FINAL REPORT TO FRDC

Introduction and development of overseas technology for production-scale maturation of penaeid prawns

Project No.: 89/52

DPI QUEENSLAND
DEPARTMENT OF PRIMARY INDUSTRIES

BRIBIE ISLAND AQUACULTURE RESEARCH CENTRE

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1. Project Description

PROJECT TITLE: Introduction and development of overseas technology for production-scale maturation of penaeid prawns

PROJECT NUMBER: 89/61

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1.1 Project Justification

The original project was entitled 'Introduction and development of overseas technology for production-scale maturation of penaeid prawns'. The project arose out of requests by the industry to solve the problem of the unpredictable supply and frequent poor quality of *Penaeus monodon* broodstock captured in Australian waters.

To achieve this end, and to explore the potential of other species for aquaculture in Australia, the following objectives were set:

(i) to introduce and adapt overseas methods for maturing *P. monodon* and *P. japonicus* in captivity; and
(ii) to develop maturation techniques for *P. esculentus*.

Soon after the commencement of the project, advances made independently by CSIRO in the maturation of *P. esculentus* satisfied this second objective. As regards the maturation of *P. monodon* two problems immediately became apparent. The first, following enquiries overseas, was that there was no 'off the shelf' technology available for transfer to resolve problems relating to *P. monodon* broodstock in Australia. Secondly, the huge variability in spawner performance in Australia made it apparent that identification of causative factors would require research on a large scale. In providing sufficient replication to ensure significant treatment effects could be identified, this project was to become one of the largest ever carried out in this field of research.

The work described in this report falls into the category of both 'basic' and 'applied' research. It became obvious very early in the project that improvements in *P. monodon* maturation were not to be found through trial and error modifications of existing techniques, but rather in a greater understanding of the mechanics of the maturation process. By understanding changes that take place in a prawn undergoing ovary maturation, it was hoped that the processes involved could be manipulated either through dietary input or the administration of hormones. It is this level of control that is required to not only see improvements in the reproductive performance of wild caught *P. monodon* but also to make the use of pond reared broodstock a commercial reality. Using pond grown animals to seed the next generation, or 'closing the life cycle', is a necessary step in the true domestication of penaeid prawns.

The broodstock maturation and hatchery phases involved in the culturing of *P. monodon* are recognised as being difficult. It was hoped that knowledge of broodstock stress reduction, dietary requirements and the hormonal processes involved in ovary development, would both directly and indirectly benefit those in the aquaculture industry involved not only in the culture of *P. monodon* and *P. japonicus* but of other penaeid species as well.

2. Project Summary

The study tour confirmed that research emphasis on penaeid broodstock has shifted from manipulating environmental parameters to promote ovary maturation. Research efforts now tend to be concentrated on the dietary requirements of broodstock and hormonal control of the maturation process. Despite world-wide attention progress in both areas has been relatively slow.

Much of the work to identify the specific dietary requirements for broodstock prawns has, until recently, involved a process of trial and error. Understanding the nutritional requirements of broodstock is complicated by the fact that, in addition to the needs of the ovary during maturation, growth and tissue maintenance needs must also be met. Similarly, the work on hormones is an area of great complexity. It is only with advances in techniques such as immunoassays and peptide extraction that the degree of interplay between hormones involved in the control of prawn reproduction has become evident.

The studies on the hormonal control of ovarian development in penaeid prawns, carried out as part of this project, have provided information considered essential prior to embarking on full scale research in this area. As a result of this project the roles played by the two major hormone groups (stimulatory and inhibitory) and their modes of action have been better defined giving direction to future research.

In addition to the studies assessing the effect of various hormone extracts, a number of studies in this project examined the effect of unilateral eyestalk ablation on reproductive performance and broodstock tissue composition. The practice of eyestalk ablation to induce ovary development in prawns represents a crude form of hormonal manipulation. Ablation works by reducing the level of vitellogenesis inhibiting hormone (VIH). Results indicate that VIH either directly or indirectly has an effect on previtellogenic ovaries. This is contrary to published models which propose that a drop in VIH is only effective once ovaries have undergone early (primary vitellogenesis) stages of development.

Research on the dietary requirements of Australian *P. monodon* focussed on the biochemical composition of both potential foodstuffs and broodstock tissues directly involved with reproduction. Visits to overseas laboratories highlighted the problems associated with studies involving biochemical analysis. Preliminary work for this project showed that *P. monodon* tissues were highly variable in their biochemical composition, and that to be able to assess significant treatment effects a large number of replicates were required. Effort was therefore put into selecting and refining techniques to ensure a high degree of accuracy and correct interpretation of results. Techniques were also modified to enable efficient handling of the large number of small samples with high water content.

To determine their potential as ingredients in broodstock diets, nutritional composition was assessed for a range of natural marine foods that are available in Australia. Most foodstuffs were found to have similar essential amino acid profiles and therefore were likely to be equally as good as protein sources. Fatty acid concentration varied significantly and hence lipid quality differed amongst the foodstuffs. On the basis of quality and concentration of protein and lipid, squid and green lip mussel were selected as basic ingredients for a fresh/frozen broodstock diet.

Much of the subsequent work on dietary requirements focussed on fatty acids. It was determined that the ratios of many fatty acids in the ovary tissue reflected maternal dietary intake. Compositional changes occurring in the ovary, eggs and non-feeding larvae (that is up to the first zoeal stage, Z_1) were measured to examine the pattern of fatty acid usage. The potential contribution of the ovary to the energy needs of the prawn prior to the onset of ovary development was considered important. Through starvation trials it was shown that the ovary does play a minor role as a storage tissue. The ovary's contribution of fatty acids was found to be selective and to reflect the roles that the various fatty acids play within the ovary tissue.

The fatty acids of greatest interest were arachidonic acid (20:4n-6), eicosapentanoic acid (20:5n-3) and docosahexanoic acid (22:6n-3). While all three are apparently used in membrane structure and as storage products in the ovary, 22:6n-3 makes the greatest contribution to the energy requirements of the prawn and of the developing larvae. Fatty acid 20:4n-6 seems more important as a membrane component than as an energy source, while 20:5n-3 is important for both.

To help account for the high levels of variability in broodstock survival and reproductive performance, consideration was also given to the effect of stress during capture and handling. Ionic changes were shown to be a good indicator of stress in penaeid broodstock. Results confirm that length of trawl time and moult stage affect initial stress levels of broodstock prawns. Stress due to trawling remained high during the holding period on board the boat, but it was found that current methods of packing and transport are not a major cause of stress in broodstock. While stress levels show positive correlation with broodstock death soon after arrival at hatcheries, the relationship between stress levels and the reproductive performance of female prawns is yet to be determined. To minimise the stress that prawns are subjected to the optimum trawling time and holding period prior to packaging are currently being investigated.

While the majority of this work was carried out on *Penaeus monodon,* a second species, *P. japonicus*, is also cultured in Australia. It is only recently (1991) with improvements in packaging live *P. japonicus* for export to Japan, that culturing this species has become popular. It was predicted that with increased hatchery demand for broodstock, and only one small population of wild *P. japonicus* available, a reliance on pond reared broodstock would soon develop. With this in mind, a study was carried out to assess the reproductive capacity of pond reared prawns. Results have shown that while mating may be a problem in tanks, this can be improved by supplying a sand substrate, or can be avoided by using mated females from the pond (where mating success is high). The use of pond reared broodstock appears to be a commercially viable proposition for *P. japonicus* hatcheries.

Thus, the project has made significant contributions to the body of information existing on penaeid broodstock maturation. While further research is needed, a considerable amount of the information compiled here has direct applications. Information on the dietary requirements is now being used to modify the soft pellet maturation diet currently being trialed at BIARC. The results from hormonal studies have directed ongoing research, while the information on stress reduction in broodstock and on the maturation conditions for pond reared *P. japonicus* have been made available, through newsletters and publications, directly to the industry.

3. Overseas study tour of prawn maturation research facilities

3.1 Aims

The aim of visiting a number of overseas research institutes was to determine the current directions of research into prawn reproduction and to detail the methods employed by these institutes. The establishment of collaborative links was also considered to be vital to the successful development of the BIARC prawn maturation project. Research on the control of reproduction was obviously moving into the realm of high technology and the prompt notification of any significant advances would save both time and money by preventing the duplication of research and ensuring the use of the latest techniques.

3.2 Outcome

Knowledge gained from the study tour confirmed that research into the control of reproduction in penaeid prawns was concentrating on two major areas:

(i) broodstock nutrition
(ii) hormonal manipulation

It was largely considered that optimum environmental parameters for the culture of traditional aquaculture species, including *P. monodon*, had been established and that while these parameters could be refined, the most profound improvements to broodstock maturation would be gained through nutritional improvements and hormonal manipulation.

3.2.1 Broodstock nutrition

Following the study tour it was concluded that the dietary requirements for penaeid broodstock were yet to be determined. Visits to Kagoshima University, Japan (Dr Teshima and Dr Kanazawa) and Texas A & M University Experimental Station,USA (Dr Lawrence) verified that artificial maturation diets gave inferior results to the commonly used but relatively inconvenient fresh feeds. It was established that the most common approach to developing a suitable artificial diet was to examine the composition of prawn tissues (in particular the ovary) and replicate this in the maturation diet. Alternatively, and equally popular, was the technique of analysing the natural foodstuff of the broodstock and using this to form the basis of an artificial diet. These two methods had met with some success and resulted in the development of artificial diets that could replace a percentage of the fresh dietary components.

To better understand the dietary requirements of penaeid broodstock, and to evaluate what role they may play in reproduction, Teshima and Lawrence were both supervising more fundamental research. Their work involves the manipulation of protein, lipid and specific fatty acid levels in diets to assess their effect on reproductive performance. Optimum protein levels were estimated to be between 55% and 60% and lipid levels between 10% and 12% on an organic dry matter basis. The fatty acids found to be

essential for prawn growth were also considered to be of great importance in the broodstock diet.

As a result of the study tour it was decided that to benefit the industry in the short term it was necessary to first establish the best fresh diet available to hatchery operators in Australia. At the same time it was considered important to carry out more fundamental research to identify prawn dietary requirements and appropriate levels of inclusion for the future formulation of an artificial diet. It was hoped that this dual approach would see an initial improvement in reproductive performance through the use of a better combination of fresh feeds than was currently used by the industry, followed by a gradual replacement of the fresh feeds with a high quality artificial diet. The knowledge gained through the more fundamental research on the transport and storage of nutrients in broodstock prawns was also considered important to the understanding of the roles that hormones play in controlling ovary development.

3.2.2 Hormonal manipulation

It was found that research on the hormonal control of reproduction was divided. Work being carried out at The National University of Singapore (Dr Khoo) and at the National Research Institute of Aquaculture in Mie, Japan (Dr Yano), was focused on the use of stimulatory hormones to induce maturation. In contrast, laboratories visited at the University of California (Dr Chang), and the University College of North Wales, UK (Dr Webster), were investigating the role played by inhibitory hormones which prevent the onset or completion of ovary maturation. From the study tour there was strong evidence to suggest that both stimulatory and inhibitory hormones were involved in prawn reproduction.

In light of these findings it was decided that the project should include hormone research as part of the work aimed at improving the control of prawn reproduction. Initial work would take a more applied approach and begin by verifying Yano's recent finding that the implantation of thoracic ganglion and brain extracts of lobsters into penaeid broodstock stimulated gonad growth. If this gonad stimulating hormone (GSH) could be shown to be effective in controlling maturation in *P. monodon* it was then planned to isolate and purify the compound in preparation for implantation and dosage determination. It was the opinion of Khoo, Yano and Chang that in practical terms, the use of a stimulatory hormone implant that released a compound which acted directly on the ovary would be preferential to working with an inhibitory hormone.

The trip also revealed that a large body of research was being carried out on the broad field of crustacean neuropeptide hormones. The 'European Network of Laboratories', which originally gained research funding because of the proposed use of hormones as insecticides, sees itself as having made significant advances in this field. As a result, hormones involved in the inhibition of reproduction and moulting in crustaceans have received a great deal of attention. The top priorities of this research are to understand the relationship between the molecular structure of neuropeptides and their physiological significance and then to determine how these peptides control physiological processes within the animal.

Thus the work on inhibitory hormones is highly theoretical, as target tissues and hormone interactions have yet to be clearly established. The research involves peptide extraction and sequencing combined with bioassays and ELISA immunoassay techniques. Despite the inherent difficulties of research at this level, the control of reproduction in prawns through the manipulation of inhibitory hormones such as the Gonad Inhibiting Hormones (GIH), remains a viable option. According to Dr Webster, 'one possible application of the work (although a distant aim), would be to use solid phase antibody implants in cultured prawns. These would bind circulating GIH and thus reduce circulating titres'. Dr Chang adds that 'despite the complexity of the endocrine system, it is certainly possible that a single endocrine factor (hormone) may regulate yolk production'.

It was therefore decided that, in conjunction with examining the role of stimulatory hormones, the project would benefit from research into the inhibitory neuropeptides involved in prawn reproduction. Rather than embarking on research of this scale alone it was considered that developing strong collaborative links with Dr Webster and his team would be advantageous. With the available expertise, BIARC would initially concentrate on improving bioassay techniques such as *in vitro* tissue culture whilst also developing some expertise in the sequencing of peptides and becoming familiar with techniques used at the University College of North Wales. Having refined the bioassay methodology, BIARC would then be in a position to assess the specificity of extracted compounds and help establish their mode of action.

These broad areas of research, the supply and mobilisation of dietary nutrients and the development of techniques for hormonal control of maturation, dictated the direction of the prawn maturation program at BIARC.

4. Hormonal control of ovarian development in penaeid prawns

4.1 Introduction

During the overseas study tour it became clear that there were two main areas in prawn maturation research that had the potential to enhance ovarian development (see section 3). The first was broodstock nutrition and the second was hormone manipulation. As a result of the study tour, it was decided to initially focus the hormone research on the factor contained in neural tissue which stimulated ovarian development in prawns. If this stimulatory effect could be demonstrated the next logical step would be to fractionate the neural tissue using high pressure liquid chromatography (HPLC) and using a bioassay, determine which fraction contained the ovary stimulating peptides.

4.2 Stimulatory neural peptides

4.2.1 Background

Otsu and Hanoaka (1951) first postulated that a factor produced by the central nervous system of decapod crustaceans promotes ovarian growth. They observed that ovarian weight in immature females of the crab *Potamon dehaani* did not increase after eyestalk removal and concluded that a stimulating factor was necessary for ovarian development. Since then several investigators have reported that addition of thoracic ganglion stimulated precocious vitellogensis *in vitro* (Otsu, 1963; Gomez, 1965; Oyama, 1968; Hinsch and Bennet, 1979). Yano *et al.*, (1988) showed that the vitellogenesis stimulating hormone (VSH) released from *Homarus americanus* neural tissue is not species specific. Thoracic ganglia from lobsters with vitellogenic ovaries stimulated vitellogenesis when implanted into immature prawns *Penaeus vannamei*. Cytological studies on the neurosecretory cells of the thoracic ganglion have shown a correlation between secretory activity and seasonal development of the ovary (Matsumoto, 1962; Babu *et al.*, 1980).

Har (1991) found that crude thoracic ganglion extracts from spent spawners were ineffective in stimulating vitellogenesis in immature eyestalk - ablated or non ablated *Penaeus monodon*. However, Mohamed and Diwan (1991) found that the majority of neurosecretory cells in the thoracic ganglia of spent *Penaeus indicus* were still in the secretory phase and presumably should still be releasing VSH. *In vitro* studies have indicated that VSH released from neural tissue affects the ovary directly (Takayanagi *et al.*, 1986), but it is not known whether the hormone acts directly on oocytes or causes follicle cells to secrete a second maturation factor. Kulkarni *et al.*, (1991) found that the addition of brain, suboesophageal ganglion or thoracic ganglion tissue to the *in vitro* culture of *Procambarus clarkii* ovary resulted in a significant increase in protein synthesis in the organ. The rate of protein synthesis was higher in ovary tissue incubated with brain tissue than in ovary tissue incubated with other neural tissue.

4.2.2 Methods and discussion

To measure the effect of neural tissue on ovary development *in vitro* a bioassay system had to be developed. It was decided that the best non isotopic method would be based around the increase in size of oocytes *in vitro*. Mud crab oocytes (*Scylla serrata*) were used initially as each female produces large numbers of oocytes which are easily separated and hence can be accurately measured under a microscope to determine if growth has occurred. Because oocytes had to be cultured for 72 hours to obtain adequate growth a medium capable of sustaining the oocytes during this period had to be developed. After trialing four different types of media a modified Reddy-Wyatt insect tissue culture medium (Table 4.1) was found to be superior and was used for further work. Unfortunately, it was found that during the 72 hour incubation a number of oocytes died, but under the microscope these were indistinguishable from live oocytes. Because this introduced a bias in estimating growth it became necessary to develop a method which differentiated live and dead oocytes. After researching several alternative methods a simple staining protocol to determine the state of the oocytes was developed (Table 4.2). Those oocytes alive at the end of the 72 hour culture period were stained and measured. After a satisfactory bioassay had been developed, brains (cerebral ganglia) from vitellogenic crabs were added to cultures of oocytes to determine if, as the literature suggests, oocyte growth would occur. It was subsequently found that the addition of brain tissue to cultures of oocytes resulted in no significant increase in oocyte diameter ($P>0.05$). To further test this finding brains from vitellogenic mud crabs were implanted into *P. monodon* females with stage 0 ovaries and each day the prawns were checked for signs of ovary development. No development was observed, however previous workers have found that the factor in neural tissue was not species specific so some ovary development had been expected.

Thus this preliminary work saw the successful refinement of existing *in vitro* bioassay techniques suitable for hormonal research in penaeid prawns. It also demonstrated that the results achieved with implanting neural tissue from a female of one family undergoing ovary development into a female of another family could not be repeated. The reasons for this are not clear but include the possibility that the families are too far removed and the hormone is more specific than first thought. Another possibility is that the hormone is only active for a very short period of the developmental cycle and if not extracted from the donor and administered to the recipient at appropriate times ovary development would not proceed. Details of this nature are not known and the work carried out overseas may have inadvertently 'timed it right'. The overseas work was also carried out with very low replication introducing the possibility that ovary development was not due to the treatment hormone.

4.3 Juvenile hormones

4.3.1 Background

Recent research by workers in the US had determined that juvenile hormones and their precursors which control reproduction in insects are also present in crustaceans. This finding led to further work showing that methyl farnesoate (MF) a precursor to juvenile hormone III had a role in the initiation of vitellogenesis in the spider crab *Libinia emarginata* (Laufer *et al.*, 1987). This compound was then discovered in a range of

different crustaceans, but the most interesting result was by Tsukimura and Kamemoto (1991) who found that MF increased the diameter of *P. vannamei* oocytes *in vitro*. This positive finding with a compound which is relatively easy to procure made this area of research attractive. If MF did in fact stimulate ovarian development it could be used by industry immediately. Therefore, the hormone research at BIARC moved towards juvenile hormones and experiments to examine the effect of these compounds on prawn ovaries *in vivo* and *in vitro* were developed.

Table 4.1 Composition of the modified ½ x Reddy-Wyatt medium used for the *in vitro* culture of *Scylla serrata* oocytes (per 100 mls)

45 mls	2 x Crustacean Saline
28.1 mls	Distilled water
15 mls	Fetal Calf Serum (CSL 09701901)
5 mls	50 x essential amino acids (Flow 16-011-49)
2.9 mls	$NaHCO_3$ (7.5% w/v)
1 ml	100 x Eagles MEM Vitamins (CSL 986.19)
1 ml	Glutamine (CSL 09871901)
1 ml	Phenol Red (1% solution)
0.9 g	Glucose
0.5 ml	HEPES (1M CSL 07391901)
0.5 ml	100 x Non essential amino acids (CSL 09751301)

2 x Crustacean Saline (g/l)

NaCl	54.7
KCl	2.62
$CaCl_2\ 2H_2O$	10.96
$MgSO_4\ 7H_2O$	4.44
$MgCl_2\ 6H_2O$	3.26

* Osmolarity of the medium was approximately 1000 mOsm and pH was adjusted to 7.4 to 7.5 with sterile 1 M NaOH

Table 4.2 Staining procedure to determine the viability of *Scylla serrata* oocytes cultured *in vitro* for 72 hours.

- 36 mg Neutral Red dissolved in six drops ethanol
- 60 mls filtered seawater or Crustacean saline (1000mOsm) added
- 2 mls of stain solution added to each 10 ml culture
- staining proceeds for one hour
- those oocytes stained red were considered to be alive and were counted and measured

4.3.2 Methods and discussion

Two hormones had been shown to have a stimulatory effect on *P. vannamei* oocytes, MF and JHIII, therefore these hormones and methoprene (a synthetic juvenile hormone used to control mosquitos) were tested. Due to a shortage of *P. monodon* females at that time, *P. japonicus* were used for experimentation. A positive result with *P. japonicus* would then have lead to further work with *P. monodon*. For the *in vivo* trials female *P. japonicus* with stage I ovaries were obtained from a commercial farm and maintained under the usual maturation conditions (see Section 6). Two days after moulting, prawns received an injection of either peanut oil (control), JHIII, methoprene or Altosid (water soluble microcapsules of methoprene). JHIII and methoprene were dissolved in peanut oil and Altosid was dissolved in sterile seawater, doses were calculated to give 100 nM concentration of hormone in haemolymph. Hormones were injected every four days until the prawn moulted and the stage of ovary development was checked every day. Five prawns were unilaterally eyestalk ablated to determine whether they had the potential to mature and spawn.

Unfortunately, mortality was very high during the experiment (Table 4.3) and appeared to be due to the peanut oil carrier. Altosid had no effect on ovary development whereas the ablated group had a significantly higher level of ovary development ($P < 0.05$). Because of the complicating effect of the peanut oil carrier it is difficult to determine whether the hormones might have increased ovary development, but Altosid which contains methoprene did not, suggesting that this analogue is ineffective. The positive response of the ovary to eyestalk ablation indicated that the animals were reproductively competent.

Table 4.3 Survival and roe stage of *P. japonicus* injected with juvenile hormones or subjected to unilateral eyestalk ablation.

		CONTROL	JHIII	MF	ALTOSID	ABLATED
Time to mort (days)		9.4 ± 3.4	11.0 ± 2.4	10.3 ± 2.5	9.0 ± 5.1	
Roe stage	START	1.2 ± 0.8	1.1 ± 0.7	1.2 ± 0.7	1.3 ± 0.6	1.0 ± 0.7
(I - IV)	FINISH	1.1 ± 0.7	0.9 ± 0.3	1.1 ± 0.3	1.2 ± 0.6	3.0 ± 0.7
Survival (%)		0	0	0	70	80

The hormones MF, JHIII and methoprene were also tested to determine whether they would increase the protein synthesis of *P. japonicus* ovary *in vitro*. The hormones were tested at concentrations between 100 nM and 100 μM on stage I and stage III ovary tissue cultured in a modified eagles minimum essential medium (Table 4.4). Methoprene was also combined with 20-OH ecdysone (this moulting hormone is required in insect organ culture for methoprene to be effective) in equimolar concentrations. None of the treatments was effective in significantly increasing the protein synthesis in *P. japonicus* ovary *in vitro* ($P > 0.05$). This conflicts with the finding of Tsukimura and Kamemoto (1991) but the isotopic method used here should be regarded as a more accurate technique than the measurement of oocyte diameter

which is open to other influences, eg. osmotic pressure of the culture medium. Tsukimura and Kamemoto (1991) could not demonstrate a dose-response effect of hormone in a concentration range of 100 pM to 100 nM, further weakening their conclusions.

Table 4.4 Composition of the modified Eagles minimum essential media used to measure the *in vitro* protein synthesis of *Penaeus japonicus* ovary (per 100 mls).

46 mls	2 x Crustacean saline
40.5 mls	HPLC grade distilled water
10 mls	Eagles MEM deficient (Gibco 041-0189)
1 ml	Non-essential amino acids (100X) (Gibco 043-01140)
1 ml	Glutamine (100X) (Gibco 043-05630)
0.5 ml	HEPES buffer 1M (Gibco 043-05630)
0.5 ml	Gentamicin (Gibco 043-05710)

During the validation of the bioassay in the juvenile hormone project it was found that adding sinus gland extract (2.0 eyestalk equivalent (ESE)/culture) to undeveloped ovary tissue in culture resulted in a significant reduction in protein synthesis ($P<0.05$). In ovaries undergoing development (stage III) the same dose of sinus gland extract did not effect protein synthesis (Hewitt, unpublished data). The reduction in ovarian protein synthesis resulting from the addition of sinus gland extract has been noted before in the literature and is thought to be due to a vitellogenesis inhibiting hormone (VIH) stored in the sinus gland. Because the VIH had an effect on previtellogenic ovaries and not on the stage III ovaries which were undergoing secondary vitellogenesis, these results are consistent with the hypothesis that the VIH released from the sinus gland prevents the initiation of ovary development. Once vitellogenesis has commenced, the ability of VIH to repress ovarian development is greatly diminished as the ovary comes under the influence of stimulatory hormones.

If the sinus gland peptides are fractionated on HPLC it should be possible to determine in which fraction the VIH activity resides by measuring the depression of ovarian protein synthesis caused by each fraction. Identifying the fraction may enable an antibody to VIH to be created or for the compound to be identified for alternative intervention strategies. This is the aim of current prawn hormone research at BIARC.

