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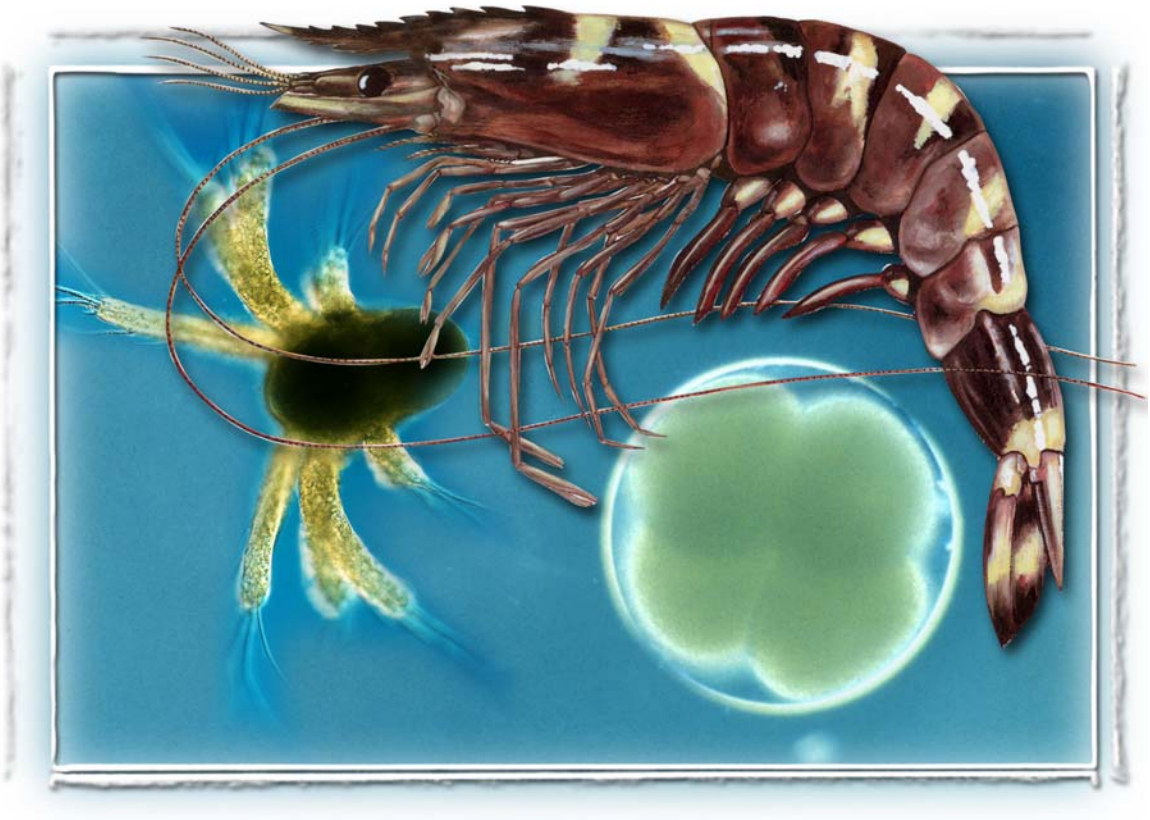

High Quality Eggs and Nauplii for the Australian Prawn Industry

Michael R. Hall, Rebecca Mastro, Neil Young, Carol Fraser, Jan Strugnell and Matt Kenway

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M.R. Hall, R. Mastro, N. Young,
C. Fraser, J. Strugnell & M. Kenway

Australian Institute of Marine Science
PMB No 3, Townsville Qld 4810

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Non Technical Summary

95/166 High Quality Eggs and Nauplii for the Australian Prawn Industry

PRINCIPAL INVESTIGATOR: **Dr Michael R. Hall**

ADDRESS: **Australian Institute of Marine Science**
PMB No. 3
Townsville Mail Centre Q 4810
Tel: (07) 4753 4308 Fax: (07) 4772 5852
e-mail: m.hall@aims.gov.au

OBJECTIVES:

1. To determine the physiological requirements for successful vitellogenesis or yolk production.
2. To identify and chronicle the substances that are accumulated during egg development.
3. To compare egg quality between wild and captive-reared broodstock.
4. To demonstrate the value of supplementing broodstock diets with carotenoids.
5. To demonstrate the value of supplementing broodtsock diets with ecdysteroids.

SUMMARY

Prawn farming is the most valuable aquaculture sector in Queensland and is a priority development industry for the State Government. Marine prawns have provided the major growth in this industry with a value of $37 million in 2000/01, or over 66% of the value of Queensland aquaculture. Nevertheless several issues need to be addressed to ensure the industry continues to grow and is economically and environmentally sustainable.

The supply and performance of broodstock is seen as the weakest link in the production cycle, both overseas and nationally, and is viewed as a major research priority by the Australian Prawn Farmers Association (APFA). Presently the marine prawn sector is dominated by *Penaeus monodon* production, the broodstock of which are obtained from the wild fishery in a few near-shore coastal areas in northern Queensland. Recent screening of wild-sourced broodstock for viruses has revealed that nearly all of them, from the traditional areas of supply, are carriers of potentially pathogenic forms of viruses. In response, the industry has developed a strategic goal of becoming a true farming sector by shifting its reliance on wild broodstock toward totally closed life cycle production. Research providers and industry are now committed to developing domesticated broodstock that will eventually be used in selective breeding programs to produce specific strains or breeds

This project placed special emphasis on strategic issues, to develop solutions to current and future challenges for prawn hatcheries, and applied issues, with effort directed towards clear and identifiable objectives in improving broodstock reproductive performance. Strategic issues included developing specific research tools to investigate some of the basic mechanisms that underpin reproductive capability and its physiological control in *P. monodon* (Objectives 1 and 2). This information is essential if informed knowledge-based improvements in broodstock management are to develop in a vigorous manner. Applied issues included the objective of comparing reproductive performance of captive-reared broodstock that had been grown to sexual maturity in either tanks or ponds (Objective 3). The final objective was directed to examining supplementation of specific compounds in broodstock maturation diets and their impacts on subsequent reproductive performance (Objective 4).

Outcomes achieved:

Two research tools have been produced that should benefit development of broodstock managerial regimes to improve reproductive performance of *P. monodon* broodstock. It is now possible to monitor the females' reproductive system in response to manipulating environmental factors to stimulate spawning activity. The development of an enzyme-linked immunosorbent assay (ELISA), as a joint project in prawn reproduction research with the Aquaculture CRC Ltd, is also of value in the endeavor to develop an alternative technology to unilateral eyestalk ablation to induce spawning of broodstock on demand. The other research tool, two-dimensional polyacrylamide gel electrophoresis (2D PAGE), has allowed the identification and comparison of gene products found in 'good' and 'poor' quality eggs.

Comparisons between wild and captive-reared, including pond-reared and tank-reared, broodstock, revealed that captive-reared females are eventually capable of egg production equivalent to that of their wild conspecifics. The trials included spawnings produced by natural matings and the use of artificial insemination for the production of post-larvae of known parentage. Captive-reared female broodstock have less reproductive competence compared to wild ones and require several reproductive cycles until they fully mature. A major problem is sperm quality and fertilization capability by the males. Captive-reared males produce spermatophores that are morphologically different from their wild conspecifics and generally contain fewer sperm bundles. Some also develop an infection of the reproductive tract that results in the inability to fertilize eggs at rates required for commercial hatchery production requirements.

Dietary supplementation of broodstock diets with the carotenoid astaxanthin significantly improved reproductive performance measured as an increase in the number of larvae (nauplii) produced per female. It may also improve the health status or quality of the larvae. The supplementation of ecdysone, a hormone produced naturally by the female and reported to influence molt, reproductive physiology and egg development, did not improve reproductive performance of female broodstock.

KEYWORDS: Penaeus monodon, aquaculture, egg quality, fertility

Background

Breeding biology of *Penaeus monodon*

The age of first breeding in *P. monodon* is unknown. However, it is apparent that they go through a period of adolescence before passing through puberty and finally becoming sexually mature (Kenway and Hall 1998). Anecdotal evidence supports the conclusion that a combination of age and body size influences the onset of sexual maturity. Induced maturation and spawning has been attained by unilateral eyestalk ablation in individuals as young as 5 months of age (Primavera 1978). On rare occasions, intact females have been found in grow out ponds with developed ovaries. Based on the experience of specialist trawl operators that supply wild broodstock to hatcheries, it is estimated that the age of first breeding ranges from 5 to 12 months (Kenway and Hall 1998). It is believed that, once broodstock enter coastal waters females will breed on a seasonal basis for the rest of their lives. Although the life span of *P. monodon* is not known with certainty, it is probable that some females survive to at least 2 years of age.

The average size of *P. monodon* broodstock varies according to geographic location and may have a genetic as well as an environmental basis. Large males, 80 grams or larger, are preferred by hatcheries for captive matings as they generally produce higher hatch rates compared to those obtained from smaller males typically utilized as broodstock as matings with large males results in higher fertilization rates compared to small males (Pratoomchat et al. 1993). In Australia, wild female broodstock are typically within the range of 110 to 160 grams, whereas in the more equatorial regions of Thailand broodstock range from 150 to well over 200 grams. Commercial hatcheries prefer larger females to smaller ones in order to maximize egg production per spawner utilized. However, it is reported that very large females, over 150 grams, which are assumed to be older females, often do not perform well in hatcheries. These very large females typically exhibit high mortality immediately after unilateral eyestalk ablation or develop an intense reddish colouration that is believed to be an indicator of stress and such females may be approaching senescence.

Upon reaching sexual maturity, the female is typically inseminated by a male each time she molts. The spermatophores are stored in the closed thelycum of the female, which is a modification to the posterior sternal plates and is a flesh-covered chamber that can maintain viable sperm throughout the inter-molt period. However, in extremely elongated inter-molt periods, such as those that may occur through winter, the viability of the sperm held in the thelycum is probably of inferior quality. In such cases hatcheries, in order to optimize fertilization rates, should induce these females to molt in a tank containing males, before ablating them. Insemination only occurs within a few hours of the female molt when the thelycum is still soft and can receive the spermatophore. The stored spermatophore is lost each time the female molts so the female must be re-inseminated by a male to maintain fertility. If a hatchery maintains female broodstock through a molt cycle, it is essential males also be held in the same tank to ensure fertilization.

Mating in *P. monodon* has only been observed in captive broodstock. Mating takes place at night immediately after the female molts. The female is followed by one or more males and eventually swims into the water column where a male will swim parallel to her. Swimming activity may occur for several hours before mating or may only take a few minutes. The male bends his body and orientates perpendicular to the female, making ventral-ventral contact. The paired spermatophores are released from the male's ampullae and are inserted through the lateral slits of the female thelycum into the chamber beneath. The thelycum is divided into two chambers, one for each spermatophore pair, on either side of the thelycum ridge.

Further matings may be prevented by the unraveling of the spermatophore tail into a fan that protrudes outside the thelycum, temporarily blocking the opening. Courtship and mating varies from 0.5 to 3 hours and requires parallel swimming over several metres which has implications for the design of broodstock holding tanks (Motoh 1981). Successful matings occur in large rectangular tanks as well as 4 metre diameter circular tanks of 1 metre depth (Primavera 1988).

In female penaeid prawns the ovaries are paired, but partially fused in the cephalothorax (head and thorax region), and consist of a number of lateral lobes which continue along the entire length of the tail. The determination of ovarian development by hatchery technicians is made by illuminating the internal body organs of the female by means of a bright underwater torch beam being passed along her side (Fig. 1). However, due to the density of the cephalothorax, the only portion of the ovary seen by illumination is that within the tail. This appears as a dark shadow, due to the dense lipid composition and pigmentation, and can be ranked through a series of developmental stages from I to V (Fig. 2).

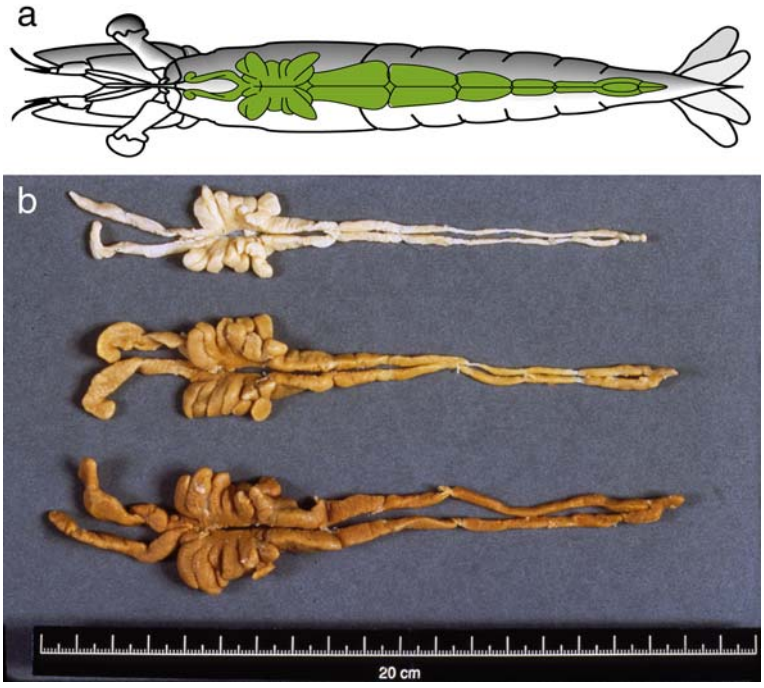

FIGURE 1. (a) Line drawing of female with ovary that extends the entire length of the prawn. (b) Complete ovaries removed from Stage II, III, and IV (top to bottom) broodstock.

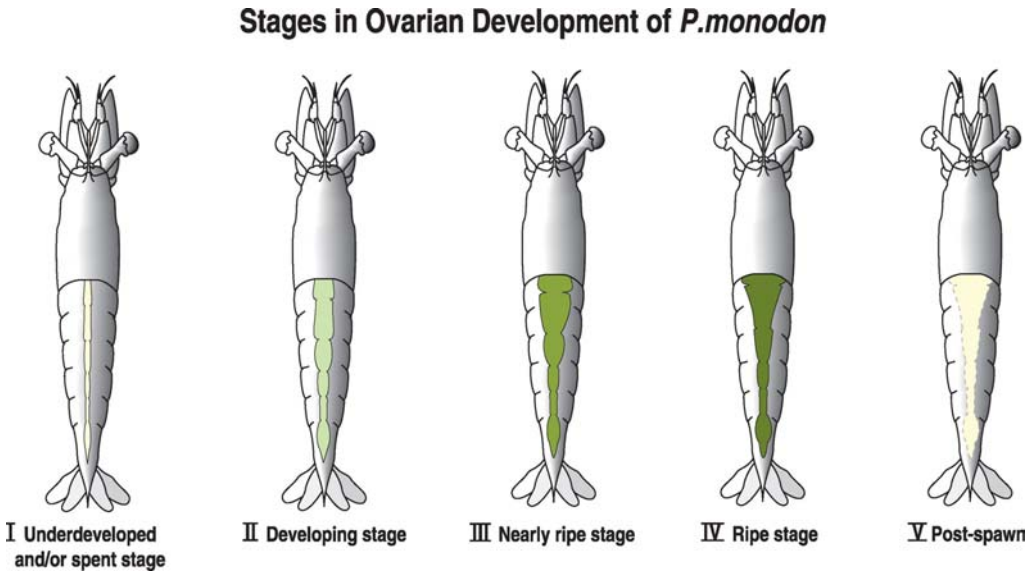

FIGURE 2. The view observed by hatchery operators when female broodstock are graded for ovarian development by torchlight.

In the undeveloped state, the ovary either does not cast a shadow or a thin opaque line is seen along the length of the tail. Such an ovary is scored as Stage I (Fig. 3).

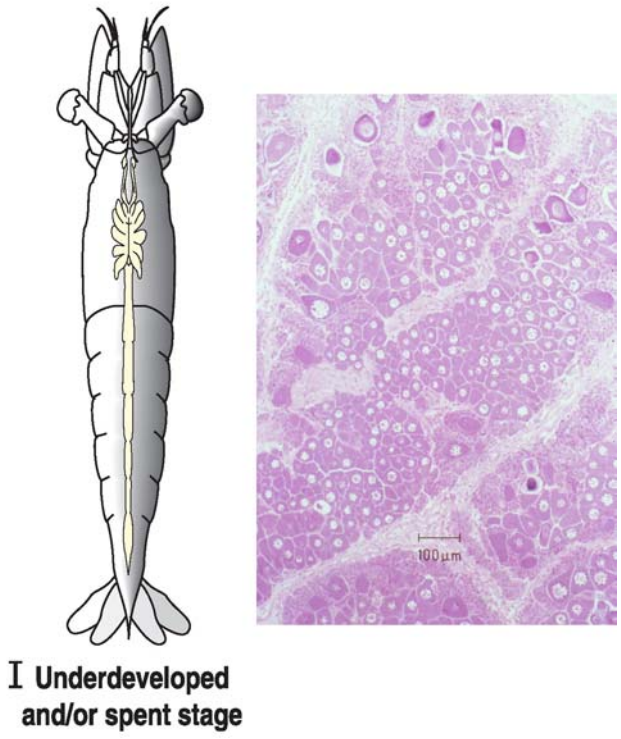

FIGURE 3. Stage I, undeveloped ovary as observed in a molting female or before ovarian maturation beings showing the zone of proliferation where egg recruitment begins.

At this stage the ovary comprises a connective tissue capsule surrounding a soft vascular area containing the future eggs, called primary oogonia, and accessory cells, also called follicle or nurse cells. The internal wall of the ovary capsule is lined with epithelial cells (called the

germinal epithelium) containing oogonia. Once the female is sexually mature, the germinal epithelium will produce oogonia by mitotic division throughout the reproductive life of the female. The eggs develop from oogonia in an area known as the zone of proliferation (Fig. 3). As the oogonia develop they increase in size and enter the first stage of meiotic division and henceforth are irreversibly destined to become haploid, with only one set of maternal chromosomes. At this point, although the developing eggs are increasing in size, they do not produce vitellogenin (yolk), and are known as previtellogenic oocytes. At this stage, the ovary can be visualized with a torch beam as a thin centrally located opaque rope-like structure and is classified as Stage II (Fig. 4).

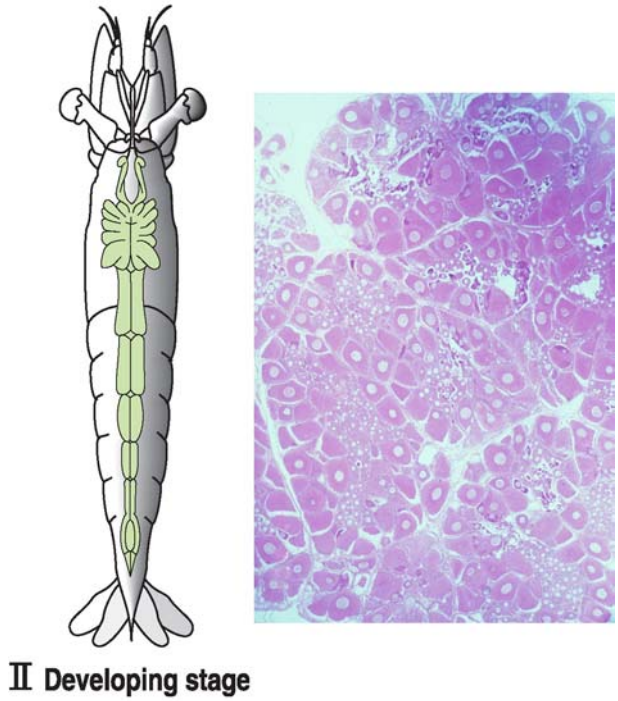

FIGURE 4. Stage II, developing ovary with some oocytes beginning to fill with vitellin (yolk).

As the oocytes develop further they migrate towards the margins of the ovarian lobes in preparation for final maturation and ovulation. During this migration, follicle cells are attached to the periphery of each oocyte and it has been demonstrated that these cells are the source of yolk production, or vitellogenesis, in the ovary, with the yolk protein being subsequently transferred to the maturing oocytes (Tsutsui et al. 2000). Cloning and expression analysis of the vitellogenin mRNA has demonstrated that there is also an extraovarian site of vitellogenin synthesis, namely the hepatopancreas (Tsutsui et al. 2000). As vitellogenesis proceeds, oocytes mature synchronously in the individual ovarian lobes and, as yolk accumulates, the ovaries develop a dark green colour as a result of co-deposition of carotenoid pigments. These absorb light and hence produce the shadow seen by hatchery technicians when the bodies of females are illuminated by torchlight. At this point of ovarian development, females are scored as Stage III (Fig. 5).

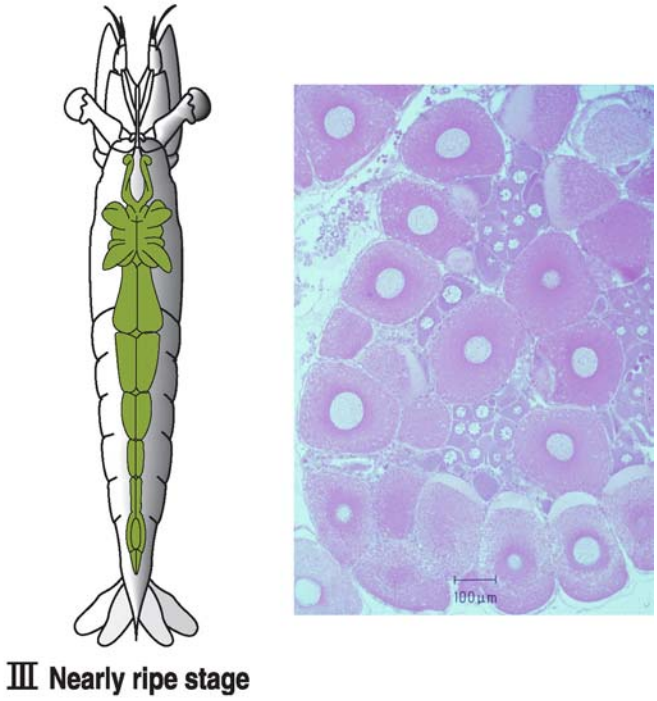

FIGURE 5. Stage III, mid- to late developing ovary with large stores of vitellin appearing in oocytes.

Towards the end of vitellogenesis, the eggs develop cortical granules, which can only be seen in histological preparations. The cortical granules, or crypts, are filled with a jelly-like substance that forms a temporary protective layer immediately after spawning, prior to formation of the hatching envelope. When the ovarian shadow is large and visible as a distinct thick dark region extending the length of the abdomen, an enlarged bulbous region called the saddle is usually visible directly behind the posterior edge of the carapace. This is scored as a gonadal index Stage IV (Fig. 6).

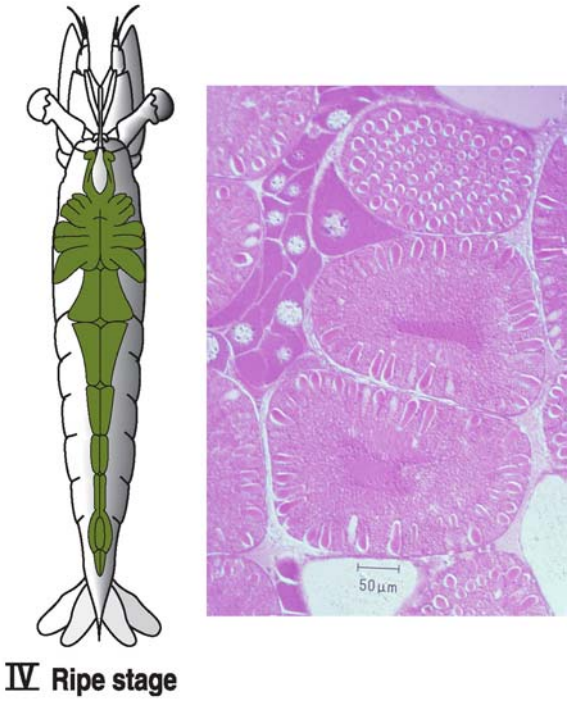

FIGURE 6. Stage IV, late developing ovary immediately pre-spawning with large cortical crypts (jelly-like substance).

Females scored with Stage IV ovaries will either spawn the same or the following night. Spawning is marked by release of the fully mature oocytes (eggs) into the oviduct at ovulation and is rapidly followed, within minutes, by the release of eggs at ovipositioning, or spawning. At spawning the female swims continuously in the water column. Increased activity may occur for hours before spawning or may suddenly develop immediately before spawning. At the moment of spawning, the last three pairs of pleopods are held tightly together and flapped vigorously. The unfertilized eggs are extruded from the paired ovipores located at the base of the third periopods. At the same time stored sperm is released in an anterior direction from the thelycum. Both eggs and sperm are ejected with considerable force. Their union can be visualized as a blending of the pale green stream of eggs with a whitish stream of sperm directly below the female as she swims forward. As the sperm are entirely non-motile they must come into contact by passive collision with the surface of the egg at the moment of ejection. It is believed that the vigorous flapping of the female's pleopods creates an intense vortex that maintains a high egg-sperm density with intense mixing, increasing the likelihood that fertilization occurs. Upon release into seawater the nuclear membrane of the egg disappears in readiness for fusion between the two (haploid) pronuclei, one in the egg originating from the female and the second from the male. Fertilization marks the beginning of embryogenesis.

The entire process lasts approximately 2 minutes, over which up to 500,000 eggs of 0.3 mm diameter are shed through the two ovipores resulting in a maximum ejection flow rate of 2.25 km/h. Immediately after spawning the fertilized eggs are either slightly positive or neutrally buoyant. Within a few minutes, after the extrusion of the jelly-like substance with the cortical crypts, the eggs gradually hydrate and drift down through the water column and collect on the bottom. Spawning can occur in females that have not been inseminated, and though egg output per female may appear adequate, fertilization will not occur and hence none of the eggs will hatch.

A female can spawn several times within a single molt cycle, with the first spawn usually occurring just a few days into the normal 16 to 18 day molt cycle exhibited by captive broodstock in tanks. If the female spawns repeatedly within a molt cycle, the same reservoir of sperm within the thelycum, which derives from the initial insemination immediately after molting, is used repeatedly. As females approach a new molt, irrespective of whether they have spawned during their current inter-molt period, the ovaries regress. Following the molt a new population of primary oogonia are recruited into the next spawning event.

Aspects of female broodstock in hatcheries

In wild broodstock, the shadow of a stage IV spawner has a distinct saddle immediate below the edge of the carapace. Typically, however, in pond- reared broodstock, this saddle is less developed or absent. It is also reduced in females that have been ablated and already had consecutive spawnings within the same molt cycle. In immediate post-spawning females, a vague shadow may be seen which is the area the previously enlarged ovary occupied. However, in a proportion of post-spawners, a dark shadow is seen intermittently along the length of the tail interrupted by a shadow outline. Such females are partial spawners where only a proportion of the oocytes ovulated and were ovipositioned (spawned). The exact reason for this is unknown, but may be due to non-synchronised development of the entire ovarian mass or may occur when females have been interrupted during spawning. In captive-reared broodstock partial spawnings following ablation may result from the female spawning for the first time when her ovaries have undergone their first maturation (Fig. 7). Typically, the portion of the ovary where ovulation did not occur will ovulate within the following 24 hours.

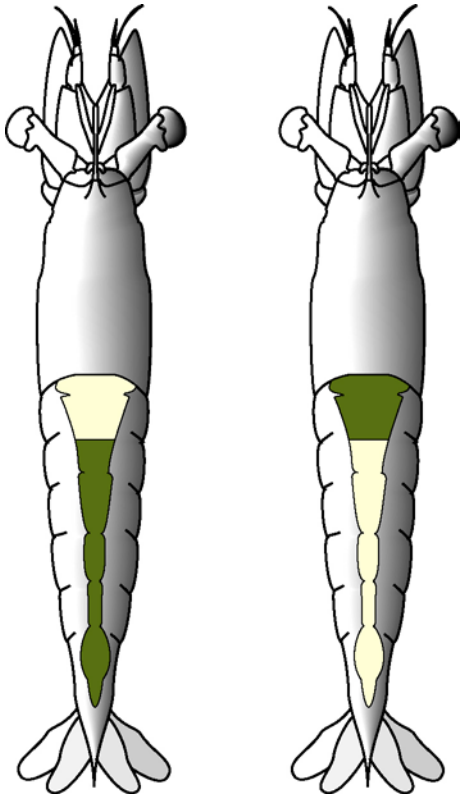

FIGURE 7. Partial spawnings are typically only observed in the tail region, where either the anterior or posterior proportion of the mature eggs are spawned. Partial spawning may also occur in the cephalothorax region but this can only be observed after dissection.

However, the unovulated eggs may be re-absorbed. Potentially, it may prove worthwhile for hatcheries to keep track of complete and partial spawners, as partial spawnings may be indicative of viral infection. It has been reported that oocytes infected with virus, such as white spot syndrome virus (WSSV), do not fully mature and hence do not ovulate (Kuo and Lee 1998). Although WSSV infected oocytes may not ovulate, virus particles may be released during the spawning event into the water. In addition, oocytes that are re-absorbed may release virus particles that could adhere to uninfected eggs which are ovulated. This could result in vertical transmission of virus from mother to offspring, and hence potentially produce post-larvae that are viral carriers with the potential to lead to an epizootic during the grow-out period.

Eyestalk ablation

It is believed that the initiation to Stage II is due to the release of the oocytes from inhibitory hormones which prevent ovarian maturation. These hormones originate in the eyestalk from an X-organ-sinus gland (XO-SG) neurosecretory complex (Fig. 8), from where they are released into the haemolymph (blood). It is not known exactly how this release from inhibition occurs in the wild.

Spontaneous spawning of either captive-reared or captive-held *P. monodon* is rare. Although the exact reason for this is unknown, it may be due to the female experiencing suboptimal environmental conditions, or to poor nutritional status. Nevertheless, spawning can be induced in these animals through unilateral eyestalk ablation, which results in the partial removal of the source of the inhibitory hormone that prevents spawning in intact females. Although prawns are bilaterally symmetrical, i.e. with two eyes, and hence unilateral eyestalk

ablation only results in the removal of one of the sinus gland complexes, it usually results in the induction of ovarian maturation and spawning in 60% to 80% of wild females. As a result unilateral eyestalk ablation is the most widely used technique to induce and synchronise spawnings for hatchery production cycles.

Maturation diets

Wild female broodstock are preferentially sourced as stage III or IV animals. These prawns generally spawn on the night of delivery to the hatchery, or within the first couple of nights, without eyestalk ablation. Hatchery managers generally consider that such females produce the highest quality eggs and nauplii. Such broodstock have obtained their entire nutritional requirements from a wild diet and spawn without the stress caused by unilateral eyestalk ablation.

Sourcing stage III or IV animals from the wild is not always possible and either ovarian maturation has to be completed within the hatchery or the female goes through a molt and the entire ovarian maturation takes place within the hatchery environment. In these latter cases, a high quality maturation diet is necessary for the female to optimize egg production and egg quality for the production of vigorous larvae and post-larvae (juveniles). The quality of the egg at the time of formation and the packaging of critical molecules into the egg may be a major determinant of larval quality. The success of fertilization and development of the embryo through to hatching, and the subsequent six non-feeding naupliar stages, is mainly determined by egg quality. The quality of larval feeds only becomes important at the first self-feeding stage of zoea 1.

This project set out to develop the research tools necessary to examine aspects of broodstock performance in relation to egg and nauplii quality. Furthermore, the project objectives were to identify factors that influence the quality of eggs and nauplii and to recommend potential broodstock management strategies to optimize broodstock performance.

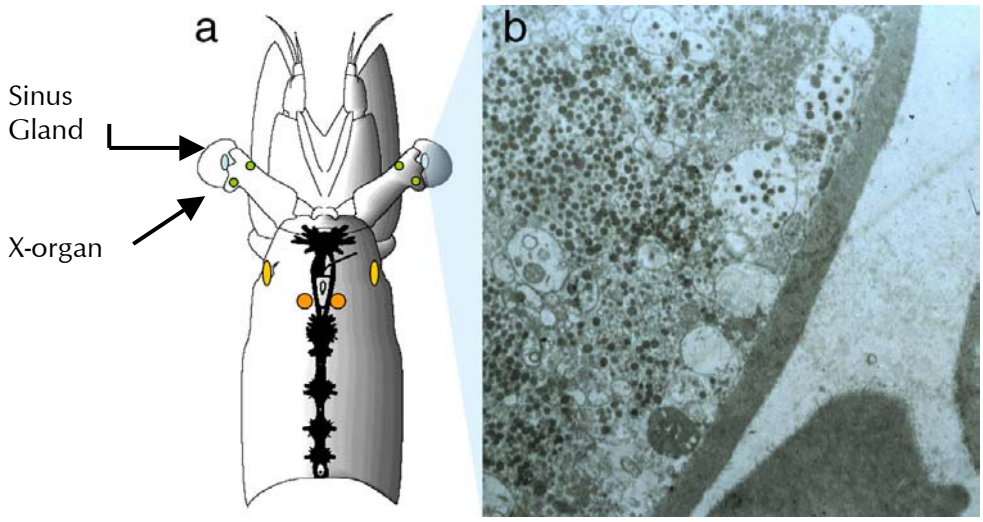

FIGURE 8. (a) Schematic diagram of the major endocrine organs in *P. monodon*. The sinus gland is composed of the terminals of neurons whose cell bodies originate in the X-organ and brain. (b) Electron microscopy section of the sinus gland demonstrating hormone filled vesicles (dark circles) which fuse with the cell membrane and release their hormone content into the blood sinus. Magnified 8500x.

Need

Major priorities for the Australian prawn-farming industry include improvements in broodstock performance and closure of the life cycle for domestication, selective breeding and biosecurity (Australian Prawn Farmers' Association Research and Development Plan 1996-2005, Macarthur Consulting). In a more recent survey of the industry, 10 research areas were given priority, with improvement of broodstock performance being the second highest priority, after improved access to spawners (APFA R&D Plan 2000).

Despite several years of research on broodstock maturation diets in Australia and overseas, only marginal improvements in spawner productivity have been achieved. The FRDC has previously provided support for related projects including:

Project 89/52 Introduction and development of overseas technology for production-scale maturation of penaeid prawns.

Project 89/78 Improving Prawn Hatchery Production by Reducing Losses due to Bacterial Diseases

Project 92/51 Factors Affecting the Reproductive Performance of Captive and Wild Broodstock Prawns

These FRDC-funded projects yielded valuable data on gross protein and lipid requirements for broodstock diets, and on the formulation of maturation diets. However, despite 15 years of research on maturation diets overseas, and more recently in Australia, only marginal improvements in spawner productivity have been achieved. These projects illustrate that nutritional state modulates spawner performance, but also emphasize that ovarian maturation and spawning is a complex physiological event. The results from these studies demonstrate the need for studies into the control of egg production, especially parameters of egg quality.

Internationally, it is acknowledged that hatcheries are the weakest link and most inefficient stage in prawn production (Rosenberry 2000). In Australia, it presently requires more than 7 spawners on average to stock a 1 hectare pond at a typical initial stocking density of 350,000 to 400,000 post-larvae/ha. For example, in 1992/93 3,000 spawners were required to produce 123,000,000 post-larvae (PLs or fry), which represents an output of 41,000 PLs per initially purchased spawner. This represents a success rate of less than 15%, i.e. survival from egg to post-larvae day 15 (PL15), assuming that the average spawner produces 350,000 eggs per spawning. With wild-caught spawners fetching prices as high as $150 each, this could represent more than $1,000/ha in spawner costs alone for Australian producers. Pond-reared broodstock performance is reported to be significantly poorer. Clearly, increases in broodstock efficiency would be highly desirable.

TABLE 1. *P. monodon* and prawn broodstock demand by the Australian industry. * = data combined with other penaeid species except *P. japonicus* (from Lobegeiger R. Reports to Famers, QDPI).

Year	Spawners Purchased	Spawners Used	Post-larvae Produced (million)	Post-larvae Sold (million)
94/95	n.a.	2,359	129.5	85.0
95/96	n.a.	2,680	120.8	66.3
96/97	n.a.	2,901	155.0	81.6
97/98	3,721	3,368	175.2	138.9
98/99*	3,553	3,218	269.0	157.3
99/00*	3,730	4,696	217.0	105.7
00/01*	5,547	4,222	278.3	125.1

The overall objective of this study was to gain an understanding and insight into the major determinants of broodstock quality and specifically those involved in egg and nauplii quality. Several key processes were examined (Fig. 9).

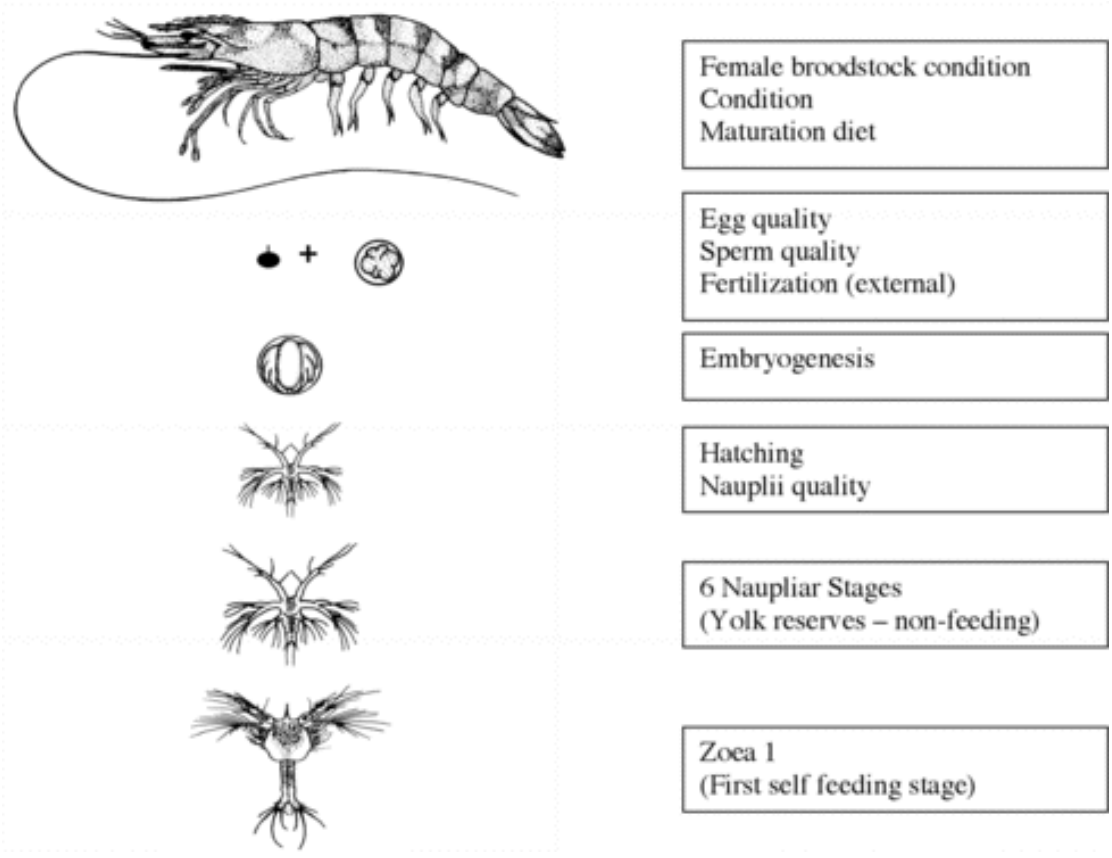

FIGURE 9. Flow diagram of major components of female broodstock spawners determining egg and nauplii quality. Not to scale.

OBJECTIVE 1

To determine the physiological requirements for successful vitellogenesis or yolk production

Introduction

Ovarian development and egg maturation vary considerably among crustaceans, but as a general rule, can be divided into two phases. The first phase is primary vitellogenesis and is characterized by the recruitment of primary oocytes from oogonia in the germinal layer, or zone of proliferation, within the ovary, resulting in primary and secondary oocytes. The process continues at various times throughout adult life in crustaceans (Charniaux-Cotton and Payen 1988). The degree to which primary vitellogenesis is under hormonal control is poorly known (Quackenbush 1991).

The next phase, secondary vitellogenesis is bridged by a transitional platelet stage during which oocytes increase in size, micropinocytosis becomes apparent and cytoplasmic yolk granules begin to appear (Anderson et al. 1984). This transitional stage is rapidly followed by full fledged secondary vitellogenesis with the appearance in the nucleolus of granular and fibrous zones. In penaeid prawns the oocytes increase in diameter to approximately 250 – 300 µm, which is mainly due to the accumulation of cytoplasmic egg proteins and vitellin. During the final stages of vitellogenesis large cortical granules or crypts develop and are destined to form a temporary protective jelly layer after spawning (Charniaux-Cotton and Payen 1988). Vitellin is by far the predominant egg protein and may constitute 60% – 90% of total egg protein (Eastman-Reks and Fingerman 1985).

Secondary vitellogenesis is modulated by hormones originating from the X-organ-sinus gland (XO-SG) neurohaemal complex within the eyestalk. A principle neurohormone involved in secondary vitellogenesis is the vitellogenesis inhibiting hormone (VIH), which has also been termed gonadal-inhibiting hormone (GIH) (Quackenbush 1991). The neurohaemal nature of the XO-SG as the source of VIH was first demonstrated by Panouse (1943). Unilateral eyestalk ablation, and hence the excision of the XO-SG, results in precocious ovarian development and ovulation in the majority of sexually mature female Crustacea. Several other hormones, including some from the XO-SG, are also thought to play a role in ovarian development (for reviews see Adiyodi 1985; Keller 1992; Van Herp 1992; Laufer et al. 1993 and Webster 1998).

The major cellular activity during ovarian development is yolk protein production, i.e. secondary vitellogenesis, during which the gene encoding vitellogenin (Vg) is activated (Tsutsui et al. 2000). The mature Vg molecule is a dimer formed from two dissimilar molecular weight monomer units of 158-170kD, the A-subunit, and 74-82 kDa, the B-subunit respectively (Chen and Chen, 1994, Thurn and Hall 1999, Fraser 2000). However, in nearly all studies, intermediate molecular weight molecules are found, typically some less than 74 kDa and others in the range of 90 to 110 kDa (Vazquez-Bouchard et al. 1986, Cariolou and Flyzanis 1993, Quinitio et al. 1990, Chang, Lee and Huang 1993, Chen and Chen 1993). The recent isolation of the Vg gene from *P. japonicus* has revealed that these subunits are all protein cleavage products of a single gene product (Tsutsui et al. 2000). The mature vitellogenin molecular in heavily post-translationally modified, with extensive phosphorylation and glycolslation, and hence some of the different molecular weight subunits observed could reflect differential post-translational modification.

Vitellogenin is a precursor molecule that circulates in the blood and is eventually accumulated within the developing oocyte as a lipo-glyco-carotenoprotein, when it is known as vitellin (Vt). A possible function of Vg is to act as a lipophilic chaperone during oocyte packaging, to ensure it contains essential components for successful embryogenesis and early larval development (Bownes 1992).

Vitellogenic tissues have been identified based on vitellogenin (Vg) or vitellin (Vt) content. The significance and role of the extra-ovarian sites of Vg production was highly controversial prior to isolation of Vg encoding genes (Tom and Ovadia 1987; Paulus and Laufer 1987; Quackenbush and Keeley 1986). Overall, the evidence indicates that the major site of Vg synthesis in penaeids is within the ovary (Vazquez-Boucard et al. 1986, Yano and Chinzei 1987; Quackenbush 1989; Rankin et al. 1989; Franzilber et al. 1989; Browdy et al. 1990; Fainzilber et al. 1992; Shafir et al. 1992). *In situ* hybridisation of the *P. japonicus* Vg gene indicates that mRNA is synthesized in the follicle cells surrounding the oocytes, and the presumption is therefore that the follicle cells are the site of ovarian synthesis with subsequent uptake of vitellin by the developing oocytes (Tsutsui et a. 2000). The use of molecular hybridisation probes has also confirmed the hepatopancreas as a site of extrovarian synthesis in both *P. monodon* and *P. japonicus* (Tsutsui et al., 2000, Tseng et al. 2001)

There have been a few reports on Vg and *in vivo* vitellogenesis in *P. monodon* (Quinitio et al. 1990; Chang et al. 1994; Chen and Chen 1993; Thurn and Hall 1999). Each of these studies report different molecular weight proteins but they fall into small (58,70 and 80 kDa), medium (96, 100, 110 kDa) and large (165, 180 and 210 kDa) molecular weight classes. In a previous *in vitro* study by Thurn and Hall (1999), only five prominent bands, 70, 78, 80, 180 and 210 kDa were found, corresponding to the low and high molecular classes of the other studies. Two of the bands, 80 and 180 kDa, corresponded to Vg subunits of 82 and 170 kDa identified by Chang et al. (1994). The majority of the other bands were vitellin subunits, corresponding to the 74, 83, 90, 104 and 168 kDa bands by Quinitio et al (1990). Although other ovarian protein bands are masked by the prominent bands of haemocyanin, reported to be 70, 77 and 85 kDa, in *P. monodon* (Ellerton et al. 1979), the Vg bands can be confirmed by immunoprecipitation (Thurn and Hall 1999, Fraser 2000).

In this section, we set out to purify vitellogenin from *P. monodon*, and to use this to develop monoclonal antibodies (MAbs) against the *P. monodon* Vg. This was then developed into a quantitative ELISA assay, which was used to assess variation in haemolymph vitellogenin levels in female broodstock at different stages of gonadal development. This provides a bioassay for assessing the impact of various exogenous treatments on the reproductive status of female *P. monodon* broodstock.

Methods

ISOLATION OF VITELLOGENIN FOR IMMUNIZATION TO PRODUCE ANTIBODIES

Vitellogenin was isolated from a pool of haemolymph from a Stage II female by ultracentrifugation using the method of Lee and Puppione (1988) as described in Fraser (2000). The density of haemolymph was adjusted to a specific gravity of 1.2 g/mL with sodium bromide. Equal volumes of aqueous sodium bromide solutions of specific gravity of 1.14 g/mL and 1.10 g/mL, respectively, were overlaid to give a final tube volume of 12 mL and this was centrifuged at 180,000 *g* for 40 hours at 4°C. Twelve 1 mL samples from top to the bottom of the centrifuge tube were removed after centrifugation and total protein content determined.

POLYCLONAL ANTIBODY PRODUCTION

Purified vitellogenin aggregates in aqueous solution over time, as do lipoproteins in general, and it was therefore emulsified as soon as possible after purification with adjuvant. Approximately 50 µg of purified vitellogenin in 1 mL was emulsified with 1 mL complete Freund's adjuvant by rapid repeated extrusions through a syringe with a 25-gauge hypodermic needle. To aid complete homogenization, 2 mL of a 2% Tween 80 solution was added to complete the emulsification.

Polyclonal antibody production was performed in sheep and Balb/c mice. A sheep was injected intramuscularly with a 4 mL emulsion of vitellogenin in complete Freund's adjuvant with all subsequent injections made with emulsions of vitellogenin in incomplete Freund's adjuvant (Table 1.1).

TABLE 1.1. Polyclonal antibody production schedule.

