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JAMES COOK UNIVERSITY AUSTRALIA

THE EFFECT OF NUTRITION ON REPRODUCTIVE PARAMETERS IN MALE BARRAMUNDI, *Lates calcarifer* (Bloch).

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for the degree of Doctor of Philosophy
in the School of Marine Biology and Aquaculture
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TO MY BELOVED PARENTS

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ABSTRACT

Fish reared in aquaculture must be provided with an adequate diet for growth and reproduction. Although there is a paucity of literature on broodstock nutrition generally, particularly for male fish, it is known that nutrition has profound effects on reproductive physiology. Most of the available information documents dietary effects on direct reproductive outcomes, such as fecundity, spawning performance, egg and larval quality. Information on the mechanisms by which these processes are regulated is rare and this lack of understanding is one of the major constraints to improved broodstock management.

Barramundi, *Lates calcarifer* (Bloch), also known as Asian sea bass or giant perch, is a highly valued food fish forming the basis of major fishery and aquaculture industries in Australia and south-east Asia. However, hatchery production of barramundi continues to be problematic, with unpredictable male fish performance in hatcheries. There is no published literature on the relationship between nutrition and reproduction in barramundi and brood fish are generally fed trash fish supplemented with vitamins. In order to contribute to our understanding of brood stock nutrition, particularly of male barramundi, this study has investigated the effect of three major nutritional regimes - feeding frequency (food deprivation), dietary protein:energy ratio and dietary fatty acid profile - on standard nutritional indices such as growth and body composition and on plasma steroid concentrations, GSI and histological stage of the gonads of male barramundi.

To test the effect of feeding frequency, groups of relatively small(700 ± 25 g) or relatively large (1000 ± 70 g) male barramundi were fed daily (D), every three days (3D) or every seven days (7D) for 24 weeks. A fourth group of each size was starved for 12 weeks and refed for a further 12 weeks. Starvation resulted in loss of body weight and tissue nutrients, while nutrients were regained and compensatory growth occurred during the refeeding period. Low feeding frequency (7D) resulted in reduced or no growth in fish, whilst fish fed more frequently (D or 3D) showed higher growth. These findings were also reflected in improved body composition of fish fed more frequently. In general, gonads were found to be cycling with a range of stages of development including development of gonia cells and appearance of spermatocytes and spermatids apparent in any particular population (treatment group) of fish.

However, a larger proportion of testes from barramundi that were starved were immature compared with those of fish in other treatments. Feeding regime affected the hormone production in the smaller and larger fish differently. Although starvation or low nutrient intake did not influence the low Oestradiol-17β (E_2) levels in small fish, the relatively higher E_2 level in larger fish was clearly reduced by starvation. In contrast, relatively high Testosterone (T) level of small fish was reduced by starvation but low T level was not influenced by starvation in large fish. In the less extreme treatments, the effects were not as clear. The large fish in D or 3D regimes increased plasma T level although not significantly and plasma E_2 level significantly decreased at week 18. A period of refeeding after starvation clearly influenced the concentration of plasma steroid hormones in both size groups.

To test the effect of protein:energy ratio, groups of male barramundi were presented with diets containing 50% protein and either 15 $MJ.kg^{-1}$, 18 $MJ.kg^{-1}$, 21 $MJ.kg^{-1}$ or 24 $MJ.kg^{-1}$ by varying dietary lipid for a 24 week period. Comparable growth was observed in all animals with low energy diets being consumed at a greater rate to compensate for the lack of energy. Body composition analysis showed that high dietary energy led to greater fat storage. As with the starved animals, gonads of fish fed the lowest energy diet were consistently found to be at an early developmental stage with tightly packed gonia cells and gonads of fish in other treatments showed evidence of normal cycling. Dietary energy level did not significantly affect the plasma hormone levels in male barramundi. Plasma E_2 was relatively low throughout and did not show differences between treatments. Plasma T level reduced with time and similarly did not vary between treatments. Plasma 11keto Testosterone (11kT) did not show any particular trend with dietary protein:energy ratio, even in the lowest energy diet.

To test the effect of dietary fatty acid levels, groups of fish were fed with one of four diets (50 % protein, 21 $MJ.kg^{-1}$) containing either linseed oil (enhanced levels of 18:3 n-3), soybean oil (enhanced levels of 18:2 n-6), fish oil (enhanced levels of 20:5 n-3 and 22:6 n-3) or Aquagrow (enhanced levels of 20:4 n-6) for 18 weeks. Fish fed a diet high in short chain n-3 fatty acids derived largely from linseed oil had lower growth than those of other treatments. Fish fed short chain n-6 and long chain n-3 fatty acids had intermediate growth and fish fed long chain n-6 had the highest growth. Tissue fatty acid profiles were highly correlated with the dietary fatty acid

profile, but evidence was also obtained that barramundi preferentially accumulate long chain HUFA into the gonad. No apparent effect on the stage of gonadal development as a result of dietary fatty acid profile was observed. Nor did dietary fatty acid profile affect the circulating concentration of plasma T or E_2. Plasma E_2 level was low at week 18 and plasma T level showed similar changes in all treatments. Plasma 11kT level clearly declined with time and it is suggested that this may have been in response to the high dietary fatty acid levels negatively affecting hormone production. Alternatively, high dietary fatty acid levels used in this study may have inhibited plasma T production with subsequent decreases in production of 11kT from its precursor T.

The principle conclusion from this study is that extreme cases of nutrition (starvation, refeeding, low dietary energy) impact on male barramundi reproductive development, but under less extreme conditions, there appeared to be little effect. This is in agreement with some of the data in the literature, which indicates that male fish expend less energy on reproduction and so are less affected by moderate changes in nutritional conditions. Conclusions regarding the effects of the moderate treatments are constrained since there appeared to be other circumstances impacting upon the experiments. Difficulties experienced with poor quality feeds and resulting long acclimation periods, disease events and the fact that relatively small gonad sizes observed in all experiments, even in the presence of gametogenesis indicate that the conditions may not have been ideal for reproductive development.

Thus, this study must be considered a preliminary investigation. It does nevertheless provide a significant platform for future work regarding the effects of nutrition on male barramundi broodstock development and teleost reproduction in general.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Abbrevation used	Full name
11KT	11 keto Testosterone
E_2	Oestradiol-17β
GnRH	Gonadotropin releasing hormone
GtH I	Gonadotropin I
GtH II	Gonadotropin II
HCG	Human Chorionic Gonadotropin
LHRHa	Luteinizing hormone releasing analogue
PG	Prostaglandin
T	Testosterone
GABA	δ-amino-butyric acid
SCC	Side-chain clevage system
LH	Luteinizing hormone
FSH	Follicular stimulating hormone
AA	Arachidonic acid
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
ALA	α-linolenic acid
LA	Linolenic acid
HUFA	Highly unsaturated fatty acids
PUFA	Poly unsaturated fatty acids
ETA	Eicosatrienoic acid
17,20β-DP	17,20β-Dihydroxy-4-pregnen-3-one

GLOSSARY OF COMMON AND SCIENTIFIC NAMES

Common name	*Scientific name*
African catfish	*Clarias gariepinus*
Arctic charr	*Salvelinus alpinus*
Atlantic herring	*Clupea harengus*
Atlantic salmon	*Salmo salar*
Barramundi	*Lates calcarifer*
Black bream	*Acanthopagrus butcheri*
Black porgy	*Acanthopagrus schlegeli*
Blue cod	*Parapercis colias*
Brown trout	*Salmo trutta*
Chinook salmon	*Oncorhynchus tshawytscha*
Chub	*Leuciscus pyrenaicus*
Chum salmon	*Oncorhynchus keta*
Cod	*Gadus morhua*
Common carp	*Cyprinus carpio*
Crucian carp	*Carassius auratus*
Demoiselle	*Chromis dispilus*
English sole	*Parophrys vetulus*
European eel	*Anguilla anguilla*
European sea bass	*Dicentrarchus labrax*
Gilthead seabream	*Sparus aurata*
Gold fish	*Carassius auratus*
Greenback flounder	*Rhombosolea tapirina*
Grey mullet	*Mugil cephalus*
Japanese eel	*Anguilla japonica*
Macquarie perch	*Macquaria australasica*
Masu salmon	*Oncorhynchus masou*
New Zealand snapper	*Pagrus auratus*
Nile tilapia	*Oreochromis niloticus*
Orange mouth carvina	*Cynoscion xanthulus*
Plaice	*Pleuronectes platessa*
Red drum	*Sciaenops ocellatus*
Rainbow trout	*Oncorhynchus mykiss*
Siberrian sturgeon	*Acipenser baeri*
Sole	*Solea vulgaris*
Spiny damselfish	*Acanthochromis polyacanthus*
Three spined stickle back	*Gasterosteus aculeatus*
Turbot	*Scophthalmus maximus*
White bass	*Morone chrysops*
White grouper	*Epinephelus aenus*
White perch	*Morone Americana*
Yellow perch	*Perca flavescens*

CHAPTER 1
LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Barramundi, *Lates calcarifer* (Bloch), also known as Asian sea bass or giant perch, is a large percoid fish (Reynolds and Moore, 1982), distributed in the tropical to semitropical waters of the Indo-Pacific region (Davis 1982, Moore, 1982) from the Arabian Gulf to China, Taiwan, Papua New Guinea and Northern Australia (Kailola *et al.*, 1993, Guiguen *et al.*, 1994). It is a freshwater, estuarine and coastal fish and migrates downstream for spawning (Russell and Garrett, 1985; Grey, 1987). In Australia, barramundi are distributed from the Ashburton river (22° 30'S) in Western Australia along the northern coast to the Noosa river (26° 30'S) in Queensland. This species is a highly valued food fish throughout its natural distribution and supports both commercial and recreational fisheries (Russell and Garrett, 1985; Grey, 1987; Guiguen *et al.*, 1994), forming the basis of a major inshore gill-net fishery throughout Northern Australia. Commercial fisheries for barramundi have come under increasing regulation, and fisheries' effort is being reduced to sustain breeding stock levels (Kailola *et al.*, 1993).

Barramundi have been the subject of considerable research over the last decade and a half, most of which is clearly identifiable as either providing information relevant to wild stock management (Moore, 1979, Davis, 1982; Davis, 1984a; Davis, 1985; Copeland and Grey, 1987; Garrett, 1987, Rimmer and Russell, 1998; Doupe *et al.*, 1999) or supporting the development of aquaculture (Copeland and Grey, 1987; Guiguen *et al.*, 1994, Barlow *et al.*, 1996).

The fast growth rate, large size and delicious flavoured flesh of barramundi have made it an excellent fish for aquaculture. In addition, the fact that this fish is an euryhaline species which can be grown in salinities ranging from fresh water to sea water, is a valuable attribute for a species being cultivated in areas subject to monsoonal conditions (Kailola *et al.*, 1993).

Techniques for culturing barramundi were first developed in Thailand in the early 1970's (Maneewong, 1987) and considerable progress in aquaculture techniques has been achieved since this time. The culturing of barramundi has expanded throughout the Indo-Pacific region over the last few years and this species is now being farmed in Thailand, the Philippines, Indonesia, Malaysia, Singapore, Burma,

India, Vietnam, Taiwan, China, Australia (Grey, 1987) and Israel (Anderson, pers. comm.).

In Australia, research into culturing barramundi began in 1984 and these fish are now being farmed throughout the country with the exception of Tasmania (O'Sullivan, 1998). Eggs for culture operations initially came from wild broodstock, a practice which was expensive, unreliable, highly seasonal and conflicted with wild stock resource management (Barlow *et al.,* 1996). In addition, the variability of supply of fish seed from year to year increased costs and limited the growth of the culture industry Therefore, most hatchery operations now retain captive broodstock (Kailola *et al.*, 1993).

Barramundi are protandrous hermaphrodites (Moore, 1979, Davis, 1982), undergoing sex inversion during their life cycle. Most barramundi mature first as males and function as males for at least one spawning season before undergoing sex inversion (Kailola *et al.*, 1993). Sex inversion usually takes place between 6 - 8 years of age after several spawnings as males (Moore, 1979; Davis, 1982; Guiguen *et al.*, 1994). Kailola *et al.* (1993) reported that sex inversion occurs in fish aged between 4 and 8 years and between 3 and 7 years in the Northern Territory and Gulf of Carpentaria respectively.

There is an apparent shift to younger ages for sex inversion in cultured stocks of barramundi. It has been reported that significant numbers of cultured fish harvested at less than 18 months are females while cultured males often last only a single spawning before changing sex at 2 years of age (Australian Barramundi Farmers Association (ABFA), pers. comm.). Thus, it is difficult to keep mature males in the broodstock for extended periods resulting in difficulties in managing hatcheries and in conducting breeding programs. Exacerbating this problem is poor male performance, further limiting the efficient operation of commercial hatcheries (ABFA, pers. comm.).

In this review I will present information on the reproductive biology of barramundi and the hormonal and nutritional regulation of reproduction in fish.

1.2 Reproductive biology of barramundi

.1.2.1 Life cycle

Studies in Northern Australia and Papua New Guinea have identified that barramundi have a complex life history (Moore, 1979, 1982; Moore and Reynolds, 1982; Davis, 1982; Russell and Garrett, 1985; Griffin, 1987; Garrett, 1987), being both protandrous and catadromous. Mature barramundi can be found in estuaries and associated coastal areas or in the lower reaches of the rivers, whereas larvae and young juveniles live in brackish temporary swamps associated with estuaries. Post larvae enter into the coastal swamps which are filled with fresh water as the wet season develops and juveniles remain in this relatively safe and productive environment. As these temporary habitats dry up juveniles move into the main stream to migrate upstream to fresh water habitats (Kailola *et al.*, 1993). Generally these young fish remain in fresh waters up to 3-4 years of age and maturing males migrate downstream to spawn at the end of the dry season.

1.2.2 Breeding season

Although barramundi have a single annual spawning season (Garrett, 1987), the timing and duration of the breeding season vary between regions and river systems in Australia (Davis, 1985). The breeding season is synchronised with the monsoon rains. so the juveniles can take advantage of the resultant aquatic habitats in nearby coastal swamps. In Queensland, the spawning period extends from October to March (Garrett, 1987). In Western Australian rivers, spawning occurs between September and February after monsoonal rainfall (Kailola *et al.*, 1993). Davis (1985) observed that the breeding season was from September to February (1978/79) in Van Diemen Gulf and from November to March (1979/80) in the Gulf of Carpentaria with spawning occurring just before the summer monsoon. In Papua New Guinea, the spawning season of barramundi commences in October and the peak season is from November to January (Moore, 1982).

1.2.3 Factors affecting spawning

Migration to spawning areas and maturation of gonads is thought to be triggered by increasing water temperatures which occur at the end of the dry season

(Grey, 1987). Relatively high salinity is an essential factor for spawning. In Northern Australia and Papua New Guinea, spawning occurs in saline waters (28 - 36‰) (Moore, 1979; 1982; Moore and Reynolds, 1982; Davis, 1982; 1985) near the mouths of rivers. Fish with running-ripe gonads have been found only in the lower estuaries of rivers and their adjacent foreshores in Queensland, Australia (Russell and Garrett, 1985; Garrett, 1987) and in coastal waters in Papua New Guinea (Moore, 1982). Davis (1985) observed that in Northern Australia, spawning was localised near river mouths on coastal mudflats or upstream in rivers where salinities were relatively high (30‰).

No matured fish with ripe gonads have been observed in non-flowing fresh water or landlocked regions (Dunstan, 1959 cited in Garrett, 1987). Furthermore, Davis (1985) observed that gonad development was retarded in landlocked freshwater barramundi in the Gulf of Carpentaria. Moore (1982) examined nearly 5200 fish from fresh, brackish and salt water in Papua New Guinea, and observed that gonad development does not normally proceed beyond developing stage (not maturing) in inland habitats. Thus, available evidence indicates that increased temperature and salinity are necessary conditions for spawning to occur.

1.2.4 Spawning grounds

The migration patterns of barramundi in Papua New Guinea showed that adult barramundi from an extensive area of western Papua migrate to a specific spawning ground during the spawning season and return back to inland waters subsequently (Moore, 1982; Moore and Reynolds, 1982). The inadequate salinities associated with high discharge of freshwaters in local areas in western Papua necessitate the catadromous migration of adults to a specific spawning ground with suitable salinity (Moore, 1982). However, there was no evidence of coastal migration of barramundi to specific spawning grounds in the Van Diemen Gulf and the Gulf of Carpentaria in Australia (Davis, 1985). The rivers of north-eastern Queensland have an insignificant discharge of fresh water, thus allowing conditions for spawning in localised areas (Russell and Garrett, 1985). Maturing male barramundi migrate downstream from fresh water habitats to brackish waters at the start of the wet season to spawn with females which usually inhabit estuaries (Grey, 1987).

1.2.5 Spawning stimulus

A close relationship between spawning and the start of the wet season has been observed in Papua New Guinea (Moore, 1982). If the wet season is delayed, then the spawning is also likely to be delayed suggesting that the fish are responding to some environmental stimulus. Moore (1982) suggested that a very strong odour from decomposing vegetation of swamps associated with the initial rains could act as a stimulus to adult fish to spawn.