5. Capture and transport stress in broodstock *Penaeus monodon*

5.1 Introduction

The capture and spawning of wild *P. monodon* broodstock is presently the only method used in Australia for the production of fry for stocking prawn farm ponds. Broodstock prawns are captured in restricted areas on the north east coast of Australia by means of an otter trawl. Captured prawns are held on board the trawler for up to 24 hours before transport. Prawns are then packed in styrofoam boxes using standard methods for transport of live fish, and shipped to hatcheries throughout Australia. The whole process of capture and transport can be quite stressful to the animals and sometimes prawns die within a few days of reaching the hatchery. Although the number of prawns dying as a result of these stresses is low, generally in the order of 2 to 5 percent, it can be higher for some shipments. It appears that there may be a seasonal influence on the susceptibility of prawns to these stresses. There is also the possibility that the stress associated with current capture and transport procedures results in a decrease in productivity of the spawners, causing further losses to hatcheries.

The aim of this study was to investigate the stresses associated with the capture and transport procedure of broodstock *P. monodon*, with a view to determining the conditions which create stress and to reduce their occurrence.

5.2 Materials and Methods

The first objective of this study was to find a suitable index of stress for *P. monodon*. This index would have to be indicative of the overall stress level as broodstock prawns are subjected to a variety of stressors including hypoxia, physical exhaustion, crushing in the trawl net, exposure to air and sudden temperature fluctuations. Mortality tests are usually indicative of the general well-being of an animal, but the high cost and low numbers of these animals prohibited such an approach. Therefore the study focused on changes in physico-chemical parameters in the haemolymph of the animal in response to stress. A breakdown of normal osmoregulatory function has been observed in other crustaceans under stress. Other potential stress indices such as glucose levels, haemolymph pH and haemolymph osmolality were also investigated.

Once a suitable index of stress was established, it was possible to determine the amount of stress to which the prawns were subject at each step of the capture and transport procedure. The three main areas of interest were:

(i) The trawling of the prawns, with an emphasis on the effect of varying the trawl time and the effect of the moult stage of the prawn.

(ii) The holding of the prawns on board the trawler, focusing on the length of the holding period, the amount of crowding in the tubs and the importance of the moult stage.

(iii) The transport of the prawns, emphasising the effect of the deterioration of the water quality during transport.

The stress index could then be used to determine if there was a seasonal effect on the susceptibility of the prawns to stress, and to determine if there was any correlation between how stressed the prawn was upon arrival at the hatchery and its later reproductive success. This data is still being collected by measuring the stress levels of prawns at regular intervals throughout the year and comparing their reproductive performance with prawns held in controlled conditions.

5.3 Results and Discussion

5.3.1 Determination of a stress index

A methodology was first developed for obtaining and handling prawn haemolymph suitable for analysis. The best method tried was to extract haemolymph from the pericardial cavity with a syringe and centrifuge the sample immediately. The supernatant serum was then collected and could be used in the assays without clotting.

A consistent pattern of ionic change was obtained in prawns subjected to a variety of stressors. The observed change was an increase in haemolymph magnesium titre and, to a lesser extent, an increase in sodium and chloride titres in prawns under stress. These changes came about when the prawns were subjected to exposure to air (Figure 5.1), simulated transport (Figure 5.2) or to anoxia (Figure 5.3).

Figure 5.1 Changes in the haemolymph titre of *P. monodon* spawners for 7 stress indicators during 2 times of exposure to air.

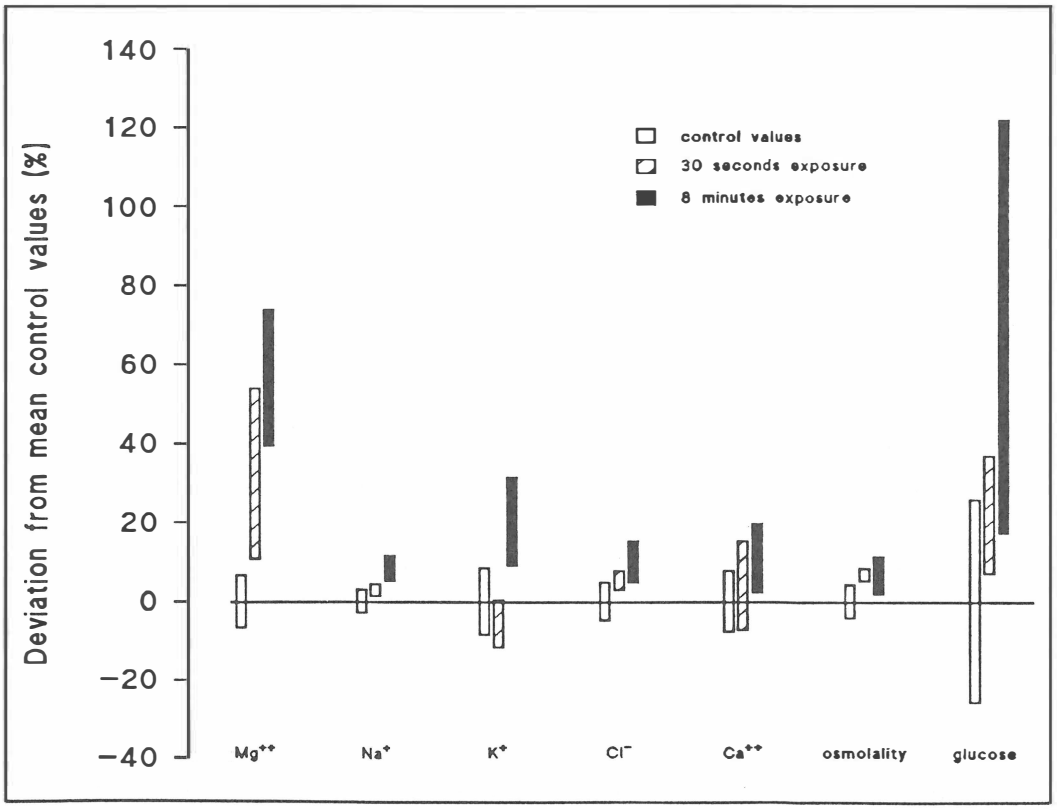
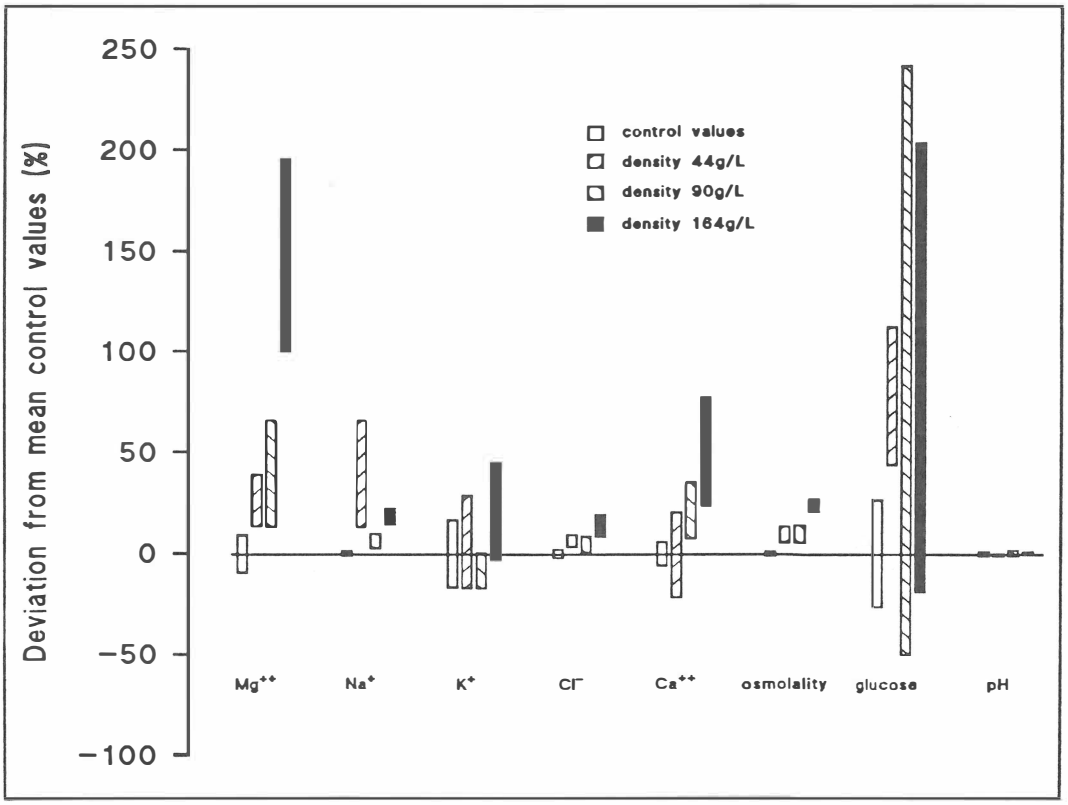


Figure 5.2 Changes in the haemolymph titre of *P. monodon* spawners for 7 stress indicators during simulated transport at 3 different densities.



Prawns arriving at BIARC also had elevated levels of these ions compared with rested prawns (Figure 5.4), and these ions only reached normal resting levels after prawns recovered in fibreglass tanks for one week (Figure 5.5).

Prawns that were assayed and subsequently died also had very high levels of these ions in the haemolymph. Magnesium proved to be the most reliable index of stress in *P. monodon* as it is tightly regulated at rest (around 5 to 7 mM) and increases sharply during stress to values over 20 mM. Mortality of prawns was generally observed when haemolymph magnesium values reach 16 mM.

5.3.2 Stresses associated with capture and transport

Trawling

There was a significant correlation between the length of the trawl and the amount of haemolymph magnesium and sodium in captured prawns, indicating that trawl time has a strong influence on the stress level of prawns. Controlled trawling experiments are needed to further confirm and quantify this result. Non-intermoult prawns (outside moult stages C-D_1) also appeared more susceptible to trawling stress, but again more data is needed to confirm this finding.

Figure 5.3 Haemolymph levels of 4 stress indicators in *P. monodon* broodstock before and after different levels of anoxia
a.Calcium b.Magnesium c.Sodium d.Chloride

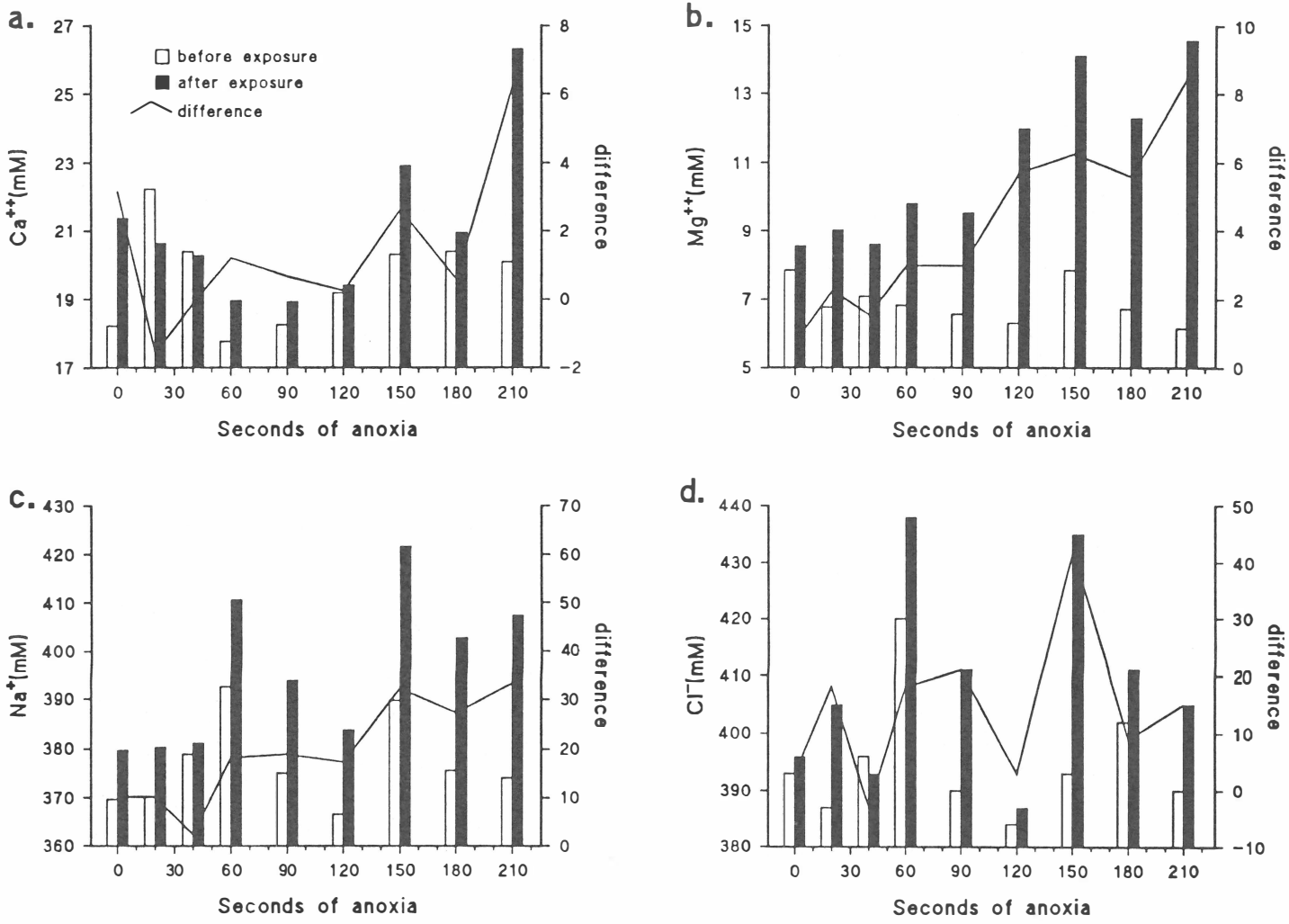
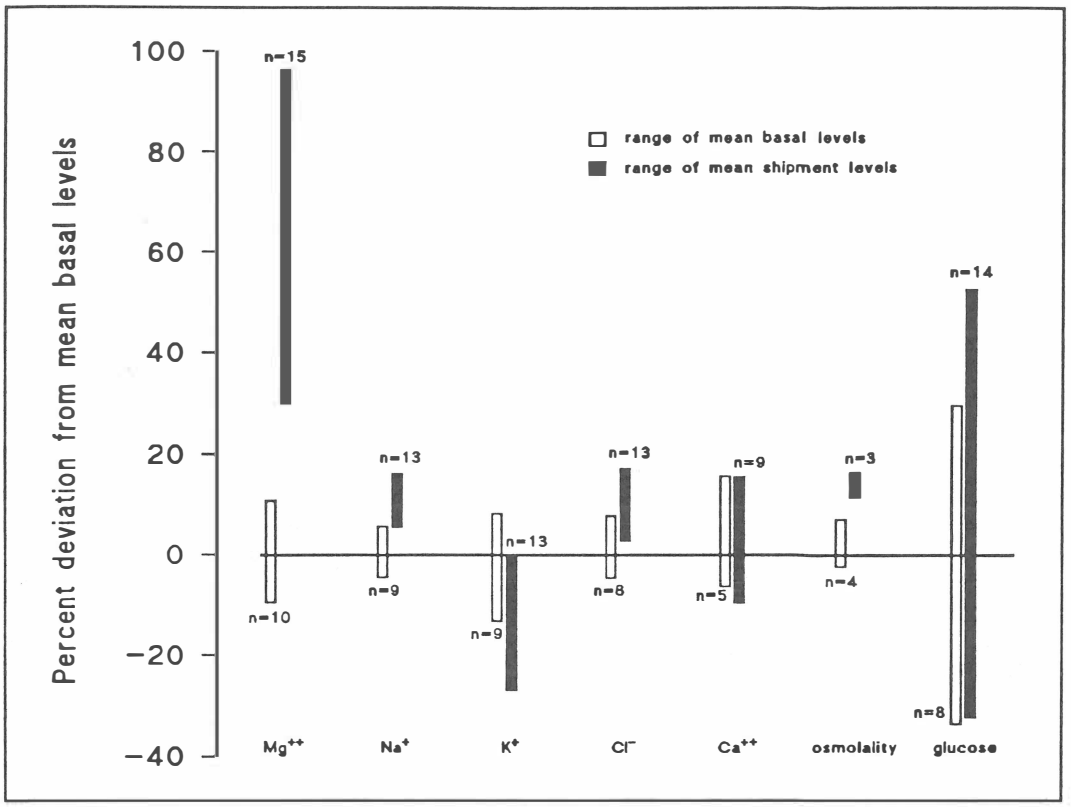


Figure 5.4 Changes in the haemolymph titre of *P. monodon* spawners for 7 stress indicators during simulated transport compared to basal levels of control prawns.



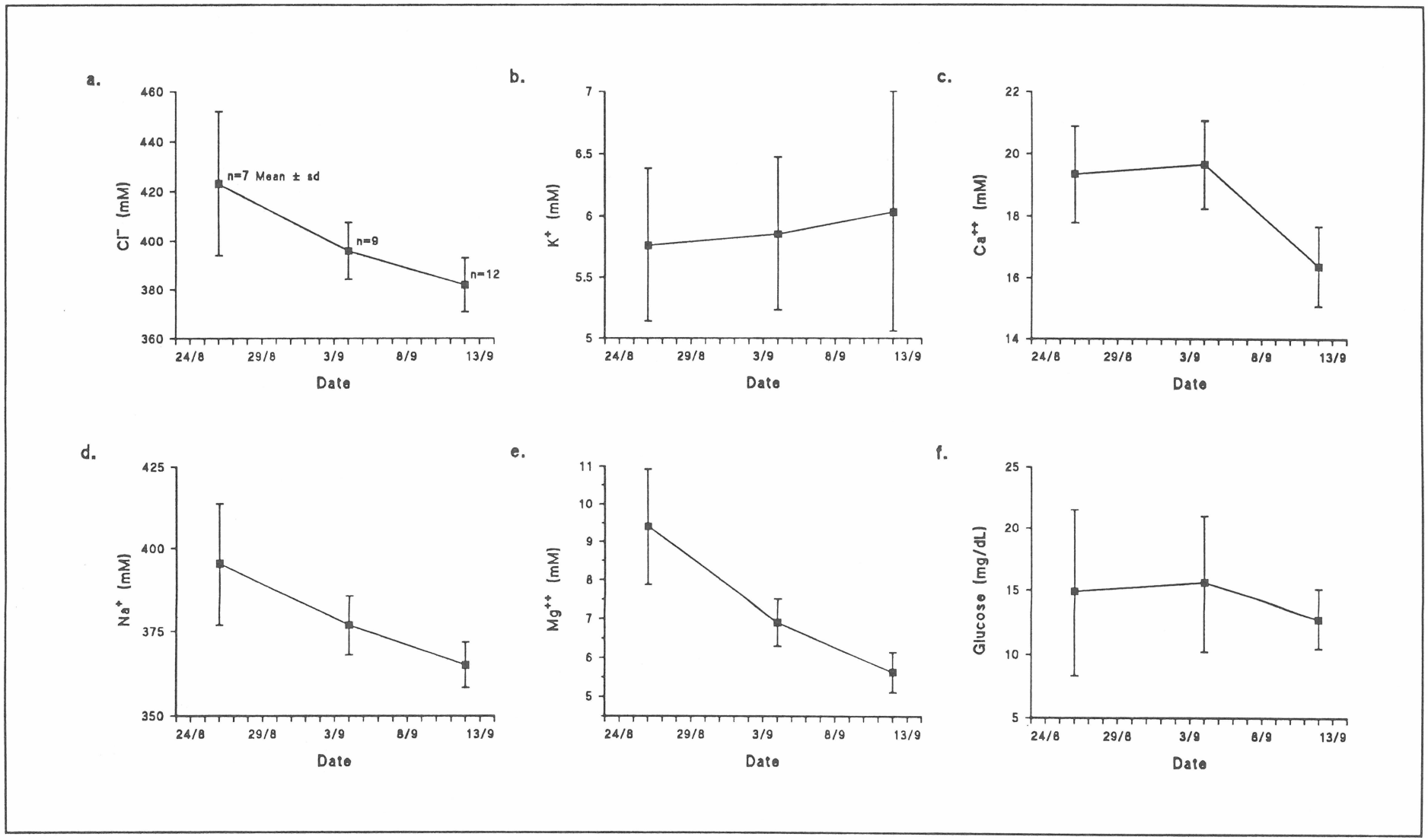
Holding

The ionic changes initiated by the stress of trawling continue for some time during the holding period on board the trawler, resulting in sharp increases in sodium and chloride, and a decrease in the haemolymph potassium and calcium. The amount of time required for the prawns to start recovering from those ionic changes whilst still on the trawler remains to be determined. Non-intermoult prawns also show greater ionic changes in the haemolymph during the holding period.

Transport

The transport procedure in itself is not very stressful to the prawns under normal circumstances. It would seem, therefore, that stress to the prawns occurs mainly as a result of trawling. It takes a prawn several days to recover from being captured and the high stress levels of prawns when they reach the hatchery is primarily due to the stress of trawling. It also seems that the prawns that do die after arrival at the hatchery are much more stressed than the other prawns. An apparent 'runaway effect' is observed in transported prawns; this means that prawns which are already highly stressed after capture become even more so during transport. However prawns with relatively low stress levels seem to recover slightly in the time between packing and reaching the hatchery. In summary, the high level of stress in prawns that subsequently die after reaching the hatchery may result either from an insufficient holding time on board the trawler or a prolonged length of trawl, or both.

Figure 5.5 Haemolymph titres of 6 stress indicators in *P. monodon* broodstock over time **a.**Chloride **b.**Potassium **c.**Calcium **d.**Sodium e.Magnesium **f.**Glucose



Seasonal effects and spawning stressed *P. monodon*

Work is still continuing on the last two aims of this study. Prawns are being assayed for haemolymph levels of stress indicators, particularly magnesium, throughout the year. However, it is too soon to report any trends in this data. Similarly records are continuing to be collected on the stress level of prawns arriving at BIARC, and the correlation between stress level and reproductive success. Already it is apparent that very high levels of stress (>16 mM magnesium) generally result in the death of the prawn. However the relationship between slightly elevated magnesium levels and subsequent reproductive performance is not yet known.

6. Maturation and spawning of *Penaeus japonicus*

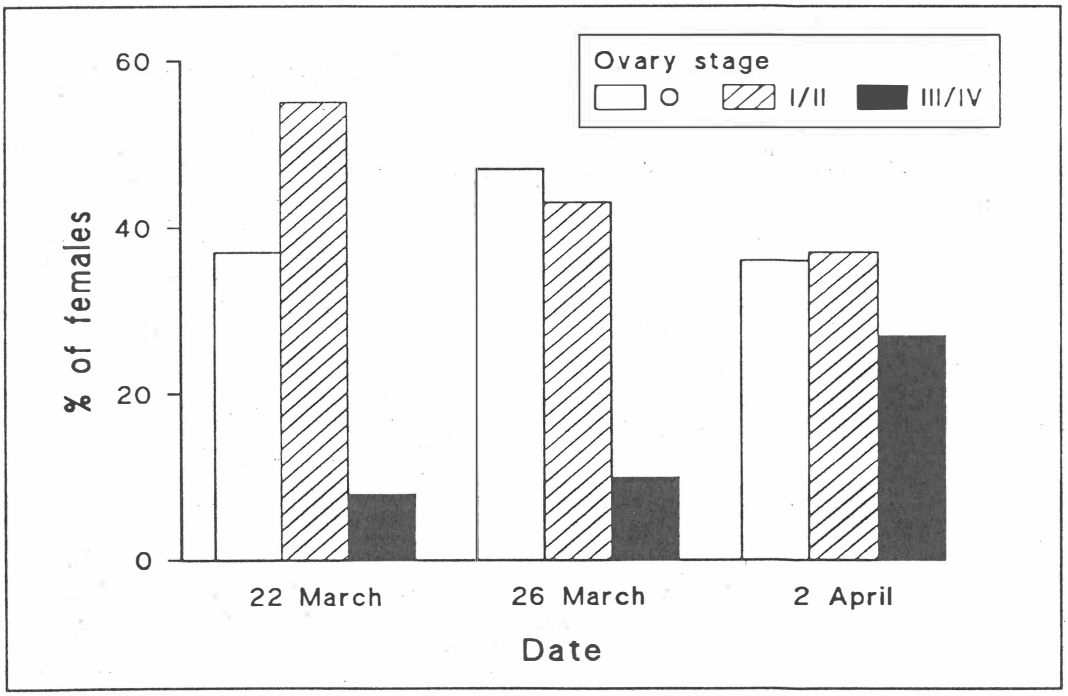
6.1 Introduction

A number of Australian prawn farmers are now beginning to culture the Japanese king prawn, *P. japonicus*, due to the high price this species attracts on the Japanese live seafood market. *P. japonicus* is not found in large numbers in Australian waters, so it is likely that a reliable supply of broodstock will depend on the use of pond reared prawns.