Day	Vitellogenin injected (µg)	Bleed (mL)
0	60	10
25	200	--
38	--	10
54	160	10
64	--	10
70	--	250

Balb/c mice were also used to produce polyclonal antibodies against vitellogenin. Six mice were inoculated intraperitoneally with complete Freund's adjuvant containing 45 µg of vitellogenin. Twenty days later, a further 48 µg was injected in incomplete Freund's adjuvant, followed by 0.2 mL of mouse sarcoma 180 cells (TropBio, Townsville) to promote ascitic fluid production. On day 35 the mice were killed by carbon dioxide poisoning and the ascitic fluid collected aseptically. This fluid was centrifuged at 1500 g for 10 minutes and the supernatant collected and stored at –70°C.

POLYCLONAL ANTIBODY CHARACTERIZATION

Polyclonal antibodies were screened against stage II female haemolymph, an extract of mature ovary (Stage IV), male haemolymph, and purified vitellogenin by dot blot. Normal sheep and mouse serum were run as controls to examine non-specific binding. As an additional comparison, a rabbit anti-vitellin, from *P. monodon*, supplied by Dr Emilia Quinitio, was also tested.

Nitrocellulose membranes were pencilled into a duplicate grid pattern, and 2 µL of purified vitellogenin was blotted onto each grid. The membranes were treated with blocking buffer (5% non-fat milk with 0.2% Tween 20 in 0.05 M PBS) and incubated with a 1/1,000 dilution of antiserum or ascitic fluid followed by standard procedures for immunoblotting (Harlow and Lane 1999).

Polyclonal antibodies were also screened by Western blot. Haemolymph from male and female *P. monodon* was separated by 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). The proteins from these gels were transferred onto nitrocellulose membranes by standard Western blot methodology (Harlow and Lane 1999). The membranes were stained with 0.2% Ponceau S, diluted 1 to 10 in 0.05 M phosphate buffer saline, from a stock of 2% Ponceau S in 30% sulfosalcylic acid, to visualize the protein

bands and the individual lanes cut into strips for immunostaining. These were destained in phosphate buffered saline and blocked with coating buffer and afterwards incubated with 1/10,000 dilutions of sheep or rabbit serum. Bands were visualised by standardized procedures (Harlow and Lane 1999).

DEVELOPMENT OF INDIRECT ELISA

Individual wells of 96-well ELISA plates (Sarsted) were coated with 50 µL of purified vitellogenin diluted in carbonate buffer. The plates were incubated at room temperature overnight in a humid atmosphere. The plates were washed once with 25 mM tris(hydroxymethyl)aminomethane (Tris) buffer saline with 0.45% Tween 20, followed by the addition of 50 µL of primary antibody diluted in 25 mM Tris buffer containing 0.2% casein and incubated for 1 hour at room temperature (Harlow and Lane 1999). This was followed by 3 washes of Tris buffer saline. Subsequently 50 µL of horseradish peroxidase conjugate (HRPO-conjugate) for the relevant species, i.e. sheep, rabbit or mouse, diluted in Tris buffer saline with 0.2% casein buffer, was added to each well and again incubated for 1 hour (Harlow and Lane 1999, Fraser 2000). After incubation the wells were washed 3 times with Tris buffered saline, followed by 100 µL of 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulphonic acid) peroxidase substrate (ABTS) (Harlow and Lane 1999). After 1 hour incubation in the dark at room temperature, absorbance was read on a plate reader at 415 and 490 nm corresponding to near the absorbance peaks of ABTS of 410 and 650 nm.

To optimize dilutions of vitellogenin and polyclonal antibodies, purified vitellogenin was serially diluted to cover a range from 1/25 to 1/6,400 in carbonate coating buffer and used to coat the wells of 96-well microtiter plates. Sheep antibody was serially diluted in the above buffer to cover a range from 1/500 to 1/64,000. The rabbit anti-sheep HRPO-conjugate was used at a concentration of 1/1000 throughout in the initial titration, but once the vitellogenin and sheep polyclonal antibody titrations were optimized it was also titrated through the range 1/1,000 to 1/16,000. The mouse antibodies were titrated as above except that vitellogenin dilutions were from 1/25 to 1/100 and mouse serum dilutions from 1/200 to 1/51,200.

To examine the affinity of the polyclonal antibodies for *P. monodon* haemolymph from male and female prawns, 96-well microtiter plates were coated with haemolymph serially diluted from 1/4000 to 1/128,000 with the sheep or mouse antibody at a dilution of 1/1,000.

As haemolymph was sampled by diluting neat haemolymph 1:1 in 2% EDTA in sterile seawater, possible interference from EDTA was examined. Wells of a 96-well plate were coated in a serial dilution of neat blood and haemolymph containing anticoagulant from 1/1,000 to 1/512,000. Antisera and HRPO-conjugate were used at a concentration of 1/1,000.

PRODUCTION OF MONOCLONAL ANTIBODIES

Ten male Balb/c mice were inoculated intraperitoneally with a 200 µL dose of 45 µg vitellogenin in complete Freunds' adjuvant as described above. A booster injection was given 21 days afterwards with 45 µg of vitellogenin in incomplete Freunds' adjuvant. A test bleed was made 49 days after the original injection and the blood diluted 1/100 in Tris Tween buffer containing casein. Antibody titers were assessed by indirect ELISA. Four days before fusion the mice were inoculated intraperitoneally with 15 µg of vitellogenin in 30 µL PBS and 10 µg in 20 µL into the tail vein.

Myeloma cells (Sp2/0) were cultured and harvested by standard methods (Liddell and Cryer 1991, Fraser 2000). The day prior to fusion, 5×10^5 Sp2/0 cells/mL in 20 mL Modified

Dulbecco's Medium (MDM) with 10% calf bovine donor serum (BDS) were used to provide a monolayer of approximately 80% confluence with 90% viability. Immediately prior to fusion the mouse was killed and the spleen aseptically removed with the splenocytes (B cells) purged by repeated injections of serum-free MDM with a syringe and 25-gauge hypodermic needle. The splenocytes were collected in 30 mL of serum free MDM, and counted in a haemocytometer before centrifugation at 100 *g* for 20 minutes. Sp2/0 cells and splenocytes were suspended in 10 mL MDM each, then pooled and diluted to 50 mL before centrifuging at 100 *g* for 10 minutes. Polyethylene glycol (PEG) 4000, which had been autoclaved then cooled to 37°C, was added at a concentration of 500 mg to 500 µL of serum free MDM. The supernatant from the centrifuge tube containing the Sp2/0 and splenocytes was discarded and the PEG solution slowly added to the pellet over 1 minute with gentle mixing. The cell suspension was mixed for a further 1 minute to complete fusion, and then 10 mL serum free MDM was added over 3 minutes with gentle mixing. The tube was centrifuged for 5 minutes at 100 *g*, the supernatant discarded and the remaining cell pellet resuspended in 300 mL MDM with oxaloacetate pyruvate insulin (OPI) media supplement and hypoxanthine aminopterin thymidine (HAT) media (Fraser 2000). The resultant mixture of putative hybridoma cells was plated into thirty-one 96-well plates at 100 µL/well and incubated at 37°C in a humid 95% oxygen:5% carbon dioxide atmosphere. An additional 100 µL/well fresh media was added after 4 days of incubation. On day 9, 50µL of media from individual wells were removed for testing, and replaced with fresh media. The culture-containing media was tested for the presence of antibodies and screened in wells coated with vitellogenin (4.3 µg/well), adult female haemolymph (1/1000 dilution) and adult male haemolymph (1/200 dilution). Those wells that reacted with vitellogenin and female haemolymph, but not with male haemolymph, were selected for further cloning. These cells were transferred to 24-well culture plates and incubated in the same media but lacking the addition of aminopterin. Cloning by limited dilution was undertaken and the resulting colonies screened by indirect ELISA for monoclonal antibody production. Hybridomas were recloned until all colonies tested positive for anti-vitellogenin.

Male Balb/c mice were injected intraperitoneally with 200 µL pristane and 11 days later the mice were injected intraperitoneally with 200µL of serum free MDM containing 1 to 10^6 hybridoma cells. The resultant ascitic fluid production was removed from the mice and the fluid centrifuged at 500 g for 10 minutes to pellet cells. The supernatant was titrated for monoclonal antibodies with the indirect ELISA. The determination of immunoglobulin isotyping was as described in Fraser (2000). Monoclonal antibodies were purified with a Protein A IgG purification kit (Pierce Chemicals) following the manufacturer's instructions.

MONOCLONAL ANTIBODY CHARACTERISATION

Each monoclonal antibody was screened against adult female and male haemolymph, ovary extract and purified vitellogenin by dot blot analysis. A nitrocellulose membrane was divided into grids and a small volume of each extract was blotted onto each grid. The membrane was blocked with coating buffer for 1 hour and incubated for 1 hour with 2 mL of hybridoma supernatant. The membrane was developed for analysis as previously described (Fraser 2000). Western blot analysis was carried out on adult female and male haemolymph, diluted 1/20 in PBS, run on 7.5% SDS PAGE and then transferred to nitocellulose membranes (Harlow and Lane 1999). The membranes were stained with Ponceau S and cut into strips for immunostaining. One strip was used for female and male samples for each MAb. After destaining and blocking the strips were incubated in one of the six MAb cell culture supernatants. After washing, the strips were incubated with goat anti-mouse HRPO-conjugate and 3,3'-diaminobenzidine tetrahydrochloride-4-chloro-1-napthol (DAB/4CN) substrate solution (Harlow and Lane 1999, Fraser 2000). Colour development was stopped by the addition of deionised water. Dot blots and indirect ELISA screening of ovarian extracts from

Metapenaeus endeavouri, Penaeus merguiensis, and *Penaeus longistylus* was performed as described previously to check for cross-reactivity with other species (Fraser 2000).

DEVELOPMENT OF SANDWICH ELISA

The mouse monoclonal antibodies of clone 2D3 and the sheep polyclonal antibody were tested for suitability as the capture antibody. Wells were coated with serial dilutions of monoclonal mouse antibody 2D3 from 1/2000 to 1/64,000 or of polyclonal sheep antibody from 1/1,000 to 1/8,000. They were then incubated with haemolymph from a stage IV spawner. Finally, for the mouse monoclonal 2D3 coated wells sheep polyclonal antibody was titrated at the same dilutions as for the capture antibody, and for the polyclonal sheep coated wells mouse monoclonal antibody was similarly titrated. Rabbit anti-sheep antibody HRPO-conjugate or goat anti-mouse antibody HRPO-conjugate was used for the mouse or sheep capture antibody titrations, respectively. A maximal absorbance of 2 or higher was sought.

RELIABILITY OF SANDWICH ELISA

Purified vitellogenin in Tris-Tween-casein buffer was serially diluted from 1,500 to 75 ng/ml. Potential interference from biological samples was tested by diluting female haemolymph for comparison against the standard curve of vitellogenin in buffer only. In addition, male haemolymph was spiked with known amounts of purified vitellogenin. Reproducibility of the assay within a batch was determined by calculating the variability of a sample of haemolymph assayed 20 times in duplicate in a single plate, and was expressed as a coefficient of variation. Reproducibility between batches was determined by the coefficient of variation of the same haemolymph sample assayed in duplicate in 20 plates run on different days.

ASSAY OF SPAWNER HAEMOLYMPH IN RELATION TO GI

Haemolymph samples from 96 spawners at different stages of gonadal maturity were assayed in duplicate. Each assay included a known haemolymph standard containing 340 ng/ml of vitellogenin as a positive control, and a male haemolymph sample at a 1/1,000 dilution as a negative control. The standard curve was done in triplicate whereas all unknowns were assayed in duplicate.

IMMUNOAFFINITY PURIFICATION OF VITELLOGENIN

Vitellogenin was purified from haemolymph obtained from ovarian Stage II female *P. monodon* broodstock by ultra centrifugation as described above. The mouse monoclonal antibody PmVg-2D3 was purified on a Protein A column (Pierce Chemicals) as per the manufacturer's instructions. The resultant purified PmVg-2D3 antibody was coupled to a 2 mL hydrazide gel column with a Affi-gel Hz Immunoaffinity kit (Pierce Chemicals) as per the manufacturer's instructions. The efficiency of antibody coupling to the hydrazide gel column was calculated and the elution buffer conditions optimised as described in Fraser (2000). Ultracentrifuge-purified vitellogenin, containing 3.27 mg/5 mL in dialysis buffer was loaded on the column. The column eluant was monitored for protein content by UV absorption. Initially the column was washed with 2 bed volumes of PBS plus 0.5 M NaCl, pH 7.0 followed by 2 bed volumes of PBS only. A further 2 bed volumes of 0.1 M glycine-HCl elution buffer, pH 4.0, were added and 2 mL fractions collected. Fractions containing vitellogenin were identified with the sandwich ELISA. The vitellogenin purified by this approach was used as the standard for the sandwich ELISA as well as for amino acid sequencing.

The purified vitellogenin was run on 2D PAGE. Individual protein spots corresponding to the A- and B-subunits were N-terminally sequenced by the Australian Proteome Analysis Facility

(APAF) at Macquarie University. Another sub sample was sent for internal sequence analysis to Auspep Pty Ltd for tryptic digestion and internal amino acid sequencing.

Results/Discussion

ISOLATION OF VITELLOGENIN

In ovarian stage II broodstock, the early stages of ovarian development can be observed histologically with yolk accumulating in the developing oocytes correlating with a rapid increase in oocyte diameter. This stage can be identified overtly by examination of broodstock with a torch, revealing a thickening dark outline along the central line of the tail and carapace of broodstock, representing the accumulating yolk within the developing ovary. These observations indicate that it is during stage II that vitellogenin production is most active. For this reason, stage II broodstock were selected from which to isolate vitellogenin from the haemolymph. Examination of haemolymph from such females by electrophoresis results in five prominent bands that can be accounted for as vitellogenin subunits and haemocyanin.

The ultracentrifugation of female stage II haemolymph resulted in the separation of putative vitellogenin from haemocyanin, which is the predominant protein in haemolymph. Due to vitellogenin being a lipoprotein, its density is significantly less than haemocyanin, The density gradient produced during ultracentrifugation resulted in a major proteinaceous band isolated within fractions 5 to 7 (ranging through fractions 5 to 10) of the 12 mL mixture (fractions 1-12 from top to bottom), tentatively identify as vitellogenin, with a heavy precipitate of haemocyanin (ranging through fractions 8 to 11). A sample of male haemolymph did not result in a distinct proteinaceous band within fractions 5 to 7. Analysis of female fractions 5 to 7 by SDS PAGE revealed 2 major molecular weight proteins of 83 and 168 kDa, representing the two monomer units of the vitellogenin dimer, as A- and B- subunits. Due to potential contamination of vitellogenin, (fractions 5 to 10) with haemocyanin (fractions 8 to 11) only fraction 6 was used as antigen for vitellogenin antibody production after dialysis against 0.19 M NaCl, 1 mM EDTA, pH 8 and was preliminarily identified as the lipoprotein vitellogenin.

POLYCLONAL ANTIBODY PRODUCTION

Purified vitellogenin was titrated against anti-vitellogenin sheep polyclonal antibody with a constant 1/1,1000 dilution of HRPO-conjugate (Fig. 1.1). Dot blot analysis of sheep and mouse polyclonal antibodies revealed cross-reaction with female *P. monodon* haemolymph. The antibodies also cross-reacted to some degree with male haemolymph but the sheep polyclonal less so than the mouse polyclonal.

The sensitivity of the indirect ELISA was tested by titrating haemolymph using a constant concentration of anti-vitellogenin sheep polyclonal antibody and a constant concentration of HRPO-anti-sheep IgG conjugate (Fig. 1.2). Western blot analysis of both antibodies showed binding to both A- and B-subunits of vitellogenin in female haemolymph. Both antibodies bound to some proteins in male haemolymph, but this effect was less strong for the sheep antibody. The cross-reactivity with male haemolymph may be due to carbohydrate moieties, in which vitellogenin is particularly rich, but which are common on glycoproteins in general. These are found in quantity in haemolymph of both sexes. Cross-reactivity to male haemolymph proteins has been reported previously, and was dealt with by adsorbing such antibodies against male haemolymph to remove cross-reacting antibodies, thereby increasing the specificity of the antibody to vitellogenin alone (Quinitio et all 1990).

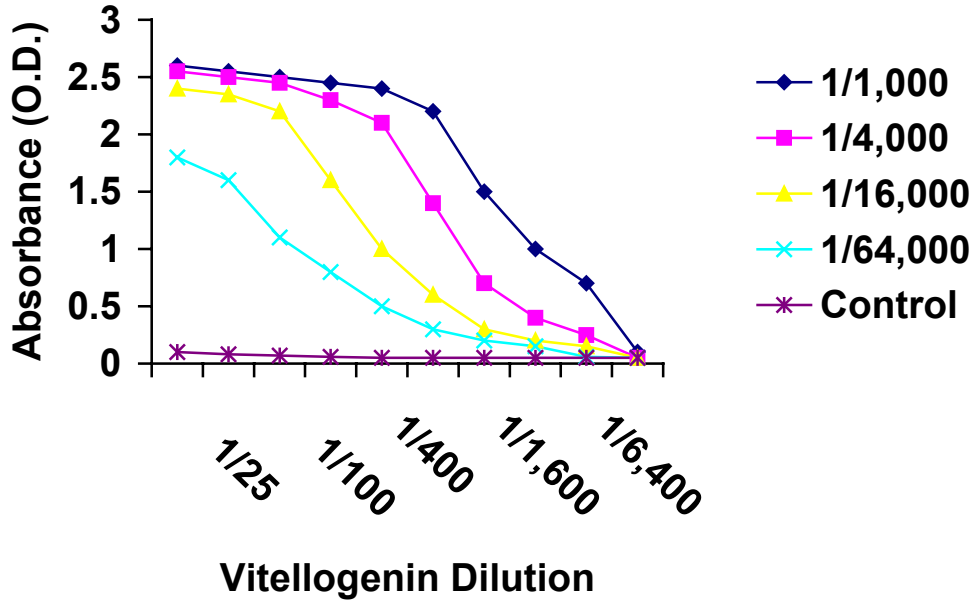

FIGURE 1.1. Optimization of indirect ELISA titrating purified vitellogenin against anti-vitellogenin sheep polyclonal antibody with a constant 1/1,000 dilution of HRPO-conjugate. O.D. = optical density.

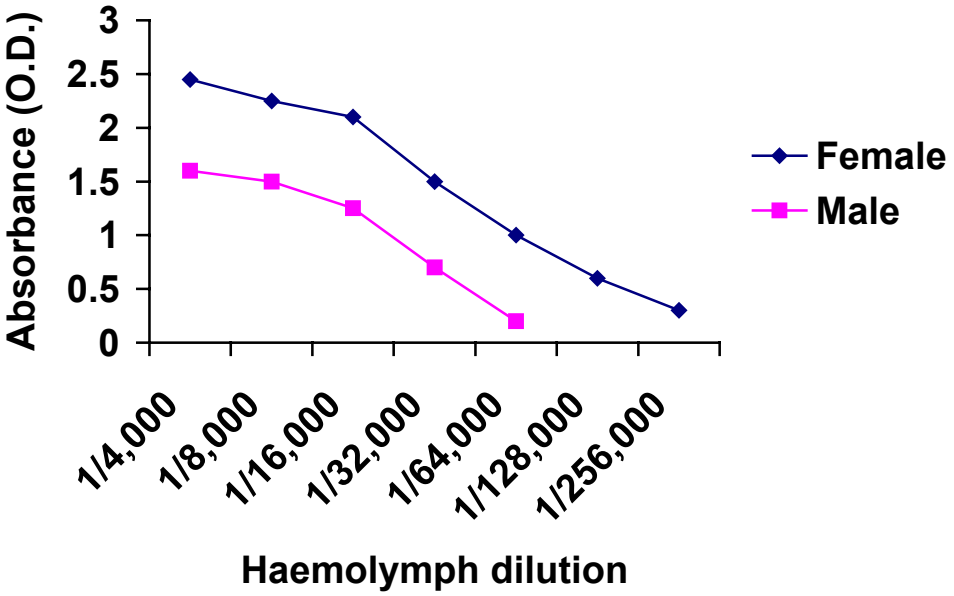

FIGURE 1.2. Sensitivity of indirect ELISA in titrating haemolymph using a constant concentration of anti-vitellogenin sheep polyclonal antibody (diluted 1/8,000) and a constant concentration of HRPO-anti-sheep IgG conjugate (diluted 1/1,000). O.D. = optical density at 415 nm.

INDIRECT ELISA

The indirect enzyme-linked immunoasorbent assay (ELISA) is the simplest of the configurations to measure antibody titers. Antigens are absorbed onto the wells of microtiter plates, and unlabelled primary antibody is added followed by a secondary antibody conjugated to an appropriate enzyme for colour development and quantification. In this configuration the antigen and secondary antibody are held constant, and only the concentration of the primary antibody is varied such that changes in the optical density of colour development reflect the concentration of primary antibody. The major disadvantage of the indirect ELISA is a lack of specificity due to possible impurities in the antigen. Nevertheless it is a useful method for the initial development of studies requiring ELISA and offers a lead into the development of sandwich ELISAs.

Polyclonal or monoclonal antibodies can be used in ELISA systems. Polyclonal antibodies are readily produced and often have high affinity for antigen. They are effective in forming large antibody-antigen aggregations suitable for immunoprecipitation, immunostaining, and as an amplification step to increase sensitivity in ELISAs. However, they do not typically exhibit specificity. For this reason, the indirect ELISA using polyclonal antibody was not developed further as the ELISA configuration of choice. However, it proved useful in the screening of monoclonal antibody hybridomas. In addition, due to the high affinity of the sheep polyclonal antibody, this was deemed suitable for use as a capture antibody in the development of a sandwich ELISA.

MONOCLONAL ANTIBODY PRODUCTION

Of the original thirty-one 96-well plates inoculated and screened by antibody-capture in the indirect ELISA, 504 wells were reactive against vitellogenin. These were further screened against adult female and male haemolymph, following which hybridomas from 76 wells were processed further by passage into 24-well plates. Of these, eight hybridomas continued to produce antibodies against vitellogenin and, following cloning, six remained active in the indirect ELISA against vitellogenin. Ascitic fluids from six mice, inoculated respectively with PmVg-1B4, PmVg-1B5, PmVg-2B4, PmVg-2D3, PmVg-3D2, and PmVg-3D5, were examined for antibodies in the indirect ELISA. Only one monoclonal antibody, PmVg-2D3, produced absorbance levels between 2.4 to 3.0 at a dilution of 1/64,000. Due to its high affinity, this antibody was used in the development of the sandwich ELISA. All clones were stored in liquid nitrogen for further development and use. Ascitic fluid and cell culture supernatants were stored as aliquots at either –20°C or –70°C.

The same 6 antibodies were examined for immunoblotting detection in dot blots and Western blots. At dilutions of 1/1,000 only the antibodies PmVg-1B4, PmVg-1B5, PmVg-2B4 and PmVg-2D3 exhibited high affinity titers. None of the antibodies cross-reacted with male haemolymph constituents. In Western blots, in which vitellogenin from adult female haemolymph is separated by SDS PAGE in a reduced state to separate A- and B-subunits, only antibody PmVg-2B4 exhibited any binding and only to the A-subunit. Monoclonal antibodies bind to a single epitope which can be either a short stretch of the amino acid sequence of the entire molecule, i.e. a linear epitope, or can be discontinuous regions of the primary amino acid sequence which are bought into close proximity in the proteins folded structure, i.e. a conformational epitope. Only antibody PmVg-2B4 recognized a linear epitope and hence is of potential use in immunohistochemical studies where the fixation process denatures proteins with concurrent lose of conformational binding sites.

SANDWICH ELISA

In this ELISA configuration a capture antibody, raised against the antigen of interest, is attached to the wells of microtiter plate followed an antigen, then a secondary antibody

which recognizes a different epitope on the antigen is added, and finally the plate is treated with an indicator antibody conjugated to an enzyme for colour development and quantification. The concentrations of capture antibody, secondary antibody and indicator conjugated antibody remain constant such that changes in measured optical density reflect changes in the antigen concentration. Preferentially, the secondary antibody is a monoclonal antibody that binds to a different epitope of the antigen than does the capture antibody. This configuration is typically more sensitive and specific than the indirect ELISA.

For the sandwich ELISA assay to measure vitellogenin, the mouse Mab PmVg-2D3 was chosen as the capture antibody, at a coating dilution of 1/4,000 to ensure complete capture of all vitellogenin in a sample. Once coated, the plates were stable when stored at 4°C for up to 2 weeks. The sheep polyclonal antibody was optimized at a dilution of 1/2,000 as the secondary antibody (Fig. 1.3). Rabbit anti-sheep HRPO-conjugate was purchased from a commercial supplier. Each batch was titrated separately but was typically used at 1/1,000.

The standard curve of purified vitellogenin was linear in the range of 75 to 700 ng/mL (Fig. 1.4). Serial dilutions of spawner haemolymph parallel the standard curve to an upper limit of 900 ng/mL. Unknown haemolymph samples were assayed in duplicate over a range of dilutions, with only those falling within the range of 75-700 ng/mL being deemed appropriate. Total processing time was approximately 4 hours and it was possible to process up to 360 samples in duplicate per day. Samples of juvenile female and male haemolymph, from dilutions of 1/100 to 1/1,000, were not significantly different from background, i.e. no vitellogenin was detected. Male haemolymph samples spiked with vitellogenin gave recoveries of between 100 – 103%. Intra-assay and inter-assay coefficients of variation were 6.1% and 9.4% respectively.

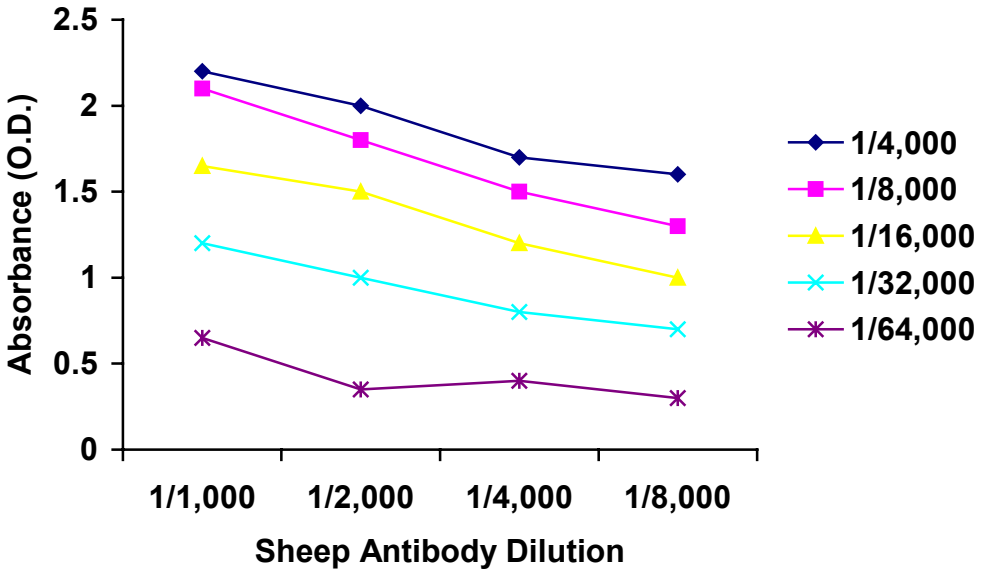

FIGURE 1.3. Optimization of sandwich ELISA titrating monoclonal antibody PmVg-2D3 as capture antibody between 1/4,000 and 1/64,000 against sheep antibody at 1/2000 with the anti-sheep HRPO-conjugate at a 1/1,000 dilution. O.D. = optical density at 415 nm.

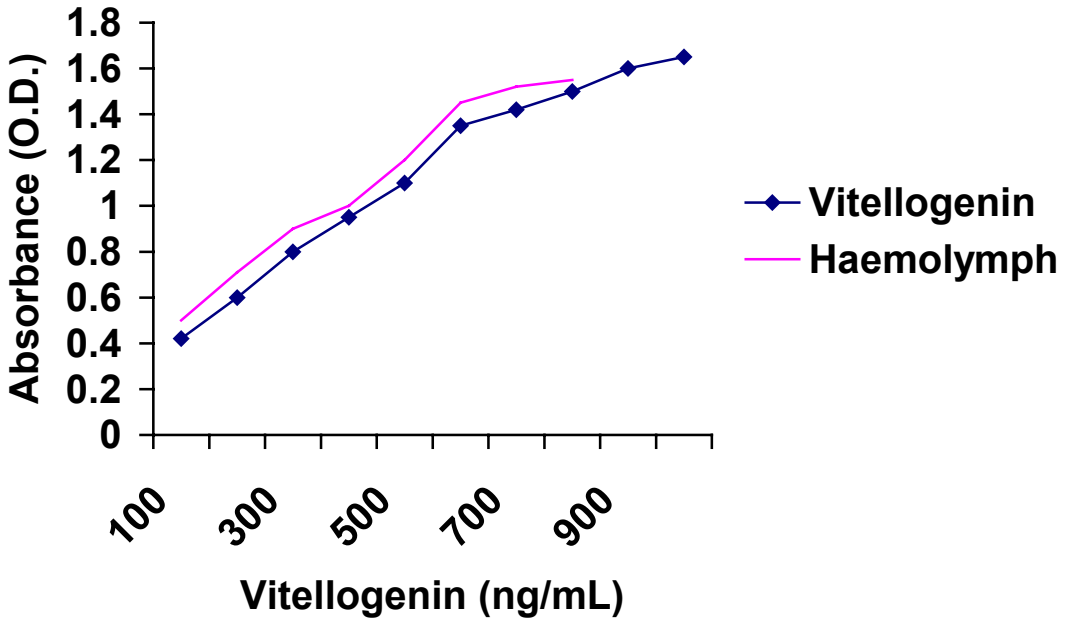

FIGURE 1.4. Sandwich ELISA standard curve of purified vitellogenin. Serial dilutions of female haemolymph are also shown, demonstrating parallel dilution.

ELISA QUANTIFICATION OF *P. MONODON* HAEMOLYMPH FOR VITELLOGENIN

Haemolymph vitellogenin levels ranged from 380 µg/mL in stage I broodstock (n=20, SEM = ± 58 µg/ml) to a maximum of 1,748 µg/mL in stage II broodstock (n=30, SEM = ± 109 µg/ml), afterwards falling to a mean of 1288 µg/mL in stage III (n=29, SEM = ± 107 µg/ml) and 878 µg/ml in stage IV broodstock (n=12, SEM ± 188 µg/ml) prior to spawning (Fig. 1.5). Differences between groups were significant by one-way ANOVA ($F=26.799$, $p<0.01$ with 3 and 87 d.f.).

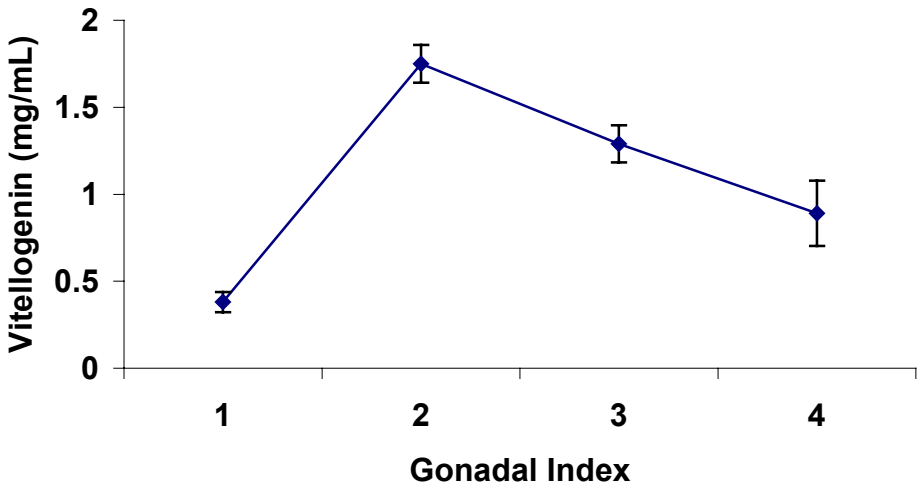

FIGURE 1.5. Variation in haemolymph vitellogenin concentration at each stage of ovarian maturation as determined by sandwich ELISA. Means ± SEM.

Other investigations of changes in haemolymph levels of vitellogenin in penaeid prawns are difficult to directly compare with this study, as a variety of standards are used in other reports of ELISAs for penaeid prawns (Table 1.2). In particular, a number of these studies used purified vitellin rather than vitellogenin as the standard. Since the molecular weight of vitellin is significantly different from vitellogenin, and the vitellin molecule represents a further chemical modification (including a cleavage event) of vitellogenin in the haemolymph, it is not necessarily possible to directly compared 'relative' haemolymph vitellogenin levels based on vitellin as the standard against levels based on vitellogenin as the standard. Nevertheless, this is the only comparison possible with most published studies.

In *P. vannamei*, 'vitellogenin' levels in the haemolymph rose from a base of 3 µg/mL in stage I to 920 µg/mL in stage IV females, thereafter returning to baseline levels (Quackenbush 1989). In contrast, another study on female *P. monodon* reported values that only ranged from 1,008 to 1,478 µg/mL through stages I to IV, with levels only dropping to 360 µg/mL in stage V spent females (Vincent et al. 1996). It is problematic to interpret the results from that study. In this study there was no significant correlation between vitellogenin haemolymph levels and total egg production. It appears that if a female responds to unilateral eyestalk ablation with ovarian maturation through stages I to IV, which can be determined by overt examination by torchlight, then spawning will typically occur. The quality of the eggs, and whether they hatch, is due to other factors beyond vitellogenin haemolymph levels.

TABLE 1.2. Sources of antigen for antibody production and standard as used in other published ELISAs for penaeid prawns.

Antigen for antibody production	Standard	Reference
'light subunit' of vitellin	purified vitellin	Derelle et al. 1986
vitellin	purified vitellin	Vincent et al. 1996
97kDa subunit	purified vitellin	Quackenbush 1989
purified vitellin	purified vitellin	Lee and Watson 1994
purified vitellogenin	purified vitellogenin	Mendoza et al. 1993
purified vitellogenin	purified vitellogenin	this study

In conclusion a reliable, accurate and precise sandwich ELISA is capable of measuring vitellogenin in haemolymph. The assay allows repeated non-destructive sampling of individuals through a spawning cycle and would be valuable in studies examining variability in vitellogenesis capability of individuals, and as an investigative tool in further understanding hormonal, nutritional and environmental impacts on broodstock (Fraser 2000).

Amino acid sequencing of vitellogenin

The work described in the present project was undertaken prior to publication of any penaeid vitellogenin gene sequences, and hence effort was made in this project to obtain partial gene sequence of the purified vitellogenin. The A- and B- sub-units separated well by 2D PAGE by molecular weight. Both subunits exhibited a typical phosphorylation train across the pH dimension indicating that the subunits are well phosphorylated. The 83 kDa B-subunit was selected for N-terminal analysis. After 15 cycles the following amino acid sequence was obtained:

Ala-Pro-X-Gly-Als-Asp-Leu-Pro-Arg-X-X-Thr-Glu-X-Pro/Lys.

The unknown amino acids, labelled X, may be modified amino acid residues or unmodified cysteine or glycosylated residues.

Another sample was forwarded to Auspep Pty. Ltd, who undertook tryptic digestion and separation of fragments to obtain the following internal sequence, with multiple sequence ambiguities shown in brackets:

Ser(Gly/Leu)-Val(Gln/Ala)-Leu-Tyr-Thr-Ala(Phe)-Gln-Leu-Tyr-Leu(Ile)-Thr-Glu(Pro)-Arg

These sequences were used to design degenerate primers, in an attempt to isolate the *P. monodon* Vg gene. Attempts were unsuccessful, possibly due to the large size of the transcript (>7000 bp in *P. japonicus*) making it extremely difficult to purify the full length mRNA that would be required for detection of the Vg gene (in a typical mRNA isolation experiment, the majority of mRNAs isolated are 1500 bp or less).

However, the recent publication of the *P. japonicus* sequence (Tsutsui et al. 2000) allows verification of the partial amino acid sequence obtained in the present project.

The N-terminal sequence that we obtained aligns with the N-terminal sequence of the *P. japonicus* Vg deduced amino acid sequence as follows (the one-letter amino acid code has been used for ease of alignment):

```
P. mon. APXGADLPRXXTE
        || |||||| ||
P. jap. APWGADLPRCSTE
```

The internal sequence obtained for the *P. monodon* Vg aligns with amino acid residues 375 to 386 of the mature protein as follows (where there are ambiguities in the *P. monodon* sequence, the homologous amino acid has been selected where identified):

```
P. mon. ALYTAQLYLTPR
        ||||| ||||||
P. jap. ALYTAALYLTPR
```

These alignments illustrate both the success of the partial amino acid sequencing of the *P. monodon* vitellogenin, and the remarkable conservation of amino acid sequence between the two species *P. monodon* and *P. japonicus*.

OBJECTIVE 2

To Identify & chronicle the substances that are accumulated during egg development

Introduction

Spawnings from wild-caught, as well as captive-reared, broodstock can result in greatly varying fertilization and hatching rates (see objective 3). The implication is that batches of eggs vary significantly in "quality" and it is likely that this variation in egg quality can be attributed, in part, to the biochemical substances that are laid down during vitellogenesis. The goal of this objective was therefore to develop a method for identifying a large component of the biochemical substances that are laid down in the developing oocytes, and to use the developed method to try to identify biochemical differences between "good quality" and "poor quality" eggs.

Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) is the only method currently available which is capable of separating the complexity of the expressed genome into its individual parts. The method utilizes two separation technologies. In the first dimension proteins are separated according to charge by isoelectric focussing (IEF). Charged proteins migrate along a pH gradient in a gel matrix exposed to a voltage gradient until they reach their isoelectric point (pI), at which point they have a net charge of zero, cease migrating and hence focus in a discrete band. In the second dimension, the gel matrix containing the proteins focussed by charge is further separated orthogonally by sieving polyacrylamide electrophoresis, which separates the proteins by their mass. To facilitate separation by mass, the proteins are run in the second dimension on denaturing PAGE in the presence of sodium dodecyl sulphate (SDS). SDS binds to the surface of the proteins and neutralizes any residue intrinsic charges, ensuring that all the proteins have the same charge density and thus that separation is by mass alone.

A critical step in 2D PAGE is the preparation of the sample, as this can determine the final resolution. Since 2D PAGE relies on the separation of proteins by charge and size, the sample must be processed to optimize the powerful separation capabilities of the technique. For optimal IEF, sample preparation needs to ensure optimal surface charges of the proteins, allowing them to migrate freely, and according to their true pI during focussing. Sample preparation must remove substances like lipids, nucleic acids, pigments, polysaccharides, and salts, which interfere with the isoelectric focusing (IEF) function of 2D PAGE. Lipids are typically extracted using chromatography, ultracentrifugation, acetone precipitation, or organic solvent extraction. Salts are removed using dialysis, TCA precipitation, or acetone precipitation. Acetone precipitation also extract pigments. Nucleic acids are removed using DNAse and RNAse, ultracentrifugation, or ultrafiltration. Polysaccharides are removed by either ultracentrifugation or precipitation. The method(s) of removal is dependent on the combination of substances contained in the homogenate of interest and the level of protein yield desired.

The analysis of prawn eggs by 2D PAGE is very challenging. Eggs have very high concentrations of lipids, accounting for 60-70% of total dry matter. Extraction of eggs must remove the majority of lipids while retaining the integrity of the proteins, since lipids may mask charged sites on the protein and have drastic effects on the power of resolution. Other problems with IEF can include horizontal streaking of proteins. This is a function of protein

solubility in solubilization buffer, protein solubility during isoelectric focusing, and other isoelectric conditions.

The development of 2D PAGE for *P. monodon* eggs enables the analytical comparison of proteins contained in different egg populations. Analytical comparisons using 2D PAGE demand consistent maximal recovery of proteins in aqueous form from each sample since protein loss can cause potentially misleading results when maps or individual spots are comparatively analyzed. Consistent maximal recovery and high resolution of protein are dependent on the methods used to solubilize proteins and to remove interfering substances. Hence extensive effort was put into optimizing these aspects of the methodology. Once methods had been optimized, they were applied for comparison of the proteome content of batches of "good" and "poor" quality eggs.

Methods

Details of methodology can be found in Mastro R. and Hall M. R. (1998) Methods for two-dimensional polyacrylamide gel electrophoresis (2D PAGE) analysis of marine eggs. AIMS/FRDC Report No. 29. 78pp.

HOMOGENISATION TRIALS

Current lipid extraction methods maximize lipid recovery thereby confining their ability to deliver maximal protein recovery in aqueous form. Traditional methods include lipid extraction using organic solvents, protein precipitation using organic solvents, reverse phase chromatography, and ultracentrifugation. Trials focused on modified versions of traditional delipidation approaches. Three basic approaches were utilised to delipidate aqueous egg homogenates and recover maximal protein recovery in aqueous form suitable for 2D PAGE: a) extraction of lipids from aqueous homogenates, b) extraction of protein by protein precipitation, and c) combined methods.

P. monodon eggs were homogenized in a buffer with 200 strokes of a dounce type homogenizer at room temperature in all trials. In addition to homogenization, trials 1, 2(a-c) and 3(a-d) were sonicated for 10 minutes at room temperature whereas trials 4, 5(b&c), 6(a&b), 7(a-c) used a 15 minute incubation at 35°C as a final step after homogenization.

One of three homogenization buffers was used in any one trial:

Buffer A (8M urea, 100 mM NaCl),
Buffer C (8M urea, 100 mM DTT, 4% CHAPS, 45 mM Tris),
Buffer E (0.1% SDS, 100 mM DTT)
Buffer H (8M urea, 100 mM NaCl, 100 mM DTT, 5% NDSB, 45 mM Tris).
Buffer N (100 mM Tris, 50 mM MgCl, 1 mg/mL DNAse I, 10 mg/mL RNAse II)

All homogenization buffers included the protease inhibitory cocktail 'Complete' (Boehringer Mannheim) at the concentration suggested by the manufacturer. Table 2.1 describes the buffer used in each trial and the egg to buffer ratio.

EXTRACTION OF LIPIDS FROM AQUEOUS HOMOGENATES

Trial 1 - Lipid extraction by centrifugation. The egg homogenate was centrifuged at 14,000 rpm for 10 min at 25°C to separate aqueous sample from pelleted debris and upper lipid layer. This was repeated until no upper lipid layer was observed.

Trial 2(a-c) - Lipid extraction using organic solvents. The egg homogenates were centrifuged at 14,000 rpm for 15 min at 4°C and 2 volumes of the following solvents were added to the supernatant: 2a) ice cold TNBP (Gardner, 1996), 2b) hexane:isopropanol (3:2), and 2c) heptane:isopropanol (3:2). They were then gently mixed by rocking at 4°C for 16 hr. Homogenate-solvent mixtures were centrifuged at 14,000 rpm at 4°C for 15 min to separate aqueous and solvent phases. The aqueous phase was centrifuged through a 100 kDa molecular cut-off ultrafiltration unit (Sartorius) per manufacturer's instructions to remove DNA and RNA. Filtrates containing molecules less than 100 kDa were collected and then dialyzed against 8 M urea. Two volumes of Buffer C were added to dialyzed samples.

Trial 3(a-d) - Lipid extraction using LH20 chromatography beads after (Gardner 1996). The egg homogenates were centrifuged at 14,000 rpm for 15 min at 4°C. The supernatants were added to 0.1 g of LH20 chromatography beads (Pharmacia) swollen with either: 3a) TNBP, 3b) chloroform:ethanol (99:1), 3c) hexane:isopropanol (3:2), or 3d) heptane:isopropanol (3:2). Sample-bead mixtures were gently agitated for 16 hr at 4°C and then centrifuged at 14,000 rpm for 5 min at 4°C to separate phases. DNA and RNA were removed as described in Trial 2. Samples were then dialysed against Milli-Q H_2O. Two volumes of Buffer C were added to dialyzed samples.

Trial 4 - Lipid extraction using HAP chromatography beads. The egg homogenate was centrifuged at 10,000 *g* for 15 min at 4°C. The supernatant was adjusted to pH 14 with 10 M NaOH. 0.1 g of HAP (prepared as described in Kragh-Hansen, 1993) was then added to the supernatant and gently agitated at 4°C for 90 min. The mixture was centrifuged at 3,500 rpm for 15 min at room temperature to remove HAP beads. Additional HAP was added to the supernatant and gently agitated for 16 hr at 4°C. The supernatant was collected by centrifugation as above and DNA and RNA were removed as described in Trial 2. The sample was dialyzed against 8 M urea, 0.1% SDS, 100 mM DTT, 45 mM Tris. Finally four volumes of Buffer C were added to the sample.

EXTRACTION OF PROTEIN BY PROTEIN PRECIPITATION

Trial 5(a-b) - Acetone/Methanol protein precipitation. The egg homogenate was precipitated using 2 volumes of acetone:methanol (7:2) and gently agitated for 16 hr at 4°C. It was then centrifuged at 14,000 rpm for 15 min at 4°C. The precipitate was dried under vacuum briefly. Protein was boiled in Buffer E and then incubated in ten additional volumes of either: 5a) Buffer C at 35°C for 15 min or 5b) Buffer C for 15 min. DNA and RNA were subsequently removed as described in trial 2.

COMBINED METHODS

Trial 6(a-b) - Lipid extraction using centrifugation / HAP & protein precipitation using acetone/methanol. The egg homogenate was incubated in Buffer N for 10 min at 4°C to remove DNA and RNA. To clear homogenate of lipids and debris, it was centrifuged at 10,000 *g* for 15 min at 4°C. 100 mg HAP beads (prepared as described in trial 3) was added to the supernatant and gently agitated for 90 min at 4°C. After removing the beads (as described in trial 4) 10 volumes of acetone:methanol (7:2) were added to the sample and incubated at -20°C for 30 min. The sample was centrifuged at 2,500 rpm for 15 min at room temperature. The supernatant was discarded and the protein pellet was air dried. The pellet was boiled for 5 min in Buffer B and then cooled on ice. One volume of Buffer C was then added and the sample vortexed briefly. Unsolubilized protein was pelleted by centrifugation at 10,000 x g for 30 min at 4°C and the supernatant collected.

Trial 7(a-b) - Lipid extraction using centrifugation and organic solvents and protein precipitation using acetone / methanol. DNA and RNA were removed from Trial 7 (a-b)

homogenates, and debris and lipids were removed by centrifugation as described in trial 6. 14 volumes of either 7a) hexane or 7b) heptane were added to the supernatant and gently agitated for 90 min at 4°C. The mixture was centrifuged at 2,500 rpm for 15 min at room temperature to separate aqueous and solvent phases. Proteins were precipitated, recovered, dried, and solubilized as described in trial 6.

Trial 8(a-c) - Lipid extraction using centrifugation and protein precipitation using acetone/methanol/tri-n-butylphosphate/heptane. Nucleic acids were removed from Trial 8(a-c) homogenates as described in trial 6. Debris and lipids were removed by centrifugation as described in trial 6. 14 volumes of solvent mixtures were added to the supernatant give a final acetone concentration of 80%, specifically either 8a,c) TNBP:Acetone:Methanol (1:12:1) or 8b) Heptane:Acetone:Methanol (1:12:1), and incubated at 4°C for 90 min. The sample was centrifuged at 14,000 rpm for 10 minutes at 25°C. The precipitate was washed sequentially with 1 ml of TNBP, methanol, and acetone, respectively. Precipitates were then recovered dried and solubilized as described in trial 6. Sample 8c was incubated at -20°C overnight (Table 2.1) .

TABLE 2.1. Initial homogenization conditions of egg samples. Homogenates were treated further, depending on the trial, after initial homogenization (for details see text).

Condition	Trial						
	1	2a-c	3a-d	4	5a&b	6a&b, 8a-c	7a&b
Homogenization Buffer	H	A	A	H	H	H	H
Egg : Buffer Ratio	0.5:1	0.3:1	0.45:1	1:1	1:10	1:1	1:10

ELECTROPHORESIS

Samples from the trials were passively loaded onto 11 cm 4-7 pH isopycnic gradients (IPGs) at room temperature for 6-16 hr. Isoelectric focusing ranged from 60 kVhr - 310 kVhr. IPGs were then equilibrated in Equilibration Solution (0.375 M Tris, 6 M urea, 30% glycerol, 2% SDS) with 65 mM DTT for 15 min followed by Equilibration Solution with 260 mM iodoacetamide for 15 min. The second dimension was 12% PAGE using a Tris-Glycine buffer system. The gels were silver stained to detect proteins (Giometti et al. 1991).

OPTIMIZATION OF IEF

P. monodon egg samples were prepared as described in Trial 8c. Protein content of samples was quantified (Bio-Rad's Protein Assay) according to the manufacturer's instructions. In Trials 9a and 10a, 0.3 mg of protein was made up in a total of 250 µl with Buffer C only. In Trials 9b and 10b, 0.3 mg of protein was made up in a total of 250 µl Buffer C plus 1% Ampholines 4 - 6.5 (Pharmacia). All samples were passively loaded onto 11 cm, linear 4 - 7 pH IPGs (Pharmacia) overnight at room temperature (Rabilloud et al. 1994a). IEF was conducted under the following conditions:

Trial 9(a&b): IPGs were run at 300 V for 3 hr then 3000 V for 21 hr for a total of 63.9 kVhr.

Trial 10(a&b): IPGs were run at 300 V for 3 hr and then at 3000 V for 45 hr for a total of 135.9 kVhr.

After IEF the IPGs were equilibrated in equilibration solution (0.375 M Tris, 6 M urea, 30% glycerol, 2% SDS) with 65 mM DTT for 15 min and then in equilibration solution with 260 mM iodoacetamide for 15 min. The SDS PAGE second dimension was run on a 9 - 17% polyacrylamide slab gel using a Tris-glycine buffer system (Mastro and Hall 1998) and run at

25 mA / gel, 200V limit, for 1 hr and then at 35 mA / gel, 400 V limit for 4 hr. The resolved proteins were visualized using a modified silver stain (Rabilloud et al. 1994b).

OPTIMIZATION OF PROTEIN LOADS

Trial 11(a-d): *P. monodon* egg samples were prepared as described for Trial 8c. Protein content of samples was quantified as above. Four concentrations of protein loading were tested: 0.1 mg (Trial 11a), 0.2 mg (Trial 11b), 0.3 mg (Trial 11c), 0.4 mg (Trial 11d), all made up to a total of 250 µl each with Buffer C and 1% (v/v) Ampholines 4 - 6.5 (Pharmacia). Each sample was passively loaded overnight at room temperature onto an 11 cm, linear 4 - 7 pH IPGs (Pharmacia). Isoelectric focusing was conducted for 300 V for 3 hr and then 3000 V for 45 hr for a total of 135.9 kVhr.

After IEF, the IPG strips were equilibrated in equilibration solution (0.375 M Tris, 6 M urea, 30% glycerol, 2% SDS) with 65 mM DTT for 15 min and then in equilibration solution with 260 mM iodoacetamide for 15 min. The SDS PAGE second dimension was run on a 9 - 17% polyacrylamide slab gel using a Tris-glycine buffer system at 25 mA / gel, 200 V limit, for 1 hr and then at 35 mA / gel, 400 V limit for 4 hr. The resolved proteins were visualized using a modified silver stain (Rabilloud et al. 1994b).

OPTIMIZATION OF POLYACRYLAMIDE GEL ELECTROPHORESIS

Egg extracts were prepared as described for Trial 8c. Samples were loaded into 11 cm pH 4 - 7 IPGs by passive loading overnight at room temperature. IEF was performed for a total of 135 kVhr. After IEF IPGs were equilibrated in equilibration solution with 65 mM DTT for 15 min and then in equilibration solution with 260 mM iodoacetamide for 15 min. IPGs were embedded onto one of the following PAGE gels:

a. 12% T resolving gel with a 4% stacking gel
b. 12% - 20% T gradient resolving gel
c. 10% - 20% T gradient resolving gel
d. 9% - 17% T gradient resolving gel

Resolving gels were made from 30% acrylamide; 0.8% piperazine diacrylamide (PDA). Stacking gels were made from 30% acrylamide; 0.8% bis acrylamide. Gel buffers for homogeneous and gradient gels are those described previously (Mastro and Hall 1998). Electrophoresis and silver staining was run using Tris/Glycine buffer under those conditions described previously (Mastro and Hall 1998).

MINIMISATION OF VERTICAL STREAKING

Eggs were prepared as described for trial 8c. Protein concentration was measured using the Bradford Coomassie brilliant blue G-250 protein assay according to the manufacturer's directions (Bio-Rad). An egg extract containing 0.2 mg protein was adjusted to a volume of 250 µl with Buffer C and loaded onto a 11 cm pH 4 - 7 IPGs by passive loading overnight at room temperature. IEF was performed for a total of 135 kVhr. After IEF IPGs were equilibrated in Equilibration Solution (0.375 M Tris, 6 M urea, 30% glycerol, 2% SDS) with 'non-streak' concentrations of DTT and iodoacetamide; 100 mM DTT for 15 min and then with 400 mM iodoacetamide for 15 min. IPG's were embedded onto a 0.1% SDS; 4% Bis/Acrylamide 'stacking' gel that overlaid a 9% - 17% PDA/Acrylamide gel. PAGE was conducted using Tris/Glycine buffer as described in Mastro and Hall (1998). Gels were then silver stained (see below).

SILVER STAINING TRIALS

A concentration range from 10 - 10,000 ng each of phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa) was run on 12% acrylamide / PDA PAGE gels using a Tris/glycine buffer system as described above. Gels were run at 100 V for 1.5 hr and then silver stained by various methods.

Trial 1: Rapid Silver Nitrate (Rabilloud, Carpentier, and Tarroux 1988)**.** After electrophoresis gels were fixed in 40% methanol with 10% acetic acid, for a total of 90 min with three fixative changes. Gels were rinsed with 20% ethanol for 10 min, followed by Milli-Q H_2O for 10 min. Gels were sensitised in 0.03% $Na_2S_2O_4$ for 1 min and then rinsed two times with Milli-Q H_2O for 1 min each. Gels were impregnated with 0.2% $AgNO_3$ for 1 hr and then washed with Milli-Q H_2O, first briefly for 20 sec followed by another wash for 5 min. Gels were developed in 3% $NaCO_3$; 0.05% HCOH, 0.001% $Na_2S_2O_4$ for 5 min. Development was stopped by soaking the gel in 5% Tris with 1% acetic acid for 30 min. Finally, gels were washed with Milli-Q H_2O.

Trial 2: Rapid Silver Nitrate (Merril, Dunau, and Goldman 1981)**.** After electrophoresis gels were fixed in 40% methanol with 10% acetic acid for a total of 90 min with 3 fixative changes. Gels were washed in a solution of 25% methanol and 10% acetic acid for 60 min, followed by a wash with 5% acetic acid for 60 min, and then washed with 1% acetic acid for 30 min. Gels were sensitised by bathing in a 0.004% DTT solution for 30 min. Gels were then washed with Milli-Q H_2O twice for 20 sec and then twice for 10 min. Gels were then impregnated with a solution of 0.2% $AgNO_3$ for 1 h. Gels were finally briefly washed with Milli-Q H_2O for 20 sec followed by an extended wash of 5 min. Gels were developed in a solution of 3% $NaCO_3$; 0.05% HCOH, 0.001% $Na_2S_2O_4$, for 5 min. Development was stopped by bathing the gel in a solution of 5% Tris with 1% acetic acid for 30 min. Gels were then washed with Milli-Q H_2O.

Trial 3: Rapid Silver Nitrate (Blum, Bejer, and Gross 1987)**.** After electrophoresis gels were fixed in a solution of 40% methanol with 10% acetic acid for a total of 90 min with 3 fixative changes. Gels were then washed in a solution of 25% methanol with 10% acetic acid for 60 min, followed by a wash in 5% acetic acid for 60 min, and then in 1% acetic acid for 30 min. Gels were then sensitised in a 0.02% $Na_2S_2O_4$ solution for 15 min. Gels were washed in Milli-Q H_2O twice for 20 sec and then for 10 min. Gels were impregnated in a solution of 0.2% $AgNO_3$ for 1 hr. This was followed by a wash with Milli-Q H_2O for 20 sec and then 5 min. Gels were developed in a solution of 3% $NaCO_3$; 0.05% HCOH, 0.001% $Na_2S_2O_4$, for 5 min. Development was stopped by bathing the gel in a solution of 5% Tris with 1% acetic acid for 30 min. Gels were finally washed with Milli-Q H_2O.

Trial 4: Long Silver Nitrate (Heukeshoven and Dernick 1986). After electrophoresis gels were fixed in a solution of 40% methanol with 10% acetic acid for 3 x 45 min incubations. Gels were sensitised overnight by bathing in a solution of 30% ethanol, 0.2% $Na_2S_2O_4$, 6.8% NaAc. The next day gels were rinsed in Milli-Q H_2O for 4 x 30 min each. Gels were then soaked in a solution of 0.2% $AgNO_3$ for 1 hr. Gels were then briefly washed with Milli-Q H_2O for 20 sec followed by a 5 min wash. Gels were developed in a solution of 3% $NaCO_3$; 0.05% HCOH, 0.001% $Na_2S_2O_4$ for 5 min. Development was stopped using 5% Tris with 1% acetic acid for 30 min. Gels were finally washed with Milli-Q H_2O.

2D-PAGE analysis of "good quality" and "poor quality" egg batches

'Good" egg populations were classified as those which had hatching rates >50% while 'poor' egg populations were those which had hatch rates of between 1 to 10%. *P. monodon* eggs (0.1g) were homogenized in 1 mL 8 M urea, 5% NDSB-195 (Calbiochem), 0.1 M dithiothreitol, 0.1 M NaCl, 0.045 M Tris and 4% protease inhibitor cocktail (Complete,

Boehringer Mannheim). After homogenization the mixture was incubated for 15 minutes at 35°C. After cooling on ice for 10 minutes, DNAase I (1mg/mL, Sigma) and RNAase II (10mg/mL, Sigma) in 0.1M, pH 7 and 0.05 M magnesium chloride hexahydrate was added to the homogenate and then incubated on ice for 10 minutes. The homogenate was centrifuged for 15 minutes at 10,000 g at 4°C and the aqueous phase was collected from between the upper lipid and lower cellular debris phase. Fourteen mLs of ice-cold tri-n-butylphosphate:acetone:methanol (1:12:1) was added to the extract for a final acetone concentration of 80% and incubated at 4°C for 90 minutes. The precipitate was pelleted by centrifugation at 2,800 for 15 minutes at 4°C, washed sequentially with 1 mL of tri-n-butylphosphate, acetone and methanol and air dried. For the first dimension run each sample of 0.2 mg of protein, in a final volume of 250 µl Buffer C with 1% (v/v) Ampholines (4 - 6.5) (Pharmacia), was applied to a 11 cm 4 - 7 linear pH IPG using passive sample loading at room temperature for 16 hr, followed by IEF at 300 V for 3 hr and then 3000 V for 45 hr.

After isoelectric focusing, immobilized pH gradients were equilibrated for 15 minutes in 0.375 M Tris, 6 M urea, 30% glycerol, 2% sodium dodecyl sulfate and 0.1 M dithiothreitol followed by a second 10 minute equilibration in 0.375 M Tris, 6 M urea, 30% glycerol, 2% sodium dodecyl sulfate and 0.4 M iodoacetamide. The second dimension PAGE slab (190 x 160 x 15 mm) was a linear gradient electrophoresis stacking gel of 4% T, 2.6% C (Bis) and a linear gradient gel of 9-17% T, 2.6% C (piperazine diacrylamide, Bio-Rad) containing 0.005 M $Na_2S_2O_3$ but not sodium dodecyl sulfate. Electrophoresis was conducted at 25 mA per gel fo 1 h and then at 35 mA per gel for 4 hours. After electrophoresis, proteins were visualized using the silver staining method as described in silver staining trial 4. Images of gels were made by scanning gels on an Agfa Arcus II flat bed scanner before gel drying. Gels were analysed with the Phoretix 2D software program.

Results / Discussion

Table 2.3 outlines the results of these trials, which were judged on the following criteria:

1) presence of focused proteins.
2) maximum protein recovery.
3) presence of horizontal streaking.

'Focused proteins' were rated 'yes' when proteins focused into a distinct pattern and 'no' when proteins did not focus and little or no distinct pattern emerged. Figure 2.1 exhibits unfocused proteins that resulted in no distinct pattern; instead horizontal lines, vertical lines and 'haze' were visualized.

'Maximum protein recovery' was rated as 'yes' or 'no' in Table 2.3. Methods removing lipid from aqueous homogenates were given a 'yes' rating when unwanted protein precipitation did not occur. Methods using protein precipitation were rated 'yes' when there was complete solubilization of precipitate in buffer and there was no evidence of debris after centrifugation of the sample.

'Horizontal streaking' was ranked as either relatively 'high' or relatively 'low' as compared to the horizontal streaking in the other gel patterns. Horizontal streaking was not rated for trials that showed no focused proteins, which were therefore marked n/a.

Trial 1. Although ultracentrifugation of samples before 2D PAGE can produce lipid-free aqueous protein solutions, this was not tested in this study as access to an ultracentrifuge was limited. However, normal centrifugation (10,000 x g) of aqueous egg homogenates described in trial 1 resulted in a floating lipid layer that was easily separated from the aqueous homogenate. While protein recovery from trial 1 was maximal, it did not result in focused proteins .

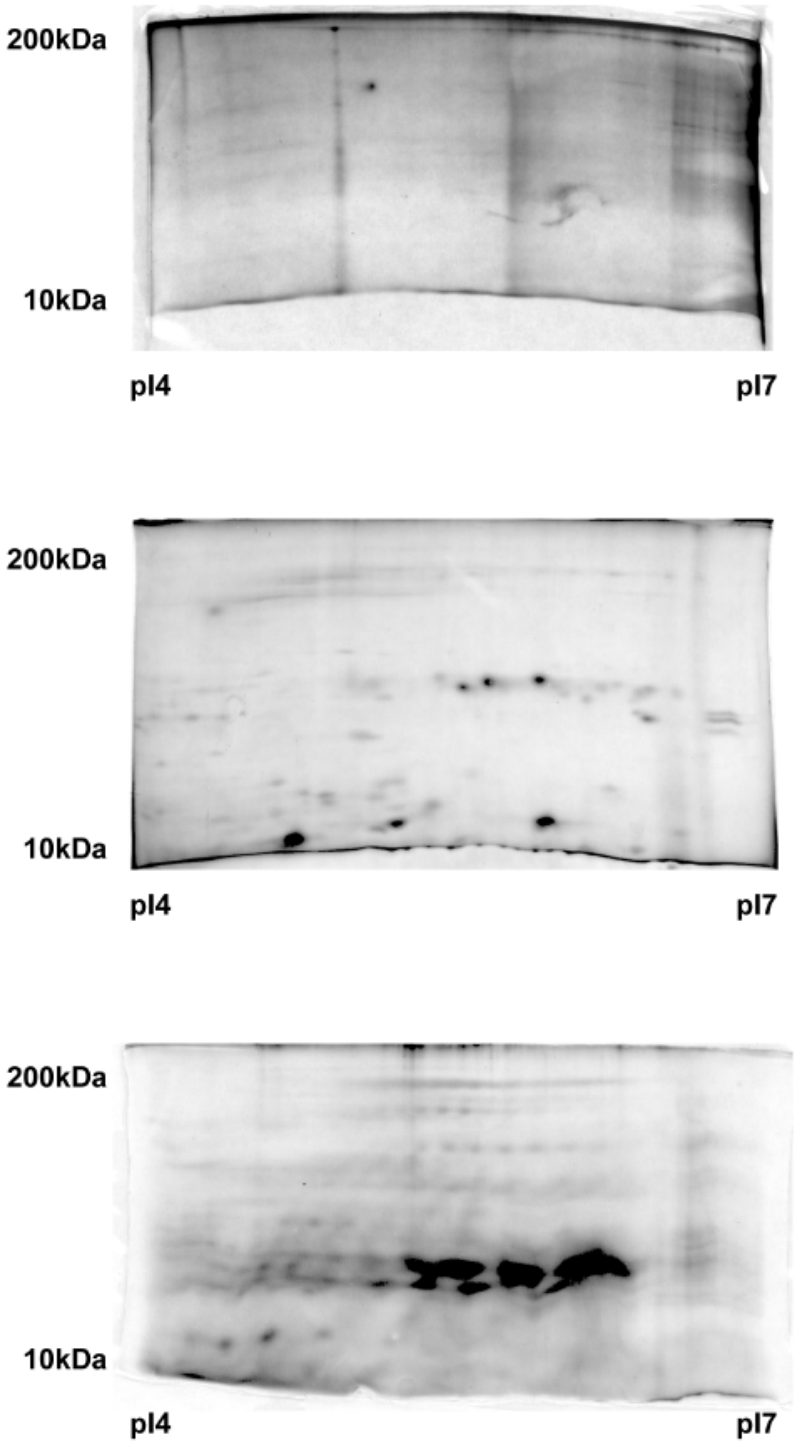

FIGURE 2.1. Effects of various sample treatment and extraction methods. Some treatments did not result in protein focussing (top). Treatment with TNBP gave distinct protein spots but poor protein solubility (middle) Treatment at high alkalinity resulted in carbamylation and 'charge trains' of proteins (bottom).

Lipid extraction methods vary considerably and include the traditional lipid extraction methods of Folch et al (1957) that utilize a chloroform and methanol extraction. Chloroform/methanol methods, however, resulted in a high degree of protein precipitation caused by solvency differences between the polar aqueous homogenate and the nonpolar chloroform solution. This protein precipitate was insoluble in the solubilization buffers tested.

Trial 2(a-c). These trials attempted to delipidate aqueous homogenates and reduce protein precipitation by extracting with reduced amounts of nonpolar solvents. Table 2.2 summarizes solvency in water for some common solvents and was used as a guide when choosing alternative solvents.

TABLE 2.2. Solvent Miscibility (Budaavari 1989)

Solvent	Solubility in Water
Acetone	100
Chloroform	0.815
Ethanol	100
Heptane	0.0003
Hexane	0.001
Methanol	100
Isopropanol	100
TNBP	0.606

Table 2.3 shows that lipid extraction with neither hexane:isopropanol (3:2) nor heptane:isopropanol (3:2) resulted in focused proteins (trials 2b and 2c). Delipidation using TNPB resulted in focused proteins with low horizontal streaking. Maps from TNBP delipidation are illustrated in Figure 2.1. All three solvents used in trial 2 produced undesirable protein precipitation.

TABLE 2.3. Ranking of focused proteins, sample preparation and horizontal streaking after trials. n/a = not applicable.

Trial Number	Focussed Proteins	Maximum protein recovery	Horizontal Streaking
Lipid Extraction Method		Protein Precipitation	
1	No	No	n/a
2a	Yes	Yes	Low
2b	No	Yes	n/a
2c	No	Yes	n/a
3a	No	Yes	n/a
3b	No	No	n/a
3c	No	No	n/a
3d	No	No	n/a
4	Yes	Yes	High
Lipid Extraction Method		Protein Precipitation	
5a	Yes	No	Low
5b	Yes	No	High
6	Yes	No	High
7a	Yes	No	Low
7b	Yes	No	High
8a	Yes	No	Low
8b	Yes	No	Low
8c	Yes	Yes	Low

Trial 3(a-d). Reverse phase partition chromatography using lipophilic beads yielded protein in an aqueous medium that was unacceptable for IEF. Using modifications to Gardner (1996,) lipophilic chromatography beads were added directly to the protein homogenate to adsorb lipids away from the aqueous solution without causing protein precipitation. Sephadex LH20 is a cross-linked dextran hydroxypropylated chromatographic bead with both hydrophilic and lipophilic characteristics. The beads swell to different sizes dependent on the solvent conditions. According to Gardner (1966) more fully swollen beads remove a greater amount of triglycerides than lesser swelled beads, indicating that internal adsorption may be more important for lipid adsorption than external surface adsorption. Table 2.4 summarizes the degree of swelling in a variety of solvents for LH20 and was used as a guide when choosing solvents. Table 2.3 indicates that while trial 3(a-d) resulted in maximal protein recovery, it failed to result in focused proteins. This methodology appears to be insufficient to delipidate egg homogenates such that the residual lipids interfere with IEF. Multiple incubations of egg homogenates using fresh LH20 beads was not investigated but could have future potential.

Trial 4. HAP, another lipophilic chromatography bead, was tested using a modified method described by Kragh-Hansen (1993). By increasing the alkalinity of the sample to approximately pH 12, proteins are partially denatured and unfold to release lipids free into solution. Under these conditions, the 2D PAGE gel resulted in numerous rows of repeating spots or 'charge trains' (Fig. 2.1). Charge trains are typically caused by heating proteins in urea above 60°C resulting in carbamylation, although this did not occur in the present trial. However, samples were not heated in urea at these temperatures for this trial. It appears likely that the extreme pH environment caused extensive protein deamidation (Wilkins et al. 1997). Deamidation results in shifts of the isoelectric point of proteins that can result in 'charge trains'. It was concluded that this method was unsuitable for 2D PAGE.

TABLE 2.4. Sephadex LH20 volumes swollen in different solvents.

Solvent	Bed volume (ml / g dry gel)
Chloroform	3.8 - 4.1
Ethanol	3.6 - 3.9
Isopropanol	3.3 - 3.6
Heptane	?
Hexane	?
TNBP	3.9

An alternative approach to achieve maximal protein recovery is precipitation using organic solvents, such as acetone and alcohols. Acetone lowers the dielectric constant of the homogenate solution, thereby reducing the solution's solvating power resulting in protein aggregation (Harris & Angal, 1989). Traditional protein precipitation methods are capable of consistently delivering maximal protein yields in precipitate form and historically have been utilised for 2D PAGE. Unfortunately the protein precipitate may not be completely nor consistently soluble using traditional solubilization buffers. In the case of analytical 2D PAGE, where quantitative comparisons between populations is desired, a complete resolubilization of the protein precipitate is necessary so that all precipitated protein species are represented in the same ratios as they are *in vivo*. Incomplete solubilization may preferentially represent proteins that solubilize better under the prescribed conditions than other proteins, and precipitated proteins are another source of horizontal streaking in the final 2D PAGE gel. A suitable resolubilization method was examined in trials 5, 6, 7, and 8, in which a consistent egg extract pool was precipitated and resolubilized under different treatments.

Trials 5(a-b) - Different methods for complete protein precipitate solubilization after acetone/methanol precipitation was examined. Trial 5a used a combination of traditional methods including heat and 0.1% SDS, 100mM DTT (Buffer E) followed by an incubation in a typical 2D PAGE buffer containing urea, CHAPS and DTT (Buffer C) (Fig. 2.1). After heating protein in Buffer E the protein pellet did not completely solubilize. The use of Buffer C and heating also resulted in incomplete solubilization. Theoretically the use of such buffers in combination with heat should disrupt both disulphide bonds and macromolecule aggregative forces. Further tests with additional amounts of heat and buffer did not improve protein solubility. Table 2.2 indicates that horizontal streaking for 5a was lower than 5b, suggesting that the degree of heating (100°C vs. 35°C) and different detergents (SDS vs. CHAPS) may result in different degrees of protein solubilization. These results indicated that the traditional methods were failing to completely solubilize the protein precipitate from *P. monodon* egg extracts and that a new approach to protein precipitation and resolubization was required.

Penaeid eggs are reported to contain neutral lipids and phospholipids, with the latter making up ~47% of the total lipid content (Chahu et al. 1994; Chahu et al. 1995). Table 2.5 reveals that most lipid extraction methods remove only one class of lipids. To enable complete solubilization of protein precipitates, however, the removal of both neutral lipids and phospholipids is necessary. Table 2.5 indicates that acetone extracts glycolipids while methanol extracts phospholipids. Preliminary trials indicated that an acetone-methanol mixture mainly resulted in the removal of phospholipids from extracts of prawn eggs. However, the remaining neutral lipids created a hydrophobic environment that minimized the ability of aqueous buffers to resolubilize all of the protein. Additional measures to remove neutral lipids are needed to improve the solubility of the protein precipitate. Subsequent trials focused on the use of a combination of methods to remove both neutral and phospholipids.

TABLE 2.5. Lipid Classes Extracted Using Different Lipid Extraction Methods

Solvent	Extracted Lipid	Source
Chloroform	neutral lipids	Gunstone et al. 1994
Methanol	phospholipids	Gunstone et al. 1994
Acetone	glycolipids & sulpholipids	Gunstone et al. 1994
Hexane	neutral lipids	Gunstone et al. 1994
TNBP	neutral lipids	Gardner 1996

Trials 6 and 7(a,b) - Centrifugation is the simplest and quickest method of removing large amounts of lipids from egg homogenates and was therefore used as a preliminary 'skimming' step in trials 6 and 7(a-c) before complete delipidation with organic solvents. While delipidation methods used in trial 6 resulted in focused proteins (Fig. 2.1) there was incomplete protein precipitate resolubilization (Table 2.3). Unlike all other precipitates from other trials, the pelleted precipitates were easily broken up with pippeting action. Pre-extraction using heptane or hexane caused protein precipitation that was collected and then treated with acetone/methanol. However, protein resolubilization was incomplete with either precipitation method. Trial 6 was also problematic because of the high degree of streaking in the resulting 2D PAGE gels.

Trials 8(a-c) - Further trials were directed towards a one-step method that extracted neutral and phospholipids as well as precipitating proteins in the same solvent solution. Two solvents examined, TNBP and heptane, are large non polar molecules. The size and structure of these molecules are such that they would be predicted to maintain their physico-chemical properties within the acetone-methanol environment and result in extracted neutral lipids from aqueous egg homogenates. While trials 8a and 8b resulted in incomplete solubilization

(Table 2.3), these extraction solutions resulted in protein precipitates that solubilized with greater ease using less heat and mechanical homogenization than any of the previous trials. Prolonged incubation at -20°C (Trial 8c) resulted in a completely solubilized protein precipitate. TNBP was chosen over heptane because of its high flashpoint (146° C) compared to heptane (-4° C).

The resultant gel patterns from the various sample homogenization trials are shown in Figure 2.1. When proteins from samples did not focus there was little or no distinct pattern; instead only horizontal lines, vertical lines and 'haze' were visualized (Fig. 2.1, top panel). Delipidation treatment with TNBP gave distinct protein spots and exhibited only slight horizontal streaking (Fig. 2.1, middle panel). However, TNBP resulted in unacceptable amounts of protein precipitation. Exposing samples to high alkalinity, pH 12, resulted in numerous rows of repeating spots or 'charged trains' due to extensive protein deamidation resulting in staged shifts of the isoelectric point of proteins (Fig. 2.1, bottom panel).

The presence of nucleic acids can also result in sub-optimal protein resolution on 2D PAGE gels. Poor separation of proteins in the IEF dimension, horizontal streaking in the SDS PAGE dimension, and a loss of high molecular weight proteins, are often associated with nucleic acid contamination of protein sample. The nucleic acids do not focus in the IEF first dimension, which results in horizontal streaking of nucleic acids and a masking of focused proteins, since nucleic acids are also visualized by silver staining. Although incompletely solubilized proteins can cause streaking, nucleic acids cause high molecular weight streaking which is non-specific. In addition, high molecular weight nucleic acid complexes block the polyacrylamide pore matrix and so impede or prevent high molecular weight proteins from entering the IPG gel matrix. This phenomenon is indicated by a lack of high molecular weight proteins visualized in stained 2D PAGE gels. While protease activity is also indicative of a lack of high molecular weight proteins, the addition of protease inhibitors controls for such protease activity. Removing DNA and RNA from aqueous homogenates can be accomplished using ultrafiltration or enzymatic methods. Both methods were tested and both performed equally well. Enzymatic removal was the method of choice for the final optimized 2D PAGE protocol because of its ease of use and short processing time.

The optimization of sample preparation for *P. monodon* egg extracts for 2D PAGE, necessitated the use of a combination of methodologies to remove the variety of substances that interfered with the high resolution IEF and SDS PAGE. The combination of nucleic acid removal by enzymatic methods, a centrifugation step at 10,000 x g to pellet the protein and remove the majority of lipids, followed by an acetone/methanol/TNBP treatment resulted in optimal protein recovery and 2D PAGE resolution. The protein precipitation method uses a number of organic solvents to simultaneously remove both neutral and phospholipids and precipitate proteins. The acetone component also removes salts and pigments in the solvent supernatant, which is advantageous when working with penaeid eggs because of their high salt and pigment content. The methanol component removes glycolipids. The use of precipitation also concentrates the proteins, which allows the operator to resolubilize at the working concentration required. This optimized method results in a protein precipitate that can be fully solubilized using an aqueous buffer compatible with IEF. The final 2D PAGE gels result in well-focused proteins with little or no horizontal streaking. These high resolution gels are necessary for analytical 2D PAGE software program analysis and the comparison of a large data set of egg samples from a population.

OPTIMIZATION OF ISOELECTRIC FOCUSING

IPG technology was created to overcome problems associated with CA-IEF. IPG-IEF uses non amphoteric weak acids and bases with the general chemical composition of CH_2=CH-CO-NH-*R*. *R* represents either an acidic carboxyl group or a basic tertiary amino group (Wilkins et al. 1997). The p*K* for the carboxyl group is: 3.6, 4.4, or 4.6 and the p*K* for the amino group is: 6.2, 7.0, 8.5, or 9.3 (available from Pharmacia as "Immobiline" or Fluka, provides this same class of amphoteric compounds ranging from *pK* 2.5-11, as "pI-Select"). For the sake of convenience and clarity these amphoteric compounds will be termed 'immobilines'. Immobilines are co-polymerised with the acrylamide matrix resulting in an immobilized pH gradient. The immobiline molecules are stationary and do not migrate within the acrylamide matrix when current is applied. Gradients can be constructed for both broad (i.e. 3 - 10 or 4 - 7), narrow (range over 1 pH unit, i.e. 3.8 - 4.8), and ultra-narrow (close to 0.1 pH unit) ranges. Methods and calculations for producing linear IPGs are reviewed in detail by Righetti (1990). Pharmacia offers precast IPGs at 4 - 7 and 3 - 10 broad ranges. For ease and consistency commercially available IPGs are recommended when working within the 4 - 7 or 3 - 10 ranges. However, custom made IP's can be produced and the slope and shape of the gradient can be manipulated to generate better resolved protein maps. If a linear gradient results in overcrowded regions, a non linear gradient may be a better option. Biological samples usually fall within the broad 4 - 7 pH range. Analysis of complex samples usually begins with broad IPG-IEF. If necessary, analysis by narrow and ultra-narrow gradients are used to increase protein banding resolution. Ideally proteins should be well resolved and dispersed evenly over the entire IPG length. The final running conditions should be judged on the resulting resolution of spots and the degree of horizontal streaking. Focusing conditions, protein load, and ampholyte concentration should be determined empirically for each sample type. Other criteria that should be considered are ampholyte cost and amount of sample available. IPG-IEF methodology was used for the first dimension of *P. monodon* egg 2D PAGE. Protein load, focusing conditions, and carrier ampholytes concentration were manipulated to optimize protein resolution and reduce horizontal streaking.

Trials 9 (a,b) and 10 (a,b) - Protein mobility during IEF determines the degree of individual spot resolution and horizontal streaking in resulting 2D PAGE maps. Interference of protein mobility during IEF can be caused by substances such as lipids, nucleic acids, pigments, salts, and carbohydrates that are commonly found in biological preparations. Protein load, focusing conditions (kVhr) and ampholyte concentration also affect the mobility of proteins during IEF. The IEF of *P. monodon* eggs was optimized by first eliminating interfering substances, such as lipids, followed by optimizing protein load, focusing conditions (kVhr); and ampholyte concentration. Resulting 2D PAGE maps were judged on individual spot resolution and levels of horizontal streaking. Trial 9 (a&b) and Trial 10 (a&b) all used the same pooled extract, prepared as described for Trial 8c. The ampholyte concentration and focusing conditions were optimized with this sample. Table 2.6 ranks spot resolution and horizontal streaking results from Trials 9 and 10. The ranking varied from 1, being the most highly resolved pattern, to 4, which was the most poorly resolved pattern.

P. monodon egg samples did not resolve well when focused without ampholytes. The addition of ampholytes at a final concentration of 1% improved spot resolution and decreased overall horizontal streaking (Table 2.6). Increased focusing time from 63.9 kVhr to 135.9 kVhr without ampholytes resulted in little or no changes in spot resolution or horizontal streaking whereas the addition of ampholytes significantly improved resolution.

TABLE 2.6. 2D PAGE resolution quality with and without ampholytes at different focusing times.

IEF running conditions	Spot resolution (ranked)	Horizontal streaking (ranked)
Trial 9		
63.9 kVhr w/o ampholytes	4	4
63.9 kVhr w/ ampholytes	2	2
Trial 10		
135.9 kVhr w/o ampholytes	3	3
135.9 kVhr w/ ampholytes	1	1

Poor protein resolution and horizontal streaking may be caused by a variety of factors, such as inherent protein property, IPG property, or IPG-protein interaction. Because Immobilines p*K*s: 6.2, 7.0, 8.5, and 9.3 are more hydrophobic than Immobilines p*K*s: 3.6, 4.4, and 4.6, zones of hydrophobic areas on the IPG may be formed, which may interact with hydrophobic proteins. Such IPG-protein interactions may retard protein migration and result in poor protein resolution (Wilkins et al. 1997). The addition of carrier ampholytes to both the sample and the IPG matrix increases solubilization of proteins (such as those associated with biological membranes) and may 'shield' hydrophobic areas of the IPG by interfacing with Immobiline molecules and sample proteins allowing hydrophobic proteins to migrate freely and resolve more fully (Righetti 1990). Many of the egg proteins from prawns may be hydrophobic and interact with the hydrophobic regions of the IPG and hence not focus completely without ampholytes. The addition of ampholytes to egg samples and IPG, as well as increasing kVhr, all improved spot resolution and decreased horizontal streaking (Fig. 2.2 AND Table 2.6). These results indicate that there are interactions between the ampholytes, proteins, and IPGs in the egg extract. However, the use of carrier ampholytes is dependent on the sample proteins' characteristics and therefore may not be useful in all instances. The ampholyte pH range, final ampholyte concentration, and focusing conditions should be determined empirically for each type of protein sample.

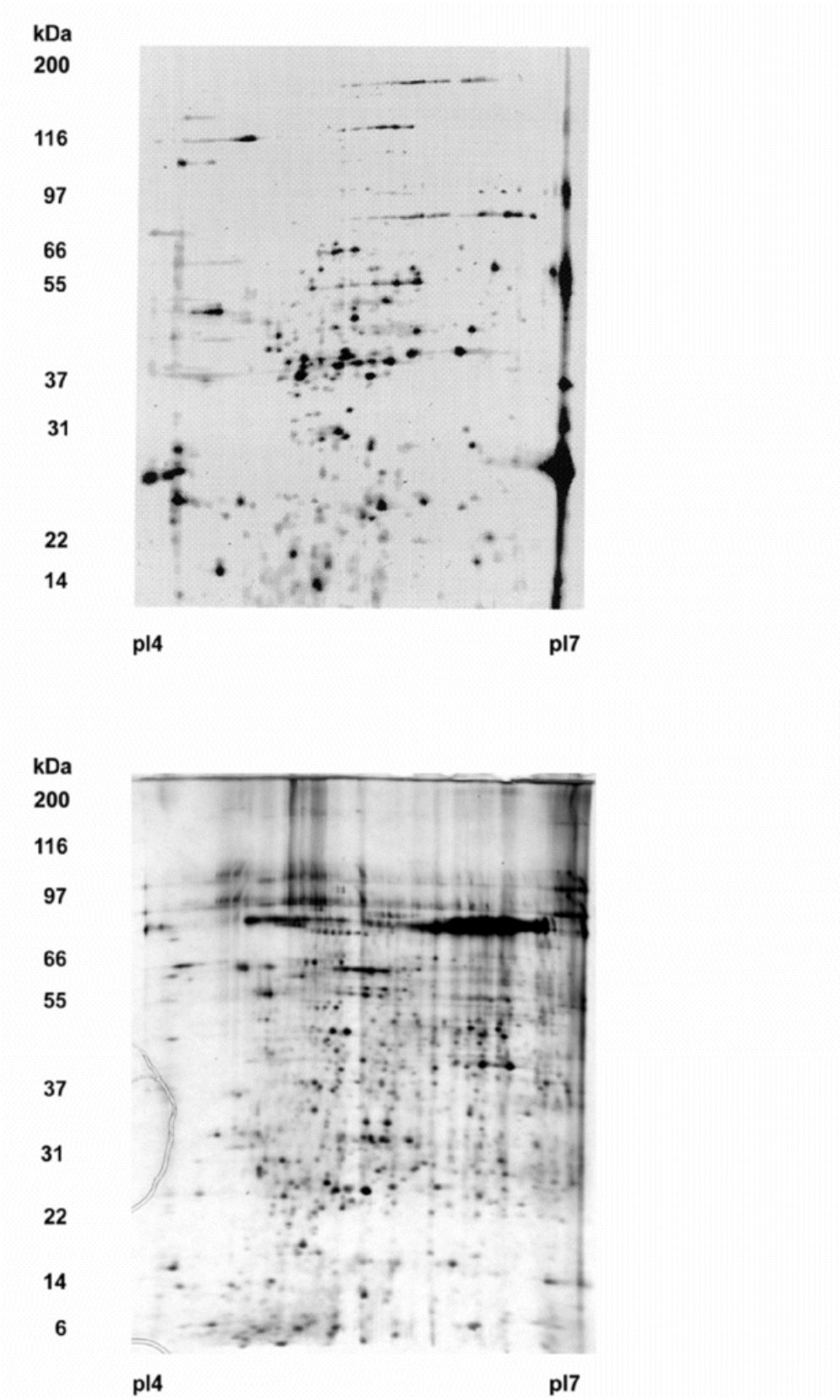

FIGURE 2.2. Protein separation pattern after TNBP/acetone/methanol precipitation and –20°C incubation (top) and after the addition of ampholytes (bottom) to sample before IEF.

OPTIMIZATION OF PROTEIN LOADS

Trials 11 (a-d) - The effects of increasing protein load on a constant ampholyte concentration, 1% final concentration (v/v) and constant focusing conditions (135.9 kVhr), were tested in Trial 11 (a-d). As the amount of protein loaded per 11 cm IPG, increases, the spot resolution decreases, and the horizontal streaking increases. Protein loadings of 0.1 - 0.2 mg / gel resulted in 2D PAGE maps which had good resolution and little horizontal streaking. In contrast, protein loading in excess of 0.2 mg / gel resulted in decreased spot resolution and increased horizontal streaking.

Protein load should be deduced empirically. Size of gels (width, height, and thickness), staining sensitivity, and concentration of individual proteins, determine optimal protein load. Under certain conditions adjustments may need to be made between total protein load and individual protein concentrations. For example, in *P. monodon* eggs vitellin, at a pI of approximately 6, is an extremely prominent band that is by far the most abundant protein in the extract. In gels loaded protein concentrations of 0.1, 0.2, 0.3, and 0.4 mg / gel, the vitellin concentration is so great that it overloads the gel resulting in poor resolution of vitellin. Proteins which may co-migrate to a similar area of the gel would be masked by the vitellin. However, a 0.1 to 0.4 mg / gel loading is required to have sufficient loading so as to detect the rarer proteins in the extract. Gels loaded with 0.1 mg / gel separate and resolve better than at 0.3 or 0.4 mg / gel because signals do not blend into one another but at 0.1 mg / gel proteins of lesser concentrations begin to loose signal. For these reasons 0.2 mg / gel was chosen as the standard for *P. monodon* eggs analysis by 2D PAGE since this loading is the best compromise between resolution and detection of rare proteins. Because 'vitellin' represents a large percentage of the mixture gels a lower total protein concentration would have to be run to resolve 'vitellin' satisfactorily. However, if rare proteins are being sought, a normal protein loading may result in the major protein species overwhelming any signal from the rare proteins. Selective removal of the major proteins might be necessary under these conditions. Alternatively, several gels at different concentrations could be run so that all proteins are represented for analysis. There are no hard and fast rules for this - it simply a matter of experimentation and judgment.

OPTIMIZATION OF POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

The ultimate goal of SDS PAGE is to achieve optimal resolution of the proteins of interest. In this study optimization of protein separation and resolution was investigated through manipulation of the acrylamide concentration of gradient gels. In addition, optimal conditions to minimize horizontal streaking by manipulating equilibration conditions and gel conditions were also investigated.

P. monodon egg homogenates are a complex mixture with proteins ranging from less than 6 to ~200 kDa. Resolving gels with different acrylamide concentrations were tested to find which produced the best separation and resolution. As discussed earlier, gradient gels are the best option for resolving such a wide range of molecular masses. Table 2.7 outlines those %T (polyacrylamide gel concentration defined as percentage total monomers (acrylamide plus cross linking agent, g/100mL)) best suited to different mass ranges. While gradient gels are superior to homogenous gels for resolving complex mixtures, they require greater time and equipment investments. A 12% (T) homogeneous resolving gel resulted in good separation but protein resolution became fuzzy at 30 kDa and less (Fig. 2.3). Different gradient combinations were then tested to compensate for resolution loss while retaining optimal separation (Figure 2.3b, c, and d). A 12% - 20% gel retarded proteins and resulted in poor separation (Fig. 2.3b). Under such conditions a larger pore size would give better resolution. When an egg extract was separated over the entire length of the 10% - 20% gel, the great majority of the proteins were condensed into one region resulting in an uneven separation (Fig. 2.3c). A larger pore size gel of 9% - 17% resulted in good separation and resolution (Fig. 2.3d).

TABLE 2.7. Corresponding Molecular Weight Ranges well-resolved and %T

%T	High kDa	Low kDa
4	200	116
7.5	200	65
10	200	21
12	200	14
15	200	6.5
4 to 15	200	40
4 to 20	200	6.5
9 to 17	200	10
10 to 20	100	6.5

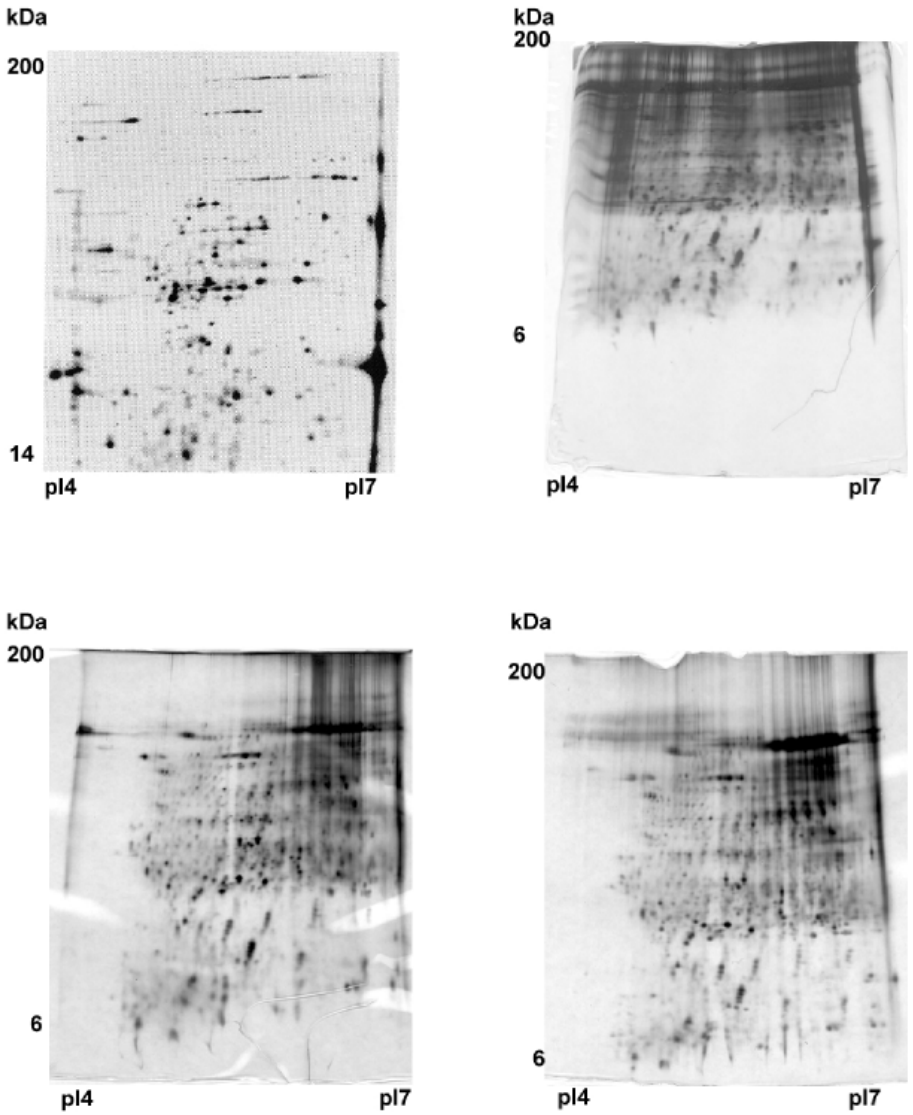

FIGURE 2.3. Protein separation patterns of egg extracts from different polyacrylamide gel (PAGE) concentrations (T%): 12% (upper left), gradient from 12% to 20% (upper right), gradient 10% to 20% (lower left), and gradient 9% to 17% (lower right).

A 9% - 17% gradient gel resulted in the best separation, with the greatest number of focused proteins, from *P. monodon* eggs and produced an acceptable level of resolution for all those proteins resolved. The range of proteins separated and resolved using a 9% - 17% gradient gel at pH 8.8 is approximately 6 to ~200 kDa.

MINIMIZATION OF VERTICAL STREAKING

Vertical streaking is often a problem in the SDS PAGE second dimension of 2D PAGE. There are two types of vertical streaking associated with 2D PAGE. Point streaks are those streaks not associated with any particular protein and are most often caused by dirt on glass plates. Plates must therefore be cleaned scrupulously. If plates are wiped using fibrous toweling, small fibers adhere to the plates and stain with silver staining and appear as point streaks. To avoid point streaking plates should be rinsed with alcohol rather than wiped with toweling. Vertical streaking can also be associated with proteins themselves. This type of streaking is most often caused by renaturation of proteins upon entering PAGE, protein overload, or glycoproteins. In complex biological extracts, such as egg samples, the presence of glycoproteins can result in poor resolution due to streaking. Resolution of some individual bands may need to be sacrificed for the overall sample resolution. If glycosylation is suspected to be causing extensive streaking, the investigator should give consideration to running additional gels that are suited for glycosylated protein separations.

