1996; Conover, 1999). Kendall tests were used to examine predictability

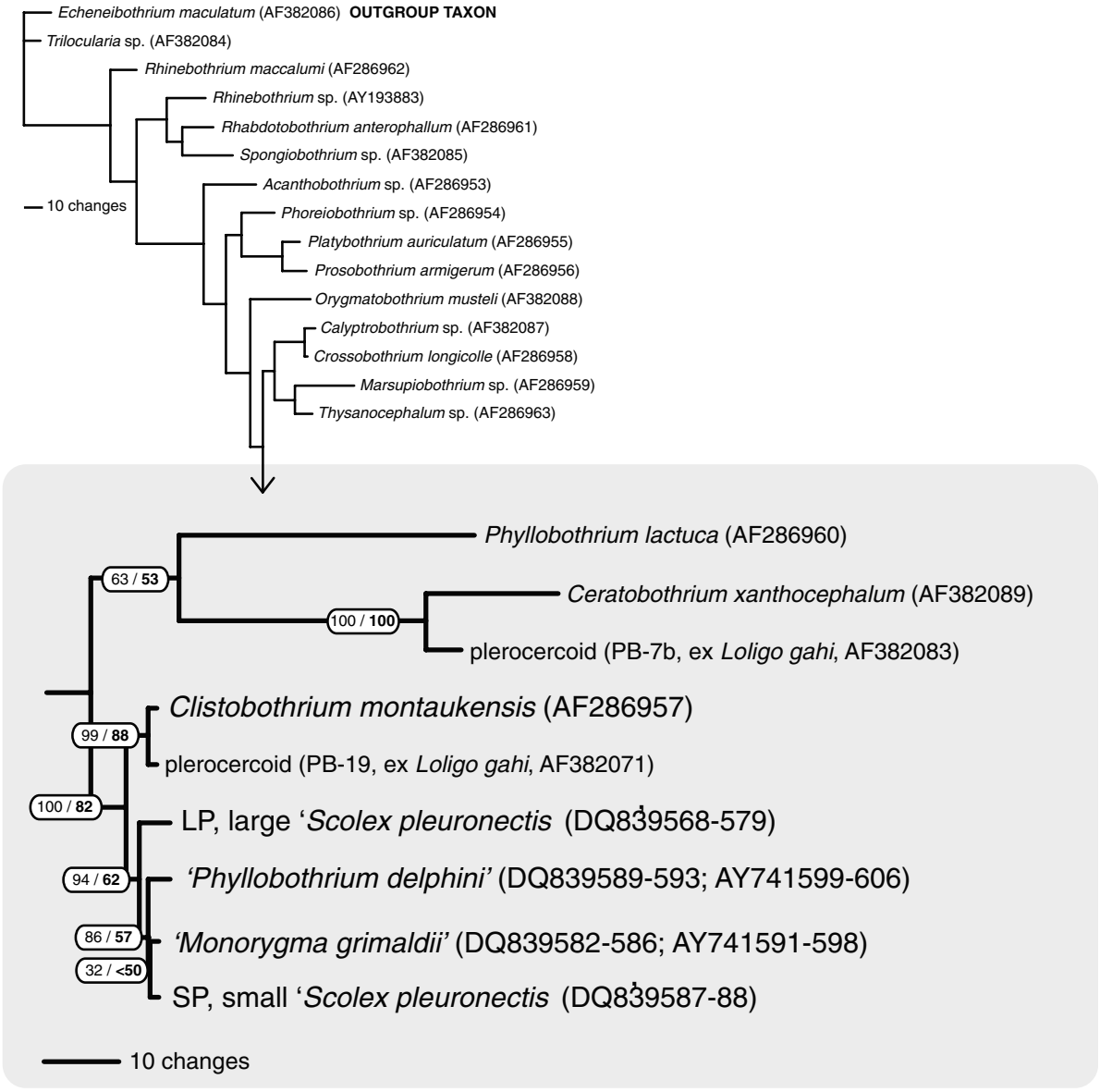

Fig. 1. Phylogenetic position of the larval tetraphyllideans based on Bayesian inference of the D2 region lsrDNA (515 characters); clade containing larval taxa enlarged to illustrate short internal branch lengths. Nodal support shown as posterior probabilities/bootstrap percentages (bold). Relative branch lengths based on Bayesian inference with scale showing absolute character differences. GenBank sequence accession numbers are shown parenthetically.

in site selection (W: Kendall's coefficient of concordance) (Zar, 1996; Conover, 1999). $P < 0.05$ was considered statistically significant. In multiple comparisons, probability values were corrected by the sequential Bonferroni Procedure (Rice, 1989). Ecological terminology follows Bush et al. (1997). Analyses were carried out with the package SPSS v.12.0 for Windows. The free software Quantitative Parasitology, v. 2.0 (Rózsa et al., 2000) was used to calculate infection parameters (Tables 3 and 4).

2.4. Patterns of host-parasite relationship

All available records of LP and SP are summarized in Agustí et al. (2005a). We conducted an exhaustive bibliographical search of reports of *P. delphini* and *M. grimaldii*. Host species were divided into three categories: (i) not sufficiently analysed, when the number of host individuals analysed was <5; (ii) accidental, when the prevalence of *P. delphini* and/or *M. grimaldii* was <10%; (iii) common, when prevalence was ⩾10%. Our goal was to test whether *P. delphini* and *M. grimaldii* tended to occur more frequently in marine mammal species living in specific marine habitats and/or with specific diets. Assignment of a host species to a given category was based on information pooled from all existing reports in order to reveal global patterns. However, we separated existing reports in which the same species of marine mammal were collected from different habitats and/or fed on different prey types. For brevity, we report only key papers that summarize the majority of references. We followed Evans and Raga (2001) for current

Table 3
Data on prevalence and intensity index (INI: see Section 2) of *Phyllobothrium delphini* and *Monorygma grimaldii* in three dolphin species from the Western Mediterranean (striped dolphins, *Stenella coeruleoalba* (Sc), Risso's dolphins, *Grampus griseus* (Gg) and bottlenose dolphins, *Tursiops truncatus* (Tt))

Host:	Sc		Gg		Tt	
Larval type:	*P. delphini*	*M. grimaldii*	*P. delphini*	*M. grimaldii*	*P. delphini*	*M. grimaldii*
Prevalence	100.0 (92.5–100.0)	96.0 (86.3–99.3)	50.0 (19.3–80.7)	87.5 (50.0–99.4)	75.0 (24.9–98.7)	75.0 (24.9–98.7)
INI	3 (2–3)	2 (2–3)	1	1 (1–2)	1	1 (1–2)

Prevalences are expressed as percentages with the 95% confidence interval in parentheses. The INI is expressed by the median value, with the range in parentheses.

Table 4
Data on prevalence of 'small' (SP) and 'large' (LP) plerocercoids in three dolphin species from the Western Mediterranean (striped dolphins, *Stenella coeruleoalba* (Sc), Risso's dolphins, *Grampus griseus* (Gg) and bottlenose dolphins, *Tursiops truncatus* (Tt))

Larval type	Host	Site of infection					
		Stomach			LPD[d]	Intestine	
		FS[a]	MS[b]	PS[c]		I (1–19)[e]	I (20)[f]
SP	Sc	–	13.3 (2.4–39.7)	100 (77.8–100.0)	–	52.0 (37.9–66.1)	94.0 (83.3-98.3)
	Gg	–	–	50.0 (19.3–80.7)	–	62.5 (28.9–88.9)	75.0 (36.5–95.4)
	Tt	–	25.0 (1.3–75.1)	50.0 (9.8–90.2)	–	NA[g]	75.0 (24.9–98.7)
LP	Sc	4.0 (0.7–13.7)	2.0 (0.1–10.7)	14.0 (6.7–26.8)	92.0 (81.2–97.2)	58.0 (44.0–71.2)	100.0 (92.5–100.0)
	Gg	–	–	–	37.5 (11.1–71.1)	12.5 (0.6–50.0)	75.0 (36.5–95.4)
	Tt	–	–	–	–	NA[g]	75.0 (24.9–98.7)

Prevalences are expressed as percentages with the 95% confidence interval in parentheses. Sample size for each site of infection is shown in Table 1.
[a] Forestomach.
[b] Main stomach.
[c] Pyloric stomach.
[d] Liver, pancreas and hepatopancreatic duct.
[e] Intestine (sections 1–19).
[f] Intestine (section 20).
[g] NA: organ not available.

nomenclature of host species, with the updates of Dalebout et al. (2002). Data regarding the habitat and diet of cetaceans and pinnipeds was obtained from Ridgway and Harrison (1981a,b, 1985, 1989, 1994, 1999).

3. Results

3.1. Molecular analysis

Each of the four larval morphotypes exhibited a unique lsrDNA signature regardless of the host species or site of infection in which they were found; no variation in the D2 region was observed within each morphotype, and raw sequence divergence among the four types was small. Unexpectedly, SP differed by only a single transition (G ↔ A) from *M. grimaldii* (99.8% similarity), whereas SP differed from LP by four G ↔ A and two T ↔ C transitions (98.8%). The *P. delphini* isolates were most similar to *M. grimaldii* (99.4%), then to SP (99.2%) and least similar to LP (98.5%).

A total of 515 characters were included in the analysis of the D2 region of the 24 adult and larval tetraphyllidean taxa of which 215 characters were parsimony informative. Interrelationships of the adult taxa based on Bayesian inference (Fig. 1) were effectively the same as those found in Agustí et al. (2005b) and the four larval types formed a clade closest to *C. montaukensis*. To emphasize graphically the small divergence among the larval taxa and *C. montaukensis*, Fig. 1 enlarges the clade including these taxa and its sister clade comprising *Phyllobothrium lactuca*, *Ceratobothrium xanthocephalum* and two larval tetraphyllidean collected from a longfin Patagonian squid, *Loligo gahi* (see Brickle et al., 2001). Bayesian posterior probabilities and bootstrap values were generally low owing to the small number of differences among the sequences.

3.2. Site selection analysis

The four larval types were found in all host species with moderate to high prevalences (Tables 3 and 4). In all host species, *P. delphini* occurred primarily in the s.c. blubber around the anal-genital slit. However, in moderately to heavily parasitized striped dolphins (INI = 3; see Table 3), the distribution extended into the blubber up to the dorsal fin and the caudal peduncle. In all host species, individuals of *M. grimaldii* were found in the peritoneum of the abdominal cavity, especially around the genital region, occurring also in the mesenteries of the uterus, testes and colorectal portion of the intestine.

Individual LPs were found free in the lumen of the intestine, in bile ducts, inside anal crypts, and rarely in the

lumen of stomach. The sites of infection were similar among the three host species, except that no worm was found in the bile ducts of bottlenose dolphins (Tables 4 and 5). In the striped dolphin, there were highly significant differences in the occurrence frequency among sites of infection (Cochran test, $Q = 133.1$, $n = 31$, $P < 0.001$). The post hoc pattern of differences (McNemar tests, $P < 0.05$) was as follows: anal crypts = bile ducts > intestinal sections 1–19 > stomach chambers (Table 4). A comparison of INI between the three former sites of infection (Table 5) indicated that the number of LP in anal crypts > bile ducts > intestinal sections 1–19 (Friedman test with post hoc comparison, $P < 0.002$). The Kendall test indicated that this ordination was highly conserved from host to host ($W = 0.89$, 2 df, $P < 0.001$). In some hosts, over 1000 worms were found inside anal crypts (Table 5).

The sites of infection of SP were similar among the three host species (Tables 4 and 5). Worms were found free in the lumen of the glandular part of the stomach (i.e., the main and pyloric stomach) and the intestine. However, the majority of individuals were found buried in the mucosa of the pyloric stomach and the intestine, with particularly large concentrations, sometimes over several thousand individuals, in the terminal colon and rectum (Table 5). No worm was found in the bile ducts of any host. In the striped dolphin, the occurrence frequency differed significantly among sites of infection. A Cochran test with post hoc MacNemar tests ($P < 0.05$) showed that the occurrence in the pyloric stomach > main stomach = forestomach. Pairwise MacNemar tests ($P < 0.05$) between pyloric stomach, intestinal sections 1–19 and section 20 showed that the occurrence in the intestinal section 20 = pyloric stomach > intestinal sections 1–19 (Table 4). A comparison of INI between the three latter sites of infection (Table 5) indicated significant differences only between sections 1–19 and section 20 (Friedman test with post hoc comparison, $P < 0.001$). The Kendall test indicated that infection levels per site of infection were similarly ordered from host to host ($W = 0.53$, 2 df, $P < 0.001$). In section 20, the maximum concentration of SPs occurred in the terminal colon adjacent to the rectum of 29 of 32 striped dolphins, two of five Risso's dolphins and three of three bottlenose dolphins. Sixteen striped dolphins, one bottlenose dolphin and three Risso's dolphins harboured SPs in both the terminal colon and rectum.

3.3. Patterns of host-parasite relationship

Occurrence of tetraphyllidean merocercoids in cetaceans and pinnipeds are shown in Table 6. Three patterns are apparent. First, *P. delphini* or *M. grimaldii* have never, or rarely, been reported in most species of mysticetes; the only exception is the study of Rice (1977) on *Balaenoptera borealis*. Second, in odontocetes, there is a broad segregation between inshore species/populations (harbouring few or no tetraphyllidean larvae) and offshore species/populations (in which these larvae are common) (Table 6). There are few apparent exceptions to this pattern (Best and Abernethy, 1994; Slooten and Dawson, 1994). Finally, in pinnipeds, tetraphyllidean merocercoids are uncommon, with few exceptions (i.e., George-Nascimento and Carvajal, 1981; Lauckner, 1985; Soares M.L.R., 1986. Ocorrencia de *P. delphini* (Bosc, 1802) Gervais, 1882 (Phyllobothriidae: Cestoda) em lobos marino *Arctocephalus australis* Zimmerman, 1783 e *Arctocephalus tropicalis* Gray, 1872 (Pinnipedia: Otariidae) no litoral norte do Río Grande do Sul, Brasil. In: Anais da 2[a] reuniao de trabalho de especialistas em mamíferos aquáticos da América do Sul. Book of Abstracts. Fundaçao Brasileira para a Conservaçao da Natureza, Brasil, p. 21; Bester, 1989).

Table 5
Data on intensity index (INI) (see Section 2) of 'small' (SP) and 'large' (LP) plerocercoids in three dolphin species from the Western Mediterranean (striped dolphins, *Stenella coeruleoalba* (Sc), Risso's dolphins, *Grampus griseus* (Gg) and bottlenose dolphins, *Tursiops truncatus* (Tt))

Larval type	Host	Site of infection					
		Stomach			LPD[d]	Intestine	
		FS[a]	MS[b]	PS[c]		I (1–19)[e]	I (20)[f]
SP	Sc	–	(1–2)	3 (2–3)	–	2 (1–4)	3 (2–4)
	Gg	–	–	2	–	2 (1–2)	2 (1–4)
	Tt	–	1	3	–	NA[g]	3
LP	Sc	1	2	1 (1–2)	2 (1–3)	1 (1–2)	3 (2–4)
	Gg	–	–	–	1 (1–2)	1	2 (1–3)
	Tt	–	–	–	–	NA[g]	(2–3)

The INI is expressed by the median value, with the range in parentheses. Sample size for each site of infection is shown in Table1.
[a] Forestomach.
[b] Main stomach.
[c] Pyloric stomach.
[d] Liver, pancreas and hepatopancreatic duct.
[e] Intestine (sections 1–19).
[f] Intestine (section 20).
[g] Organ not available.

Table 6
Reports of *Phyllobothrium delphini* and *Monorygma grimaldii* in cetaceans and pinnipeds worldwide

Host family	Host species[b]		
	Not sufficiently analysed	Accidental	Common
Cetacea			
Mysticeti[a]			
Balaenidae	Euj	Eua, Eug, Bm	–
Eschrichtidae	–	Esr	–
Balaenopteridae	Bpbn, Bpe	Bpa, Bpm, Bpp, Mn	Bpbr
Neobalaenidae	Cm	–	–
Odontoceti[a]			
Physeteridae	–	–	Pm
Kogiidae	–	–	Kb, Ks
Ziphiidae	All except three species	–	Zc, Mee, Mem
Monodontidae	Mom	Dl	–
Delphinidae	Ce, Det, Lrau, Lrcr, Lip, Ob, Pee, Psc, Sop, Sot, Seb, Stf, Ta	Cc, Lral, Slf, Soc, Tt (inshore population)	Dec, Ded, Fa, Glma, Glme, Gg, Ldh, Lrac, Lrol, Lros, Chv, Chc, Lib, Oo, Sta, Stcl, Stco, Stl, Tt (offshore population)
Phocoenidae	Phd, Phsi	Np, Pdd (inshore population), Php, Phsp	Pdd (offshore population)
Pontoporidae	–	Pb	–
Carnivora			
Pinnipedia[a]			
Otaridae	Atw, Ap	All except three species	Otb, Aa, Atr,
Odobenidae	–	Odr	–
Phocidae	Omr, Ps, Mm	All except one species	Ml

[a] References for Mysticeti: Skrjabin, 1970, 1972; Rice, 1977; Raga, 1994; Uchida et al., 1998. References for Odontoceti: Delyamure, 1955; Williams, 1968; Dailey, 1971; Skrjabin, 1972; Machida, 1974; Testa and Dailey, 1977; Brownell, 1989; Mead, 1989; Van Waerebeek et al., 1990, 1993; Alfaro, J., Van Waerebeek, K., Van Bressem, M., Reyes, J., 1994. Parásitos de *Delphinus capensis* en el Pacífico suroriental. In: Ximenez, A., Simoes-Lopes, P.C., (Eds.), Anais da 6ª reuniao de trabalho de especialistas em mamíferos aquáticos da América do Sul. Book of Abstracts. Universidade Federal de Santa Catarina, Centro de Ciencias Biológicas, Departamento de Biología, p. 80; Aznar et al., 1994; Best and Abernethy, 1994; Raga, 1994; Slooten and Dawson, 1994; Corcuera et al., 1995; Measures et al., 1995; Santos et al., 1996; McAlpine et al., 1997; Rogan et al., 1997; Abollo et al., 1998; Gibson et al., 1998; Mignucci-Giannoni et al., 1998; Perrin, 1998; Kuramochi et al., 2000; Parsons and Jefferson, 2000; Parsons et al., 2001; Perrin, 2001; Walker, 2001; Jefferson and Curry, 2003; Siquier and Le Bas, 2003; Colom-Llavina, M.M., 2005. Metazoan parasites of marine mammals from the Caribbean and the western coast of North America. Unpublished Master Thesis, Department of Marine Sciences, University of Puerto Rico, Mayagüez, Puerto Rico; Melo et al., 2006; Berón-Vera (personal communication for *Cephalorhynchus commersonii*). References for Pinnipedia: Dailey, 1975; George-Nascimento and Carvajal, 1981; Lauckner, 1985; Soares, M.L.R., 1986. Ocorrencia de *Phyllobothrium delphini* (Bosc, 1802) Gervais, 1882 (Phyllobothriidae: Cestoda) em lobos marino *Arctocephalus australis* Zimmerman, 1783 e *Arctocephalus tropicalis* Gray, 1872 (Pinnipedia: Otariidae) no litoral norte do Río Grande do Sul, Brasil. In: Anais da 2ª reuniao de trabalho de especialistas em mamíferos aquáticos da América do Sul. Book of Abstracts. Fundaçao Brasileira para a Conservaçao da Natureza, Brasil, p. 21; Bester, 1989; Raga, 1992.

[b] Abbreviations for host species: Aa, *Arctocephalus australis*; Ap, *Arctocephalus philippii*; Atr, *Arctocephalus tropicalis*; Atw, *Arctocephalus townsendi*; Bm, *Balaena mysticetus*; Bpa, *Balaenoptera acutorostrata*; Bpbn, *Balaenoptera bonaerensis*; Bpbr, *Balaenoptera borealis*; Bpe, *Balaenoptera edeni*; Bpm, *Balaenoptera musculus*; Bpp, *Balaenoptera physalus*; Cc, *Cephalorhynchus commersonii*; Ce, *Cephalorhynchus eutropia*; Chc, *Cephalorhynchus hectori*; Chv, *Cephalorhynchus heavisidii*; Cm, *Caperea marginata*; Dec, *Delphinus capensis*; Ded, *Delphinus delphis*; Det, *Delphinus tropicalis*; Dl, *Delphinapterus leucas*; Esr, *Eschrichtius robustus*; Eua, *Eubalaena australis*; Eug, *Eubalaena glacialis*; Euj, *Eubalaena japonica*; Fa, *Feresa attenuata*; Gg, *Grampus griseus*; Glma, *Globicephala macrorhynchus*; Glme, *Globicephala melas*; Kb, *Kogia breviceps*; Ks, *Kogia simus*; Ldh, *Lagenodelphis hosei*; Lib, *Lissodelphis borealis*; Lip, *Lissodelphis peronii*; Lrac, *Lagenorhynchus acutus*; Lral, *Lagenorhynchus albirostris*; Lrau, *Lagenorhynchus australis*; Lrcr, *Lagenorhynchus cruciger*; Lrol, *Lagenorhynchus obliquidens*; Lros, *Lagenorhynchus obscurus*; Mee, *Mesoplodon europaeus*; Mem, *Mesoplodon mirus*; Ml, *Mirounga leonina*; Mm, *Monachus monachus*; Mn, *Megaptera novaeangliae*; Mom, *Monodon monoceros*; Np, *Neophocaena phocaenoides*; Ob, *Orcaella brevirostris*; Odr, *Odobenus rosmarus*; Omr, *Ommatophoca rossi*; Oo, *Orcinus orca*; Otb, *Otaria byronia*; Pb, *Pontoporia blainvillei*; Pdd, *Phocoenoides dalli*; Pee, *Peponocephala electra*; Phd, *Phocoena dioptrica*; Php, *Phocoena phocoena*; Phsi, *Phocoena sinus*; Phsp, *Phocoena spinipinnis*; Pm, *Physeter macrocephalus*; Ps, *Phoca sibirica*; Psc, *Pseudorca crassidens*; Seb, *Steno bredanensiss*; Slf, *Sotalia fluviatilis*; Soc, *Sousa chinensis*; Sop, *Sousa plumbea*; Sot, *Sousa teuszii*; Sta, *Stenella attenuata*; Stcl, *Stenella clymene*; Stco, *Stenella coeruleoalba*; Stf, *Stenella frontalis*; Stl, *Stenella longirostris*; Ta, *Tursiops aduncus*; Tt, *Tursiops truncatus*; Zc, *Ziphius cavirostris*.

4. Discussion

The molecular analysis shows four unique genetic signatures that correspond with the four morphological types of tetraphyllidean larvae thus far described from cetaceans. Within each type, signatures were identical regardless of host species or site of infection. Several authors have reported different morphotypes of *P. delphini* in the same locality and have suggested that they represent different species (see Testa and Dailey, 1977; Dailey, 1985). Although we also found substantial morphological variability in specimens of *P. delphini* from the same locality (Agustí et al., 2005b), the present study confirms previous suggestions that this most likely represents intraspecific variability (see Siquier and Le Bas, 2003; Agustí et al., 2005b).

The partial phylogenetic tree of the Tetraphyllidea (Fig. 1) indicates that the four larval types occurring in cetaceans are closely related. Contrary to what had been hypothesized previously (Skrjabin, 1972; Agustí et al., 2005a), molecular analysis indicates that SP and LP represent different taxonomic entities, and that LP is not the larval stage of either *P. delphini* or *M. grimaldii*. However, the analysis cannot rule out that SP might be the plerocercoid stage of *M. grimaldii*, albeit it may represent a distinct population from those larval forms provisionally identified as *M. grimaldii* herein. The previous stage of development of *P. delphini* remains unknown. To clarify this issue, it would be necessary to do a more exhaustive sampling of larvae, e.g., of SP from sites of infection not analysed in the present study (i.e., the pyloric stomach).

Considering the morphological variation present among these larval types (see also Agustí et al., 2005a,b), it is surprising how little variation is present in this gene region compared with other genera in the analysis (cf relative branch lengths in Fig. 1). However, with so few tetraphyllidean species characterized genetically, we are far from knowing whether it will be possible to apply a genetic 'ruler' with which to predict taxonomic boundaries within the group. Moreover, the Tetraphyllidea is a species-rich group containing many forms that parasitize large, pelagic sharks with life cycles comparable to that of *Clistobothrium* in makos. Nevertheless, divergences among the larvae are consistent with these forms being members of the genus, at least compared with other parasitic platyhelminths for which such data are available (Olson et al., 1999, 2002; Reyda and Olson, 2003; Tkach et al., 2003; Olson and Tkach, 2005; Marques, J., Santos, M.J., Gibson, D.I., Cabrali, H.N., Olson, P.D., unpublished data). As the prevailing concept of species in the parasitic flatworms relies primarily on the morphology of adult characteristics, it would have little utility to make nomenclatural assignments on the basis of DNA alone. Genetic characterization of the tetraphyllidea of pelagic sharks in the Mediterranean would very likely recover exact matches for at least some of the larvae parasitizing cetaceans and would allow for their proper taxonomic placement. In particular, molecular characterization of the other two described members of *Clistobothrium*, *C. tumidum* and *C. carcharodoni*, is critical as these species parasitize the great white shark, *Carcharodon carcharias* (Linton, 1922; Dailey and Vogelbein, 1990; Ruhnke, 1993), which is one of the main shark predators of marine mammals around the world (Cortés, 1999; Compagno, 2001).

Our study shows that each larval type occurs predictably in specific sites of infection within the host regardless of dolphin species. The only obvious difference among hosts is that the LP were found in the bile ducts of striped and Risso's dolphins but not in those of bottlenose dolphins. However, this difference could result from a bias related to the very small sample size of bottlenose dolphins examined.

The sites of infection of *P. delphini* and *M. grimaldii* described in this study, i.e., the s.c. blubber and the abdominal peritoneum and mesenteries, mainly of the anal-genital region, are the same as those reported previously in most species of cetaceans and pinnipeds (George-Nascimento and Carvajal, 1981; McColl and Obendorf, 1982; Bester, 1989; Norman, 1997; Gibson et al., 1998; Walker, 2001 and references therein). The question remains how these cystic larvae arrive at these sites. Skrjabin (1972) suggested that *P. delphini* and *M. grimaldii* would enter cetaceans as plerocercoids and would use the blood system, the lymphatic system, or both, to reach their final sites of infection where they transform into merocercoids. Our study shows that individual SPs concentrate in a specific area of the colorectal region where the mucosa and submucosa are rich in lymphoid nodules (Simpson and Gardner, 1972). Interestingly, this region of the intestine is directly connected with the peritoneum of the body cavity through a short mesentery harbouring mesocolic nodes (Cowan and Smith, 1999). This short mesentery would be the shortest way for SP from the colorectal mucosa to reach the sites of infection of *M. grimaldii* (peritoneum of the body cavity) through the lymphatic system. Significant concentrations of SP also occurred in the wall of the pyloric stomach, the submucosa of which contains well-developed lymphatic nodules (Simpson and Gardner, 1972). This site of infection could serve as an alternative site for the plerocercoid stage of *M. grimaldii* to enter the lymphatic system. The small number of SPs found in the lumen of other parts of the gut would be suggestive of larvae migrating throughout the gut.

Individual LPs tended to accumulate mainly within anal crypts in all dolphin species. The presence of very low numbers in the lumen of the stomach and intestine would suggest that larvae migrate passively throughout the gut. Individual LPs were also observed, in moderate amounts, as free larvae in the lumen of bile ducts of striped and Risso's dolphins. There are two alternative ways through which the larvae could reach this site of infection. Perhaps the individual LPs from anal crypts could enter the underlying blood vessels. If so, it would be possible that some larvae could arrive at the liver, migrating through the portal system. Alternatively, individual LPs could enter the hepatopancreatic duct from its distal opening, which occurs in the first intestinal section in the three cetacean species analysed. In any event, the reason why the same type of larvae tend to accumulate in these two disparate sites of infection is unclear (see the last section).

Although tetraphyllidean larvae appear to be ubiquitous in the marine realm, they show two broad patterns of infection in marine mammals. First, our results, and those by Agustí et al. (2005a), clearly suggest that tetraphyllideans usually infect marine mammals feeding on fish and/or cephalopods. This would explain why the occurrence in baleen whales feeding on krill is incidental (see also Skrjabin, 1972): larvae only appear when fish make up a substantial portion of whales' diet (Rice, 1977). Second,

tetraphyllidean larvae mostly occur in marine mammals inhabiting offshore waters. With a single exception, all records of SP and LP (Agustí et al., 2005a) are restricted to offshore marine mammals, and a similar tendency can be observed in *P. delphini* and *M. grimaldii*. The pattern is particularly well illustrated in the case of host species with populations inhabiting inshore and offshore waters. For instance, the infection levels of *P. delphini* have been used to distinguish inshore and offshore stocks of *T. truncatus*, as the inshore stocks show lower prevalence (Walker, 1981; Van Waerebeek et al., 1990; Hoelzel, 1998). Likewise, the prevalence of *P. delphini* seems to be significantly lower in coastal than oceanic populations of *Phocoenoides dalli* (Machida, 1974; Walker, 2001). The rarity of tetraphyllidean infections in fish-eating pinnipeds might therefore be related to the usual coastal foraging of these hosts, rather than to the inability of the larvae to survive in pinnipeds. For instance, sub-Antarctic fur seals, *A. tropicalis*, harbour frequent infections with *P. delphini* when these hosts forage in oceanic areas (Bester, 1989). In summary, the occurrence of tetraphyllidean larvae in marine mammals appears to be driven mainly by ecological factors, i.e., host habitat and diet.

Two lines of evidence have traditionally supported the hypothesis that marine mammals are intermediate hosts, and large predatory and/or scavenger pelagic sharks are the most probable definitive hosts, for *P. delphini* and *M. grimaldii* (Southwell and Walker, 1936; Johnston and Mawson, 1939; Dollfus, 1964; Testa and Dailey, 1977; Walker, 2001; Agustí et al., 2005b). First, *P. delphini* and *M. grimaldii* are morphologically specialised larvae that only appear in marine mammals; therefore, some ontogenetic change must be postulated to occur within these hosts. The peculiar morphological modification as a fluid-filled bladder is exceptional among the Tetraphyllidea (Chervy, 2002), and is thought to have some adaptive value in transmission (Norman, 1997). Second, marine mammals represent important trophic resources for large sharks (Long and Jones, 1996; Cortés, 1999; Heithaus, 2001). Moreover, sharks often consume specific parts of marine mammals (Martin et al., 2005), particularly the abdominal and caudal region (Long and Jones, 1996; Walker, 2001). Interestingly, these regions correspond to the sites of infection selected by both *P. delphini* and *M. grimaldii* in marine mammals, which could be viewed as a strategy to enhance transmission to their putative definitive hosts (Geraci and Aubin, 1987; Garippa et al., 1991; Walker, 2001). Results from this study strongly support this hypothesis. First, the molecular and site selection analyses suggest that *M. grimaldii* would enter dolphins as plerocercoids (SP) and would follow a predictable migratory pathway to reach the sites of infection where they would transform into the cystic (merocercoid) stage. Second, as indicated by molecular analysis, there was a close relationship, probably congeneric, between the larval types occurring in dolphins and a species of *Clistobothrium*. Species of *Clistobothrium* are restricted to large pelagic sharks of the family Lamnidae (Ruhnke, 1993). As noted above, the great white shark, *C. carcharias*, could be a definitive host for these larvae. In the Mediterranean Sea, large juveniles and adults of this shark feed mainly on odontocetes and large pelagic fish (Fergusson, 1996; Fergusson et al., 2000). This impression is reinforced by the observation that *P. delphini* and *M. grimaldii* are common among offshore marine mammals, thus suggesting that the life cycle is normally completed in this environment.

How could the relationship between these tetraphyllidean larvae and marine mammals have evolved? Phylogenetic studies suggest a great age (>100 million years) for the origin of association between tetraphyllideans and chondrichthyans (Hoberg et al., 1999). The ancestors of cetaceans and pinnipeds entered the sea much later, in the Eocene (55–34 million years) and Late Oligocene epochs (27–25 million years), respectively (Berta, 2002; Fordyce, 2002). These events altered the structure of trophic webs because marine mammals could act as both new top predators and potential prey. Within this scenario, many tetraphyllidean larvae would now end up in marine mammals which, in turn, might be consumed by some shark species. If the cost of survival in marine mammals was low and the shark predation rate was sufficiently high, a 'downward incorporation' (Parker et al., 2003a) of marine mammals in the established life cycle of tetraphyllideans could have been advantageous. First, marine mammals could accumulate far more larvae than fish or cephalopods because they are large, long-lived, endotherm predators exhibiting high consumption rates (Williams et al., 2001). Therefore, larvae could avoid the mortality associated with marine mammal predation and could increase the transmission rate to the definitive host (Parker et al., 2003a). In addition, a large accumulation of larvae might increase the probability of finding mates in the definitive host (Brown et al., 2001), promoting genotype mixing in the intermediate host (Rauch et al., 2005; note, however, that intraspecific competition in the definitive host could also increase). Finally, due to the large size of marine mammals, larvae could reach larger sizes and develop further without being lethal to the host (Poulin, 1998; Parker et al., 2003b). A major benefit of this strategy is that the larvae could reproduce immediately upon arrival in the definitive host. Interestingly, in the merocercoid stage of *P. delphini*, the scolex seems to be fully developed and some individuals have even begun proglottization (Agustí et al., 2005b).

The role of Mediterranean dolphins in the life cycle of the LP is more difficult to establish. Our results indicate that the LP is a close relative to species of *P. delphini* and *M. grimaldii* but would not transform into a merocercoid in dolphins. Since tetraphyllideans are usually found at the plerocercoid stage in fish, cephalopods and other marine invertebrates (Euzet, L., 1959. Recherches sur les cestodes tétraphyllides des sélaciens des côtes de France. Ph. D. Thesis, University of Montpellier; Threlfall, 1970; Wojciechowska, 1993; Caira and Reyda, 2005), it is possible

that individual LPs are simply plerocercoids accidentally acquired with prey. However, in dolphins these larvae were found to be highly prevalent and abundant, and underwent predictable migrations to specific sites of infection, namely, the anal crypts and bile ducts. This behaviour is not well understood and raises a number of questions, but would not be expected in larvae ingested accidentally. One might speculate that the LP has adapted to avoid the strong flow associated with digestion by selecting safe sites, particularly the anal crypts. This would somehow imply that dolphins might act at least as paratenic hosts for these parasites. The fact that LP clusters together with a *Clistobothrium* species and occurs only in offshore cetaceans (Agustí et al., 2005a) would support this contention.

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