The first prawn farm to stock *P. japonicus* in this country was Moreton Bay Prawn Farm (Cleveland, Qld) which stocked with larvae spawned from wild caught animals in late 1989. At that time a good quality high protein *P. japonicus* diet was not available in Australia. Consequently growth rates were poor and prawns did not reach harvestable size until 70 weeks post stocking. At that time farm workers reported to BIARC that a number of females had mature ovaries.

Prawns were initially sampled and collected for spawning over three separate nights in March/April 1991. Almost 1000 prawns were weighed and females examined for stage of ovarian development and evidence of mating. In excess of 99% of females were mated, but the percentage of mature females varied over the time period studied (Figure 6.1). The population sex ratio was close to 1:1 (male:female) but females were significantly (30%) larger than males (Figure 6.2).

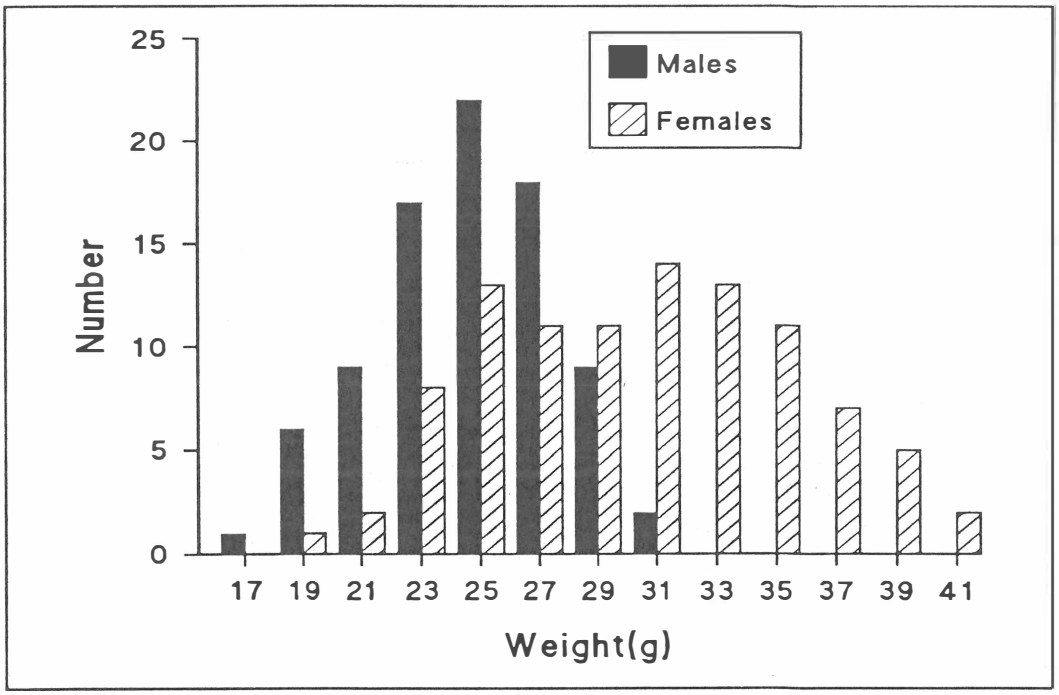
Figure 6.1 Percentage of female *P. japonicus* at various stages of ovarian development over 3 successive examination times



A total of 28 mature (stage IV) females were returned to BIARC but only four spawned. This represented the first time that pond-reared *P. japonicus* were successfully spawned in Australia. Hatch rates and larval survival rates were high (>80% and >90% respectively), but the low success in spawning apparently mature animals suggested that unilateral eyestalk ablation may be necessary to produce reliable supplies of eggs and larvae. This was similar to the results of overseas studies of *P. japonicus* (Lumare, 1981; Caubere *et al.*, 1979).

P. japonicus is generally active at night and will burrow into a soft substrate, if available, during the day. It is normal practice to supply this species with a sand substrate in maturation tanks so that it can burrow (Primavera, 1985). It is difficult for hatcheries to establish and maintain sand bottom maturation tanks and it is not easy to detect the maturation stage of a prawn that is buried. For this reason an experiment was designed to measure the effect of sand versus no sand in maturation tanks on the spawning success of *P. japonicus*.

Figure 6.2 Population distribution of *P. japonicus* from a south-east Queensland prawn farm



6.2 Materials and methods

Two five tonne maturation tanks were established with water flow through, heated to 28°C and controlled low level light. Tanks were identical except that one had an undergravel filter (necessary in a tank with substrate) and sand substrate to a depth of 5 cm. The tanks were stocked with 10 ablated females, 10 non-ablated females and 10 males and then maintained for 20 days. Prawns were fed a measured diet of squid mantle, green lip mussel and pipi. Mature prawns were spawned in separate

drums and larvae grown to the first zoeal stage (Z_1). After the 20 days prawns were removed, the undergravel filter and sand moved to the opposite tank and both tanks restocked with new animals.

6.3 Results and discussion

None of the non-ablated prawns spawned on either substrate. A total of 99 spawns were recorded from ablated prawns, 34 from the sand tanks and 65 from the hard bottom tanks; 57 of these spawns were assayed. From the assayed spawns the hard bottom tank produced 6.26 million eggs and 2.56 million Z_1; the sand tank produced 1.72 million eggs and 1.23 million Z_1. There was no significant difference in fecundity, hatch rate or larval survival on the two substrates (Table 6.1).

The higher rate of prawn maturation on the hard bottom reflected the higher rate of food consumption, which was also evident in the weight gain of non-ablated prawns (Table 6.2). Prawns held on sand buried and did not eat during the day, while those on a hard substrate remained active and ate both night and day. Mating success in both tanks was low, with less than 30% of female moults resulting in mating.

Table 6.1 Mean fecundity, hatch rate and larval survival from assayed spawns of *P. japonicus* held on sand and hard substrates.

	Substrate	Mean number of eggs[A]	Mean HR (%)	Mean survival to Z_1 (%)
$Time_1$	Sand	198 600 (153 700 - 255 400)	81.2	77.9
	Hard	140 600 (79 100 - 233 500)	84.9	73.2
$Time_2$	Sand	153 300 (72 500 - 270 900)	76.5	52.2
	Hard	160 300 (62 000 - 293 000)	70.9	71.4

[A] range in parenthesis

Table 6.2 Ratio of food consumption, spawning success and weight gain of *P. japonicus* held on sand and hard substrates.

	Daily food consumption	No. of spawns	Total eggs spawned[A]	Weight gain[B]
Ratio (sand:hard)	1 : 1.9	1 : 1.8	1 : 2.15	1 : 2.1

[A] estimated, including non-assayed spawns
[B] does not include eye-stalk ablated females

It is considered that the most efficient maturation system for hatcheries not able to rely on consistent supplies of wild caught *P. japonicus* spawners would be the combined use of a large tank or small pond holding facility with a sand bottom for mating and a hard bottom maturation tank. Successfully impregnated female prawns could be removed in early post-moult (stage C), ablated and transfered to the maturation tank. Such animals could then be expected to spawn an average two or three times before they next moulted. The results of this study indicate that although fecundity would decrease significantly over successive fertilised spawnings larval quality would not decrease significantly. Using such a system it would be possible to maximise egg and larval production per prawn, limiting spawner waste and thus keeping hatchery costs down.

Thus while successive generations of this species have been cultured successfully in a number of other countries (Caubere *et al.*, 1979; Lumare, 1981; Nakamura, 1988) it is reassuring that *P. japonicus* from Australian waters can produce high quality larvae in the next generation (that is, from pond reared spawners). Fertilisation and/or mating is more of a problem for this species than for *P. monodon.* However if the protocol suggested above is adopted then the use of pond grown *P. japonicus* spawners is a viable option for commercial hatcheries in Australia.

7. Development of techniques for biochemical analyses

The majority of the analytical support for this project was provided by the QDPI Nutrition Laboratory located at the Animal Research Institute, Yeerongpilly. Much of this work involved the preparation of lipid extracts from prawn tissues and other marine animals and the analysis of those extracts for total lipid, neutral and polar fatty acid profiles and cholesterol levels. Samples were also analysed for dry matter, crude protein, crude fat and amino acid profile.

A range of techniques and procedures applicable only to the analysis of marine samples had to be developed. The large number of small samples and the high lipid levels of these samples necessitated the adaptation of existing equipment and current analytical methods. The systematic approach now employed at ARI was specifically designed for the analysis of lipid and protein in marine tissues and provides a level of accuracy comparable to overseas companies specialising in this area. Cross checking the efficacy of these techniques by analysing a number of subsamples has established that inter replicate variability is very low.

7.1 Marine Samples

Very small samples with high water content posed special challenges and required the development of specific handling techniques. Special homogenisers were purchased to ensure the homogeneity of samples while procedures were developed to ensure that samples stayed frozen to minimise tissue breakdown. As there was often very little sample available, a system was developed to allow the same extract to be subsampled for a number of different analyses. The one subsample was sometimes used sequentially for more than one analysis. For example the same material used for dry matter analysis was also used for protein analysis, and that used for total lipid determination was also used for phospholipid analysis.

Long term storage of marine tissue at very low temperatures, such as -80°C (and occasionally -170°C) coupled with inert gas flushing, was used to prevent the enzymatic degradation of tissue samples that was noted when tissues were held for even short periods at room temperature.

7.2 Advances in Techniques

As there have been many modifications to the lipid extraction procedures of Folch *et al.* (1957) and Bligh and Dyer (1959), procedures suitable for our samples had to be selected and adapted. Techniques to separate lipids into their neutral and polar fractions were developed using pre-packed silica columns to avoid the tedious and time consuming thin layer chromatography procedures originally used. References were later found in the literature which supported our findings. Use of the columns significantly accelerated the preparation process. However it was found that care had to be taken in the selection of solvents because different brands gave different chromatography results. Chloroform from different manufacturers appeared to be particularly variable.

A method for the analysis of cholesterol by gas chromatography was successfully adapted from a number of older methods and incorporated into the suite of analyses used. Instrumentation, particularly gas chromatographs, were improved during the project from very old machines requiring hand injection, to modern instruments using auto-samplers which left staff free for other tasks. These instruments were also better suited to split injection and to the fused silica capillary columns used. Steady improvements in amino acid analysis has provided, for example, a practical alkaline hydrolysis method for tryptophan analysis using reverse phase HPLC.

7.3 Data Handling

Techniques for data handling and the presentation of results have been improved over the life of the project. Fatty acid analyses generate a large amount of data which was initially calculated by hand and took about a day and a half per run to complete. After some experimenting with a number of small spreadsheets and old computers a high performance 80486 PC and a modern spreadsheet, QUATTRO PRO 4.0 were acquired. This drastically reducing calculation time to two hours per run and presented results already in a form suitable for statistical analysis. Graphical presentation of results is now used where applicable.

7.4 Analytical procedures

7.4.1 Proximate Analyses

The moisture content of each sample was determined by oven drying to constant weight at 105°C. Using freeze-dried material, crude protein (N x 6.25) was derived from Kjeldahl nitrogen analysis using copper and selenium as catalysts (AOAC, 1990, method 988.05). Ash was determined as the residue after muffle furnace ignition at 600°C for 2 hours (AOAC, 1990, method 942.05) and ether extract by Soxhlet extraction with petroleum ether (bp 40-60°C) for 6 hours (AOAC, 1990, method 960.39). Ether extract was used as a measure of total lipid content.

7.4.2 Fatty acid analysis

For fatty acid analysis, the lipids were first extracted from pooled samples of each tissue by the method of Folch et al., (1957) using the suggested modifications of Christie (1982). An aliquot of the lipid extract so obtained was separated into polar and nonpolar fractions using Sep-Pak silica cartridges (Waters Associates, MA, USA). The nonpolar fraction was eluted with 15mls chloroform and the polar fraction with 20mls of methanol (Christie, 1982). The solvent was removed from each fraction by rotary film evaporation and the lipids esterified to fatty acid methyl esters (FAME) by the method of Van Wijngaarden (1967). FAME were separated by capillary gas chromatography using split injection on a 30m x 0.25mm i.d. fused silica column coated with 0.25um of Durabond-23 (J and W Scientific, Folsom, California). Column temperature was held at 160°C for 10 minutes and then elevated at 3°C/minute to 210°C where it was held until all FAME of interest had been eluted. FAME were quantified by comparison with the response of an internal standard (heneicosanoic acid methyl ester). FAME were identified by comparing their retention times with those of authentic standards (Sigma Chemical Company, St. Louis, Missouri).

7.4.3 Amino acid analysis

Prior to amino acid analysis, 200mg of finely ground freeze dried sample was hydrolysed with 20ml 6N HCl at 110°C under a nitrogen atmosphere for 18 hours (Finlayson, 1964). Methionine was converted to methionine sulphone by performic acid oxidation prior to acid hydrolysis (Moore, 1963). Amino acid composition was determined by high performance liquid chromatography (Waters 840 Liquid Chromatography System, Millipore Australia, Sydney). Separation was achieved in a protein hydrolysate cation ion exchange column using sodium citrate buffers (Speckman *et al.*, 1958). Detection followed post column derivatization with ninhydrin reagent and measurement of the resultant coloured complexes at 546 nm.

8. Nutritional composition of some species of marine animals and their potential as food for *Penaeus monodon* broodstock

8.1 Introduction

In general prawn hatcheries rely largely on live or fresh-frozen natural foods (Muthu and Laxminarayana, 1982; Harrison, 1990). The choice of diet usually depends on local availability and subjective assessments of reproductive performance. In Australia there tends to be a reliance on fresh-frozen sources of squid, bivalves and polychaete worms.

The nutritional composition of a range of natural marine foods available in Queensland was assessed. Seven species of marine animal (Table 8.1) were sampled and subjected to proximate, amino acid (AA) and fatty acid (FA) analyses. These nutritional profiles were then compared to that of *P. monodon* ovaries for both published results (Dy-Penaflorida and Millamena, 1990) and local prawns. Comparison of nutritional value concentrated on the essential amino acids (EAAs) and the long chain polyunsaturated fatty acids (PUFAs). The analytical techniques used were as described in section 7.

8.2 Results and discussion

Proximate analysis results are presented in Table 8.1, AA levels and EAA indexes (EAAIs) in Table 8.2 and FA content (for 14 to 22 carbon FAs) in Table 8.3. EAAIs are used for evaluating the nutritional value of the protein of a food source on the basis of its AA composition in comparison to the animal tissue of interest, in this case prawn ovary. The EAAI is the n^{th} root of the product of the ratios (food:ovary) of each EAA.

Table 8.1 Moisture, ash, crude protein and lipid levels for some natural marine feeds.

	Species	% Moisture	% Dry matter basis		
			Ash	Crude protein	Lipid
1.	*Loligo chinensis* (Squid)	81.8 ± 0.5	6.6 ± 1.9	84.9 ± 1.8	6.0 ± 1.24
2.	*Perna canaliculus* (Green mussel)	78.3 ± 2.6	10.2 ± 1.4	57.5 ± 1.8	10.4 ± 1.00
3.	*Maryphysa sanguinea* (Mud worm)	81.5 ± 3.7	18.4 ± 3.3	69.2 ± 3.6	4.3 ± 0.41
4.	*Donax deltoides* (Pipi)	80.9 ± 3.1	19.9 ± 3.1	62.5 ± 5.6	3.9 ± 0.90
5.	Chironomid larva (Blood worm)	92.2 ± 2.3	16.9 ± 1.0	63.0 ± 0.07	5.5 ± 0.56
6.	*Amusium balloti* (Scallop)	82.0 ± 1.8	14.4 ± 1.9	83.5 ± 1.2	1.9 ± 0.30
7.	*Onuphidae sp* (Beach worm)	81.3 ± 4.3	14.0 ± 0.80	75.4 ± 2.1	3.3 ± 1.08

Means (n=4) ± standard deviation

Table 8.2 Essential amino acid content (% dry organic matter) for species analysed Means (n = 4) in columns with same superscript are not significantly different (P > 0.05).

Species	Arginine	Histidine	Isoleucine	Leucine	Lysine	Methionine	Phenylalanine	Threonine	Valine	EAAI
Squid	7.41	1.54^{cde}	3.77^{c}	6.95	6.77	2.10^{b}	3.18^{b}	3.69	3.57^{c}	0.94
Green Mussel	4.85^{ab}	1.95^{b}	2.40^{a}	3.83	3.87^{abc}	1.19^{a}	2.36^{a}	2.51	2.48^{b}	0.99
Mud worm	5.89^{a}	1.59^{c}	2.86^{a}	5.34^{ac}	4.82^{a}	1.22^{a}	2.61^{a}	3.01^{a}	2.96^{a}	0.95
Pipi	5.90^{a}	1.19^{ab}	2.73^{a}	5.07^{a}	4.24^{abc}	1.33^{a}	2.33^{a}	3.00^{a}	2.84^{ab}	0.94
Chironomid larva	4.75^{b}	1.69^{c}	3.62^{bc}	6.12^{b}	4.26^{a}	1.33^{a}	3.49^{b}	3.10^{a}	3.50^{c}	0.97
Scallop	8.65	1.31^{ad}	3.19^{ab}	5.96^{bc}	5.81	1.81^{b}	2.72^{a}	3.00^{a}	3.01^{a}	0.91
Beach worm	5.95^{a}	1.18^{af}	2.77	5.21^{ad}	4.34^{abc}	1.37^{a}	2.40^{a}	3.06^{a}	2.90^{a}	0.94
S.E.	±0.374	±0.085	±0.169	±0.217	±0.316	±0.128	±0.153	±0.116	±0.110	
**P. monodon*	6.34	2.31	4.60	7.76	6.54	2.80	5.75	4.79	5.69	

*(Dy-Penaflorida and Millamena, 1990 [% Dry Matter])

Table 8.3 Fatty acid content (% dry organic matter) for species analysed Means (n = 4) in columns with the same superscript letter are not significantly different (P > 0.05).

Species	14	16	16:1	18	18:1	18:2	18:3	20	20:1	20:4	20:5	22:6	n-3:n-6 Ratios
Squid	0.11^{cd}	0.92^{be}	0.02^{b}	0.21^{a}	0.14^{bc}	$-.^{a}$	$.-^{a}$	$.-^{a}$	0.08^{c}	0.14^{b}	0.41^{c}	1.35^{c}	12.6
Green Mussel	0.35	1.25	0.51^{a}	0.31	0.29	0.14	0.13^{b}	$.-^{a}$	0.17	0.10^{a}	1.05	1.29^{c}	10.3
Mud worm	0.03^{abd}	0.33^{ace}	0.05^{b}	0.16^{a}	0.20^{c}	0.06^{b}	0.03^{a}	$.-^{a}$	0.04^{cb}	0.18^{b}	0.28^{bc}	0.03^{b}	1.4
Pipi	0.70^{a}	0.40^{a}	0.49^{a}	0.21^{a}	0.12^{ac}	0.02^{ab}	0.03^{a}	$.-^{a}$	0.02^{ab}	0.07^{a}	0.17^{ab}	0.43^{a}	7.0
Chironomid larva	0.19^{c}	0.81^{b}	0.40^{a}	0.43	0.74	1.16	0.16^{b}	0.06	$.-^{a}$	0.10^{a}	0.10^{a}	0.02^{b}	0.2
Scallop	0.01^{b}	0.17^{c}	$.-^{b}$	0.09^{b}	0.05^{a}	$.-^{a}$	$.-^{a}$	$.-^{a}$	$.-^{a}$	0.06^{a}	0.09^{a}	0.35^{a}	7.3
Beach worm	0.03^{ab}	0.17^{c}	0.03^{b}	0.10^{b}	0.08^{ab}	0.02^{ab}	$.-^{a}$	$.-^{a}$	0.06^{c}	0.08^{a}	0.15^{ab}	0.2	3.5
Average S.E.	±0.011	±0.056	±0.091	±0.020	±0.030	±0.019	±0.017	±0.002	±0.007	±0.014	±0.055	±0.040	
P. monodon	0.25	2.30	1.15	0.87	2.25	0.13	0.0	0.0	0.1	0.78	1.25	1.45	3.0
S.E.	±0.03	±0.224	±0.114	±0.054	±0.148	±0.021	±0.0	±0.0	±0.035	±0.041	±0.074	±0.09	

The percentage lipid, in comparison to protein levels, was low for all species. Green lip mussel had the highest lipid level and scallop the lowest, while protein levels varied from 84.9% (squid mantle) to 57.5% (green lip mussel). Despite statistically significant differences between species for single EAA's the profiles of all the species show a similar pattern. All seven species had a high EAAI (>0.90) and are therefore considered as good protein sources (Murai *et al.*, 1984). Squid mantle, however, was the most concentrated source of EAAs and, of all species analysed, it may best meet the protein requirements of *P. monodon* broodstock.

FA profiles were markedly different between species, particularly the levels of PUFAs. *P. monodon* ovaries are high in 20:4n-6, 20:5n-3 and 22:6n-3 FAs. Squid mantle and green lip mussel FA profiles most closely resemble that of *P. monodon* ovaries as both are relatively high in these three PUFAs (Table 8.3).

In conclusion, on the basis of their EAA and FA profiles, a combination of squid mantle and green lip mussel appears to provide a good foundation for the fresh component of a *P. monodon* maturation diet. Both of these food sources are readily available in Australia in bulk packs of fresh-frozen product and are used as a base diet for the maturation studies conducted at BIARC.

9. The effect of starvation and ablation on the composition of ovary and hepatopancreas tissues in *Penaeus monodon* broodstock

9.1 Introduction

A number of studies have examined the qualitative and quantitative changes in body composition occurring in crustaceans as a result of starvation. (Cuzon *et al.*, 1980; Regnault, 1981). Most species deplete reserves of protein, lipid and carbohydrate, however, the relative importance of each appears to depend on the duration of starvation (Cuzon *et al*, 1980) and to be species dependant. The extent to which various tissues contribute reserves has also been a subject of research (Barclay *et al.*, 1983; Schirf, *et al.*, 1987). Comparisons of the composition of tissues including the ovary have been done for penaeid broodstock (Millamena and Pascual, 1990; Dy-Penaflorida and Millamena, 1990), however, data on ovary tissue compositional changes with starvation is limited.

Ablation significantly increases the growth rate of *P. monodon* (Nan *et al.*, 1993). It also promotes ovary development (Primavera, 1984), decreases blood glucose level (Keller *et al.*, 1985) and increases respiratory rates (Rosas *et al.*, 1991).

The ovary behaves as a sink for nutrients under normal circumstances, a situation that is exacerbated by ablation. However, during starvation the ovary might conceivably act as a source of nutrients for the prawn. Imposing both ablation and starvation simultaneously may shed light on the mechanics underlying ablation. It would be of interest to know whether ablation can influence the composition of the early stage ovary while there is a net loss of nutrients from both the ovary and hepatopancreas due to starvation. Also, identifying those components such as fatty acids which are readily lost from the ovary during starvation may assist in the interpretation of spawning results from prawns fed maturation diets that are poor in those components.

This study examined the effect of short term (10 day) starvation on the composition of ovary and hepatopancreas tissues of ablated and non-ablated *P. monodon* broodstock.

9.2 Materials and methods

P. monodon broodstock between 90 and 100 grams in size were captured from waters off Cairns in northern Queensland. Prawns which exhibited no visual signs of ovary development were air freighted to the Bribie Island Aquaculture Research Centre and upon arrival were weighed and moult staged. Twenty prawns in the postmoult and early intermolt (B-C) stage were selected for the experiment, four of these were killed immediately to act as controls and their ovary and hepatopancreas tissue removed for biochemical analysis. The remaining prawns were individually housed in black polyethylene tanks (0.9 l x 0.5 d x 0.6 w) with seawater of 36 ppt and 28°C continuously exchanged at a rate of 100% per day. Each prawn was then randomly allocated to one of four treatment groups: fed, fed and ablated, starved, starved and ablated. There were eight prawns per treatment. The fed prawns received pipi (*Donax deltoides*) mantle to *ad lib*. and food intake was monitored to confirm that feeding was occurring. On day 10 treatment prawns were killed and their ovary and

Table 9.1 Mean level (mgs per 100 g prawn) of protein, lipid and cholesterol in the protein and ovary tissues and GSI and HSI values for each treatment group; F = fed; FA = fed and ablated; S = starved; SA = starved and ablated. Superscript denote treatments that are **not** significantly different.

Treatment	Ovary					Hepatopancreas					GSI	HSI
	Dry matter (%)	Protein (mg)	Lipid (mg)	Cholesterol (mg)	(%)	Dry matter (%)	Protein (mg)	Lipid (mg)	Cholesterol (mg)	(%)		
Control	27.8^{a}	632	200	8.2	7.7^{a}	31.5	198^{a}	173^{a}	1.8^{a}	4.8^{a}	3.7 ± 0.5	2.4^{ab} ± 0.1
F	24.1^{c}	346^{a}	74^{bc}	4.0^{a}	7.9^{a}	37.8^{a}	353^{b}	345^{b}	5.0^{b}	6.1^{a}	2.2^{a} ± 0.3	2.6^{b} ± 0.2
FA	26.6^{ca}	400^{a}	99^{c}	4.8^{a}	8.9^{a}	37.5^{a}	320^{ab}	300^{ba}	3.2^{a}	4.4^{a}	2.4^{a} ± 0.5	2.3^{a} ± 0.1
S	19.2^{b}	134^{b}	15^{a}	1.5	7.5^{a}	20^{b}	189^{a}	30^{c}	2.5^{ca}	9.4^{b}	1.2 ± 0.1	1.6 ± 0.1
SA	20.3^{b}	283^{ba}	38^{ab}	3.2^{a}	7.5^{a}	21.6^{b}	297^{ab}	43^{c}	4.0^{cb}	9.3^{b}	2.0^{a} ± 0.3	2.1^{a} ± 0.3

tissues were removed for biochemical analysis. The ovary and hepatopancreas of each prawn was weighed so that somatic indices could be calculated. The Gonad Somatic Index (GSI) and the Hepatopancreas Somatic Index (HSI) were calculated as the wet weight of the respective organs, expressed as a percentage of the wet weight of the prawn. Moult stage was determined by a modified scheme of Drach and Tchernigovtzeff (1967). Biochemical analysis of tissue samples was carried out according to the methods previously outlined in Section 7. Statistical analysis involved analysis of variance (ANOVA) to assess treatment effects.

9.3 Results

Table 9.1 shows the mean levels (mg per 100g prawn and percentage in dry tissue) of protein, lipid and cholesterol in the ovary and hepatopancreas of prawns in each treatment group. The mean GSI and HSI are also presented in this table.

Figure 9.1 shows graphically the percentage of protein and lipid in the ovary and the hepatopancreas for each treatment group. Ablation had the effect of maintaining tissue size in both the ovary (GSI) and hepatopancreas (HSI) of starved prawns (Table 9.1). While the corresponding average lipid and protein levels were also higher these were not significant ($P > 0.05$). Ablation had no significant effect on hepatopancreas reserves of the fed prawns, however in the ovary of fed prawns ablation caused a significant increase in the percentage of lipids.

Tables 9.2 and 9.3 show the mean levels of fatty acids in the neutral and polar lipid fractions of the ovary and hepatopancreas tissues respectively, in each treatment group. Absolute levels (expressed as mg per 100g prawn), show that all fatty acids in both tissues were depleted during starvation. However, some fatty acids were depleted more than others and the percentage compositions reflect these relative changes. The fatty acids whose ratio increased following starvation represent those that were preferentially retained or were in less demand as an energy source by the prawn.

Figure 9.2 shows the percentage composition of 20:4n-6, 20:5n-3 and 22:6n-3 in the polar and neutral lipid fractions of the ovary and hepatopancreas tissues in each treatment group. These three fatty acids are of particular interest because they showed the most differences between treatments and are considered to be essential in the diets of penaeid prawns (Kanazawa, 1981).

9.4 Discussion

Both the ovary and hepatopancreas tissues lost protein and lipid as a result of starvation. In comparison to the hepatopancreas, the ovary lost more protein than lipid. On average the ovary contributed 212mg of protein and 69mg of lipid (per 100g prawn) during the ten days starvation. The hepatopancreas contributed only 94 mg of protein but 315mg of lipid. The ovary and hepatopancreas contributed equal quantities of cholesterol during starvation.

Thus, while reserves of lipid in the ovary were depleted at a proportionately greater rate than protein, they made a relatively small contribution in absolute terms. The

Table 9.2. Mean fatty acid levels (% of total fatty acids) and total levels (mg per 100 g animal) polar and neutral fraction of ovary tissue from treatment groups (0 = <0.5%). Means with same superscripts are **not** significantly different.

Lipids	Treatment	14	16	16:1	18	18:1n-7	18:1n-9	18:2	18:3	20:1n-7	20:1n-9	20:1n-11	20:4n-6	20:5n-3	22:6n-3
Neutral (mg)	C	1.35^{a}	11.39^{a}	7.51^{ac}	2.69^{a}	2.21^{a}	7.37^{a}	0.47^{a}	0.10^{a}	0.35	0.25^{a}	0.43	2.72	4.79	6.05^{a}
	F	0.45^{cb}	4.84^{ab}	2.34^{b}	1.02^{bc}	0.84^{b}	3.32^{bc}	0.17^{a}	0.07^{ba}	0.13^{a}	0.10^{ab}	0.17^{a}	0.84^{a}	1.45^{a}	2.77^{bc}
	FA	0.70^{ca}	7.64^{ba}	3.13^{cab}	1.92^{ca}	1.06^{ab}	4.93^{ba}	0.22^{ab}	0.05^{ba}	0.12^{a}	0.17^{a}	0.19^{a}	1.16^{a}	1.73^{a}	5.37^{ca}
	S	0.02^{b}	0.24	0.07^{b}	0.09^{b}	0.03^{c}	0.15	0.01^{b}	0.0^{b}	0.0^{a}	0.0^{b}	0.0^{a}	0.07^{a}	0.08^{b}	0.09^{b}
	SA	0.13^{b}	1.79^{b}	0.65^{b}	0.41^{b}	0.34^{c}	1.30^{c}	0.06^{b}	0.0^{b}	0.04^{a}	0.03^{b}	0.06^{a}	0.51^{a}	0.66^{ba}	0.59^{b}
Polar (mg)	C	0.48	7.47	3.72	4.53	2.31	7.34	0.49	0.03^{a}	0.14^{a}	0.27^{a}	0.46	4.63	6.11	5.29^{a}
	F	.14^{a}	3.19^{a}	1.25^{a}	1.80^{a}	0.73^{a}	2.92^{a}	0.16^{a}	0.03^{a}	0.06^{ba}	0.11^{b}	0.11^{a}	2.19^{a}	2.61^{a}	2.63^{b}
	FA	0.17^{a}	3.55^{a}	1.36^{a}	2.16^{a}	0.73^{a}	3.29^{a}	0.16^{a}	0.03^{a}	0.05^{b}	0.15^{ab}	0.13^{a}	1.94^{a}	2.49^{a}	3.30^{ba}
	S	0.02^{a}	0.72^{b}	0.14^{a}	0.40	0.17^{b}	0.55	0.04^{a}	0.0	0.0^{b}	0.0^{c}	0.0	0.72^{b}	0.66^{b}	0.35^{c}
	SA	0.06^{a}	1.95^{ba}	0.55^{a}	1.17^{a}	0.50^{ba}	1.64^{a}	0.08^{a}	0.0	0.0^{b}	0.04^{a}	0.06^{a}	1.52^{ba}	1.47^{ba}	0.88^{c}
Neutral % FA	C	1.9^{a}	19.9^{a}	10.2^{a}	4.9^{a}	4.5^{a}	13.9^{a}	1.0^{a}	0.2^{a}	0.8	0.5^{a}	1.0^{a}	5.5^{a}	9.3^{a}	10.7^{ba}
	F	1.8^{a}	24.6^{c}	8.3^{a}	6.6^{ab}	4.0^{a}	15.9^{b}	0.5^{b}	0.1^{ba}	0.5^{a}	0.3^{ba}	0.7^{ba}	5.4^{a}	6.2^{cb}	12.2^{a}
	FA	1.8^{a}	22.6^{ca}	8.9^{ca}	5.8^{a}	3.4^{ba}	14.8^{ba}	0.6^{ba}	0.2^{a}	0.3^{ab}	0.5^{a}	0.5^{b}	3.8^{a}	5.4^{b}	16.0
	S	1.9^{a}	26.3^{b}	4.9^{b}	11.0	2.5^{b}	15.4^{b}	0.1	0.0^{b}	0.0	0.0^{c}	0.1	10.6^{b}	8.9^{a}	7.7^{b}
	SA	1.7^{a}	23.6^{cba}	6.4^{cb}	8.0^{b}	3.3^{ba}	15.4^{b}	0.5^{b}	0.0^{b}	0.3^{b}	0.2^{cb}	0.4^{b}	8.5^{b}	8.2^{ca}	8.2^{b}
Polar % FA	C	0.9^{b}	14.5^{c}	7.1	8.2^{a}	4.5^{a}	13.9^{ca}	0.9^{a}	0.1^{a}	0.4	0.5^{a}	0.9	9.1^{a}	11.7^{a}	9.9
	F	0.6^{ca}	15.1^{cb}	4.9^{ba}	8.3^{a}	3.4^{b}	12.4^{b}	0.8^{ba}	0.1^{a}	0.1^{a}	0.5^{a}	0.4^{a}	11.2	12.3^{ba}	11.5
	FA	0.7^{bc}	15.3^{b}	5.3^{a}	8.5^{a}	3.1^{b}	13.4^{cba}	0.7^{b}	0.1^{a}	0.1^{a}	0.6^{a}	0.4^{a}	9.6^{a}	11.7^{a}	13.2
	S	0.5^{a}	16.5^{ab}	3.2^{c}	9.1^{a}	3.8^{c}	12.4^{b}	0.9^{a}	0.0^{b}	0.0^{a}	0.1^{b}	0	16.5	14.6	7.8^{a}
	SA	0.5^{a}	17.1^{a}	4.1^{cb}	9.3^{a}	4.2^{ca}	13.3^{ba}	0.8^{ba}	0.0^{b}	0.0^{a}	0.2^{b}	0.3^{a}	14.3	13.2^{b}	8.1^{a}

hepatopancreas was more important than the ovary as a source of lipid, while the ovary made a significantly higher contribution to the protein requirements. In terms of energy, using an average energy density for lipids of 9.5 kcal per gram and 4.5 kcal per gram for protein (Gordon *et al.*, 1977), the ovary contributed a total of 1609 kcal and the hepatopancreas 3425 kcal to the prawns total energy requirements during starvation. Thus, in *P. monodon* broodstock, both tissues provide energy substrates during short periods of dietary deprivation.

Ablation maintained the size of the ovaries countering the tendency of the GSI to fall during starvation. This suggests that Vitellogenesis Inhibiting Hormone (VIH), the level of which is reduced by ablation, is involved at the very early stages of ovary development and possibly may initiate it. This is contrary to the theory that the reduction in VIH caused by ablation was only effective at the secondary stages of vitellogenesis and that a stimulating hormone was required as an initial cue (Santiago,1977). The study on compositional changes occurring during ovary development also examined the effect of ablation and showed that ablation may have accelerated the onset of secondary vitellogenesis (Section 11). It would therefore appear that levels of VIH have an effect on undeveloped ovaries and may be involved, with or without a stimulatory hormone, in initiating ovary development.

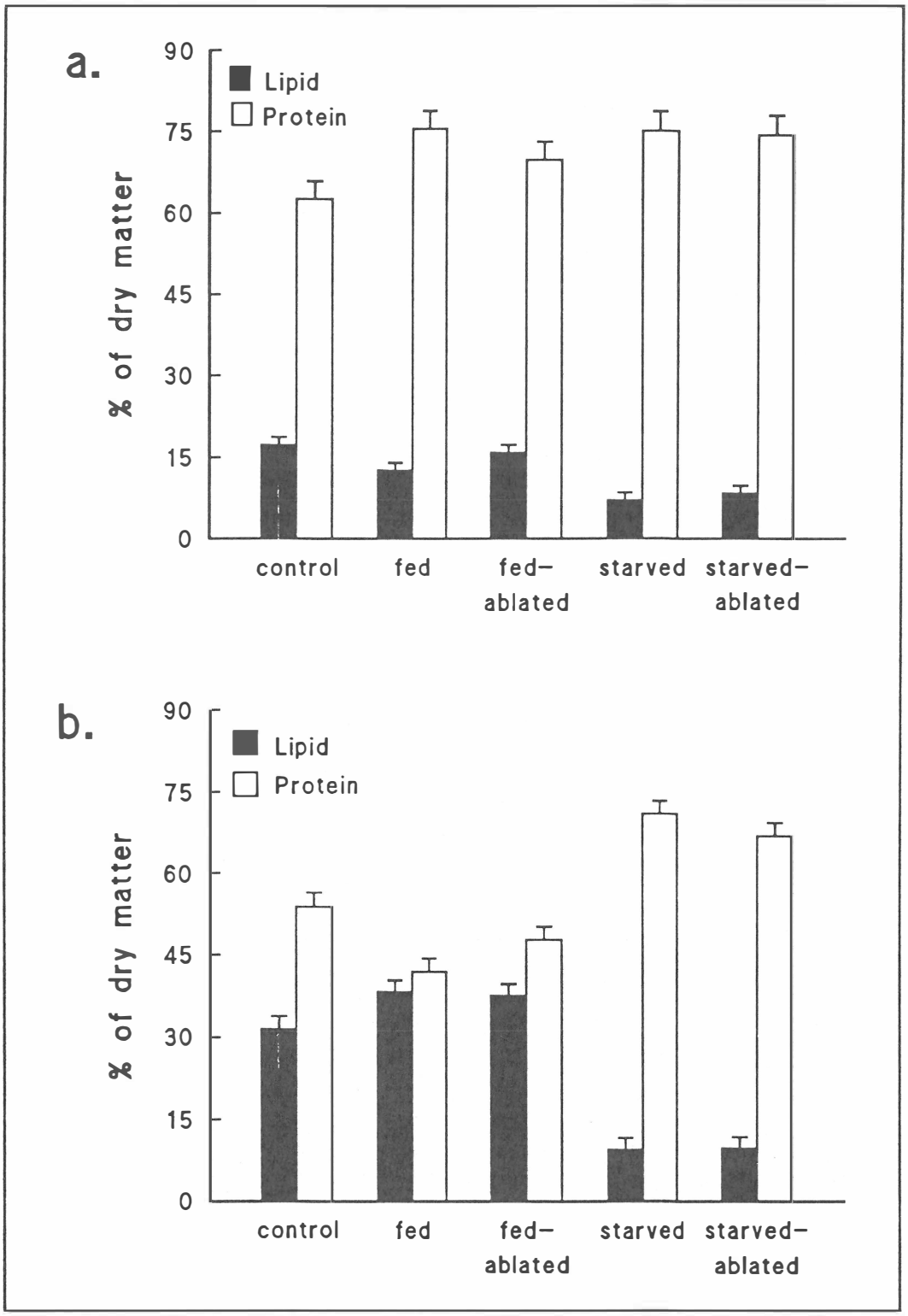
At a more detailed level, starvation resulted in significant changes in the ratios of some fatty acids in both the ovary and hepatopancreas tissues. Interesting trends were apparent for 20:4n-6 (arachidonic acid), 20:5n-3 (eicosapentanoic acid) and 22:6n-3 (docosahexanoic acid). The 20:4n-6 and 20:5n-3 fatty acids tend to have the same response as each other to the various treatments. When the prawn was starved, the ovary appeared to retain both polar and neutral classes of these fatty acids in preference to 22:6n-3. The hepatopancreas also retained neutral and polar 20:5n-3 and neutral 20:4n-6 fatty acids during starvation though polar 20:4n-6 is depleted in this tissue. In contrast, the 22:6n-3 fatty acid was selectively depleted from polar and neutral lipids in the ovary and hepatopancreas.

The selective retention of 20:4n-6 and 20:5n-3 fatty acids by the tissues during starvation can be interpreted in two ways. Firstly, these fatty acids may be acting in a structural capacity and are not readily mobilised or secondly, there is little demand for these particular fatty acids by the prawn during starvation. The preferential use of 22:6n-3 may reflect its suitability as an energy source, its lack of a structural role in the tissues or its greater abundance relative to other fatty acids.

The quantities of each of these fatty acids supplied by the tissues also indicated a preferential use of 22:6n-3 followed by 20:5n-3 then 20:4n-6 fatty acids. While the hepatopancreas provides five times as much from the neutral lipid fraction as from the polar, the ovary provided slightly more 20:4n-6 and 20:5n-3 fatty acids from the polar fraction and roughly equal amounts of 22:6n-3 from the polar and neutral fractions.

The large supply of fatty acids from the neutral lipid fraction of the hepatopancreas is in keeping with its role as a major lipid storage tissue in prawns (Gibson and Barker, 1979). Neutral lipids, particularly the triacylglycerides (Section 10) are a concentrated source of stored energy (Tacon, 1990). The significant contribution from the polar lipids in the ovary may relate to the weight of protein that was depleted. The depletion of both indicates that, rather than just the use of storage products to supply energy, tissues are also being broken down to meet energy requirements.

Figure 9.1 The effect of starvation and ablation on the proportion of lipid and protein in *P. monodon* a.Ovary tissue b.Hepatopancreas tissue



Ablation had no significant effect on the percentages of these three fatty acids in the hepatopancreas of fed or starved prawns. However, in the ovary tissue of the fed prawns, ablation significantly ($P < 0.05$) increased the percentage of the 22:6n-3 fatty acid in both the neutral and polar lipid fractions. Also, when prawns were starved, ablation resulted in small but significant ($P < 0.05$) depletion of 20:4n-6 and 20:5n-3 fatty acids from the polar lipids in the ovary, with no change in the percentage of 22:6n-3. This would indicate that following hormonal cues to commence development the ovary retains or accumulates the 22:6n-3 fatty acid preferentially.

While there were differences between the control group and the fed group in tissue size and lipid to protein ratios of both the ovary and hepatopancreas, only a few fatty acids showed significant differences in percentage composition. This was surprising given that diet has been shown to affect fatty acid ratios in tissues (Millamena, 1989) and that the diet of the control group prawns was likely to be different to the pipi diet of the fed group (See Section 8 for composition).

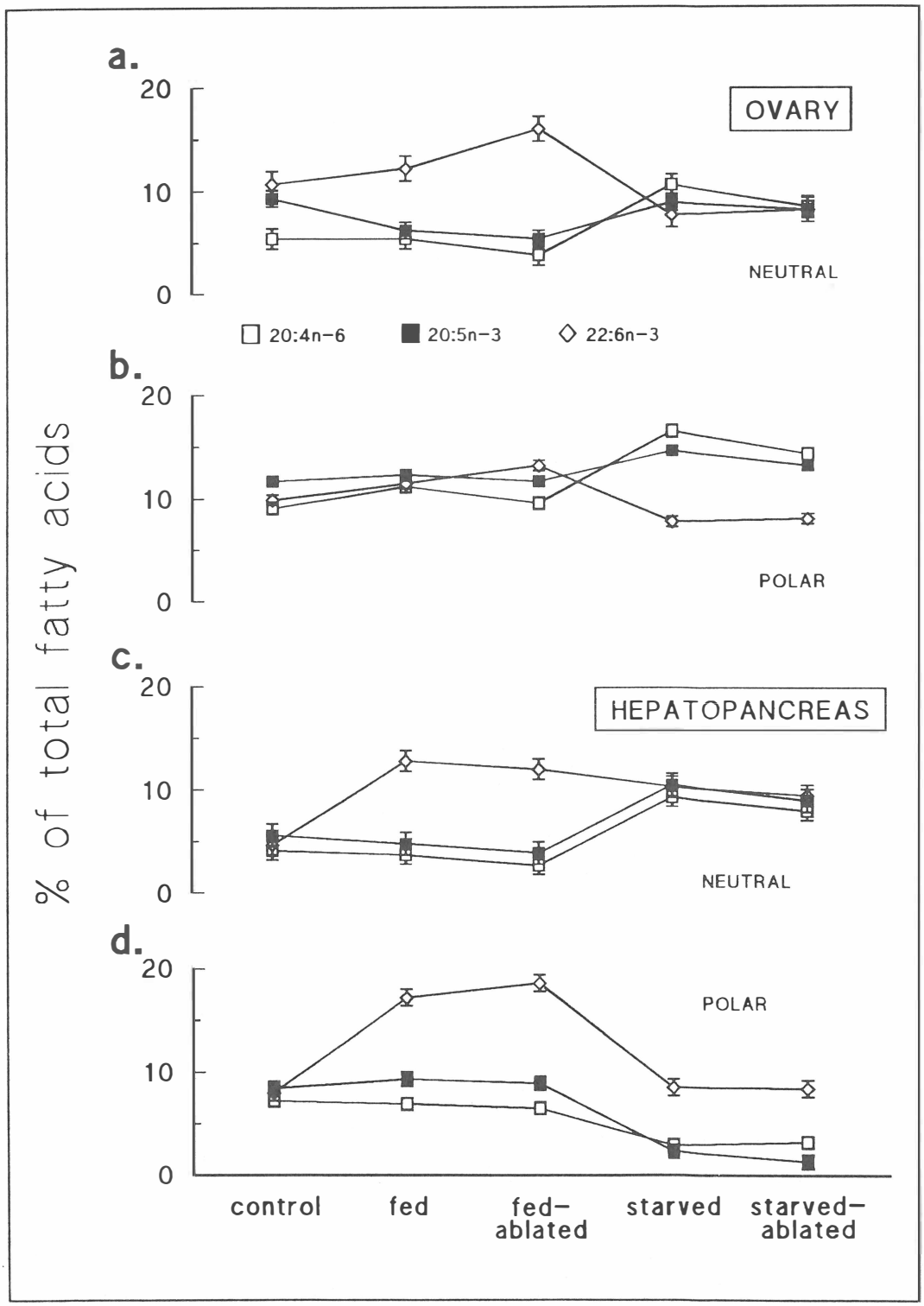
Fatty acids 20:4n-6 and 22:6n-3 were lower in the polar lipids in the ovaries of the control group than they were in the ovaries of fed prawns. The ovaries of the control group prawns contained higher levels of the 20:5n-3 in the neutral lipids than the ovaries of fed prawns. The hepatopancreas' of control group prawns contained less 22:6n-3 fatty acid in both neutral and polar fractions than prawns fed the experimental diet. While the prawns in the control group were at a more advanced stage of ovary development, these observed differences in fatty acid levels in the hepatopancreas and ovary are more likely to be a result of diet than developmental stage as it has been shown that fatty acid ratios change little with ovary development (Teshima *et al.*, 1989). Other prawns captured at the commencement of this experiment proved to be good broodstock in terms of egg quality (BIARC, unpublished data), thus these results may indicate that the higher percentage of 20:5n-3 in the ovary is of importance to the development of good quality eggs.

9.5 Conclusions

The ovary is used as a storage tissue by *P. monodon*, however during short periods of starvation it contributes only half the energy contributed by the hepatopancreas. Even when the ovary is acting only as a storage tissue, that is, prior to ovary development, it has a pattern of nutrient accumulation that is different to other storage tissue. The pattern of fatty acid accumulation in undeveloped ovaries is influenced by diet but the ratio of lipid to protein in the tissue appears to be more conservative. Unlike the hepatopancreas, the ovary stores equal portions of lipids in the polar and neutral classes, because of its primary role as a reproductive tissue. Major storage tissues, such as the hepatopancreas, respond to a dietary surplus by increasing the amount of stored neutral lipids rather than polar lipids.

During a ten day starvation period the levels of all fatty acids in both the hepatopancreas and ovary tissue decreased, but the magnitude of the change was greater for particular fatty acids. During starvation the greater reduction in the level of 22:6n-3 relative to 20:4n-6 and 20:5n-3 fatty acids in the ovary and hepatopancreas could be interpreted as, (i) stored fatty acid 22:6n-3 being used preferentially by the prawn to satisfy metabolic requirements when dietary supply is

Figure 9.2 The percentage composition of fatty acids 20:4n-6, 20:5n-3 and 22:6n-3 in *P. monodon* tissues **a.** Ovary neutral fraction **b.** Ovary polar fraction **c.** Hepatopancreas neutral fraction **d.** Hepatopancreas polar fraction



lacking, or (ii) the retention of 20:4n-6 and 20:5n-3 may indicate their importance to the integrity of the tissue and hence their preferential conservation, or (iii) the dietary supply of 22:6n-3 fatty acid to captive prawns was higher than that of 20:5n-3 and

20:4n-6 fatty acids so the surplus stored in the ovary was readily mobilised from the tissue and used to satisfy energy needs.

Thus, the ovary does provide a source of nutrients for prawns, however ablation reduces this role triggering a retention and/or accumulation of nutrients in undeveloped ovaries, presumably as a first step in the process of vitellogenesis.

While ablation reduces the level of vitellogenesis inhibiting hormone (VIH) the mode of action and target tissues of the hormone have not been clearly identified. The results from this study (as well as studies presented in Section 4 and 11) suggest VIH is operative at the very early stages of vitellogenesis and its reduction may initiate ovary development. Further work is needed to clarify the role of VIH, but these results are contrary to the model proposing that a stimulatory hormone, acting directly on the ovary tissue, is responsible for initiating ovary development (Khoo, 1988). If a stimulatory hormone is involved it is active either in conjunction with VIH at the early stages or is only operative at later stages of ovary development, such as during secondary vitellogenesis.

As a result of this work a clearer picture has been developed of how broodstock diet effects ovary tissue and, in particular, how the ovary responds to a dietary deficiency in fatty acids. Through a better understanding of the role of particular fatty acids, steps can be taken towards further refining broodstock diet formulations. The response of the ovary tissue to ablation has lent support to our developing model of the hormonal control of vitellogenesis and will add focus to our future research in this area.

Table 9.3 Mean fatty acid levels (mg per 100 g prawn + % of fatty acids) in the polar and neutral fraction of hepatopancreas tissue from treatment groups. For each column values with a common superscript letter are not significantly different ($P>0.05$).

Lipids	Treatment	14	16	16:1	18	18:1n-7	18:1n-9	18:2	18:3	20:1n-7	20:1n-9	20:1n-11	20:4n-6	20:5n-3	22:6n-3
Neutral (mg)	C	2.27^{a}	23.23^{a}	6.49^{ca}	6.67^{c}	5.38^{a}	7.04	0.79^{a}	0.06^{ba}	1.30^{a}	0.73^{a}	2.12^{a}	4.93^{a}	7.52^{a}	6.89^{a}
	F	3.85^{a}	56.11^{a}	13.23^{cb}	15.41^{b}	6.44^{a}	16.23^{a}	1.34^{a}	0.42	2.13^{a}	2.74^{b}	3.18^{a}	8.19^{a}	10.99^{a}	29.16^{b}
	FA	3.00^{a}	51.34^{a}	11.32^{ba}	13.19^{cb}	6.01^{a}	14.60^{a}	1.15^{a}	0.26^{b}	1.85^{a}	2.48^{b}	2.62^{a}	5.08^{a}	7.92^{a}	22.68^{b}
	S	0.07	1.43^{b}	0.29^{d}	0.51^{a}	0.36^{b}	0.51^{b}	0.05^{b}	0.0^{a}	0.11^{b}	0.06^{a}	0.16^{b}	0.32^{b}	0.34^{b}	0.36
	SA	0.07	0.85^{b}	0.27^{d}	0.51^{a}	0.35^{b}	0.48^{b}	0.06^{b}	0.0^{a}	0.11^{b}	0.06^{a}	0.13^{b}	0.38^{b}	0.41^{b}	0.47^{a}
Polar (mg)	C	0.21^{ba}	2.56^{ca}	1.34^{ab}	1.40^{ca}	0.75^{bca}	1.63^{a}	0.14^{cab}	0.0^{a}	0.0^{a}	0.02^{ca}	0.05^{a}	1.32^{a}	1.74^{ac}	1.71^{a}
	F	0.32^{a}	6.11^{b}	2.49	3.27^{b}	1.08^{b}	3.44^{b}	0.25^{c}	0.04^{a}	0.12	0.32^{b}	0.32	2.35^{b}	3.34^{b}	6.20^{b}
	FA	0.24^{ab}	5.08^{ba}	1.83^{a}	2.55^{cb}	0.80^{cab}	2.70^{ba}	0.11^{ab}	0.04^{a}	0.04^{a}	0.18^{cb}	0.08^{a}	1.81^{ba}	2.51^{cb}	5.13^{b}
	S	0.04	1.19^{c}	0.51^{b}	0.65^{a}	0.34^{c}	0.84^{c}	0.06^{b}	0.0^{a}	0.02^{a}	0.05^{a}	0.03^{a}	0.92^{a}	0.88^{a}	0.63^{a}
	SA	0.06^{b}	1.94^{c}	0.72^{b}	1.00^{a}	0.63^{bca}	1.40^{ac}	0.11^{b}	0.0^{a}	0.06^{a}	0.07^{ca}	0.13^{a}	1.54^{a}	1.28^{a}	1.00^{a}
Neutral % FA	C	1.7^{a}	21.8^{a}	5.3^{a}	6.1^{ba}	4.8^{ba}	6.3^{a}	0.7^{a}	0.1^{a}	1.6^{a}	0.7^{a}	2.4^{a}	4.1^{a}	5.6^{a}	4.7
	F	1.6^{a}	26.2^{b}	5.8^{a}	7.0^{ba}	3.3^{a}	7.5^{ab}	0.6^{a}	0.1^{ba}	1.0^{ba}	1.3^{b}	1.5^{ba}	3.7^{a}	4.8^{a}	12.8^{a}
	FA	1.5^{ab}	27.5^{b}	5.5^{a}	7.9^{bc}	2.9^{a}	7.5^{ab}	0.6^{a}	0.2^{b}	1.0^{ba}	1.3^{b}	1.4^{b}	2.7^{a}	3.9^{a}	12.0^{ba}
	S	0.7	20.0^{a}	5.3^{a}	9.9^{d}	5.5^{b}	10.5	0.4^{a}	0.0^{a}	0.5^{b}	0.4^{a}	0.9^{b}	9.3^{b}	10.5^{b}	10.3^{ba}
	SA	1.2^{b}	17.9^{a}	4.6^{a}	9.1^{dc}	5.1^{a}	8.5^{b}	0.6^{a}	0.0^{a}	1.2^{ba}	0.7^{a}	1.5^{b}	8.0^{b}	9.0^{b}	9.5^{b}
Polar % FA	C	1.2	20.1	6.6^{a}	7.7^{a}	4.5^{a}	9.2^{a}	0.6^{ba}	0.0^{a}	0.5^{a}	0.3^{a}	1.0^{a}	7.2^{a}	8.4^{a}	8.0^{a}
	F	0.8^{a}	17.3^{b}	6.7^{a}	9.3^{a}	3.1^{b}	9.7^{a}	0.7^{ba}	0.1^{a}	0.3^{a}	0.9^{a}	0.9^{a}	6.9^{a}	9.3^{a}	17.2^{b}
	FA	0.8^{a}	17.7^{b}	6.3^{a}	9.2^{a}	2.7^{b}	9.4^{a}	0.3^{b}	0.1^{a}	0.1^{a}	0.7^{cb}	0.4^{bc}	6.5^{a}	8.9^{a}	18.6^{b}
	S	0.6^{ba}	15.3^{a}	7.7^{a}	8.9^{a}	4.9^{a}	12.2^{b}	0.7^{ba}	0.0^{a}	0.1^{a}	0.7^{cb}	0.3^{b}	2.9^{b}	2.3^{a}	8.5^{a}
	SA	0.5^{ba}	15.7^{a}	6.2^{a}	8.5^{a}	5.0^{a}	11.9^{b}	0.8^{a}	0.0^{a}	0.3^{a}	0.6^{ca}	0.8^{c}	3.2^{b}	1.3^{b}	8.4^{a}

10. The fatty acid composition of the eggs and larvae of *Penaeus monodon* and *Penaeus esculentus*

10.1 Introduction

Larval quality, and hence later prawn growth and survival, is not only affected by dietary intake but also the availability of stored energy reserves. Newly hatched prawn larvae rely completely on yolk reserves accumulated during egg development. These reserves, which originate from the spawner, are systematically used during the hatching process and the early larval stages, until first feeding occurs. This pattern of usage will reflect the early requirements of the larvae.

Because of the high energy density of lipids and their recognised importance in prawn maturation, the relationship between maternal dietary lipids and egg and larval quality was examined in *P. monodon* and *P. esculentus.* Larval quality was measured in terms of the percentage of eggs that hatched and the rate of larval development. The more robust larvae spent shorter periods in each of the larval stages. In addition the ovary tissue, eggs, nauplii and zoea were compared for captive and wild *P. monodon* to assess the changes in the percentage composition of selected fatty acids.

10.2 Materials and methods

Two sources of female broodstock were used, (i) gravid females caught from the wild, and (ii) females (ablated and non-ablated) matured in captivity on a diet of known lipid and fatty acid content.

Prawns were held in maturation tanks prior to spawning (ablated and non-ablated treatments) and fed a mixture of pipi muscle (*Donax deltoides*) and squid mantle (*Loligo sp.*) offered to slight excess.

After spawning a sample of eggs was taken for biochemical analysis and the remainder reared through to the first zoea stage (Z_1). Eggs from three *P. monodon* spawnings were also sampled at the time of spawning and at three hourly intervals until hatching and analysed for fatty acid (FA) content. Larvae were counted to assess hatch rate and survival at 20, 42 and 60 hours post-spawning. For vigorous larvae these times equate to first nauplius (N_1), fourth nauplius (N_4) and first zoea (Z_1) (Treece and Yates, 1988). Upon reaching Z_1 (stage of first feeding) a sample of the Z_1 were taken for FA analysis.

Chemical analysis followed the same procedure outlined in Section 7 except that lipids were also separated into triacylglyceride (TG) and phospholipid (PL) classes. Statistical analysis involved analysis of variance (ANOVA) and Tukeys test to assess species and treatment effects. Results were regarded as significant at the 5% level.

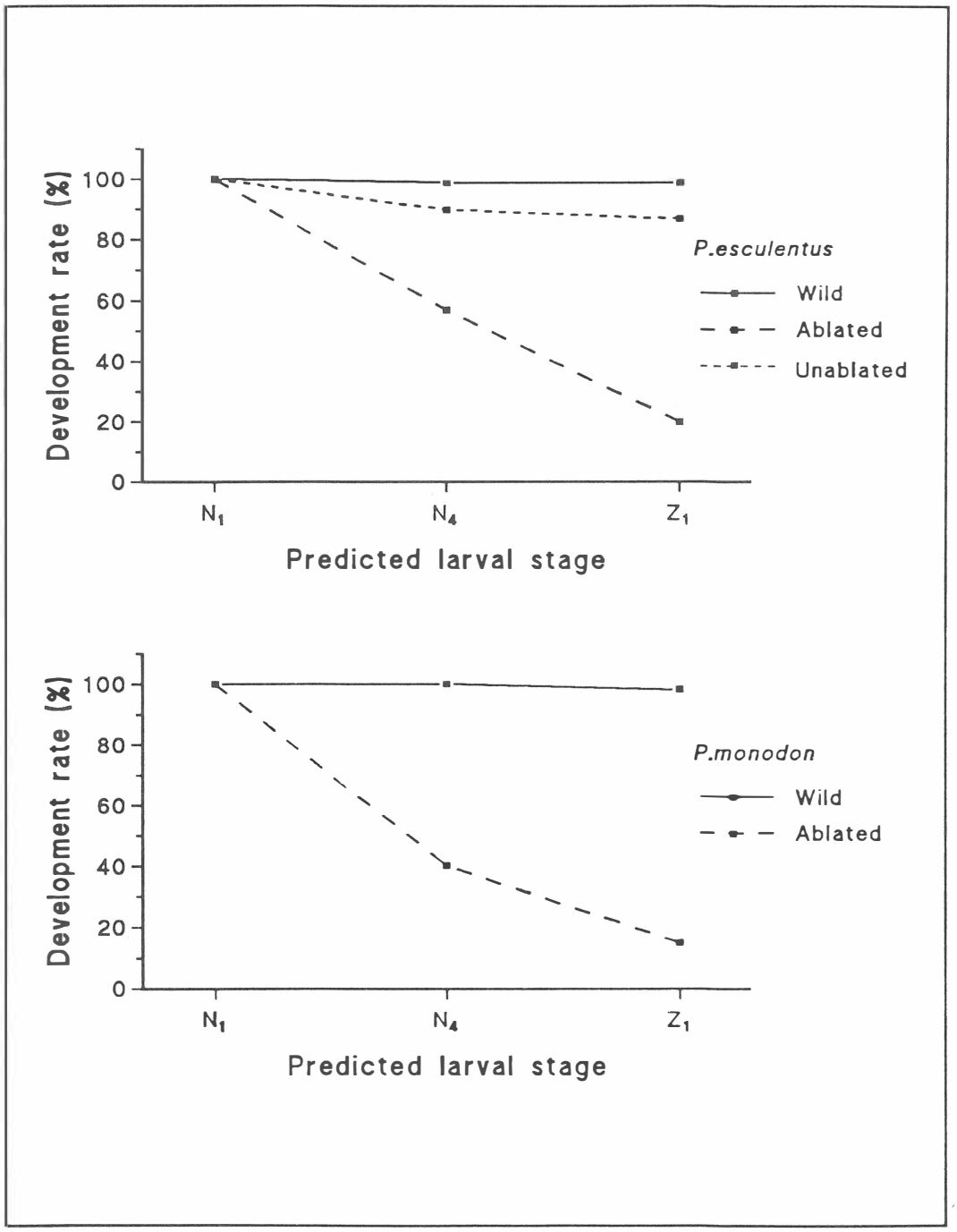
10.3 Results and discussion

Changes that occurred in *P. monodon* eggs from the time of spawning to hatching related mainly to egg size and moisture content, no significant depletion of reserves

was evident. FA profiles remained constant in both the PL and TG lipid classes (data not shown).

Egg hatch rates were significantly higher for wild prawns (mean = 40%) than for captive prawns (mean = 28%) for both species. Larval development rates were calculated as the percentage of larvae to reach each larval stage within the 42 and 60 hour time limit. The larvae of wild caught females of both species had significantly higher development rates than eggs and larvae of tank held animals (Figure 10.1).

Figure 10.1 Development rate of larvae from captive and wild prawns



The level of total lipid in the eggs and zoea of the five prawn groups are shown in Figure 10.2, while their FA profiles are shown in Figure 10.3. As eggs developed through to zoea, total lipid level was halved due mainly to the depletion of the triacylglyceride fraction. For this reason only the FA profile for the phospholipid fraction is shown for the zoea (Figure 10.3b).

Eggs from the ablated prawns were significantly lower in 18:0, 18:1n-9, 20:5n-3 and 20:4n-6 in both the PLs and TGs, than eggs from wild caught prawns and also produced the poorest quality larvae (data not shown). Fatty acid 20:5n-3 is regarded as an essential fatty acid for *P. monodon* (Kanazawa *et al.*, 1979) and in addition to 20:4n-6 is considered important for the production of prostaglandins which are precursors of reproductive hormones (Middleditch *et al.*, 1980). It is generally regarded that higher levels of fatty acids from the n-3 family, relative to the n-6 family, are important to prawn reproductive performance (Millamena *et al.*, 1984). It was surprising then that the prawns with PLs having a relatively high concentration of 20:4n-6 and 20:5n-3 fatty acids and TGs with a low concentration of the 22:6n-3 fatty acid produced the most robust larvae.

Despite the apparent conservation of PLs relative to TGs, approximately 60% (per unit dry matter) of the total phospholipid FA's were depleted (data not shown). As the degree of depletion was not equal for all FA's, profiles of the phospholipids changed significantly with development from egg to zoea (Figure 10.3b). The species and ablation treatment had no significant effect on the fatty acid profile of the phospholipid fraction. In all treatment groups the percentage of polyunsaturated PLs (and to a lesser extent the long-chain saturated PLs) tended to increase with larval development (Figure 10.2). This probably reflects the preferential use of monoenes both for energy and/or their desaturation and chain elongation to form polyunsaturated FAs (PUFAs) in the zoea.

Figure 10.4 shows the percentage composition of selected HUFAs in the neutral and polar lipids in the ovary, eggs and zoea of ablated *P. monodon* fed a diet of squid and mussel. There was a significantly higher percentage of neutral and polar 20:5n-3 and 22:6n-3 fatty acids in the egg than in the ovary. This implies that 20:5n-3 and 22:6n-3 fatty acids were, relative to other fatty acids, more important in the oocytes than as structural components or transport lipids in the surrounding ovary tissue.

The subsequent decrease in the level of the 22:6n-3 fatty acid in the polar lipids as the egg hatches and develops into a first stage zoea, indicates the preferential use of this FA as an energy source, and /or its inclusion in the hatching envelope. The hatching envelope is discarded during the hatching process (Clarke, 1982). The level of polar fatty acid 20:5n-3 also shows a significant drop from egg to zoea, however, its reduction is not as great as that observed for the 22:6n-3 fatty acid and suggests that the 20:5n-3 fatty acid is a more important constituent of membranes and/or more active as a transport lipid than 22:6n-3. The percentage of the polar 20:4n-6 fatty acid remains constant indicating that it's level declines at a rate similar to that of the total fatty acids. In a manner similar to the 20:5n-3 fatty acid, though quantitatively at a lower level, the polar 20:4n-6 fatty acid may be membrane bound and only used if the cells and organelles are broken down for energy.

Figure 10.2 Comparative lipid composition in the eggs and zoea of *P. monodon* and *P. esculentus* **a.**Total lipid **b.**Monoenoic phospholipid FA's c.Saturated phospholipid FA's **d.**Poly-unsaturated phospholipid FA's

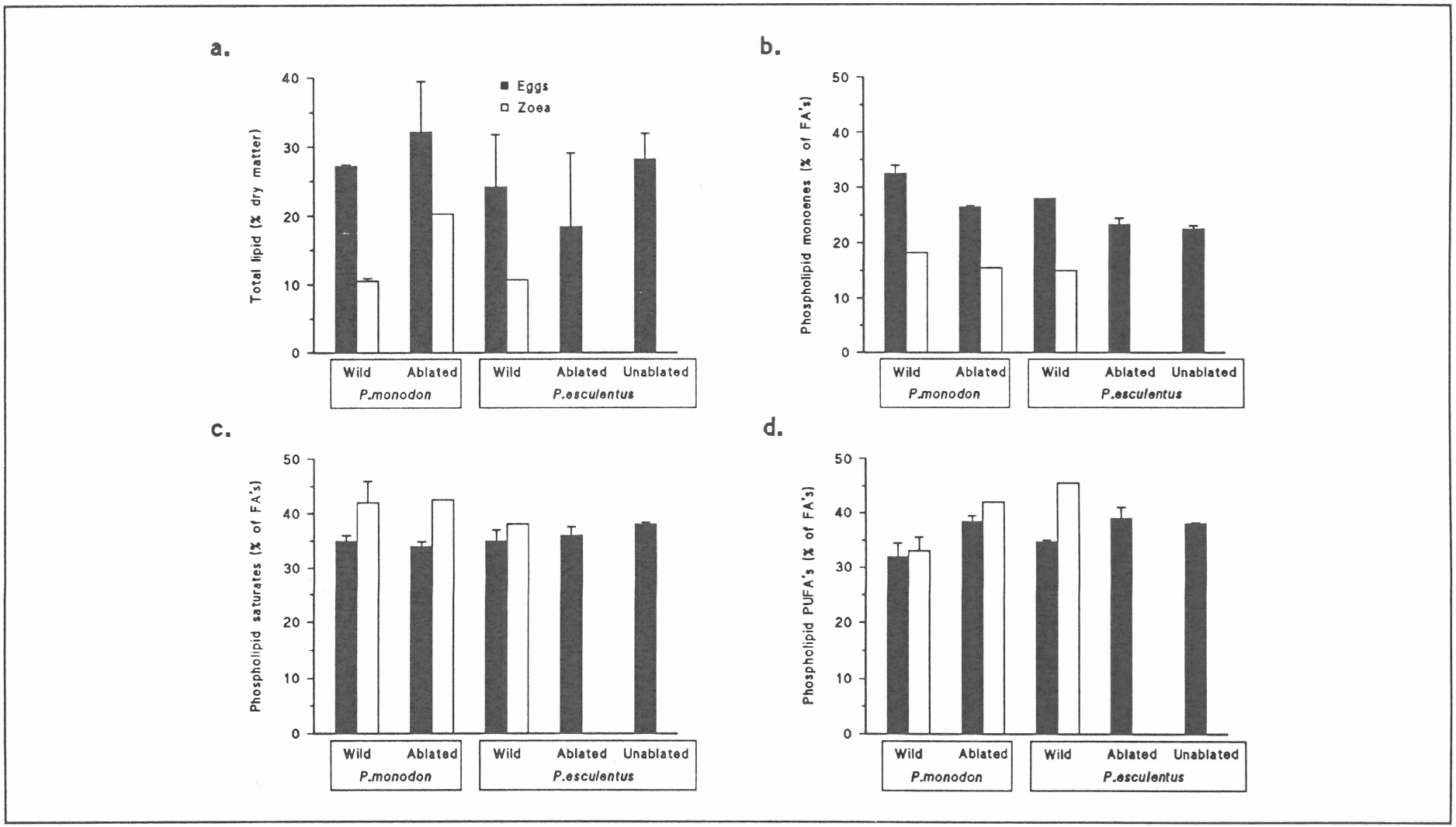


Figure 10.3 Levels of **a.** Triacylglycerides in eggs and **b.** Phospholipids in eggs and zoea of *P. monodon* (means + standard error)

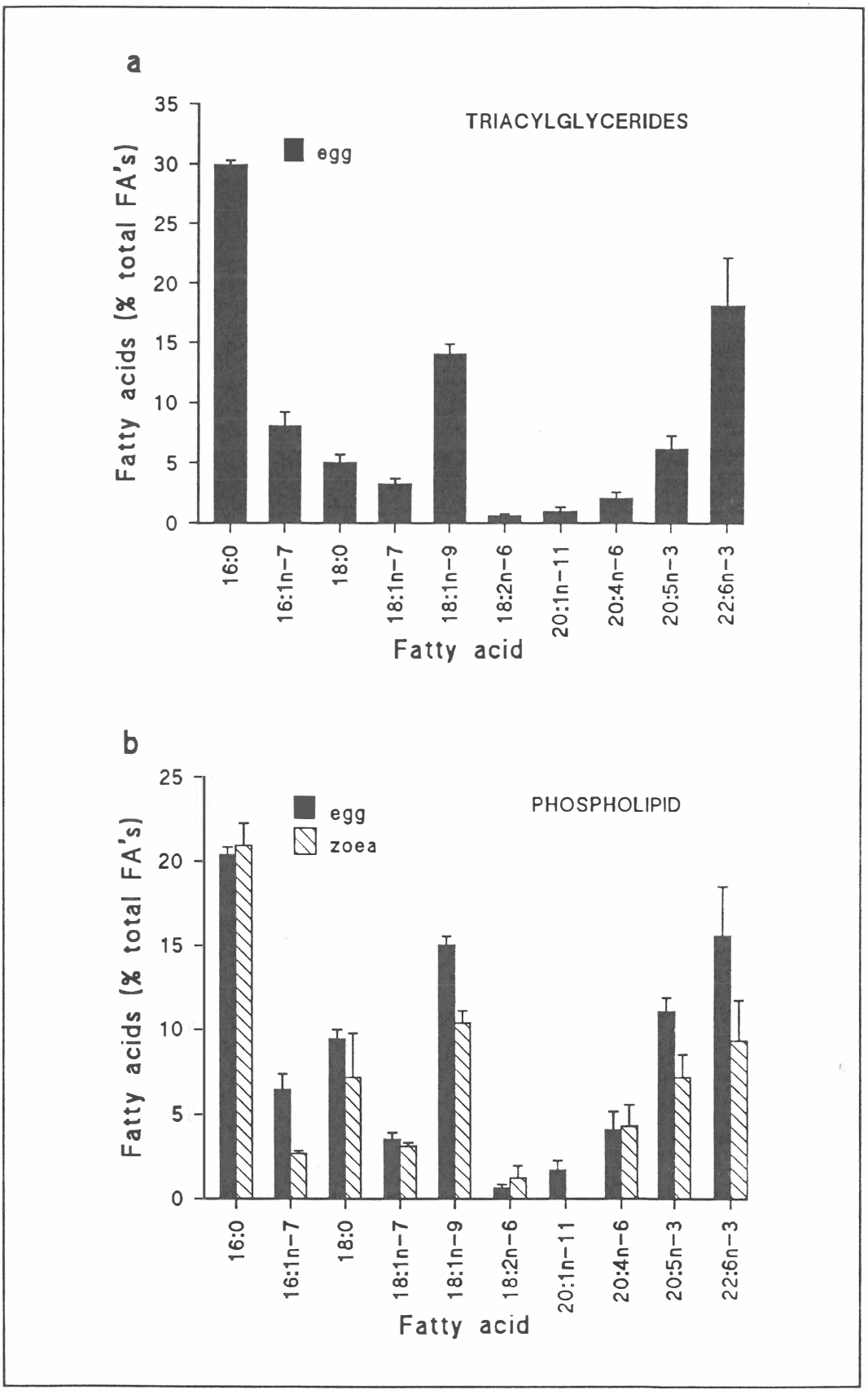
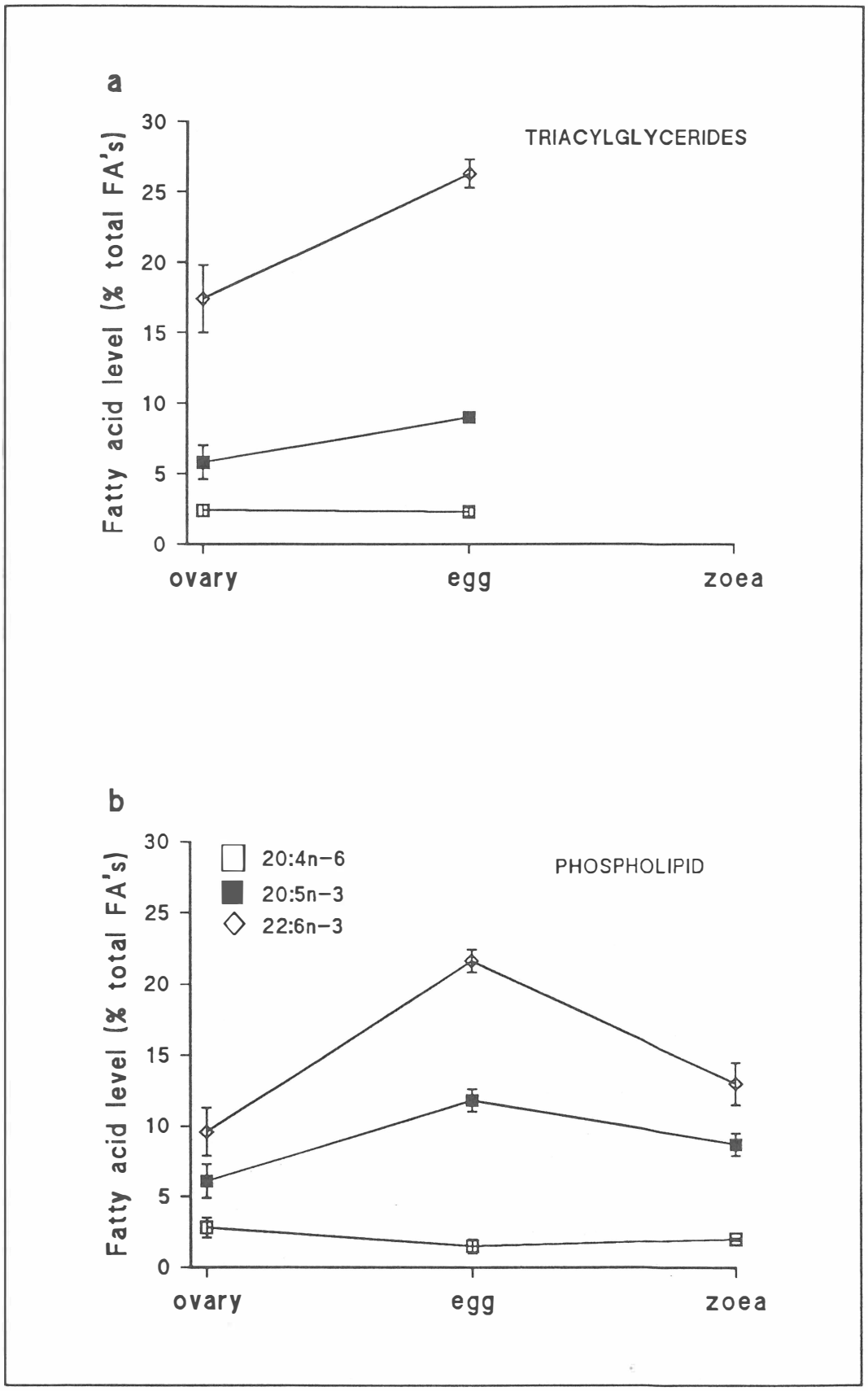


Figure 10.4 Levels of a number of fatty acids in the **a.** Neutral and **b.** Polar lipid fraction of *P. monodon* ovary, eggs and zoea.



10.4 Conclusions

The diet (and/or environmental conditions) available to spawners maturing in the wild, resulted in the production of larvae of higher quality than those produced from spawners matured in captivity. Results indicate that a maternal diet lower in the 22:6n-3 fatty acid and higher in 20:4n-6 and 20:5n-3 fatty acids than that provided by a diet of pipi and squid would result in higher larval development rates. This is contrary to findings for *P. chinensis* (Xu, 1993) which showed a positive correlation between the level of the 22:6n-3 fatty acid in the egg and the hatching rate of those eggs.

The patterns of FA depletion with larval development appear to be common to the two species studied, and not to be affected by hormonal changes brought about by ablation. Most of the energy required during the early larval stages is provided by TGs, however the PLs do make a significant contribution. This disparity in the patterns of FA use may reflect the different functional roles of these two lipid classes. Triacylglycerides are used primarily for energy metabolism, while phospholipids are conserved within the larval body for the maintenance of cellular and structural integrity.

The contribution of PLs to the energy requirements of the larvae is made largely from the monoene class and the polyunsaturated 20:5n-3 and 22:6n-3 fatty acids. The 20:4n-6 fatty acid is conserved which indicates that it remains in a structural capacity rather than being used as a source of energy to fuel larval development.

Thus, while further work needs to be carried out to validate these results, from this study it would appear that the 22:6n-3 fatty acid is used preferentially for energy and a diet comprising of pipi and squid supplies this fatty acid in excess. The 20:5n-3 fatty acid makes a significant contribution to energy production but along with 20:4n-6 it also plays a role in membrane structure and/or lipid transport. Both 20:4n-6 and 20:5n-3 fatty acids may need to be provided at a higher level than that available from this experimental diet.

11. Ovary development and associated changes in the biochemical composition of ovary and hepatopancreas tissues in *Penaeus monodon*

11.1 Introduction

Spawning in penaeid prawns is generally predicted by an external visual examination of the size and shape of the ovary. This technique is employed in the study of wild populations and in the management of aquaculture broodstock. More detailed studies using tissue biochemical composition (Millamena and Pascual, 1990; Spaargaren and Paul, 1994) and histology (Tan-Fermin and Pudadera, 1989) have been made with the overall aim of understanding the processes involved in ovarian maturation and oocyte development. Identification of these processes and the factors that control them may ultimately enable aquaculturists to increase production of high quality eggs and larvae. Advances in this field are important because the production of high quality larvae to stock growout ponds remains one of the major impediments to the growth of the prawn aquaculture industry world wide (Csavas, 1993).

The main aim of the present work was to investigate and quantify the compositional changes occurring with ovary development in *Penaeus monodon*. Few studies have assessed the magnitude of these changes in terms of the amount of a particular nutrient accumulated (Teshima *et al.*, 1989; Rankin *et al.*, 1989). This information would be useful for determining nutrient partitioning in broodstock and in quantifying the dietary requirements of prawns undergoing ovarian maturation.

A further aim of this work was to investigate the suitability of different methods of ovary classification. In this study ablated prawns were used to illustrate the limitations of classifying ovary development on the basis of GSI. Comparative studies of compositional changes are best understood in light of accompanying structural changes. Histochemical observations which describe cell and tissue structure and the distribution of materials within, are rarely accompanied by data on compositional changes.

11.2 Methods

Mature female *P. monodon* (90 to 130 g weight) were collected by beam trawl in Cook Bay, northern Queensland and air freighted to BIARC in southern Queensland. Upon arrival prawns were allocated to one of two groups; captive (ablated) or wild (non-ablated).

11.2.1 Ablated Treatment

After arrival at the Centre 85 prawns were tagged and randomly allocated to one of four groups which would be sacrificed at a different stage of development. These four groups represented the four stages of development (I to IV) based on external appearance as per Primavera (1982).

Prawns were held in 4 m diameter tanks at a density of less than 2 per m^2 with water exchanges at a rate of 200% per day. Seawater (33 ppt salinity) was filtered to 20 μm and temperature maintained at 28°C. A diet of fresh-frozen squid mantle

(*Loligo sp.*) and mussel (*Perna canaliculatus*) was fed *ad libital*. After a two day acclimation period (during which time prawns with ovaries showing signs of development resorbed their ovaries), prawns were subjected to unilateral eyestalk ablation.

Daily examination of ovary development was carried out using a submerged flashlight to view the shadow of the ovary through the dorsal side of the prawn body. Upon reaching the ovary stage denoted by their group number, the prawns were dissected, moult staged as per Smith and Dall (1985) to ensure they were intermolt, and the hepatopancreas and ovary tissue removed, weighed and stored at -70°C pending biochemical analysis.

Following dissection, a Gonad Somatic Index (GSI) was calculated for each individual to provide an objective system of ovary classification. The GSI was calculated as the wet weight of the gonad expressed as a percentage of the prawn wet weight.

11.2.2 Non-ablated Treatment

As spontaneous ovary development in non-ablated *P. monodon* is rare in captivity, the 63 intermolt prawns in the non-ablated treatment group were dissected immediately upon arrival at BIARC (10 to 20 hours post capture). Numbers in each GSI group depended on availability. Ovary and hepatopancreas tissues were removed and stored at -70°C prior to biochemical analysis.

11.2.3 Histology

To determine whether maturation of oocytes was proceeding in the same manner as described previously by Tan-Fermin (1992) in wild *P. monodon*, ovaries from prawns in the ablated treatment were examined histologically. Each ovary was weighed and a portion was removed from the anterior abdominal region and fixed in 10% formalin and seawater. Samples were cross sectioned (6 μm) and stained with haematoxylin fuscin (Hamason, 1972). Ovary sections were examined microscopically and classified into the three ovarian development groups as per Tan-fermin and Pudadera (1989).

11.2.4 Biochemical Analysis

The moisture content of each tissue sample was determined by oven drying a sub-sample to constant weight at 105°C. Using freeze dried material, crude protein (N x 6.25) was derived from Kjeldahl nitrogen analysis, with copper and selenium as catalysts (AOAC 1990, method 988.05). Ether extract, used here as a measure of total lipid content, was determined by Soxhlet extraction with petroleum ether (bp 40 to 60°C) for six hours (AOAC 1990, method 960.39). These techniques are described in some detail in Section 7.

11.2.5 Statistical Analysis

The effect of ovary development (GSI) on tissue levels of lipid and protein was examined using analysis of variance least squares method for unequal subclass numbers.

11.3 Results

11.3.1 Biochemical analysis

Table 11.1 shows the contribution of protein and lipid to the percentage composition of ovary and hepatopancreas in each GSI class of ablated and non-ablated *P. monodon*. The number of prawns in each GSI class were unequal as the GSI value could not be established prior to dissection.

The dry matter of the ovary tissue consisted mostly of protein and lipid which together comprised between 87.9% and 90.3% in ablated prawns and between 82.6% and 88.0% percent in non-ablated animals (Table 11.1). As lipid and protein were the major components of the ovary tissue there was a high negative correlation between levels of these the two in both ablated (r^2 = 0.95) and non ablated (r^2 = 0.62) prawns.

The percentage dry matter and lipid levels in ovary tissue only showed significant ($P<.05$) increases with ovary development for ablated prawns (Table 11.1). The percentage of protein in the ovary showed no significant change with ovary development for either ablated or non-ablated prawns (Table 11.1).

Figures 11.1 and 11.2 show the amounts of lipid and protein in the tissues at each GSI value in ablated and non-ablated prawns as milligrams of dry matter per 100 gram wet weight prawn. Expressed in this way changes can be quantified for a standardised prawn.

Absolute amounts of ovary protein and lipid increased significantly ($P<.05$) with ovary development (Figure 11.1) in both ablated and non-ablated prawns. The ovaries of both groups accumulated approximately 1 gram of protein and 380 mgs of lipid up to GSI stage 6 of ovary development. The dry matter of the hepatopancreas also consisted mainly of protein and lipid which together totalled between 83.8% and 85.6% in ablated prawns and between 82.8% and 91.0% in non-ablated prawns.

The percentage of lipid and protein in the hepatopancreas of both ablated or non-ablated prawns showed no significant change with GSI but a non significant ($P>0.05$) decrease occurred in hepatopancreas protein levels at GSI 3 in the ablated group. The only significant change in absolute quantities of lipid and protein was a decrease in lipids at GSI 2 in ablated prawns of approximately 465 mgs.

To clarify what is happening, Figure 11.3 shows the quantity of lipid and protein that accumulated in ovary tissue between successive GSI stages (up to GSI 6) for ablated and non-ablated prawns expressed as mgs of dry matter per unit GSI. This is a novel way of plotting the data and when studying this figure it is well to remember that GSI is not a real time axis, so the 'rate' of change shown here is only relative to the GSI categories.

Figure 11.3a shows that in ablated prawns the period of maximum accumulation of lipids in the ovary tissue occurs between GSI stages 2 and 3. By GSI 4 the rate of accumulation has halved. In non-ablated prawns the period of highest lipid accumulation does not occur until between GSI stages 3 and 4 after which it remains relatively constant.

Table 11.1 Mean values of dry matter, lipid and protein in the ovary and hepatopancreas tissues in ablated and non-ablated *P. monodon* at different stages of ovary development (GSI).

Component	Treatment	Tissue	GSI							
			1	2	3	4	5	6	7	9
Sample size	Non-ablated		n = 8	n = 12	n = 11	n = 18	n = 10	n = 8	n = 0	n = 0
	Ablated		n = 15	n = 4	n = 13	n = 15	n = 16	n = 14	n = 8	n = 0
Dry matter (% of wet weight tissue)	Non-ablated	H	35.7	26.8	32.2	33.6	37.3	31.6		37.0
		O	29.3	25.61	26.1	29.4	32.6	31.1		29.3
	Ablated	H	39.6	30.9	37.2	40.9	42.4	39.9	40.4	
		O	21.6	22.7	27.4	29.3	29.3	30.7	30.4	
Lipid (% dry matter)	Non-ablated	H	38.5	22.8	30.3	33.2	47.3	31.0		28.8
		O	14.1	16.0	17.2	20.9	21.9	21.8		18.0
	Ablated	H	50.0	35.2	46.5	46.9	49.2	44.0	43.9	
		O	9.6	10.9	17.5	20.6	20.2	20.2	20.0	
Protein (% dry matter)	Non-ablated	H	46.4	60.0	58.4	50.6	43.7	51.9		52.6
		O	70.7	69.2	65.4	67.0	64.4	66.2		68.3
	Ablated	H	35.0	48.6	38.5	38.1	36.4	40.0	41.7	
		O	78.3	78.0	70.5	69.1	70.1	67.7	67.9	

Figure 11.3b shows that the rate of protein accumulation in the ovaries of ablated prawns is at its highest during the early GSI stages of development. It then drops slightly between stages 3 and 4 and remains constant up to GSI 6. In contrast non-ablated prawns showed an initial slow accumulation of protein with an increase between GSI 3 and 4 onwards and a maximum rate between GSI stages 5 and 6.

11.3.2 Regression Analysis

A decrease in hepatopancreas lipid content was noticed for ablated and non-ablated prawns at GSI 2 which was not significant at 5% (Figure 11.1). Because GSI classes pool prawns together over a relatively wide range of ovary size (eg.GSI 2 includes values from 1.5 to 2.4), this may involve pooling hepatopancreas data from prawns at different stages of vitellogenesis. A change in lipid content could be hidden behind the variation caused by the lack of sensitivity of the GSI method. In an attempt to circumvent this, a regression analysis was conducted on the raw data using GSIs of individual prawns accurate to one decimal place.

Thus, to more closely examine the relationship between this drop in hepatopancreas and increases in ovary lipid reserves, GSI values within each GSI category were expressed to one decimal place. A regression analysis was then carried out for each GSI, of the ovary and hepatopancreas lipid content of the individual prawns. For mgs of lipid a positive relationship was found at GSI 1 ($r^2 = 0.62$, $n = 20$) and a negative relationship at GSI 2 ($r^2 = 0.61$, $n = 17$).

11.3.3 Histology

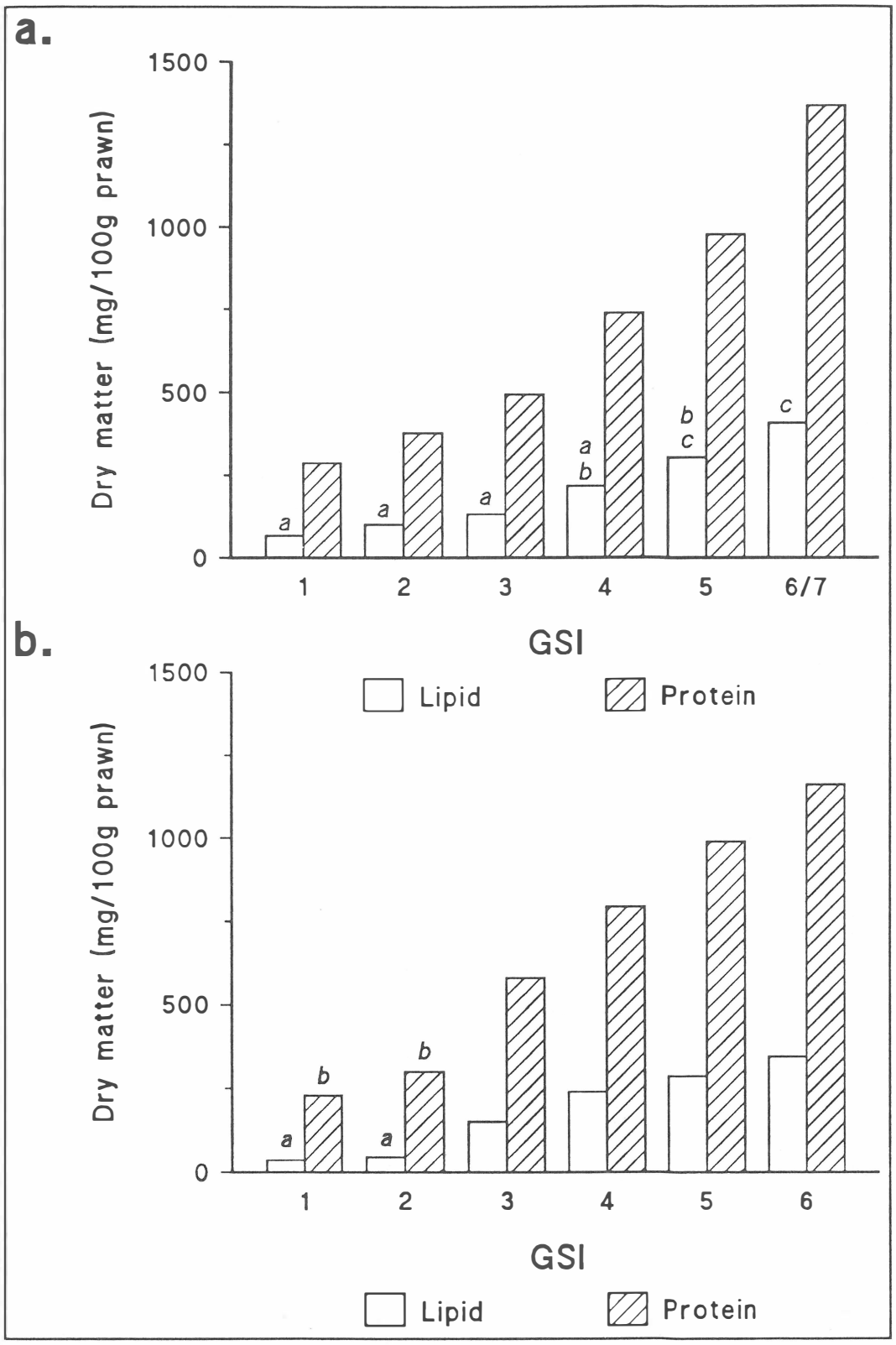
Table 11.2 shows the mean GSI value for non-ablated and ablated *P. monodon* for each of the first four developmental stages recognised histologically by Tan-Fermin (1989). Stage IV which represents the 'spent' ovaries, was not examined in this study. The data for the non-ablated prawns was obtained from Tan-Fermin (1989). Mean GSI values of ablated prawns were similar to those non-ablated in the previtellogenic stage but were slightly lower in the vitellogenic and cortical rod stages.

Table 11.2 Mean (± SE) GSI values for previtellogenic, vitellogenic and cortical rod stage ovaries in ablated and non-ablated *P. monodon* ovaries.

Developmental stage*	GSI	
	Ablated (n = 15)	Non-ablated* (n = 5)
Previtellogenic	1.4 ± 0.5	1.4 ± 0.1
Vitellogenic	4.4 ± 1.0	5.1 ± 0.3
Cortical rod	5.9 ± 1.0	8.5 ± 1.2

* Tan-Fermin and Pudadera 1989

Figure 11.1 Lipid and protein levels in the ovary of *P. monodon* for **a.** Non-ablated and **b.** Ablated spawners at successive GSI values. Values labelled with a common letter are not significantly different.



11.4 Discussion

The biochemical composition of ovary tissue and eggs has been determined for a number of species of penaeid prawns including *Parapenaeopsis hardwickii* (Kulkarni and Nagabhusuanam, 1979), *P. japonicus* (Teshima and Kanazawa, 1983), *P. aztecus* and *P. setiferus* (Castile and Lawrence, 1989), *P. vannamei* (Rankin *et al.,* 1989), *P. stylirostris* (Lawrence *et al.*, 1979) and *P. monodon* (Millamena, 1989). In each case the percentage of lipid increased as the ovary developed.

In this study the percentage of lipid in the ovaries only showed significant increases for ablated prawns (Table 11.1). Levels were similar to those found for ablated *P. monodon* by Millamena (pers com. cited in Primavera, 1985) except for immature ovaries where our results were slightly higher (9.6 vs 7.5%). The non-ablated group showed higher ovarian lipid levels than those reported by Millamena and Pascual (1990) for non-ablated prawns. This was particularly evident in immature ovaries where lipids were only 5.8% of the dry matter as opposed to 14.1% as found in this study.

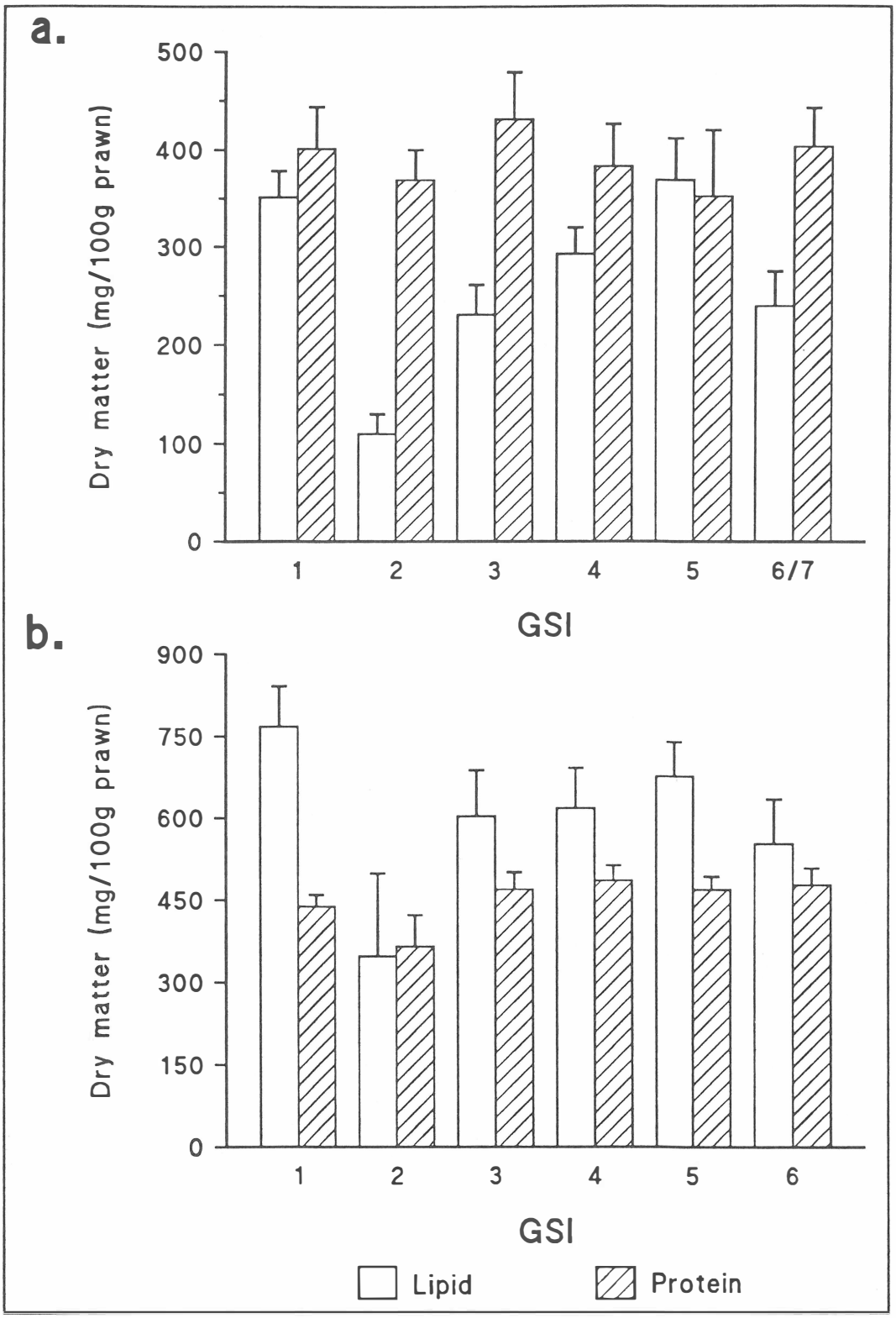
The percentage protein in the ovary tissue of wild non-ablated *P. monodon* was similar to levels found by Dy-Penaflorida and Millamena (1990). This level was nearly twice that found for *P. setiferus, P. vannamei* and *P. stylirostris* which had average protein contents of around 40% (Lawrence *et al.*, 1979). Dy-Penaflorida and Millamena (1990) found levels to be significantly lower in immature *P. monodon* ovaries than in developing ovaries. However, our results showed no significant change ($P<.05$) in the protein concentration of ovaries at any GSI stage.

These results indicate possible differences in diet or moult stage between the groups of prawns in each study. Alternatively, as Millamena's prawns were collected in Philippine waters and the prawns used in this study were from Australian waters, population differences may have developed. Some differences would be expected between the two groups as distinct populations of *P. monodon* have been shown to exist in Australian waters by The Australian Institute of Marine Sciences (Benzie, Personal communication). Another possible explanation for these differences is the potential variability in analytical techniques between the different laboratories conducting the studies.

Where the nutrients that accumulate in the ovary originate is the subject of much study and some debate. This is particularly important to investigations involving prawn maturation. While changes in tissue size are occurring, values expressed as percentages of tissue composition fail to indicate the real rate of accumulation or depletion of nutrients. To determine the contributions from different sources the results presented here have been expressed as both absolute quantities and percentage composition.

While the percentage of protein in the ovary did not change, significant quantities of protein were accumulated as the ovary developed (Figure 11.1). Rankin *et al.* (1989) found that the amount of protein in *P. vannamei* increased by approximately 400 mg during ovary development. Non-ablated *P. monodon* in this study, which were twice the body weight of *P. vannamei*, gained over 1700 mg between GSI 1 and GSI 9. The total amount of protein that accumulates in the ovary is probably species dependent, with prawn size a major determinant of the level of nutrients accumulated. From

Figure 11.2 Lipid and protein levels in the hepatopancreas of *P. monodon* for **a.** Non-ablated and **b.** Ablated spawners at successive GSI values



this study *P. monodon* accumulate more protein per unit weight of the female, than does *P. vannamei*. It seems that *P. monodon* broodstock may have a higher requirement for protein (per unit weight of female) than *P. vannamei* during periods of ovary development.

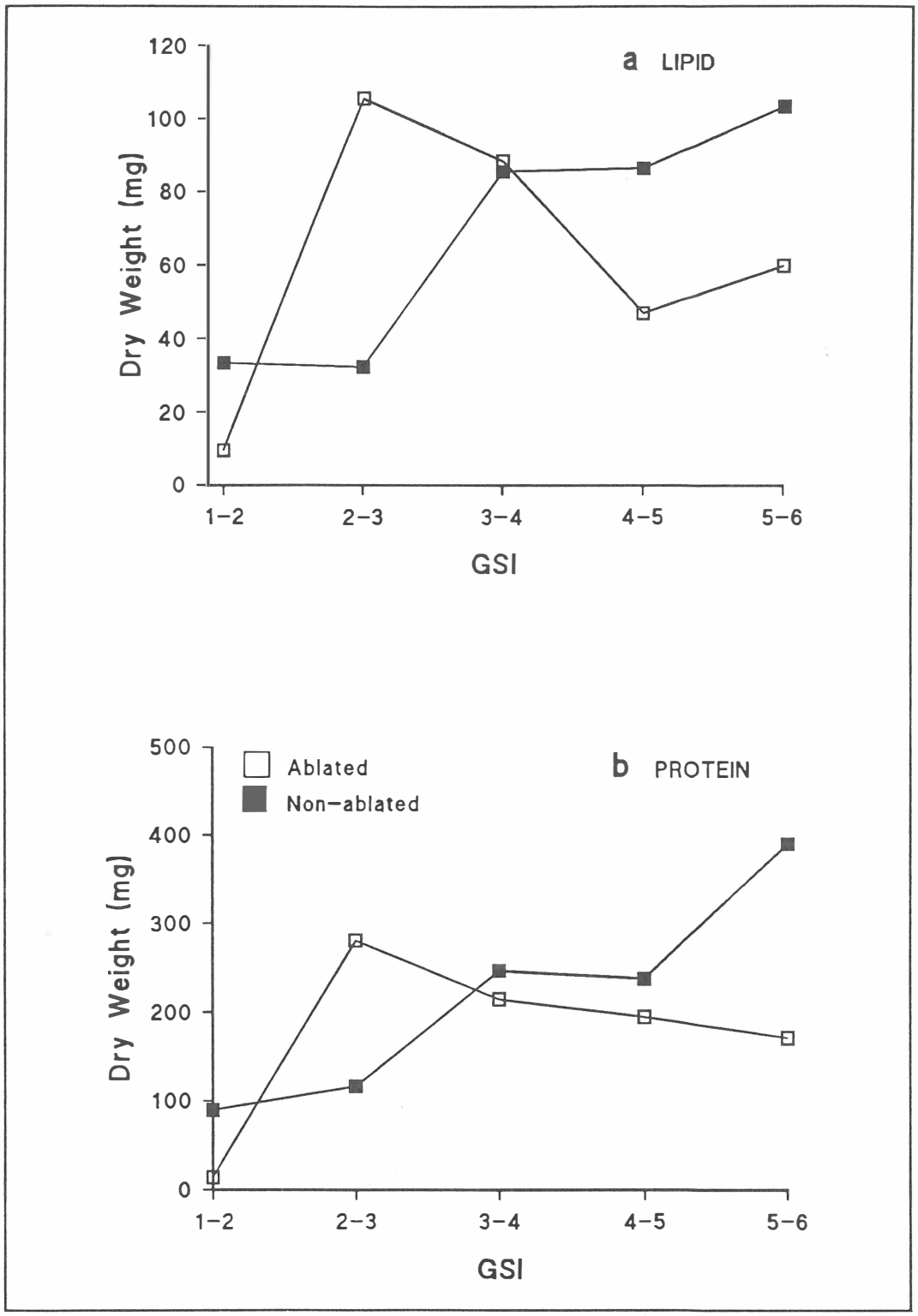
Lipid levels in the hepatopancreas of prawns vary with diet (Castille and Lawrence, 1989) and stage of the moult cycle (Read and Caulton, 1980). Ovary development has also been shown to affect hepatopancreas lipid levels (Teshima *et al.*, 1989; Castile and Lawrence, 1989). The extent to which the hepatopancreas lipid decreases appears to be species dependent (Clarke, 1982). Species that have little or no fluctuation in food availability may not require the transfer of stored nutrients to the ovary as nutritional requirements can be met by immediate dietary intake (Clarke, 1982). Millamena and Pascual (1990) found a drop in the percentage of lipid in the hepatopancreas of late maturing wild *P. monodon*, implying a loss of lipid from the hepatopancreas to the ovary. The present study showed a decrease in the absolute levels and percentage of lipid in the hepatopancreas in the early stages of ovary maturation.

In ablated prawns the decrease in hepatopancreas lipid levels at GSI 2 was associated with a corresponding increase in ovary lipid reserves. In non-ablated prawns the increase was slightly delayed and did not occur until between GSI 3 and 4. A synchronised decrease in hepatopancreas and increase in ovary lipid levels was also found for ablated *P. japonicus* by Teshima *et al.*, (1989) who proposed that rapid ovary development, induced by ablation, made the transfer of hepatopancreas reserves necessary. However Kulkarni and Nagabhushanam (1979) found concomitant increases in ovary lipid levels of *Parapenaeopsis hardwickii* that were non-ablated.

In ablated *P. monodon* broodstock, the quantities of lipid transfered from the hepatopancreas between GSI stages 1 and 2 (429mg) was sufficient to account for all the lipid accumulated by the ovary during its development (380 mg). Castille and Lawrence (1989) found the maximum potential contribution of mobilised hepatopancreas lipids to ovary stores to be only 57% in *P. setiferus.* Further studies are needed to determine how much of the mobilised hepatopancreas lipids are accumulated in the ovary tissue. However, there is enough evidence to recommend that while trying to determine the quantities of and frequency at which dietary lipids need to be fed to broodstock, the storage capacity of the hepatopancreas must be taken into account.

The hepatopancreas has also been implicated in the supply of proteins to the ovary. However, in this study hepatopancreas protein levels showed no significant changes with ovary development, which confirms the findings of Dy-Penaflorida and Millamena (1990) for *P. monodon*. This indicates that protein is not transfered from the hepatopancreas to the ovary of maturing *P. monodon* as has been suggested for other species (Kulkarni and Nagabhushanam, 1979; Castille and Lawrence, 1989). The prawn hepatopancreas has a high rate of protein turnover (up to 60% per day in juvenile *P. esculentus* (Hewitt, 1992)), which means that protein may be transfered to the ovary with no apparent decrease in the hepatopancreas protein level. The immunological evidence that the hepatopancreas plays a role in the production of protein subunits that end up in the yolk vitellin (Quackenbush, 1989; Quintio and Millamena, 1992) suggests that protein transfer occurs at some stage of ovary development. However problems associated with labelling the protein subunits

Figure 11.3 Changes between GSI stages in the level of **a.** Lipid and **b.** Protein in ablated and non-ablated *P. monodon*



have made it difficult to show conclusively that the hepatopancreas is an important site for the production of ovarian protein (Rankin *et al.*, 1989).

The origin of nutrients accumulating in the developing ovary is of particular importance to the study of vitellogenesis in prawns. The process of vitellogenesis is the production and accumulation of lipovitellin (LV), the major high density egg yolk lipoprotein (Harrison, 1990). In prawns this process has been divided into two phases (Charniaux-Cotton, 1985; Vogt *et al.*, 1989). The sites for the production of the composite units are still debated (Yano, 1987; Chinzie, 1987; Quackenbush, 1989; Vogt *et al.*, 1989; Lui *et al.*, 1974) however it is considered that during the primary phase the units are produced endogenously (Lui *et al.*, 1974) while during the secondary phase exogenous sources such as the hepatopancreas (Vogt *et al.*, 1985) and the sub-epidermal adipose tissue (Tom *et al.*, 1987) produce units that are subsequently transported to the ovaries via the haemolymph.

The data presented here supports the hypothesis that the process of vitellogenesis in prawns can be divided into two phases. In non-ablated prawns, primary vitellogenesis, could be seen to occur between GSI stages 3 and 4 with a two fold increase in the previtellogenic rate of protein and lipid accumulation (Figure 11.3). A second increase is evident between GSI values 5 and 6 (when cortical rod formation begins) and could be interpreted as secondary vitellogenesis.

Primary vitellogenesis is considered to be a slow and often lengthy process relying on the local synthesis of nutrients (Harrison, 1990). The gradual nature of the second increase in quantities of both protein and lipid recorded here (Figure 11.3), is uncharacteristic of secondary vitellogenesis which is normally marked by a sudden increase in nutrient accumulation. However, it must be recognised that the 'rate' referred to in this study is not a 'time rate'. It is difficult to attach a time scale to ovary development as the ovaries of individual prawns mature over time periods of different length. If GSI stages 5 and 6 were brief relative to the other GSI stages, then the second phase in nutrient accumulation could be considered as a 'sudden increase'.

The rate of nutrient accumulation in ablated prawns shows a different pattern to non-ablated animals. Only one peak occurred between GSI 2 and 3 followed by a sharp decline in lipid levels (Figure 11.3a) and a slow decline in protein accumulation (Figure 11.3b).

This absence of a second peak in nutrient accumulation could be explained by non-uniform ovary development. Ablated *P. monodon* have a tendency to spawn prematurely, that is when the oocytes from the anterior lobes are mature but the remaining tissue is underdeveloped (Primavera, 1985). This incomplete development may be attributed to changes in hormonal balance resulting from ablation (Lumare, 1979; Primavera, 1985) and/or to stress factors associated with captive environment (Browdy, 1992; Treece and Fox, 1993). With incomplete ovary development, vitellogenesis in ablated prawns is likely to occur at lower mean GSI values. A greater variation around the early mean GSI values could also be expected in ablated prawns, as the extent of incomplete development varies between individuals. This explanation is further supported by the finding that in ablated prawns vitellogenic and cortical rod stages of development have lower mean GSI values than for wild *P. monodon* (Tan-Fermin and Pudadera, 1989). Also, in this study, the range of GSI values for ablated prawns with vitellogenic oocytes was large compared to the range for previtellogenic

oocytes. This large range indicates that the vitellogenic oocytes were occurring in prawns that had different sized ovaries. Thus the nutrient accumulation data should be treated cautiously. Trends that are apparent when examining the mean levels of nutrients in the ovary tissue at a given GSI, may be masking the presence of the two step nutrient accumulation shown by non-ablated prawns.

Alternatively, the differences in accumulation patterns in the ovaries of ablated and non-ablated prawns may be due to factors associated with ablation and/or captivity. These unknown factors may significantly alter the pattern of nutrient accumulation such that primary vitellogenesis is shortened or bypassed. This change could in part account for the reported decrease in quality of successive spawns in ablated prawns (Beard and Wickins, 1980). Endogenous and exogenous components of yolk apparently play specific roles in egg development. Goudeau and Lachaise (1980) demonstrated that endogenous yolk was a component of cortical granules while exogenous components contributed to the 'yolk' proper. Cortical granules, which are membrane bound structures in the cortex of oocytes, contain material that is destined to become the surface coat or hatching envelope of the egg (Clarke *et al.,* 1980; Section 10). This surface coat appears to provide a suitable microenvironment for developing embryos (Guraya, 1982) and may play a role in fertilisation (Rankin and Davis, 1990).

The diet of captive and wild caught prawns may also contribute to differences in reproductive performance (Sections 10 & 12). Gut analysis of *P. monodon* broodstock indicate a diverse diet (Marte, 1980; Mohauty, 1975) while captive prawns generally have a more limited selection (Primavera, 1985). Dietary lipid levels have been shown to affect reproductive performance in *P. stylirostris* (Bray and Lawrence, 1990). Direct dietary input to oocyte lipid accumulation has been demonstrated in *P. indicus,* and was shown to influence the fatty acid composition of egg lipids in that species (Galois, 1984).

Thus the accumulation pattern displayed by ovaries of captive ablated *P. monodon* may indicate deficiencies due to an inadequate diet or a reduced ability by the prawns to digest, assimilate or synthesise required nutrients after ablation. Consequently, eggs from ablated prawns could be deficient in storage, structural or energy components. Also the reduced time interval between successive spawnings in ablated prawns (Vogt *et al.*, 1989) may limit the ability of the hepatopancreas to store nutrients that would normally be transfered to the ovary during periods of peak demand.

11.5 Conclusions

This study illustrates that the classification of ovary tissues according to a size index may not give a true indication of changes occurring within the tissue. Such classification may not be an appropriate basis on which to compare compositional changes in tissues of different individuals or species. It is also worth noting that, in a tissue that is expanding in size, compositional changes require examination in terms of absolute values as well as on a percentage basis.

Despite the limitations imposed by difficulties in classifying ovary development some interesting conclusions can be drawn from this study. While protein and lipids are the

major components of prawn ovary and hepatopancreas tissues, their percentage composition varies with species. In this study it was shown that *P. monodon* accumulates approximately three times as much ovary protein as lipid during ovary maturation. Also there is strong evidence to suggest that *P. monodon* is a species that uses lipids stored in the hepatopancreas to partly satisfy ovary requirements. Information gained from this study on the nutrient accumulation patterns of ovaries in wild, non-ablated and captive, ablated *P. monodon* help explain differences noted in the reproductive performance of the two groups. If, as appears to be the case, ablation is modifying the nutritional status of oocytes resulting in poor quality eggs, then an alternative to this severe method of hormonal manipulation needs to be found. In terms of understanding the mode of action of the Vitellogenesis Inhibiting Hormone (VIH), results from this study support evidence that it is involved at the very early stages of ovarian development (Section 4).

12. Reproduction of eyestalk ablated *Penaeus monodon* fed four different diets

12.1 Introduction

From the study of natural foodstuffs described in Section 8 a basal diet of squid and mussel was recommended to supply protein, lipid and fatty acid requirements of prawns undergoing ovary development. Subsequent studies shed some light on the role of various fatty acids in the ovary and eggs of penaeid prawns (Sections 9 & 10) and indicated that the modification of some fatty acids levels in broodstock diets may improve spawning performance.

Two diet trials were carried out to assess the effect of specific diets on the spawning performance of *P. monodon* and to relate performance criteria to ovary and hepatopancreas tissue composition. Spawning performance was measured in terms of broodstock survival, maturation and spawning rate, as well as fecundity, egg hatch rate and larval survival.

12.2 Materials and methods

The study was conducted in two parts; one experiment to determine the changes in tissue composition resulting from the broodstock diet (Compositional analysis trial), the other to assess the spawning performance of the prawns on those diets (Spawning performance trial). One diet was different for the two experiments as is explained in section 12.2.1 below.

12.2.1 Compositional analysis

Four diets were selected on the basis of earlier work. Diet A consisted of fresh frozen whole pipi (*Donax deltoides*); Diet B of fresh frozen squid mantle (*Loligo sp.*), and fresh frozen whole mussel (*Perna cannaliculatus*) offered on alternating days. Diet C consisted of squid mantle, whole mussel and a dry commercial pelleted feed (*P. japonicus* finisher diet) offered at alternating feeds. Diet D1 consisted only of the dry commercial pellet feed. All diets provided similar levels of protein and differed mainly in their lipid component. The squid and mussel combination (Diet B) was chosen as it provided a concentrated source of protein and lipid from the squid and mussel respectively. Also the fatty acid profile of the mussel matched that of the mature ovary tissue of wild caught spawners (Sect 8). The addition of the commercial pellet to the squid/mussel combination (Diet C) was to provide an extra source of n-6 fatty acids. Pipi (Diet A) was lower in 22:6n-3 and higher in 20:5n-3 and 20:4n-6 than Diet A. As both 20:5n-3 and 20:4n-6 appear to be lacking in the eggs of captive prawns and 22:6n-3 appears to be in excess, pipi was potentially a better diet than Diet B. Diet D1 was chosen for its high protein content and because it had been recommended as a maturation diet for *P. monodon* (personal communication from the manufacturer). Food was offered in measured amounts twice daily at 0900 and 1700 and the amount of food eaten each day recorded.

Prawns were sourced from Cook Bay and, after a one week acclimation period, were unilaterally eyestalk ablated. Thirty females (no males were required as spawning was not to occur) were stocked in fibreglass maturation tanks (4.0 m in diameter and 0.8 m water depth). Seawater (33 ppt salinity) supplied to the tanks was filtered to 20 μm, heated to 28°C and exchanged at a rate of 200% per day. Controlled light

was provided by suspended fluorescent fittings wrapped in green 70% 'shade cloth' (Dindas Lew Cat No 5C7036 BL) to reduce light intensity to 5 lux as measured at the water surface using a Licor light meter (model L1-185B) fitted with a photometric sensor (Licor model PH4432). Day length was 14L:10D, with a ramp period of 20 minutes.

Ovarian development was monitored daily using a submerged light to reveal the shadow of the ovary on the dorsal exoskeleton. Prawns were removed when their ovaries reached maturity and the ovary and hepatopancreas were dissected out. Both tissues were subjected to biochemical analysis as described in section 7.

12.2.2 Spawning performance

Diets A, B and C were the same as those described in section 12.2.1. Diet D1 was not used because of the low rate of maturation achieved in the compositional analysis trial. It appeared highly unlikely that broodstock would spawn on a diet composed entirely of dry pellets. Instead a soft moist pellet manufactured at BIARC, diet D2, (see Table 12.1 for formulation) was trialed.

Table 12.1 Composition of the experimental moist pellet diet.

Ingredient	Weight (g/100 g dry diet)
Squid (*Loligo* sp.) meal	41.0
Minced mussel (*P. canaliculatus*)	23.0
Fish roe (*Hoplostethus atlanticus*)	12.0
Cod liver oil	2.4
Binder mix	10.0
Milled mollusc shell (*D. deltoides*)	2.0
Lecithin	3.0
Vitamin mix[1]	5.0
Mineral mix[2]	3.0
Cholesterol[3]	1.1
Astaxanthin	40.0ppm
β-carotene	40.0ppm

[1] Vitamin composition (mg/100 g diet) ρ-amino benzoic acid, 15.8; biotin, 0.63; inositol, 632.0; nicotinic acid, 63.2; Ca-pantothenate, 94.8; pyridoxine HCl, 19.0; riboflavin, 12.6, thiamine HCl, 6.32; folic acid, 1.26; cyanocobalamin, 0.13; choline HCl, 948.0; menadione, 6.34; Na ascorbate, 3160.0; calciferol, 1.9; α tocopherol, 50.0.

[2] Mineral composition (g/100 g diet) $K_2H_2PO_4$, 0.70; $Ca_3(PO_4)_2$, 0.95; $MgSO_4.7H_2O$, 1.1; $NaH_2PO_4.2H_2O$, 0.28.

Broodstock prawns for spawning trials were collected from Cook Bay in September and again in November. Each diet treatment consisted of one maturation tank in the first trial and two tanks in the second trial, except diet B which consisted of three tanks in September and one in November. Twelve females and six males were randomly stocked in each tank and all females were ablated after a one week acclimation period. Trials were carried out over 42 days under the same environmental conditions as described earlier (Section 12.2.1).

Mature females were removed and spawned in 0.15 m^3 rectangular drums in 130 l seawater at 28°C. Water was filtered to 0.5 μm and exchanged in spawning drums at a rate of 1000% per day. Egg and larvae numbers were estimated by vigorously stirring the spawning drums and counting four 100 ml subsamples. Larvae were left to develop in the spawning drum and counted at the first nauplius (N_2) and first zoeal (Z_1) stage.

The survival rate of female prawns was expressed in terms of the mean percentage of the total possible number of prawn days (42) survived by each female in each tank. Maturation rate was expressed as the mean number of spawns per 30 prawn-days per tank. Female survival rate, maturation rate and fecundity as well as egg hatching rate and larval survival rate were compared by analysis of variance to isolate time and treatment effects.

12.3 Results and discussion

On a wet weight basis prawns fed Diet A consumed an average of 11.4% of their body weight (BW) per day. Prawns fed Diet B had an average intake of 8.4% BW of squid and 6.1% BW of mussel per day while prawns fed Diet C consumed, on average, 5.5% BW of squid, 4.9% BW of mussel and 0.5% BW dry pellet. Prawns fed diet D1 in the compositional analysis trial ate negligible amounts of dry pellet and in the spawning trial prawns consumed an average 8.3% BW of BIARC pellet (D2) per day. Moisture content was not significantly different for Diets A, B and C (mean = 80%) therefore dry weight consumed was highest for prawns fed Diet B followed by those Diet A and C. Despite the lower wet weight consumption of Diet D2, on a dry weight basis consumption was equal to that of Diets B and C as the moisture content of diet D2 was lower (mean = 75%). This finding suggests that the amount of food consumed is driven by the animals nutrient or energy requirement rather than by gut distension which would result in a higher intake of food with a lower water content. However, food intake is also related to diet palatability and this may be lower for the diets which do not consist of fresh material.

12.3.1 Compositional analysis

Results from the proximate analysis of the experimental diets are summarised in Table 12.2 The table also shows the ratio of neutral to polar lipids and the n-3:n-6 ratios of fatty acids in the neutral and polar lipid fractions for each diet.

The difference in the total lipid levels and the level of lipid calculated on the basis of the fatty acids extracted (Table 12.2) illustrates the need to define the term 'lipids'. The discrepancy between the percentage of 'total lipid' and the 'lipids with fatty acids'

in the pellet (Diet D1) and the BIARC diet (Diet D2), indicates that the diets have other components which are extracted by the process used (See Section 7). These include cholesterol and in the case of a pelleted diet may include binding ingredients.

Results from the compositional analysis of the diets showed no significant ($P<0.05$) difference in percentage protein except for Diet D2 which had less protein than the other diets (Table 12.2). This lower dietary protein level was reflected in the lower level of protein in the hepatopancreas of prawns fed Diet D2, however the protein level in the ovary tissue was not significantly different to that in ovaries of prawns fed the other diets (Table 12.3). Diet A had a significantly ($P< 0.05$) lower lipid level than the other diets, however, there was no difference ($P> 0.05$) in the lipid level of tissues from the prawns fed this diet compared to those fed the other diets.

Thus, the composition of mature ovary tissue was not affected by a dietary protein content ranging from 52 to 62%. The amino acid profile of prawn tissue protein is conservative and does not change with diet (Tacon, 1990), therefore it is the quantity rather than the quality of protein in the prawn tissues that is affected by dietary intake. If protein quality is poor in terms of amino acid ratios then a higher protein intake will be necessary to satisfy amino acid requirements. In terms of amino acid profiles the protein in each of the treatment diets was considered to be of high quality (Section 8). The results therefore suggest that dietary protein levels as low as 52% are adequate to support the ovarian development of *P. monodon* broodstock, however, further work is required to verify this finding.

Table 12.2 Mean levels (%) of protein and total lipid and lipid class composition of the experimental diets.

Diet	Crude protein (%DM)	Total lipids (%DM)	Lipids with fatty acids (%DM)	N:P	n-3:n-6	
					Neutral	Polar
A	62.5[a]	3.9	4.0	0.7	6.4	5.8
B	62.7[a]	9.0[a]	6.7[a]	0.3[a]	15.9	26.6
C	60.0[a]	9.8[a]	7.2[a]	0.4[a]	9.4	18.2
D1	59.6[a]	11.2	7.0[a]	4.6	2.3	3.0
D2	52.2	15.0	7.5[a]	2.5	4.5	3.0

Values in each column with a common superscript letter are not significantly ($P<0.05$) different.

The ratio of neutral to polar lipids varied between diets (Table 12.1) however diet did not appear to effect the ratio in the ovary or hepatopancreas tissues (Table 12.2). Ovary tissue was composed of equal quantities of neutral and polar lipids while the hepatopancreas had on average twice the level of neutral as polar lipids. Each tissue showed similar fatty acid profiles in its neutral lipid fraction as it did in their polar (Figures 12.1 and 12.2, Table 12.5).

Table 12.3 Mean levels of protein, lipid and cholesterol in *P. monodon* ovary and hepatopancreas tissue components for Diet A = pipi; Diet B = squid mantle and mussel; Diet C = squid, mussel and a commercial pellet; Diet D1 = commercial pellet; Diet D2 = BIARC pellet.

Diet	% Dry matter				mg/g	
	Protein		Lipid		Cholesterol	
	Ovary	Hepatopancreas	Ovary	Hepatopancreas	Ovary	Hepatopancreas
A	69.0	40.1[b]	19.9	43.0	7.4	3.9[abc]
B	69.5	38.2	20.0	47.0	7.2	5.0[a]
C	68.9	36.5	19.9	48.0	6.8	4.3
D1	69.5	41.4[a]	18.9	46.1	6.9	4.8[b]
D2	69.7	32.4[ab]	19.9	47.2	7.2	4.9[c]

Values in each column with a common superscript letter are not significantly ($P<0.05$) different.

Table 12.4 Mean levels (mg per 100 g prawn) of neutral and polar lipid fractions and phospholipids in the polar fraction of ovary and hepatopancreas tissues.

Diet	Neutral lipids		Polar lipids	
	Ovary	Hepatopancreas	Ovary	Hepatopancreas
A	76.7[b]	267.5[a]	80.4[a]	134.4[a]
B	86.4[a]	294.0[a]	86.5[a]	138.2[a]
C	86.2[a]	321.9[a]	85.7[a]	128.5[a]
D1	67.9	317.0[a]	72.5	173.8
D2	80.6[bca]	299.0	82.7	56.6

Values in each column with a common superscript letter are not significantly ($P<0.05$) different.

The quality of a lipid is determined mostly by the ratios of its composite fatty acids. Profiles of some of the major fatty acids which showed differences between treatment diets, are shown in Figure 12.3. A comparison of this data with that presented in Figure 12.1 and 12.2 shows that profiles of the neutral and polar lipid fractions in each diet closely match those of the ovary and hepatopancreas of prawns fed the respective diets. The HUFA ($\geq$20 carbon) concentrations in the tissues appear to vary far less with diet than the shorter chain fatty acids. This would indicate that tissues are to some extent conservative with regards to HUFA concentrations.

Table 12.5 Mean levels (mg per 100 g prawn) of neutral and polar fatty acids in ovary and hepatopancreas tissues. Diets with same superscript are significantly different ($P<0.05$).

Fatty acids	Neutral Lipids								Polar Lipids							
	Hepatopancreas				Ovary				Hepatopancreas				Ovary			
	Diet				Diet				Diet				Diet			
	A	B	C	D1	A	B	C	D1	A	B	C	D1	A	B	C	D1
14	3.8^{abc}	6.7^{a}	7.1^{b}	6.5^{c}	1.4^{ba}	1.7^{a}	1.6^{b}	1.3^{ab}	1.0	1.7	1.6	2.2	0.43	0.48	0.53	0.4
16	65.6	75.8	78.5	59.6	15.9^{a}	19.4^{b}	17.8^{c}	12.5^{abc}	15.3^{a}	17.7	15.4^{b}	19.5^{ab}	7.4^{ba}	8.9^{a}	8.8^{b}	7.8
16:1n-7	15.0	17.0	19.0	14.9	5.7^{a}	5.5^{b}	5.1^{c}	2.9^{abc}	8.0	8.4	8.9^{a}	6.9^{a}	3.0^{a}	2.8^{b}	2.8^{c}	1.5^{abc}
18	19.2^{a}	14.3^{a}	15.6	7.6	4.3^{bac}	3.7^{a}	3.6^{b}	2.3^{abc}	6.3^{a}	5.5	5.2	4.8^{a}	5.2^{a}	5.2^{b}	5.1^{c}	4.2^{abc}
18:1n-9	17.3	12.7	14.2	32.3	9.9^{bac}	8.4^{a}	8.1^{b}	8.0^{c}	7.9^{bac}	5.8^{a}	5.3^{b}	13.6^{abc}	7.2^{bac}	6.5^{a}	6.5^{b}	6.6^{c}
18:1n-7	8.6	10.6	11.4	13.1	2.0^{a}	2.4^{a}	2.3	2.3	3.3	4.0	3.2	5.5	1.5^{bac}	1.8^{a}	1.8^{b}	2.0^{c}
18:2n-6	2.5^{a}	2.6^{b}	3.5^{c}	29.9^{abc}	0.7^{a}	0.7^{b}	0.9^{c}	4.6^{abc}	1.1^{a}	1.1^{b}	1.3^{c}	11.9^{abc}	0.5^{a}	0.6^{b}	0.7^{a}	3.1^{ab}
18:3n-3	1.5^{a}	1.6^{b}	1.9^{c}	2.5^{abc}	0.4^{a}	0.4^{b}	0.4^{c}	0.3^{abc}	0.63	0.6^{a}	0.7	1.0^{a}	0.2	0.2	0.2	0.1
18:4n-3	1.7^{a}	2.4^{a}	2.9^{ab}	1.4^{ab}					0.7	0.70	0.8	0.6				
20:1n-9	4.3	6.9	7.1	6.5	0.5^{ba}	0.7^{a}	0.71^{b}	0.5^{ba}	1.3^{bac}	2.2^{a}	2.1^{b}	2.2^{c}	0.5	0.7	0.7	0.6
20:1n-7	2.5^{ab}	3.7^{a}	3.9^{b}	2.8^{b}	0.2^{ab}	0.4^{a}	0.37^{b}	0.3^{ba}	0.6^{bac}	1.0^{a}	1.0^{b}	0.9^{c}	0.1	0.3	0.2	0.2
20:2n-6	3.3^{abc}	2.1^{a}	2.3^{b}	1.9^{c}	0.4^{bac}	0.2^{a}	0.22^{b}	0.2^{c}	1.2^{ba}	0.8^{a}	0.7^{b}	0.9	0.5	0.3^{a}	0.6^{a}	0.4
20:4n-6	6.3^{a}	6.8^{b}	7.0	4.4^{abc}	1.8^{a}	2.1^{b}	1.9^{c}	1.2^{abc}	2.9	2.7	2.4	2.4	2.8^{a}	2.8^{b}	2.5	2.1^{ab}
20:5n-3	12.6^{bac}	21.2^{a}	24.4^{b}	24.6^{c}	3.9^{bac}	5.8^{a}	5.6^{b}	6.0^{c}	5.5^{bac}	6.9^{a}	6.7^{b}	8.7^{c}	5.0^{b}	6.1^{a}	5.6^{ba}	6.6^{c}
22:1n-6	3.9^{abc}	1.3^{a}	1.4^{b}	0.4^{abc}					1.1^{bac}	0.2^{a}	0.2^{b}	0.1^{c}				
22:5n-3	4.7^{bac}	3.2^{a}	3.5^{b}	2.8^{c}	1.3^{bac}	0.8^{a}	0.7^{b}	0.6^{c}	1.4^{ba}	0.8^{a}	0.8^{b}	0.9	0.9^{bac}	0.5^{a}	0.5^{b}	0.4^{c}
22:6n-3	31.3^{bac}	49.1^{a}	52.4^{b}	26.0^{abc}	11.8^{abc}	17.4^{a}	15.7^{b}	6.5^{abc}	11.2^{a}	15.0^{bac}	12.8^{b}	9.5^{abc}	7.5^{bac}	9.6^{a}	9.3^{b}	4.8^{c}
n-3:n-6	3.2	7	6.5	1.6	5.8	8.0	7.5	7.9	3.0	5.0	4.7	1.3	3.5	4.4	4.3	2.8

Figure 12.1 Levels of some fatty acids in the **a.** Neutral and **b.** Polar lipid fraction of the ovary of *P.monodon* (means + standard error)

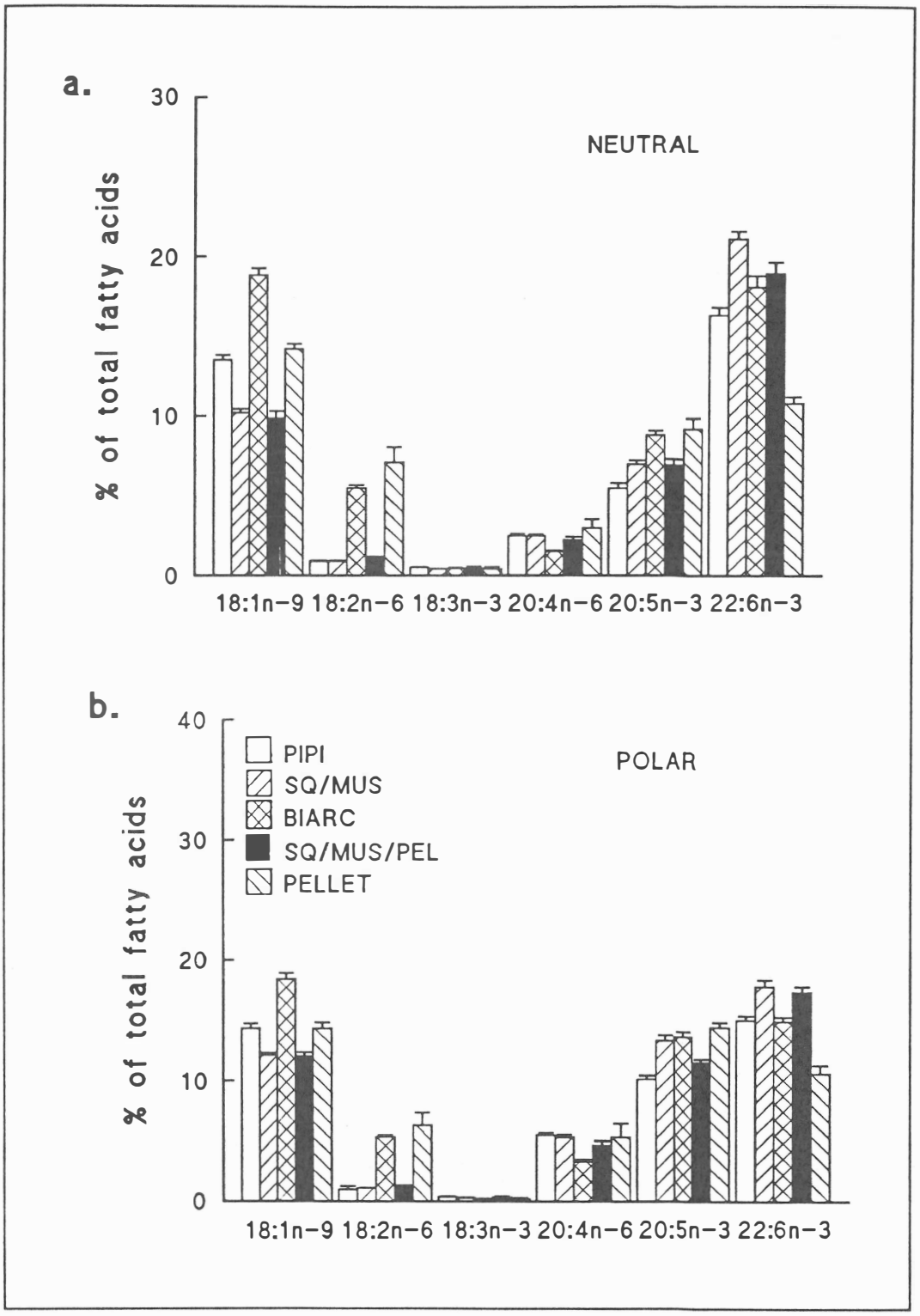
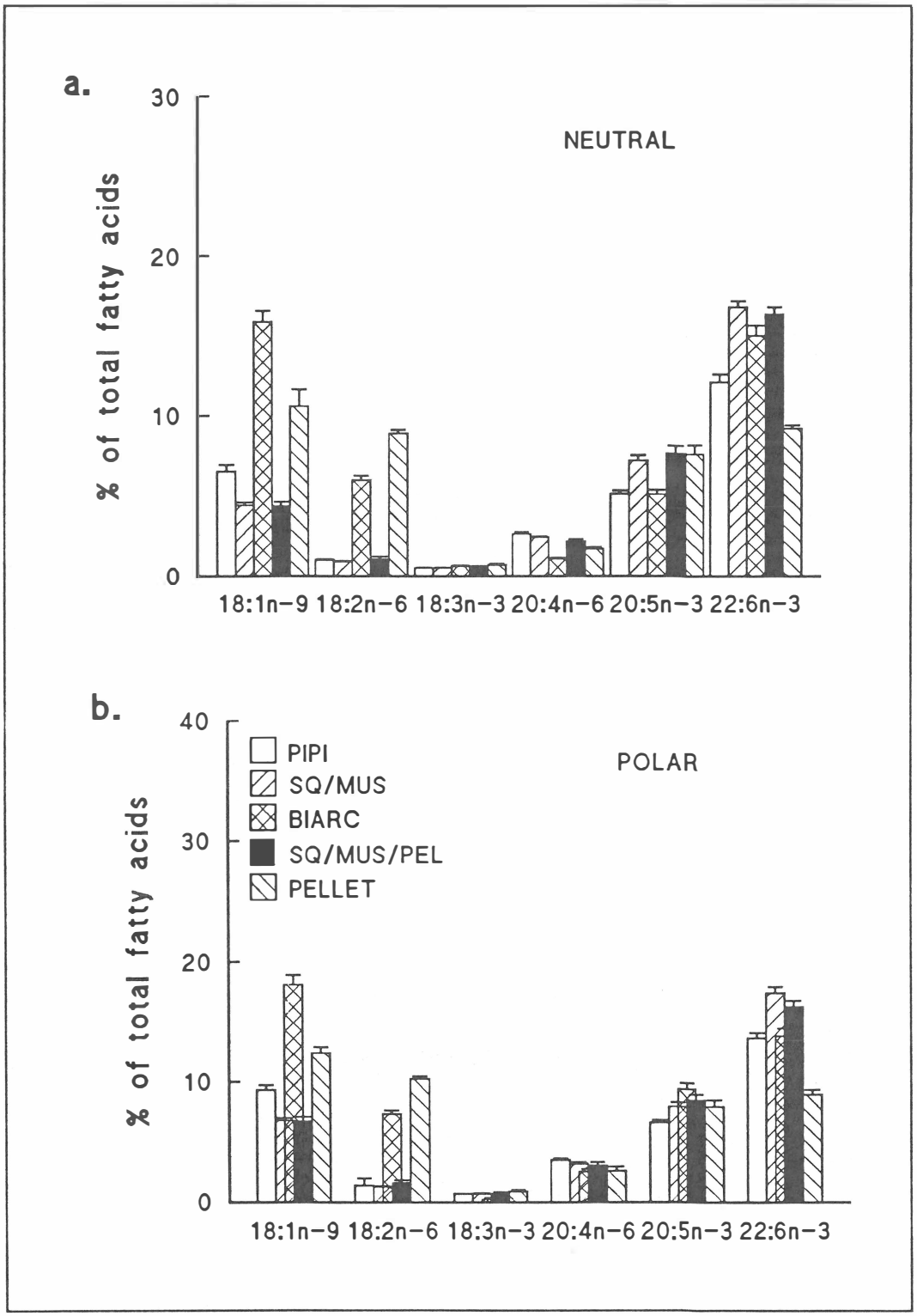


Figure 12.2 Levels of certain fatty acids in the **a.** Neutral and **b.** Polar lipid fraction of the hepatopancreas of *P. monodon* (means + standard error)



The level of dietary fatty acids was, in general, reflected in the level of fatty acids in the tissues. One exception to this trend was the neutral fatty acid 20:5n-3 which, despite being present at a higher level in the pipi diet (Diet A) than in the other diets, was present at a lower level in the hepatopancreas and ovary of prawns fed this diet than in the tissues of prawns fed the other diets. This drop may be explained by the preferential incorporation of 20:5n-3 into phospholipids (Kanazawa 1981) which are the major polar lipid class in prawns. As Diet A was relatively low in polar 20:5n-3, the fatty acid in the neutral lipid fraction may have been transfered to the polar to make up for the shortfall. This transfer may also, in part, explain the levels of 18:1n-9 and 20:5n-3 in the hepatopancreas of prawns fed the BIARC diet (D2) relative to their levels in tissues of prawns fed the other diets. The ovary of prawns fed the BIARC diet show an unexpectedly high level of 20:5n-3 relative to that in prawns fed the other diets which is difficult to explain.

Lipid accumulation by prawn tissues is not conservative in terms of lipid quality. Prawn tissues reflect the quantity and quality of dietary lipids, particularly in relation to the fatty acid content. Rather than tissues accumulating a predetermined concentration of individual fatty acids, as they do with amino acids, the tissues tend to accumulate fatty acids as they become available through the diet. The low level of lipids (with fatty acids) in Diet A had no effect on ovary lipid levels suggesting that levels as low as 4% of dry matter supply sufficient quantities of lipid during ovary development. However, the quality of the ovary tissue and hence the developing oocytes, will still be effected by the fatty acid content of the dietary lipids.

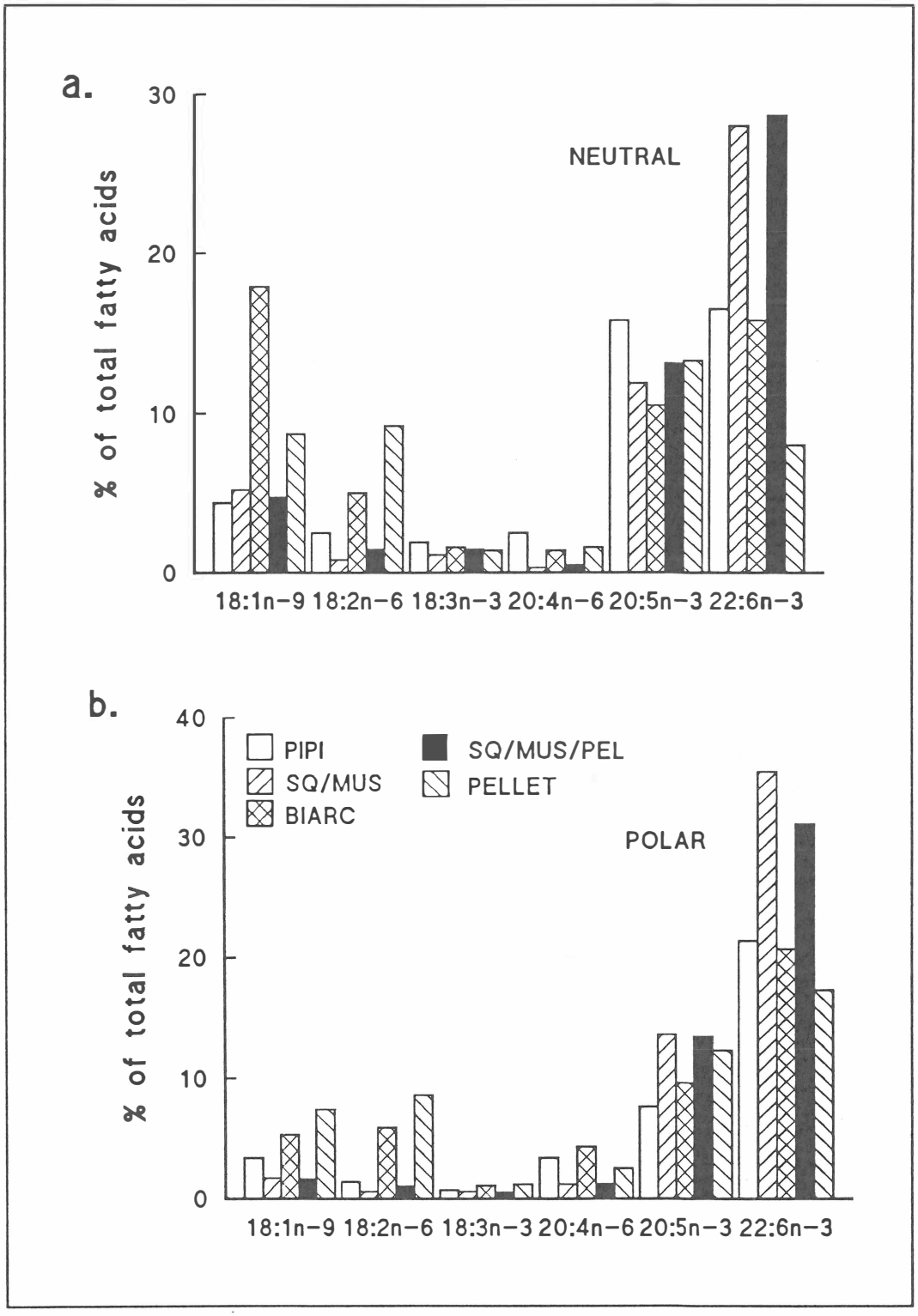
Thus, while increasing the quantity of poor quality protein in a diet may be a means of satisfying protein requirements, this principal does not apply to lipids. Poor quality dietary lipids will directly effect the quality of the lipid component of the ovary tissue and hence, egg and larval quality.

12.3.2 Spawning performance

Table 12.6 shows the mean values for spawning performance of prawns fed each of the four diets. Results were also pooled to enable a comparison of spawning performance in each of the two time periods and are presented in Table 12.7 A total of 185 spawns were assayed. There was no significant difference between any of the parameters measured for the two experiments, except for larval survival which was significantly higher for the November group. The cause of this difference is unknown but the potential significance to the Australian prawn industry indicates that further investigation is warranted.

Based on the biochemical composition of the feeds offered (Section 8) and the ratios of components consumed estimated from daily intake over the 42 day period, the fatty acid profiles were estimated for each of the four diets (Figure 12.3). As the dietary components (squid, mussel and pellet) in Diets B & C were consumed in similar ratios in all trials the results presented in Figure 12.3 are representative of both.

Figure 12.3 Levels of some fatty acids in the **a.** Neutral and **b.** Polar lipid fraction of the experimental diets (means + standard error)



Between diets there were significant differences in fecundity, egg hatch rate, larval survival and female maturation rate (Table 12.6). A number of these differences can be related to the diets eaten by the prawns.

Table 12.6 Summary of rates of maturation, female survival, fecundity, egg hatch rate and larval survival of *Penaeus monodon* fed four different diets.

Diet[1]	Total spawns	Assayed spawns	Female survival (% p.d)$_{2,3}$	Maturation rate$_4$	Fecundity (eggs/spawn)	Hatching rate (%)	Larval survival (%)
A	56	45 (80%)	75.5^{A} (n=24)	2.11^{AB} (n=24)	353 440^{A} (n=44)	37.1^{A} (n=42)	46.6^{A} (n=30)
B	102	69 (68%)	85.2^{A} (n=48)	1.81^{AB} (n=48)	309 760^{B} (n=69)	21.1^{B} (n=65)	24.2^{B} (n=39)
C	71	49 (69%)	86.6^{A} (n=24)	2.20^{B} (n=23)	380 250^{A} (n=49)	19.7^{B} (n=48)	31.1^{AB} (n=32)
D2	29	18 (62%)	84.5^{A} (n=24)	1.22^{A} (n=24)	259 210^{B} (n=18)	41.2^{A} (n=17)	26.2^{AB} (n=12)

[1] Diet A: Pipi (*D. deltoides*), Diet B: Squid (*Loligo* sp.) mantle, and whole mussel (*P. cannaliculatus*); Diet C: Squid mantle, whole mussel and dry pellet (*P. japonicus* finisher); Diet D: experimental moist pellet.

[2] Prawn-day

[3] (total p-d/total possible p-d) x 100

[4] Number of spawns per female per 30 p-d.

Within columns values with a common superscript letter are not significantly different

Table 12.7 Summary of rates of maturation, female survival, fecundity, egg hatch rate and larval survival of *P. monodon* in two experimental periods.

Month	Total spawns	Assayed spawns	Female survival (% p.d)	Maturation rate	Fecundity (eggs/spawn)	Hatching rate (%)	Larval survival (%)
September	142	93 (65%)	79.8^{A} (n=72)	1.83^{A} (n=71)	309 760^{A} (n=92)	27.3^{A} (n=87)	21.4^{A} (n=48)
November	116	88 (76%)	86.7^{A} (n=48)	1.82^{A} (n=48)	338 560^{A} (n=87)	32.2^{A} (n=84)	42.6^{B} (n=64)

Within columns values with a common superscript letter are not significantly different.

The level of neutral 20:5n-3 fatty acid was highest in Diet A, declined through Diets B and C and was present at the lowest level in diet D2. The same ranking was apparent in the maturation rate and fecundity criteria of spawning performance (Table 12.6). This positive relationship was not however evident between fecundity, maturation and the level of 20:5n-3 in the ovary tissue. It is possible that the positive effect of 20:5n-3 was not from its structural or storage roles in the ovary tissue but rather its role in the transportation of lipids and in the activation of certain enzymes (Kanazawa, 1981).

This relationship between dietary levels of the 20:5n-3 fatty acid and fecundity was also demonstrated by Xu (1993) for *P. chinensis.* However, Xu (1993) also found a positive correlation between dietary levels of the 22:6n-3 fatty acid and hatch rate. In this study the reverse was found. Hatch rates were highest for eggs from prawns fed Diets A & D2 which had the lowest levels of the 22:6n-3 fatty acid. As differences in dietary levels of this fatty acid were also reflected in the polar and neutral lipids of the ovary tissue, it would seem that the 22:6n-3 fatty acid has a significant role in the membranes and/or energy production within the developing egg. Xu's (1993) results may simply indicate a difference in 22:6n-3 fatty acid requirement between a temperate species (*P. chinensis*) and a tropical prawn species (*P. monodon*).

Larval survival showed no relationship to the level of any dietary component, however, a negative relationship existed between larval survival and the level of cholesterol in the hepatopancreas. Diet A contained significantly ($P < .05$) lower levels of cholesterol than Diet B and the larvae of prawns fed Diet A had a significantly higher survival rate than larvae from prawns fed Diet B. One explanation for this negative relationship is that the cholesterol in the hepatopancreas is used in the production of prostaglandins (Kanazawa 1981) and steroid hormones thought to be involved in the control of ovarian development (Section 4).

When interpreting these results it must be recognised that not using purified diets means that other, unknown variables may be influencing the results. Vitamin, mineral and pigment composition are three other factors which are likely to have been different for the diets examined. Despite this, these results, if used in conjunction with other outcomes outlined in this report, help to define the role of specific fatty acids in prawn maturation.

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