Excessively high concentrations of particular proteins can overload the SDS PAGE at the point of entry causing streaking. The streaking is a result of the protein renaturing as it enters the gel. Streaking of 'vitellin' and other egg proteins occurred under the conditions described in the protein load trials. Even at concentrations of 0.1 mg / gel, proteins were streaking vertically. To keep proteins denatured as they entered the gel two approaches were examined. First, the DTT concentration in the equilibration buffer was increased to denature any proteins that might renature in the IPG. Secondly, a 4 % stacking type acrylamide gel containing 0.1% SDS was layered on top of the 9% - 17% gradient to reduce the renaturation of proteins as they enter the acrylamide gel. SDS was not added to the gradient gel because it contributes greatly to high background from silver staining. Both these approaches significantly reduced the degree of vertical streaking associated with 'vitellin' and other abundant proteins (Fig. 2.4).

SILVER STAINING

Silver staining was introduced in 1979 by Switzer et al. as a new method of protein staining 100 times more sensitive than Coomassie brilliant blue R250. In the early 1980s silver staining was plagued with numerous problems such as cost, background, and surface staining (Rabilloud 1992). Present day silver staining methodology has solved many of these problems giving sensitivities in the 1 to 10 ng range. However, silver stains do have a number of drawbacks, bring reasonably laborious and expensive. In addition some proteins stain poorly or appear negative and some non-specific staining of DNA, lipopolysaccharides and polysaccharides may occur. The linear dynamic range and sensitivity of silver staining can be over a 40-fold range in concentration, but at microgram levels the silver image becomes saturated and can result in bands that negatively stain. Advances in silver staining from the 1980s have increased sensitivity to the sub-nanogram per mm^2 range allowing more proteins to be visualized on any one gel.

There are two types of silver staining: silver nitrate and silver diammine (silver complexed with ammonia) (Rabilloud 1992). These methodologies are distinguished primarily by the pH of the silver solution and the subsequent development solution. The choice of methodology is dependent on pH of proteins, time constraints, convenience, resulting sensitivity, background, chromatic consistency, and the performer's willingness to work with hazardous chemicals. Because of the high incidence of hazardous chemicals, diammine methods were

not used in this study. Instead the most sensitive silver nitrate methods were modified to yield sensitivity thresholds <10 ng. Because *P. monodon* egg samples have proteins predominantly in the pH range 4 - 7 silver nitrate methods can be used.

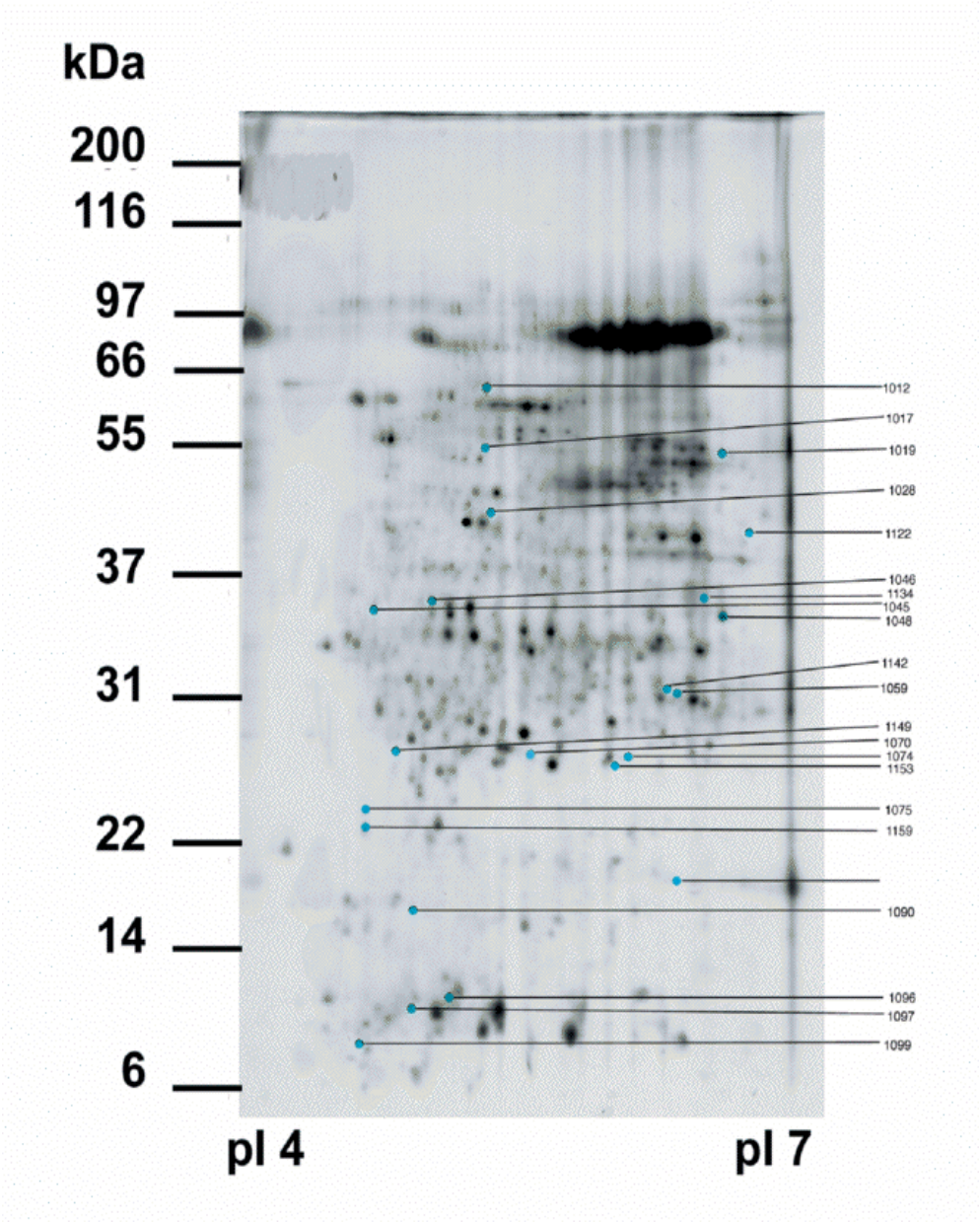

FIGURE 2.4. 2D PAGE profile of 'good' eggs.

While silver nitrate methods are reported to have sensitivity thresholds of 5 – 20 ng the results from this study demonstrated that with modifications to Heukeshoven and Dernick's procedure, and the addition of sodium thiosulfate to PAGE gels, the silver nitrate method can reach sensitivity thresholds of 10 ng or less.

The variations to the silver nitrate method were judged on background, sensitivity and chromatic consistency. Background and sensitivity are closely related in silver staining. As background, or non-specific gel binding, is reduced, and sensitivity, or specific protein binding, is increased, contrast increases. Increases in contrast result in better resolution of bands or spots. The balance between background and sensitivity are balanced by a well

timed rinse after the silver impregnation stage and just before the development stage. Background results from the development of silver ions loosely bound to the gel matrix. Rinsing the gel with high quality water after silver impregnation releases these loosely bound silver ions from the gel matrix, thus reducing background. The rinse needs to be timed so that silver remains bound to the proteins, allowing development to occur, which allows the attainment of good sensitivity. Silver bound proteins should develop into darkly stained monochromatic proteins with low levels of background during the development stage (Rabilloud 1994). In this study, sensitivities were approximately 10 ng with low backgrounds for all trials (Fig. 2.4).

Staining results from Trial 1 and 2 exhibited multi-chromatic staining while Trial 3 resulted in reverse staining for the lanes loaded with 50 ng and 1 µg of protein. Protein was present in a gaussian type pattern with higher concentrations in the center and lesser concentrations at the edges. Higher concentrations of protein bind silver ions more tightly than proteins at low concentration (Rabilloud 1994). The tighter the protein-silver complex, the less reactive the silver ion. When development of a focused protein band begins, the outer edges of the protein begin to stain before the inner core of the band or spot resulting in darker edges and lighter centers. This results in multi-chromatic staining with different shades of gold, brown, and black. In extreme cases reverse staining occurs because the protein-silver complex is so tight that reduction of the silver ion never occurs (Yuksel and Gracy 1985, Rabilloud 1992, 1994). An optimal silver stained gel results in monochromatic dark brown to black bands or spots. The high quality wash between silver impregnation and development contributes to monochromatic staining by loosening the protein-silver complex in the highly concentrated protein areas just enough so they develop at the same rate as those in the less concentrated protein areas (Rabilloud 1994). The silver staining from Trial 4 resulted in very dark brown monochromatic staining (Fig. 2.3). Because proteins appear in a range of different concentrations on 2D PAGE maps, monochromatic staining is important not only for an individual protein but for all proteins mapped.

Trial 4 staining results in sensitivity thresholds of 1 - 10 ng. This increase in sensitivity from Heukeshoven and Dernick's original protocol is related to the reduction in background and an increase in silver reduction. The purpose of sensitization of gels is to treat the gel with a reagent that exhibits affinity for the protein and forms silver sulfide, in the case of silver nitrate methods. This sensitization increases the speed at which silver is reduced, thus improving sensitivity and contrast. Heukeshoven and Dernick's original protocol used gluteraldehyde combined with sodium thiosulphate as a sensitiser. In Trial 4 gluteraldehyde was eliminated from the sensitizing solution because it has been reported that gluteraldehyde is only effective in diammine methodologies (Rabilloud et al. 1994b). Gluteraldehyde has also been found to cause high backgrounds in silver staining if rinsing is inadequate. Sodium thiosulfate, when used with long incubation times and without gluteraldehyde, is a sufficient sensitiser for silver nitrate methods and results in low background staining and good sensitivity, <10 ng (Fig. 2.3). Formaldehyde was also used in the original Heukeshoven and Dernick protocol, in the silver nitrate and development solutions. However, formaldehyde, like gluteraldehyde, can cause high background. In Trial 4 formaldehyde was used only in the development solution. The removal of formaldehyde from the silver nitrate solution resulted in gels with low backgrounds but did not affect the overall sensitivity (Fig. 2.3). The modified method used in Trial 4 results in sensitivity thresholds less than those reported by Rabilloud (1992). The addition of sodium thiosulphate to the developer cannot be used in diammine methodologies. The acidic developer has a lower reduction speed that increases the chances of thiosulfate sequestering silver bound to proteins and preventing image formation. To overcome this problem, sodium thiosulfate can be added to the gel matrix prior to polymerization where it can bind to silver ions that settle on the gel and prevent silver reduction (Heukeshoven and Dernick 1986). To reduce background further, sodium thiosulfate can be added to the developer solution that results in decreased backgrounds

(Fig. 2.4). These modifications result in little or no background and an increase in sensitivity to 1 ng. The final methodology used in Trial 4 had greater contrast, greater resolution, and greater monochromatic staining than the other silver staining methods tested. The background, sensitivity, contrast, resolution, and chromatic features of this methodology are optimal for 2D PAGE protein maps and subsequent densitometry analysis for proteins of neutral and acidic proteins like those found in *P. monodon* eggs. A modification of the silver nitrate method of Heukeshoven and Dernick (1986) was finally chosen for this study.

COMPARISON OF 'HIGH' AND 'POOR' QUALITY EGGS

Four sets of 'good' and 'poor' quality eggs were analyzed by 2D PAGE (Table 2.8). Each egg population was spawned from a wild female mated with a wild male. 'Good" egg populations were classified as those which had hatching rates >50% while 'poor' egg populations were those which had hatch rates of between 1 to 10%.

TABLE 2.8. Source of 'good' and 'poor' eggs and their respective hatching rates.

Date Spawned	Total Eggs	Total Nauplii	% Hatch
9 April	284,400	14,400	5.1
6 April	198,000	3,200	1.6
18 April	168,000	2,000	1.2
5 May	159,200	8,000	5.0
24 March	264,400	148,000	56.0
29 April	490,400	298,400	60.8
29 March	205,600	173,600	84.4
29 March	228,000	138,400	60.7

Analysis of the gels was made with the Phoretix 2D software program using, in order, the specific macros for intensity calibration, spot detection, spot measurement, background subtraction, edit image, select reference gel, match spots, adding to reference gel and analyzing data. Intensity calibration ensures measurements are real-world values and compensates for variation in conditions of image capture. The binary images of each protein pattern were calibrated using a 20 point density curve ranging from 0 to 63639 pixels was created using the densitometry strip provided by Agfa. Spot detection selects 'real spots' by identifying them according to specific parameters, as selected by the user, best suited to each image with optimal results being those with low background and edges of binary images only around spots. Spot measurements are made on the volume of each spot according to the values set out in the intensity calibration. Background subtraction is an average measurement of each spot's boundary and is calculated and removed from the measurement value of that spot. The binary images were edited to remove artifacts and make any adjustments to the binary image where the binary image may not reflect the real life image. Spot measurement and background subtraction must be repeated after editing. A reference gel is composed from the image with the greatest number of spots and with the best quality separation. This process is based on the best estimate of the user and is usually a compromise between number of spots and separation quality. An image from the sample of 9 April was used as the reference gel for this experiment. Spot matching is made by comparison of the reference gel to the experimental, or slave, gel. An algorithm matches spots of different gels according to the parameters set by the user. Optimal matching should show vectors flowing smoothly and logically. After spot matching, all matches are manually checked by the user and edited if clear mismatches are observed. Spots that appear on the experimental (slave) gel which are not matched to the binary image of the reference gel are added to the reference gel database allowing all spots to be matched gel to gel consistently. Any one spot must have occurred in at least three gels from one group to be considered significant and not a random occurrence. A two-tailed student's t-test was performed on each of the unmatched protein

spots and a set of zero values to determine if there was a significant difference between the spots measured volume less background and no measurement. It is acknowledged that spots that are not visible on a gel may still be present in undetectable levels.

The 2D PAGE that was finally adopted had the ability to separate proteins with isoelectric focusing values between 4 to 7 and a molecular weight range between 6 kDa to approximately 66 kDa. The average Rf value value for each molecular weight protein marker was determined followed by the calculation of the relative molecular weight of each novel protein spot using the nearest relative Rf values and calculating the average. The average Rf value for each pI marker in a carbonic anhydrase carbamylate calibration kit was determined followed by the calculation of the relative pI of each novel protein spot using the two nearest relative carbamylyte marker and calculating the average. Swiss-Prot data base was then searched using the relative molecular weight to aid in identifying each protein spot.

The analysis of the egg populations resulted in the identification of 804 'real' protein spots (Table 2.9). These protein spots ranged in molecular weight from about 6kDa to 200kDa within isoelectric points from pI 4-7. Both good and poor egg populations had a total of 763 protein spots identified with a balance of 42 unmatched protein spots in each egg population (Figs. 2.4 & 2.5). Of the 42 unmatched poor egg population protein spots only 13 spots matched known proteins in the Swiss-Prot database (Tables 2.10 & 2.11). Of the 42 unmatched good egg population protein spots only 23 spots were matched to Swiss-Prot database entries (Table 2.12). Quantitative analysis showed that only one unmatched spot, reference number 1097, was significantly different in good egg populations from that of poor egg populations. No unmatched protein spots from poor egg populations proved to be present in significant amounts.

TABLE 2.9. Gene products isolated from good and poor quality *P. monodon* eggs.

	Good Eggs	Poor Eggs	All Eggs
	65.5% Hatch	3.2% Hatch	
'Real' Protein Spots	715	763	804
Unmatched before qualitative analysis	42	46	88
Unmatched after qualitative analysis	22	13	35
Unmatched after quantitative analysis	1	0	1

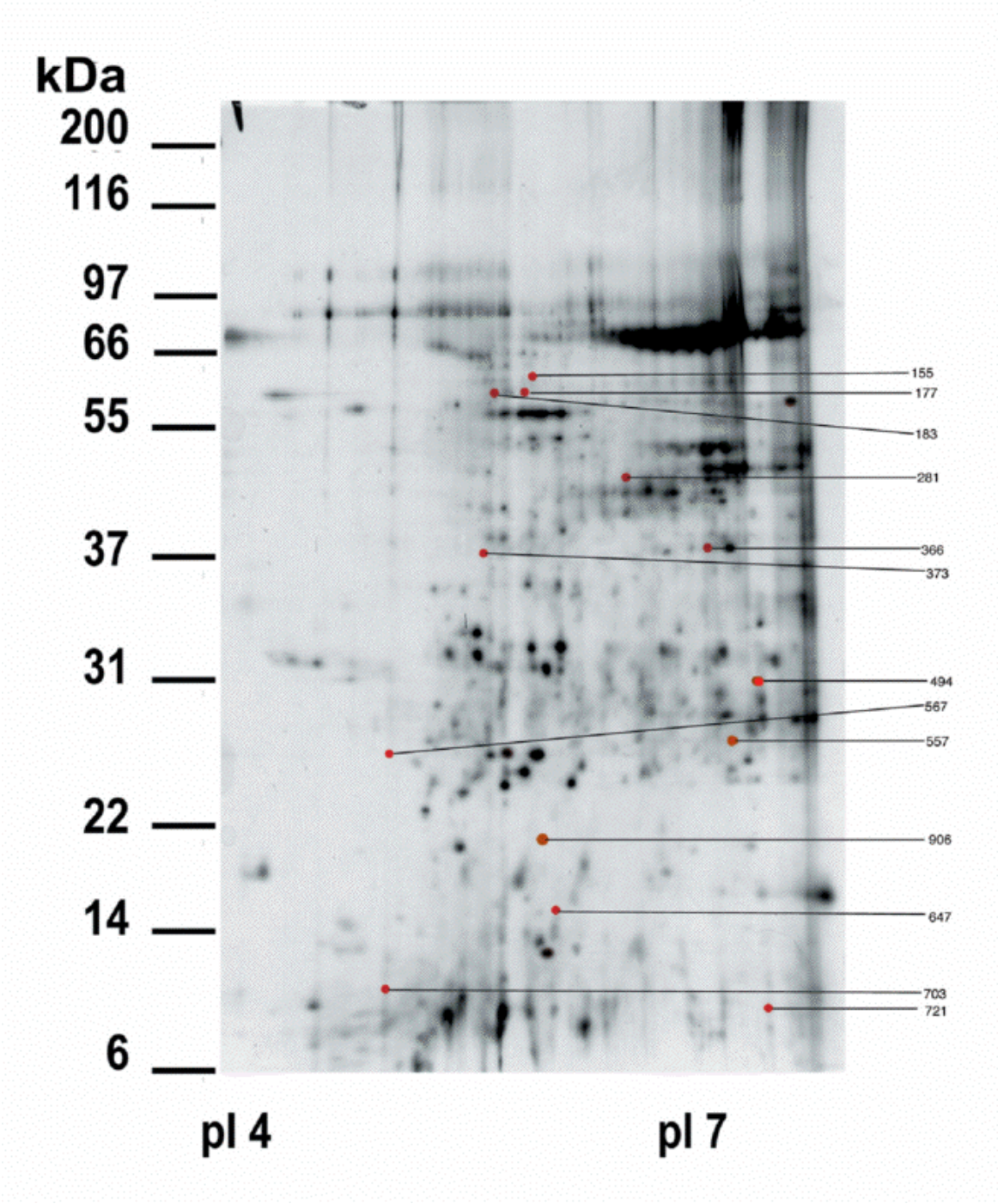

FIGURE 2.5. 2D PAGE profile of 'poor' eggs.

TABLE 2.10. Proteins identified from 2D PAGE as being unique to 'good' eggs.

Spot Reference Number	Relative MW (Da)	Relative pI
1012	59,690	5.65
1017	48,302	5.85
1019	47,592	6.55
1028	44,040	5.75
1045	41,073	5.25
1046	41,073	5.55
1048	31,000	6.65
1059	27,706	6.55
1070	47,854	6.05
1074	22,669	6.35
1075	21,396	5.15
1085	17,384	6.55
1090	15,378	5.45
1096	8,618	5.65
1097	8,003	5.45
1099	8,003	5.15
1122	41,909	6.65
1134	67,403	6.56
1142	27,706	6.56
1149	23,298	5.35
1153	22,039	6.35
1159	20,058	5.15

TABLE 2.11. Proteins identified from 2D PAGE as being unique to 'poor' eggs.

Spot Reference Number	Relative MW (Da)	Relative pI
155	58,690	5.95
177	55,400	5.95
183	49,723	5.85
281	44,040	6.25
366	28,336	6.55
373	33,702	5.75
494	25,817	6.65
557	22,669	6.55
567	22,039	5.25
647	12,312	5.95
703	6,000	5.25
721	4,909	6.65
906	18,721	6.05

TABLE 2.12. Commonality of gene products ('spots') between good and poor eggs.

Good Egg Spot Reference	Poor Egg Spot Reference	Swiss-Prot Description
–	373	ELAC protein capsid scaffolding protein
–	366	floral homeotic protein AGL1
494	–	nonstructural protein NS1
647	-	protein translation factor SUI1
–	721	none
–	703	none
906	–	olfactory neuronal specific protein R-phycoerythrin beta chain
1028	281	none
1045,1046	–	acyl-coa desaturase
1048	–	STO-2 protein
1059, 1142	–	none
1074	557	sensor protein hydh
1075	–	tetraspan membrane protein IL-TMP transforming protein P21/K-RAS
1085	–	40S ribosomal protein S15
1090	–	ribosomal protein S17
1096	–	none
1097,1099	–	none
1122	–	actin, macronuclear
1149	–	2-CYS peroxiredoxin BAS1 precursor
1153	567	none
1159	–	50S ribosomal protein L5P hypothetical 20.1k protein C13C5.06C

Whereas 2 out of 20 protein spots of good eggs were not identified 4 out of 7 spots were not identified in the poor eggs. As there was no N-terminal sequencing of the protein spots, identification is solely based on molecular weight (kDa) and isoelectric point (pI). Because of this any protein match to the Swiss-Prot database is not conclusive evidence that the protein is correctly classified. Protein identification with high accuracy can be achieved in many ways but this does not mean that the best-matching data exactly represents the protein. Isoforms of proteins, which can be widespread in some protein types, may go undetected. The protein N- and C-termini are frequently not defined and variations in mRNA splicing may be overlooked. Developments are underway to address these issues (Wilkins et al. 1997). Of particular interest in this study is the observation that one of the gene products (spot 373) of poor eggs was of viral origin and may indicate that eggs with this protein are infected with virus.

OBJECTIVE 3

To compare egg quality between wild and captive-reared broodstock

Introduction

A major goal in the developmental pathway of *P. monodon* aquaculture is to close the life cycle and use fully captived-reared broodstock to supply post-larvae (PLs) for stocking commercial production ponds. While closed life cycle stock have been produced on a research scale this has not been achieved on a commercial scale. Once achieved, however, there is the potential for true domestication and selective breeding with significant production gains for the industry. To date it has not proven possible to achieve sufficiently reliable spawnings from tank or pond-reared broodstock for commercial purposes compared to those obtained from wild sourced broodstock. The purpose of this objective was to obtain detailed data on the difference in performance between wild-caught and pond or tank-reared stock, and to additionally evaluate the impact of diet on maturation and spawning performance of broodstock.

Methods

SUPPLY AND AGE ESTIMATION OF WILD BROODSTOCK

Wild adult *Penaeus monodon* were obtained from coastal waters between Townsville and Cairns in north Queensland, Australia (17-19°S, 146-147°E) by commercial broodstock trawler operators. The wild-sourced broodstock were either used in the experiments directly or were used as the parent stock for the production of tank or pond-reared broodstock. Wild broodstock were typically procured in May to June to avoid direct competition with industry during the late winter/spring period when demand from hatcheries was high.

While the age of tank and pond-reared broodstock was known, the exact age of wild broodstock could not be determined due to the lack of a robust technique for achieving this. An estimate of the age of wild broodstock can be made from comparisons of their body weight to a database of body size, weight and age of tank-reared and pond-reared broodstock (Figs. 3.1, 3.2, 3.3).

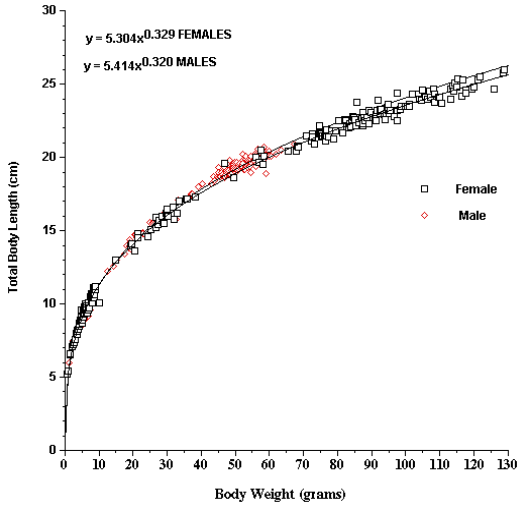

FIGURE 3.1. Relationship between body weight and total body length, from juvenile to adult, in male and female tank-reared *P. monodon*.

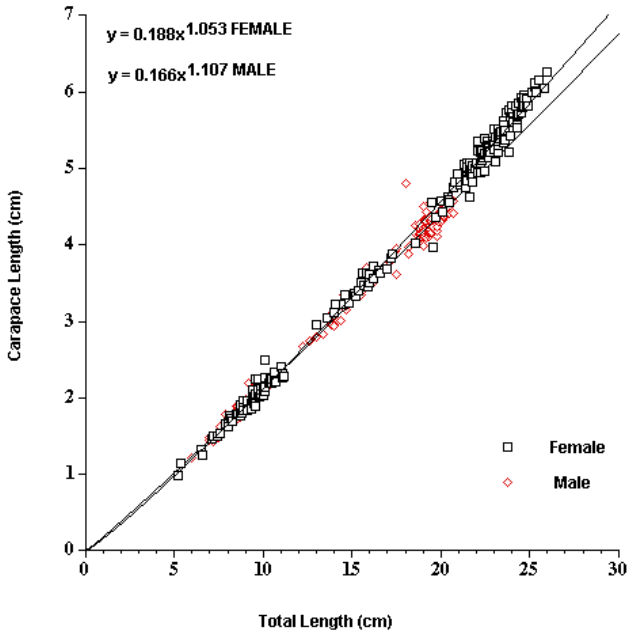

FIGURE 3.2. Relationship between total body length and carapace length, from juvenile to adult, in male and female tank –reared *P. monodon*.

Differences in body weight between females and males increased over time, with a 3%, 12%, 24% and 28% difference between them, respectively at 3,6,9 and 12 months (Fig. 3.2).

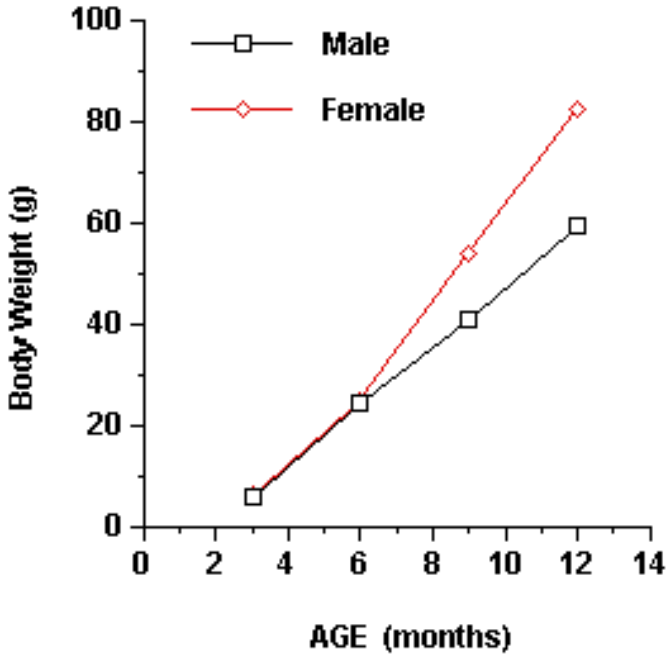

FIGURE 3.3. Changes in body weight in female and male tank-reared broodstock over time. Standard errors were small and did not register on this graph.

Actual growth rate, and hence body weight at a given age, is dependent on a wide range of environmental factors, such as temperature, salinity and food availability, and hence the extrapolation of age vs growth data is only indicative. For example, broodstock collected at the same time of year, between mid-May and early June, have significantly different body weights depending on the location of capture, and presumably the area in which they grew up (Fig. 3.4). Therefore, the use of body weight data is only presented as one indication of age.

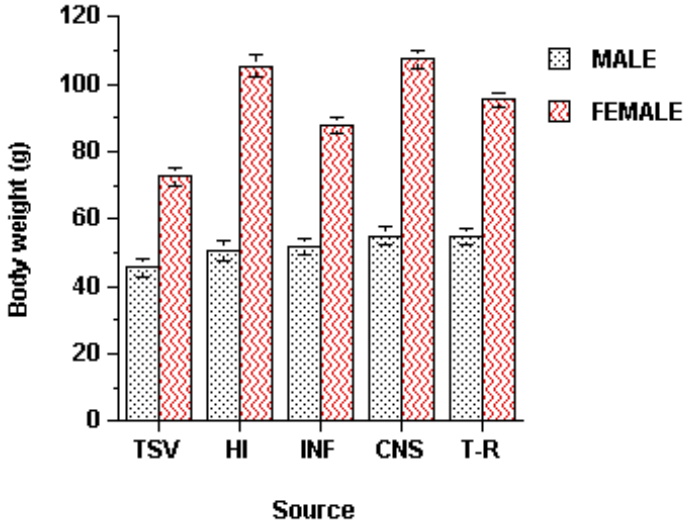

FIGURE 3.4. Differences in body-weight of wild and tank-reared broodstock collected at different locations at the same time of year. Source of prawns were, TSV = Townsville area, HI = Hitchenbrook Island area, INF = Innisfail area, CNS = Cairns area, T-R = tank-reared broodstock at AIMS.

PERFORMANCE OF WILD BROODSTOCK USING NATURAL MATINGS

Wild broodstock were supplied to the Australian Institute of Marine Science (AIMS) (19°16.3' S) within 2-3 days of capture. Indoor holding tanks at AIMS were circular, 4 m in diameter, with a capacity of 10,000 L with a bottom area of 12.6 m^2. Tanks were maintained on a flow-through system (2 x 10,000 L per day) and recirculating (6 x 10,000 L per day). The seawater was treated with ozone and ultraviolet sterilisation with each recirculating pass. Timer-controlled incandescent lamps illuminated the tanks, with an average intensity of 2 lux, for 14.5 hours per 24 hours. Water temperature and salinity varied between 28°- 31°C and 33 - 35 ppt, respectively.

Prawns were fed twice daily at a rate of 5-10% of total broodstock biomass per tank per day. The first feed in the morning was a mixture of whole fresh- frozen mollusc. The afternoon feed was a proprietary maturation diet that was a minced paté of molluscs, vitamin and mineral mix, ß-carotene, ß-glucan, cholesterol, fish oil and binders.

Each new batch of broodstock contained female broodstock at various stages of ovarian development. After noting their ovarian condition each female was weighed, marked with an eye-tag identification ring and tagged on the carapace to determine the time of the following molts. Before being used in the experiments, female and male broodstock were housed together and acclimatized in indoor tanks until they molted. Natural matings would occur around the time of female moult. Two days after molting the females were unilaterally eyestalk ablated to initiate ovarian development. At the time of molting the ovary is fully

regressed and therefore all broodstock used in the experiments started from the same reproductive state, regardless of their ovarian development at the time of capture. Their reproductive performance in the experiment should mainly reflect the captive environment and experimental design rather than their condition due to the natural environment.

Females were monitored for ovarian growth on a daily basis in the afternoon. The portion of the ovary within the tail region of the female can be observed by illuminating this region by passing a bright underwater torch beam, attached to the end of a 2 metre long pole, along the side of the female. This measurement can be done without handling or disturbing the female. Ovarian development is ranked on a score from I to V as a gonadal index (G.I.) (Table 3.1) (see Background section for further information).

TABLE 3.1. Ranking scores used for the determination of female reproductive condition.

Gonadal Index	Stage of Development
I	No ovarian development
II	Early developing ovary
III	Mid- to late developing ovary
IV	Immediate pre-spawning
V	Post-spawning

When females had a G.I. of IV they were removed from the holding tank in the afternoon and placed individually into 125 L spawning tanks for the night. Most spawning occurred between 2300 and 0200 hours. The spawning tanks were maintained on a flow-through recirculating system, with aeration occurring in the central sump reservoir, with approximate turnover rates of 1 tank volume every 15 minutes. On the morning following spawning, each batch of eggs was harvested and screened to remove excess debris and broodstock fecal material. A sub-sample of eggs was then collected to determine the total number of eggs spawned. The remaining eggs from each batch were placed in separate trays and incubated in a flow-through hatching system.

All the nauplii from each batch were automatically harvested. A sub-sample of nauplii was taken from each batch to estimate the total number of nauplii from each spawning. From this sub-sample the percent hatch rate and total naupliar output by each female was calculated.

PERFORMANCE OF POND-REARED BROODSTOCK USING ARTIFICIAL INSEMINATION.

As part of a domestication program, post-larvae were obtained from known matings of wild broodstock for the production of specific family lineages. The resultant post-larvae were grown to adulthood in small grow-out ponds on a commercial farm in north Queensland. Prawns were stocked in January in a 0.1 ha pond at an initial density of 0.8 prawns per m^2 and grown-out under normal practice. After almost 12 months in the ponds these prawns were harvested in mid-December and transported by road to AIMS.

Upon arrival, males and females were held in separate tanks. All female broodstock had fully regressed ovaries with gonadal index (G.I.) scores of I. Each female prawn was weighed, marked with an eye-tag identification ring and tagged on the carapace to determine the time of the following molts. Before being used in the experiments, female and male broodstock were acclimatized in indoor tanks until they molted. Prawns were fed twice daily at a rate of 5-10% of total broodstock biomass per tank per day. The first feed in the morning was a mixture of whole fresh frozen mollusc. The afternoon feed was a propriety maturation diet that was a minced paté of molluscs, vitamin and mineral mix, cholesterol, fish oil and binders.

As a first test of reproductive performance and maturation diet, 130 pond-reared females were divided into two groups. One group of 52 females was fed the normal diet as described above and another group of 72 females was fed the same diet supplemented with bloodworms (*Marphysa sanguinea*). Males for the respective groups were held separately and given the base maturation diet.

The experiment was designed to examine the performance of pond-reared broodstock at 15 months of age. As this experiment was part of a domestication program, it required the production of post-larvae of known parentage. Matings were therefore performed using artificial insemination, for which a spermatophore was collected from a known male (Tave and Brown 1981, Sandifer et al. 1984, Lin and Ting, 1986). This was scheduled for each female 2 days after molting, when the exoskeleton had sufficiently hardened to allow handling. Immediately after artificial insemination, each female was unilaterally eyestalk-ablated to initiate ovarian development. At the time of molt the ovary is fully regressed and therefore all broodstock used in the experiments started from the same reproductive state. Following unilateral eyestalk ablation, each female was monitored for ovarian development. Each afternoon females that had reached an ovarian development of Stage IV were transferred to individual spawning tanks in preparation for spawning that evening. Egg and nauplii samples were collected as per the wild broodstock.

ISOLATION AND STAINING OF PRAWN EGG NUCLEI FOR FLOW CYTOMETRY

Isolation of the nuclei followed modified protocols from Darzynkiewicz *et al*. (1984). Initially non-ionic detergents were used to try and disaggregate the egg to release the nuclei. However, the shell membranes were not disrupted with detergents and physical disruption of the hatching envelope was used. Eggs were homogensied with a glass tissue grinder with 101.6 to 152.4 µm side clearance (1 ml working capacity) in 1 mL lysis buffer (3 mM magnesium chloride, 2 mM calcium chloride, 0.32 M sucrose, 3 mL 10 mM PIPES buffer, and 2 mL 0.2% Nonidet P40 per liter) at 4°C. The homogenate was centrifuged at 300 *g* at 4°C, the pellet was collected, resuspended in lysis buffer (as above but without Nonidet P40) and centrifuged. The resultant pellet was layered onto a 20% Percoll gradient in marine phosphate buffered saline (0.19 M sodium chloride, 2.68 M potassium chloride, 7.37 mM sodium dihydrogen orthophosphate and 1.15 mM potassium phosphate) and centrifuged at 200 *g* for 30 min at 4°C to pellet nuclei and hatching envelopes. The pellet was then re-suspended in 10 µl of RNAase (1 mg/ml) and left to stand for 20 min at room temperature. To this suspension 100 µL of acid solution (368 µL 0.2 M hydrochloric acid, 438 mg 0.15 M sodium chloride and 50 µL 0.1% Triton X-100 per 50 mL solution) was then added and left for 30 sec. at 4°C.

For fluorescence staining 1 ml of propidium iodide staining solution (75 µg/mL propidium iodide, 2 mM magnesium chloride, 3 mL 0.1M PIPES buffer and 50 µL 0.1% Triton X-100 per 50 mL) was added and the solution stored at 4°C for 20 minutes.

FLUORESCENCE MICROSCOPY AND FLOW CYTOMETRY

The DNA in isolated nuclei was observed using an Olympus Vanox fluorescent microscope using a reflected light fluorescence attachment with the green excitation range (dichroic mirror DM580; excitation filter BP545; barrier filter 0590). The nuclear DNA content was determined by means of a Becton-Dickinson FacScan flow cytometer. Fluorescence was excited using an argon-ion laser tuned to 488 nm. Data was collected and interpreted using Cell Quest on an Apple Mac processor.

Chicken erythrocytes, when stained and analyzed with the flow cytometer have a low coefficient of variation and are routinely used as a haploid cell standard. Chicken red blood cells were stained as per the isolated nuclei as a control. Samples were then either examined using fluorescent microscopy or analyzed with the flow cytometer.

STATISTICAL ANALYSIS

Results were analysed with a computer statistical program using analysis of variance (ANOVA) or analysis of covariance (ANCOVA) after checking normality and variance homogeneity, transformed by arcsine where necessary. Percentage data were converted to arcsine values prior to analysis but only untransformed data are presented. If the *F*-statistic indicated significance, analysis was followed by a Tukey test for unequal N post-hoc mean comparisons. When one or both tests failed, ANOVA on ranks (Krushall-Wallis) was applied, followed by the Student-Newman-Keuls test for significant difference. Fertility and the number of nauplii in relation to spawning order were analyzed by an ANCOVA because of missing data (Sokal and Rohlf 1981). Results are reported as mean and ± standard error (SE). Significance was preset at $P<0.05$.

Results

PERFORMANCE OF POND-REARED BROODSTOCK USING ARTIFICIAL INSEMINATION ON DIFFERENT DIETS.

There were no significant differences in the inter-molt period between the female broodstock maintained on a basal maturation diet and those supplemented with bloodworms (Table 3.2, Fig. 3.5). There was, however, a significant lengthening of the inter-molt period over time, averaging 16.4 days between the first and second molts compared to 18.7 days between the sixth and seventh molts.

TABLE 3.2. Number of pond-reared female broodstock artificially inseminated within each inter-molt period.

Crosses	Inter-molt Period					
	1st	2nd	3rd	4th	5th	6th
Basal Diet	60	58	56	53	40	6
Plus Worms	62	59	53	52	34	10

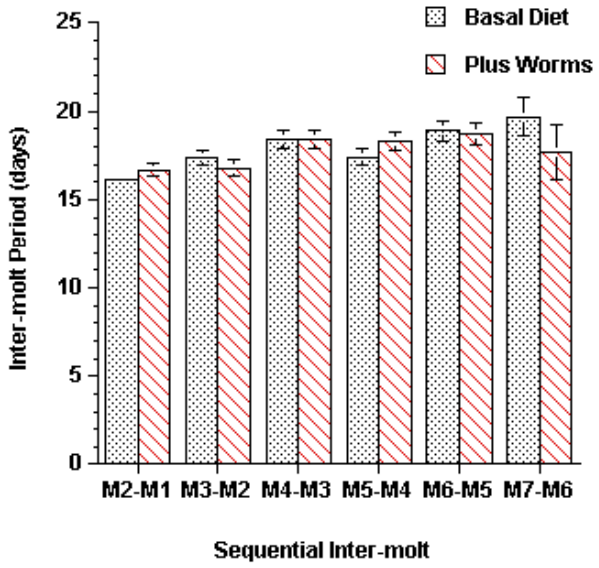

FIGURE 3.5. The inter-molt period through 7 molt cycles of the two groups of female pond-reared broodstock.

There was a progressive increase in the proportion of the broodstock population that spawned in each successive inter-molt period (Fig. 3.6).

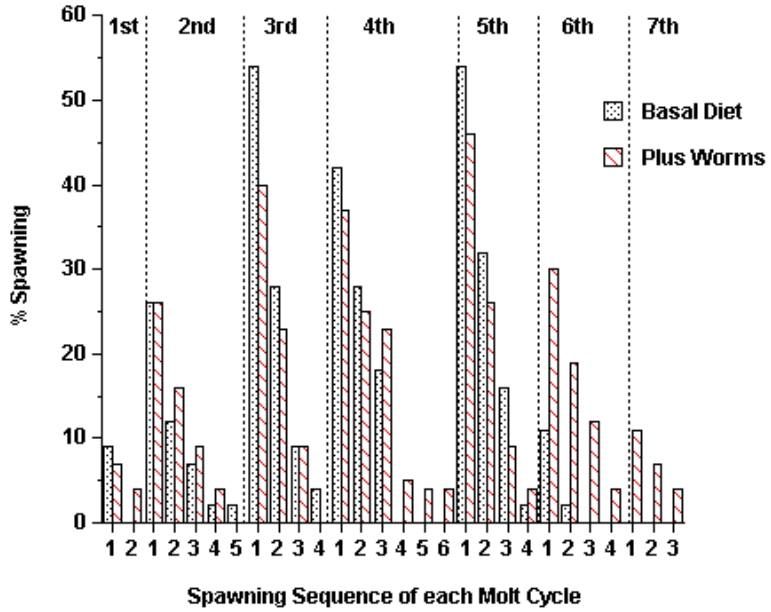

FIGURE 3.6. The percentage of the broodstock population spawning once, or more than once, during each of the seven inter-molts. The bars indicate the proportion spawning one, two, three, four, five or six times within an inter-moult.

Less than 10% of the population spawned in the first inter-molt period. The most productive periods spanned the third to fifth inter-molt period. Following the first spawning in each inter-molt period there was a progressive decrease in the proportion of subsequent spawnings within the population (Fig. 3.7). Nearly 90% of broodstock spawned at least once in the group fed the basal maturation diet whereas 62% did so in the diet supplemented with worms.

After the first spawning, there were insignificant differences between the groups. In both cases the number of broodstock spawning more than once decreased from 89% to 50% and 62% to 46% in the basal diet and that supplemented with worms, respectively. By the fifth inter-molt period less than 10% of the remaining broodstock population continued to spawn.

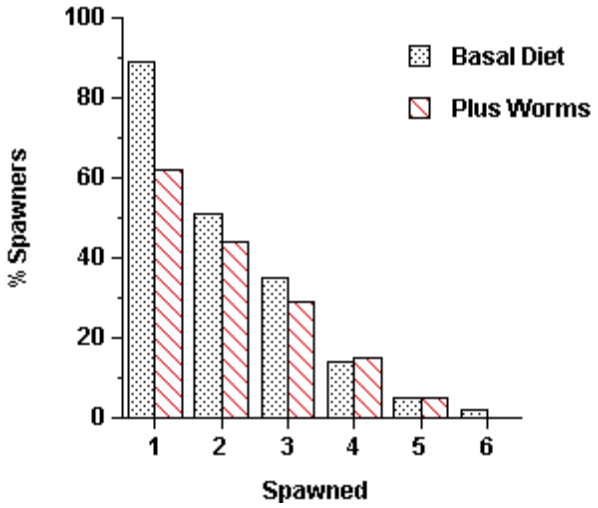

FIGURE 3.7. The percentage of the broodstock population that spawned between one and six times.

The majority of wild broodstock that are unilaterally eyestalk-ablated spawn within the first molt cycle. It is also common for cohorts of wild broodstock to exhibit varying degrees of ovarian development upon arrival at a hatchery. Despite this range most wild females spawn within a few days after unilateral eyestalk ablation. It is likely that the wild broodstock have well conditioned ovaries, having spawned several times before capture and shipment to hatcheries. In contrast, none of the pond-reared broodstock exhibited any signs of ovarian development when harvested. It is possible that none of these captive-reared females had ever gone through a complete reproductive cycle, with spawning, in the pond. This may be reflected in the percentage of pond-reared broodstock that did not successfully spawn until several molt cycles after unilateral eyestalk ablation (Fig. 3.8). Within the first molt cycle 10% or less of the broodstock spawned. In the second, third and fourth molt cycle, the proportion of the population that comprised first time spawners was 20%, 25% and 10%, respectively.

At the time of molt the ovary is in a regressed state. With each new molt cycle the female must be re-inseminated to produce fertilized eggs during that inter-molt period. One indication of reproductive maturity and/or performance is the time required from molt to spawning. In the pond-reared broodstock used in this experiment it required 11 to 13 days to spawn from the time of molt, or 9 to 11 days from the time of unilateral eyestalk ablation for the basal maturation diet and that supplemented with worms, respectively (Fig. 3.9). This period decreased to 6.4 and 8.2 days in the second molt second, followed by 7.0 and 7.2 days in the third and 5.6 and 4.9 days in the fourth molt cycle. This could indicate that the female broodstock were breeding for the first time and offers one possible explanation why captive reared broodstock of *P. monodon* are reported to be inferior to their wild conspecifics. That is to say, wild broodstock may have already gone through many successful reproductive cycles before being captured, and hence are more reproductively competent, in contrast to captive-reared broodstock, which may only be going through their first reproductive cycle 'forced' by the unilateral eyestalk ablation.

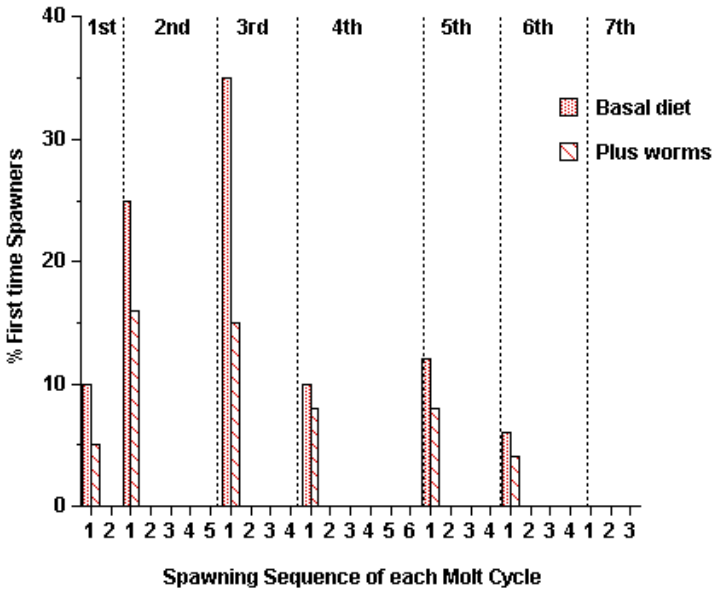

FIGURE 3.8. The percentage of the broodstock population that spawned for the first time within a specific inter-molt period.

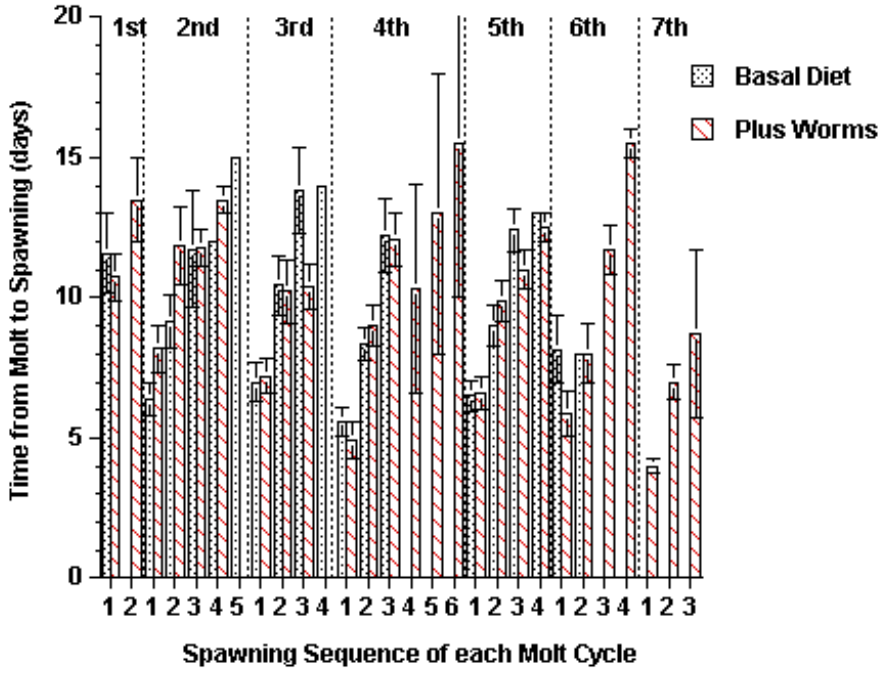

FIGURE 3.9. The time between sequential molts and spawning for pond-reared broodstock.

Further evidence of the immaturity of the pond-reared broodstock is also demonstrated with the increasing improvement of egg production per successive spawning and molt cycle (Fig. 3.10). In those females that spawned once within the first molt cycle, egg production was only 67,200 and 156,000 eggs per spawning for the basal maturation diet and worm supplemented groups, respectively. Only few females spawned within the first molt cycle. Egg production per female did not reach maximal levels until the fourth and fifth molt cycles, when average egg production per spawning ranged between 326,000 and 393,958 eggs.

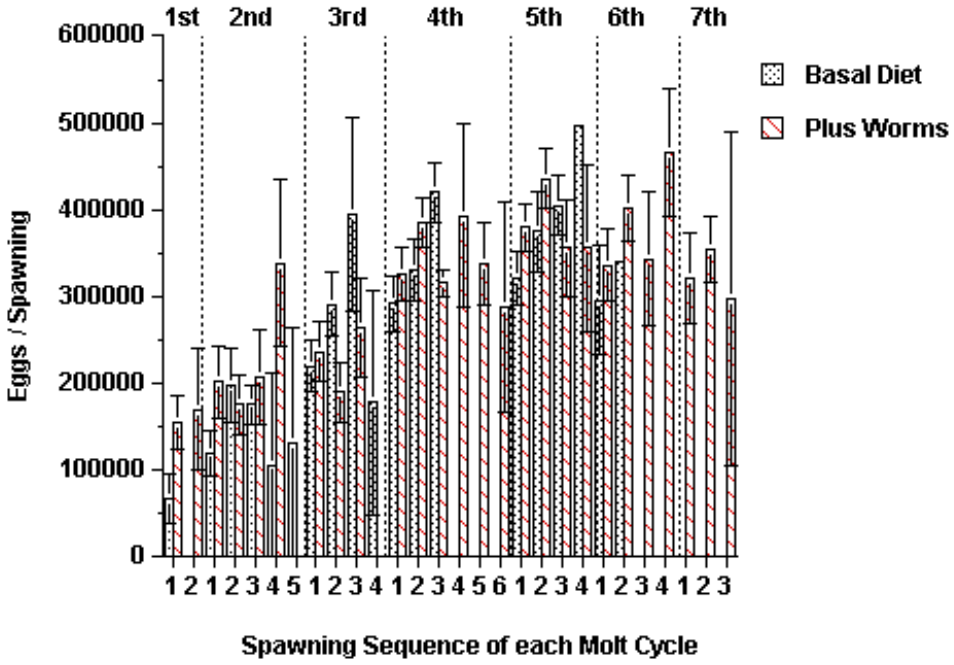

FIGURE 3.10. Eggs produced per spawning within each subsequent spawning of pond-reared broodstock through the seven inter-molt periods.

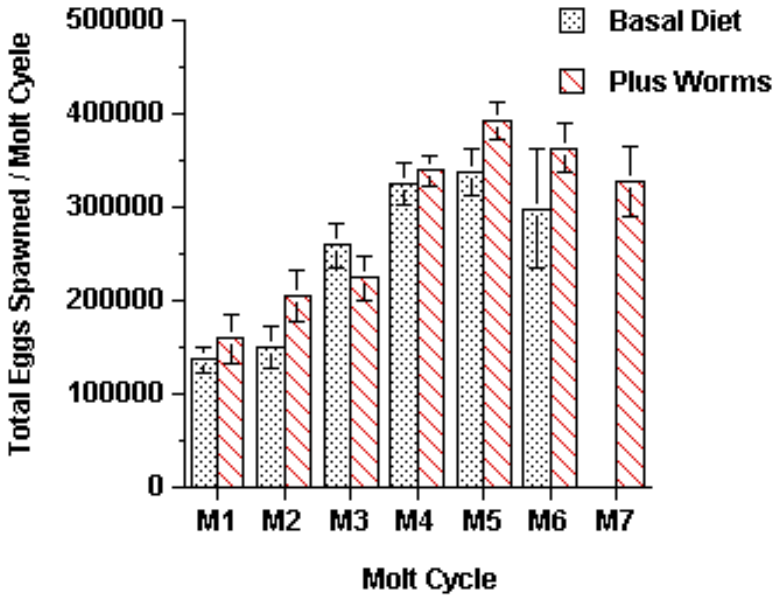

FIGURE 3.11. Average number of eggs produced per spawning per pond-reared female within each molt cycle.

The trend in progressive egg production per molt cycle is perhaps best illustrated by averaging egg production per spawning for all spawnings within a molt cycle (Fig. 3.11).

As another indicator of progressively increasing reproductive competence, the percentage hatch improved over time (Figs. 3.12 and 3.13). However, overall the hatch rate was very poor, being only 14% for animals fed the basal maturation diet and 8% for those fed the diet supplemented with bloodworms. A significant decrease in the hatching rate occurred in the fourth molt cycle compared with the third and fifth. Considering that egg production per female progressively increased with each molt cycle, the very low hatch rate was probably due to poor fertilization.

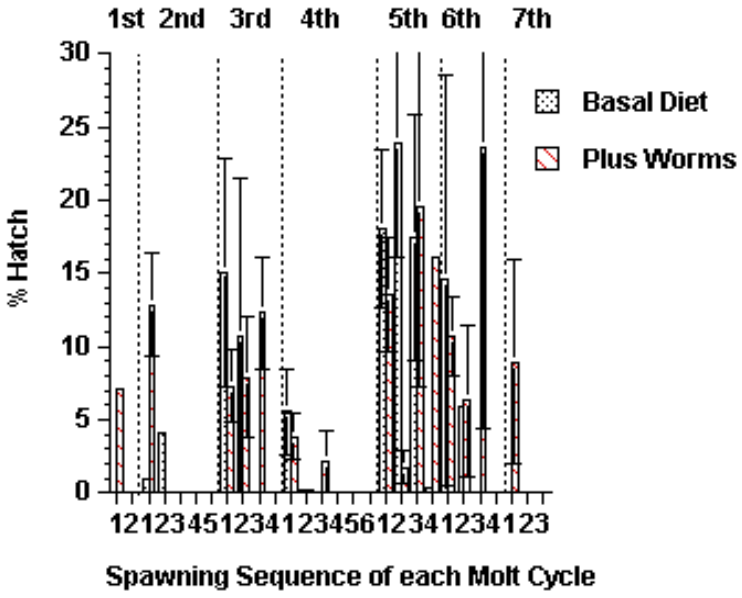

FIGURE 3.12. Percentage hatch of each subsequent spawning of pond-reared broodstock through the seven inter-molt periods.

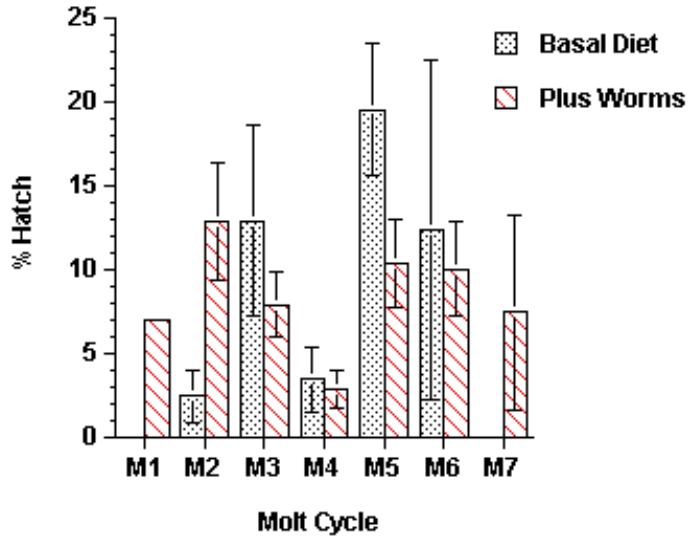

FIGURE 3.13. Percentage hatch of all spawnings for pond-reared broodstock within each molt cycle.

Nauplii production per spawning within successive molt cycles also reflected the poor hatch rate (Figs. 3.14 and 3.15). The same trend was observed, with a progressive increase in nauplii production with each molt cycle.

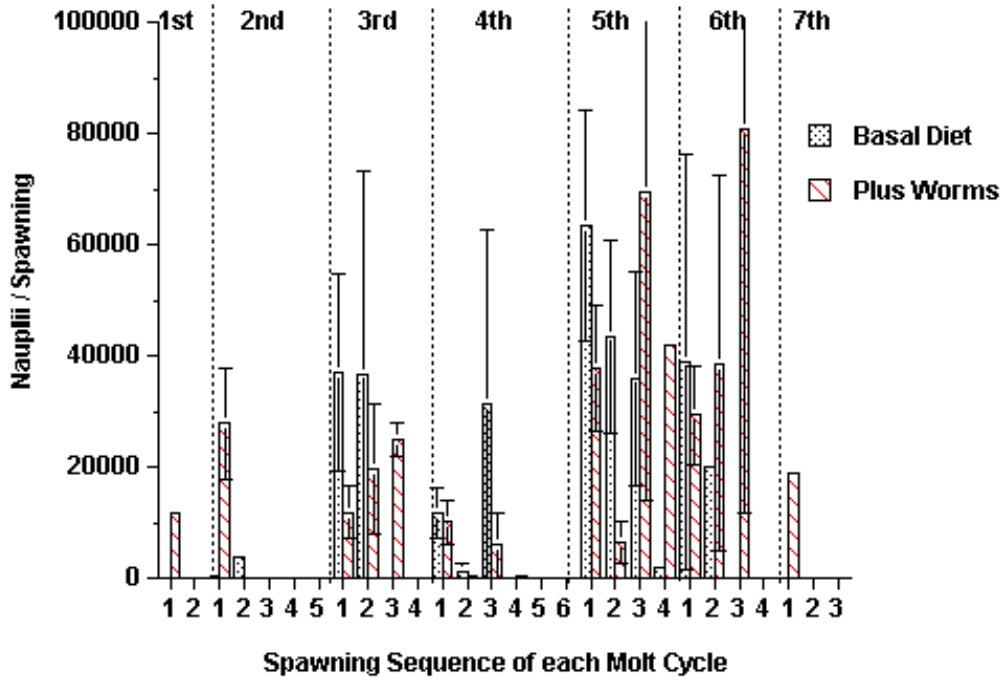

FIGURE 3.14. Nauplii production of each subsequent spawning by pond-reared broodstock through the seven inter-molt periods.

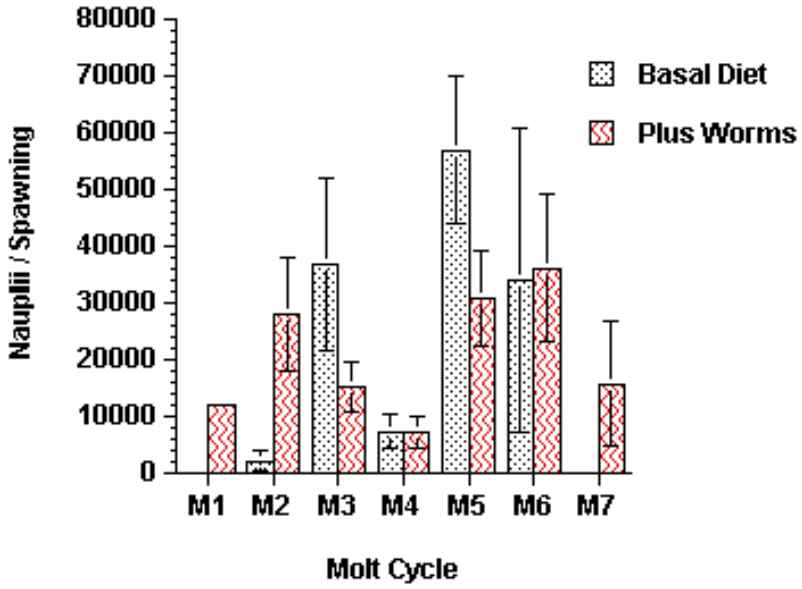

FIGURE 3.15. Nauplii production in each molt cycle.

COMPARISON BETWEEN POND-REARED AND WILD- CAUGHT BROODSTOCK USING ARTIFICIAL INSEMINATION

The results from the first experiment on pond-reared broodstock indicated that the females may have been 'virgin' females, which may require several ovarian cycles before egg production per spawning approaches that of wild caught females. Moreover, the very poor hatch rate indicated either poor egg quality, due to the female, or poor fertilization, due to the male. To test this, all possible crosses between wild and pond-reared females and males were undertaken (Table 3.3). Pond-reared adult *P. monodon* were collected from a commercial farm from ponds that were used to grow-out *P. japonicus*. The origin of the *P. monodon* was believed to be from natural wild *P. monodon* post-larvae that had been pumped into the ponds at the time of *P. japonicus* stocking. The age of the *P. monodon* was unknown but was believed to be approximately 1 year.

Broodstock were collected in early May and held and processed as described in Methods. However, females were only held through three molt cycles after eyestalk ablation. Artificial insemination (AI) was performed on 285 crosses between wild and pond-reared broodstock (Table 3.3).

There was a significant difference in the inter-molt period between pond-reared and wild female broodstock (Fig. 3.16). It was shorter in the pond-reared females, being 15.3 and 13.9 days between the first and second molt and the second and third molt, compared to the wild females that had inter-molt periods of 16.6 and 17.4 days, respectively. This is unusual, as the inter-molt period is generally related to the size of the prawn. In this experiment the body weight of the pond-reared females, 112.1±3.6 g, was significantly greater than the wild females, 88.9±1.3 g.

TABLE 3.3. Number of mating crosses made using artificial insemination in groups of pond-reared or wild *P. monodon* broodstock. F x M = female x male cross. P = pond-reared, W = wild-caught.

Cross	AI's			
Female x Male	**1st Molt**	**2nd Molt**	**3rd Molt**	**Total**
P x P	29	19	11	58
P x W	39	9	3	51
W x P	57	37	2	96
W x W	39	38	3	80
Total	164	103	18	285

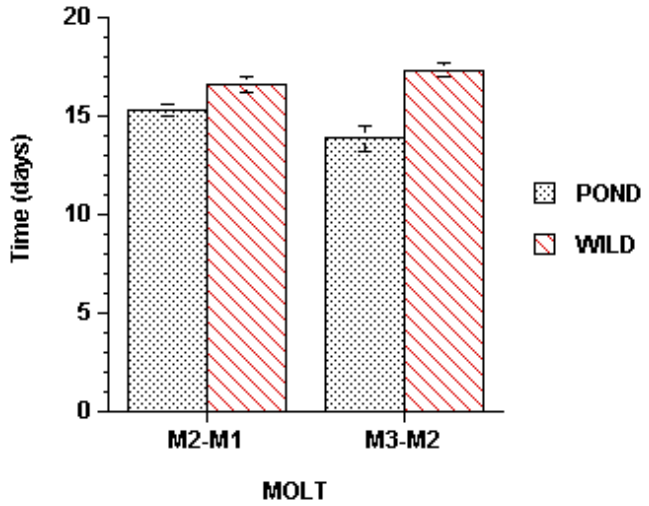

FIGURE 3.16. The inter-molt period between pond-reared and wild female broodstock.

Although the pond-reared females were larger than their wild conspecifics, they had highly significantly lower spawning rates in the population (P<0.001) (Table 3.4). A similar pattern of increasing reproductive competence occurred with each progressive molt cycle as in the first experiment in pond-reared broodstock. Surprisingly, there were significantly more spawnings in the female:male pond x pond crosses compared to the pond x wild ones. The reason why there should be a difference between female pond-reared spawning successes when crossed with pond-reared males compared to wild conspecifics is unknown.

TABLE 3.4. Percentage of female broodstock population that spawned within each inter-molt period for the various crosses of pond-reared or wild *P. monodon* broodstock.

Cross				
Female x Male	1st Molt	2nd Molt	3rd Molt	Total
P x P	3.4%	31.5%	36.3%	19%
P x W	7.7%	11.1%	0.0%	8%
W x P	67.0%	67.6%	100.0%	68%
W x W	100.0%	71.0%	71.0%	88%

Significantly more spawnings occurred in both of the wild female groups of crosses compared to the pond-reared females within all molt periods (Table 3.5). Whereas most wild female broodstock spawned two to three times within each molt cycle, all but one of the pond-reared females spawned only once per molt cycle, even though the pond-reared females were larger than their wild conspecifics (Fig. 3.17).

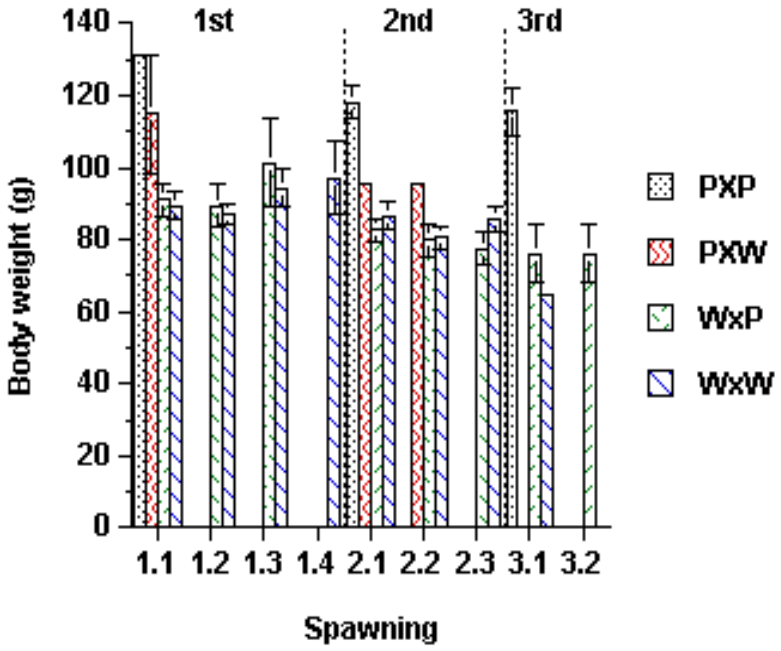

FIGURE 3.17. Body weights of the female broodstock that spawned. Number to the right of the decimal point represents the spawning number within the specific inter-molt period.

TABLE 3.5. Number of spawnings throughout each molt cycle in the various groups.

Cross	1st Molt				2nd Molt			3rd Molt	
F x M	1	2	3	4	1	2	3	1	2
P x P	1	0	0	0	6	0	0	4	0
P x W	2	0	0	0	1	1	0	0	0
W x P	38	18	7	0	25	12	3	2	2
W X W	39	27	10	4	29	14	7	1	0

The cohort of wild females collected in May and used in this experiment were small 88.9 ± 1.3 g and would not have been selected by commercial hatcheries. However, despite their small size, they would have already spawned in the wild during the summer/autumn spawning season after being flushed out of the estuaries to the near-shore spawning grounds in February and March. It again appeared that the pond-reared females may have been breeding for the first time, as the pond females required 11 and 13 days to spawn first, compared to only 5 and 6 days for the wild females (Fig. 3.18).

Egg production per spawning averaged 420,000 ± 31,370 eggs for pond x pond spawnings, compared to 336,575 ± 72,652 for pond x wild, 477,093 ± 22,959 for wild x pond and 480,652 ± 20,912 for wild x wild matings (Figure 3.19). Few spawnings occurred in the pond-reared females. However, in those that spawned more than once there was an increase in egg production with each spawning. This evidence again suggests that the pond-reared females were breeding for the first time. Nevertheless, the few pond-reared females that did spawn produced nearly the same number of eggs as did their wild conspecifics.

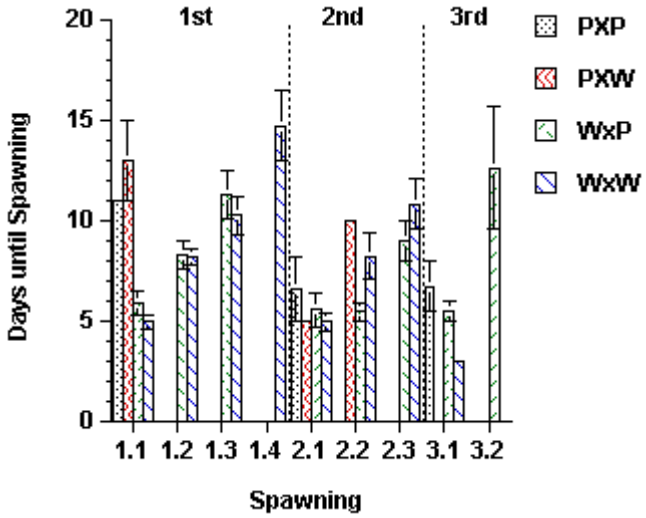

FIGURE 3.18. The time between the day of each molt and spawning in the various groups of spawners. The females were not ablated until 2 days after the first molt, hence the first period included is not directly comparable to the second and third molt period.

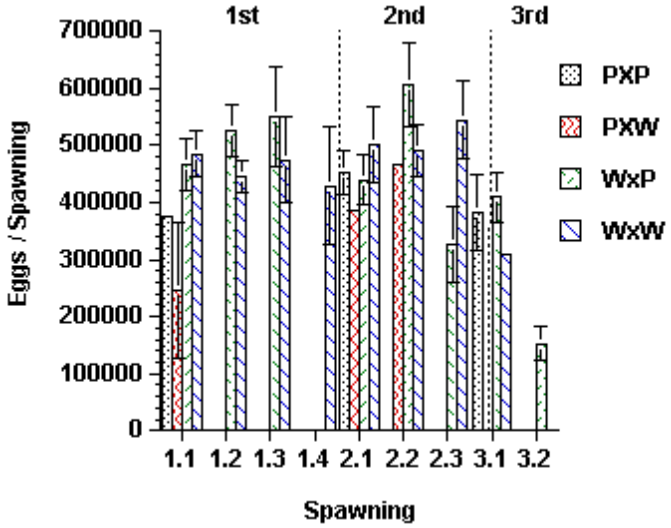

FIGURE 3.19. Number of eggs per female per spawning throughout the experiment.

Artificially inseminated females only received one spermatophore each time, compared to the normal complement of 2 spermatophores received during mating, which may partially account for the poor hatch rate for all spawnings (Fig. 3.20). The highest hatch rate was obtained in the wild x wild crosses, but only reach a maximum of 41% in the first spawning of the first molt cycle and averaged 30% for all spawnings in this group. The hatch rate for all spawnings in the wild x pond crosses was significantly lower at only 16.5% (P<0.01). Although only based on a few spawnings, the pond x wild crosses only produced an average hatch rate of 17%. Thus is possible that a combined female and male effect occurs. The wild males would be expected to have the same sperm quality whether they were crossed with wild or pond-reared females, and hence could potentially produce spawnings with the same hatch rate. Although little is known of the sperm maturation that occurs in penaeid females of closed thelycum species after the female is inseminated, it is known that the thelycum produces a 'copulatal fluid' which gives sperm full competence (Lin and Hanyu 1989). It is possible that the pond-reared females do not have full functionality of the thelycum, and this leads to inferior quality sperm.

Due to the low hatch rate, nauplii output was also low (Fig. 3.21). The highest nauplii production occurred in the wild x wild cross in the first spawning of the first molt cycle, with an output of 203,684 ± 28,461 nauplii per spawning. Nauplii output was under half that in the second spawning of the first molt cycle, at only 101,219 ± 21,906 and did not improve significantly in the first spawning of the second molt at 122,391 ± 23,182. Overall, nauplii output was only 71,918 ± 22,156 nauplii per spawning in the wild x pond crosses, 33,600 ± 16,271 in the pond x wild, and 55,200 ± 24,771 in the pond x pond crosses, respectively.

Data on reproductive performance of the wild x wild crosses in this experiment were sufficient to examine the relationship between female body size and egg production (Fig. 3.22). There was a highly significant increase in egg production per female with increasing size (P<0.0001).

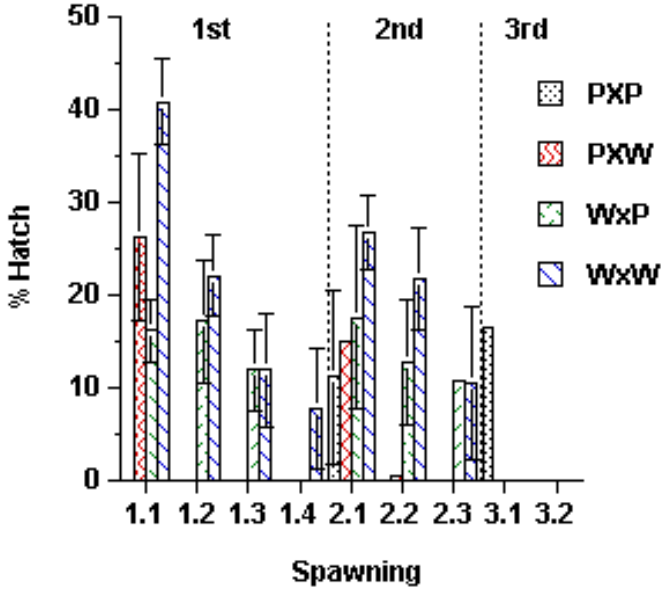

FIGURE 3.20. Percentage hatch of each spawning in the various groups.

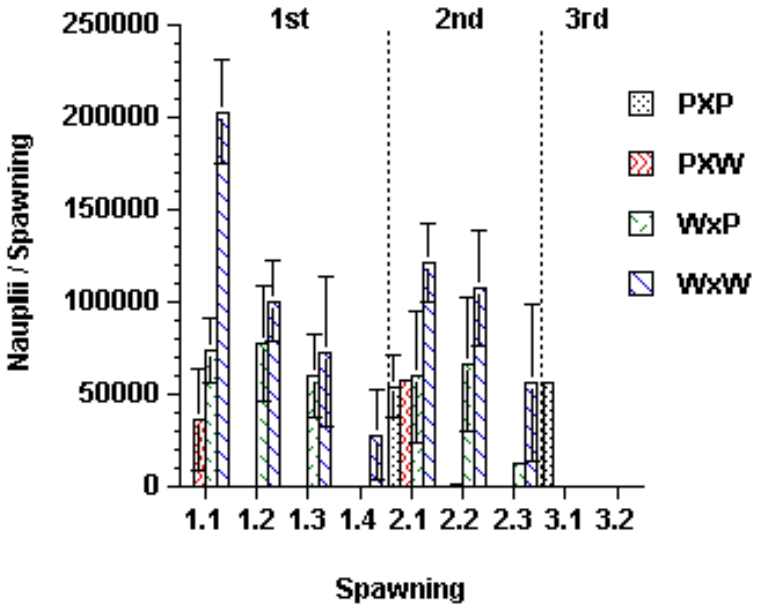

FIGURE 3.21. Nauplii production per spawning in the various groups.

It has previously been noted that female reproductive performance can deteriorate over time while held in captivity (Browdy 1992). In this experiment, however, there was no indication in a decrease of egg production per spawning associated with time held in captivity (Fig. 3.23).

There was, however, a significant decline in the hatching performance from wild x wild crosses over time held in captivity ($P<0.005$) (Fig. 3.24).

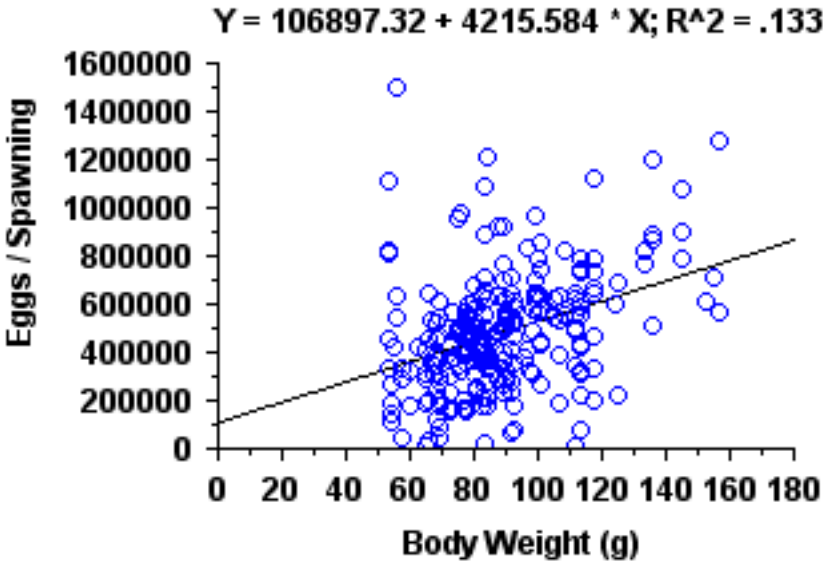

FIGURE 3.22. The relationship between egg production per spawning and female body weight of the wild spawners for wild x wild crosses. Linear regression formula shown at the top of the figure.

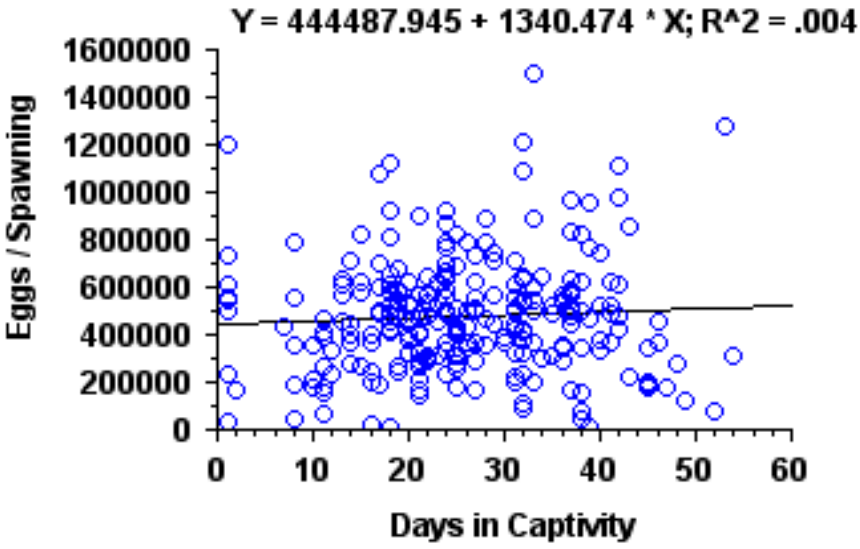

FIGURE 3.23. Changes in egg production per spawning in the wild females for wild x wild crosses over the 50 days they were held in captivity.

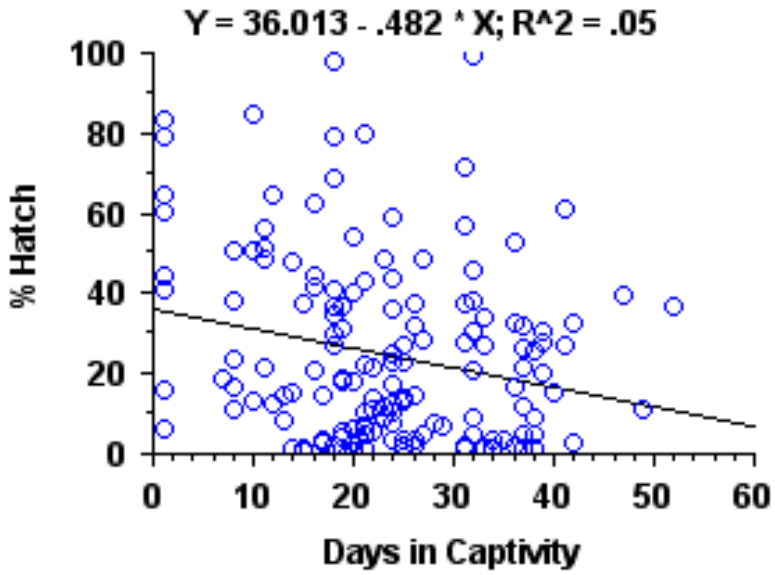

FIGURE 3.24. Changes in hatch rate in the wild females for wild x wild crosses over the 50 days they were held in captivity.

There was also a significant decline in the nauplii production per spawning over time held in captivity (P<0.02) (Fig. 3.25).

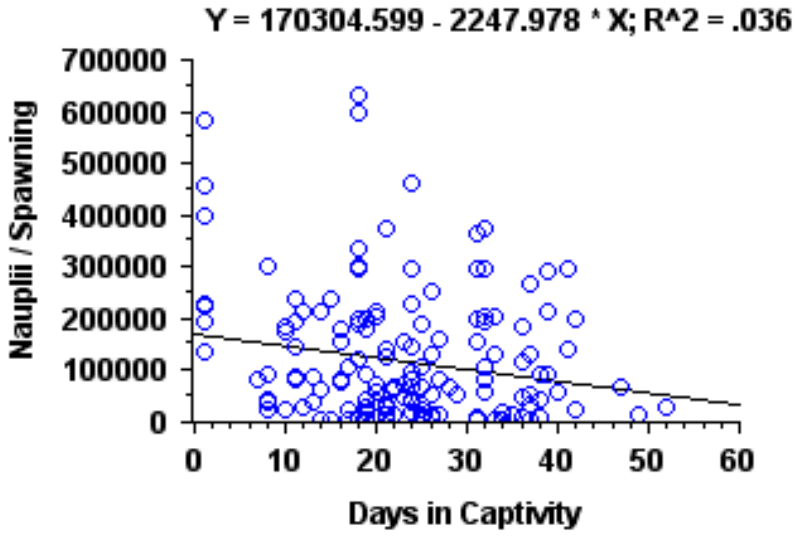

FIGURE 3.25. Changes in nauplii per spawning in the wild females from wild x wild crosses over the 50 days they were held in captivity.

DETERMINATION OF FERTILITY RATE

Variability in hatch rates necessitated the need to further understand each component of the process, from fertilization of the egg by the immotile spermatozoa immediately after spawning, to the normal development of the embryo through to the first naupliar stage and then to the developed nauplii hatching from the egg. If hatch rate alone is used as the

measure of spawning success, the underlying causes of poor hatch rates cannot be accurately identified.

Fertilisation rates are dependant on factors relating to the quality of both the sperm and eggs, their interaction over a short, crucial period of time, and numerous external factors relating to the environment in which spawning occurs. To differentiate causality of poor hatch rates we examined the use of flow cytometry as a tool to quantify fertility rates of egg batches.

Flow cytometry involves the examination of multiple physical characteristics of particles using a laser that illuminates individual particles, and selected characteristics of each particle are collected using photomultiplier tubes. Data are usually displayed using histograms and dot plot analysis. Dot plots show two characteristics such as the size of the particle, measured as the side scatter, plotted against another characteristic such as the amount of fluorescence emitted by the particle if stained with a specific fluorochrome.

To examine fertility, it is necessary to differentiate between ploidy states in the eggs. Fertilised eggs will be diploid, unfertilised eggs will be haploid. To examine this it is necessary to stain the cells with a fluorochrome that specifically stains DNA. A fluorochrome widely used in routine flow cytometry is propidium iodide, a membrane impermeant dye that intercalates with the nucleic acids of both DNA and RNA. Once bound to the nucleic acids, their fluorescence is enhanced 20 to 30 fold when excited by the laser of a flow cytometer. As each cell is excited by the laser, the amount of fluorescence emitted is proportional to the amount of DNA in the cell. Hence flow cytometry offers a rapid quantifiable technique of examining whether an egg is fertilised (diploid) or unfertilized (haploid).

The large size of the prawn eggs (250-330 μm diameter), and the impermeable hatching envelope which develops after fertilization, makes it impractical to pass eggs directly through the flow cytometer. Therefore, it is necessary to isolate the nuclei for ploidy determination. Nuclear isolation is a common method of quantitatively determining the DNA content of cells, differentiating cells that are undergoing a normal cell cycle from those that are aneuploid (variable amount of DNA).

All egg samples used were obtained from wild-caught *Penaeus monodon*. Samples were stored using a variety of methods to examine their effect on nuclei isolation and nuclei staining. Some samples were collected and snap frozen in liquid nitrogen prior to storage at -70°C. A second set were collected less than one hour after spawning, and immediately fixed in 5% formalin or 70% ethanol. The third set were collected less than one hour after spawning, stored temporarily at 4°C and immediately processed.

Repeated refining of the methodologies resulted in the isolation of the nuclei from surrounding tissues (Fig. 3.26a). Centrifugation steps to isolate the nuclei failed to separate all nuclei from disrupted hatching envelopes, and in some cases the nuclei remained within the envelope (Fig. 3.26b). RNAase treatment of the samples was effective in reducing background staining and nuclei stained intensely in comparison to the hatching envelope and other particles present in the preparation (Fig. 3.27a). However, the diameter of isolated nuclei was variable with little consistency in size in samples prepared. The shape of the nuclei also showed considerable variability with eccentric shapes being common in the preparations (Fig. 3.27b).

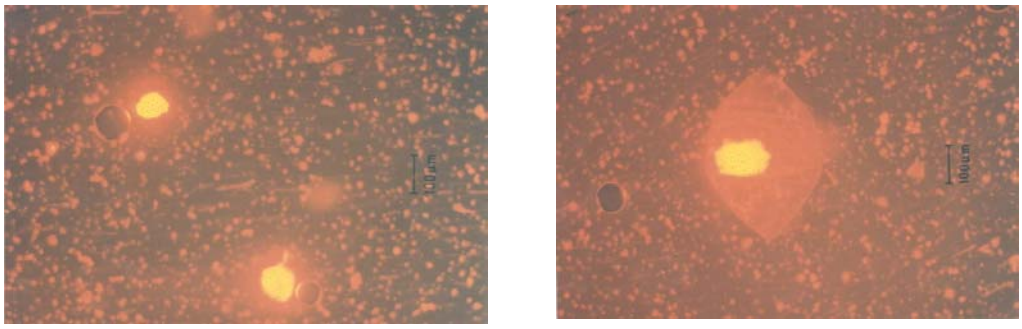

FIGURE 3.26A,B. a) Isolated nuclei from unfertilized eggs stained with propidium iodide for flow cytometry analysis (left), b) isolated nucleus with hatching envelope (right).

Various protocols were attempted to ensure the stability of the nuclear membrane. However, fresh or frozen samples showed similarly variable nuclei shapes. Of the different egg storage methodologies applied, the best results were obtained from fresh eggs or those snap frozen and stored at -70°C.

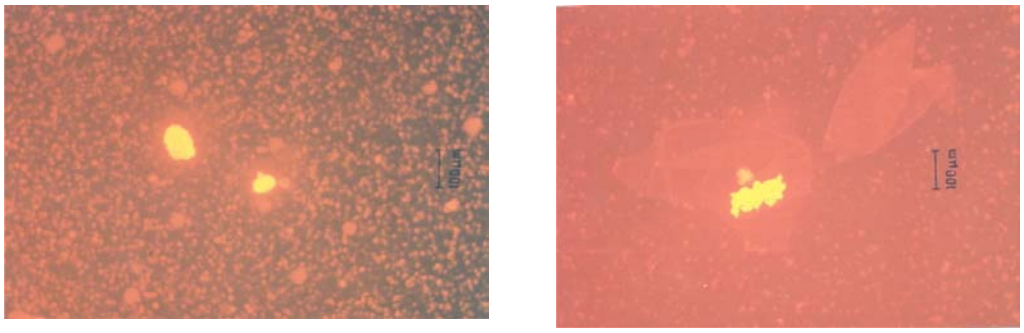

FIGURE 3.27A,B. a) Isolated nuclei free of egg envelope debris (left), b) example of variation in isolated nucleus shape and size (right).

Ethanol treatments failed to separate the nuclei from the contents of the disrupted egg and clumping of debris on the nuclei was common. The variability in the nuclei isolated was very high with large differences in size and in intensity of fluorescence. When these samples are compared to the chicken red blood cell haploid standard there is a vast difference. The chicken red blood cell nuclei are smaller and they fluoresce brighter than the nuclei isolated from the prawn eggs.

Analysis of the chicken red blood cells produced two to three populations of cells separated out by side scatter (Fig. 3.28a). The nuclei populations combined gave a reasonably low coefficient of variation as all cells emitted fluorescence at the same intensity. In contrast, unfertilised prawn egg nuclei preparations produced no clear population of haploid nuclei distinguishable in the dot plot (Fig. 3.28b). An important factor in flow cytometry is that the fluorescence must be intense and uniform for a quantitative signal to be produced. Compared to the chicken red blood cells, the nuclei from the prawn eggs stained above background particles but their intensity was much lower. The further to the left of the scattergraph, the more difficult it was to differentiate the population of nuclei from background noise. Additionally the nuclei isolated were much larger than those of the chicken red blood cells. The large size may have resulted in a weaker signal being produced (similar quantity of DNA dispersed over a greater area).

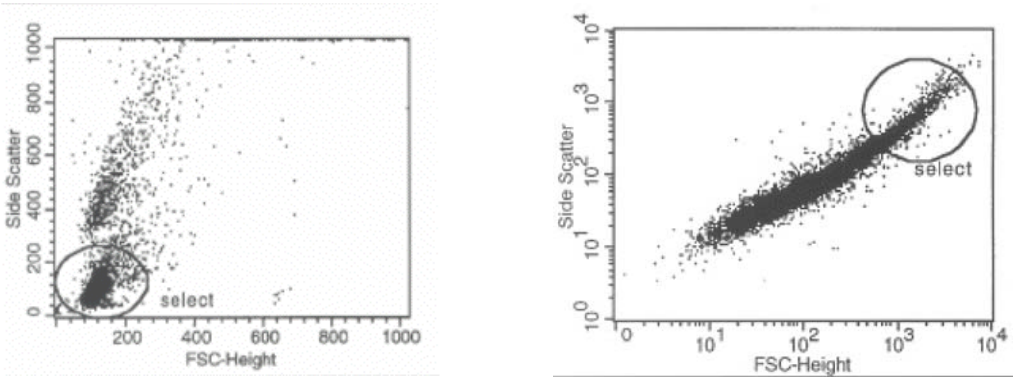

FIGURE 3.28A,B. a) Flow cytometric analysis of chicken red blood haploid cells stained with propidium iodide (left), b) unfertilized egg nuclei of *P. monodon* (right).

Although the use of flow cytometry may be advantageous in processing a large number of samples, it was not applicable to prawn eggs due to the variability in the state of the nucleus during high mitotic activity early on in the development of the prawns (cell division occurs independent of fertilization). Additionally the inability to differentiate the signal produced from the isolated nuclei from the background noise made the technique even more difficult.

The most affordable and appropriate method for a commercial hatchery to determine fertility rates would be by observation of eggs under a stereo low-power (40X) dissector microscope. Eggs are preferentially sampled 1.5 hours after spawning, but sampling after 2-3 hours is also possible to obtain accurate fertility rates. When eggs are collected for counting the morning following spawning, there is little agreement on estimates of fertility rate by different observers. This is because unfertilized eggs still divide several times and differentiating these from normal fertilized cell divisions can prove difficult many hours after spawning (Table 3.6). For further information see Hall MR, Young N, and Kenway M. Manual for the Determination of Egg Fertility in *Penaeus monodon* (http://www.aims.gov.au/pages/research.html).

If a permanent collection is desired, the eggs may be fixed and stored in seawater with 5% formalin. Eggs may be added to a Bogorov tray (a plastic tray with routed lanes used for counting plankton). However, any clear bottom container, such as a glass petri dish will suffice. One to two hundred eggs should be scored to obtain an accurate reflection of fertility rate. Use a microscope with illumination from below the sample. It is best to first make a total count of eggs in the container under low magnification. Afterwards, using a higher magnification, count the number of eggs that have a symmetrical cleavage pattern.

TABLE 3.6. Characteristics distinguishing fertilised from unfertilised eggs.

$$\text{Fertility rate} = \frac{\text{Number of eggs with symmetrical cleavage}}{\text{Total number of eggs sampled}} \times 100$$

Example: Three samples of eggs collected from a single spawning. Eggs collected 1 hour 15 minutes after spawning.

COUNT 1		COUNT 2		COUNT 3	
Total	Fertilised	Total	Fertilised	Total	Fertilised
385	235	430	270	597	385

Average total count	(385 + 430 + 597) / 3	=	471
Average fertilised eggs	(235 + 270 + 385) / 3	=	297
Fertility rate	297 / 471 x 100	=	63%

Characteristic	Fertilised Egg	Unfertilised Egg
Cleavage Pattern	Symmetrical Pattern	Asymmetrical Pattern
Post-1 hour development	2^{ND} – 3^{RD} Cleavage 4 – 8 Cell Stage	1^{ST} Cleavage Undivided or 2 Cell Stage
Other	Rapid Cell Division After 1^{st} Cleavage	Erratic cell division After 1^{st} Cleavage

INFLUENCE OF MALE SIZE ON OVERALL REPRODUCTIVE PERFORMANCE OF FEMALE BROODSTOCK

Female broodstock in the first experiments produced spawnings that averaged 300,000 to 400,000 eggs per spawning. However, few eggs actually hatched, and fewer still produced nauplii, and those that did only produced 30,000 to 40,000 nauplii. Such results could indicate poor performance of the males in their ability to produce viable sperm. However, the matings were also done by artificial insemination. Although artificial insemination gives complete control for the production of known male to female crosses, the technique might also result in a poor fertilization rate from otherwise good quality males. To examine some of these aspects, we examined whether male body size resulted in improved nauplii output per spawning. This experiment was done with natural matings to eliminate potential effects of the use of artificial insemination.

