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Influence of squid extracts on the triggering of secondary vitellogenesis in *Penaeus vannamei*

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Abstract

A series of five experiments were carried out to find an alternative to eyestalk ablation for inducing and controlling vitellogenesis in penaeid shrimps. Several extracts from squid were tested as supplements to a basal diet. Polar components of hydro-alcohol (ethanol/dicloromethane/water, 2:2:1:8) soluble and lipid squid fractions (Bligh & Dyer), when incorporated in formulated feed at low doses, trigger secondary vitellogenesis in 15–35 g female *Penaeus vannamei*, showing maturations of the same order of magnitude as the eyestalk-ablated controls. Achievement of vitellogenesis was estimated by a homologous ELISA-vitellogenin test. Even though the nature of the active molecules was not completely elucidated, the results obtained indicate that they may probably be steroid-like molecules of cephalopods, acting in a heterologous way.

KEY WORDS: feed, induction, shrimp, squid, vitellogenesis

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Introduction

Eyestalk ablation is the only endocrinological control practised on penaeid shrimp to stimulate the onset of vitellogenesis. This method allows the triggering and development of secondary vitellogenesis in species in which maturation cannot be obtained in captivity. At present most of the commercial production of postlarvae relies on this technique, which enables producers to coordinate maturation, artificial insemination and spawning, to adapt the production to a regular schedule.

The immediate consequences of unilateral eyestalk ablation are a precocious maturation and a reduced period between spawnings, but it also leads to other physiological anomalies, due to the removal of an organ producing and stocking a number of hormones controlling moulting, carbohydrate metabolism, cardiac frequency, pigmentation, etc. (Browdy 1992). Among the main drawbacks attributed to this operation, provoking a sudden mobilization of energy resources directed towards reproduction, are: the scarce mating rate of eyestalk-ablated females (Browdy 1992), the deterioration with time of spawning quality (Emmerson 1983) and irregularity of yolk deposition in oocytes (Anilkumar & Adiyodi 1980).

On the other hand, some authors (Brown *et al.* 1979; Chamberlain & Lawrence 1981a; Tan-Fermin 1991) have reported that eyestalk-ablated females show a regular ovarian development and that they produce good-quality larvae in reasonable periods of time. Certainly there is a great demand for energy associated with multiple spawning during a short period of time: this implies new and high nutritional requirements that must be covered by feeding and/or by body reserves of the animal. The commercial operations rely on fresh food in a proportion varying from 20% to 50% of the maturation diets in order to obtain successful maturations (Bray & Lawrence 1992). Among the fresh feed most commonly fed to the adults, molluscs and particularly squid have been used to promote maturation and enhance egg quality, as complement in semipurified diets or incorporated in these (AQUACOP 1977; Lumare 1986). Some of these studies have concluded the superiority of squid compared with other animals (Chamberlain & Lawrence 1981b; Rodriguez-Marín *et al.* 1986). In addition, the positive effect of squid on development of vitellogenesis seems to have a wide range of zoological specific action, as has been demonstrated in *Pagrus major* (Luquet & Watanabe 1986).

From these different points emerges the need for an alternative to the practice of eyestalk ablation, to stimulate and control vitellogenesis in order to allow a better management of the broodstock. For this purpose, a series of experiments was carried out

directed at elucidating the effect and nature of different squid fractions on triggering secondary vitellogenesis in *Penaeus vannamei*.

Material and methods

All the experiments were performed following the same protocol. Different fractions extracted from squid were incorporated into a basal diet (Table 1) in amounts corresponding to 100 g kg^{-1} of freeze-dried squid. Care was taken to maintain constant the lipid and protein components as well as the energy value. Animals were fed each diet, twice a day at 3% of the biomass during a 15-day experimental period.

