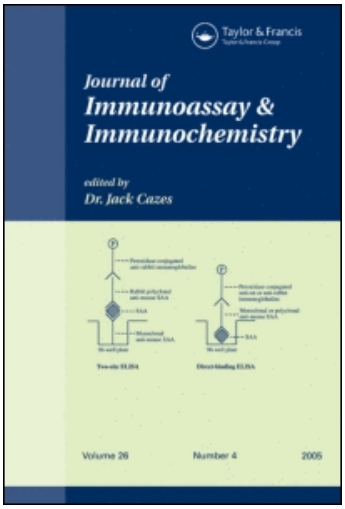

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DETECTION OF ZOOPLANKTON PREY IN SQUID PARALARVAE WITH IMMUNOASSAY

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ABSTRACT

Sustainable management of economically important squid requires monitoring of changes in their abundance, which are related *inter alia,* to their success in the food chain. The highest mortality is expected in the paralarval stages, which are prone to starvation. Causes of starvation may be linked to the lack of suitable prey. A multiple detection system was developed for the simultaneous identification of five putative zooplankton prey in the guts of paralarval Chokka squid, *Loligo vulgaris reynaudii,* by employing polyclonal rabbit antisera in conjunction with solid phase immunoassays. Specificities of antisera were validated by ELISA screening against different zooplankton taxa. Cross-reactions observed with ELISA were minimized through manipulation of antibody and antigen concentrations resulting in more specific detection of target prey antigens when used in an immunodot assay. Application of this optimised immunoassay detected multiple predation in paralarval squid samples collected from diverse areas in the Agulhas Bank ecosystem on the south coast of South Africa.

(KEY WORDS: Immunoassays, polyclonal antibodies, predation, squid paralarvae).

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INTRODUCTION

Starvation is suspected to be a major cause of mortality during the planktonic paralarval stage of Chokka squid, *Loligo vulgaris reynaudii,* a key component in the trophic structure of the Agulhas Bank ecosystem (1) and impacts on an important commodity of the South African jigging fishery (2, 3). After hatching, the paralarvae can survive on yolk for 4 days after which they have to find food (2). Vecchione (4) has proposed that the cause of starvation in loliginids during this stage may be due to their underdeveloped tentacles which impede prey capture and that it therefore requires high prey concentrations. Identification of the preferred prey during the paralarval stage may help to ascertain the relationship between prey abundance/availability and paralarval survival.

Augustyn *et al.* (2) quoted evidence that paralarvae may capture *Artemia* and *Calanus* species in the laboratory. Predation on copepods, palaemonid shrimps, mysid shrimps and fish larvae by *Loligo* paralarvae in the laboratory was described by Hanlon (5). Using microscopy, Vecchione (6) identified remnants of copepods, hyperiid amphipods and decapod zoea in paralarvae collected from the sea. Prey in an advanced stage of digestion could not be accurately identified by this method which was also regarded to be labour-intensive and time-consuming (6).

Problems encountered with microscope analysis were successfully surmounted using a diverse spectrum of immunological methods in studies of several other non-related ecological predator-prey relationships. Pickavance (7) used the Ouchterlony immunodiffusion technique for the detection of prey in the guts of

planarians. Greenstone (8) demonstrated the use of passive haemagglutination for prey identification. Boyle *et al.* (9) used Rocket Immunoelectrophoresis for semi-quantitative detection of five prey taxa in adult squid. The same method was applied by Kear (10) for immunodetection of various euphausiid species in adult squid.

Although prey remnants in the gut could be determined by means of polyclonal antisera with no or little cross-reactivity among closely related species by using any of the above mentioned techniques, large amounts of antigen and/or antibody, specialized equipment in the case of immunoelectrophoresis and a critical antigen/antibody ratio for immunoprecipitation are required (11). Due to these disadvantages, ecologists have harnessed solid phase immunoassays such as ELISA in predation studies. McIver and Tempelis (12) were able to distinguish between predation on two different plant bugs in spiders by using ELISA and polyclonal antibodies. Theilacker *et al.* (13) used the same method in their efforts to determine the effect of euphausiid predation on fish larval survival. Cross-reactivity of antibody probes can be reduced, or even eliminated, by preparation of monospecific, polyclonal antiserum as was demonstrated by Ragsdale (14) in a study where predation on a stink bug by various predators was determined using ELISA. Comparative sodium dodecyl sulphate (SDS) electrophoresis of proteins from closely related specimens can reveal unique bands that may be cut out and used as antigen to prepare monospecific antibodies for improved species-specific analysis of prey. Although such an approach may be rewarding, it demands large

amounts of antigen and often yields antibodies directed against the SDS denatured proteins, not necessarily recognising the native form (15, 16).

Greater specificity is obtained when using hybridoma technology (17) which entails the fusion of antibody-producing B-cells with their malignant counterparts or myeloma cells in order to produce monoclonal antibodies (mAbs), *i.e.* antibodies that are homogeneous in immunoglobulin subclass, specificity and affinity. Lenz and Greenstone (18) developed a monoclonal antibody against arylphorin, an abundant storage protein in the hemolymph of larval corn earworm and used this mAb to identify predators of the latter (19). Hagler *et al.* (20) produced a mAb against vitellin protein in the egg of the Lygus bug and used it in an ELISA to determine predators of the latter's eggs. Despite all its virtues, production of mAbs is labour intensive, expensive and time consuming, factors which constrained its use for multiple prey detection (21). Furthermore, the extreme specificity of a mAb may be the cause of low sensitivity if it is directed towards a denaturation vulnerable epitope where polyclonal antibodies may still be the better option (15). This is a crucial point to consider for detection of labile epitopes in the destructive environment of the digestive tract of predators. Monospecificity of a mAb is not guaranteed and cross-reactions with non-target antigens can also occur for reasons described by Lane and Koprowski (22) and also encountered by Demers *et al.* (23) when mAbs were used to identify early stages of scallops.

In view of the above considerations, polyclonal antisera were generated against putative prey of Chokka paralarvae and the sensitivity, specificity and potential of

the antibodies to detect their target antigen in the paralarvae were assessed with ELISA and immunodot assay.

METHODS

Samples

Zooplankton specimens used for antibody production or screening of antibodies were collected during three research cruises of the FRS *Africana* on the Agulhas Bank to the south of South Africa during June 1994, May 1995 and April 1996. Field collections were made at various stations between 33° and 35°S and between 22° and 27°E. The taxa and the level to which they were identified, are indicated in Table 1. Squid paralarvae were obtained at three stations between 34° and 37°S and between 21° and 25°E. Their sizes were not accurately recorded but were estimated to be between 3 and 4 mm.

Preparation of Antigen

The various zooplankton specimens used as antigen are shown in Table 1. Those used for antibody production are indicated by an asterisk. All these samples and the squid paralarvae were suspended in equal volumes of saline solution and homogenized in a Potter Elvejhem homogenizer for 1 minute at 4°C. Homogenates were subsequently centrifuged at 10000 g for 10 minutes after which the supernatants were distributed in aliquots and stored at -70°C. Protein concentrations were determined by measurement of absorbance at 205 nm of representative aliquot dilutions of each specimen. A 10 µg/ml solution of Bovine

TABLE 1

ZOOPLANKTON TAXA used for ANTIBODY PRODUCTION or SCREENING of ANTIBODIES.

Phylum	Subphylum	Class	Order	Species	Stage
Cnidaria		Hydrozoa	Siphonophora	Various spp.	
Ctenophora				*Pleurobrachia* sp	
Annelida		Polychaeta		Various spp.	Larvae and adults*
Arthropoda	Crustacea	Ostracoda		Various spp.	
		Copepoda		Various spp.	
				*C. agulhensis**	
		Malacostraca	Cumacea	Various spp.	
			Isopoda	Various spp.	
			Amphipoda	Various spp.	
			Euphausiacea	Various spp.	calyptopis, furcilia, juveniles, adults*
			Decapoda	Various spp.	
		Cladoceran		*Evadne* sp.*	
Chaetognatha					
Mollusca	Gastropoda				
	Cephalopoda		Teuthoidea	*Loligo vulgaris reynaudii*	Paralarvae
Chordata	Tunicata	Thaliacea	Salpida	Various spp.	
		Appendicularia		*Oikopleura* sp.	
	Pisces	Teleostomi		Various spp.	Egg
				Various spp.	Larva *

* Taxa towards which antibodies were produced.

Serum Albumin was used as a standard for measuring the various protein concentrations (24).

Antibody Production

Five female New Zealand White rabbits were immunized separately with antigen of *Calanus agulhensis,* euphausiid, cladoceran, polychaete and fish larvae. Rabbits were injected intramuscularly and subcutaneously with a total dosage of 100 µg protein of each target species in Freund's complete adjuvant (1:1). Three booster injections of 100 µg of protein in Freund's incomplete adjuvant (1:1) were administered at 3-week intervals. A week after the final booster injection, the rabbits were bled and the antisera stored at - 70°C.

ELISA

An indirect ELISA was used to determine immunoglobulin class, antibody titer and specificity for each antiserum, and to screen four squid paralarvae for predation on plankton.

Protein antigens of the five target plankton groups were diluted to 10 µg/ml in PBS (pH 7.4) and used to coat 96-well polystyrene microtiter plates (Sero-Wel, Sterilin, Sterilab) at 100 µl per well. The plates were incubated at 4°C overnight after which the solvent was flicked out and non-specific binding sites were blocked for 1 hour at room temperature with 0.5% Casein/PBS, pH 7.4 at 200 µl/well. Blocking, dilution and washing were done by using the same buffer. After blocking, the wells were filled with each antiserum dilution in quadruplicate at 50 µl per well and incubated at room temperature for 45 minutes. Plates were washed

and filled with monoclonal anti-rabbit IgG (γ-chain specific) peroxidase conjugate (Sigma-Aldrich) at a 1000 fold dilution for 30 minutes at room temperature. After final washing, plates were developed by adding 50 µl/well of the substrate solution consisting of 10 mg o-phenylenediamine and 8 mg urea-hydrogen peroxide in 10 ml citrate buffer (0.1 M, pH 4.5). Colour development was measured at 450 nm using a multi-channel spectrophotometer (SLT 340 ATC).

Specificity of each antiserum and screening of squid paralarvae for predation on the target taxa were also determined by an indirect ELISA. Antisera were diluted 1:100 with PBS and screened in quadruplicate against 1 µg of coated protein/well of each of the eighteen taxa (including squid paralarvae) listed in Table 1. Sera obtained from the rabbits prior to immunisation were used as negative controls. ELISA was performed as described above.

Immunodot Assay

Antigens showing cross-reactions with ELISA were subsequently examined with immunodot assay. The immunodot assay was conducted by means of a Bio-Dot microfiltration apparatus (Bio-Rad). A poly(vinylidenedifluoride) or PVDF membrane was cut to the size of the 96 well template of the Bio-dot apparatus, wetted with cold methanol, washed with CAPS, *i.e.* 3-(cyclohexylamino)propanesulfonic buffer (pH 9.6, 10 mM) and submerged in the latter for 15 minutes before it was inserted in the Bio-dot apparatus. CAPS was loaded at 100 µl per well and was removed by aspiration before loading with antigen. Antigens were diluted to a protein concentration of 25 µg/ml with PBS,

loaded at 100 µl per well and allowed to filtrate by gravitation for 30 minutes before the remaining fluid was suctioned through. The membrane was washed 3 times with CAPS before it was removed from the apparatus and allowed to air-dry. Blocking of non-specific protein binding sites was performed by incubation of the membrane in a solution of 1% non-fat milk powder in 0.05% Tween-20/TBS (Tris Buffered Saline, 0.02M, pH 7.4) for 20 minutes at room temperature. The membrane was cut into 3 strips for screening of each strip against a different antiserum. Each strip was placed in a different dish containing antiserum diluted 1:400 with blocking buffer and incubated overnight at 4°C with mild agitation on a shaker. Unbound antibodies were subsequently removed by washing 3 times for 5 minutes each time with 0.1% non-fat milk powder in TBS. Antibodies reacting with antigen were detected by incubating with monoclonal anti-Rabbit IgG (γ-chain specific) peroxidase conjugate (Sigma) at a 1000-fold dilution in incubation buffer for 2 hours at room temperature. After final washing, the strips were developed with substrate solution consisting of 0.05% 4-chloro-1-naphthol in cold methanol (17% v/v) and 0.015% hydrogen peroxide in TBS. The reaction was stopped by washing in deionized water.

The immunodot assay was subsequently used to screen six paralarvae. Five replica PVDF membranes were prepared for immunodot assay. Each membrane carried immobilized quadruplicate target antigens at 0.25 µg protein per position of the five different antisera as well as antigens at 5 µg, 2.5 µg and 1 µg protein per position of all six paralarvae. Extracts were screened against anti-euphausiid,

anti-*Calanus agulhensis*, anti-cladoceran and anti-fish sera 1:400 while anti-polychaete serum was 1:700.

RESULTS

Characterization of Polyclonal Antisera

Antibody titer and class of the various antisera were determined by an indirect ELISA. All the antisera screened positive for immunoglobulin G at different dilutions which is an indication of proper maturation of the antibody response. Signals became undetectable at 1600-fold dilutions of all the antisera.

Specificities of Polyclonal Antisera

As detection of minute amounts of prey antigen may be best achieved with lower antibody dilutions, a 100-fold antiserum dilution was selected in order to determine which of the available zooplankton specimens cross-reacted with antisera raised against the target antigens. Results are summarized in Table 2. The positive-negative threshold (25) for each antiserum was set at $\bar{n}$ +3 sd where $\bar{n}$ is the average of quadruplicate absorbance values of the naive serum (negative control) and sd is the standard deviation against the target antigen. Average ELISA signals of antisera equal to or below the threshold value were taken as negative and scored 0 in the table while average values above the cut off value were taken as positive. The relative signal strength of each antiserum for the various antigens, is depicted as the ratio of the average of quadruplicate ELISA signals of an antiserum obtained against an antigen to the average ELISA signal of

TABLE 2

SPECIFICITIES of POLYCLONAL RABBIT ANTI-PLANKTON SERA as determined by an ELISA.

Relative Elisa signal strength of polyclonal antisera against various plankton specimens

Antigen	Antisera				
	Anti-euphausiid	Anti-*Calanus agulhensis*	Anti-fish larvae	Anti-polychaete	Anti-cladoceran
Euphausiid	3+	0	0	0	2+
C. agulhensis	0	4+	0	0	0
Fish larvae	0	0	8+	0	0
Polychaete	2+	1+	0	6+	1+
Cladoceran	4+	0	0	0	9+
Siphonophore	0	0	0	0	0
Ctenophore	2+	3+	0	0	0
Ostracod	2+	2+	0	1+	4+
Copepod	0	1+	0	0	0
Cumacean	3+	1+	0	0	2+
Isopod	3+	2+	0	0	2+
Amphipod	4+	1+	0	0	3+
Decapod	3+	0	0	0	3+
Chaetognatha	0	0	0	0	0
Gastropod	1+	0	0	2+	3+
Salp	0	3+	0	0	0
Oikopleura	1+	0	0	0	0
Fish egg	0	0	0	0	0

Scale: See Text

the naive serum against the target antigen of the antiserum, rounded off to whole numbers.

Anti-euphausiid serum gave stronger ELISA signals to cladoceran and amphipod than to euphausiid (Student t-test, P>0.01), while it gave similar signals for euphausiid, cumacean, isopod and decapod and reacted stronger with euphausiid than with the remaining cross-reacting taxa. Coefficients of variance (CV), *i.e* the standard deviations expressed as percentages of the average absorbance values were determined for each antiserum. CV were between 2% and 39% for all cross-reactions of anti-euphausiid serum. Anti-*Calanus agulhensis* serum reacted with eight different taxa (CV between 6% and 26%). It reacted four times stronger with *Calanus agulhensis* antigen than with antigen of other copepods. Although it could not distinguish *Calanus agulhensis* from salp, it did resolve the former from all the other cross-reacting taxa (P>0.02). Anti-fish serum was highly specific against larval fish (CV=6%) with no cross-reactions with other antigens. Anti-polychaete serum gave three positive reactions (CV between 12% and 17%) and it reacted more strongly against polychaete antigen than ostracod and gastropod (P>0.01). Apart from cladoceran, anti-cladoceran serum recognised eight other taxa (CV between 2% and 14%). The difference between the absorbance mean against cladoceran antigen and the absorbance mean of the closest cross-reacting antigen, ostracod, was highly significant (P>0.01).

Specificities of antisera were also determined by immunodot assay (Fig. 1). By using a higher anti-euphausiid serum dilution (1:400) and lower antigen concentration (0.25 μg per spot) in an immunodot assay, cross-reaction to

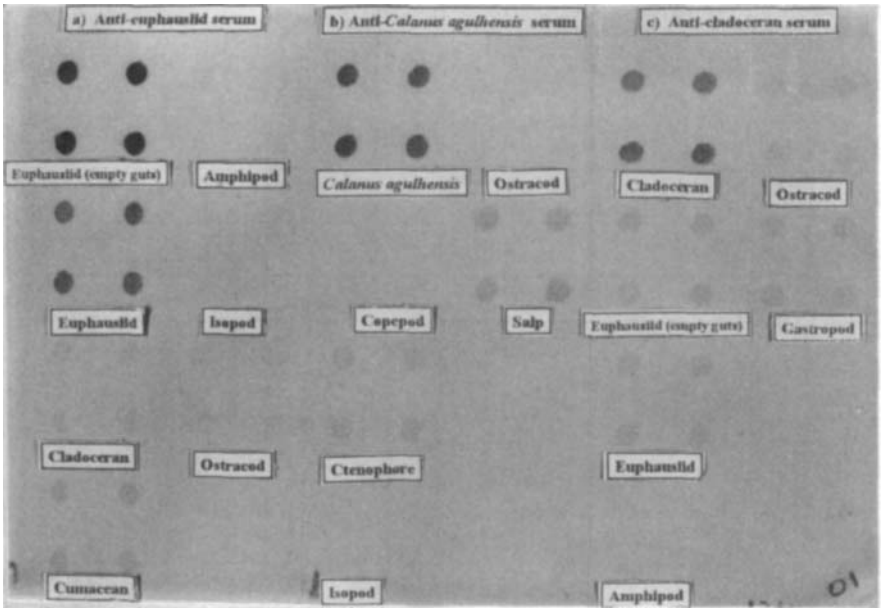

FIGURE 1. Immunodot assay of zooplankton antigens with (a) anti-euphausiid serum, (b) anti-*Calanus agulhensis* serum and (c) anti-cladoceran serum. Antisera were diluted 1:400 with incubation buffer. Antigens were coated at 0.25 µg of protein/spot.

amphipod, isopod and ostracod disappeared completely, while it was quenched against cladoceran and cumacean (Fig. 1a). Cross-reactions of anti-*Calanus agulhensis* serum with copepod, isopod and ostracod, as displayed by ELISA, were insignificant with the immunodot assay but persisted with salp and ctenophora, although the intensities of spots obtained with the latter were significantly lower than those which emerged for *Calanus agulhensis* target antigen (Fig. 1b). Previous cross-reaction of anti-cladoceran serum with amphipod

TABLE 3

ELISA SCREENING of SQUID PARALARVAE with POLYCLONAL RABBIT ANTI-PLANKTON SERA.

Relative ELISA signal strength of polyclonal antisera against 4 squid paralarvae					
	Antisera				
Antigen	Anti-euphausiid	Anti-*Calanus agulhensis*	Anti-fish larvae	Anti-polychaete	Anti-cladoceran
Paralarva 3	2+	1+	0	3+	1+
Paralarva 4	1+	2+	0	2+	1+
Paralarva 5	0	1+	0	1+	2+
Paralarva 6	2+	2+	0	2+	2+

Scale: See Text

disappeared while the rest of the specificity pattern observed with the ELISA was sustained in the immunodot assay (Fig. 1c).

Screening of Paralarvae :

ELISA was employed for screening of four squid paralarvae against the various antisera (Table 3). With the exception of anti-fish serum, all the other antisera could detect their target antigens in the paralarvae tested. The variation in signal strength among the paralarvae for each antiserum may be ascribed to predation on the target prey rather than cross-reaction with squid antigen since the same amounts of squid protein homogenate (1 μg of protein per well) were used in the ELISA screening.

Six paralarvae of which four corresponded with the four used in the ELISA, were probed for ingested prey with immunodot assay (Fig. 2). Anti-euphausiid serum gave bright dots for euphausiid antigen with fainter dots against cladoceran and no dots against the target antigens of the other antisera. Although dots were obtained for all paralarvae, the brightest dots were obtained with paralarvae 3 and 6. Dots were obtained with paralarvae 1, 3, 5 and 6 at a protein quantity of 1 μg (Fig. 2a). In the panel of accompanying antigens, anti-*Calanus agulhensis* serum rendered intense dots with its target antigen and cross-reacted slightly with copepod. It recognised antigen in paralarvae 1, 2, 4 and 6 at 1 μg of paralarval protein per spot (Fig. 2b). In contrast with anti-euphausiid serum, anti-*Calanus agulhensis* serum reacted with paralarvae 2 and 4 at a protein quantity of 1 μg while anti-euphausiid serum detected antigen in 1 μg protein of paralarva 3. Although both antisera gave dots at 1 μg of paralarval protein for paralarvae 1 and 6, dots obtained with anti-*Calanus agulhensis* serum were more intense for paralarva 1 while anti-euphausiid serum reacted more strongly with paralarva 6. These results indicate that the antisera do not cross-react with each other's target antigens. With the exception of its target antigen, anti-fish serum did not recognise any of the other target antigens and did not detect fish antigen in any of the paralarvae (Fig. 2c). As anti-polychaete serum did not cross-react with any of the other target antigens, it was concluded that it recognised polychaete antigen in paralarvae 1, 3, 4, 5 and 6. The absence of any dots with paralarva 2 suggests that anti-polychaete serum does not cross-react with paralarval antigen (Fig. 2d). Anti-

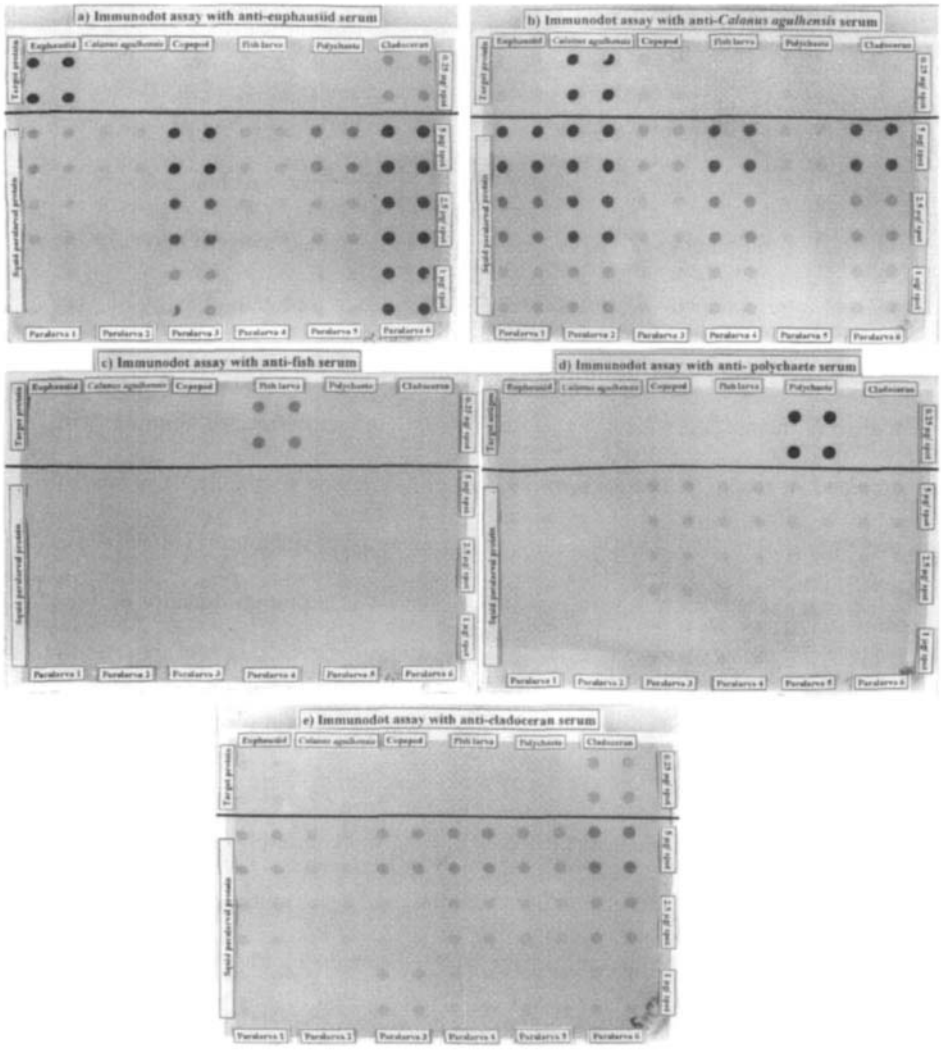

FIGURE 2. Immunodot assay of 6 paralarvae against dilutions of: (a) anti-euphausiid serum; 1:400, (b) anti-*Calanus agulhensis* serum; 1:400, (c) anti-fish serum; 1:500, (d) anti-polychaete serum; 1:700 and (e) anti-cladoceran serum; 1:400. The top panel of each membrane is covered with target antigens of the antisera at protein quantities of 250 ng/spot in quadruplicate. The bottom panels are coated with protein homogenates of 6 paralarvae in quadruplicate at quantities of 5 µg, 2.5 µg and 1 µg/spot.

cladoceran serum gave dots for paralarvae 1, 3, 4, 5 and 6 at 1 μg protein of squid homogenate (Fig. 2e).

DISCUSSION

Squid paralarvae can select prey among various planktonic species which abound on the Agulhas Bank. Among the crustaceans, the copepods, especially the dominant copepod, *Calanus agulhensis,* euphausiids, mysiid shrimps and cladocerans are regarded as the most important potential prey-taxa (26). This hypothesis has to be tested by identification of prey residues in the guts of paralarvae that have fed in their natural habitat. To this end, polyclonal rabbit antisera were generated against five of the seven mentioned taxa for gut analysis of squid paralarvae.

Polyclonal antiserum was preferred to mAb as antibody probe in the light of the greater sensitivity of polyclonal antiserum towards denaturation-vulnerable epitopes (15). For this particular study, the degree of specificity required is, with the exception of *Calanus agulhensis,* at the level of order and class. Various authors have demonstrated the capacity of polyclonal antisera to resolve phylogenetic relationships on order (10), family (7, 9, 12) and even species (8, 13, 14, 38) level, when used in predation studies.

The specificities of the five antisera produced in this study were assessed by screening each antiserum against seventeen taxa by means of an ELISA. Table 2 revealed cross-reactions of anti-euphausiid, anti-*Calanus agulhensis,* anti-cladoceran and anti-polychaete sera, whereas anti-fish serum reacted only with

fish antigen. Decapods and amphipods are reported to be carnivorous (27) which may explain cross-reactions of anti-euphausiid and anti-cladoceran sera with these taxa. Euphausiids (28), polychaetes and ostracods (29), ctenophores and gastropods (27) are omnivorous which may account for some of the cross-reactions among these groups. Anti-*Calanus agulhensis* serum gave lower ELISA signals to its closest relatives, other copepods, than with salp and ctenophores which both belong to different phyla than *Calanus agulhensis.* This apparent relatedness may, however, be ascribed to *Calanus agulhensis*, salp and ctenophore sharing a diet consisting of the same phytoplankton. The same reason may also account for cross-reactions of anti-cladoceran serum and the remaining cross-reactions observed with anti-euphausiid serum. Phytoplankton are antigenic as were demonstrated by Vrieling *et al.* when they used monoclonal antibodies for enumeration of *Gyrodinium aureolum* (30). In the current study, plankton with unknown feeding regimes were used for immunizations. Cross-reactions may therefore be reduced further by evacuation of plankton gut contents of specimens before immunization.

Specificity of an assay may be improved by elimination of cross-reactions due to intrinsic properties of polyclonal antiserum or the particular assay system. Multimeric interactions allow low-affinity antibodies to bind more tightly to antigen, thereby causing cross-reaction. By lowering the concentration of both antigen and antibody, antigen-antibody complex formation of antigen with low- affinity antibodies can be decreased (16). The advantage of a solid-phase immunoassay, such as ELISA, over liquid-phase

immunoassays resides in the more rapid binding of antibodies to protein when the latter is adsorbed on a solid-phase instead of being free in solution. Djavadi-Ohaniance and Friguet (31) proposed that the slow binding in solution is due to antigenic determinants that are hidden in the native protein but become exposed when the protein is adsorbed on the solid- phase. Equilibrium of binding of antibodies to surface-immobilised antigen can be established quicker when the antibody solution is agitated during incubation. Furthermore, it was shown that agitation promotes binding of high-affinity antibodies over low-affinity antibodies thereby increasing specificity (32). Membranes may possess a greater diversity of binding sites and a larger surface area in comparison with microtiter wells (33) to improve sensitivity and reproducibility (34). In addition, membranes are amenable to flow-through sample application which accelerates antigen-antibody reaction (35) and have potential for multianalyte applications (36). Immunodot assays do not need expensive photometers and provide a permanent record of results (34). Gut analysis of predators with immunodot assays were shown to be more rapid and cost-effective (19, 37). By using an immunodot assay at lower antigen concentration and agitation during incubation with more diluted antisera, cross-reactions observed with ELISA (Table 2) were significantly reduced (Fig. 1).

Screening of paralarvae with immunodot assay (Fig. 2) confirmed the screening results obtained with ELISA (Table 3). Multiple predation on euphausiid, *Calanus agulhensis* and polychaete was observed in paralarvae 1 and 6, predation on both

euphausiid and polychaete was seen in paralarvae 3 and 5, paralarva 4 screened positive for both *Calanus agulhensis* and polychaete whereas paralarva 2 fed mainly on *Calanus agulhensis* (Fig. 2). The immunodot assay may be simplified further by determination of detection limits for each antiserum, thereby eliminating the protein dilution series used for each paralarva which will facilitate screening of more paralarvae per membrane. Dots that still emerge, due to cross-reaction, may be diminished further by optimisation of exposure time to substrate solution during membrane development.

Although more extensive field trials will be necessary to identify the preferred prey of paralarval Chokka squid, this study demonstrates how the desired level of specificity with an immunodot assay and polyclonal antisera could be acquired by simple manipulations in order to detect prey in paralarvae within the constraints imposed by time, cost, availability of reagents and equipment.

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