Wild broodstock were collected from the inshore breeding grounds in the Cairns to Innisfail region in May to June. Females of similar weight were selected and divided into two groups of 10-12 prawns each, termed the “little” and “big” groups according to which group of males with which they were mated. The average body weight of these groups was 138 ± 3.9 g and 139 ± 5.6 g. Males were also divided into 2 groups based on weight, a ‘little’ group of 63 ± 1.1 g and a ‘big’ group 81 ± 1.2 g (Figure 3.29).

The inter-molt period was 22.0 ± 1.15 days and 21.8 ± 1.63 days for females in the groups mated with the little and the big males respectively. The inter-molt period was not determined for the males as they were handled as infrequently as possible.

The time between molt and the first spawning in the first inter-molt period was 7.1 ± 1.0 and 8.8 ± 0.78 for the little- and big-mated groups respectively (Fig. 3.30).

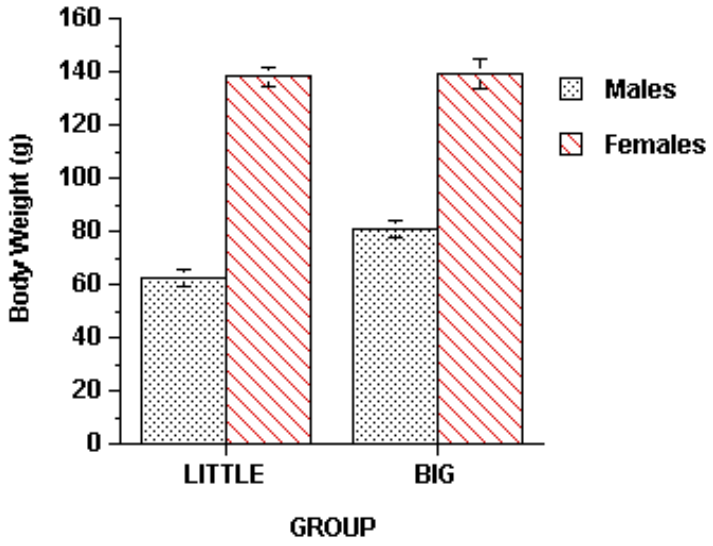

FIGURE 3.29. Body weight of males and females in the 'little' and 'big' groups.

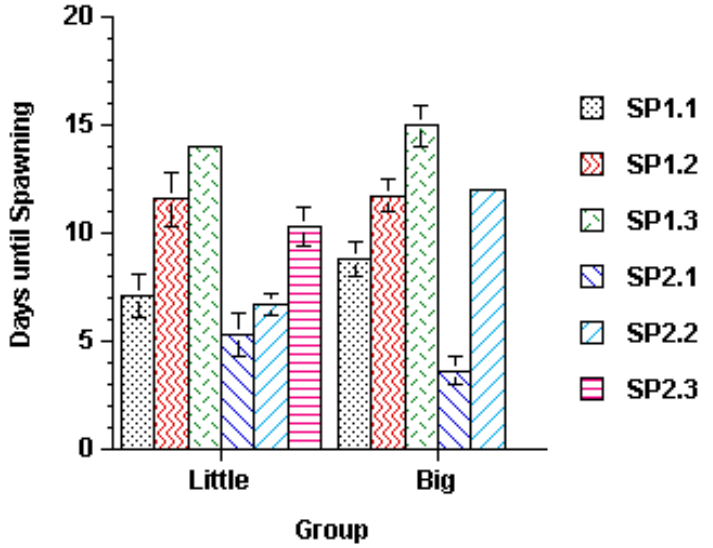

FIGURE 3.30. Time between molt and spawning for the two groups. SP1 and SP2 = 1st or 2nd inter-molt period.

The majority of females spawned at least once, with 89% and 60% of females spawning in the little and big group respectively (Fig. 3.31). Only about a quarter of the females continued to spawn more than once.

Egg production per spawning was reasonably poor, being 290,342 ± 70,058 and 258,600 ± 36,178 eggs per spawning for the first spawning in the first inter-molt period for the 'little' and 'big' group respectively (Fig. 3.32). Egg production remained relatively constant regardless of the number of spawns within a molt cycle, as well as for the first and second inter-molt period.

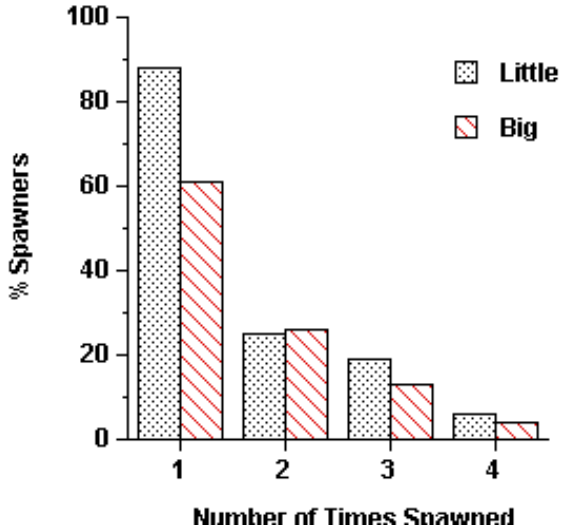

FIGURE 3.31. The percentage of spawners spawning once or more times during the experiment.

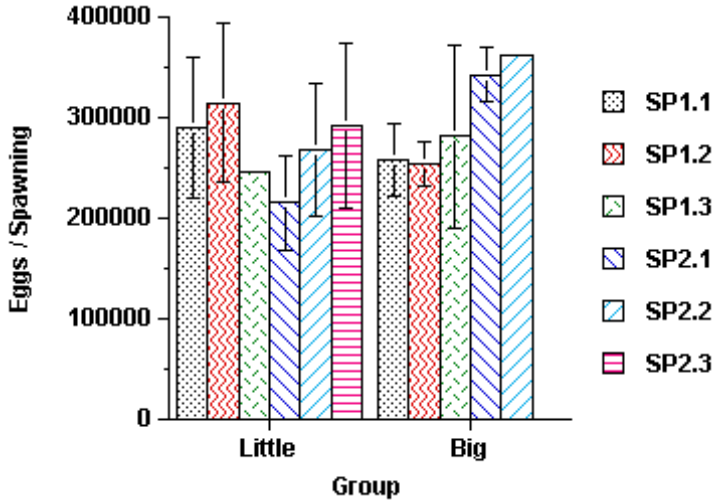

FIGURE 3.32. Eggs produced per spawning in both groups during the experiment. See Fig. 3.30 for nomenclature.

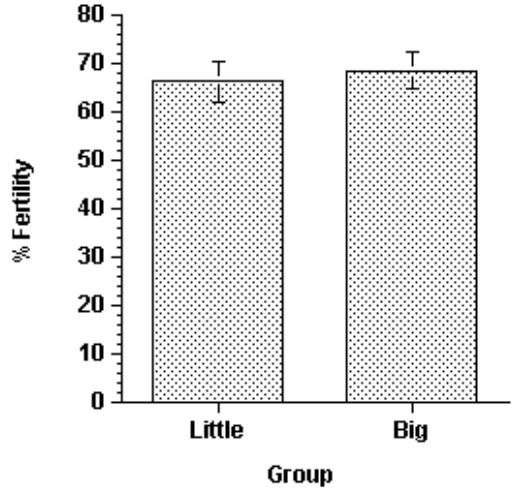

FIGURE 3.33. Percentage fertility between the groups.

A sub-sample of spawnings was examined for fertility, which was 68.6 ± 3.8 and 66.5 ± 4.1 percent in the little and big group respectively (Fig. 3.33). Less than half of these fertilized eggs actually hatched (Fig. 3.34).

There was no significant difference in the fertility or hatch rate between the groups. Overall the hatch rate was poor with an overall average of 28.9 ± 7.3 and 32.8 ± 6.7 percent in the little and big group respectively.

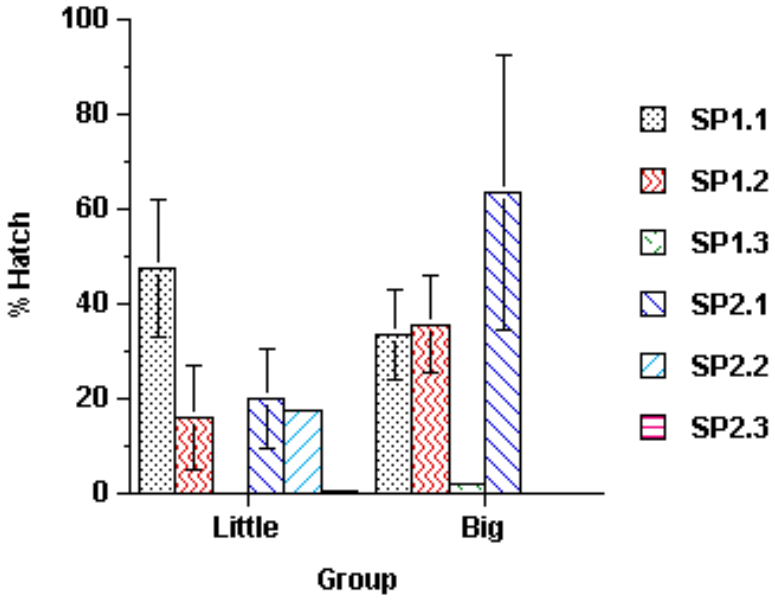

FIGURE 3.34. Hatch rate of the groups for the various spawnings. See Fig. 3.30 for nomenclature.

Nauplii output per spawning did not differ between the groups (Fig. 3.35) Overall, the average nauplii output for all spawnings was 96,462 ± 28,183 and 88,722 ± 20,404 for the little and big group respectively. This may have resulted from sourcing wild broodstock at the start of winter, when these animals become less active and cease spawning.

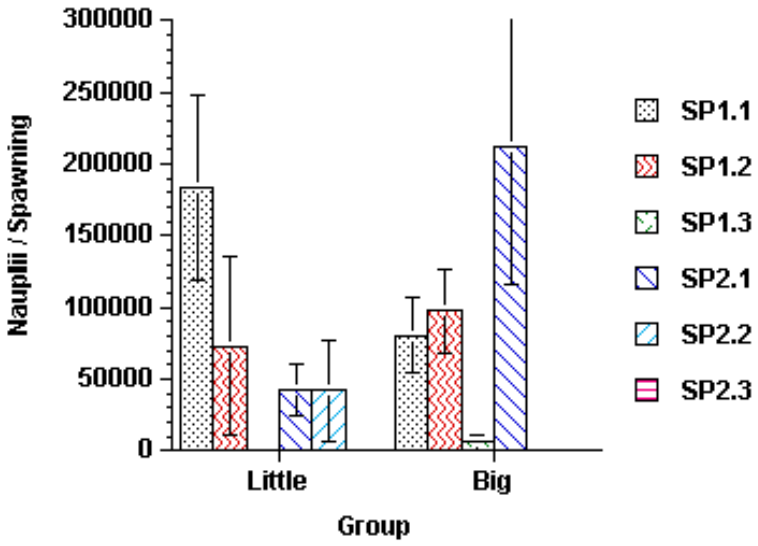

FIGURE 3.35. Nauplii production for each spawning of the groups. See Fig. 3.30 for nomenclature.

INFLUENCE OF MALE SPERMATOPHORE QUALITY ON REPRODUCTIVE PERFORMANCE

Results from the previous experiment established that there was no difference in reproductive performance due to male body size with respect to egg production, hatching success or nauplii production. An experiment was therefore designed to examine whether variation in nauplii production was due to spermatophore quality from wild or tank-reared males (Table 3.7). It has been reported that male quality is a major influence on final reproductive output and that tank-reared males are usually of inferior quality compared to wild captured males.

Wild broodstock were collected in early April. Tank-reared males were 16 months old. Broodstock were only held for one molt after unilateral eyestalk ablation and only the first spawning was measured. Artificial insemination was used throughout.

TABLE 3.7. The experimental design to examine the role of spermatophore origin in relation to final reproductive performance in female broodstock.

Group	Males N =	Females N =	Treatment
1	25 Wild, Used fresh	25	Wild caught males. No treatment. Using spermatophore that was formed under natural conditions.
2	25 Tank-reared, Fed Maturation diet	25	Tank-reared males. Males electro-ejaculated before placing into experimental holding tank.
3	30 Wild, Squid only diet	25	Wild caught males. Males electro-ejaculated upon arrival and fed on squid only.
4	30 Wild, Maturation diet	25	Wild caught males. Males electro-ejaculated upon arrival and fed on maturation diet.

TABLE 3.8. BODY WEIGHTS OF THE VARIOUS GROUPS.

Group	Mean body weight (g) ± SEM
1 - Females	86.2 ± 3.63
2 - Females	81.5 ± 3.63
3 - Females	95.5 ± 5.65
4 - Females	80.9 ± 5.00
Wild males	54.7 ± 4.44
Pond-reared males	53.9 ± 4.06

There was no significant difference between the groups for the time between artificial insemination and spawning, being 5.9 ± 0.97, 5.86 ± 0.64, 5.84 ± 0.68 and 5.29 ± 0.99 days for groups 1 to 4 respectively (Fig. 3.36).

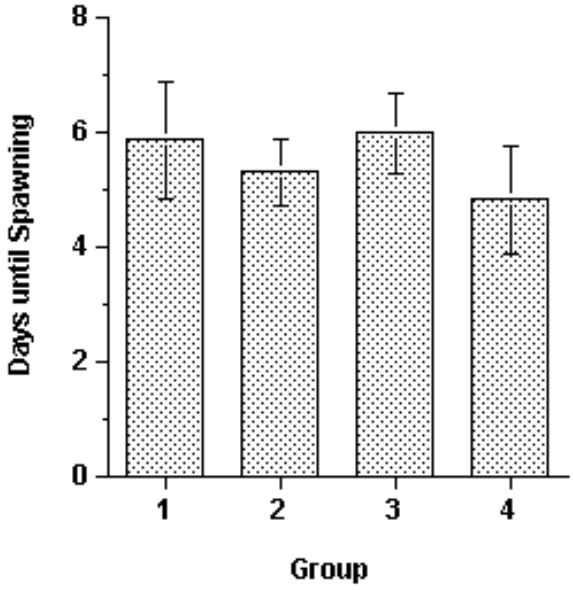

FIGURE 3.36. Days between artificial insemination (2 days post-molt) and spawning.

There was an apparent progressive increase in egg production per spawning in groups 1 to 4 being 202,900 ± 22,601, 229,184 ± 23,296, 279,000 ± 43,838 and 305,900 ± 68,493, respectively (Fig. 3.37). However, there were no significant differences between the groups.

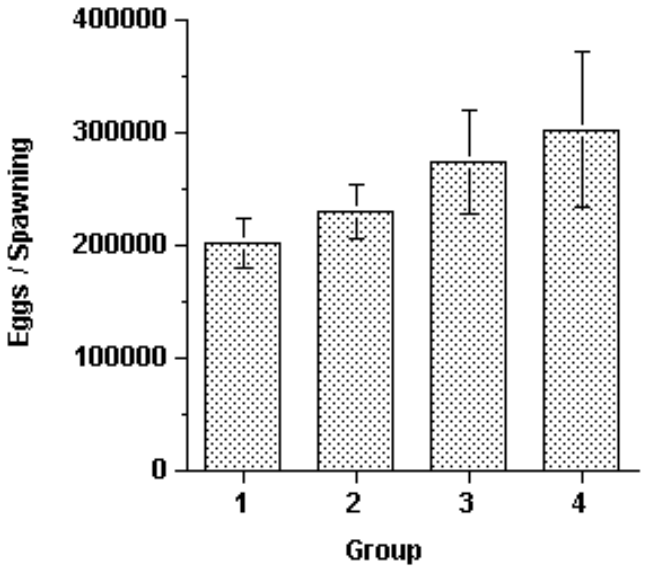

FIGURE 3.37. Egg production per female per spawning for the groups.

Although the percentage hatch was lowest in the matings with the tank-reared males (Group 2), being 18.4%, it was not significantly different from the other groups, which ranged from 27% to 35% (Fig. 3.38).

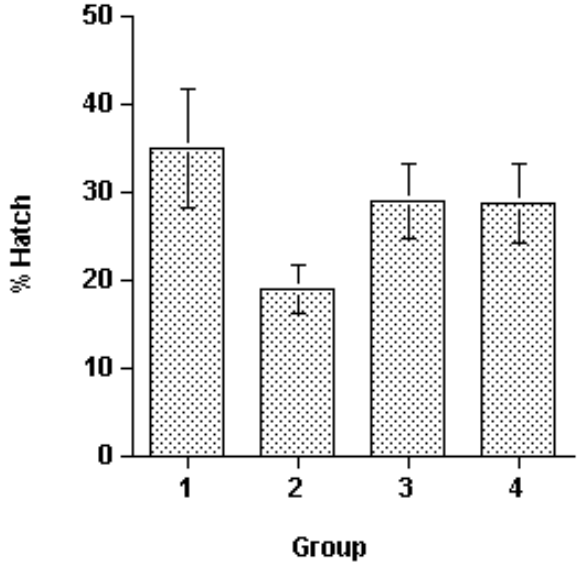

FIGURE 3.38. Percentage hatch for the spawnings in groups 1 to 4.

There was no significant difference in nauplii production per spawning between the groups (Fig. 3.39).

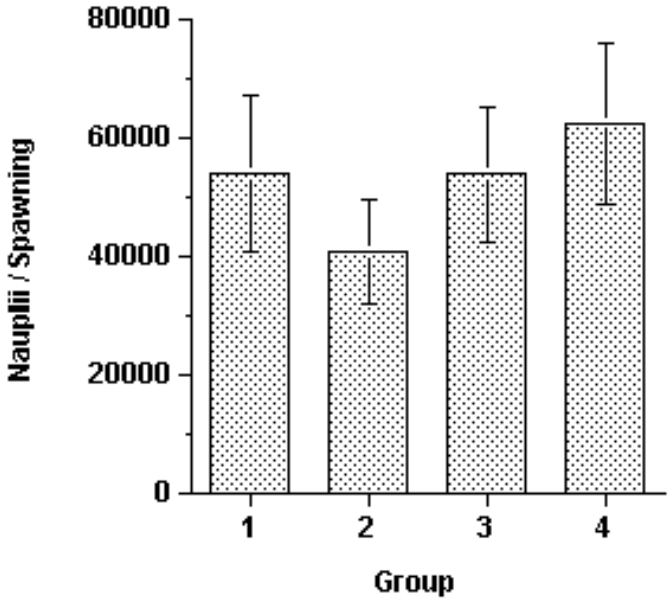

FIGURE 3.39. Nauplii production per spawning in groups 1 to 4.

Although not significantly different, there was variation in the average body mass of females between the 4 groups (see Table 3.8). Both egg and nauplii production per spawning per spawner was plotted on a per gram body weight of spawner to correct for any influence of female body size on reproductive performance (Figs. 3.40 and 3.41). In neither case were there any significant differences between the groups.

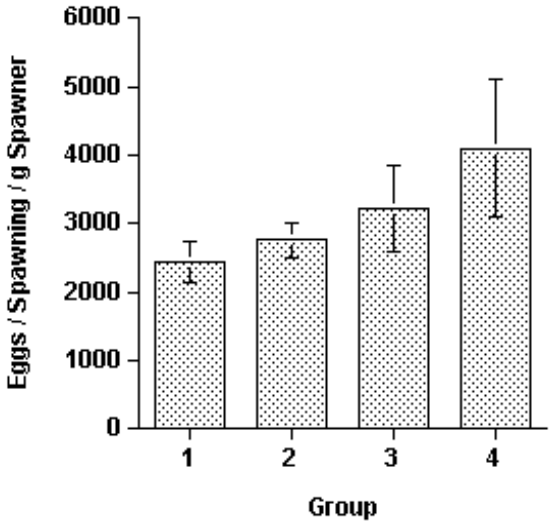

FIGURE 3.40. Reproductive performance plotted as eggs produced per spawning per gram of spawner body weight.

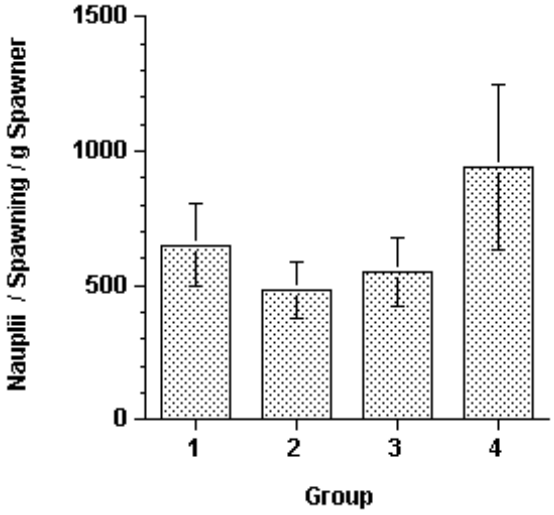

FIGURE 3.41. Reproductive performance plotted as nauplii produced per spawning per gram of spawner body weight.

This experiment used a total of a 100 wild female broodstock, each assessed over a single molt cycle. Some of these females were used within the first molt cycle upon arrival in captivity, and consequently were ablated within this initial molt cycle. Others could not be used until their second molt cycle in captivity and hence were only ablated after molting. Only the first spawning of the molt cycle in which the females were ablated was used in this experiment. To examine any change in reproductive performance due to time held in captivity, egg production per spawning was plotted against time in captivity (Fig. 3.42). Although there was a tendency for a decrease in egg production per first spawning, it was not significant (P=0.0502).

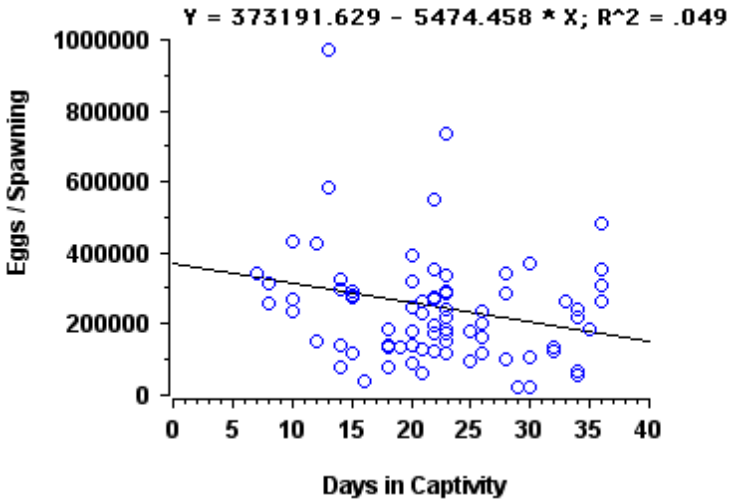

FIGURE 3.42. The linear regression of egg production per spawning against time held in captivity for all females.

There was also no significant decline in nauplii output with time in captivity. (Fig. 3.43).

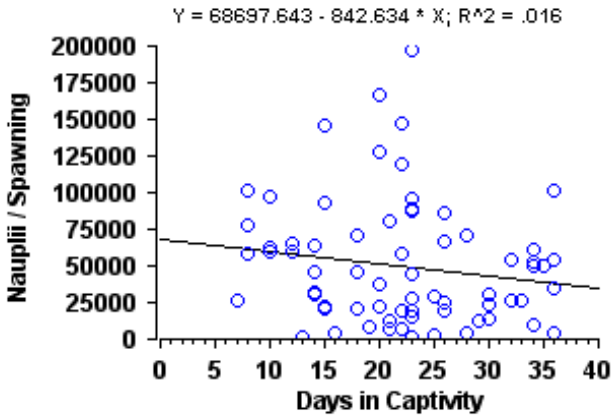

FIGURE 3.43. The linear regression of nauplii production per spawning against time held in captivity for all females.

COMPARISON OF TANK-REARED AND WILD BROODSTOCK BY NATURAL MATINGS.

This experiment was designed to make a direct comparison of the reproductive performance of tank-reared females and males compared to wild conspecifics by natural matings. Wild broodstock were collected in mid-May to June. Tank-reared broodstock were 12 months of age. Broodstock performance was measured through two complete molt cycles.

The inter-molt period was 18.1 ± 0.9 and 20.1 ± 0.7 days in the tank-reared and wild females respectively. There were no significant differences between body weights of the tank-reared (96.6 ± 4.5 g) and wild (88.6 ± 3.1 g) female broodstock (Fig. 3.44).

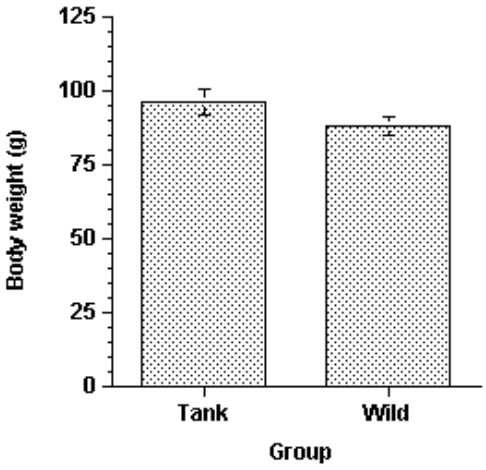

FIGURE 3.44. Body weights of the tank and wild female broodstock.

There was no significant difference in the time between molt and spawning in the tank-reared and wild female broodstock (Fig. 3.45).

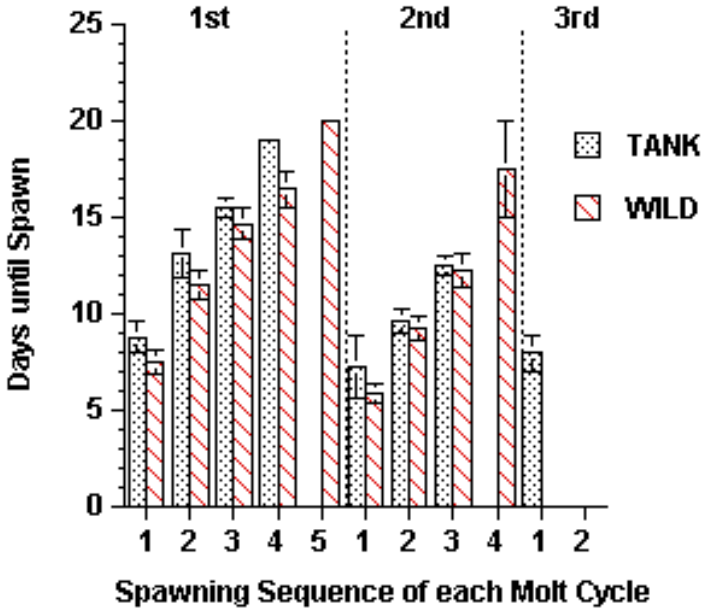

FIGURE 3.45. Days between molt and spawning of tank-reared and wild female broodstock.

A significantly higher proportion of the wild broodstock population spawned compared to the tank-reared females (Fig. 3.46).

Egg production per female per spawning was not significantly different between tank-reared and wild broodstock (Fig. 3.47).

Average egg production per female for all spawnings, however, was significantly greater in the wild females compared to the tank-reared ones ($P<0.0006$) (Fig. 3.48). Wild females produced an average of 236,174 eggs per spawning, averaged over all spawnings, compared to 152,708 for tank-reared females. The tank-reared females appeared to be reproductively exhausted sooner than their wild conspecifics as there were no significant differences between them in the first two spawnings of each molt cycle, but differences became apparent with third spawnings onwards (Fig. 3.47).

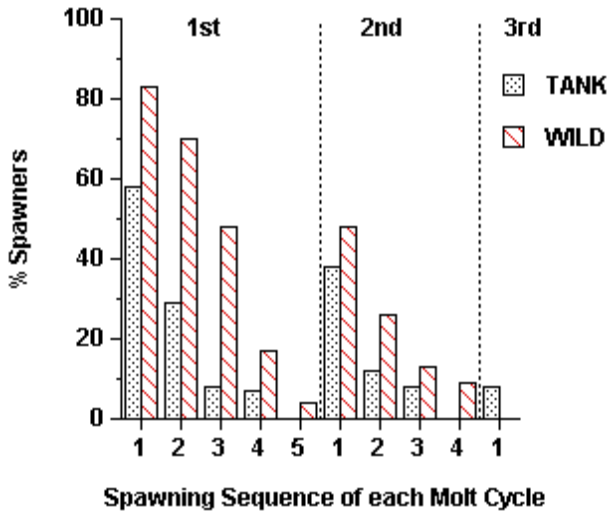

FIGURE 3.46. Percentage of the broodstock population that spawned through the molt cycles.

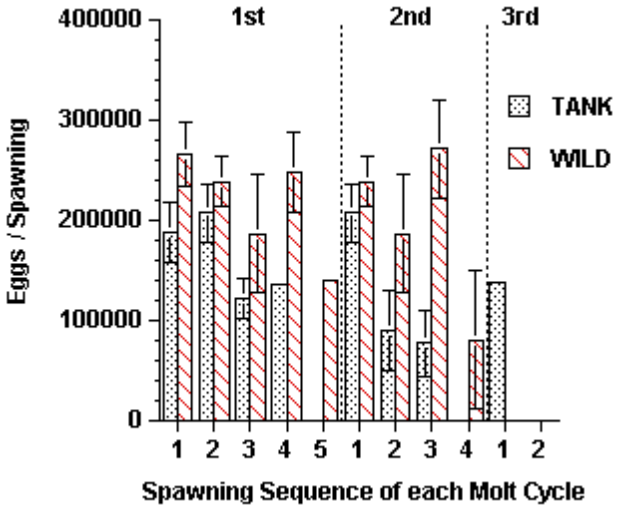

FIGURE 3.47. Egg production per spawning for the tank-reared and wild broodstock.

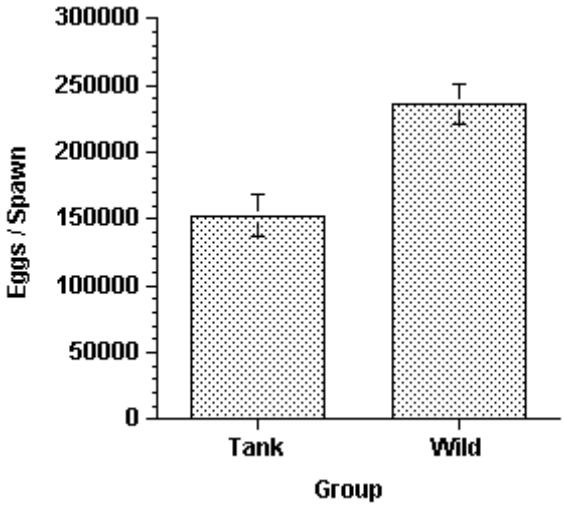

FIGURE 3.48. Average egg production for all spawnings within each group.

There was no significant difference in percentage hatching between tank-reared and wild broodstock in the first two spawnings of each molt cycle (Fig. 3.49). A significant difference was apparent, however, for the average hatch rate for all spawnings (P<0.009) (Fig. 3.50). The average hatch rate for the tank-reared females was 50.7% compared to 70% for their wild conspecifics.

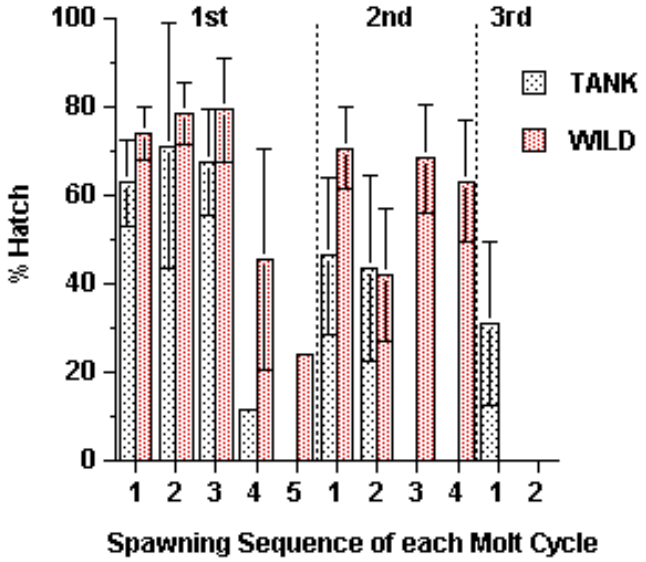

FIGURE 3.49. Percentage hatch for each spawning through the molt cycles for the tank-reared and wild broodstock.

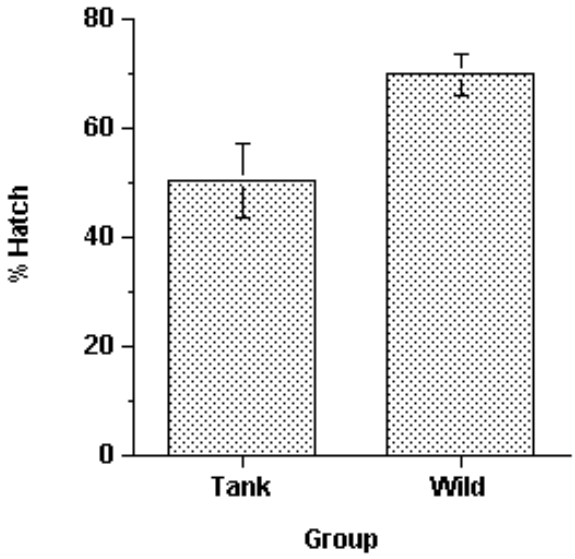

FIGURE 3.50. Average percent hatch for all spawnings within each group

Nauplii production was significantly less in the tank-reared broodstock for the first spawnings of each molt cycle (P<0.05) (Fig. 3.51). Nauplii production was 120,430 ± 22,855 and 45,867 ± 22,396, respectively, for the first spawning of the first and second molt cycle of the tank-reared broodstock. This compared to 206,947 ± 33,467 and 206,700 ± 29,167 for the wild broodstock. The tank-reared broodstock did not appear capable of maintaining a consistent production of nauplii. Their nauplii production was more than halved between the spawnings of the first and second molt cycle. There was also a highly significant difference for the average nauplii output per spawning in a molt cycle (P<0.0001) (Fig. 3.52). The tank-reared broodstock averaged 80,548 ± 13,762 nauplii compared to 177,833 ± 15,978 for their wild conspecifics.

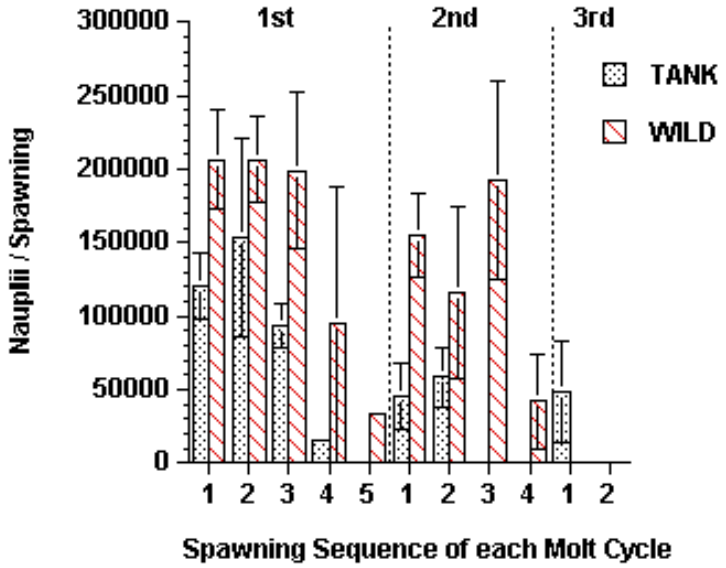

FIGURE 3.51. Nauplii output for each spawning through the molt cycles for the tank-reared and wild broodstock.

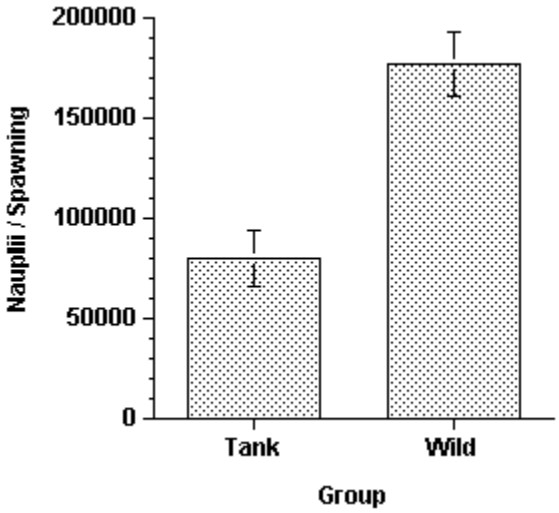

FIGURE 3.52. Average nauplii output for all spawnings within each group.

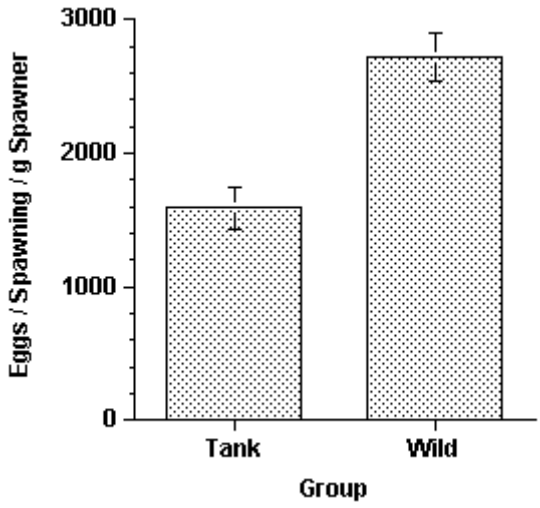

FIGURE 3.53. Average egg production for all spawnings expressed as per gram female body weight by each group.

The poorer reproductive performance of the tank-reared females was further accentuated when egg production was re-analysed on the basis of egg production per gram female body weight (Fig. 3.53). The average production for tank-reared females was 1,593 ± 156 eggs per gram spawner body weight compared to 2,718 ± 181 for their wild conspecifics, which was highly significant (P<0.001).

The same trend was apparent in nauplii output (Fig. 3.54). The tank-reared broodstock only produced an average of 846 ± 149 nauplii per gram spawner body weight compared to 2,068 ± 194 for their wild conspecifics (*P*<0.0001).

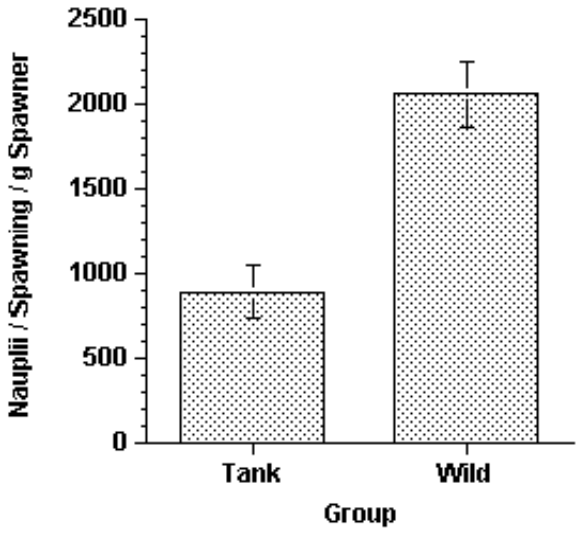

FIGURE 3.54. Average nauplii production for all spawnings expressed as per gram female body weight by each group.

Discussion

Comparison between wild and captive, either pond or tank, reared female broodstock indicates that the captive-reared females are capable of eventual equivalent egg production as their wild conspecifics. Direct comparison between wild and captive-reared broodstock may be misleading as the evidence suggests that captive reared broodstock are 'virgins' when first used in a hatchery. Wild broodstock have probably undergone several ovarian cycles before being captured and used in a hatchery and typically produce good spawnings of several hundred thousands of eggs per spawn. In those cases where tank-reared females produced an equivalent number of eggs per spawning, the females exhibited reproductive exhaustion within the first molt cycle.

The inferior performance of the captive-reared broodstock did not appear to be so much due to female reproductive performance but rather to poor sperm quality. Hatchability tended to be significantly lower in spawnings produced by matings with captive-reared males. The morphological appearance of the spermatophore following electro-ejaculation from captive-reared males was typically different from that of wild males (Fig. 3.55, Table 3.9). A normal spermatophore of penaeid prawns is composed of a sperm mass bundle and a wing structure. The sperm bundle is rich in organic substances necessary for sperm metabolism (Subramoniam 1991). The chemical composition of the gel bundle is composed of primarily neutral and acidic mucopolysaccharides (Hinsch 1991). The wing like structure is composed of a gelatinous protein that tends to swell and dissolve in water and may aid in the adhesion and attachment of the spermatophore in the thelycum. Within the sperm bundle are

packages of aflagellate and non-motile sperm (Fig. 3.56). The size of the sperm mass bundle was typically reduced in captive-reared males. In addition, either by gentle homogenization and microscopy analysis or by histological preparation, actual sperm numbers were greatly reduced in captive-reared males.

TABLE 3.9. Key morphological differences between spermatophores from wild caught and captive-reared male broodstock.

Parameter	Wild	Captive-reared
Size	-	Smaller than wild
Colour	Whitish	Opaque/Milky
Sperm bundles	Clearly visible through transparent envelope of spermatophore	Not as visible and generally fewer bundles
Spermatophore tails	Long, at least 3 times length of body of spermatophore	Short, usually less than length of body of spermatophore
Unravelling of the tail to form fans	Unravel to form fans within 3 minutes exposure to seawater	Tail too short to form fans

It is believed that the sperm bundle matrix provides energy-yielding substances for sperm maintenance during storage in the thelycum and may contain substances that protect against microbial infection during storage (Subramoniam 1991). As the insemination of the female is essential for successful reproduction, it is essential to understand the mechanisms of spermatophore quality, sperm storage and sperm release during fertilization (Bauer and Min 1993). The reason for poor fertilization by captive-reared males is not known. It could be due to a deficiency in actual sperm production or infection of the male reproductive tract (Talbot et al. 1989). This area requires further research to improve the performance of the captive-reared males to that of their wild counterparts.

FIGURE 3.55. Example of paired spermatophore morphology of wild (above) and captive-reared (below) male broodstock. The sperm are embedded within the main body of the spermatophore (cf Fig. 3.53). Note the tails on the spermatophore of wild males that unravel to form fans soon after exposure to seawater. These fans probably function to prevent additional matings after females are first inseminated. The spermatophores of captive-reared males generally have small tails that do not unravel to form fans after exposure to seawater.

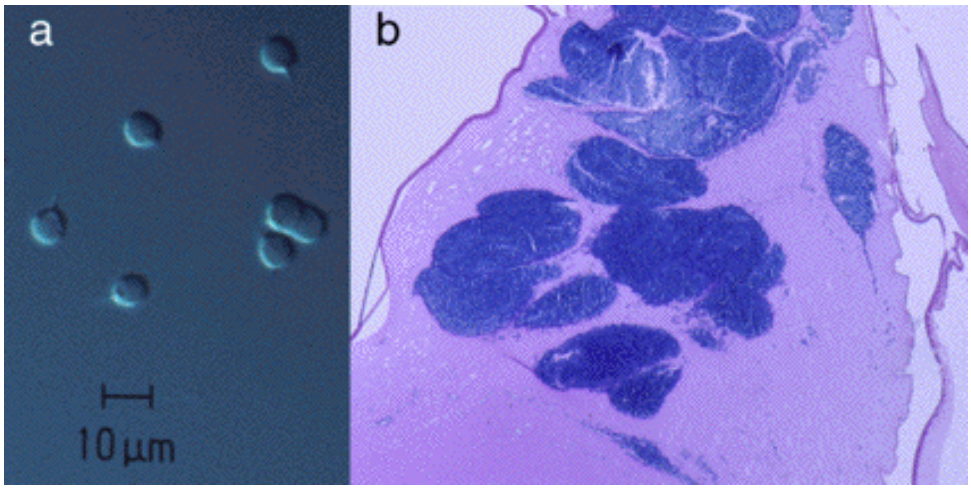

FIGURE 3.56. (a) Individual sperm cells of *P. monodon*. The sperm are completely non-motile and must make physical contact with the egg at the time of spawning with appropriate mixing to ensure contact with eggs. Magnification 400x. (b) Section of the spermatophore. The sperm cells appear darkly stained compare to the mucopolysaccharide spermatophore matrix in which the sperm packets are embedded. Magnification 40x.

Objective 4

To demonstrate value of supplementing broodstock diets with different concentrations of carotenoids

Introduction

Carotenoids are natural lipid soluble pigments and are nearly universally distributed in bacteria, plants and animals. However, carotenoids can only be produced de novo by some microbes and plants and hence prawns are dependent on an exogenous supply of carotenoids to meet nutritional requirements (Castillo et al. 1982, Kleppel 1988). Although not fully understood, carotenoids appear to be critical for general metabolism and maintenance of health. Current evidence indicates that carotenoids function as anti-oxidants, have provitamin A activity, leading to immune enhancement, and play a beneficial role in reproduction and egg development as well as general growth and survival (Miki 1991). There is an apparent relationship between low levels of carotenoids in tissues and onset of opportunistic pathogens, correlated with loss of resistance to disease (Meyers and Latscha 1997). However, the current main use of carotenoids in prawn aquaculture is purely cosmetic and is used in finishing diets, especially in *P. japonicus* farms, for colour enhancement of the final product. The 'blue disease' or 'blue colour syndrome' of farmed penaeid prawns is due to a nutritional deficiency in carotenoids. In *P. monodon* 'blue' individuals had a concentration of total carotenoid of 4 – 7 ppm, whereas normal coloured wild specimens had levels of 26 ppm in the shell (Howell and Mathews 1991, Menasveta et al. 1993). Although normal colouration can be obtained with dietary carotenoid supplementation, carotenoid deposition can be due to poor lipid nutrition.

At least 600 natural carotenoids have been identified and are responsible for a wide range of reds, yellows, blues greens, purples, browns and blacks in marine organisms (Latscha 1991). The predominate carotenoid in penaeid prawns is astaxanthin which can account for 86-98% of total carotenoid present in penaeid prawns (Katayama et al. 1972, Tanaka et al. 1976, Katagiri et al. 1987). Whereas the carotenoid astaxanthin in its free form is red, it is typically esterified to long chain fatty acids or complexed with proteins as carotenolipoproteins in the epidermal tissue or shell, and hence can produce a range of shell colouration (Zagalsky et al. 1990). Although the carotenoids are highly diverse, only a limited number of specific forms can be utilised by prawns (Meyers and Latcha 1997). Carotenoids can be products of catabolic degradation or anabolic oxidation, with the 40-carbon xanthophylls being the dominant form in crustaceans, with the bicyclic mono-, di-, and poly-forms of hydroxycarotenoids and ketocarotenoids being the most relevant (Latscha 1990). As food additives, the simple hydrocarbon beta-carotene and the hydroxycarotenoids, such as beta-cryptoxanthin, lutein and zeaxanthin are the most common (Liao et al. 1993). The most common form within prawns themselves are the further oxidised ketocarotenoids, with one, two or more carbonyl groups, and include echinenone, adonixanthin, phoenicopterone, adonirubin, canthaxanthin and astaxanthin (Tanaka et al. 1976).

Astaxanthin is the main end-product carotenoid in prawns and is found in the shell, blood, hepatopancreas, midgut, ovary and eggs. In *Penaeus japonicus* fed a range of dietary carotenoids, including beta-carotene, astaxanthin, and canthaxanthin, all were deposited in the tissue as astaxanthin esters, with astaxanthin being the most readily accumulated (Yamada et al. 1990, Negre-Sadargues et al. 1993). Similar studies in *P. monodon* revealed the same trend (Okada et al. 1994). Penaeid prawns have levels of total body carotenoids ranging between 80 to 200 mg/kg with large intraspecific differences of at much as 300%

(Meyers and Latscha 1997). In *P. monodon* the carapace may contain 86-98% of total carotenoids (Okada et al. 1994). Whereas carotenoid lost at the time of molting can be as high as 85%, it is more typical for approximately 30% to be lost at molting in *P. monodon* (Meyers and Latscha 1997). However, with as much as 92% of carotenoids being lost during extended culture (120 days), replacement of carotenoids is dependent on appropriate diets. If dietary carotenoid needs are not met, *P. monodon* will develop a variety of bluish hues (Howell and Matthews 1991).

The role of carotenoids in penaeid prawn reproductive performance and egg quality is not fully understood. Although the main form of astaxanthin in the carapace of adult *P. monodon*, *P. esculentus* and *Cherax quadricarinatus* is etherified astaxanthin, the main form in the ovaries is unesterified and may, therefore, have an active function in egg production (Scheidt 1990, Dall et al. 1995, Sagi et al. 1995). In *Penaeus schmitti* the carotenoid content of ovaries increased from 39 µg/g in undeveloped ovaries to 463 µg/g in fully matured ovaries (Vincent et al. 1988). In contrast, total carotenoid concentrations in ovaries of *P. esculentus* increased from 5 µg/g to 40 µg/g between immature and mature ovaries (Dall et al. 1995). The absolute concentration of carotenoids in penaeid prawns varies and there is a consistent multi-fold increase in total carotenoid content during ovarian maturation. Between spawning and hatching approximately 50% of the original egg content of free astaxanthin, but not mono- or di-esters, is metabolized or lost in *P. japonicus* before the first self-feeding stage of the protozoea (Dall 1995). Despite the apparent association between increasing carotenoid content during egg development and its utilization during early planktonic life, the exact role and importance of carotenoids remains largely unknown. However, three possible roles are suggested where carotenoids: 1) act as a protectant against electromagnetic radiation, 2) act as an antioxidant or provitamin A both in the larvae and egg, where vitellin serves as a carrier of the hydrophobic astaxanthin acting as an antioxidant during embryogenesis and 3) act as a general reserve for pigmentation in the early larval stages in the construction of the cuticle. The ability of *P. japonicus* larvae to metabolize carotenoids by oxidative pathways and esterification reactions does not fully develop until the late post-larval stages. In *P. monodon* there is evidence that astaxanthin gives a significant improvement in stress resistance, but not disease resistance, in post-larvae (Merchie et al. 1998, Petit 1993).

The experiments conducted in this study were designed to examine the proposed beneficial aspects of carotenoids, specifically astaxanthin, as an enhancer of maturation and reproduction, including fecundity and embryonic and larval development (Torrissen 1990).

Methods

GENERAL

Adult *Penaeus monodon* were obtained between mid-September and mid-October from coastal waters between Townsville and Cairns in north Queensland, Australia (17-19°S, 146-147°E) by commercial broodstock trawler operators. The prawns were processed and held under the same conditions as outlined in Objective 3.

Their exact age was unknown but was believed to be over 1 year of age. Average measurements are shown in Table 4.1.

TABLE 4.1. Total body weight, body length and carapace length of female and male broodstock used in the experiments.

Measurement	Females	Males
Total body weight (g)	129.3 ± 1.96 g	50.7 ± 0.9 g
Total body length (cm)	22.7 ± 0.22 cm	19.3 ± 0.11 cm
Carapace length (cm)	5.27 ± 0.07 cm	4.24 ± 0.03 cm

Prawns were fed twice daily at a rate of 5-10% of total broodstock biomass per tank per day. The first feed in the morning was a mixture of whole fresh frozen mollusc. The afternoon feed was a propriety maturation diet that was a minced paté of molluscs, vitamin and mineral mix, ß-carotene, ß-glucan, cholesterol, fish oil and binders. Synthetic astaxanthin, in the form of carophyll pink (Hoffmann-La Roche Ltd.), was added as a fine powder to fish oil in a glass container and thoroughly mixed. The astaxanthin-oil mix was then added in small aliquots during the mincing and mixing of the paté to ensure a homogenous blend. The maturation diet was made in batches, divided into small blocks and frozen. On a daily basis the required amount of maturation diet was thawed and used the same day.

Each new batch of females contained broodstock at various stages of ovarian development. All prawns were weighed, marked with an eye-tag identification ring and tagged on the carapace to determine the time of the following molts. Before being used in the experiments, female and male broodstock were acclimatised in indoor tanks until they molted. During this time the female and male broodstock were fed on their respective diets with or without astaxanthin. Two days after molt the females were unilaterally eyestalk ablated to initiate ovarian development. At the time of molt the ovary is fully regressed and therefore all broodstock used in the experiments started from the same reproductive state regardless of their ovarian development at the time of capture. Their reproductive performance in the experiment should therefore reflect the effects of the experimental design and those of a captive environment.

This experiment was designed to examine the effect of three dosages of astaxanthin on reproductive performance in female *P. monodon* broodstock. The dosages were 0.25, 2.5 and 25 g of astaxanthin per kilogram of feed. Each group comprised 20-25 females and 10-12 males. Controls received the same amount of maturation paté, but without the addition of astaxanthin.

An outline of the experimental design is shown in Figure 4.1. Since astaxanthin could influence several physiological and behavioral processes in crustaceans, due to the potential multi-functional nature of carotenoids, several variables were monitored during the experiment (Figure 4.2).

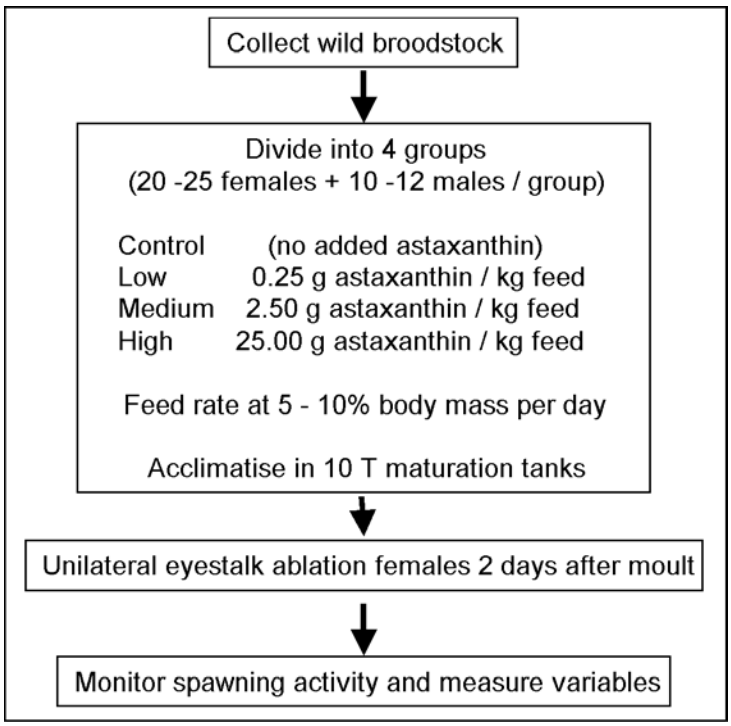

FIGURE 4.1. Outline of experiment design to examine the effects of astaxanthin on reproductive activity in *P. monodon* broodstock.

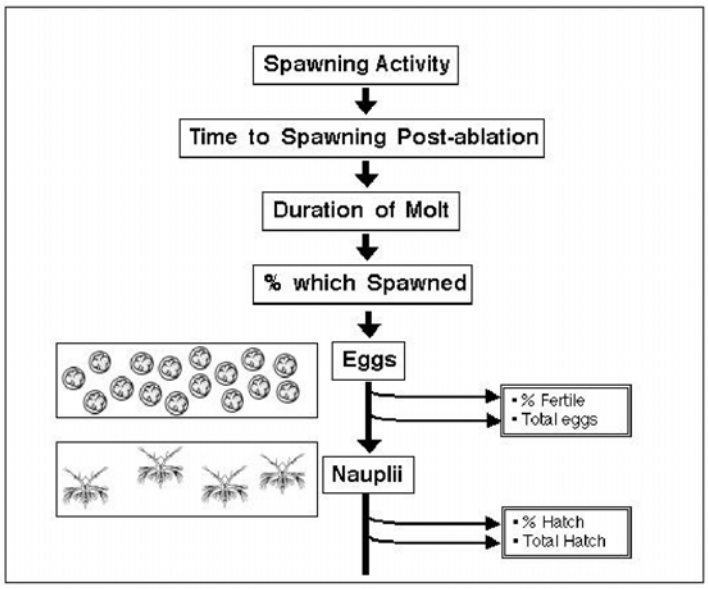

FIGURE 4.2. Variables measured for the examination of the effects of astaxanthin on the reproductive performance of *P. monodon*.

When females, which were examined each afternoon, had a G.I. of IV they were removed from the holding tank and placed individually into 125 L spawning tanks for the night as *P. monodon* spawns at night. Spawning tanks were maintained on a flow-through recirculating system, with aeration occurring at in the central sump reservoir, with approximate turnover rates of 1 tank volume every 15 minutes. Some spawning tanks were equipped with an electronic monitoring system that allowed the determination of time of spawning. If a spawning was detected, a sub-sample of eggs was automatically collected and fixed 1 hour

and 15 minutes after spawning. This sub-sample was used to determine the fertilisation rate of the spawned egg mass the morning after spawning each batch of eggs was harvested into a 4 litre container. Sub-samples were taken to estimate the total number of eggs in each batch. The remaining eggs were placed in hatching trays and incubated in a flow-through system. The nauplii hatched were collected and a sub-sample taken. From this sub-sample the percent hatch rate and total naupliar output was calculated. The variables measured are summarised in Figure 4.2.

Results/Discussion

All treatment groups were fed their respective astaxanthin diets while being held until their first molt in captivity. Differential ovarian development was not observed in the females before unilateral eyestalk ablation. There was no intact non-ablated female astaxanthin-fed control group.

The average body mass of female broodstock for the control group was 139.7 ± 4.2, for the high astaxanthin group it was 125 ± 3.4 g, for the medium group 130.4 ± 5.2 g and for the low group it was 122 ± 3.5 g.

Unilateral eyestalk ablation can result in the death of a small proportion of individuals, possibly due to post-surgery trauma. As ovarian development and spawning typically requires 7 days, females that died within 1 week of ablation due to post-operative stress were not included in the analysis. Post-surgery deaths for each group include 3 in the controls, and 2, 2 and 3 in the low, medium and high group, respectively. Most females spawned in all groups (Table 4.2).

Treatment with astaxanthin did not alter the time of spawning in females (Fig. 4.3). Lights came on at 0600 hours in the morning and turned off at 2030 hours. Spawning occurred five hours into the dark phase, on average between 0100 and 0200 hours. There was a considerable range, however, with some spawnings occurring as early as 2200 hours and some as late as 0530 hours.

Stage IV broodstock were weighed when they were transferred from their maturation tank into a spawning tank. The following morning all broodstock that had spawned overnight were weighed again as they were transferred back to their respective maturation tank. In this experiment, in approximately 30% of cases the female did not spawn on the predicted night. On average 5.8 g were lost per spawning, representing the egg mass spawned and associate loss of ovarian fluids and spermatophore (Fig. 4.4). As the measurements were wet weight there was considerable variation between the two weight determinations. Nevertheless, there was approximately 1 g of body mass loss in non-spawners in the 16 hours the potential spawner was held in the spawning tank. Hence, the actual weight loss attributed to spawning *per se* is about 5 g per spawning event.

TABLE 4.2. Percent of females that spawned at least once. Excludes females that died within 7 days of unilateral eyestalk ablation from post-surgery trauma.

Group	% Females which spawned at least once
CONTROL (No astaxanthin)	100%
LOW (0.25 gm / kg food)	93%
MEDIUM (2.50 g / kg food)	96%
HIGH (25.00 g / kg food)	100%

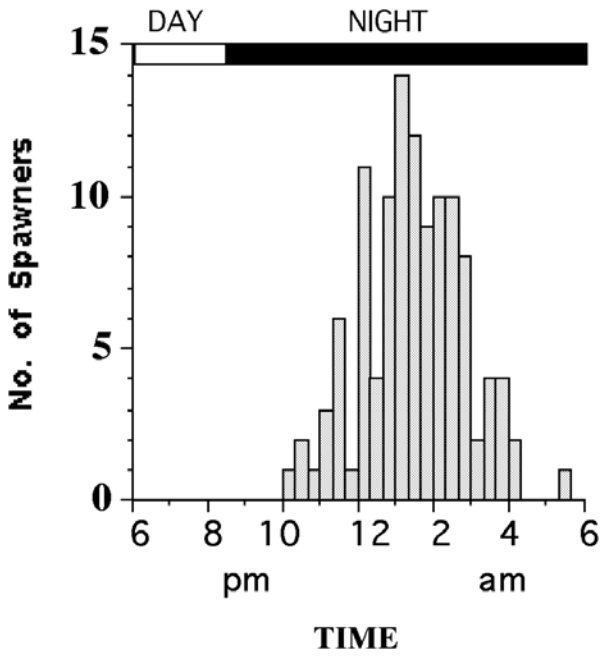

FIGURE 4.3. Distribution of the time of spawning in *P. monodon*. The time of light and darkness is shown by the horizontal bar.

Almost all broodstock spawned at least once within the first molt cycle (Fig. 4.5). In the control group over 80% of broodstock spawned, whereas all spawned in the low and high groups and all but one in the medium group. Overall, there was a high incidence of second spawnings within the first molt cycle. On average there was a halving in the incidence of spawning for a third time. Very few broodstock spawned for a fourth time during the first inter-molt period. Ovarian development was extremely rapid in those females that spawned 3 times or more within a molt cycle (Fig. 4.6). Approximately half of all broodstock spawned in the second molt cycle, which was less than the number that spawned once or twice within the first molt cycle.

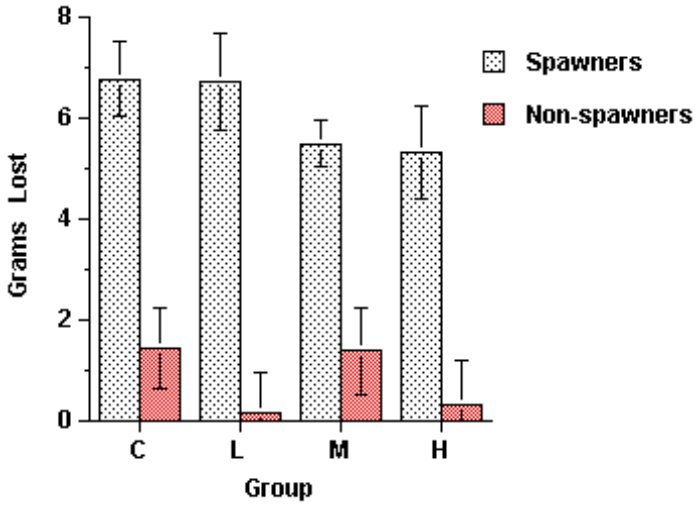

FIGURE 4.4. Loss of body mass between spawners and non-spawners during overnight incubation in a spawning tank.

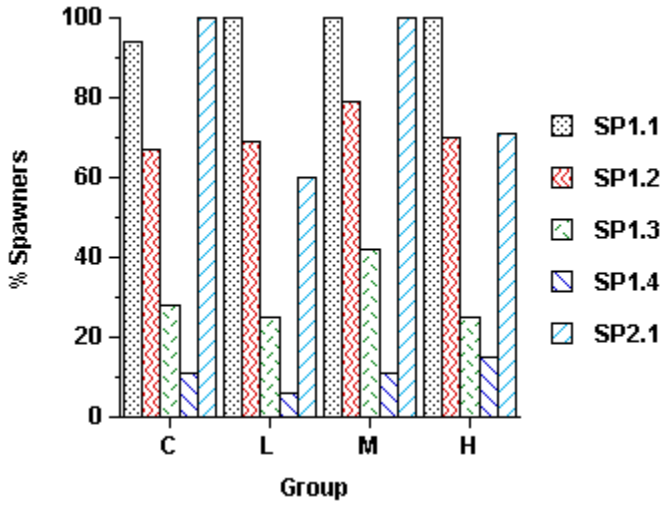

FIGURE 4.5. Percentage of broodstock population that spawned one (SP1.1), two (SP1.2), three (SP1.3) or four (SP1.4) times in the first molt cycle and those that spawned once (SP2.1) in the second molt cycle.

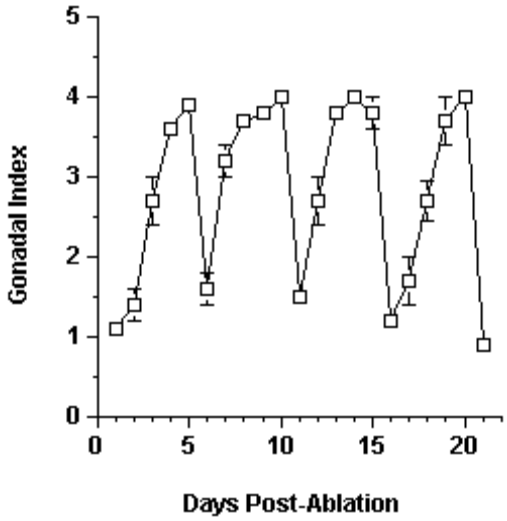

FIGURE 4.6. The cycling of gonadal (ovarian) index in broodstock that spawned up to four times within the first molt cycle. Molt period was an average of 19 days.

There was no significant difference in egg production per spawning amongst the groups (Fig. 4.7). When egg production for all spawners, regardless of group, was pooled, there was a progressive increase in egg production from the first through to the third spawning in the first molt cycle (P<0.01) (Fig. 4.8). There was a significant decrease in egg production in the second molt cycle compared to the first (P<0.05).

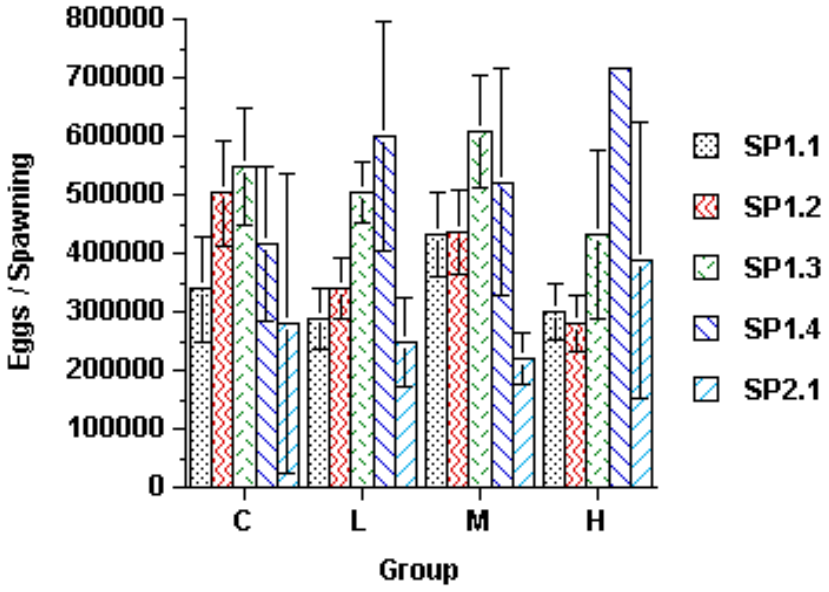

FIGURE 4.7. Number of eggs per spawning event for the four spawnings throughout the first molt cycle and the first spawning after the second molt.

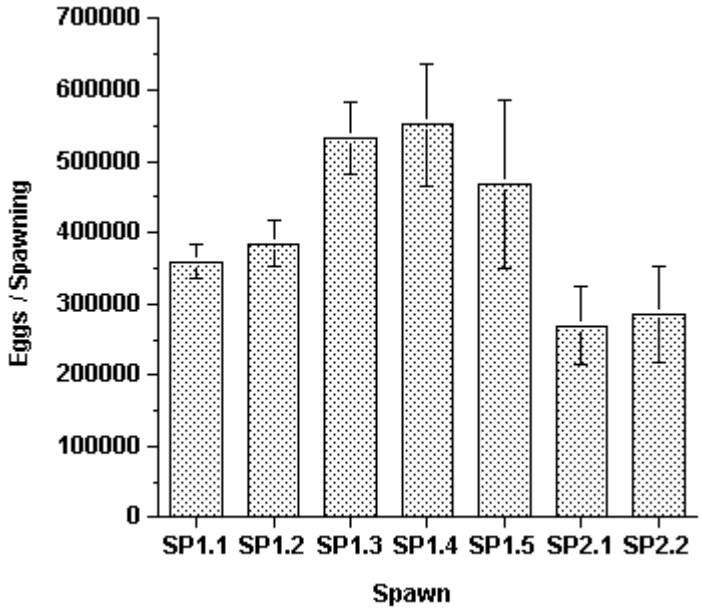

FIGURE 4.8. Average number of eggs for all spawnings. Data are combined for the controls and three experimental groups.

Total eggs spawned per spawning correlated significantly to female body weight (P<0.001) (Figure 4.9). In order to minimise the variance due to the interaction between female body mass and total eggs spawned, although there were no significant differences in female body weight between the groups, the data were expressed as eggs spawned per gram of female

broodstock weight (Figure 4.10). There were no significant differences between the controls and experimental groups.

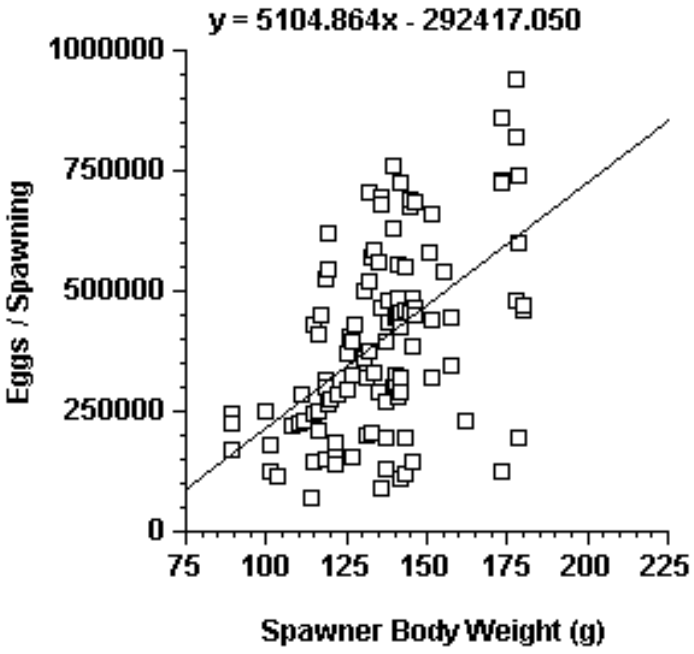

FIGURE 4.9. The relationship between spawner body weight (mass) and eggs spawned per spawning event. The regression formula is shown in the upper left-hand corner.

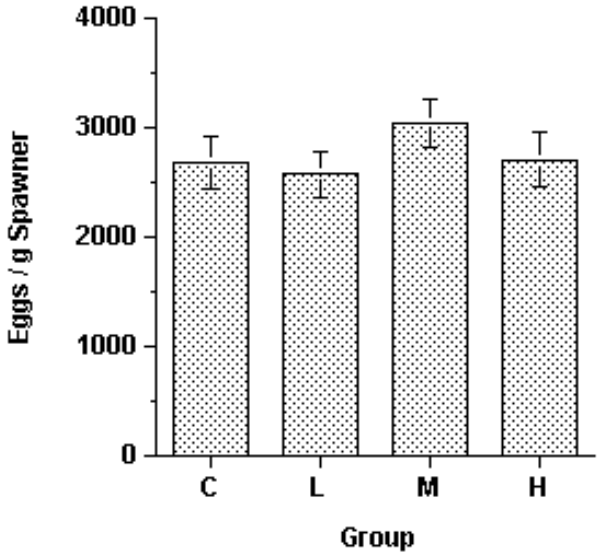

FIGURE 4.10. Average egg output for all spawnings within the first molt cycle expressed as per gram of spawner body weight.

There were no significant differences in fertility between the groups. Overall, average fertility was 68% (Figure 4.11). The fertility rate of the controls was 64.8% whereas that of the low, medium and high treatment group was 65.9%, 72% and 65.5%, respectively.

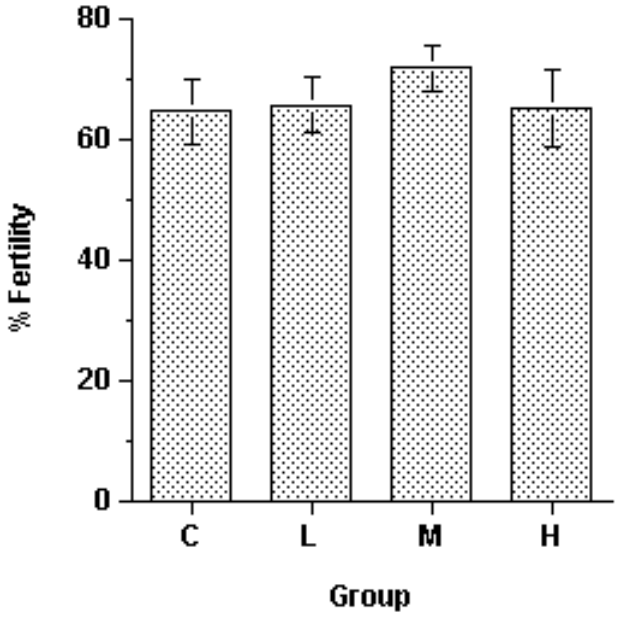

FIGURE 4.11. Fertility rates of eggs spawned from the control and three experimental groups.

When all groups were pooled there was a significant decline in fertility between spawnings in the first and second molt period (P<0.01) (Fig. 4.12).

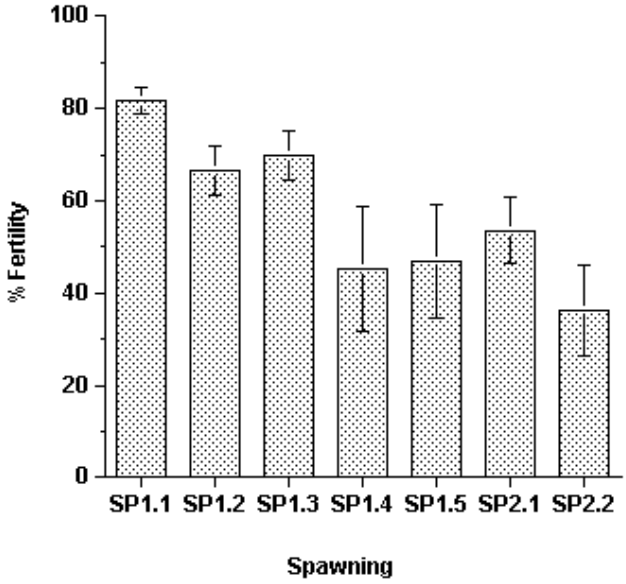

FIGURE 4.12. Fertility rates of all groups pooled throughout the molt cycle.

There were no significant differences between the groups in hatching rate for each separate spawning (Fig. 4.13). However, when average hatch rates for all spawnings were calculated for each group, there appeared to be an improved hatching rate in the medium and high treatment groups compared to the controls and low group. However this was not statistically significant (Fig. 4.14).

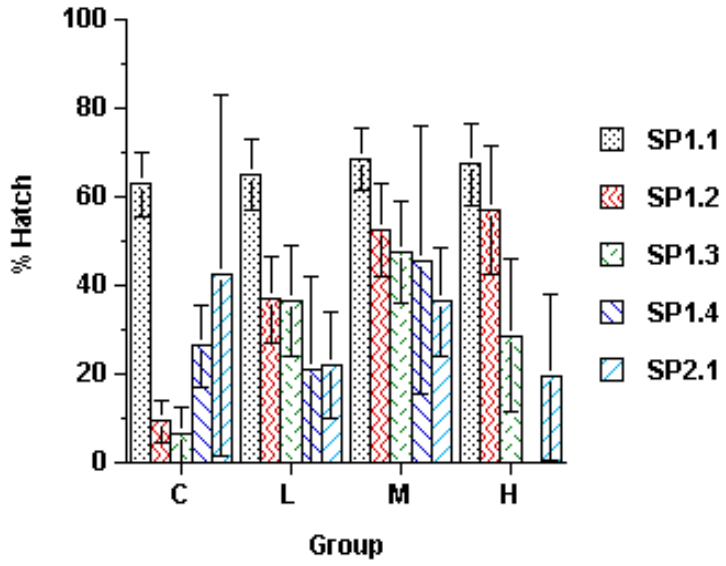

FIGURE 4.13. Percentage of eggs hatching, and hence eggs successfully fertilized which completed embryogenesis and hatched as nauplii, for the first four spawning events throughout the first molt cycle, and the first spawning after the second molt.

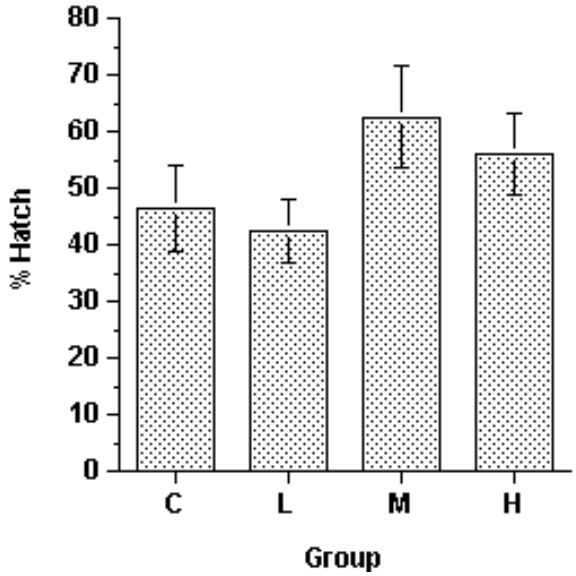

FIGURE 4.14. Percentage hatch rate for all spawnings for each group.

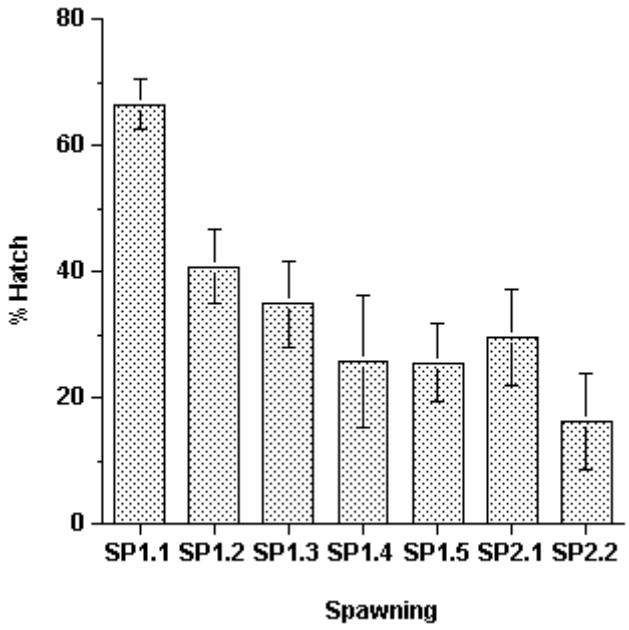

FIGURE 4.15. Change in hatching rate, of all groups combine, with spawning sequence.

When all the hatching rates are pooled and plotted against spawning sequence there is a significant decline with each sequential spawning ($P<0.01$) (Fig. 4.15).

Fertility and hatching rate are independent measures of reproductive performance. Fertilization occurs at the moment of spawning when the female releases her eggs into the water column. Simultaneously, sperm stored in the thelycum are released to fertilise the eggs. Embryogenesis occurs over the following 12 to 13 hours. A wide variety of factors influence whether or not an egg will complete development and hatch as a nauplius. Egg quality *per se* depends in part on efficient nutritional packaging during egg development within the ovary, as well as other essential factors. Environmental conditions can also influence embryonic development. In controlled experiments, such as this, any environmental influence should be consistent across all spawnings from all groups. If the eggs are of high quality there is the potential for hatch rates to be equivalent to fertilization rates. However, this is rarely the case (Fig. 4.16). Very few fertilized eggs have a 100% hatch rate. The actual regression of fertility rate against hatch rate parallels that of the theoretical 100% success rate, indicating that, in general, about 20-30% of fertilized eggs do not hatch. It would be desirable to identify the factors yielding eggs of the type that occur in the upper right-hand corner of the graph. At present these are unknown, but if the causal factors are heritable ones then any selective breeding program should select for both high fertility, even though part of this is a male trait, and high hatchability.

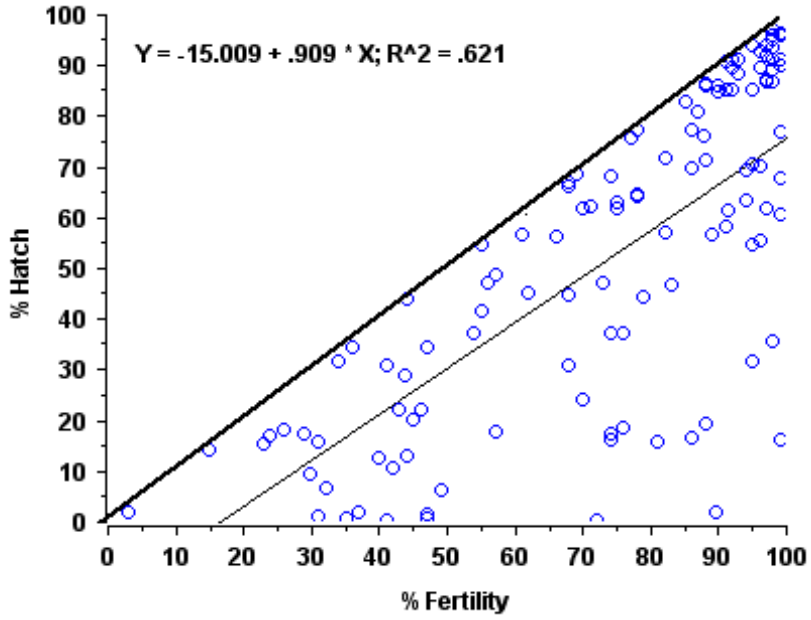

FIGURE 4.16. Relationship between percentage of eggs fertilised and hatching. The dotted line represents a 1:1 relationship and would indicate that all eggs fertilised actually hatched. The regression line of the data is below this, indicating embryonic mortality.

Nauplii production rapidly declines with sequential spawning in the control group, whereas in the various treatment groups it remained relatively constant (Fig. 4.17).

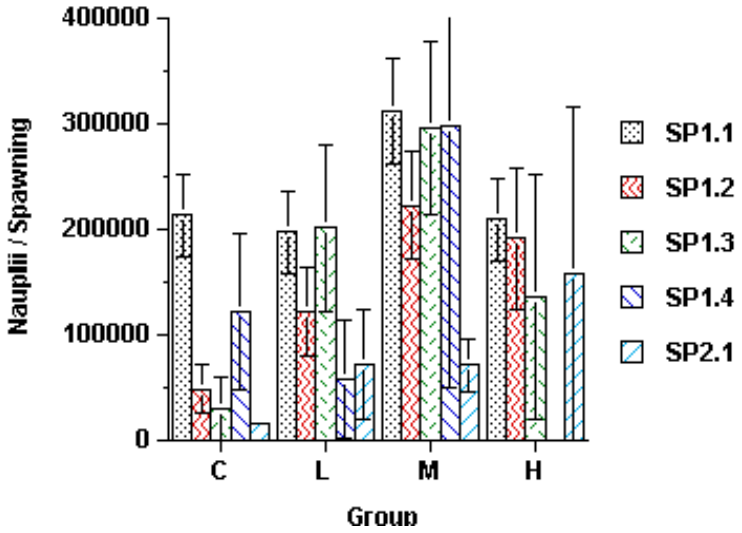

FIGURE 4.17. Number of nauplii per spawning event for the first three spawnings throughout the first molt cycle and the first spawning after the second molt.

There was a significant increase in nauplii output in the medium treated group compared to the low, high and control groups (P<0.05) (Figure 4.18).

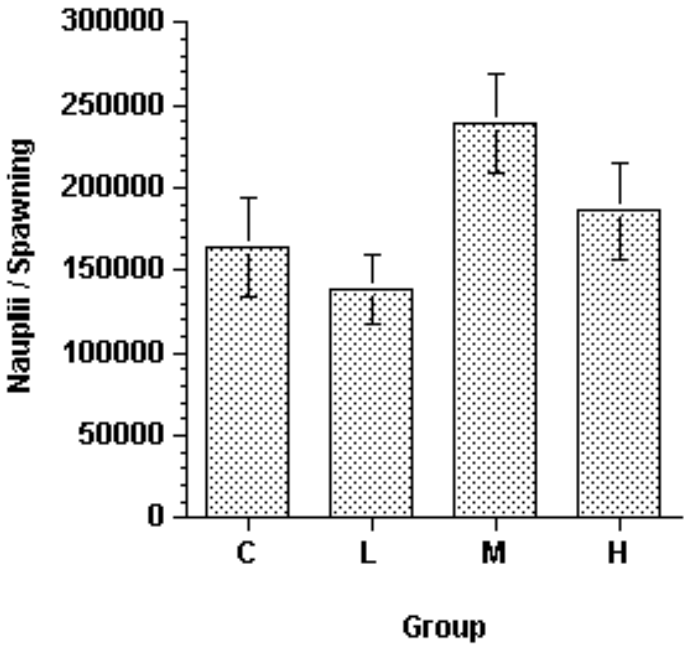

FIGURE 4.18. Number of nauplii per spawning produced from all spawnings for each group.

Nauplii output was also examined in relation to female body weight and expressed as nauplii output per gram spawner (Fig. 4.19).

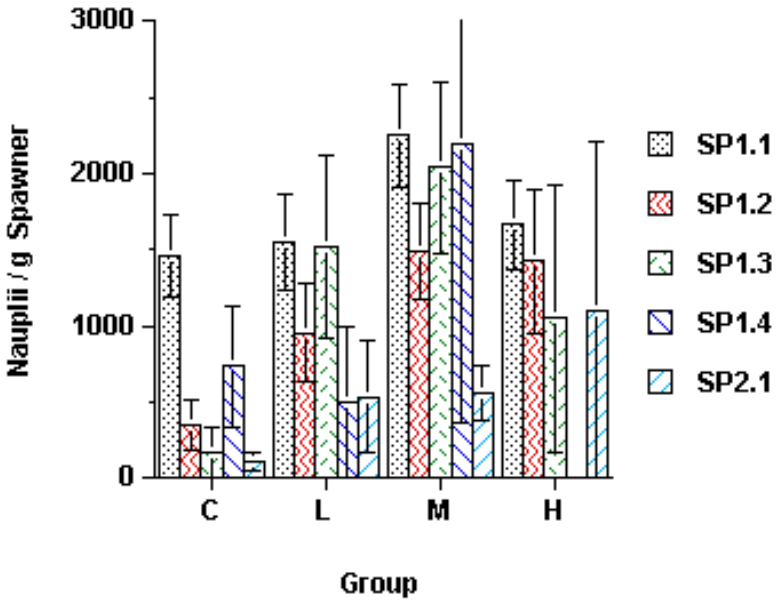

FIGURE 4.19. Number of nauplii per spawning per gram spawner body weight for each group through the molt cycle.

There was a significant increased in nauplii output for all spawnings in the medium treated group, compared to the controls (P<0.01) (Fig. 4.20).

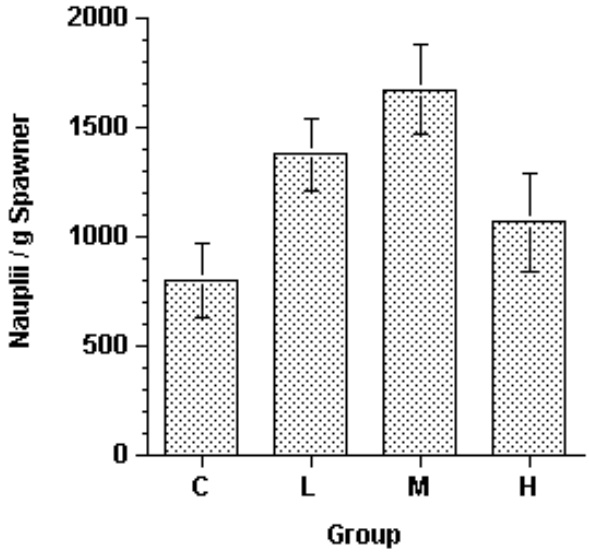

FIGURE 4.20. Number of nauplii produced in all spawnings in various groups expressed as per gram of female spawner weight.

The experiments conducted in this study were designed to examine the proposed beneficial aspects of carotenoids, specifically astaxanthin, as an enhancer of maturation and reproduction, including fecundity and embryonic and larval development (Torrissen 1990). During grow-out, or time in captivity, the diet should contain 30-70 ppm astaxanthin to maintain normal pigmentation and health. Both synthetic and naturally occurring carotenoids can be used in the diet. Astaxanthin (3,3'-dihyroxy-beta, beta – carotene-4,4'-dione) and canthaxanthin (beta, beta-carotene-4,4-dione) are available synthetically, Natural sources include krill oil, *Spirulina*, *Phaffia rhodozyma* yeast, microalga, such as *Haematococcus pluvialis*, marigold, capsicum, and xanthophyll containing vegetables meals such as oats, wheat and corn (Johnston and An 1991, Liao et al. 1993, Maoka et al. 1985, Meyers 1994, Meyers and No 1995, Storebakken 1988). Carotenoids are inherently chemically unstable, are subject to a range of chemical alterations and oxidative degradation, and are sensitive to light, heat, oxygen, oxidants and other chemicals. Hence, depending on the processing method, feed composition and length and mode of storage conditions, feedstuffs can have limited value as a consistent source of carotenoids. This study demonstrated that the supplementation of 2.5 g/kg carotenoid in the broodstock maturation diet, in the form of astaxanthin, is beneficial with respect of an increase output of nauplii per spawner.

OBJECTIVE 5

To demonstrate value of supplementing broodstock diets with different concentrations of ecdysteroids

Introduction

Broodstock are considered to be the weakest link in the sustainable production cycle of the most commercially important species, *P. monodon*. As *P. monodon* production still depends on the procurement of wild stock a key bottleneck is simply achieving a reliable supply of healthy arrivals from the wild. Hatcheries preferentially obtain females close to the time of spawning as they are considered to perform better than females induced to mature and spawn in captivity. Although part of the difference in broodstock performance could be attributed to some form of stress associated with being held under captive conditions and the opportunistic development of pathogens, it is likely that nutrition has a major influence.

The prawn farming sector is evolving towards full domestication of the target species to obtain long term success and sustainability of the industry. A key constraint in the shift from wild broodstock to domesticated broodstock is the breeding performance of domesticated stock compared to their wild conspecifics. For penaeid prawns in general, the availability of an optimal broodstock diet has been identified as a priority in reproductive success, especially for captive reared broodstock (Kawahigashi 1998). However, there is still limited understanding of the interaction between nutrition and reproductive performance or even a knowledge of specific nutrient requirements for successful reproduction.

There are several components to the development of a broodstock diet. The diet must provide adequate nutrition for the promotion or induction of a reproductive capability. Secondly the diet must be able to support the rapid, precocious gonadal development associated with the maturation technique of unilateral eyestalk ablation. The diet must be able to support rapid maternal nutritional uptake as the ovary matures from a regressed state to one of fully mature eggs with complete energy reserves. In addition, the diet must also provide sufficient nutrients and energy reserves up to the first stage protozoea and first self-feeding.

In broad nutritional terms the broodstock diet must contain a suitable mix of lipids, proteins and carbohydrates. For lipids, the broodstock diet should contain a total lipid content of between 8 – 11%, with levels above 14% potentially interfering with efficient ingestion and assimilation (Bray et al. 1990). Of the fatty acids, the *n*-3 highly unsaturated fatty acids (HUFA) are the most important and especially the 20:5*n*-3 and 22:6*n*-3 forms (Wouters et al. 1999). In addition, arachiodonic acid (20:4*n*-6) is probably critical, as well as a ratio of 2 to 1 to 3 to 1 between *n*-3/*n*-6 fatty acids (Ravid et al. 1999). There is also a requirement for sufficient triacylglycerides (TAG), which appear to be specifically incorporated into the eggs as an energy source to support embryogenesis, hatching and naupliar development. (Palacios et al .1999). A source of phospholipids is also essential, such as 1.5% soybean lecithin (Alava et al. 1993). Finally cholesterol must also be provided for in the diet (Harrison 1990, 1997). Protein is required, and although optimal protein levels or amino acid profiles are not known for broodstock diets, a natural diet would be over 50% protein (Wouters et al. 2001). There is no additional carbohydrate requirement over and above what would be present in other ingredients. Although the minimal vitamin requirements for broodstock are yet to be defined, the fat-soluble vitamins A, D and E are essential (He et al. 1992). The addition of thiamin, riboflavin, niacin, vitamin B6, vitamin B12, choline, inositol and ascorbic acid are also beneficial (D'Abramo and Conklin 1992). Although mineral deficiencies could impact on

reproductive performance the minimal requirements are unknown, but diets are typically fortified with calcium, phosphorus, magnesium, sodium, iron, manganese and selenium (Chamberlain 1988, Alava et al. 1993, Marsden et al. 1997, Xu et al. 1994). As discussed in Objective 4, carotenoids are essential, as prawns cannot synthesize these chemicals *de novo*. According to the present study, 2.5 g astaxanthin per kilogram of broodstock diet is optimal.

Nutrient composition, *per se*, does not entirely explain the beneficial aspects of natural food items and hence it is believed that there are additional stimulatory and/or essential factors that are, as yet, unknown. For example, bloodworms (marine polychaetes *Glycera dibranchiat*a and *Americonuphis reseii*) are considered indispensable for optimizing broodstock reproductive performance (Kawahigashi 1998). The nutritional benefit of bloodworms is considered to be due to their HUFA composition; however, these fatty acids are the precursors to prostaglandin hormones, which are required for successful reproduction as well as methyl farnesoate (Harrison 1990, Laufer et al 1997, Hall et al. 1999). Hence, it may be that the bloodworms are acting as immediate precursors to reproductive hormone synthesis. Other reproductive stimulatory factors may also originate from the diet, such as 5-hydroxytryptamine (5-HT), which has been shown to stimulate gonadal maturation in crustaceans (Fingerman, Sarojini and Nagabhushanam 1998). Squid and molluscs are often used in broodstock diets as they are believed to improve reproductive performance (Wouters et al. 2001). However, it has been suggested that only sexually mature squid and other molluscs are actually beneficial, whereas juveniles or non-breeding ones are not (Mendoza et al. 1997). It is believed that these dietary items are not only a rich source of cholesterol but also reproductive steroid hormones, with the latter being the actual beneficial component.

Wild *P. monodon* broodstock that are collected in the final stages of ovarian maturation, i.e. Stage IV, often spawn spontaneously, either on the night of arrival at a hatchery or within the first few days. If spontaneous spawning does not occur, it becomes necessary to unilaterally eyestalk ablate females to complete ovarian development and induce spawning. Ablation is also necessary to synchronise broodstock to meet production schedules and to induce broodstock that are sourced outside of their natural spawning seasons. In effect, unilateral eyestalk ablation removes one of the two hormone organs (X-organ sinus gland complex, with one in each eyestalk) that normally inhibit reproduction and spawning. Unilateral eyestalk ablation results in the partial removal of the reproductive inhibitory hormone (RIH) to a sufficient degree to allow the completion of ovarian development and spawning in most broodstock. However, the X-organ sinus gland complex is also the source of several other hormones that regulate various physiological processes in the prawn. One of these, molt-inhibiting hormone (MIH), is responsible for the inhibiting the secretion and/or synthesis of ecdysteroids by the Y-organ (another hormone organ). There are over 250 forms of ecdysteroids but only a few are found in crustaceans, with the most physiologically active one being 20-hydroxy ecdysone (Lafont and Wilson 1996, Subramoniam 2000). The ecdysteroids influence aspects of molt and reproductive physiology and egg development (Subramoniam 2000). Eyestalk ablation is known to disrupt the normal pattern of secretion of ecdysteroids in reproductively active female crustaceans (Lachaise et al. 1992).

As ecdysteroids are known to be important in aspects of reproduction, this study was designed to examine whether the application of exogenous ecdysteroids, in the form of 20-OH ecdysone, would influence reproductive performance and specifically egg quality.

Methods

GENERAL

Adult *Penaeus monodon* were obtained from coastal waters between Townsville and Cairns in north Queensland, Australia (17-19°S, 146-147°E) by commercial broodstock trawler operators. The broodstock were collected in late April to early June towards the end of their autumn spawning period. Their exact age was unknown but based on their size estimated to be under 1 year of age. Average measurements are shown in Table 5.1.

TABLE 5.1. Total body weight, body length and carapace length of female and male broodstock used in the experiments.

Measurement	Females	Males
Total body weight (g)	108.0 ± 1.72 g	45.4 ± 0.7 g
Total body length (cm)	20.1 ± 0.19 cm	18.3 ± 0.09 cm
Carapace length (cm)	5.40 ± 0.06 cm	4.00 ± 0.03 cm

Prawns were fed twice daily at a rate of 5-10% of total broodstock biomass per tank per day. The first feed in the morning was a mixture of whole fresh frozen mollusc. The afternoon feed was a propriety maturation diet that was a minced paté of molluscs, a vitamin and mineral mix, βeta-carotene, β-glucan, cholesterol, fish oil and binders. Twenty-hydroxy ecdysone (20-OH ecdysone), purchased from the Institute of Biology Komi Science Centre, Russia, was dissolved in 100% ethanol and diluted with water to give a final 20% w/v 20-OH ecdysone solution in 20% ethanol. This solution was added gradually during the mincing and mixing of the paté to ensure a homogenous blend. The maturation diet was divided into small blocks and frozen. On a daily basis the required amount of maturation diet was thawed and used the same day.

All arrivals of batches of wild caught broodstock contained females at various stages of ovarian development. At stocking each prawn was weighed, marked with an eye-tag identification ring and tagged on the carapace to determine the time of the following molts. Before being used in the experiments, female and male broodstock were acclimatized in indoor maturation tanks until they molted. During this time the female and male broodstock were fed on their respective diets with or without 20-OH ecdysone. Two days after molt the females were unilaterally eyestalk ablated to initiate ovarian development. At the time of molt the ovary is fully regressed and therefore all broodstock used in the experiments started from the same reproductive state, regardless of their ovarian development at the time of capture. Their reproductive performance in the experiment should largely reflect the effects of the experimental design and those of a captive environment.

This experiment was designed to examine any effect of three dosages of 20-OH ecdysone on reproductive performance in female *P. monodon* broodstock. The dosages were 0.1, 1.0 and 10 mg of 20-OH ecdysone per kilogram of feed. Each group comprised 20-25 females and 10-12 males. Controls received the same amount of maturation paté but without the addition of 20-OH ecdysone.

An outline of the experimental design is shown in Figure 5.1. Females were monitored for ovarian growth on a daily basis in the afternoon as described in Objective 4. Fertility rate was determined using the method described previously (Objective 3). The female broodstock were monitored throughout the first inter-molt period post-ablation and up to the first two spawnings in the second inter-molt period.

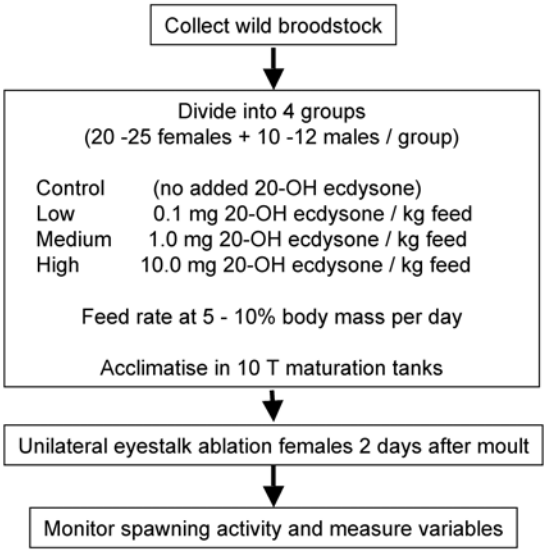

FIGURE 5.1. Outline of experiment design to examine the effects of 20-OH ecdysone on reproductive activity in *P. monodon* broodstock.

Results/Discussion

All the experimental groups were fed their respective 20-OH ecdysone diets upon arrival and while being held until their first molt in captivity. Significant ovarian development, that is over a G.I. II, was not observed in the females before unilateral eyestalk ablation. There was no intact non-ablated female 20-OH ecdysone fed control group.

The average body weight of female broodstock did not differ significantly between the groups. Body weight for the control group was 107 ± 3.8g, for the low group 105 ± 4.6 g, the medium group 113 ± 4.7 g and the high group 108 ± 4.8 g.

The mean inter-molt period was 20.9 days (Fig. 5.2). The controls had a mean inter-molt period of 22.8 days whereas the low, medium and high treated groups had means of 19.6, 20.4, and 21.4 days respectively. There was a significant shortening of the inter-molt period between the controls and the treatment groups ($P<0.05$).

On average 6.4 days elapsed between unilateral eyestalk ablation and spawning (Figure 5.3). There was no significant change in time to spawning between the controls and the treatment groups. The results showed that ovarian development could be completed within a few days in those females that spawned several times within a molt cycle.

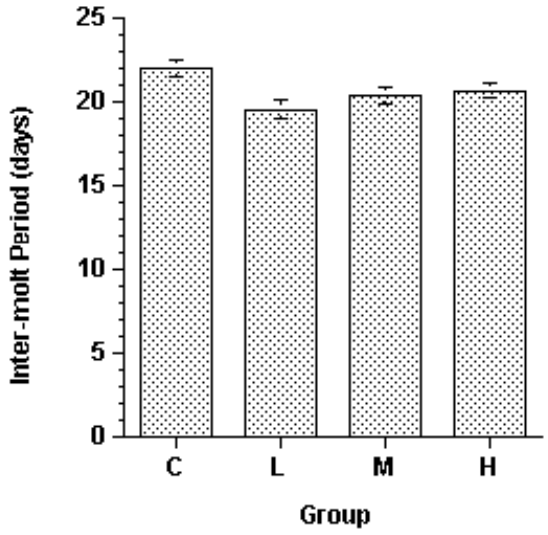

FIGURE 5.2. Inter-molt period for the controls and three experimental groups: Low (L) 0.1 mg 20-OH ecdysone /kg feed, medium (M) 1.0 mg 20-OH ecdysone / kg feed, and high (H) 10.0 mg 20-OH ecdysone / kg feed.

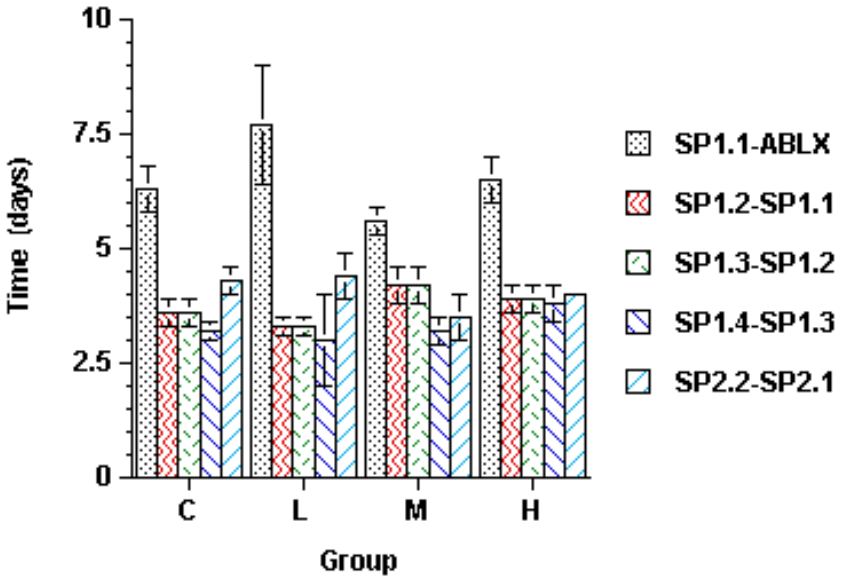

FIGURE 5.3. The time between day of unilateral ablation (2 days post-molt) until the time of spawning (SP1.1-ABLX) and thereafter time between sequential spawnings.

Unilateral eyestalk ablation resulted in the death of a small proportion of individuals by post-surgery trauma. As ovarian development and spawning typically requires approximately 7 days, females that died within 1 week of ablation due to post-operative stress were not included in the analysis. Post-surgery deaths for each group include 1 in the controls, and 3, 2 and 2 in the low, medium and high group, respectively.

TABLE 5.2. Mortality and survival rates of the broodstock used in the experiments.

Group	Mortality	Survival (days)
Control	42%	17.4±3.4
Low	40%	23.4±2.5
Medium	39%	24.7±2.5
High	30%	17±2.2

Sampling for gill associated virus (GAV), a known pathogenic virus of *P. monodon* wild stock, has shown that it can be ubiquitous in the natural population (Walker et al. 2001, Cowley et al. 2002). It has also been shown that the broodstock used in this study were GAV positive (unpubl.data). Although the mechanism of pathogenesis of GAV is not fully understood, we have consistently observed mass mortalities that eventually occur in ablated *P. monodon* that appeared otherwise healthy. The mortality rate varied considerably and mortalities may not be observed for months, while in other groups, such as the broodstock used in this experiment, it may begin within a few weeks of captivity, particularly following unilateral eyestalk ablation (Table 5.2). As commercial hatcheries do not typically hold wild broodstock, intact or ablated, for long periods of time, and given that most hatcheries only use the first, and sometimes second, egg hatches from these females, mortalities are not necessarily observed as expired broodstock are culled before death due to pathogenesis.

Nearly all females spawned in all groups (Table 5.3).

TABLE 5.3. Percent of females that spawned at least once.

Group	% Females which spawned at least once
CONTROL (No 20-OH ecdysone)	89%
LOW (0.1 mg / kg food)	89%
MEDIUM (1.0 mg / kg food)	100%
HIGH (10.0 mg / kg food)	100%

Most to all broodstock spawned at least once within the first molt cycle (Figure 5.4). In the control and low group nearly 90% of all broodstock spawned, whereas all spawned in the medium and high groups. There was a high incidence of second spawnings within the first molt cycle. On average there was a halving in the incidence of spawning for a third time, and even fewer spawned a fourth time.

Egg output per spawner per spawning event did not vary significantly throughout the first molt cycle (Fig. 5.5). There was no evidence of a decrease in egg output per spawning event even up to the third spawning within the first molt cycle. As there were no significant differences between the groups the data were replotted for clarity by combining all egg output per sequence of spawning event (Fig. 5.6).

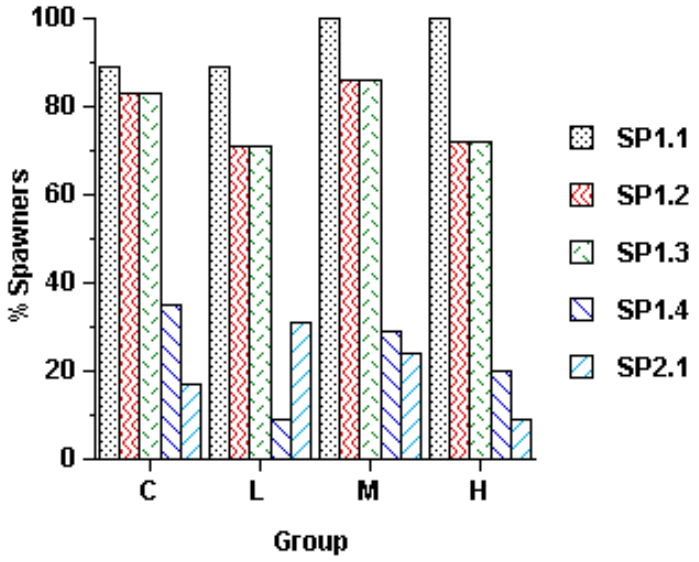

FIGURE 5.4. Percentage of broodstock population which spawned one (SP1.1), two (SP1.2), three (SP1.3) or four (SP1.4) times in the first molt cycle and those which spawned once (SP2.1) in the second molt cycle. The data are inclusive, such that an animal that spawned three times in the first molt cycle will be represented in SP1.1, SP1.2 and AP1.3.

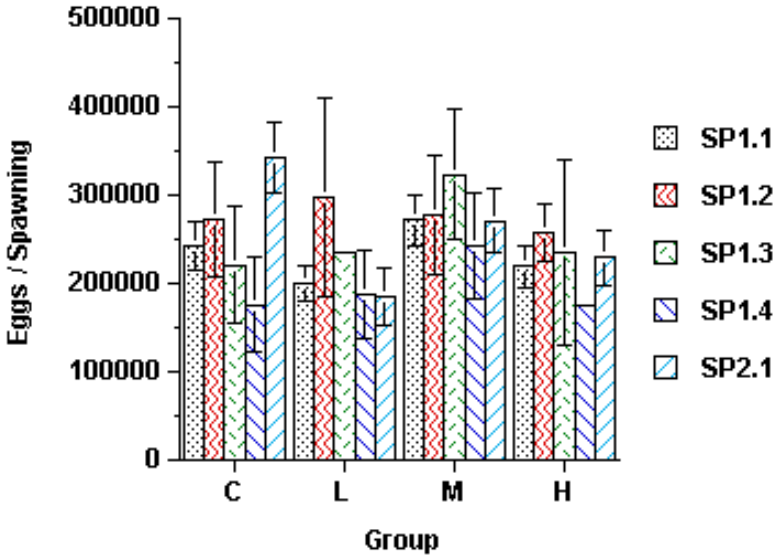

FIGURE 5.5. Number of eggs per spawning event for the four spawnings throughout the first molt cycle and the first spawning after the second molt for the control and experimental groups.

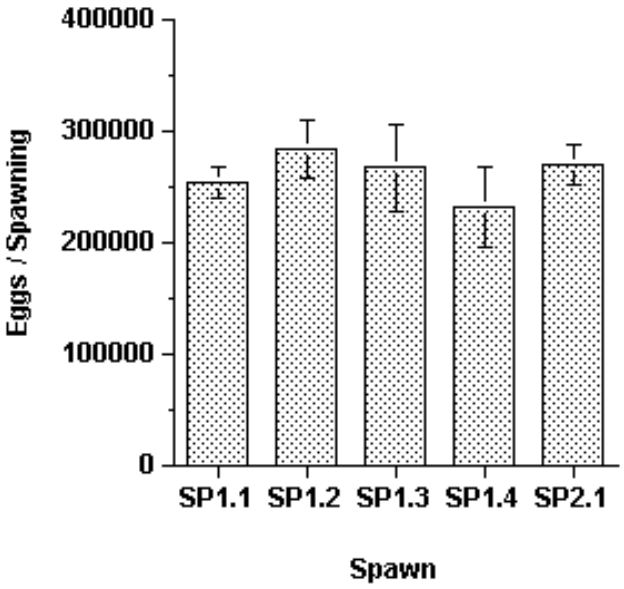

FIGURE 5.6. Average number of eggs for each spawning combining all groups.

Total eggs spawned per spawning event correlate significantly to female body weight (Fig. 5.7). In order to minimise the variance due to the interaction between female body mass and total eggs spawned, the eggs per spawning data set was expressed as eggs spawned per gram of female broodstock weight (Fig. 5.8). There were no significant differences between the controls and experimental groups.

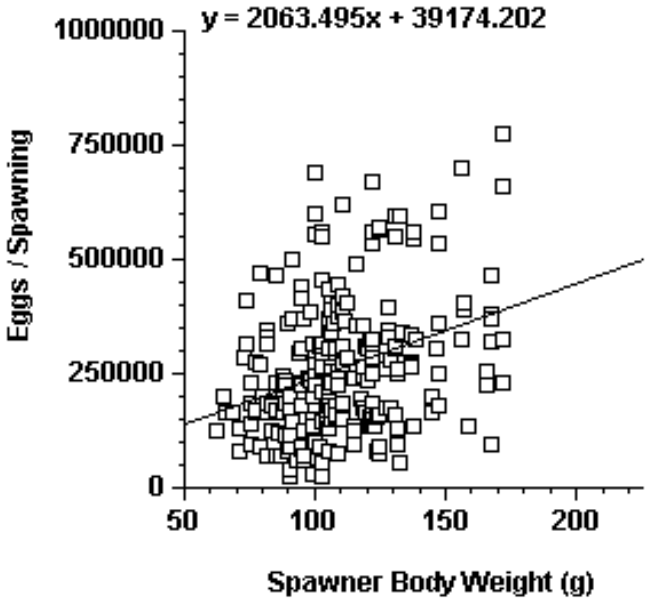

FIGURE 5.7. The relationship between spawner body weight and eggs spawned per spawning event.

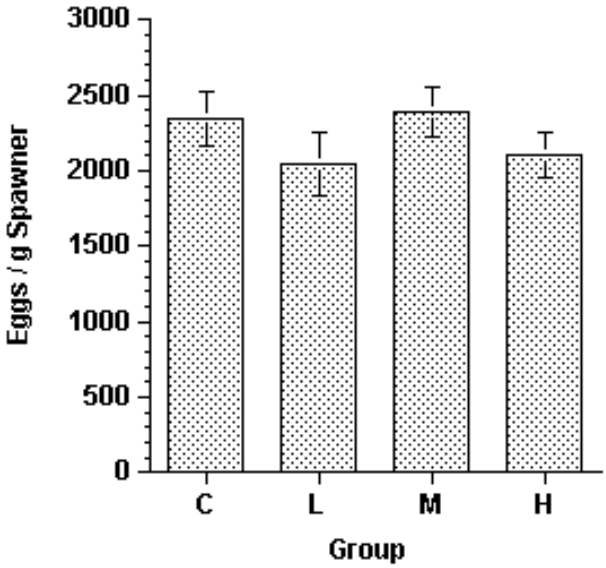

FIGURE 5.8. Average egg output for all spawnings within the first molt cycle expressed as per gram of spawner body weight.

Mean fertility was 68% for all spawnings with no significant differences between groups (Figure 5.9). The fertility rate of the controls was 62% whereas that of the low, medium and high treatment groups were 61%, 72% and 77%, respectively.

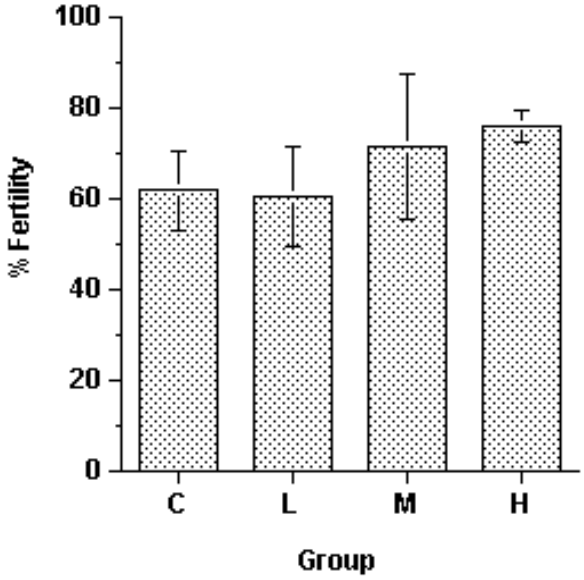

FIGURE 5.9. Fertility rates of eggs spawned from the control and three experimental groups.

Fertility did not vary significantly throughout the molt cycle (Fig. 5.10).

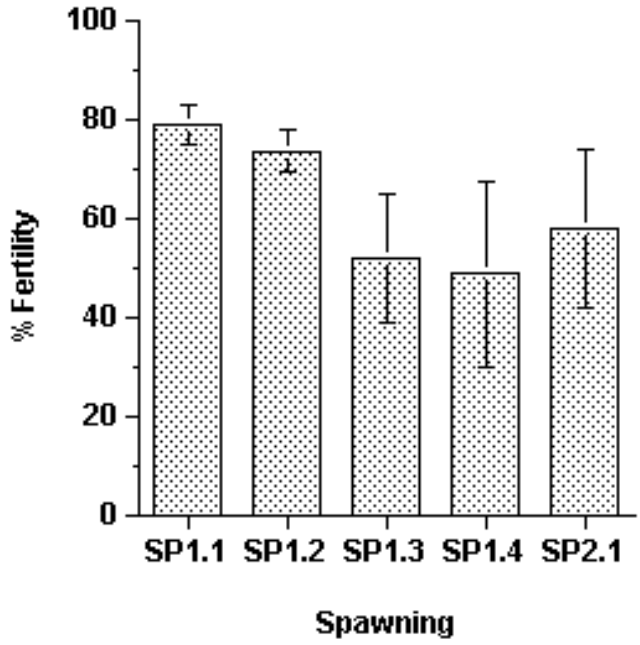

FIGURE 5.10. Average fertility for all groups in sequential spawns.

There was no significant difference in hatching between the groups (Figs. 5.11 and 5.12).

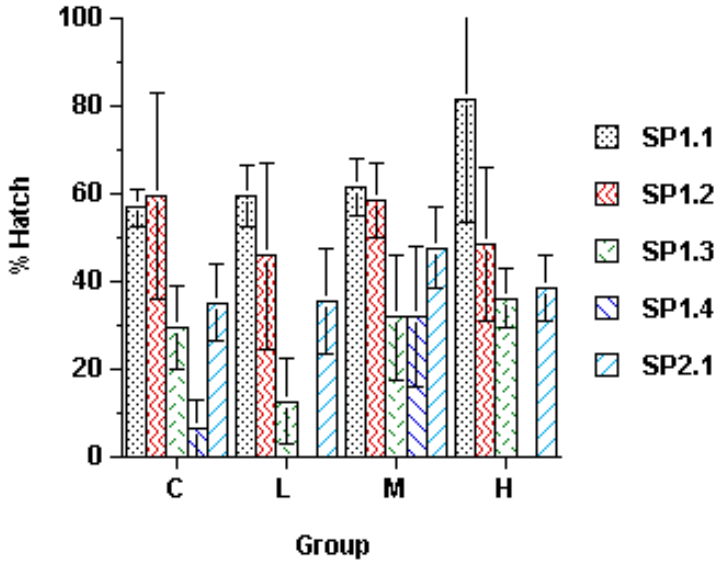

FIGURE 5.11. Percentage of eggs hatching for the spawning events throughout the first molt cycle and the first spawning after the second molt.

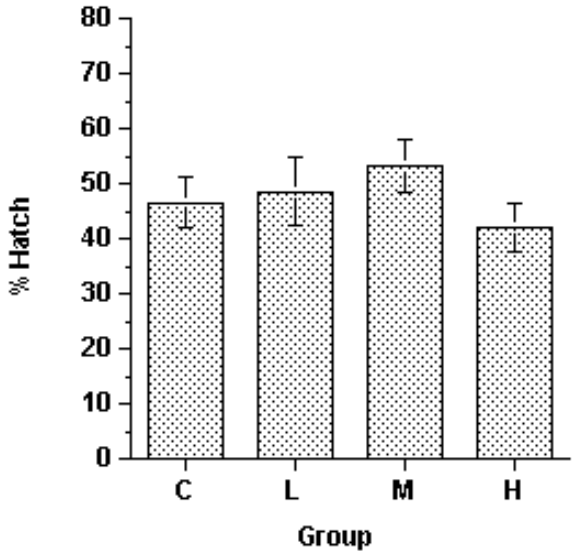

FIGURE 5.12. Combined hatching rates of eggs spawned from the control and three experimental groups.

When the hatch rate was pooled from all groups there was a significant decline between the first and fourth spawning of the first molt cycle, which probably represents exhaustion of the sperm from the thelycum (P<0.5) (Fig. 5.13). The significant difference was lost with the first spawning in the next molt cycle, when the female would have been re-inseminated.

Due to technical difficulties with the egg collection devices, only a subset of spawnings could be determined for percent fertility compared to percent hatching for individual females. In nearly all cases there is not complete hatching success (Figure 5.14).

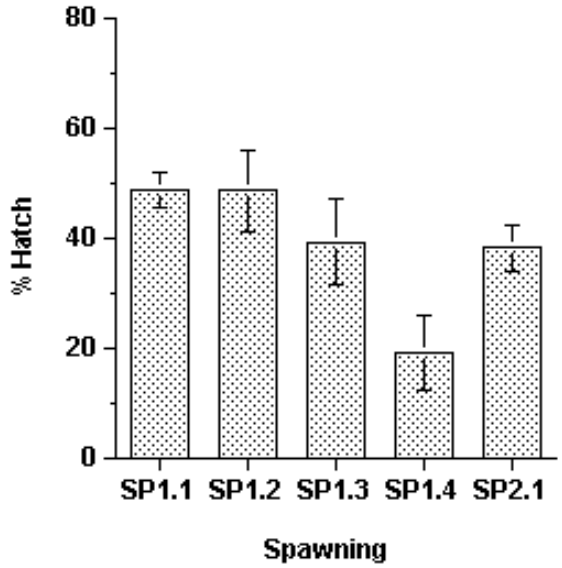

FIGURE 5.13. Average hatch for all groups in sequential spawns.

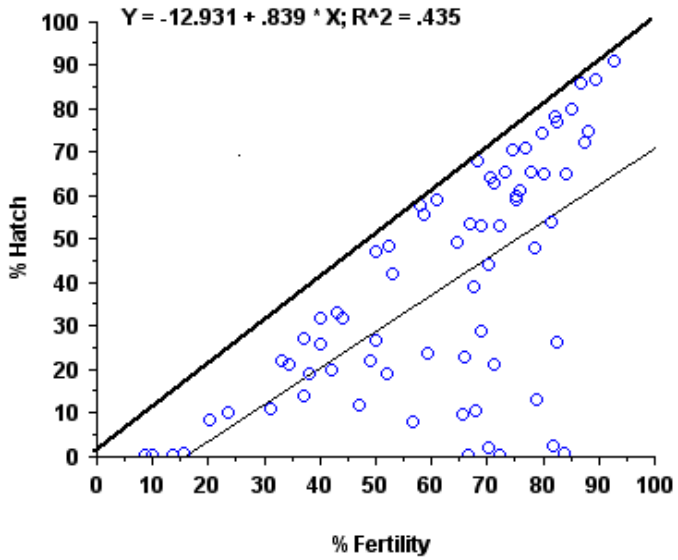

FIGURE 5.14. Relationship between percentage of eggs fertilised and hatching. The dotted line represents a 1:1 relationship and would indicate that all eggs fertilised actually hatched.

Despite the majority of spawnings having over 50% fertility, there are considerable losses due to failure to hatch. The reason for such high failure is unknown but is one area that may be worthy of further study.

There were no significant differences in nauplii output per spawning between the groups (Fig. 5.15). With all spawnings combined, the medium treated group had the highest production of nauplii, expressed as total nauplii output on a per gram spawner basis, but this was not statistically significant (Fig. 5.16).

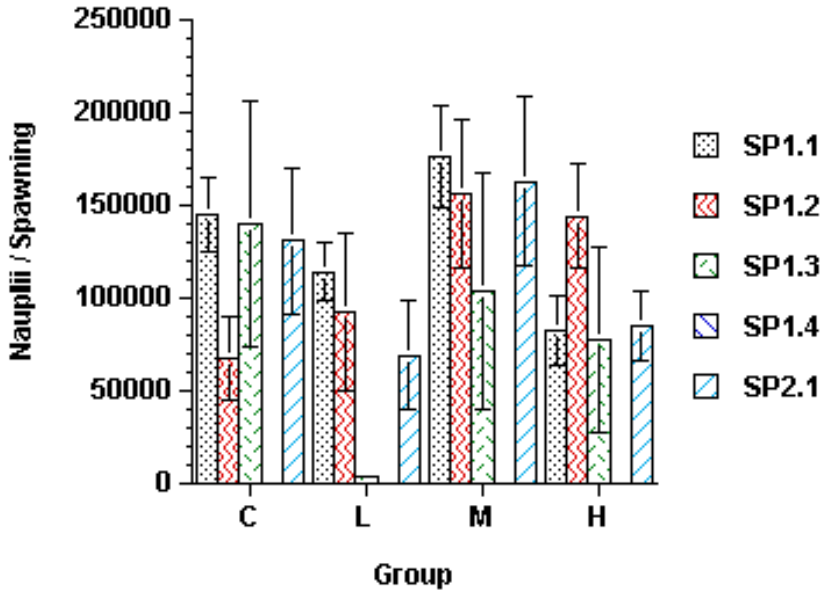

FIGURE 5.15. Number of nauplii per spawning event for the first three spawnings throughout the first molt cycle and the first spawning after the second molt.

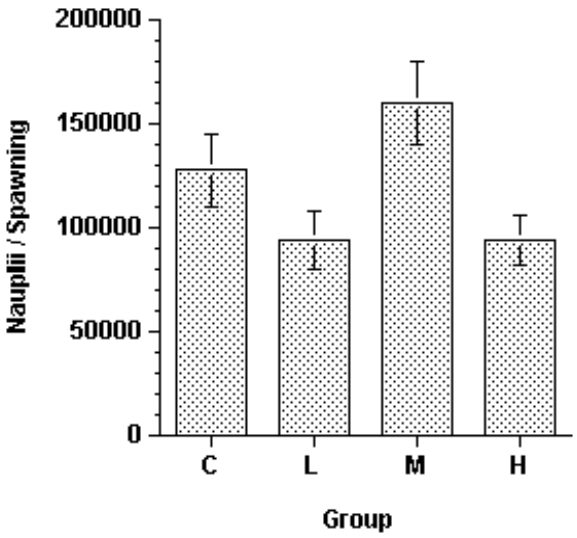

FIGURE 5.16. Number of nauplii produced in all spawnings.

Nauplii production, expressed as per gram of spawner body weight was also not significant between the groups (Figs. 5.17 and 5.18).

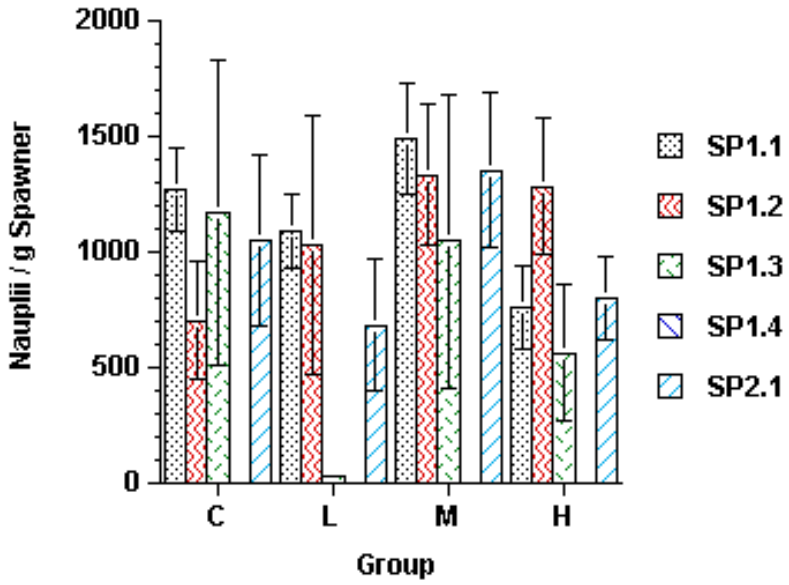

FIGURE 5.17. Number of nauplii per spawning event for the first three spawnings throughout the first molt cycle and the first spawning after the second molt expressed as per gram of female spawner weight.

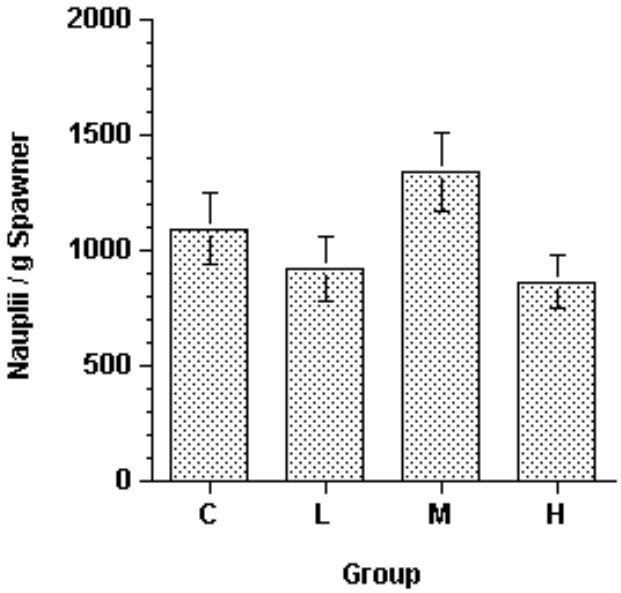

FIGURE 5.18. Number of nauplii produced in all spawnings in controls and the three experimental groups expressed as per gram of female spawner weight.

This study was designed to examine whether the application of exogenous ecdysteroids, in the form of 20-OH ecdysone, would influence reproductive performance and specifically egg quality. Although there was no significant impact on reproductive performance, there was a significant reduction in the inter-molt period. This result demonstrates that ecdysteroid supplementation influences at least one physiological parameter. However, ecdysteroid supplementation in a broodstock maturation diet does not offer any clear benefit to reproductive efficiency.

Benefits

The beneficiaries will be *P. monodon* prawn hatcheries and benefits consist of data of value to assist in the development of domestication programs. Of immediate benefit to hatcheries is the finding that the dietary supplementation of carotenoids in broodstock diets improves nauplii output. The project has also identified that male quality, especially sperm production and/or spermatophore quality, appears to be a major determinant in the typically observed poorer reproductive efficiency of pond or tank-reared broodstock, compared to wild ones. The data obtained indicate that the design of broodstock grow-out facilities needs to allow the reproductive cycling of stock in the grow-out area before they are taken into the hatchery, if female broodstock performance is required at the level obtained from wild broodstock.

The scientific benefits include research tools that are now available for use in further understanding of reproductive performance in penaeid prawns. This includes the development of an enzyme-linked immunoabsorbent assay (ELISA) for detection of vitellogenin, which was developed in associated with the Aquaculture CRC Ltd., and the ability to analyse the biochemical composition of eggs to examine determinants of egg quality.

The project has resulted in the production of materials over and above this final report to benefit the operational training of hatchery personnel. This has included a manual for the determination of egg fertility in *Penaeus monodon*, as a hard copy and website document (http://www.aims.gov.au/pages/research.html). The issue of the life history of *P. monodon* and its consequences for sustainable wild broodstock supply for the industry has also been published and is available on the web at (http://www.aims.gov.au/pages/research/prawns/tiger-prawns/btp-broodstock/btp-broodstock-00.html).

Flow of Benefits

FISHERIES MANAGED BY	COMMERCIAL SECTOR	RECREATIONAL SECTOR	OTHER FISHERIES BENEFICIARIES
NSW	15	-	-
NT	5	-	-
QLD	80	-	-
WA	-	-	-
AFMA	-	-	-
TOTAL	100	-	-

Non-Fisheries Beneficiaries

Summary Flow of Benefits

Total Commercial Sector	100
Total Recreational Sector	-
Total Other Fisheries Beneficiaries	-
Total Non-Fisheries Beneficiaries	-
Summary Flow of Benefits	100

Further Development

The black tiger prawn (*Penaeus monodon*) continues to be the dominant prawn species farmed in Australia, accounting for more than 60% of production in volume. Nevertheless, this sector still relies entirely upon the procurement of wild-broodstock. Such dependence has repeatedly caused production problems for this sector as the supply from the wild is seasonal, and of unknown health status, as well as exhibiting considerable annual variability in supply. At times there have been severe shortages in broodstock supply to the extent that ponds can remain fallow or be stocked late, having longer term supply impacts, or are stocked with alternative species. Although it can be debated whether the wild supply is sustainable, sole dependency upon it does not lead to industry stability. In addition, in recent years it has become apparent that wild broodstock are a vector for the pathogenic gill associated virus (GAV) and the broodstock can pass this virus to the post-larvae, which in turn can have profound consequences on production yields if non-optimal conditions occur during grow-out in the ponds. Because of these risks, the industry is evolving towards the closed-life cycle breeding of *P. monodon* with domestication and eventually selective breeding of specific strains. This will require production of captive-reared broodstock that are capable of sufficient reproductive performance to supply industry's requirements of post-larvae. The understanding and removal of the barriers to the domestication of *P. monodon* is an area that FRDC has committed support to.

Attempts to produce closed-life cycle *P. monodon* broodstock have been made over the past decade in Tahiti, the Philippines, Thailand, Vietnam, Indonesia and the USA. To our knowledge, none of these have succeeded in supplying broodstock on a commercial scale to industry on a sustained basis. The main challenge is still to produce domesticated broodstock that can be induced to perform to a degree sufficient to supply post-larvae on a commercial needs basis.

Issues related to domesticated broodstock reproductive performance that require further improvement are:

- Production of specific pathogen free (SPF) post-larvae for grow-out to broodstock.
- Induction of reproductive cycling in captivity to obtain optimal spawning performance before transfer to the hatchery, i.e. to precondition the ovaries so that the first spawning post-ablation is not the female's first spawning.
- Develop alternatives to unilateral eyestalk ablation as females have limited use if this technique is used, with only first and sometimes second spawnings used in commercial hatcheries.
- Identifying the factors that influence male insemination capability, especially with reference to the production of high quality spermatophores.

Planned Outcomes

The main planned outcomes of the project included research and development (R&D) of a strategic nature, to develop solutions to current and future challenges for the prawn industry. Based on information supplied by the industry itself, the annual publication of industry statistics by the government agencies (QDPI Aquaculture Production Survey – Report to Farmers), indicates that the overall efficiency of broodstock performance is approximately 15%. For example, a reported 2,680 spawners supplied to industry resulted in 121 million post-larvae being sold onto farms. Nevertheless, this has been sufficient to generally meet the industry's requirements. With the pending shift to the use of domesticated broodstock, reproductive performance equivalent to that of wild-sourced ones is required. As captive-reared broodstock typically under-performed in comparison to their wild conspecifics, improvement in broodstock reproductive output is necessary.

Two research tools have been produced which should benefit development of broodstock managerial regimes to improve reproductive performance. The ability to accurately monitor the female response in her ability to initiate egg production is now possible through the use of an assay (enzyme linked immunosorbent assay, ELISA) allowing the measurement of the production of yolk, which is a key component in the development of the ovary to the point of spawning. This assay is also of value in the endeavor to develop an alternative technology to unilateral eyestalk ablation to induce spawning of broodstock on demand. The other research tool that has been developed is a method (two- dimensional polyacrylamide gel electrophoresis, 2D PAGE) to examine the detailed biochemical composition of eggs.

Reproductive performance between captive-reared, including pond-reared and tank-reared, and wild broodstock was compared. These trials included the use of natural matings and artificial insemination, which is of use in the development of families of know lineage for selective breeding. One of the outcomes of these investigations was the demonstration that females require several cycles of ovarian maturation before their egg production is equivalent to that of wild broodstock. Another critical outcome was the demonstration that captive-reared males are often extremely poor at fertilization. The evidence indicates that there is either a significant difference in quality of the spermatophores, based on overall morphology compared to the spermatophore produced by wild caught males, a decrease in sperm production per spermatophore or an infection of either the male reproductive tract or of the spermatophore itself which results in very low fertilization rates, and hence minimal nauplii output from an otherwise adequate (with respect to egg production) spawning.

The project has resulted in a number of publications and manuals, including web based documents, concerning broodstock management and hatchery issues which are listed in Appendix 3.

Conclusion

The project had five major objectives. The objectives were directed towards: the development of research tools to aid investigations into aspects of reproductive performance of *Penaeus monodon* broodstock; aspects of captive versus wild broodstock performance; and dietary supplements to improve spawner performance.

1) To determine the physiological requirements for successful & high vitellogenesis or yolk production.

The major energy and biochemical source for eggs, and hence for the development of the nauplii, is yolk. Packaged in the developing egg with the yolk are other metabolically important substances, such as nucleic acids and a host of hydrolytic enzymes, micro-nutrients and hormonal substances. Yolk is a critical determinant for egg quality. Yolk, in the form of vitellogenin, is found in the blood and is produced in the ovary itself and in the hepatopancreas, before being finally packaged in the egg as vitellin. Presently the only monitoring of ovarian development in broodstock is done overtly by indexing the physical size of the ovary after examination of the female by torchlight. This objective resulted in the isolation and purification of vitellogenin, and the production of an enzyme-linked immunosorbent assay (ELISA) for the measurement of vitellogenin in prawn blood (haemolymph). During a typical ovarian maturation and spawning cycle the level of blood vitellogenin is maximal at a gonadal index of II. The assay allows repeated non-destructive sampling of individuals through a spawning cycle and is valuable in studies that attempt to improve reproductive performance of female broodstock by various means.

2) To identify and chronicle the substances that are accumulated during egg development and their importance in egg viability and larvae survival.

Eggs from batches with high (66%) and low (3%) hatches were analyzed by two-dimensional polyacrylamide gel electrophoresis (2D PAGE). Eggs from the low hatch batches had a greater number of proteins present compared to eggs from high hatch batches. One of the putative gene products identified was a capsid protein, indicative of a viral infection. Based on mortalities of spawners used in the other objectives, it is believed that the broodstock suffered from a viral infection, possibly gill associated virus (GAV), which not only potentially comprised spawner performance but could also lead to vertical transmission of GAV to post-larvae.

3) To compare egg quality between wild and captive-reared broodstock.

Comparison of wild and captive-reared, either pond-reared or tank-reared, broodstock indicated that captive-reared females are eventually capable of egg production equivalent to that of their wild conspecifics. Captive reared females initially under-perform wild broodstock and this is believed to be due to the greater sexual maturity of the wild broodstock as these prawns have probably undergone several breeding cycles before being brought into a hatchery. Captive-reared broodstock do not appear to undergo reproductive cycling until unilaterally eyestalk ablated in the hatchery. Natural matings resulted in higher rates of hatchability compared to those made using artificial insemination. The use of artificial insemination is valuable in the development of known family lines that will be required for selective breeding programs. It is not, however, a technique suitable for routine hatchery production as hatch rates are low compared to natural matings. A major problem in captive-reared broodstock is male quality. The males either produce fewer sperm or a poor quality spermatophore. The males may suffer from a reproductive tract infection, either bacterial or

viral, or inadequacies in the biochemical composition of the spermatophore leading to lower rates of sperm production and to sperm that are incapable of egg fertilization.

4) To demonstrate value of supplementing broodstock diets with carotenoids.

Carotenoids are believed to benefit general metabolism and health, function as anti-oxidants, have provitamin A activity leading to immune enhancement, and play a beneficial role in reproduction as well as general growth and survival. Their main use in prawn aquaculture is purely cosmetic and they are used in finishing diets for colour enhancement before harvesting. Broodstock maturation diets were supplemented with low (0.25 g/kg diet), medium (2.5 g/kg diet) and high (25 g/kg diet) dosages of a synthetic carotenoid in the form of astaxanthin. Controls were given a maturation diet with a minimum quantity of carotenoids which occur naturally in the fish and molluscs that make up fresh diet components. Astaxanthin is the major carotenoid found in *P. monodon*. Egg output per female reached levels of 550,000 eggs per spawning, although there was no difference between treatments. Egg fertility and hatching were not improved by any of the treatments. Although no definitive conclusions could be drawn, there were factors that led to hatching rates being below that of fertilisation, indicating inferior egg quality *per se*. Nauplii output per spawning was improved in females fed a diet containing 2.5 g/kg astaxanthin per kg of maturation diet. This was particularly apparent when examined as nauplii output per gram of female broodstock body weight. Dietary supplementation of broodstock maturation diets with astaxanthin is of benefit in improving nauplii output from female broodstock.

5) To demonstrate value of supplementing broodstock diets with ecdysone.

Ecdysteroids, including ecdysone, are known to influence aspects of molt, reproductive physiology and egg development in penaeid prawns. Broodstock maturation diets were supplemented with low (0.1 mg/kg diet), medium (1.0 mg/kg diet) and high (10 mg/kg diet) dosages of ecdysone in the form of the known active form in crustaceans, 20-hydroxyecdysone (20-OH ecdysone). Controls were given a maturation diet without ecdysone supplementation. There was a significant reduction in all treatment groups in the inter-molt period of the females. There was no significant difference in egg production, hatchability or nauplii output in any of the groups. Dietary supplementation of broodstock maturation diets with 20-OH ecdysone is of no benefit in improving reproductive output from female broodstock.

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Appendix 1: Intellectual Property

No patentable inventions or processes have been developed as part of this project. Selected results have either been published in relevant scientific articles or other public domain literature. The information contained in this report is for broad release. The intellectual property contained in this report is 32% FRDC owned.

Appendix 2: Staff

Mr Matt Kenway	Manager, Tropical Aquaculture Facilities, AIMS
Mr Don Booth	Aquarist Specialist, AIMS
Ms Carol Fraser	Ph D candidate, JCU & Aquaculture CRC Ltd.
Dr Michael Hall	Senior Research Scientist, AIMS
Ms Rebecca Mastro	Experimental Scientist, AIMS
Mr Matthew Salmon	Experimental Scientist, AIMS
Ms Jan Strugnell	Experimental Scientist, AIMS
Mr Neil Young	Experimental Scientist, AIMS
Mr Rick Willis	Mass Spectrometer/NMR Manager, AIMS
Dr Kate Wilson	Senior Research Scientist, AIMS

Appendix 3: Project Outputs

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Hall MR, Young N, and Kenway M. Manual for the Determination of Egg Fertility in *Penaeus monodon*
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