Animals: Adult *Penaeus vannamei* Boone originally from Tahiti were maintained in captivity at the facilities of the IFREMER Center at Brest, France and Tahiti, French Polynesia, where the experiments took place. Only those females not yet in vitellogenesis were selected. The animals were tagged individually with a coloured silicon ring around the eyestalk, then they were randomly allotted to the experimental tanks at a density of 10 females per tank. The assignment of the different treatments (diets) and the tanks was done at random. Unilaterally eyestalk-ablated females (positive controls) were maintained in separate tanks.

Experimental conditions: The tanks (3 m^3) were supplied with sea water pumped through a sand filter and thermoregulated to reach a constant temperature of 28°C. A water-renewal rate of 300% day^{-1} was maintained and a supplementary aeration was provided to assure oxygen saturation. Salinity remained around 35 g L^{-1}, and the pH was near to 8.2. A 14:10 h light/dark artificial photoperiod was maintained.

Table 1 Maturation basal diet

Ingredient	Dry basis (g kg^{-1})
Casein	62.5
Fish protein hydrolysate	175.0
Shrimp meal	162.0
Wheat gluten	150.0
Lactic yeast	125.5
Cellulose	25.0
Gelatinized potato starch	121.0
Sucrose	50.0
Na_2HPO_4	25.0
Cholesterol	2.5
$CaCO_3$	19.0
Vitamin mix[a]	22.5
Ascorbic acid	1.2
Lipids[b]	57.0

[a]Vitamin mix, per kg: vitamin A palmitate, 200 000 IU; vitamin D_3, 200 000 IU; α-tocopherol acetate, 3.75 g; menadione bisulphite, 200 mg; thiamine, 0.45 g; riboflavin, 2.5 g; Ca pantothenate, 5 g; pyridoxine, 0.4 g; niacin, 10 g; folic acid, 0.43 g; biotin, 0.5 g; vitamin B_{12}, 1 mg; meso-inositol, 20 g; choline chloride, 37.5 g.
[b]Cod liver oil, 38 mL kg^{-1}; soybean lecithin, 19 g kg^{-1}.

Vitellogenin determination: Haemolymphatic vitellogenin concentration was determined by a homologous vitellogenin immunoassay (ELISA), according to the method developed by Mendoza *et al.* (1993), the principle of which is the following. Purified vitellogenin (VTG) is first immobilized in a solid phase, then free VTG contained in samples or standards is added together with a polyclonal antibody (anti-VTG). The free VTG will then compete with coated VTG for the binding sites of the antibody, thus preventing a certain fraction of this antibody from being immobilized. The amount of antibody is measured in a subsequent step by an enzyme-labelled second antibody. The enzyme activity detected is inversely related to the VTG concentration in the sample. The assay is calibrated using dilutions of standard VTG as a competitor.

Quantification of vitellogenin was performed on individuals in intermolt (stage 'C') in order to avoid haemolymphatic variations which might influence the VTG concentration. VTG was measured before and after each trial and the percentage of females entering secondary vitellogenesis* was determined.

Experimental design: To study the effect of squid fractions, randomized screening experiments (experiments 1 and 2) were performed. Then for subsequent experiments (experiments 3, 4 and 5) a randomized-block design with two replicates was established. As replicates were carried out at different times they were considered as blocks. Comparisons of the mean concentrations of different treatments were accomplished by ANOVA and Duncan's new multiple range test (Steel & Torrie 1980), additionally a χ^2 test was applied to assess the difference between proportions of individuals that responded to the fractions.

Experiment 1

Three main biochemical groups obtained from squid which might potentially be implicated in the promotion of secondary vitellogenesis were tested. The three different classes were separated in a first approach by Blight and Dyer's lipid extraction technique from freeze-dried whole squid, using a mixture of ethanol/dichloromethane/water (2:2:1.8) (modified by Beninger

*Only those females with a vitellogenin concentration above a threshold of 0.1 mg mL^{-1} were considered. This level was defined taking into account the results reported by Quackenbush (1989) with *P. vannamei* and those that have found with *P. indicus* (Mendoza & Fauvel 1989).

1982); the fractions obtained were: a lipid fraction (LIP), a hydro-alcohol soluble fraction (HAS) and a protein fraction (Proteins-B&D). Due to the denaturation of the protein fraction we chose to include also squid proteins obtained by precipitation with ammonium sulphate (AS-Proteins). Soluble proteins from freeze-dried squid were first extracted in a phosphate buffer (Na_2HPO_4, 0.1 M, NaH_2PO_4, 0.1 M, pH 7.4) and then were precipitated with a saturated solution of ammonium sulphate (80% final concentration). Precipitated proteins were dialysed against the same buffer and were continuously monitored with $BaC1_2$ to indicate any residues of ammonium sulphate.

Each fraction was included in a previously tested basal diet (Table 1) that had not shown any positive effect on the onset of *P. vannamei* vitellogenesis. In this experiment, six treatments were compared in a completely randomized experimental design with no replicates, just to identify the most interesting fractions. The treatments are those specified in Table 2.

Experiment 2

Due to difficulties in performing the ammonium sulphate precipitation on a large scale, proteins and peptides were obtained by precipitation with water and ethanol following the technique described by Roberts *et al.* (1980), which allowed the separation of insoluble or precipitated proteins (PP) from soluble peptides and proteins (SP). In this experiment the fractions were extracted from freeze-dried squid, *Loligo* sp., whereas the fresh squid used was *Nototodarus sloani* Gray. A totally random design was established with two replicates per treatment.

Experiment 3

For this experiment, both the HAS and the lipid fractions were refractionated according to their polarity. This was accomplished by partition chromatography with butanol/water (1:2) according to Mangold (1984). The butanol extracts were combined, washed twice with Na_2CO_3 aqueous solution 20 g L^{-1} before being evaporated. The residues were partitioned in methanol/hexane (1:1). The methanol extracts (polar fraction) and the residuals (aqueous phase and hexane phase) were incorporated in the basal feed.

Experiment 4

In this case, the polar fractions of HAS and lipids were obtained based on the eleutropic series for the extraction of steroids (Makin 1984). The HAS and lipid fractions were saponified and the non-saponifiable phases were extracted first with chloroform and then with ethylacetate. The fractions obtained from the last solvent were pooled and dried. The rest of the fractions (acetone non-soluble fraction, non-saponifiable fraction in chloroform) were pooled to constitute the 'residual fraction'. Treatments were constituted as in the former experiment (Table 2).

Once identified, fractions showing positive effects were used in an additional experiment aimed to further elucidate their nature.

Experiment 5

The HAS fraction was partitioned into subfractions by chromatography on Sephadex G-25 (Pharmacia). The sequential

Table 2 Description of the experimental series and treatments[1]

Exp. 1	BD	BD–LIP	BD–HAS	BD–P. B&D	BD–P. AS	SQ^D		
Exp. 2	BD	BD–LIP	BD–HAS	BD–PP	BD–SP	SQ^F	BD–SQ^F	EABL
Exp. 3	BD	BD–HAS	BD–HAS/PF	BD–HAS/RF	BD–LIP	BD–LIP/PF	BD–LIP/RF	EABL
Exp. 4	BD	BD–HAS	BD–HAS/PF	BD–HAS/RF	BD–LIP	BD–LIP/PF	BD–LIP/RF	EABL
Exp. 5	BD	BD–HAS	BD–HAS/PF	BD–HAS/NPF	BD–HAS-RE	EABL		

[1]Treatments are coded as follows:

BD:	basal diet.
BD–LIP:	BD + lipids.
BD–HAS:	BD + hydro-alcohol soluble fraction after Bligh & Dyer's extraction.
BD–P. B&D:	BD + protein fraction after Bligh & Dyer's extraction.
BD–P. AS:	BD + protein fraction after ammonium sulphate precipitation.
BD–PP:	BD + precipitated peptides after precipitation by the technique of Roberts *et al.* (1980).
BD–SP:	BD + soluble peptides after precipitation by the technique of Roberts *et al.* (1980).
SQ^D:	freeze-dried squid (*Loligo* sp.).
SQ^F:	fresh squid (*Nototodarus sloani*).
EABL:	eyestalk-ablated females.
BD–HAS/PF:	BD + polar fraction from the HAS.
BD–HAS/RF:	BD + residual fraction from the HAS.
BD–LIP/PF:	BD + polar fraction from the LIP.
BD–LIP/RF:	BD + residual fraction from the LIP.
BD–HAS/NPF:	BD + non-polar fraction from the HAS.
BD–HAS-RE:	BD + reconstituted HAS fraction (HAS-polar fraction & HAS-non-polar fraction).

fractionating was carried out in an 80 × 2.5 cm column. The following elution sequence was used: chloroform/ methanol (19:1), chloroform/methanol/acetic acid (19:1:4), chloroform/ methanol/acetic acid (9:1:3), methanol/water (1:1), water. The lipid components were obtained in the first two fractions and the hydrosoluble ones in the last three. The subfractions obtained were pooled to reconstitute the HAS fraction. This was done to know if a complementary factor of the non-polar fraction (NPF) was involved in the activity of the polar fraction. The experimental design to test these treatments was based on orthogonal contrasts.

Results

Experiment 1: In the first screening experiment no significant differences regarding the VTG concentration of individuals were noted (Table 3, Fig. 1) but a large variation of the percentage of individuals stimulated was recorded ($P < 0.05$). These percentages were larger for the treatments containing proteins obtained by ammonium sulphate precipitation (AS-proteins), squid (SQ), and the hydro-alcohol soluble fraction after the Bligh & Dyer extraction (HAS). These fractions were also those which had induced the highest VTG levels. There was a clear difference in response between the protein fractions obtained after the Bligh &

Table 3 Results of the six experiments. Within experiments, means with the same superscript letters belong to homogeneous groups (separated by Duncan's new multiple range test)

Exp.	Treatment[1]	Mean	Percentage[2]	ANOVA			χ^2		
		VTG mg mL^{-1}		*F*	d.f.	*P*	χ^2	d.f.	*P*
1	BD	0.12	10.00	1.917	5,6	NS	12.10	5	<0.05
	BD–LIP	0.26	20.00						
	BD–HAS	0.36	35.00						
	BD–P. B&D	0.14	25.00						
	BD–P. AS	0.43	66.66						
	SQ^{D}	0.21	45.00						
2	BD	0.16^{c}	20.00	1.149	7,8	NS	26.00	7	<0.01
	BD–LIP	O.42^{a}	25.00						
	BD–HAS	0.34^{ab}	77.77						
	BD–PP	0.17^{bc}	25.00						
	BD–SP	0.21^{abc}	20.00						
	SQ^{F}	0.00^{c}	0.00						
	BD–SQ^{F}	0.18^{bc}	22.22						
	EABL	0.34^{ab}	80.00						
3	BD	0.07^{c}	5.55	12.31	7,7	<0.01	4.87	6	NS
	BD–HAS	0.96^{ab}	50.00						
	BD-HAS/PF	1.14^{a}	52.63						
	BD-HAS/RF	0.70^{b}	36.48						
	BD–LIP	1.21^{a}	47.37						
	BD-LIP/PF	0.71^{b}	42.10						
	BD-LIP/RF	0.62^{b}	29.41						
	EABL	1.18^{a}	62.50						
4	BD	0.00^{b}	23.53	4.01	7,7	<0.05	5.79	7	NS
	BD–HAS	0.94^{a}	42.10						
	BD-HAS/PF	0.68^{ab}	44.44						
	BD-HAS/RF	0.57^{ab}	37.50						
	BD–LIP	0.48^{b}	38.88						
	BD-LIP/PF	0.59^{ab}	41.17						
	BD-LIP/RF	0.34^{b}	25.00						
	EABL	0.98^{a}	55.55						
5	BD	0.27^{c}	50.00	9.37	5,5	<0.05	3.43	5	NS
	BD-HAS	0.83^{ab}	75.00						
	BD-HAS/PF	1.11^{ab}	58.33						
	BD-HAS/NP	0.69^{b}	41.66						
	BD-HAS–RE	1.05^{ab}	66.66						
	EABL	1.52^{a}	58.33						

[1]see Table 2.
[2]Percentage of individuals stimulated.

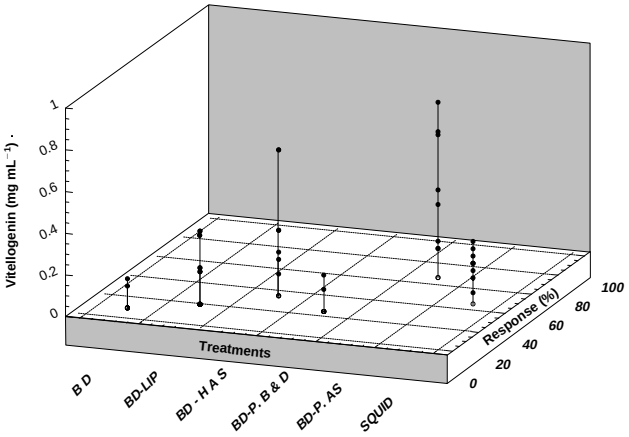

Figure 1 Effect of the main biochemical groups issued from squid on *Penaeus vannamei* vitellogenesis. Each mark ● represents an individual responding to the treatments, marks ○ are individuals not responding or showing only basal level and were not considered in the statistical analysis. (BD, basal diet; BD–LIP, BD + lipids; BD–HAS, BD + hydro-alcohol soluble fraction; BD–P.B&D, BD + proteins after Bligh & Dyer's extraction; BD–P.AS, BD + proteins after ammonium sulphate precipitation.)

Dyer extraction and those obtained by precipitation with ammonium sulphate.

Experiment 2: Figure 2 shows the difference in magnitude (VTG concentration) and in percentage of individuals stimulated in the second screening experiment. Even though no significant differences were observed, the impact caused by the HAS fraction when compared with the rest of the treatments in noteworthy.

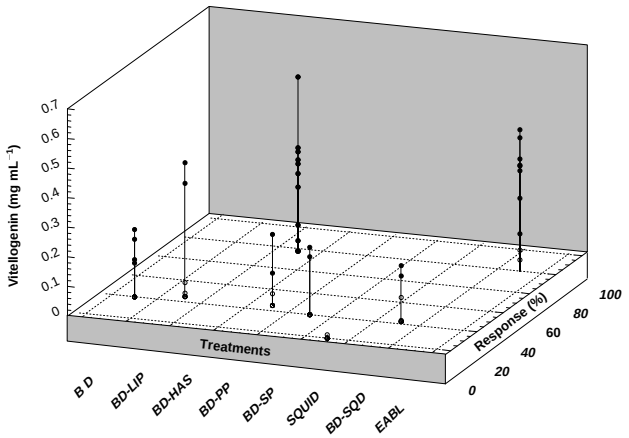

Figure 2 Effect of an insoluble and soluble peptides and proteins. Conventions as in Fig. 1. (BD, basal diet; BD–LIP, BD + lipids; BD–HAS, BD + hydro-alcohol soluble fraction; BD–PP, BD + precipitated peptides; BD–SP, BD + soluble peptides; BD–SQD, BD + squid; EABL, unilaterally eyestalk-ablated females.)

Vitellogenesis induction by this particular fraction was equivalent to that observed in eyestalk-ablated animals (Table 3). The lipid fraction was the next in order of importance, but the proportion of shrimps attaining maturation was lower (about one-third of the HAS fraction). Paradoxically the treatments involving fresh squid (SQ and basal diet + SQ) generated only very weak responses. The protein fractions evoked a response comparable only to the basal diet.

Experiment 3: The overall effect of the different fractions was superior in terms of stimulation over the negative control (Fig. 3). The best result were obtained with the HAS fraction, hydro-alcohol soluble/polar fraction (HAS/PF) and lipid fraction (LIP): these provoked almost a double concentration as that produced by other fractions. It is to be noted that the HAS/PF generated a response equivalent to that of the eyestalk-ablated organisms. In this experiment, the percentage of individuals responding positively was homogeneous.

Experiment 4: The effects of fractions obtained by the protocol of steroids extraction were in general similar to those of the former experiment (Fig. 4). The fractions HAS and HAS/PF again showed a marked effect. However in this case the lipid fraction and its polar component induced VTG levels lower than 50% of those obtained by the fractions of experiment 3.

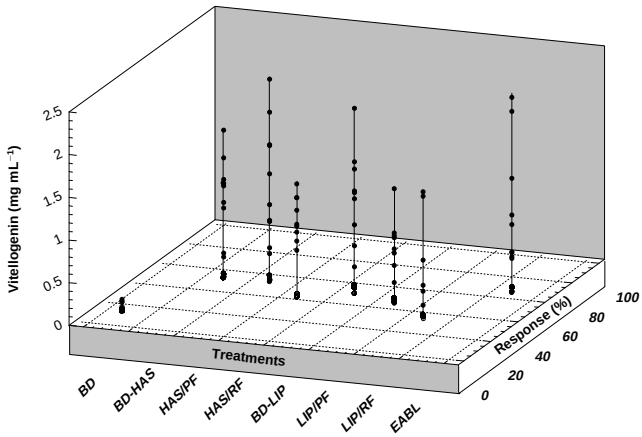

Figure 3 Effect of fractions of different polarity from the HAS and lipidic extracts, partitioned according to the protocal of Kaplanis for the extraction of ecdysteroids. (BD, basal diet; BD–HAS, BD + hydro-alcohol soluble fraction; HAS/PF, HAS polar fraction; HAS/RF, HAS residual fraction; BD–LIP, BD + lipids; LIP/PF, lipids polar fraction; LIP/RF, lipids residual fraction; EABL, unilaterally eyestalk-ablated females.)

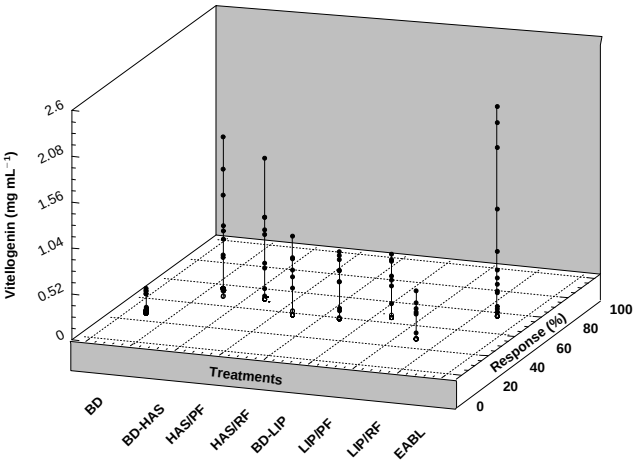

Figure 4 Effect of fractions of different polarity from the HAS and lipidic extracts, partitioned according to the eleutropic series of Makin for the extraction of esteroids. (BD, basal diet; BD–HAS, BD + hydro-alcohol soluble fraction; HAS/PF, HAS polar fraction; HAS/RF, HAS residual fraction; BD–LIP, BD + lipids; LIP/PF, lipids polar fraction; LIP/RF, lipids residual fraction; EABL, unilaterally eyestalk-ablated females.)

Experiment 5: No significant differences were found regarding the proportion of individuals showing signs of maturation. Nevertheless differences were obtained when comparing the VTG concentration of shrimps (Fig. 5). There was an especially good performance in the treatment in which the HAS polar fraction was assayed (HAS/PF), in contrast to the non-polar one (HAS/NPF). The original HAS fraction and that reconstituted (HAS/RE) showed equivalent results. Only the positive control (EABL) outperformed the HAS/PF.

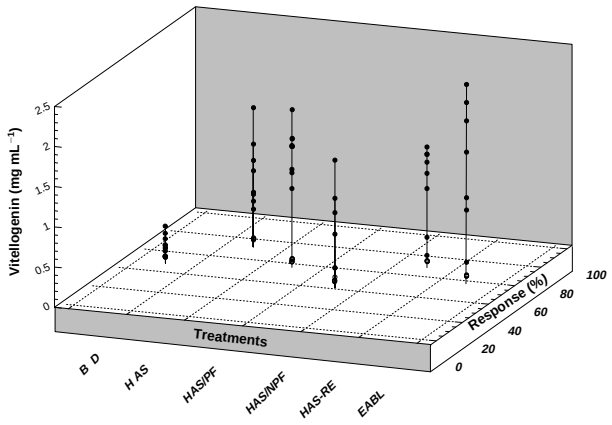

Figure 5 Effect of the polar and apolar fractions of the HAS extract and the joint effect of both fractions. (BD, basal diet; BD–HAS, BD + hydro-alcohol soluble fraction; HAS/PF, HAS polar fraction; HAS/NPF, HAS non-polar fraction; HAS–RE, reconstituted HAS fraction (BD-HAS/NPF; EABL, umilaterally eyestalk-ablated females.)

Discussion

The results of the different bioassays indicate the possibility of stimulating the vitellogenic process merely by incorporating squid extracts at low doses in the feed, thus achieving maturation levels of the same order of magnitude as the positive controls (unilaterally eyestalk-ablated females) without performing the eyestalk ablation. Another important finding was the induction of vitellogenesis in females (15–25 g) below the size currently considered as minimal for maturation (30–45 g) (Bray & Lawrence 1992). The shrimps used were from intensive cultures, while normally those animals destined to constitute a broodstock are raised under special conditions.

The heterogeneity of responses in the case of protein fractions prepared by different methods points to the hypothesis that the beneficial effect observed with the precipitated proteins might be due to molecules coprecipitated with or bound to proteins.

The results of the experiments in which different fractionation protocols were applied showed the same tendencies, the greatest stimulation being observed with the HAS and lipid extracts. Nevertheless, the intratreatment and intertrial variability observed was high and complicated the statistical analysis.

The variation observed may be attributed to the squid species and/or origin. This could be the reason for the absence of stimulation observed when fresh squid (*Nototodarus sloani*, from the South Pacific region) was used, and when the basal diet was complemented with it (experiment 2), while the extracts obtained from the freeze-dried squid (*Loligo* sp. from the English Channel region) were shown to be active. The composition of squid may change during the season and as a consequence of the different stages of their reproductive cycle. Moreover, Middleditch *et al.* (1980) showed that many lipid components pass unaltered through the trophic chain, thus the ability of squid to trigger maturation may be based equally on its own feed, and this may vary over the seasons and according to its geographical location.

The intratreatment variations noticed in the course of the experiments led us to consider some factors that might explain the differences. One of these factors is dominance. Indeed there is a certain hierarchy among the individuals sharing the same tank, as has also previously been pointed out by researchers of the Oceanic Institute (Newsline 1988). Dominance could imply a difference in consumption and thus a concentration of the stimulating effect. This factor would be accentuated by the asynchrony of the moulting cycle among the individuals during the experimental period. Finally, the low proportion of the extracts incorporated in the basal diet could have caused an uneven distribution in the feed.

Considering the stimulation observed with the HAS and the lipid fractions, we aimed our further experiments at elucidating

the nature of the active molecule(s). In the case of the HAS fraction, the component responsible for triggering the vitellogenesis appears to be of polar nature. In a similar way the results obtained after refractioning the lipid extract indicate as well that the active component would be rather polar in nature. However the second extraction has shown that it is not a phospholipid (because it is acetone-soluble).

The fact that the activity was found in different fractions and that this was not evenly conserved may be due to the following: when separating the fractions by chromatography the active part may have been shared between the HAS and lipid extracts according to its solubility in each one of them, or there might have been a partial destruction of the active factor. The possible existence of two independent factors acting at different levels should also be considered.

Even if the nature of the molecules was not completely elucidated, according to the kind of extraction achieved, it is probable that the active fractions contain, among other components, sexual steroids that may have had a direct or indirect influence on shrimp vitellogenesis. The work of Boticelli (cited by De Longcamp *et al.* 1974) supports this hypothesis. This author showed that alcoholic extracts of the gonads of *Pecten* have oestrogenic and prostagenic properties. On the other hand, even if the lipids from squid represent a good source of cholesterol (93% of sterols) (Blanchier *et al.* 1986), which shrimps are not able to synthesize and is needed as a substrate of the synthesis of steroids, it should be noted that the basal diet already contained ample amounts of cholesterol. Nonetheless, it is worth considering that cephalopods contain a broad spectrum of steroids in their gonads and an important amount of these is also found in their haemolymph (Carreau & Drowsdowsky 1977; Nikitina *et al.* 1977). Recent research results provide us with three arguments supporting the functional role of steroids in the control of vitellogenesis. First is the presence of these molecules in the various tissues involved in the vitellogenesis of crustacea (ovary, haemolymph, mandibular organ, hepatopancreas, etc.) Different steroids (testosterone, 11-ketotestosterone, 17-β-oestradiol, 17-α-hydroxy-progesterone, pregnenolone, 20-α-hydoxy-pregn-4-en3-one, etc.) have been detected by sensitive methods like RIA, GC-MS and SIM (Ollevier *et al.* 1986; Couch *et al.* 1987; Fairs *et al.* 1989; Novak *et al.* 1990).

Secondly there is strong evidence relative to the correlation between the concentration of these molecules and the development of the ovary (Van Beek & De Loof 1988; Fairs *et al.* 1989; Novak *et al.* 1990). Additionally there is a great body of evidence of many enzymatic systems that participate in the metabolism of steroids. Indeed, the capacity of bioconversion of cholesterol into progesterone and other steroids has been demonstrated in the ovaries of crustaceans (Kanazawa & Teshima 1971; Teshima & Kanazawa 1971). Since then, an increasing number of enzymes has been found (review: Swevers *et al.* 1991), revealing the importance of this specific enzymatic equipment.

The third point supporting the hypothesis of a steroid-like molecule is the possibility of induction. There are many demonstrations of sexual steroids showing positive stimulating results with different kinds of crustaceans (Caillouet 1972; Kulkarni *et al.* 1979; Nagabhushanam *et al.* 1980; Yano 1987). However, their physiological functions have not yet been elucidated. The triggering of vitellogenesis by gonadotrophic hormones allows envisaging the existence of a steroid relay as in vertebrates (Bomirski & Klek-Kawinska 1976; Zukowska-Arendaczyck 1981; Souty & Picaud 1984).

These arguments point to the existence of a component of steroid nature in the squid which very probably is located in the gonads. If this is true, this finding would explain the results reported by AQUACOP (1977) regarding the stimulation of penaeid shrimps by the ingestion of sexually mature molluscs, *Troca niloticus*.

Another possibility would be that the gonadotrophic hormone controlling vitellogenesis in cephalopods and emitted by the optical glands (Wells & Wells 1959; Richard & Lemaire 1975) could have stimulated the vitellogenesis in female shrimps in a heterologous way. This hormone has been postulated to be a steroid (Froesh 1979; Mangold 1987).

Equivalent results concerning the stimulation of shrimps, *Penaeus japonicus* Bate, with the incorporation of mollusc extracts in the feed were reported by Kanazawa (1989) employing a similar protocol of extraction to the one that we have used, the main difference being that Kanazawa used the extracts in combination with eyestalk ablation in order to achieve vitellogenesis.

These results open new perspectives from the standpoint of applied aquaculture, because they could eventually lead to the development of a ‘maturation feed’ that would allow aquaculturists a better control of reproduction.

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