1.2.6 Sexual maturity

As previously stated, most barramundi mature first as males and function as males for one or more spawning seasons before changing sex at 6 - 8 years of life (Grey, 1987). However, the time taken to first maturity varies in different areas (Kailola *et al.*, 1993). Davis (1982) reported the length of males at first maturity in the Northern territory and South-eastern Gulf of Carpentaria were 600 and 550 mm, respectively. Most fish of these areas change their sex at lengths of 900 and 850 mm, respectively. However he concluded that these size differences were due to slower growth of fish in Gulf of Carpentaria, both reproductive processes (maturity and sex change) being related to age rather than to size.

In 1984, Davis (1984a) identified a sexually precocious population of barramundi in the northern Gulf of Carpentaria. These precocious males matured in their first or second year of life at body lengths of ~ 250 mm. The stunted growth of fish in these stocks was probably the result of sexual precocity (Davis, 1984a). Sex inversion also occurred earlier; females were much smaller than normal and no males larger than 500 mm were found. Stunting is presumed to be caused by the channelling of energy into gonadal growth at the expense of somatic growth at a relatively early age, although the exact cause for the stunted population in the northern part of the Gulf of Carpentaria is not known. These northern rivers are characterised by sandy bottoms, less turbidity, and less flooding in the wet season due to smaller catchment areas, compared to the southern rivers. Also, the northern rivers flow through alluvial deposits of bauxite. Smaller females (360-400 mm) have also been found in rivers with similar conditions in Melville Bay in the Western Gulf (Davis, 1984a). This

author concluded that some combinations of these factors may be contributing to the occurrence of a sexually precocious population in the northern Gulf of Carpentaria.

1.2.7 Sex inversion

Sex inversion of barramundi was first reported by Moore (1979) in Papua New Guinea. The protandry of barramundi stocks has also been reported in different areas in Australia (Davis, 1982; 1984a; Garrett, 1987; Russell, 1987).

Testes and ovaries

A detailed investigation of the reproductive biology of barramundi was done by Moore (1979), Davis (1982) and Guiguen *et al.* (1994). The testes are paired flat, elongated organs lying between the swim bladder and the ventrolateral wall of the abdominal cavity. The ovaries are paired, hollow organs situated ventrolaterally in the abdominal cavity. Due to the strong dimorphism that exists between testis and ovary (Moore, 1979; Guieguen *et al.*, 1994), a complete reorganization of gonad structure as well as function is required for a sex inversion.

Transitional gonads

Transitional gonads are difficult to distinguish macroscopically from testes (Davis, 1982) except that there is a slight darkening of the ventral surface of the gonad due to increased blood supply (Moore, 1979). Guiguen *et al.* (1994) observed that all transitional gonads were smaller than the testis and were red-pink in colour. This is probably due to the extensive peripheral blood supply to the gonads, which can also be seen in histological cross-sections. No spermatogenetic tissue remained by the time the external appearance of the gonad had changed (Davis, 1982; Moore, 1979), so the only reliable method to detect transitional gonads is the histological examination of gonads stained with haematoxylin and eosin. Moore (1979) and Davis (1982) found few synchronous hermaphrodite gonads with well developed male and female tissue which were regarded as abnormal because they do not represent a transition stage between male and female.

Sex inversion of barramundi is strongly linked with the breeding season and the transition is completed shortly after spawning in Papua New Guinea (Moore, 1979), Australia (Davis, 1982) and Tahiti (Guiguen *et al.*, 1994). Very few

hermaphrodite gonads are detected due to the rapid transition phase from male to female (Moore, 1979; Davis, 1982). A very rapid inversion of testes to ovaries was confirmed by Moore (1979) in his biopsy experiments. A fish producing milt on 25 March 1975, had changed to a normal female without any trace of male tissue when dissected on 15 June 1975, after nearly 11 weeks. In another example, Guiguen *et al.* (1994) reported that complete sex inversion, from a flaunt male to a newly inverted female, took place in less than 17 days.

Sex inversion occurs in inland waters in Papua New Guinea (Moore, 1979) but in tidal waters in Australia (Davis, 1982). These observations have been related to the more clearly defined spawning and post-spawning migrations of barramundi in Papua New Guinea (Reynolds and Moore, 1982) which is not observed in barramundi in Australia (Davis, 1986). In Papua New Guinea, fish migrate inland from their specific spawning grounds immediately after spawning, whereas in Australia large fish tend to stay in tidal waters (Davis, 1982).

1.2.8 Maturity stages

Guiguen *et al.* (1994) studied gonadal histology of barramundi in detail and identified different maturation stages in males, females and transitional fish which are detailed in Table 1.1. Furthermore, Guiguen *et al.* (1994) reported that barramundi have longitudinal homogeneous gonads which makes possible a correct assessment of the gonadal stages using only a single cross section of the gonad.

Fecundity

Female barramundi are one of the most fecund fish producing up to 10×10^6 eggs at 100 cm length and $30 - 40 \times 10^6$ eggs at 120 cm length (Davis, 1984b). Fecundity ranging from 2.3×10^6 to 32.2×10^6 eggs/female was recorded for barramundi (weighing 7.7 to 20.8 kg) in natural environments in Papua New Guinea (Moore, 1982). Lim *et al.* (1986) observed that overall fecundity ranged from 0.8 - 3.1 $\times 10^6$ eggs/female (mean 1.73×10^6 eggs/female) when fish were induced to spawn by hormonal injection. In the Philippines, female barramundi subjected to different treatment methods, (e.g., pellets, pumps or repeated injections of gonadotropin.

Table 1.1 Maturation stages of male, transitional and female barramundi (Guiguen, *et al.*, 1994).

Sex	Stage		Description
Male	M1	- Testis gonia stage	Predominance of gonia within the testis.
	M2	- Spermatogenesis stage	Predominance of spermatocytes and spermatids.
	M3	- Spermiation stage	Large parts of the testis were filled with spermatozoa, and the lobular structure of the testis was no longer distinguishable in these areas.
	M4	- Post-spawning stage	Most of the testicular lobules were devoid of spermatozoa, but some gonia and spermatogenic cysts remained in the lobule periphery.
Transitional	T1		Degeneration of male testicular tissue
	T2		Appearance of ovarian tissue with still degenerating testicular tissue
	T3		Ovarian tissue < 50% within histological cross-section (no testicular tissue)
	T4		Ovarian tissue > 50% within histological cross-section (no testicular tissue)
Female	F1	- Previtellogenesis stage	Gonia and previtellogenic oocytes
	F2	- Early vitellogenesis stage	Vitellogenic oocytes < 50% within histological cross-section
	F3	- Vitellogenesis stage	Vitellogenic oocytes >50% within histological cross-section
	F4	- Atretic stage	Oocytes undergoing atresia

releasing hormone analogue (GnRHa)), produced variable numbers of eggs but did not show any relationship with body size. Fecundity ranged from less than 1 x 10^6 eggs/female to 9 x 10^6 eggs/female with body weights varying from 1.7 to 6 kg (Almendras *et al.*, 1988). Thus, fecundity of female barramundi has varied largely among studies, probably due to the different fish sizes and treatment methods used in these studies.

1.2.9 Induced breeding

In most countries, barramundi cultured broodstock are induced to spawn in captivity by hormonal or environmental stimulation. Successful breeding has been achieved in Singapore by inducing brooders to spawn spontaneously in captivity by a single intramuscular injection of either an analogue of the luteinizing hormone releasing hormone analogue (LHRHa) or human chorionic gonadotrophin (HCG) (Lim *et al.*, 1986). The hormones are administered intramuscularly below the dorsal fin and the dosages used vary according to the maturation conditions of the spawners and their sex.

In Australia, sexually ripe fish are induced to spawn by intramuscular injection of LHRHa (Garrett and O'Brien, 1994). In the Philippines, induced spawning of barramundi has been achieved by using HCG, crude extracts of pituitary or gonads, salmon gonadotropin or LHRHa (Fortes, 1987; Parazo *et al.*, 1998). Stripping running-ripe fish from spawning grounds and artificial fertilisation of eggs is also a common method used in barramundi breeding in Thailand (Maneewong, 1987) and in Singapore (Lim *et al.*, 1986).

1.2.10 Feeding of broodstock

Little information is available on the feeding strategies used for barramundi broodstock in commercial hatcheries, probably due to the commercial importance of this type of information. Generally, broodstock are fed with trash fish but the quality and quantity would be different in various hatcheries. In Singapore, barramundi broodstock are fed with trash fish once a day at 2 - 3% Bwt.day^{-1}, and feeding rate is reduced to 1 - 2% Bwt.day^{-1} during the spawning season (Cheong and Yeng, 1987; Lim *et al.*, 1987). In Australia, trash fish mixed with vitamin mixture is used to feed

barramundi broodstock and feeding level varied in different hatcheries from being fed to satiety daily to every 3 days (Anderson, pers. comm.).

1.3 Reproduction in fish

Reproduction is a very complex dynamic process (Sanchez-Muros *et al.*, 1991) which involves large changes and tissue reorganization in maturing gonads of animals. Gonadal maturation often involves many months of preparation and growth, with the release of gametes generally comprising a short period in the developmental process (Shepard and Bromage, 1988). The maturing process supposes a series of metabolic and structural changes that are conducted and regulated by hormones. These mechanisms and interrelationships are not understood (Sanchez-Muros *et al.*, 1991).

It is well known that some environmental and nutritional factors influence reproduction. For most wild fish, spawning is an annual event which coincides with favourable external conditions and the supply of available food, which are critical factors for the survival of fertilised embryos and developing fry (Shepard and Bromage, 1988). Most fish use seasonal patterns of changing day length and temperature, the presence of food or the onset of rainfall to time gonadal maturation and spawning accurately. Although external environmental factors are responsible for the initial induction and subsequent course of reproduction, it is the endocrine hormones which directly control this process.

1.4 Hormonal regulation of reproduction

1.4.1 Hypothalamic hormones

In response to changes in environmental factors, the hypothalamus secretes the gonadotropin releasing hormones (GnRH), which control reproduction as well as other vital functions in vertebrates such as growth and water balance (Sherwood and Coe, 1991). GnRH, a neuropeptide, is not a single molecule but a family which contains different forms of molecules known as Chicken I, Chicken II, Mammal, Catfish, Salmon, Lamprey I and III, Seabream and Dog fish GnRHs (Sherwood and Coe, 1991; Sherwood *et al.*, 1994; Kobayashi *et al.*, 1997). All of these forms except Chicken GnRH I (cGnRH I) have been isolated from brain extracts of fish (Sherwood

and Coe, 1991). Each form has ten amino acids in length and shares 50% or more sequence identity.

In teleost fish, multiple forms of GnRH are present within the tissue of a single species (Goos, 1991). For example, two forms of GnRH (Salmon and Chicken-II) were found in salmon (Sherwood and Coe, 1991), goldfish (*Carassius auratus*) (Murthy *et al.*, 1991), medaka (*Oryzias latipes*) (Powell *et al.*, 1996) and zebra fish (*Brachydanio rerio*) (Powell *et al.*, 1996). Catfish and chicken II GnRHs were found in *Claraias macrocephalus* (Thai catfish), *C. batrachus* (Walking catfish) (Ngamvongehon *et al.*, 1991) and in *C. gariepinus* (African catfish) (Goos *et al.*, 1991; Ngamvongehon *et al.*, 1991).

In 1994, Seabream GnRH (sbGnRH) was found in the brain of gilthead sea bream, *Sparus aurata* (Powell *et al.*, 1994) in addition to salmon GnRH and chicken II GnRH. This species was the first vertebrate from which three forms of GnRHs were isolated. Since then, three GnRHs were found in several other teleosts including the striped bass (Gothilf *et al.*, 1995), grass rock fish (*Sebastes rastrelliger*) (Powell *et al.*, 1996), the tilapia (Parhar and Sakuma, 1997) and red sea bream (Okuzawa *et al.*, 1997).

Biological significance and control of GnRH

The biological significance of these multiple forms of GnRHs within the same fish is not clear. It may be that distinct forms of GnRH differ in function (Sherwood *et al.*, 1994). In gold fish, two native forms of GnRH (Catfish and Chicken II) act through the same population of receptors on gonadotropes, and both forms stimulate gonadotropin (GtH) release (Murthy *et al.*, 1991). Gothilf *et al.* (1995) have shown that in gilthead seabream and striped bass, the sbGnRH producing cells were found in the preoptic area of the brain, a region known to control gonadotropin secretion, while sGnRH and cGnRH-II were found in the terminal nerve and midbrain tegument respectively. In addition, sbGnRH is the dominant form in sexually mature fish of both species, while cGnRH-II is not found in the pituitary and sGnRH is found in very low levels. Gothilf *et al.* (1995) suggested that sbGnRH is the most relevant form for the regulation of Gonadotropin II (GtH II) release in sexually mature fish.

Holland *et al.* (1998) detected both sbGnRH and cGnRH-II in the pituitary, irrespective of reproductive state or sex of gilthead sea bream. Early recrudescent fish had similar levels of both GnRHs in the pituitary. As the gonads developed, sbGnRH levels increased significantly while cGnRH-II levels remained unchanged. Mature fish also showed high plasma GtH II levels which were positively correlated with sbGnRH content, suggesting that sbGnRH is the most relevant form of GnRH in the control of reproduction in seabream. In teleosts, GnRHs are usually found in nerve endings in the pituitary near the gonadotropic cells (Goos, 1991). These GnRHs bind specifically to receptors in the pituitary. The action of GnRH on its hypophysial targets is inhibited in some species by dopamine. In gold fish, dopamine has inhibitory effects on GnRH release while norepinephrine and serotonin are stimulatory on GnRH release (Peter *et al.*, 1991b).

There is some evidence that social interaction and androgens affect GnRH activity. Larger GnRH-containing neurons were found in territorial males of *Haplochromis burtoni* (African cichlid fish) which are aggressive and have higher reproductive activity than non-territorial males (Soma *et al.*, 1996). Also, castration of territorial males caused GnRH neurones to further increase in size and this could be prevented by either Testosterone (T) or 11 ketoTestosterone (11kT) treatment. Soma *et al.* (1996) suggested that GnRH is primarily determined by social signals, but maintained by negative feedback from gonadal androgens.

An extreme olfactory sensitivity to GnRH has been found in mature rainbow trout and it has been suggested that GnRH might play another role as a reproductive pheromone (Anderson *et al.*, 1991). These examples show that the GnRHs play an important role in regulating reproduction, and this role may be dependent on the species, reproductive status and social and behavioural factors.

1.4.2 Pituitary hormones

Possession of a pituitary gland which synthesises and secretes gonadotropin (GtH) is a common characteristic of all vertebrates (Yu *et al.*, 1991). GnRH released from the hypothalamus influences the activity of gonadotropic cells in the pituitary gland to secrete GtH. In mammals, birds, reptiles and amphibians, two distinct types of gonadotropins, luteinising hormone (LH) and follicle stimulating hormone (FSH)

exist. All GtHs are glycoproteins composed of two glycosylated subunits, α and β. The α subunit is unique within a given species, and common between LH and FSH. The β subunit is specific and determines the biological activity of the hormone (Dickoff and Swanson, 1989; Yu *et al.*, 1991; Querat, 1995).

Although it was considered for many years that fish pituitaries produce only one gonadotropin (Burzawa and Gerad, 1980 cited in Kawauchi *et al.*, 1989), it is now evident that teleosts, similar to other vertebrates, possess two gonadotropins. Idler and Ng (1983) isolated two gonadotropins from chum salmon, a carbohydrate rich "maturational' gonadotropin and a carbohydrate poor "vitellogenic" gonadotropin. Although investigations have shown that two gonadotropins are present in teleosts, this does not necessarily mean that the concept of dual control of gonadal functions, as in mammals, has been established for all species (Goos, 1991).

It was thought that the concept of duality of teleost GtH was different from that of the LH-FSH relationship. Kawauchi *et al.* (1989) reported that two types of teleost GtHs are chemically homologous to LH and FSH, but neither of them is identical to LH or FSH in terms of physiochemical and functional properties. These authors found that both GtHs in chum salmon consist of two sub units, α and β. GtH I was fractioned to two α subunits (α1 and α2) and β subunit, whereas GtH II was fractioned simply into α and β sub units. GtH I α2 was identical to GtH II α, but distinct from GtH I α1. These two distinct GtH molecules are synthesised from two different cell types in salmonid pituitaries. GtH I producing cells are present prior to gametogenesis and increase in number during spermatogenesis and vitellogenesis. Appearance of the GtH II cells is associated with the onset of spermatogenesis and vitellogenesis, and their numbers increase dramatically at the time of final maturation (Kawauchi *et al.*, 1989).

Goos (1991) demonstrated that one type of GtH, referred to as Con-A I (carbohydrate poor), stimulates yolk incorporation, while the other, Con-A II (carbohydrate rich) induces oocyte maturation and ovulation. However, Con-A I appeared not to be homologous with LH and FSH and was thought not to consist of two sub units and to lack the glycogen characteristic of the mammalian GtHs (Goos 1991).

However, more recent work (Querat, 1994 cited in Davies *et al.*, 1999) on amino acid sequences confirmed that fish GtH-Iβ subunit is related to the follicle stimulating hormone type (FSH type) family, while GtH-IIβ subunit belongs to the luteinizing hormone type (LH type) family.

Biological Activity of Gonadotropins

In teleosts, gonadal function is controlled primarily by pituitary gonadotropins which bind to specific receptors in the testes and ovary to regulate steroidogenesis and gametogenesis (Xiong *et al.*, 1994). GtH stimulates spermatogenesis, spermiation and testicular steriodogenesis and it also stimulates androgen release (Idler and Ng, 1983).

In chum salmon GtH I is secreted during vitellogenesis and early stages of spermatogenesis, whereas GtH II is secreted at the time of spermiation and ovulation (Kawauchi *et al.*, 1989). These authors concluded that GtH II appears to correspond to a maturational gonadotropin while the function of GtH I was unclear. Mylonas *et al.* (1997) found that the plasma GtH II levels in male and female wild striped bass captured on their spawning grounds are associated with the progression of spermiation in males and final oocyte maturation in females. Kagawa *et al.* (1998) examined the biological activities of red seabream gonadotropins (GtH I and II) in final maturation of oocytes. They found that GtH II, but not GtH I, induces germinal vesicle breakdown by stimulating the follicle layer to produce the maturation-inducing hormone.

It has been suggested that GtH I is predominantly present during prepubertal stages, while GtH II is synthesised and released during vitellogenesis, spermatogenesis and final maturation (Goos, 1991). The function of two GtHs has been studied extensively using purified hormones. Although both GtH I and II stimulate oestradiol production from mid vitellogenic oocytes, GtH II is more active in stimulating 17α 20β dihydroxy-4-pregnen-3-one (17α,20β, DP) production in amago salmon (Xiong *et al.*, 1994) . In males, both GtH I and II are potent in stimulating 11kT production in early spermatogenetic testes, while GtH II is superior in stimulating 17α,20β, DP production in late spermatogenic testes (Reviewed by Xiong *et al.*, 1994). Studies on salmonids suggest that GtH I is primarily involved in the control of vitellogenesis and spermatogenesis, whereas GtH II controls final

maturation. Nevertheless, the specific actions of GtH I and GtH II in salmonids are still unclear and very little is known about their role in other groups of fish (Swanson, 1991).

More recently, Breton *et al.* (1998) reported that GtH I was detected in immature to postovulatory stages, and it increased significantly at the onset of exogenous vitellogenesis in female rainbow trout. GtH II remained very low until the end of the vitellogenesis but peaked on the day of maturation. However, the reason for these increases in GtH I and II before maturation is still unknown.

Regulation of gonadotropins

The regulation of GtHs is complex and includes the participation and feedback controls of the hypothalamus pituitary-gonadal axis (Xiong *et al.* 1994). The synthesis and secretion of GtHs are regulated by neuropeptides in the brain and hypothalamus (Peter *et al.*, 1991a) as well as by feedback from the gonads by both steroid and peptide hormones (De Leeuw *et al.*, 1987; Breton *et al.*, 1991; Larsen and Swanson, 1997). In African catfish, castration of adult males led to an increase in plasma GtH levels and a decrease in pituitary GtH contents (De Leeuw *et al.*, 1987). Gonadectomy in maturing coho salmon (*Oncorhynchus kisutch*) caused a decrease in plasma sex steroids and an increase in plasma GtH I and GtH II levels. However, the negative feedback of gonads on both GtHs depends on the stage of maturation (Larsen and Swanson, 1997).

Saligaut *et al.* (1998) studied the effect of catecholaminergic neurones and oestrogens on the release of GtHs in immature and early vitellogenic female rainbow trout and observed that oestrogens exert a negative feedback on the release of GtH I. In contrast, low pituitary GtH II levels in ovariectomized vitellogenic trout confirm that sex steroids have a positive feedback on GtH II synthesis at the vitellogenic stage (Saligaut *et al.* 1998). In male Atlantic Salmon the feedback control of GtH I appears to be more complex than that of GtH II (Antonopoulou *et al.*, 1999a,b). GtH I secretion is controlled by both aromatase dependent and non-aromatase dependent feedback effects, which may be positive or negative depending on the season, while the secretion of GtH II is controlled by the positive aromatase dependent feed back mechanism.

Many other factors are involved in controlling GtH production in teleosts. De Leeuw *et al.* (1987) have shown that dopamine inhibits spontaneous GtH release and suppresses the stimulatory effect of GnRH in teleosts. Peter *et al.* (1991b) have demonstrated that dopamine released by the hypothalamus acts as an inhibitor to GtH II secretion in some species including goldfish, African catfish, Chinese loach, tilapia, rainbow trout and European eel. Blocking of the synthesis of dopamine resulted in an increase in serum GtH levels (Chang *et al.*, 1983 cited in De Leeuw *et al.*, 1987). Furthermore, the dopamine agonist apomorphine caused a decrease and its antagonist pimozide caused an increase in GtH release (De Leeuw *et al.*, 1987).

Kah *et al.* (1991) found some evidence on the participation of δ-amino-butyric acid (GABA), a neurotransmitter within the central nervous system of vertebrates, in the control of pituitary GtH function. High concentrations of GABA have been found in the hypothalamo-hypophyseal complex, which is important for control of GtH release in gold fish. Furthermore, administration of GABA, or inhibitors of GABA degradation, also stimulated GtH release. These authors suggested that the effects of GABA may be mediated by direct or indirect GABA influence on GnRH secretion.

Stimulatory influence of serotonine on GtH release in sexually mature Atlantic croaker (*Micropogonias undulatus*) at the pituitary level was demonstrated by Khan and Thomas (1991). In gold fish (*Carassius auratus*), a pheromone (17α, 20β-dihydroxy-4-pregnen-3-one) and spawning stimuli (interaction with a sexually active female releasing prostaglandin pheromone) both increased serum GtH II (Zheng and Stacey, 1997).

In addition to internal factors, external factors also play an important role in the regulation of GtH. In female rainbow trout, fish exposed to ambient photoperiod showed gonadal steroid production, vitellogenin production and oocyte growth but no increase in serum GtH I levels (Davies *et al.*, 1999). In contrast, stimulatory long-short photoperiod advanced spawning by 3 - 4 months, which coincided with high levels of Oestradiol-17β (E_2), T, vitellogenin and GtH I and II levels. A high temperature stimulates GtH secretion and inhibits spermatogenesis in teleosts whereas a low temperature initiates spermatogenesis (Billard *et al.*, 1982).

1.4.3 Steroid hormones

Steroids are a group of lipids based on a molecular skeleton of four fused carbon rings, the perhydrocyclopentanephenanthrene system of which cholesterol is the major component present in animal tissue (Gower, 1988). Cholesterol is modified by the enzyme system known as the cholesterol Side-Chain Cleavage (SCC) system to produce steroid hormones in different tissues ie: the brain, adrenals, ovaries and testes (Fig. 1.1) (Martin, 1985; Gower, 1988). In these tissues production of all of the steroid hormones begins with the oxidation of carbons 20 and 22. As a result of this, cholesterol is first converted to pregnenolone and then into different sex hormones (Gower, 1988). These hormones can be categorised into different groups according to the number of carbon atoms in the fused ring. The main three groups are:

i. C21 group - Progestins, glucocorticoids and mineralcorticoids with 21 carbon atoms
ii. C19 group - Androgens with 19 carbon atoms
iii. C18 group - Oestrogens with 18 carbon atoms

The adrenal cortex can produce all three groups of hormones but progestins, glucocorticoids and mineralcorticoids are the major products. The gonads are the principal sources of androgens and oestrogens (Gower, 1988) which are important hormones in reproduction. However, the production of these hormones in the brain may have significant effects (Gower, 1988). Major functions of glucocorticoids (e.g., cortisol, corticosterone) are the regulation of carbohydrate metabolism and promotion of glucogenesis. Mineralcorticoids (e.g., aldosterone) are responsible for controlling salt and water secretion. Once the various steroids have been formed in particular tissues, they are released into the peripheral blood circulation (Gower, 1988).

Tissues of sex steroid production

Testes

The testes are the main sex steroid production site in male fish. The teleost testis, as in other vertebrates, is composed of interstitial and lobular components

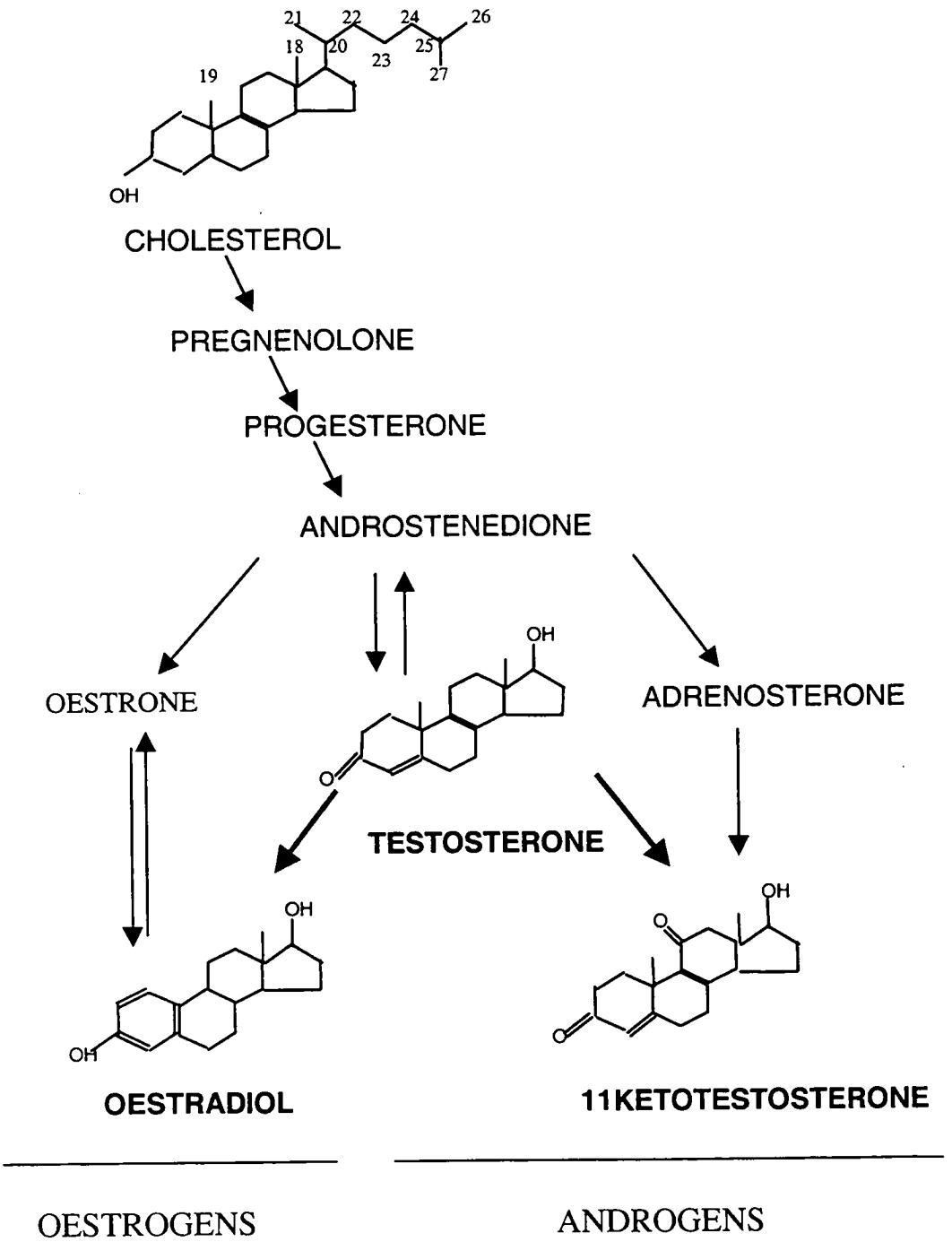

Figure 1.1 Simplified pathways of biosynthesis of androgens and oestrogens (Martin, 1985; Gower, 1988)

(Billard *et al.*, 1982; Nagahama, 1983; Yoshikuni and Nagahama, 1991). The interstitium consists of interstitial cells (Leydig cells), fibroblasts and blood vessels. The lobular component contains germ cells and sertoli cells which make the lining of the lobule (Nagahama, 1983; Nagahama *et al.*, 1982; Zohar, 1989).

It has been shown that the testis has a dual function to produce spermatozoa and sex hormones (Schulz and Goos, 1999). Although the main testicular steroidogenic site is thought to be the Leydig cells, in many teleosts sertoli cells, the sperm duct and the seminal vesicle also act as additional steroid production sites (Zohar, 1989). Spermatogenesis in teleosts has been considered to be under GtH control. In Japanese eel, a single injection of human chronic gonadotropin (HCG) induced spermatogenesis, and Leydig cells and sertoli cells were activated markedly prior to the spermatogonial proliferation (Miura *et al.*, 1991). Miura *et al.* (1991) suggested that the effect of GtH is mediated through the action of testicular somatic cells. Further studies revealed that Leydig cells produced 11 ketoTestosterone (11kT) from T which triggered sertoli cells to stimulate spermatogenesis (Yoshikuni and Nagahama, 1991). This process required physical contact between germ cells and sertoli cells (Miura *et al.*, 1996 cited in Schulz and Goos, 1999). Plasma levels of these androgens diminish after castration, indicating that testes are the major source of circulating androgens (Billard *et al.*, 1982).

Ovary

In female teleosts, the ovary, which consists of oogonia, oocytes and surrounding follicle cells, supporting tissue and blood vessels, acts as the major sex steroid production site (Yoshikuni and Nagahama, 1991). During gonad development, oocytes are surrounded by an inner granulosa cell layer and outer thecal layer and become larger in size due to the incorporation of vitellogenin. Vitellogenin, a hepatically derived precursor, is converted into yolk protein and incorporated into the growing oocyte. The process of hepatic synthesis and secretion of vitellogenin is controlled by E_2 which is produced by the ovary (Yoshikuni and Nagahama, 1991).

Yoshikuni and Nagahama (1991) proposed a two cell type model in the production of follicular E_2. The thecal cell layer, under the influence of GtH, secretes androgen substrate (probably T) which diffuses into the granulosa cell layer where the

aromatase is localised. The aromatase activity, which results in conversion of T to E_2, increases progressively during vitellogenesis suggesting that the teleost ovary produces E_2 during this period. C21 steroids are involved in the final oocyte maturation of the gonads. The thecal cell layer produces 17α-hydroxyprogesterone which is converted to 17α, 20β-dihydroxy-4-pregnen-3-one (17,20β-DP) by the granulosa cell layer. A steroidogenic shift from E_2 to 17,20β-DP has been observed in salmonid ovarian follicles immediately prior to oocyte maturation. This has been thought as a prerequisite for the growing oocytes to enter the maturation stage (Nagahama *et al.*, 1994).

Sexuality in fish

The physiological sex of a fish basically depends on the type of gonad it possesses and can be classified as either gonochorism or hermaphroditism (Yamazaki, 1983). Gonochorism is the existence of either testes or ovaries in one individual fish. Hermaphroditism is the possession of both testicular and ovarian tissue in one animal which functions both as male and female during its life cycle. Unlike in other vertebrates, hermaphroditism is a common phenomenon in teleost fish (Borg, 1994) and at least 130 species of fish exhibit hermaphroditism during their life (Yamazaki, 1983).

There are two types of hermaphroditism. In synchronous hermaphroditism, fish have ovotesis, comprised of testicular and ovarian tissue but function as male or female alternatively. In consecutive hermaphroditism, fish are either first males (protandrous) or first females (protogynous) in their life cycle. Sex reversal and hermaphroditism are presumed to be controlled by the endocrine system (Yamamoto, 1969; Reinboth, 1988).

Seasonal changes in plasma sex hormones

Correlations between seasonal variations in plasma levels of gonadal steroids and gonadal changes have been well documented in many teleosts. In fish showing well-defined seasonal breeding cycles, plasma GtH and sex steroid levels rise as the gonad matures, and decline after ovulation and spermiation. This pattern has been demonstrated in a variety of teleosts including *Salmo gairdnerii* (Scott *et al.* 1980 a,b), *Salmo salar* (Hunt *et al.*, 1982), *Heteropneustes fossilis* (Singh and Singh, 1990),

Morone americana (Berlinsky *et al.*, 1995; Jackson and Sullivan 1995), *Pygocentrus cariba* (Gurrero *et al.*, 1995), *Acanthopagrus butcheri* (Haddy and Pankhurst, 1999), *Salvelinus alpinus* (Tveiten *et al.*, 1998) and *Rhombosolea tapirina* (Barnett and Pankhurst, 1999).

Most studies investigating reproductive steroid cycles in teleosts measure only plasma concentrations of steroids (Barnett and Pankhurst, 1999). There is only limited information on the correlation between gonadal and plasma levels of steroids. Some studies have demonstrated that plasma and ovarian steroid concentrations are not always correlated (Guiguen *et al.*, 1993, Hobby and Pankhurst, 1997), probably due to the rapid metabolism and conjugation of free steroid from the plasma (Barnett and Pankhurst, 1999). Plasma and ovarian steroid levels are less likely to correlate well in species which gonadal cycles are of short duration (Hobby and Pankhurst, 1997).

In contrast with the intense research on seasonality and sex hormones in separate sexes, there are only a few studies reported on levels of steroids in the circulation and the gonadal development of hermaphrodites which include Black porgy (Chang and Yueh, 1990; Chang *et al.*, 1997), *Lates calcarifer* (Guiguen *et al.*, 1993; 1994), *Sparidentex hasta,* (Kime *et al.* 1991) and *Epinephelus morio* (Johnson *et al.*, 1998).

1.4.3.1 Physiological role of gonadal steroids

Androgens in male teleosts

Androgens are present throughout the reproductive cycle, with the highest plasma levels found during the spawning season. Thus, these hormones are potential regulators of spermatogenesis (Fostier *et al.*, 1983; Borg, 1994). Testosterone and 11kT appear to be the predominant testicular steroids in teleosts (Liley and Stacey, 1983, Borg, 1994). Steroid concentrations are highly variable, probably as a result of differences in species and assay methods used in these studies.

In most fish the T levels gradually increase during testicular development and the high levels of T in the plasma coincide with spermatogenesis. In immature male rainbow trout, plasma T level was low (2 - 3 $ng.ml^{-1}$) while the highest levels (100-150 $ng.ml^{-1}$) were found in the plasma of sexually mature fish when spermiation began. Circulating T declined thereafter (Scott *et al.*, 1980 a; Scott and Baynes, 1982).

In male snapper, T levels began to increase in early spring and remained high (1 $ng.ml^{-1}$) during the spawning period (Carragher and Pankhurst, 1993). In male *Stizostedion vitreum*, T levels rose from undetectable levels to 1.6 $ng.ml^{-1}$ by November, remained elevated throughout the winter and peaked at 2.8 $ng.ml^{-1}$ prior to spawning (Malison *et al.*, 1994). Testosterone levels exhibited biphasic patterns of increase during the reproductive cycle in white perch (*Morone americana)* and levels peaked at 1.29 $ng.ml^{-1}$ early in the spawning season (Jackson and Sullivan, 1995). Plasma concentrations of T were higher throughout spermatogenesis and fell just before spermiation in male greenback flounder (Barnett and Pankhurst, 1999).

11kT is often considered to be an important effector androgen in male fish (Billard *et al.*, 1982; Hunt *et al.*, 1982; Mayer *et al.*, 1992; Borg, 1994; Cavaco *et al.*, 1997) as plasma 11kT levels are significantly higher than those of T during most of the reproductive cycle. In breeding stickleback males, plasma 11kT levels were 43 $ng.ml^{-1}$ while T levels were 2 $ng.ml^{-1}$ (Borg and Mayer, 1985). The dominant androgen in maturing Arctic charr males was 11kT which reached levels of 90 $ng.ml^{-1}$ (Mayer *et al.*, 1992). In male snapper, the maximum concentrations of T and 11kT were 1.1 $ng.ml^{-1}$ and 2.8 $ng.ml^{-1}$ (Carragher and Pankhurst, 1993). In male African catfish (*Claris garipinus*), 11kT is the main circulating androgen while OHA is the main product of testicular steroidogenesis *in vitro (*Cavaco *et al.*, 1997). In *Tilapia zillii* Plasma 11kT levels (10 - 12 $ng.ml^{-1}$) were twice as much as T level (4.5 $ng.ml^{-1}$) (Neat and Mayer, 1999).

Plasma 11kT levels also display clear seasonal changes with maximal levels during the breeding season. In immature rainbow trout, the plasma 11kT level was low (2 - 3 $ng.ml^{-1}$) and rose slowly until a peak of 260 $ng.ml^{-1}$ in the breeding season (Scott *et al.*, 1980a). In male snapper, plasma 11kT levels followed testicular recrudescence, being lower in concentration than T during autumn, but rising and remaining high (2.8 $ng.ml^{-1}$) during the spawning period (Carragher and Pankhurst, 1993). The 11kT levels in male *S. vitreum* remained low (10 $ng.ml^{-1}$) from post spawning through January, then increased significantly and reached maximum concentrations just prior to spawning (Malison *et al.*, 1994). In breeding stickleback males, the plasma levels of 11kT are higher than those of other measured androgens and reach a distinct maximum in the breeding season (Borg, 1994). Such trend was

not observed in some teleosts. For example, in male greenblack flounder, plasma 11kT did not change with the gonadal stages (Barnett and Pankhurst, 1999).

In general, plasma T and 11kT levels increase with the gonadal development, but the concentrations fell rapidly at the onset of spermiation. This trend has been observed in male rainbow trout (Scott *et al.*, 1980a; Baynes and Scott, 1985), in male white bass (Berlinsky *et al.*, 1995), in male amago salmon (Fostier *et al.*, 1988), in snapper (Carragher and Pankhurst, 1993) and in green back flounder (Barnett and Pankhurst, 1999).

In most teleosts T reaches a maximum concentration before 11kT (Scott *et al.*, 1980a,b; Baynes and Scott, 1985; Carragher and Pankhurst, 1993) and it has been suggested that these two hormones have separate roles (Matty, 1985). The peak plasma levels of T coincide with spermatogenesis, while those of 11kT coincide with spermiation (Berlinsky *et al.*, 1995; Fostier *et al.*, 1988). However, as identified by Matty (1985), T is one of the intermediate products in the synthesis of 11kT and it is possible that the decrease of T in the plasma is related to an increase in the ability of the fish to transform T to 11kT.

Several studies have reported a possible relationship between 11kT and milt production. Higher plasma concentrations of 11kT are generally found just before or during milt production (Hunt *et al.*, 1982; Baynes and Scott, 1985; Fostier *et al.*, 1988). Chang *et al.* (1995) reported that plasma levels of 11kT were closely associated with milt volume in black porgy. In male Atlantic salmon, animals maturing as grilse (fish having spent one winter at sea) during November and December showed detectable levels of 11 kT as early as February. The 11 kT levels remained elevated until milt became expressible and then fell to low levels suggesting that this steroid has a role in the early stages of gonad development. It's precise function, however, is not clear (Hunt *et al.*, 1982).

11kT or other 11-androgens have been found to be more effective than T in stimulating secondary sexual characteristics (Zohar, 1989), influencing spermatogenesis, stimulating reproductive behaviour (Pankhurst *et al.*, 1999), dominance (Elofsson *et al.*, 2000) and in masculinizing genotypical females (Borg, 1994). In contrast, Neat and Mayer (1999) reported that plasma T and 11kT were not correlated to the dominance status of male *Tilapia zillii*. In some teleosts, plasma

11kT concentrations do not change with gonadal stage (review Pankhurst and Carragher, 1991; Barnett and Pankhurst, 1999).

The plasma levels of 11kT are generally higher in male fish than in females. This has been observed in some teleost species including rainbow trout (Scott *et al.*, 1980 a,b), three-spined stickleback (Borg and Mayer, 1995), Siberian sturgon (Cuisset *et al.*, 1995) and Nile tilapia (Toguyeni *et al.*, 1996). In Atlantic salmon and Coho salmon, 11kT concentrations varied seasonally during the reproductive cycle in both males and females (review Borg, 1994).

Androgens in female teleosts

T has been identified as the major androgen in mature females of many teleosts. In female fish, plasma T rises in parallel with oestrogens as the ovary develops, often reaching levels higher than those found in sexually mature males (Liley and Stacey, 1983; Mayer *et al.*, 1992; Borg and Mayer, 1995). Very high levels (211 $ng.ml^{-1}$) of T were found in the plasma of sexually mature female rainbow trout at the time of ovulation, when E_2 levels were minimal. In male rainbow trout, maximum levels of T can be observed in this period, but the levels (115 $ng.ml^{-1}$) were lower than found in females (Scott *et al.*, 1980b).

There are many studies showing that plasma T concentrations elevate during gonadal development in female teleosts. In female snapper (*Pagurus auratus*), plasma T levels were low during autumn and winter, rising from early spring to a maximum of 0.63 $ng.ml^{-1}$ during the spawning season (Carragher and Pankhurst, 1993). In female orange mouth corvina, T levels rose in the period of oocyte growth and declined at the onset of spawning (Thomas *et al.*, 1994). In female Artic charr, low plasma T levels (2-5 $ng.ml^{-1}$) were observed in early vitellogenesis, rapidly increasing during late vitellogenesis (Frantzen *et al.*, 1997). The levels in this species peaked at 71 $ng.ml^{-1}$ and declined rapidly during final maturation and ovulation. T levels were also lowest during spring and summer (10 - 20 $ng.ml^{-1}$) in maturing female spring chinook salmon, rising in late July to reach maximum levels (100 $ng.ml^{-1}$) by ovulation in September (Slater *et al.*, 1994).

Vitellogenic follicles of ovaries are capable of producing T which most probably are further aromatised into E_2 (Zohar, 1989). The decrease in E_2 and increase

in T at the end of vitellogenesis appears to be a common phenomenon indicating the completion of vitellogenesis (Aida, 1988). Testosterone as well as other C19 steroids have been shown to induce oocyte maturation only at high concentrations (Yoshikuni and Nagahama 1991). However, the high levels of T found in female teleosts may be due to the consequence of the role of T as a precursor for the synthesis of E_2 (Berlinsky *et al.*, 1995).

The presence of 11kT, generally considered as male specific androgen in teleosts has only been reported for few female fish species. In female fish, the plasma levels of 11kT are low throughout sexual maturation until ovulation but 11kT increase after that time. For example, in female spring chinook salmon, 11kT was detected in the plasma at low levels (< 10 $ng.ml^{-1}$) throughout spring and summer before ovulation, increasing to 10-20 $ng.ml^{-1}$ in September just prior to ovulation (Slater *et al.*, 1994). In Siberian sturgon females, fish with previtellogenic and vitellogenic stages of oocytes had very low 11kT (2-8 $ng.ml^{-1}$) in their plasma, while very high levels of 11kT (49-82 $ng.ml^{-1}$) found in maturing fish (7 yr old) at the end of the reproductive cycle (Cuisett *et al.*, 1995). Plasma 11kT levels were very low in non-migratory female eels, however, high levels of 11kT were found in migrating females which have much advanced gonadal development stages than non-migratory females (Lokman and Young, 1995). These authors suggested that 11kT may have anabolic functions that prepare animals for migration. Some teleost species are also capable of *in vitro* synthesis of 11kT (Zohar, 1989). However, the function of 11kT in females is not clear.

Oestrogens

The major physiological role of oestrogenic steroids is the control of reproduction in the female (Nagahama *et al.*, 1994). Oestradiol-17β has been identified as the major oestrogen in the plasma of a variety of oviparous teleosts (Liley and Stacey, 1983). The female teleost ovary synthesises T and converts it into E_2 by aromatisation (Matty, 1985, Nagahama *et al.*, 1994). In male teleosts, plasma E_2 levels are undetectable or present in very low levels.

Oestradiol-17β in female teleosts

Oestradiol-17β is the major ovarian steroid of most teleosts and it has been observed that E_2 levels in plasma are correlated with the gonadosomatic index (Matty, 1985). Numerous studies (Zohar, 1989; Carragher and Pankhurst, 1993; Thomas *et al.* 1994; Frantzen *et al.,* 1997) have demonstrated that in the teleosts, E_2 levels in plasma increase during vitellogenesis and rapidly decline prior to oocyte maturation. Oestradiol-17β was found to reach peak levels during the prespawning period in rainbow trout (Scott *et al.*, 1980 b) and in the fresh water teleost *Stizostedion vitreum* (Malison *et al.*, 1994). In female snapper (*Pagrus auratus*), plasma E_2 increased from low levels in sexually immature fish to a peak (0.93 $ng.ml^{-1}$) in fish undergoing ovarian recrudescence and ovulation (Carragher and Pankhurst, 1993). High levels (20 $ng.ml^{-1}$) of plasma E_2 concentrations were observed before ovulation in female spring chinook salmon, which declined significantly to 0.5 $ng.ml^{-1}$ at the time of ovulation (Slater *et al.*, 1994). In female Arctic charr (*Salvelinus fontinalis*) broodstock, plasma levels of E_2 were low during early vitellogenesis but increased significantly after the maturing oocytes entered the rapid growth phase (Frantzen *et al.*, 1997). In white bass, E_2 increases gradually during vitellogenesis with highest levels (1.35 $ng.ml^{-1}$) found at spawning time (Berlinsky *et al.*,1995).

Furthermore, it is clear that in some salmonids, plasma levels of E_2 actually decrease prior to the occurrence of ovulation and spawning (Fostier *et al.*, 1988; Scott *et al.*, 1980b). Scott *et al.* (1980b) also reported that in rainbow trout as in other teleosts, E_2 level rises several months before maturation, reaching a maximum during the period of exogenous vitellogenesis and rapidly declining prior to oocyte maturation. It has been suggested that this decline is to allow the synthesis of hormone 17α,20β-DP (Yoshikuni and Nagahama, 1991). In generally, E_2 and other C18 steroids are not effective in inducing oocyte maturation in the teleost oocytes (Yoshikuni and Nagahama, 1991).

Oestradiol-17β in males

Generally in male teleosts, plasma E_2 levels are undetectable or present in very low levels. However, many workers did not measure plasma E_2 level in male fish. Only few studies reported plasma E_2 levels in male teleosts and most of them were

done on sex reversing fish. Gurrero *et al.* (1995) found that in fresh water teleost (*Pygocentrus cariba*) plasma E_2 levels in males were very low (> 100 $pg.ml^{-1}$) when compared to those of females. Afonso *et al.* (2000) reported that it was not possible to detect E_2 in male coho salmon plasma and even if E_2 was present it could be below the detection limit. Previous studies suggested that fish testes have minimal ability to synthesise oestrogens (Colombo *et al.* 1978 cited in Afonoso, *et al.*, 2000). On the other hand, oestrogens can be formed in the brain and aromatase activity has been detected in fish brain irrespective of sex (Callard, 1978; Borg, 1994; Mayer *et al.*, 1991). This may suggest that the brain is the source of low levels of plasma oestrogens in males (Fostier *et al.*, 1983).

Although the function of E_2 in males is not very clear, there is evidence that oestrogen formation in the brain is essential for reproductive development in fish. Afonso *et al.* (2000) demonstrated that inhibition of E_2 production by aromatase inhibitor, Fadrozole, in male coho salmon brain resulted in a premature increase in 17,20βDP in the circulation in adult males. These fish showed early spermiation which may suggest that sexual maturation in males may be related to E_2 production probably at the brain level (Afonso *et al.*, 2000). On the other hand, adminstration of high levels of E_2 was found to be influence sex inversion of many juvenile male fish (Pandian and Sheela, 1995; Condeca and Canario, 1999).

Physiological role of C21 steroid hormones in teleosts

Steroidogenic shift from E_2 to 17,20β-DP in females and from androgens to 17,20β-DP in males occur under the influence of GtH at the end of the gametogenesis cycle.

Females

The presence of 17,20β-DP in plasma at the time of oocyte maturation has been demonstrated in many female teleosts. Yoshikuni and Nagahama (1991) proposed that the granulosa cell layer of oocytes convert 17α hydoxyprogesterone in to 17,20β-DP in response to GtH during oocyte maturation. In amago salmon, high plasma levels of 17,20β-DP were found only in mature and ovulated females (Yoshikuni and Nagahama, 1991). The levels were found to be very low or non-detectable (<30 $ng.ml^{-1}$) in females during the remaining period of life (Yoshikuni and

Nagahama, 1991). Slater *et al.* (1994) reported that circulating 17,20β-DP was low throughout maturation (>1ng.ml^{-1}) and increased significantly at ovulation (25 - 35 ng.ml^{-1}). Nagahama (1983) also reported that 17,20β-DP acts as an important steroidal mediator of oocyte maturation in salmonids and in a number of non-salmonid species. C21 steroids including progesterone, 17α, hydroxyprogesterone, 17,20β-DP, cortisol and deoxycorticosterone have been reported to induce oocyte maturation *in vitro* in teleosts (Foister *et al.*, 1983).

In addition, 17,20β-DP was found to be the most effective inducer of germinal vesicle breakdown (Nagahama *et al.*, 1982). King *et al.* (1995) reported that in female white perch and white bass, plasma levels of 17,20β-DP increased in association with oocyte germinal vesicle migration and germinal vesicle breakdown and decreased thereafter. In female Artic charr, plasma 17,20β-DP coincided with a rapid decline in plasma levels of T and the levels of 17,20β-DP dropped rapidly after ovulation (Frantzen *et al.*, 1997). In gold fish, 17,20β-DP has a dual role as the hormone that induces final oocyte maturation and as a pheromone that stimulates gonadotropin release, milt production and behavioural spawning competence in males (Stacey *et al.*, 1994).

In some teleosts plasma 17,20β-DP levels were very low both in males and females during reproductive cycle (eg. spiny damselfish) (Pankhurst *et al.*, 1999). These authors suggested that the low levels of plasma 17,20β-DP could be the result of rapid conjugation and excretion of this hormone.

Males

In male teleosts 17,20β-DP has been shown to be responsible for final sperm maturation (Fostier *et al.* 1988; Yoshikuni and Nagahama, 1991; Miura *et al.*, 1991; Afonso *et al.*, 2000). High concentrations of 17,20β-DP have been reported in the plasma or serum of several species of salmonid during spermiation (Fostier *et al.*, 1988). It has been demonstrated that mature spermatozoa are involved in 17,20β-DP synthesis in some fish (Billard *et al.*, 1990; Yoshikuni and Nagahama, 1991).

Presence of 17,20β-DP at the end of the gametogenesis cycle in both males and females and also findings from *in vitro* studies, have demonstrated that 17,20β-

DP is responsible for final oocyte maturation in females and final sperm maturation in males.

Changes in plasma reproductive steroids in sex reversal fish

Hermaphrodite teleosts show much more complex steriod changes during their reproductive cycle. T, 11kT and E_2 are generally found but the relationships are much more complex in these fish probably due to the complex gonadal development stages or various sex changing process (e.g., male to female, female to male, ovotestis etc.). Many endocrine studies focussed on inducing or preventing sex change of these fish and reported plasma hormone changes after administration of various hormones in order to induce/prevent sex change (Chang *et al.*, 1994; 1995a,b; Pandian and Sheela, 1995; Lau *et al.*, 1997; Condeca and Canario, 1999). There are limited studies on natural plasma hormone changes in these fish.

Protandrous change

Some protandrous fish show the normal teleost pattern of steroid levels, with higher concentrations of 11kT in males than in females (e.g., saddleback wrasse, black seabass) and /or highest gonadal biosynthesis of 11kT or other 11-androgens in males than in females (Borg, 1994). There are, on the other hand, notably many sex reversing fishes with very low or non-detectable levels of circulating 11kT in males (Borg, 1994).

Guiguen *et al.* (1993) reported plasma and gonadal levels of several steriods (T, 11kT, E_2, estrone etc.) in *Lates calcarifer*. Very low plasma levels of E_2 (mean < 68 $pg.ml^{-1}$) and estrone (mean < 42 $pg.ml^{-1}$) in males and 11kT (mean < 75 $pg.ml^{-1}$) in females did not fluctuate significantly during the reproductive cycle. Plasma T and 11kT peaked during spermiation in males and E_2, estrone and T increased during vitellogenesis in females. Females showed high E_2 (173 ± 233 $pg.ml^{-1}$) and estrone (79 ± 72 $pg.ml^{-1}$) levels than males while males had higher 11kT (153 ± 88 $pg.ml^{-1}$) and T (51 ± 91 $pg.ml^{-1}$) than females. Transitional fish always exhibit low plasma levels of these steroids. These authors also observed that plasma E_2 level was virtually undetectable (<25 $pg.ml^{-1}$) in sex changing fish. The androgen:oestrogen ratios were significantly higher in males than in transitional fish. T and 11kT concentrations were not different in testes or ovary but transitional gonads showed low concentrations.

Gonadal E_2 was nearly undetectable in testes (0.06 $ng.ml^{-1}$), low in ovaries (0.42 $ng.ml^{-1}$) but strikingly high (2.89 $ng.ml^{-1}$) in transitional gonads. These authors suggested E_2 has an important role in sex inversion of barramundi.

Chang and Co-authors (1990, 1995a, 1997) observed the changes in plasma sex steroids in black porgy (*Acanthopagrus schlegeli*), a marine protandrous hermaphrodite, during the reproductive cycle. These fish have both ovarian and testicular tissue, but function as males for the first two years of life, beginning to change to females during the third year. In spermiating black porgy, high plasma levels of T and 11kT were found. In the prespawning season, significantly higher plasma E_2 levels were observed in sex reversing fish (male to female) than in males. Plasma E_2 and T levels increased to high levels during the spawning season in juvenile males and adult females (Chang and Yueh, 1990; Chang *et al.*, 1997). Although plasma T levels were similar in males and females, low plasma 11kT levels were observed in transversing fish.

Kime *et al.* (1991) reported the plasma hormone changes of a protandrous teleost, sobaity (*Sparidentex hasta*). Plasma T levels increased during the post spawning period in both males and females sobaity. 11 kT and OHA increased sharply in the spawning season in males. E_2 levels started to increase in both sexes during the prespawning period, but levels in females continued to rise, whilst those of males had fallen to undetectable levels by the spawning season. The peak concentration of T coincided with low concentrations of 11-oxygenated androgens and E_2. These authors suggested that sex inversion during the spawning period corresponds with the drop of 11-oxygenated androgens and E_2 may be involved in the sex inversion process of this species.

Protogynous change

The relationship between gonadal development and plasma sex steroid levels for protogynous grouper (*Epinephelus morio*) was reported by Johnson *et al.* (1998). In females, seasonal increases in sex steroids (E_2 and T) were observed in the previtellogenic period whereas declining steroid levels were accompanied by ovarian regression at the end of the breeding season. In males, plasma T and 11kT levels increased while E_2 levels were low during the breeding season. In transitional fish, the

E_2 levels were lower than in females but higher than in males. The T levels of transitional fish were similar to males and females and 11kT was lower than in males. In protogynous blackeye goby, T, 11kT and E_2 are naturally occurring steroids in both female and males (Kroon and Liley, 2000). Whole body concentrations of T and 11kT of males were approximately twice as those in females, whereas E_2 in females was almost twice that in males. 11 kT is involved in the mediation of natural sex inversion of this species, which demonstrated by administration of 11kT and its precursor 11kA.

The red porgy, *Pagrus pagrus* is another protogynous marine teleost. Kokokiris *et al.* (2000) have found that plasma E_2 and T increased during vitellogenesis in females. Plasma 11kT levels were low in females and no relationships were found with female maturity. In males, plasma T and 11kT were correlated to gonadal development but E_2 levels showed no changes. Kuo *et al.* (1988) demonstrated that 2 year old female protogynous blue spotted grouper can be induced to reverse sex by oral administration of methyl testosterone. The serum E_2 levels decreased as sex reversal progressed although an elevation in serum E_2 concentration was observed during spermatogenesis.

These studies demonstrated that plasma T, 11kT and E_2 showed sex specific patterns in hermophroditic species, although the changes may be related to their particular sex inversion process.

1.5 Nutritional regulation of reproduction

1.5.1 Nutritional requirements of fish

Like all animals, fish need protein, minerals, vitamins and energy sources such as carbohydrates and lipids for their maintenance, growth and reproduction. Without intake of suitable amounts of these nutrients, fish are unable to remain healthy and productive. Therefore, formulation of nutritionally well-balanced diets and adequate feeding of fish are the most important requirements for successful aquaculture (Shepard and Bromage, 1988). Nutritional requirements of fish in general and in detail, have been well documented in the last few decades (Cowey and Sargent, 1979; Lovell, 1984; Cowey *et al.*, 1985; Shepard and Bromage, 1988; Wilson, 1991; De Silva and Anderson, 1995). The following is a brief account of this information and the reader is referred to those reviews for full detail.

Protein

As protein is the main constituent of the tissues and organs of the animal body (Shepard and Bromage, 1988), continuous and adequate supply of dietary protein is essential for growth and repair of tissue protein. The quality of proteins depends on the quality, quantity and the bioavailability of the amino acids, which are basic structural components of proteins (Shepard and Bromage, 1988).

Lipid

Lipids, in which the main component is fatty acid, represent concentrated energy sources and substrates for vitamins, pigments, hormones and essential growth factors of fish. Lipids also act as a vehicle for absorption of other fat-soluble nutrients such as sterols and vitamins (Lovell, 1984; Shepard and Bromage, 1988). The standard nomenclature for fatty acids is $C_{x:y}$(n-z), where x is the number of carbon atoms, y is the number of double bonds and z denotes the position of the first double bond from the terminal methyl group. The length of the carbon chain and the number and location of unsaturated bonds of the fatty acid determine the physical and nutritional properties of fats.

Generally, the fat stores in terrestrial animals are highly saturated (i.e., few double bonds), while fats from plants and aquatic animals are more unsaturated (Lovell, 1984). Short chain (C18) unsaturated fatty acids n-9, n-6 and n-3, are acted upon by a common enzyme system (delta desaturases) of alternating desaturation and elongation to give a series of fatty acids of increasing length and unsaturation (Henderson and Sargent, 1985). The competition for further unsatuaration of 18 carbon fatty acids is known to be in the order n-3> n-6> n-9 (Sargent *et al.*, 1989). The important features of these metabolic conversions are that they are non-interconvertable and the fatty acids of one series cannot give rise to fatty acids of another series (Bell *et al.*, 1987).

There are two series of essential fatty acids (EFA) which cannot be synthesised by animals and must be obtained by dietary intake. The n-6 series are derived from linoleic acid (18:2 (n-6); LA), and the n-3 series from α-linolenic acid (18:3 (n-3); ALA) (Steffens, 1997). The elongated and desaturated derivatives or metabolites of these fatty acids are physiologically more important than their precursors. However,

the requirements of EFA differ considerably from species to species and may be temperature dependant, because adipose and membrane lipids in fish are affected by temperature (Lovell, 1984; Shepard and Bromage, 1988).

Fatty acid composition of adipose lipid in fish is influenced primarily by diet, whereas membrane lipids are more characteristic of environment and species (Lovell, 1984). Terrestrial plants primarily synthesise C_{18} or shorter fatty acids, with the unsaturated bonds in the n-6 and n-9 positions. As a result, grain-fed fish will primarily store these types of fatty acids (Lovell, 1984). Some plant oils, however, such as linseed oil, contain significant amounts of short chain n-3 fatty acids. In marine food webs, marine algae are the primary sources of n-3 fatty acids which are elongated through the food chain (Lovell, 1984). An example of the environmental influence of the fatty acid composition of fish is the occurrence of very high levels of LA (18:2 n-6) in river-caught fingerlings of coho salmon, and a much lower content of this fatty acid in the ocean caught mature salmon (Stansby, 1967 cited in Steffens, 1997). The opposite is true for docosahexaenoic acid (DHA (22:6 n-3)) and eicosapentaenoic acid (EPA (20:5 n-3)).

It is believed that dietary lipid, particularly fatty acids, are important determinants for gonadal development. Long chain fatty acids are involved in the synthesis of vitellogenin, the precursor of the oocyte yolk proteins (Sargent *et al.*, 1989). They are also precursors of prostaglandins, which in turn are essential for steroid hormone production and GtH release (Wade and Van der Kraak, 1993; Wade *et al.*, 1994; Mercure and Van der Kraak, 1995).

Carbohydrates

Carbohydrates play an important role as either an immediate energy source or as a rapidly available energy reserve stored as glycogen in the liver and muscles (Shepard and Bromage, 1988). However, the capacity to utilise carbohydrates differ between carnivorous and omnivorous fish. Thus, omnivores such as carp or catfish can effectively utilise carbohydrate as energy sources and a relatively large quantity can be included in their diet. However, the ability of carnivores to utilise carbohydrate may vary within species (Shepard and Bromage, 1988).

Minerals and vitamins

Minerals are essential for normal animal growth, mainly as constituents for bones and teeth, cofactors of enzymes, maintaining osmotic regulations and proper functioning of muscles and nerves. Watanabe *et al.* (1997) recently reviewed the importance of minerals in fish nutrition. Vitamins are complex organic compounds which are required in trace amounts for normal growth, maintenance and reproduction of all animals, most usually as cofactors in metabolic reactions (Shepard and Bromage, 1988).

Energy requirements

Energy is required for various chemical reactions needed to build new tissues, maintain osmotic and salt balance, retain or excrete water, move food through the digestive tract, respire, reproduce and to move the muscles to provide locomotion (Lovell, 1984). The requirement for energy in fish is low when compared to the other vertebrates, as fish require less energy for protein metabolism, maintaining position and moving in water. Fish generally spend less energy on maintaining constant body temperature. In addition, the amount of energy lost in protein catabolism and in excretion of nitrogenous wastes of fish is low as they excrete most of their nitrogenous waste as ammonia through the gills (Lovell, 1984).

A number of factors influence the energy consumption of an animal over and above its basal metabolism. Basal metabolic rate is the heat energy produced during the activities which are necessary to maintain the life of an animal (e.g., cellular activity, respiration and blood circulation) (Lovell, 1984; De Silva and Anderson, 1995). The larger the fish, the smaller proportion of energy consumed in its food is required to maintain basal metabolic rate and so a greater amount of energy can be devoted to growth. However, when gonads develop, large amounts of energy are diverted from growth of muscle and other activities. The energy cost of locomotion is also considerable and varies between species, depending upon body shape, size and behavioural patterns. Animals can also cover the energetic cost of reproduction by reducing the expenditure of energy on locomotion (Lovell, 1984).

Protein: Energy Ratio

The amount of protein in a diet relative to the energy contained in that diet is termed the protein:energy ratio. A balance between dietary energy and protein is required for efficient utilization of dietary nutrients. Energy needed for maintenance and voluntary activities is satisfied before energy is available for growth. If the amount of energy in relation to protein is low, dietary protein will be used for energy (Lovell, 1984). On the other hand, a dietary excess of energy can limit food consumption and this prevents the intake of necessary amounts of protein and other nutrients for maximum growth (Lovell, 1984) because fish adjust their food intake to satisfy their need for energy (Halver, 1989). Optimal dietary protein:energy ratios vary between species, and is also dependent upon the protein source (De Silva and Anderson, 1995).

In general, nutritional requirements of fish depend on various factors such as species, stage of the life cycle (larvae, juvenile, reproductive status etc.), feeding behaviour, water temperature, season, and the availability and quality of natural foods. During the early stages of life, fish grow much faster and display high growth rates. However, after they enter into the reproductive stage, fish utilise their energy for both somatic and reproductive tissue development. Therefore, diets should provide sufficient nutrients to satisfy animals' energy requirements. The effectiveness of diets can be evaluated by measuring fish growth (weight gain), the body composition of fish and feed efficiency or food conversion (Shepard and Bromage, 1988).

1.5.1.1 Growth and body composition

Growth of animals is usually defined as a correlated increase in the mass of the body in definite intervals of time, in a way characteristics of the species (Shepard and Bromage, 1988). The growth can be either somatic or reproductive (De Silva and Anderson, 1995). Somatic growth entails an increase in the size of the body while reproductive growth entails an increase in the size of the gonads. Growth of fish is generally related to the species, fish strain, sex and stage of life cycle. In addition, dietary quality, in terms of nutrient balance, energy content and bioavailability of each nutrient, plays a significant role in affecting fish growth. Food intake is also a major limiting factor affecting growth rate in fishes (Brett, 1979). Parameters such as weight

gain or specific growth rate of fish are commonly used to evaluate growth in fish nutrition studies.

Factors affecting growth and body composition

Dietary quality and quantity, as well as feeding frequency influence growth and body composition of fish. This has been observed in natural environments as well as in experimental conditions and has been well described in the literature (Love, 1970; Dawson and Grimm, 1980; Devauchelle *et al.*, 1982; Kjorsuvik *et al.*, 1994; Collins and Anderson, 1995; Power *et al.*, 2000).

Many fish species have developed an ability to survive without food, as they are exposed to periods of food deprivation in natural environments (Love, 1970). During starvation an animal's energy reserves are mobilised and its metabolism is altered (Collins and Anderson, 1995). Carbohydrate, protein and fat stores may be differently affected and consequently the biochemical composition of different tissues can be markedly altered. Most fish use muscle protein as a major energy source, while others utilise fat and glycogen reserves and conserve their protein sources. The liver, which plays an important role in storage and distribution of energy reserves, is one of the first organs to be affected by food deprivation (Power *et al.*, 2000). Although the changes in liver weight and composition are dramatic, its contribution to the energy reserves is almost insignificant (Dawson and Grimm, 1980). Fish on restricted rations reduce energy storage and growth, redirecting energy towards maintenance metabolism. This is reflected by decreased growth rates, condition factor, organ weights (muscle, liver, and adipose tissue) and an increase in the mobilisation of glucose, lipid and amino acids from storage (Mackenzie *et al.*, 1998).

The duration of food deprivation (Collins and Anderson, 1995; Hung *et al.*, 1997; Power *et al.*, 2000), together with the species, sex (Karlsen *et al.*, 1995) and the stage of life cycle of the fish (Hung *et al.* 1997), influences the way in which energy reserves are utilised and metabolic processes are altered. For example, food restriction for 3 weeks significantly reduced liver lipid, glycogen and protein but increased water content in sea bream (Power *et al.*, 2000). In the sub yearling white sturgeon starved for 10 weeks (Hung *et al.*, 1997), viscera was the preferred tissue over muscle for energy resources and lipid was the preferred nutrient over protein for mobilisation. In

Atlantic salmon, fillet was used most, followed by viscera and liver during starvation (Einen *et al.*, 1998). Liver reserves are the initial source of energy in response to food deprivation, followed by the perivisceral adipose tissue and finally muscle component in golden perch in response to food deprivation (Collins and Anderson, 1995). During starvation, rainbow trout used visceral lipid followed by muscle lipid stores and the amount derived from the liver is much smaller (Jezierska *et al.*, 1982). In female plaice, carcass lipid level supplied the energy for metabolism during starvation (Dawson and Grimm, 1980). The way of utilising energy reserves is also dependent on sex differences. For example, female Atlantic cod deplete muscle protein and liver lipid resources to a greater extent than do males (Karlsen *et al.* 1995), probably as a result of vitellogenesis.

Refeeding/ compensatory growth

Catch-up or compensatory growth in fish after a period of starvation or restricted feeding, followed by high rations, has been well described (Miglavs and Jobling, 1989; Quinton and Blake, 1990; Rowe and Thorpe, 1990b; Russell and Wootton, 1992; Reimers *et al.*, 1993; Kim and Lovell, 1995; Collins and Anderson, 1999; Qian *et al.*, 2000). Compensatory growth can be accompanied by hyperphagy (Miglavs and Jobling, 1989), improved food conversion efficiency (Miglavs and Jobling, 1989; Russell and Wootton, 1992; Hayward *et al.*, 1997; Qian *et al.*, 2000) and reduced metabolic expenditure (Miglavs and Jobling, 1989). Compensatory growth is also characterised by alterations in hormone production which promote rapid lean growth (MacKenzie *et al.*, 1998).

Most animals regain their body weights during rapid growth phase and managed to restore their tissue nutrient levels to the same levels as animal fed continuously (Reimers *et al.*, 1993; Hung *et al.*, 1997; Collins and Anderson, 1999; Power *et al.*, 2000). However, some animals show only partial recovery. The ability of animals to show partial or complete compensatory growth depends on the severity and duration of food deprivation (Jobling *et al.*, 1993).

Numerous exogenous factors, both environmental and dietary, have been reported to affect the proximate composition of cultured fish. Environmental factors (e.g., temperature, salinity, exercise), and dietary factors (e.g., nutrients,

protein:energy ratio, as well as ration, time, feeding frequency and starvation) can influence the proximate composition of fish. This is also associated with fish size and life cycle stages (Shearer, 1994).

The form in which data is expressed makes the information available on changes in body composition in fish somewhat difficult to compare. Some workers use whole fish for studying qualitative changes in body composition while others use part of the fish and assume this to be a representative of the whole, although it is known that chemical composition can vary in different parts of the body (Love, 1970). In addition, use of percentages (% dry wt or % wet wt) can be misleading when compared to data expressed as total weight changes. To overcome these errors, some authors used whole carcass or organ weight and analysed homogenised sub samples (Dawson and Grimm, 1980). Shearer (1994) reviewed some of the problems that can occur when comparing proximate composition. For example, percent dry weight basis comparisons fail to remove endogenous factors such as fish size. When dry weight values are used, a change in one component affects the relative amount of others and this may lead to misinterpretation of results.

1.5.1.2 Evidence for a relationship between nutrient intake and hormones

Although it is well known that nutrient intake influences somatic growth, the underlying physiological mechanisms are complex and not fully understood (MacKenzie *et al.*, 1998). However, it is assumed that food intake and endocrine function are closely coupled and some evidence exists for such a relationship (MacKenzie *et al.*, 1998). Ingested nutrients can activate hormone secretion from different endocrine tissues such as the pituitary and thyroid glands, and gastric and intestinal mucosa (MacKenzie *et al.*, 1998). Alterations in diet composition have shown that nutrients influence endocrine function. Dietary protein, lipid and carbohydrate have been thought to influence fish thyroid hormone production (MacKenzie *et al.*, 1998) and specific amino acids and glucose influence pancreatic hormone regulation.

More recent research has focussed on metabolic hormones. There is some evidence that the amount of nutrient intake, as well as time of feeding, can significantly influence the plasma concentrations of hormones, such as growth

hormone and cortisol in rainbow trout (Reddy and Leatherland, 1995). Although alterations in diets (quality or quantity) or feeding regimes can influence the endocrine function in fish, endocrine function is rarely evaluated in fish nutrition studies and particularly in broodstock nutrition.

To understand complex physiological mechanisms underlying those responses, it is essential to study the endocrine functions which regulate these processes. For example, nutrient intake can influence growth and/or reproduction as secondary responses, but primarily the changes in hormones should occur to initiate and drive these processes.

1.5.2 Broodstock nutrition

Although nutrition is known to have a profound effect on gonadal growth and fecundity, there are relatively few studies on the effects of nutritional status on reproduction (Kanazawa, 1985; De Silva and Anderson, 1995; Garcia-Rejon *et al.*, 1997). Most of the research work conducted on broodstock emphasised factors which effect gonad maturation and spawning behaviour, such as induced spawning by hormone injection, selective breeding, hybridisation and environmental factors (e.g., temperature, photoperiod, stocking density and rainfall) (Watanabe *et al.*, 1985). Watanabe *et al.* (1985) evaluated the importance of broodstock nutrition in aquaculture. However, the relationship between nutrition and adult maturation is still unclear (Hardy, 1999).

Studies based on the influence of nutritional conditions on teleost reproduction reveal that in general, food quality and quantity, as well as feeding regimes, are important for spawning, fecundity, and egg and larval quality (Kanazawa, 1985; Watanabe, 1984; Navas *et al.*, 1997). These studies have focussed on a limited number of species of cultured female broodstock, such as salmonids, red sea bream, carp and tilapia (Watanabe, *et al.*, 1984; Springate *et al.*, 1985; Luquet and Watanabe, 1986; Bromage, 1995; De Silva and Anderson, 1995). Species differences are quite apparent in these studies and mostly contain conflicting information making it difficult to draw more generalised conclusions (De Silva and Anderson, 1995). Furthermore, precise information on the nutritional requirements for gonadal maturation in broodstock for many teleosts is still lacking. In addition, the metabolism

and gonad physiology of fish fed different diets has received even less attention in broodstock nutrition studies (Cerda *et al.*, 1995).

Most of the available information documents dietary effects on direct reproductive outcomes, such as fecundity, spawning performance, egg and larval quality; information on the relationship between nutrition and endocrine functions, which regulate these processes, is very rare. The lack of understanding of the factors which may regulate these processes is one of the major constraints to improved broodstock management. One aspect that has received minimal attention is the nutritional effects on reproductive performance of male broodstock.

More recently, the importance of the relationship between reproductive physiology and broodstock nutrition has been recognised. For example, low survival of sockeye salmon broodstock and difficulties in synchronising male and female maturation in captivity was a problem in broodstock development, but improvements in diet and other rearing conditions has led to increasing survival of fish and reproductive performance (Flagg *et al*, 1997 cited in Hardy, 1999). These results stress the important role of broodstock nutrition in reproduction.

Somatic and reproductive investment

In natural environments, many fish exhibit seasonal changes in growth and energy storage (Love, 1970; Dygert, 1990; Jorgenson *et al.*, 1997) as energy from diet and body reserves is partitioned between maintenance, somatic growth and reproduction (Craig *et al.*, 2000). These changes are also related to temporal variations in environmental factors such as photoperiod, food supply and water temperature (Love, 1970). Lipid, protein and ash are typically accumulated during somatic growth while protein and lipids are depleted during gonadal investment (Jorgenson *et al.*, 1997). The seasonal trends in growth and energy allocation have been described for teleosts, including plaice (Dawson and Grimm, 1980), English sole (Dygert, 1990), Atlantic herring (Bradford, 1993), Arctic charr (Jorgensen *et al.*, 1997), chub (Encina and Granado-lorencio, 1997) and Brook trout (Hutchings *et al.*, 1999).

Previous studies suggest that fish largely utilise storage lipids for non-reproductive metabolic processes and storage protein for gonadal development (Dawson and Grimm, 1980; Dygert, 1990; Bradford, 1993) and the main source of

lipid and protein reserves is the carcass (Dawson and Grimm, 1980). Although liver weight and/or liver composition show dramatic changes during the seasonal cycle in some fish, its contribution to the total energy reserves is almost insignificant (Dawson and Grimm, 1980). Nevertheless, the liver may play an important role in lipid metabolism. Some authors have suggested that the liver is the main storage organ and as maturation takes place, a significant proportion of the nutrition needed by the oocytes is supplied by the liver (Sheikh-Eldin *et al.*, 1995). Although some fish lose somatic mass during their reproductive cycle, analysis of the chemical composition of tissues revealed that most of the change in protein could be accounted for by an increase in gonadal tissues, especially in females.

Decreased growth in association with gonad development has also been observed in some teleosts, both in the wild and in captivity (Cerda *et al.*, 1994, Shearer, 1994; Stead *et al.*, 1999). It has been suggested that this could be the result of hormonal changes during later stages of gametogenesis (Stead *et al.* 1999). Some other teleosts exhibit specific patterns of growth and energy allocation in tissues during their life cycle. In *Menidia menidia*, no body growth occurred during gonad maturation and spawning whereas liver and gonad tissues showed significant weight increases at the same time (Huber and Bengison, 1999). In contrast, *M. beryllina* showed rapid weight increases in carcass, liver and gonad during the spawning season (Huber and Bengison, 1999). These authors suggested that although these two species live in the same environment the differences may be related to their different life strategies.

Many temperate fishes either do not feed or have very low food intake during the gonad maturation period. This may be due to low food availability in winter conditions (Dawson and Grimm, 1980; Dygert, 1990; Bradford, 1993) or spawning migration (Doucett *et al.*, 1999; Booth *et al.*, 1999; Slotte, 1999). However, these fish are capable of utilising body reserves for gonad maturation and migrating fish utilise most of their energy reserves for both locomotion and gonad development. Fat and proteins are the most readily available sources of energy in fish tissues, however, due to differences in life history and migration patterns and environmental factors, the preferences for energy sources vary between species. For example, storage lipid was utilised for energy by Norwegian spring herring during migration while the protein

remained relatively constant so the loss of protein from somatic tissue was balanced by the gain in gonad (Slotte, 1999). Atlantic salmon use lipid from red muscle during their migration and show little or no reduction in lipid from liver or white muscle (Doucett *et al.*, 1999). White muscle is generally not used for aerobic swimming whereas maintenance of liver lipid is presumed to be essential due to the role it plays in lipid metabolism. Therefore, it is believed that lipid is mobilised from areas of less importance (e.g., pyloric area) and transferred to the liver (Johnsson *et al.*, 1996). Shearer, (1994) showed that during gonadal maturation, protein and lipid are mobilised from the muscle and moved to gonad in Atlantic salmon. Hutchings *et al.* (1999) found a size effect on lipid depletion in adult brook trout. Larger reproductive animals lost proportionally more lipids during winter than smaller individuals; however, such an effect of body size was not evident in non-reproductive animals.

Early maturation

Precocious sexual maturation is a common phenomenon in many captive-reared fish when compared to their wild counterparts (Thorpe *et al.*, 1990a,b; Kjesbu and Holm, 1994; Karlsen *et al.*, 1995; Holland *et al.*, 1996; Craig *et al.*, 2000). For example, in captive environments, one year old male striped bass, *Morone saxatilis*, reached sexual maturity but with lower sperm motility and less milt volume per kg body weight, than wild males maturing at three years (Holland *et al.*, 1996). Red drum matured at 11/2 years in culture condition, while animals in wild matured at three to five years of age (Craig *et al.*, 2000). This is a great concern to the aquaculture industry, because in some species, maturing animals exhibit reduced somatic growth when they divert energy from muscle growth into gonadal development (Holland *et al.*, 1996). On the other hand, fish breeding programs may benefit from early maturation broodstock. Increased feeding rates and higher temperatures in captive environments can reduce age at maturity and are associated with precocious maturation of these fish (Wootton, 1982; Holland *et al.*, 1996).

1.5.2.1 Dietary effects on a reproductive performance of female broodstock

The studies available on the effects of nutritional status on reproduction are mostly focussed on the time of first maturity, fecundity, egg size and egg quality and are limited to a few cultured species (e.g., salmonids, carp, tilapia and seabream)

(Watanabe, *et al.*, 1984; Bromage *et al.*, 1992; Luquet and Watanabe, 1986; De Silva and Anderson, 1995). Although sex steroids play an important role in mediating dietary influences on reproductive output, little attention has been given to the effects of these manipulations on endocrine changes.

Time

The time taken to show the effects of dietary quality on egg and larval quality vary between different species. This may depend upon the length of reproductive cycle, particularly the vitellogenesis phase, and the spawning pattern (ie. continuous, annual spawning etc). In rainbow trout, fish fed on high rations for the first four months of the reproductive cycle had higher fecundities and a higher percentage of spawned fish than those fed on low rations over the same period (Bromage *et al.*, 1992). In contrast, high rations during the latter stages of the reproductive cycle did not appear to affect the number of eggs produced by each individual fish, but did increase the weight of the brood fish (Bromage *et al.*, 1992). In Japanese flounder the dietary fatty acid changes upto three months before and during spawning, were shown to affect larval quality (Furuita *et al.*, 2000).

Although temperate species show a clear pattern in their annual reproductive cycle, many tropical species tend to spawn a number of times throughout the year and with varying intervals. It has been reported that Nile tilapia reared on different dietary protein levels (10, 20 and 35%) for two months showed no marked differences in the oocyte stages, while those reared for four months had marked differences in oocyte development (Gunasekera and Lam, 1997). Dietary EFAs affect egg composition and spawning quality of gilthead seabream broodstock after three weeks of feeding (Fernadez-Palacios *et al.*, 1995). This may suggest that spawning quality of gilthead seabream can be improved by altering the nutritional quality of broodstock diets even during spawning (Fernadez-Palacios *et al.*, 1995). A similar trend was observed for red seabream (Shepard and Bromage, 1988). Feeding broodstock with krill, mysis, shrimp and crab wastes within a few hours before spawning resulted in pigmentation of the eggs. This indicates that pigments and other fat soluble vitamins in diets are easily incorporated into eggs and so the nutritional quality of broodstock diets shortly before spawning may effect egg quality (Shepard and Bromage, 1988).

Egg and larval quality

Good quality eggs may be defined as those that exhibit low levels of mortality through fertilisation up to first-feeding. They would also be expected to produce the healthiest and fastest growing fry, although the influence of egg quality on these characteristics may be masked by a variety of environmental and husbandry factors (Bromage *et al.*, 1994). It has also been noted that hormonal levels in eggs or larvae which passed from broodfish may be an important determinant of egg/larval quality (Lam, 1994). Furthermore, nutritional status of the broodfish has been clearly shown to influence egg quality (Watanabe *et al.*, 1985). Kanazawa (1985) reviewed the effects of dietary proteins, lipids, minerals, vitamins and pigments on the egg quality and quantity of many teleost species. Kjorsuvik (1994) studied egg quality in wild and broodstock cod, *Gadus morhua*. They concluded that the spawning result of cod is dependent on fish age, nutritional status and batch number of spawned eggs. The number of eggs produced is clearly in favour of well-fed captive fish rather than wild fish.

Food ration

Food ration modifications can significantly affect the fecundity of broodstock, their ability to mature and the size of eggs produced (Bromage, 1995). Scott (1962), Springate and Bromage (1985), Springate *et al.* (1985) and Bromage (1995) have shown that food ration affect the fecundity and egg size in salmonids. Restricted feeding during oocyte recruitment, particularly during vitellogenesis, slows oocyte growth and delays the start of spawning in turbot (*Scophthalmus maximus*) (Bromley *et al.*, 2000). Three spined stickle back females fed high rations had a higher fecundity compared to fish fed lower rations (Ali and Wootton, 1999b). Atlantic herring fed a high ration had a higher number of oocytes in the ovary and a low level of atresia than fish fed on a low ration (Ma *et al.*, 1998). In contrast, low rations induce Nile tilapia to mature earlier and produce more eggs relative their body size (Radampola, 1990).

In some teleosts, food availability can influence oocyte development and steroid hormone production even at a time when they are not normally reproductively active. Female golden perch show rapid oocyte growth after a period of starvation and refeeding when compared to normal fed fish which did not show advanced oocyte

development during the same time (Collins and Anderson, 1999). This may indicate that the cycling of food abundance stimulates oocyte development in this fish (Collins and Anderson, 1999).

Protein level

Although detailed studies on the effect of dietary protein level are fewer and contained conflicting information, it is generally agreed that an optimum protein level is required for reproductive success (De Silva and Anderson, 1995). For example, it has been shown that in Nile tilapia, the frequency of spawning increased slightly as the dietary protein level increased from 20 % to 50 % (Santiago *et al.*, 1983). In another study, the greatest proportion of female spawning occurred when fish fed 25% or 30 % protein diet (De Silva and Radampola, 1990). Gunasekera *et al.* (1995) and Gunasekera and Lam (1997) have shown that Nile tilapia fed on low protein diets showed a slower rate of ovarian recrudescence when compared to fish fed a high protein. Reduced spawning performance and egg quality, in terms of buoyancy and hatchability and a high percentage of abnormal larvae were observed in broodstock sea bass fed diets with a low level of protein (Cerda *et al.*, 1994).

Some studies focussed on the effect of dietary protein quality on reproductive performance of teleosts as efforts are concurrently being made to replace fishmeal with cheaper, more readily available grain meals. Nile tilapia fed fish meal protein had better ovarian growth and large oocytes compared to fish fed legume substituted diets which may indicate that legume meal is an inadequate source of nutrients for egg production (Cumarantunga and Thabrew, 1989). These authors attributed this difference to higher levels of vitellogenic proteins or lipids in fish meal. Santiago *et al.* (1988) found that fry production was highest for fish fed on the control diet (0% leucaena) while it was significantly low in fish fed on leucaena substituted diets, therefore, incorporation of Leucaena leaf meal negatively effected the reproductive performance of tilapia. Radampola (1990) has shown that fish meal can be substituted by a legume (*Vigna catiang*) up to a certain level (47%) without having adverse effects on reproduction in Nile tialpia. Total number of spawnings, number of females spawned and relative fecundity in nile tilapa increased with increasing dietary *V. catiang* level up to 47% level. These studies suggested that diets will have to be carefully evaluated for the effects on reproduction before they use for broodstock

Vitamins

Little information is available describing the effects of dietary vitamins on reproductive development. Milkfish broodstock fed a diet supplemented with vitamin C produce more spawnings with good quality eggs, defined as a high percentage of egg hatchability and survival rates (Emata *et al.*, 2000). Cod broodstock fed on diets with high vitamin C had higher vitamin C levels in ovaries and fertilised eggs (Mangor-Jensen *et al.*, 1994). Hatchery performance could not be observed in the latter study due to technical problems. However, it has been reported in other species that vitamin C deficiency in broodstock diets resulted in low hatchability (Soliman *et al.*, 1986). Eggs from fish fed high Vitamin C diet had low chorion strength, thus, Mangor-Jensen *et al.* (1994) suggested that the low egg strength could be associated with high hatching rate.

Fatty acids

Of the factors influencing diet quality, fatty acids have received much more attention than any other component of broodstock nutrition. Reproduction and egg quality have been found to be markedly affected by the nutritional quality of the broodstock diet (Watanabe *et al.*, 1984). The fatty acid pattern in fish egg lipids reflects that of the dietary lipids for broodstock and EFAs greatly affect egg and larval quality (Navas *et al.*, 1997).

In both marine and fresh water fish, reduced fecundity, hatchability and survival, and a higher proportion of deformed larvae were found after feeding n-3 fatty acid deficient diets (Watanabe 1982; Cerda *et al.*, 1995; Navas *et al.*, 1997; Rodriguiez *et al.*, 1998; Furuita *et al.*, 2000). Sea bass (*Dicentrachus labrax*) broodstock fed on trash fish showed high reproductive performance and high egg viability and hatching rates, when compared to fish fed on commercial diets with different fatty acid levels (Navas *et al.*, 1988). In the same species, high EFA in the broodstock diet during vitellogenesis resulted in a high number of viable eggs and low mortality rates. (Navas *et al.*, 1997; Bruce *et al.*, 1999). The n-3 highly unsaturated fatty acid (HUFA) level in the broodstock diet showed positive effects on larval quality and survival in Japanese flounder (Furuita *et al.*, 2000). Broodstock fed diets with the optimal n-3 HUFA level (0.8 %) for juvenile growth showed lower growth

than broodstock fed the higher n-3 HUFA diet (2.11 %). This suggests that the n-3 HUFA requirement for adult female flounder increases during the spawning season (Furuita *et al.*, 2000). In some teleosts, excess n-3 HUFA levels in broodstock diet caused negative effects in larval quality. Fernandez-Palacios *et al.* (1995) found that although % egg viability (hatch rates) increased with the n-3 HUFA levels in the broodstock diet, high levels of those fatty acids caused yolk sac hypertrophy and decreased larval survival. Those authors suggested that spawning quality of gilthead seabream can be improved by feeding n-3 HUFA up to 1.6%, but high levels can cause negative results.

Feeding an n-3 HUFA deficient diet resulted in lower fecundity, percentage of fertilised eggs and percentage of hatching larvae in gilthead seabream (Rodriguez *et al.*, 1998). Sea bass fed a lower dietary lipid level and/or a different fatty acid level showed dramatic reductions in fecundity and egg viability compared to fish fed a natural diet (Cerda *et al.*, 1995).

In some fish species, the biochemical composition of eggs (in terms of lipid, fatty acids and amino acids) varied significantly between first time and repeat spawners. The repeat or more matured spawners of Atlantic halibut (*Hippoglossus hippoglossus*) produced eggs with more lipids and fatty acids, and these eggs are also associated with better fertilisation and larval survival rates (Evans *et al.*, 1996).

Marine fish larvae require n-3 highly unsaturated fatty acids, mainly EPA (20:5 n-3) and docosahexaenoic DHA (22:6 n-3) acids, for normal development and survival (Bessonart *et al.*, 1999). These fatty acids play an important role as components of membrane phospholipids. Marine fish do not have an ability to synthesise EPA and DHA from LA (18:3 n-3) and it is expected that provision of these fatty acids by the mother into the egg is a fundamental process of egg development.

Free arachidonic acid (AA- 20:4 n-6) has been implicated as a major fuel in energy metabolism of halibut eggs and larvae (Fyhn, 1989 cited in Evans *et al.* 1996). Arachidonic acid has been implicated in prostaglandin formation in marine fish and eicosanoids produced from n-6 fatty acids have a critical physiological role in the gills, kidneys, intestine and ovaries (Sargent *et al.*, 1989; Castell *et al.*, 1994). The lack of response to hypophysation techniques in tank reared Macquarie perch,

compared to wild fish was attributed to low levels of n-6 fatty acids (particularly 20:4 n-6) in the diet of tank reared fish (Sheikh-Ekdin *et al.*, 1996).

Nocillado *et al.* (2000) investigated the egg quality in induced spawns of barramundi. Based on fertilisation and hatching rates, the eggs were classified as either zero fertilisation (group I) or fertilisation occurred (group II). The total lipid, EPA (20:5 n-3) and LA (18:3 n-3) were higher in the high fertilisation group. These results indicate that specific lipids of barramundi eggs correlate with the egg viability. As has already been shown, the components of the broodstock diet are reflected in the composition of the eggs, and manipulations in broodstock diet can improve egg viability and survival.

Hormone and reproduction

As described in section 1.3, reproduction is regulated by a complex series of hormones. Some information is available regarding interactions between diet and reproductive hormones. Female sea bass (*Dicentrachus labrax*) fed on either diet 1 (56% protein and 11% lipid) or Diet 2 (47% protein and 7% lipid) compared to natural diet (trash fish) differed in concentrations of plasma T and E_2 levels and showed poor spawning performance (Cerda *et al.*, 1995). In addition, fish fed on commercial diets (diet 1 or 2) had low fecundities, higher atresia and altered patterns of plasma lipid levels with respect to the females fed a natural diet (Cerda *et al.*, 1995).

Navas *et al.* (1998) further investigated the effects of dietary lipid composition on reproductive performance and plasma hormonal levels of sea bass. Fish fed commercial diets (commercial diet alone (10% lipid) or enriched with n-3 HUFA (22% lipid)) had low plasma GtH II and E_2 concentrations and lower fecundity, viability and hatching rate when compared to those fed a natural diet. This study suggests that the fatty acid composition of the diet altered E_2 synthesis and GtH II production; which in turn influence the reproductive performance of fish. Thus, diets can influence spawning performance, not only by altering egg production, but also by disrupting the hormonal pathways that control reproductive process.

Wild and captive broodstock

Many studies have found that some specific differences in biochemical composition of eggs of wild and captive reared broodstock. Devauchelle *et al.* (1982) compared the biochemical composition of eggs of wild and captive sea bass, sole and turbot. Eggs from wild fish contain more protein, lipids and polyunsaturated fatty acids compared to the spawns of captive fish. Differences in fatty acid components of eggs are prominent in previous studies. For example, higher AA (20:4 n-6) and total lipid content in wild striped bass eggs (Gallagher *et al.*, 1998), low 18:0 and high 18:1 n-9 in wild sturgeon fish eggs (Czesny *et al.*, 2000) and high n-3:n-6 ratio of eggs in wild striped trumpter (Morehead *et al.*, 2001) in comparison with their respective cultured fish were reported. Ahlgren *et al.* (1999) have shown that pelleted food resulted in unnaturally elevated and unbalance n-3:n-6 ratio in European grayling when compared to wild fish fed natural diet. As it is well known that the composition of broodstock diet influences egg composition, these findings suggest that nutritional quality of wild and captive broodstock may be different from each other and this may be cause the low spawning performance of many captive reared fish.

1.5.2.2 Dietary effects on the reproductive performance of male fish

Although many studies have demonstrated the effect of dietary quality and quantity on the reproductive performance of female broodstock, only a little information is available on the effect of diet on male broodstock performance.

Evidence for nutrition and maturation in male fish

Early sexual maturation of male Atlantic salmon in captive environments has been thought to be induced by growth rate or body size at specific times of the year (Rowe and Thorpe, 1990b). It has been suggested that fish may assess body energy stores, and that critical thresholds for size and energy storage must be met at a specific time of year to allow maturation. Otherwise maturation is inhibited (Rowe *et al.*, 1991; Thorpe, *et al.*, 1994, Silverstein *et al.*, 1999).

Reduced food intake during spring suppressed growth and energy storage and leads to reduced incidence of sexual maturity in Altlantic salmon (Rowe and Thorpe, 1990b; Thorpe *et al.*, 1990). However, Rowe *et al.* (1991) compared the seasonal cycles of depletion and replenishment of total lipids and mesenteric fat in maturing

and non-maturing male Atlantic salmon and suggested that maturation is suppressed when mesenteric fat fails to exceed an undefined level by May. These authors also proposed a model to explain the physiological link between fat accumulation during spring and the initiation of maturation. In addition, delayed spawning in Atlantic herring males fed on low rations has been reported by Ma *et al.* (1998).

Jobling *et al.* (1993) studied the effect of alternating short periods of food deprivation on maturing and non-maturing Arctic charr. However, the proportion of matured fish at the end of the experimental period did not show significant differences between treatments. In previous studies with Atlantic salmon, food restriction was applied at a particular time of the year rather than as a routine procedure (Jobling *et al.*, 1993) and this may explain why alternative food restriction did not show suppressed maturation rates.

There is some evidence that in some other teleosts, food quality is inversely related to maturation. The F1 population of wild Arctic charr matured early in rearing conditions compared to the original population (Svedang, 1991). A decrease in food quality (high cellulose) increased the frequency of maturation whereas high food quality inhibited maturation in male Arctic charr (Svedang, 1991). This is in contrast to other findings which showed reduced food intake decreased maturation (Scott, 1962; Bagnel, 1969; Bromage, 1995). Svedang (1991) suggested that the negative relationship between food quality and maturation might reflect an adaptive trait of the original population.

Some workers have studied changes in food intake, growth performance and plasma hormone levels in fish over a reproductive cycle. In Atlantic salmon, the early phase of sexual maturation was characterised by slow rising hormone levels which associated with high rates of food consumption and growth rates. In contrast, hormone levels increased more rapidly in association with slow growth rates and inappetence in the late phase (Stead *et al.*, 1999).

In a more recent study, Shearer and Swanson (2000), studied the effect of whole body weight stores on the incidence of maturation in 1+ male spring chinook salmon which had similar rate of body growth. As pituitary hormones (FSH and LH) are involved in regulating spermatogenesis in fish (Xiong *et al.*, 1994) and insulin-like growth factor (IGF-1) is regulating growth, Shearer and Swanson (2000) hypothesised

that growth may influence maturation via an interaction between these two endocrine systems. These authors observed that maturing males were significantly larger than the non-maturing fish. In addition, the percentage of maturing fish was significantly higher in fish fed higher dietary lipid (22%) level. In regard to hormone levels, pituitary FSH and LH levels increased during the reproductive cycle and plasma IGF-1 levels were significantly higher in maturing males. These authors suggested that whole body lipid levels influenced the incidence of maturation of male spring chinook salmon.

Cerda *et al.* (1994) reported that male sea bass fed diets of varying protein levels showed no changes, unlike the females of this species. Males were in running condition (release sperm on gentle pressure on the abdomen) during the spawning period. However, these authors did not measure any sperm quality parameters (e.g., sperm concentration, motility, and sperm volume) in their study. They also reported that egg viability differed with different treatments, but were unable to provide an explanation because the egg proximate compositions were not different between treatments. These authors suggested that different egg viability could be due to some other factors (e.g., amino acids, fatty acids of eggs) which were not measured in their study. However, as successful fertilisation also depends on sperm viability, it may be that low protein diets led to low sperm quality in males and consequently affected egg viability.

Holland *et al.*, (1996) observed the sperm characteristics of precocious 1-year-old male striped bass (*Morone saxatilis*). Precocious males had lower sperm motility and a significantly higher sperm concentration than adult males. The presence of precocious male striped bass reared in captivity could be the result of hatchery conditions, which would suggest the influence of environmental factors (e.g., increased feeding and/or higher temperatures) on the sexual maturation of striped bass.

Effects of diet on the sperm quality

Although sperm quality is an important factor in egg viability, less attention has been given to the effect of diets on sperm quality. There is only one site in teleost fish eggs for spermatozoon penetration: the micropyle at the animal pole. The

probability for reaching the micropyle and subsequent fertilisation depends on the density of motile sperm surrounding the egg (Ciereszko and Dabrowski, 1995). Therefore, both sperm motility and concentration are crucial factors for egg viability. Motility, the fertilising capacity and the concentration of sperm in the milt are used to evaluate sperm quality. The quality of sperm is highly variable and depends on various external factors, such as the feeding regime, the quality of feed and the rearing temperature of the males (Billard *et al.*,1982).

Many other factors also affect sperm quality, including season (Munkittrick and Maccia, 1987; Holland *et al.*, 1996; Wang and Crim, 1997; Suquet *et al.*, 1998), morphometry (Gage *et al.*, 1998; Lahnsteiner and Patzner, 1998), temperature (Emri *et al.*, 1998), the suspension solution (Krise *et al.*, 1995; Toth *et al.*, 1997) and the hormones used to induce spermiation (Stacey *et al.*, 1994; Weber and Lee, 1985).

Ration level did not significantly affect the maturation rate of males in turbot (Bromley *et al.*, 2000). However, the small size of the testes and low milt production indicate that reproductive investment is much lower in male turbots than in female turbots (Bromley *et al.*, 2000). It is expected, therefore, that the affect of diet on male turbot reproduction might be less than that on females.

In rainbow trout, dietary ascorbic acid deficiency (0%) reduced both sperm concentration and motility (Ciereszko and Dabrowski, 1995); which then diminish the fertility of rainbow trout males.

Although previous studies have suggested that dietary lipid influences egg quality, very little research has been focussed on the effect of dietary lipid or fatty acids on sperm quality. As it is well known that fatty acid composition of fish tissue reflects dietary lipid (Sargent *et al.*, 1989), it can be hypothesised that sperm quality is also altered by dietary lipids. However, these studies mainly discussed the influence of dietary fatty acid on the sperm compostion. Leray and Pelletier (1985) reported that rainbow trout fed an (n-3) deficient diet but with high LA (18:2 n-6) had high proportions of (n-6) fatty acids in their sperm. High levels of dietary 18:2 (n-6) resulted in increased amounts of 20:4 and 22:5 (n-6) fatty acids in spermatozoa, which is presumably a result of the conversion of 18:2 (n-6) to longer chain polyunsaturated fatty acids (Leray and Pelletier, 1985). Bell *et al.* (1996) studied the fatty acid composition of sperm of cultured sea bass, fed either a commercial diet or trash fish,

and those of wild fish. These authors showed that dietary lipid markedly altered the fatty acid composition of sperm in sea bass, especially with respect to the content of AA (20:4 (n-6)) and EPA (20:5 (n-3)). These effects were similar to those of the same diets on the egg composition in the same broodstock. For example, fish fed a commercial diet have elevated 20:5 (n-3) and decreased 20:4 (n-6), resulting in a greatly decreased AA/DHA ratio in their sperm when compared to fish fed either trash fish or compared to wild fish. Thus, the fatty acid profiles of a broodstock diet greatly influenced the fatty acid composition of spermatozoa. However, the aforementioned studies did not pay much attention to sperm quality such as motility, concentration or fertilising capacity.

Labbe *et al.* (1993) have shown that phospholipids of the sperm of male rainbow trout fed on a diet of either corn oil (high n-3) or cod liver oil (n-6) reflected the features of the pelleted diets. However, the ability of the sperm to freeze-thaw is not influenced by different fatty acid compositions. Pustowka *et al.* (2000) shown that dietary fatty acids affect sperm plasma membrane integrity in rainbow trout, and therefore fertility after cytopreservation. High levels of cholesterol and monounsaturated fatty acids provide spermatozoa with increased resistance to cryopreservation damage wherears low levels of monounsaturated and high levels of PUFAs may increase crypreservation damage (Pustowka *et al.*, 2000).

1.6 Objectives and aims

From the reviewed literature, it is apparent that the relationship between nutrition and reproduction in fish is far from clear. Furthermore, it is apparent that the mechanisms by which those effects are manifest are also very poorly understood (Shearer and Swanson, 2000). The period when spermatogenesis is initiated and likely to be influenced by nutritional status, is difficult to define through direct histological examination without killing the study animal. However, endocrine measurements which would indicate the activation of the reproductive axis can be measured over a relatively long period in order to understand the complex mechanisms underlying these processes.

In view of studies cited above, it is expected that nutritional status will affect the reproductive status of fish. Therefore, the objective of this study is to investigate

the impact of different dietary nutrient levels on reproductive performance of male barramundi. Three particular aspects of this relationship will be investigated to answer the following questions.

1. Does feeding level affect the reproductive performance of male barramundi?
2. Does dietary protein:energy ratio affect reproductive performance of male barramundi?
3. Does the fatty acid composition of diets affect reproductive performance of male barramundi?

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Introduction

Barramundi, *Lates calcarifer* (Bloch) reared in fresh water were obtained from commercial barramundi farms and transferred to aquaculture facilities at James Cook University, Townsville, Australia.

All experimental regimes were conducted within the guidelines of "The Australian Code of Practice for the Care and Use of Animals for Scientific Purposes" Ethical clearances were given by James Cook University animal ethics committee.

2.1.1 Experimental conditions:

Fish were held in eight 3500L circular fibreglass tanks (~10 fish/tank) with recirculating salt water for an acclimatisation period for 4 weeks to 10 weeks before the start of the experiments. Two experimental systems, each consisting of four 3500 L tanks for rearing fish, one small (~2000 L) rectangular tank (used as a reservoir), and a biological filter were used. These systems were supplied with sa

chapter. During the acclimatisation period, fish were fed to satiety in the morning between 0800 and 1000 hr, seven days per week. The diet used was a commercial barramundi pellets (15mm; Ridley Aquafeed, Brisbane, Australia) containing 48 % crude protein, 12 % lipid and 18 $MJ.kg^{-1}$ crude energy.

Generally, the fish were very aggressive, especially at the beginning of feeding, and it would take 20-30 min to feed them to satiety. During the experimental period, the amount of food put into each tank was recorded daily and was considered as food consumed by the fish. The pellets were given to fish in small amounts at a time and care was taken to ensure all fish fed to complete satisfaction.

2.1.3 Anaesthesia

Barramundi were anaesthetised by immersion in a well-aerated sea water tank containing 1 $g.l^{-1}$ of benzocaine (Ethyl-p-amino benzoate, Sigma chemicals, USA). Benzocaine was dissolved in absolute ethanol (AR grade, BDH chemicals, Australia) and was injected below the surface using a syringe to prevent precipitation. Anaesthetisation occurred within 3 - 5 min and it was considered to be adequate when the opercula and fin movements ceased. Fish generally recovered within 5 min.

2.1.4 Sampling

At the start of each experiment or at six weekly intervals, fish were netted and anaesthetised using benzocaine and their total length to the nearest cm and weight to the nearest g were recorded. Initial tagging, length weight measurements and blood sampling of fish were completed within 2-3 min. Just after sampling, the fish were placed in a 100 l recovery tank containing dechlorinated fresh water for 10 min as a prophylactic treatment before being returned to the experimental tanks.

Tagging of fish

Fish were tagged using plastic T tags of different colours for easy identification prior to being assigned to the tanks. Tags were inserted into the dorsal muscle just below the dorsal fin.

2.1.5 Blood Sampling

A 5 ml blood sample was taken from the caudal vein using an 18 gauge hypodermic needle and 5ml disposable syringe. Blood samples were immediately transferred to 2.5 ml fluoride-oxalate tubes (Sarstedt, Technology Park, SA 5095, Australia), mixed gently and kept on ice until taken to the laboratory. Blood samples were centrifuged at 14,000 g for 10 min (Eppendorf centrifuge 5415C, Hamburg, West Germany). Plasma samples were collected into 1.5 ml polypropylene tubes and stored at –20 °C until analysis.

2.1.6 Cannulation of fish

When possible, fish were cannulated to check for presence of any milt. This was done by inserting polyethylene tubing (1 mm- outside diameter) through the urino-genital opening of anaesthetised fish until it reached the gonad. A slight vacuum was applied by mouth, the tube removed and checked for any evidence of milt. Milt was collected into 1.5 ml tubes and kept on ice until taken to the laboratory. These tubes were kept in the refrigerator (-4 °C) until sperm quality was determined.

2.1.7 Final sampling

At the end of the experiment, the length and weight measurements of fish were taken and blood was sampled as previously described. In addition, five fish (unless otherwise stated) from each tank were sacrificed by cervical dislocation, and gonadal and liver weights were recorded. Samples of liver and muscle were taken for proximate analysis and stored in the freezer at –20 °C until further analysis. Gonads were preserved in formaldehyde acetic acid calcium chloride (FACC) solution (Appendix I) for further histological studies. Whole muscle weight was determined by dissection.

2.2 Growth parameters and related measurements

The following parameters were estimated according to Daniels and Robinson, (1986), Nematipour, *et al.* (1992) and De Silva and Anderson, (1995).

a) Specific Growth Rate (SGR)

$$SGR = \frac{Ln(BWt_f) - Ln(Bwt_i)}{t} x100$$

b) Percent Body Weight Gain (%BWG)

$$\%BWG = \frac{BWt_f - BWt_i}{BWt_i} x\,100$$

Where BWt_i and BWt_f are initial and final body weights (g) of fish respectively and t is the number of days between weighing.

c) Gonado Somatic Index (GSI)

$$GSI\,(\%) = \frac{Wt_{gonad}}{Wt_{body}} x\,100$$

d) Hepato Somatic Index (HSI)

$$HSI\,(\%) = \frac{Wt_{liver}}{Wt_{body}} x\,100$$

e) Muscle Index

$$\text{Muscle Index}\,(\%) = \frac{Wt_{muscle}}{Wt_{body}} x\,100$$

f) Visceral Fat Index

$$\text{Visceral fat index}\,(\%) = \frac{Wt_{Visceral\ fat}}{Wt_{body}} x\,100$$

Body weight (Wt_{body}), liver weight (Wt_{liver}), total muscle (Wt_{muscle}) and visceral fat weight ($Wt_{visceral\ fat}$) to the nearest g and gonad weight (Wt_{gonad}) to the nearest 0.1g were used for calculations.

g) Food Conversion Efficiency (FCE)

$$FCE = \frac{\text{Increase in body weight (g)}}{\text{Food consumed (g)}}$$

2.3 Proximate analysis

Proximate analysis of tissues, ingredients and diets was done using standard methods. All nutrients were expressed as total nutrients (g.kg of body weight of whole $fish^{-1}$) and energy as total amount (MJ.kg of body weight of whole $fish^{-1}$) to remove effect of fish size. In addition, relative amounts (% dry or % wet weight basis) and (MJ.g of $tissue^{-1}$) were used in some cases for comparison.

2.3.1 Sample preparation

Muscle samples were dried to constant weight at 50 °C to determine moisture (Section 2.3.2) and then ground into a fine powder using a grinder (Mikro-Feinmuhle-Culatti MFC, Ika Labor technik, D7813 Stauten 1-Br). Liver samples were first homogenised (Sorvall Omni-mixer, Dupant Company, Newtown, Connecticut 06470, USA) before drying, and the dried samples ground using a mortar and pestle. All dried samples were kept in sealed plastic vials until further analysis. Ingredients and diet samples were also dried at 50 °C to a constant weight before proximate analysis.

2.3.2 Moisture

Moisture was determined gravimetrically by drying wet samples at 50° C to a constant weight. The moisture from samples were measured in triplicate and dried samples were used for further analysis.

2.3.3 Protein

Total nitrogen was determined using the Kjeldhal method and multiplied by 6.25 to give crude protein (Crooke and Simpson, 1971). A Tecator Kjeltec system was used for the analysis. In brief, dried samples (~0.200 g) were digested with a Kjeltab (3.5 g K_2SO_4 + 3.5 mg Selenium catalyst - Tecator) and 6 ml of concentrated H_2SO_4. Tecator digestion system 4.0 (1016 digester) was used to digest the samples at 410° C for about 50 min until the solution became clear. After cooling, 25 ml of distilled water was carefully added to each tube. Ammonia was extracted from the samples using a Kjeltec system 1002 Distilling unit by adding 40% NaOH solution (~30 ml) and then distilling with steam. The distilled ammonia was collected into a flask containing 20 ml of boric acid indicator solution which was titrated against 1N H_2SO_4 solution. Each sample was analysed in duplicate. The crude protein of the sample was calculated using the formulae:

$$\text{Nitrogen (\%)} = \frac{\text{Titrant (ml)} \; x \; \text{normality of titrant}}{\text{Sample weight (g)}} \; x \; 0.014 \; x \; 100$$

$$\text{Crude protein (\%)} = \text{Nitrogen content (\%)} \; x \; 6.25$$

2.3.4 Lipid

Lipid was determined by a modified Folch *et al.* (1957) method. A sample (150 - 200 mg) was homogenised (Heidolph Electro GmbH & CoKG, Kelheim, Germany) with 5 ml of 2:1 (v:v) chloroform: methanol ($CHCl_3$:MeOH) mixture and filtered into a 25 ml screw cap glass tube through a filter paper (Qualitative no 1; Whatman, England). This step was repeated again to ensure all lipid was extracted from the sample. The original vial and filter paper were then rinsed with 5 ml of $CHCl_3$:MeOH solution and 3.75 ml of 0.88% KCl solution was added to each extract. The tubes were capped, inverted three times to mix the solutions and the solutions allowed to separate. The top layer (water) was aspirated using a vacuum and Na_2SO_4 (anhydrous) (~1 g) was added to the remaining organic layer. The salt and solvent were mixed by inverting three times. The organic layer was removed into a preweighed beaker, which was left overnight at room temperature in a fume hood to evaporate the chloroform. The beaker was weighed periodically until a constant weight was achieved. Fat content was calculated as:

$$\text{Fat}\ (\%) = \frac{\text{Weight of fat (g)}}{\text{Sample weight (g)}}\ x\,100$$

2.3.5 Ash

Samples were weighed into dry, preweighed porcelain crucibles and then heated in a muffle furnace at 550 °C overnight. The crucibles were then reweighed. Ash was calculated as:

$$\text{Ash}\ (\%) = \frac{\text{Weight of ash (g)}}{\text{Sample weight (g)}}\ x\,100$$

2.3.6 Energy

A Parr Semimicro bomb calorimeter (Parr Instrument Company, Moline, Illnois 61265, USA) was used to determine energy content of samples. The samples were compressed into pellets (~0.100 g), weighed and then ignited with excess oxygen. The heat released by complete combustion of the sample was recorded using

a Series 4500 Microscribe strip chart recorder (Houston Instruments, Austin, Texas, USA). The bomb calorimeter was calibrated using pelleted benzoic acid (energy - 26.6 $kJ.g^{-1}$; Sigma chemicals, USA).

2.4 Hormone assays

2.4.1 Oestradiol-17β and Testosterone

Radioimmunoassay (RIA) methods were used for measuring plasma T and E_2 levels. Pantex RIA kits (Testosterone direct ^{125} I- cat. no 135 and Estradiol direct ^{125} I- cat. no 174M, Pantex, Santa Monica, CA 90404) were modified and validated for use with barramundi plasma.

All reagents, with the exception of the second antibody, were diluted ten fold with (1:10) double deionised water prior to use. Apart from this, the instructions for the kits were followed (Appendix II). After mixing with second antiserum, the tubes were centrifuged at 3650 rpm for 10 min (Beckman GS-6R Centrifuge, Beckman Instrument Inc., CA 94304, USA). The supernatant was aspirated immediately and the pellets were counted using a gamma counter (Cobra TM11, Packard, Canberra, Australia) for 3 min.

Assay sensitivity was 10 $pg.ml^{-1}$ and 1 $pg.ml^{-1}$ for T and E_2 respectively. Inter-assay and intra-assay variation for T was 9.9% (n = 5) and 10.0 % (n = 7) respectively. Inter-assay and intra assay variation for E_2 was 4.5% (n = 5) and 6.1% (n = 7) respectively. Cross reaction of other steroids with anitserum to E_2 and T are minimal with percentage reactivities for all hormones below 7% (appendix II).

2.4.2 11 keto-Testosterone

Extraction of plasma

The 11kT was assayed using a modification of the method of Cuisset *et al.* (1994). Primary antibodies were supplied by Dr. David Kime, The University of Sheffield, UK.

Frozen plasma samples were thawed at room temperature and centrifuged for 10 min at 14,000 g (Eppendorf centrifuge 5415C, Hamburg, West Germany). Plasma (250 µl) was pipetted into a 5ml glass vial to which was added 1.5 ml of hexane:ethyl

acetate in a 1:1 mixture. The vials were capped tightly, vortexed for 5 min and immersed in a -30 ^{0}C ethanol bath for 2 min. The water layer was frozen and the top organic layer was quickly decanted to a second 5 ml glass vial. The extraction with hexane:ethyl acetate was repeated once to ensure all the hormones were extracted. The organic layer was evaporated under N_2 at 30 ^{0}C. The extracted samples were resuspended in 800 µl steroid assay buffer (pH 7.4) (Appendix I), vortexed for 10 min and kept at -80 ^{0}C under N_2 until further assay.

Hormone assay

The 11kT was measured by enzyme linked immuno sorbent assay (ELISA). Briefly, the method is as follows:

The 96 well microplates (Flat bottom, polystyrene; Iwaki glass, Japan) coated with goat anti-rabbit immunoglobin (10 µg/ml in potassium phosphate buffer; appendix I) were washed with wash buffer (appendix I) three times to remove any unbound second antibody. Plasma extracts or standards (100 µl) were incubated with 11kT-3-CMO-AchE tracer (25 µl; in steroid assay buffer; appendix I) and antisera (25 µl; in steroid assay buffer).

Non specific binding was determined using an incubation in which 25 µl tracer was added to 125 µl of steroid assay buffer. The maximal binding was obtained in the absence of competitor, in which 25µl of tracer and 25µl of antisera were added to 100 µl of steroid assay buffer. After incubating for 2 hr at room temperature, the plates were washed with wash buffer (3 times) and 200 µl of Ellman reagent (appendix I) were added to each well and left for 48 h in dark at room temperature to develop. After incubation, the absorbance at 414 nm was measured. A log:logit plot of the standard curve was used to extrapolate unknown concentrations.

2.5 Histology

A piece of fixed gonad was dehydrated, and embedded in paraffin for sectioning. Sections of 5µm were then stained with haematoxylin and eosin and mounted in DPX. Stained sections were examined using a binocular microscope (Olympus CH2) to determine the gonadal developmental stage. Gonadal stages were identified according to Guiguen *et al.* (1994) (Section 1.2.8).

The testes were classified as follows:

1. M1- Gonia stage
2. M2- Spermatogenesis
3. M3- Spermiation
4. M4- Post-spawning

A slight modification was made in the M1 stage on the basis of observations of gonadal stages in this study. Three sub stages were identified:

1. Stage1a - Gonia stage with no further development. All cells were identified as gonia cells and were very closely packed in the gonad.
2. Stage1b - Mostly gonia stage, but clear indications of further development of cells including primary and secondary spermatocytes and spermatids.
3. Stage1c - Gonia stage with remaining spermatids from the previous developmental cycle and with empty tubules. This was identified as a new developmental cycle.

2.6 Sperm quality

Sperm quality was determined according to Palmer *et al.* (1993). Frozen semen was thawed at room temperature and a sample of semen adhering to the tip of a fine dissecting needle was mixed with one drop of clean sea water on a microscopic slide. Motility was observed under 400 magnification (Olympus, Japan) and the intensity was assessed according to a relative 5-0 scale.

Stage of motility

5 - Most active sperms; sperm creating swirling current obscuring the movement of individual sperm across the field or view.

4 - Very active sample; all sperm visibly progressing rapidly across the field of view.

3 - Less energetic head and tail movement; most with forward motion

2 - Slow head and tail movement; some individuals progressing slowly

1 - Head movement only; no progressive motion

0 - No activity

CHAPTER 3

EFFECT OF FEEDING FREQUENCY ON THE GROWTH AND STEROID HORMONE LEVELS OF BARRAMUNDI

3.1 INTRODUCTION

Food quantity or ration has been shown to affect reproductive performance of fish, particularly the fecundity and egg quality in female broodstock (Scott, 1962; Wootton, 1973; Springate and Bromage, 1985; Bromage, 1995; Ali and Wootton, 1999; Bromely *et al.*, 2000). In many teleosts, it has been shown that the favourable/better feeding conditions induce early maturation or reduced age at maturity (Wootton, 1982; Holland *et al.*, 1996), and conversely, reduced food supplies delay maturation (Scott, 1962; Bagnel, 1969; Springate *et al.*, 1985; Reimers *et al.*, 1993; Bromage, 1995). In contrast, some teleosts have an ability to switch from growth to reproduction in unfavourable conditions. Inadequate food causes stunting and precocious breeding of tilapia in natural waters (Lowe-McConnell, 1982), as well as in experimental conditions (Radampola, 1990). This ability is probably an adaptation to harsh and changing environments that occurred during their evolution (Lowe-Mcconnell, 1982). