Vol. 41: 37–42, 2000 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published May 25

# Plasma and bile antibodies of the teleost *Trematomus bernacchii* specific for the nematode *Pseudoterranova decipiens*

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ABSTRACT: We investigated the occurrence of antibodies against protein antigens of the nematode parasite *Pseudoterranova decipiens* in the plasma and bile of the Antarctic teleost *Trematomus bernacchii*. Three different *P. decipiens* protein solutions were prepared: excreted/secreted proteins from live larvae (ESP); surface-associated proteins obtained by mild extraction of larval bodies (SAP); and cuticular soluble proteins recovered by extraction in strong reducing conditions (CSP). Using different immunoassays, these 3 preparations were tested for their ability to bind fish antibody. As determined by ELISA, the specific antibody binding activity was higher in SAP than in CSP. As determined by dot-blot immunoassay, the specific antigen binding activity versus SAP was higher in bile than in plasma antibodies. A different number of antigenic components of SAP and ESP were identified by immunoblotting performed with plasma or bile antibodies. These results led to the conclusion that *T. bernacchii* parasitism by nematodes involves plasma and bile anti-parasite antibodies. Furthermore bile antibodies were found to be more reactive and more heterogeneous than plasma.

KEY WORDS: Fish antibody · Nematode antigen · *Pseudoterranova decipiens* · *Trematomus bernacchii* · Anti-parasite antibody · Antarctic fish · Bile antibody

## INTRODUCTION

Protein A-sepharose affinity chromatography has been used to purify Antarctic fish immunoglobulins (Igs), and the relative molecular mass of both heavy and light chains of serum Igs was found to differ among the teleosts *Trematomus bernacchii*, *T. hansoni*, *T. newnesi* and *Chionodraco hamatus*, species living in the Antarctic Ocean at the constant temperature of −1.9°C (Scapigliati et al. 1997). In an earlier study, using a combination of thiophilic adsorption chromatography on T-gel and size-exclusion fast-performance liquid chromatography, we purified Ig from the plasma of *T. bernacchii*, *Notothenia coriiceps*, *Chaenocephalus aceratus*, *C. hamatus*, from eggs of *C. hamatus*, from bile of *T. bernacchii* and from cutaneous mucus of *Gobionotothen gibberifrons* (Coscia & Oreste 1997, Coscia & Oreste 1998a).

Igs in cutaneous mucus and bile are documented in many fish species. Cutaneous mucus Ig has been studied in more detail than bile Ig. The latter appears to exist in a non-covalently linked dimeric form $(H_2L_2)_2$ (Lobb & Clem 1981a) and to be due to local synthesis rather than active transport of plasma Ig (Lobb & Clem 1981b).

Helminth parasites are frequently found in fish. The most abundant in the Antarctic Ocean are nematode species of the Anisakid genera *Contracaecum* (Klöser & Plötz 1992) and *Pseudoterranova* (Palm et al. 1994). The genus *Pseudoterranova* consists of 3 species: *P. kogiae*, *P. ceticola* and *P. decipiens*. The life cycle of the latter—the only cosmopolitan species—has been described by McClelland (1990): the third larval stage occurs in benthic fish and the main sites of infestation are the liver, mesenteries and stomach walls. *P. decipiens* was first found in *Trematomus bernacchii* by Johnston in 1938 (Johnston 1938). Other fish species infested by *P. decipiens* are *Notothenia coriiceps*, *Champsocephalus*

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*gunnari*, *Chionodraco kathleenae*, *Notothenia gibberifrons*, *Pseudochaenichthys georgianus* and *Notothenia neglecta* living in the Antarctic Ocean. In the North Atlantic Ocean also commercially important species, including Atlantic cod *Gadus morhua*, are infested by *P. decipiens*. Based on the results of multilocus electrophoresis, *P. decipiens* larvae recovered from North Atlantic fish have been separated into 3 sibling, morphologically indistinguishable species, A, B and C (Paggi et al. 1991).

In an attempt to understand the molecular mechanisms of the host/parasite relationship, Politz & Philipp (1992) conducted a biochemical study of the structure of the nematode cuticle. They defined 3 categories of protein components. (1) Surface-associated proteins and glycoproteins, characterised by their ability to be solubilised from the cuticle by mild extraction procedures without reducing agents. They are expressed as the parasite colonises a new host environment, and can elicit host immune response. (2) Collagens, hydrolysed by collagenase, solubilised only with the aid of reducing agents. (3) The so-called cuticlins, cross-linked by non-reducible bonds involving tyrosine residues (Cox 1992). The extracellular globin from *Pseudoterranova decipiens* has been cloned (Dixon et al. 1991) and is thought to be antigenic in grey seals. *In vivo*, antigen shedding in nematodes could be a parasite defence mechanism against antibody-mediated immune effector processes (Apfel & Meyer 1990).

We previously reported antibodies specific for *Contracaecum osculatum* proteins in *Chionodraco hamatus* plasma, and identified the major antigen as a protein component of the body extract with a relative molecular mass of 91 kDa (Coscia & Oreste 1998b). In this study we have examined the parasitism of *Trematomus bernacchii* by *Pseudoterranova decipiens* using 3 approaches: (1) looking for *T. bernacchii* antibodies specific for different *P. decipiens* proteins (excreted/secreted proteins [ESP], surface-associated proteins [SAP], and cuticle soluble proteins [CSP]); (2) comparing the antigen-binding activity of plasma and bile antibodies; and (3) identifying the most relevant antigens.

*Trematomus bernacchii* belongs to the perciform suborder Notothenioidei, the most relevant component of the teleosts of the Antarctic Ocean; it represents 34.7% of the species, and includes 6 families and 120 species (Gon & Heestra 1990). *T. bernacchii* is the most abundant fish near the Antarctic Italian Station at Terra Nova Bay, confined to waters less than 400 m deep preferring seaweed-covered moraines. Morphological, biochemical and phylogenical data are available for this species (Ritchie et al. 1996).

## MATERIALS AND METHODS

**Biological samples.** Plasma samples were collected from 12 *Trematomus bernacchii* specimens caught in the Ross Sea, at 74° 42' S and 164° 07' E, near the Italian Station at Terra Nova Bay. The plasma samples, collected by caudal venipuncture using a heparinised syringe, were centrifuged at 2000 × *g* for 10 min, pooled and stored at −20°C until used; all the specimens were found parasitised by nematodes. To obtain a control serum pool, plasma was collected from non-parasitised fish of the Mediterranean species *Boops salpa*, caught in the Bay of Naples. The gall bladders of 5/12 *T. bernacchii* specimens were stripped of the adhering mesenteries; the free-flowing bile was recovered by puncturing the gall bladder in the terminal site. The bile was centrifuged at 2000 × *g* for 10 min and the pooled supernatants were stored at −20°C until used. A control bile pool was obtained in the same fashion from 2 specimens of *Dicentrarchus labrax* caught in the Bay of Naples.

Nematode larvae were collected from the liver of *Trematomus bernacchii* specimens; their mean length was 4.1 cm; most larvae were white; a few were blood-red coloured. They were morphologically identified as third larval stage of *Pseudoterranova decipiens*.

**Ig purification.** The Ig fraction was purified from the plasma or the bile by a combination of thiophilic adsorption chromatography and size-exclusion fast-performance liquid chromatography as previously reported (Coscia & Oreste 1998b). The final purity was confirmed by SDS-PAGE run under reducing conditions, which revealed only the heavy and light chain band. One milliliter of plasma and bile yielded 19.70 and 3.47 mg Ig respectively.

**Protein content determination.** The Bradford method (BioRad Laboratories, Richmond, CA) was used to determine protein content, and bovine γ globulin as standard.

**SDS-PAGE.** A total of 150 µg of nematode preparation was boiled for 10 min in the sample solution (10% β-mercaptoethanol, 6% SDS, 0.001% bromophenol 20% glycerol) and subjected to electrophoresis in a 10% polyacrylamide slab gel using 0.025 M Tris, 0.2 M glycine, pH 8.3 containing 0.1% SDS, as running buffer. Bands were revealed by Coomassie brilliant blue R250 staining. The relative molecular mass of individual components was determined with protein markers: hen egg transferrin (78 kDa), bovine serum albumin (BSA, 66 kDa), hen egg albumin (42 kDa), bovine erythrocyte carbonic anhydrase (30 kDa) and equine cytochrome C (12 kDa).

**Rabbit anti-*Trematomus bernacchii* Ig heavy chain antibody.** Two rabbit anti-*Trematomus bernacchii* Ig heavy chain (IgH) antisera (RbC and RbD) were raised

to purify IgG antibody specific for the antigen-antibody binding tests, ELISA, dot-blot immunoassay and immunoblotting. IgH was recovered by electroelution of the 75 kDa band at 60 mA for 5 h from 12% polyacrylamide slab gel, run under reducing conditions of purified *T. bernacchii* Ig, and dialysis against phosphate buffer solution (PBS) pH 7.2. Each specimen was injected intradermally with 100 µg of antigen in 400 µl of PBS emulsified with 500 µl of complete Freund's adjuvant. Then 5 biweekly intramuscularly injections were given, with the same amount of antigen, but emulsified with incomplete Freund's adjuvant. Seven days after the last injection, the specimens were killed; the blood, collected by auricular venipuncture, was centrifuged at 2000 × *g* for 10 min and the supernatant was recovered. RbC antiserum showed the highest IgG-*Tb*IgH titre in dot-blot immunoassay and was used to purify IgG (RbC IgG) by MabTrap™ G II affinity chromatography following the manufacturer's instructions. Purified rabbit IgG was labelled using the chloramine-T procedure. $^{125}$I-RbC antibodies were reactive with separated Ig heavy chains either from *T. bernacchii* or *Dicentrarchus labrax* or *Boops salpa*.

**ELISA.** For ELISA, 50 µl of SAP or CSP (0.7 mg ml$^{-1}$ in carbonate buffer 0.1 M, pH 9.6) were incubated for 16 h at 4°C in polystyrene microwell plates. Wells were washed 3 times with PBS, pH 7.2 containing 0.05% Tween 20. After blocking remaining sites with 75 µl well$^{-1}$ PBS, pH 7.2, containing 0.05% Tween 20, 5% BSA, for 1 h at room temperature, 50 µl of purified plasma Ig (6 mg ml$^{-1}$) undiluted or 1/3, 1/9, 1/27 and 1/81 diluted in PBS, pH 7.2, containing 0.25% BSA, were incubated for 16 h at 4°C. Wells were then washed 3 times with PBS, pH 7.2 containing 0.05% Tween 20, 1.5 mM $MgCl_2$, 2 mM β-mercaptoethanol and incubated for 1 h at 4°C with 50 µl well$^{-1}$ of IgG-*Tb*IgH (10 µg ml$^{-1}$ in PBS, pH 7.2 containing 0.25% BSA). Lastly, each well was washed, and 50 µl of alkaline phosphatase conjugate mouse IgG (anti-rabbit IgG) (BioRad) were added at a 1:3000 dilution, to each well. After 2 h, wells were washed and the enzymatic reaction was visualised with 50 µl well$^{-1}$ paranitrophenylphosphate diluted according to the manufacturer's instructions. The reaction was left to proceed for 35 min, and was stopped with 1 M NaOH; the absorbance was determined at 405 nm with an automatic plate reader.

**Dot-blot immunoassay.** Three µl of SAP or CSP (1.0 mg ml$^{-1}$) were spotted on nitrocellulose sheet (Schleicher & Schuell, BA 83, 0.2 mm); the same amount of BSA was spotted as control of unspecific binding. After 16 h the remaining reactive sites were blocked for 1 h at 37°C with 50 mM Tris-HCl pH 8.0, containing 0.9% NaCl, 3% BSA. The filter was then incubated for 16 h at 4°C with plasma or bile purified Ig (3 mg ml$^{-1}$). After several washings the filter was incubated for 16 h at room temperature with $^{125}$I-RbC IgG (200 000 cpm ml$^{-1}$). Autoradiography was performed by exposing the sheet to Fuji HR-E 30 X-ray film, with an intensifying screen, for 48 h at −80°C.

**Immunoblotting.** The nematode protein preparations were first reduced and separated by SDS-PAGE as described above, then electroblotted onto a nitrocellulose sheet (Schleicher & Schuell, BA 83, 0.2 mm), according to the method of Towbin (Towbin et al. 1980), at 20 mA for 17 h. The blocking, washings, incubations and radiodetection were as described above for dot-blot immunoassay.

## RESULTS

### Recovering nematode proteins

Twenty-nine live nematode larvae were maintained in individual vessels with 2 ml of PBS, pH 7.2, at 18°C. Twice a week the medium was changed and the larvae were kept at 37°C for 10 min. The temperature change caused an increased larvae motility. After 18 d most larvae had lost their motility; they had released similar amounts of protein in the medium, determined as absorbance at 280 nm, with values decreasing in time from 0.3 to 0.05 optical density (OD) ml$^{-1}$. Subsequently, all the recovered samples were pooled, concentrated by ultrafiltration and dialysed using a membrane with a cut off of 10 kDa. The total amount of ESP was 0.21 mg.

To obtain SAP, the dead larvae were incubated for 16 h at 37°C in PBS, pH 7.2 containing, as protease inhibitors, 0.1 mM benzidamine, 0.3 mM PMSF, 1% sodium deoxycolate, and 1 mM EDTA. After centrifugation at 15 000 × *g* for 10 min, the supernatant was dialysed at 4°C for 48 h. At this step 4.0 mg of SAP was recovered.

To collect the soluble fraction of the cuticle, the precipitate of the previous centrifugation was boiled for 10 min with 3 ml of PBS, pH 7.2 containing 2% SDS and 10% β-mercaptoethanol; the mixture was then incubated for 16 h at 37°C and centrifuged at 15 000 × *g* for 10 min; the dialysed supernatant (CSP) contained 16.0 mg of protein. The precipitate, presumably containing the cuticlin fraction, was discarded.

### Analysis of the recovered proteins

To characterise the protein components of the 3 preparations (ESP, SAP and CSP) SDS-PAGE, run under reducing conditions, was used. The number of protein components differed among the 3 preparations (Fig. 1): 26 in CSP ranging between 200 and 21 kDa; 12 in SAP; and only 6 in ESP. Four bands of CSP (58,

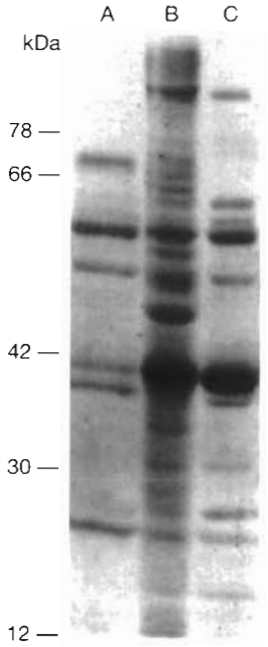


Fig. 1. Analysis of nematode protein preparations. Coomassie staining of 10% polyacrylamide SDS-PAGE of reduced ESP (lane A), CSP (lane B) and SAP (lane C); on the left: the migration of molecular weight markers

54, 40 and 25 kDa) were found also in ESP and SAP. The 40 kDa band corresponded to the main component of CSP and SAP. To better characterise this molecule, the band was blotted onto an Immobilon sheet and its N-terminal amino acid sequence was determined and found very similar to the oxygen-transporter molecule of *Pseudoterranova decipiens*, extracellular globin (Dixon et al. 1991).

### Antibody-binding activity of the protein preparations

The specific antibody-binding activity of CSP and SAP was tested by ELISA using the same protein amount of antigen coated (0.7 mg $ml^{-1}$) in the wells and different dilutions (undiluted, 1/3, 1/9, 1/27 and 1/81) of purified *Trematomus bernacchii* plasma Igs (6 mg $ml^{-1}$). Purified Ig was used instead of unfractionated plasma to avoid non Ig-mediated binding of different plasma fractions. ESP was not considered in this experiment because of the scarce amount. Igs purified from plasma of a specimen of *Boops salpa* were used as control of unspecific binding at the same dilutions (the difficulty of finding unequivocally non-parasitised Antarctic fish compelled us to use a non-parasitised Mediterranean species). As shown in Fig. 2, SAP had a specific antibody binding 1.3 times higher than CSP. The unspecific binding of control Igs was very low (about 7.7 times lower than that of *T. bernacchii* Igs).

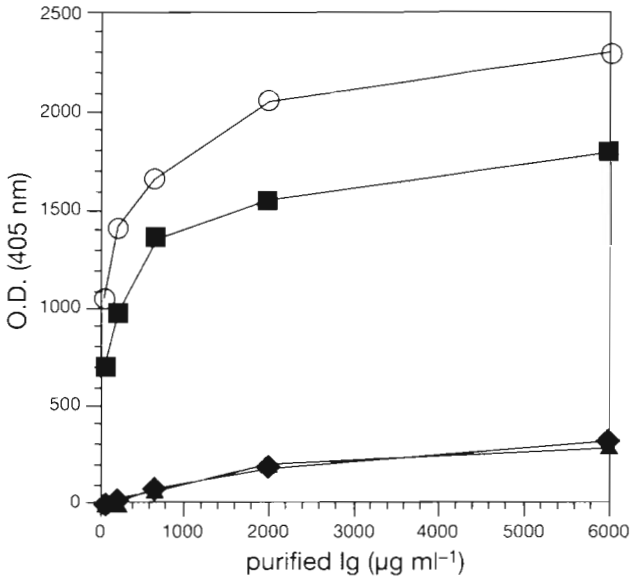


Fig. 2. ELISA of antibody binding of SAP and CSP. In each well, 35 µg of SAP was incubated with *Trematomus bernacchii* (—○—) or *Boops salpa* (—◆—) Igs at serial dilutions; equal amounts of CSP were incubated with *T. bernacchii* (—■—) or *B. salpa* (—▲—) Igs at serial dilutions

### Comparison of reactivity of plasma and bile antibodies toward SAP

We examined nematode specific antibodies in the bile and compared their ability to recognise SAP to that of plasma Igs. The same dilution of *Trematomus bernacchii* plasma or bile Igs was tested by dot-blot immunoassay against SAP, which was demonstrated, by ELISA, to be highly reactive; the unspecific binding was tested against BSA. The bile Ig-generated signal was clearly more pronounced than the plasma signal (Fig. 3). These results indicate the presence of an immune response against parasite at both systemic and secretory level.

### Specific antibody reactivity toward SAP and CSP components

We used immunoblotting to analyse the fish plasma and bile antibody reactivity toward the nematode preparations. The results obtained with purified plasma Igs are shown in Fig. 4A. Seven SAP and only 12/26 CSP protein components were identified as antigens. The greatest plasma antibody reactivity was detected toward a CSP component of a relative molecular mass of 90 kDa; in SAP preparation the corresponding reactivity was detected toward a slightly slower band. These components were difficult to detect in SDS-PAGE, presumably being in very low amounts. Plasma

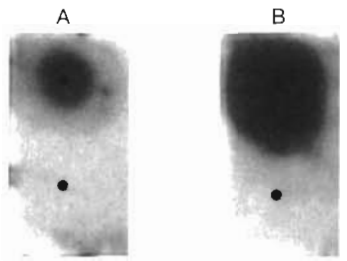


Fig. 3. Autoradiography of dot-blot immunoassay. Top: 3 µg of SAP; bottom: 3 mg of bovin serum albumin are spotted. Sheet A was incubated with 3 mg $ml^{-1}$ plasma Igs; sheet B with the same concentration of bile Igs

antibodies recognised also the 65, 58, 54, and 49 kDa SAP bands as well as that of the major protein component 40 kDa. However, the reactivity toward the latter was weaker albeit the higher amount detected by Coomassie staining.

The immunoblotting analysis was repeated for SAP using *Trematomus bernacchii* bile Igs and nematode proteins at the same concentrations as those used in immunoblotting performed with plasma Ig (Fig. 4B): the spectrum of antibody reactivity was wider. The relative intensity of autoradiographical signals was different when using bile or plasma anitibodies: bile antibodies preferentially reacted with the 49 kDa component. Overall, this implies a larger spectrum of specificities and that the specific antibody/total Ig ratio is higher in the bile than in plasma, confirming the dot-blot results.

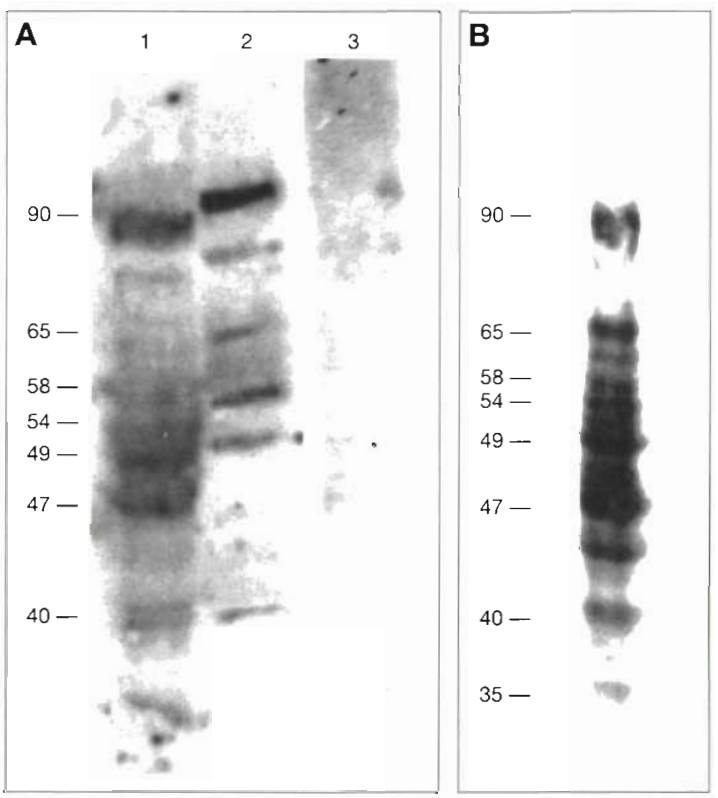


Fig. 4. Autoradiography of immunoblotting analysis. (A) Lane 1, CSP (150 µg); lane 2, SAP (150 µg); both incubated with *Trematomus bernacchii* plasma Igs (3 mg $ml^{-1}$); lane 3, SAP (150 µg), incubated with *Boops salpa* Igs at the same concentration. (B) SAP (150 µg), incubated with *T. bernacchii* bile Igs (3 mg $ml^{-1}$)

## DISCUSSION

We present evidence of occurrence of anti-parasite antibodies in parasitised Antarctic fish. The amount of Ig recovered was higher than that generally detected in fish plasma (Klesius 1990) and, probably, anti-nematode specific antibodies are predominant. In contrast to data reported so far on other fish Ig (Magnadòttir et al. 1999), *Trematomus bernacchii* Ig has been detected at higher concentrations (19.70 mg $ml^{-1}$ and 3.47 mg $ml^{-1}$ for plasma and bile respectively). This finding is relevant because *T. bernacchii* lives at a temperature of −1.9°C and the immune response induced by the helminth parasite in non-polar fish is absent at low temperature (5.5°C) (Andersen & Buchmann 1998).

Very few nematode antigens have been described so far in fish; a case in point is the antigens of the nematode *Anguillicola crassa* identified by *Anguilla anguilla* antibodies, some of which are secretory/excretory antigens (Buchmann et al. 1991, Nielsen & Buchmann 1997). In addition, to our knowledge, ours is the first report of specific antibodies in the bile of parasitised fish. High titres of bile antibodies were detected, and they recognised an antigenic repertoire larger than that recognised by plasma antibodies. This could be attributed to the greater local topic immune response at intestinal level. The high titre of secretory antibodies is confirmed by our finding of anti-SAP antibodies in the cutaneous mucus of another parasitised species, *Gobionotothen gibberifrons* (authors' unpubl. data).

We defined 3 nematode protein preparations: (1) ESP, which occurred in very small amounts and consisted of a few protein components, easily to purify by HPLC (data not shown); (2) SAP, which was highly immunogenic, and which is in line with antigen shedding as a parasite immune escape mechanism; and (3) CSP, which was a very complex mixture as observed in 2-dimensional electrophoresis (data not shown), less antigenic perhaps because of the strong conditions used, and cer-

tainly including proteins not exposed on the body surface.

Bile and plasma antibodies showed a different reactivity toward SAP components. Plasma antibodies were found to be more reactive with the 90 kDa component. Conversely, bile antibodies reacted with a larger spectrum of components and preferentially recognised the 49 kDa antigen. Thus, it cannot be stated unequivocally what the major antigen is. A further characterisation of the nematode molecules involved in the antigen-antibody reaction may clarify the point.

The *Pseudoterranova decipiens* 90 kDa antigen shows the same relative molecular mass as the antigen recognised by *Chionodraco hamatus* antibodies in *Contracaecum osculatum* protein preparations (Coscia & Oreste 1998b); also, the 47 kDa antigen could be similar between the 2 anisakid nematodes.

The role of these parasite antigens is obscure. We reported data on antigen recognition by antibodies purified from a pool of samples from different *Trematomus bernacchii* specimens. An analysis of antibody titres from individual fish of a documented level of parasitism might indicate that the host's immune response to parasite nematode exerts a protective effect.

*Acknowledgements.* We thank Drs G. di Prisco and L. Camardella, Institute of Protein Biochemistry and Enzymology, CNR, Naples, for providing fish plasma, secretions and parasites; we also thank Dr S. Mattiucci, Institute of Parasitology, University of Rome, for identification of the nematode larvae. This research was supported in part by a grant from the Italian National Program for Antarctic Research.

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*Editorial responsibility: Donald Evans, Athens, Georgia, USA*

*Submitted: August 23, 1999; Accepted: February 1, 2000*
*Proofs received from author(s): May 10, 2000*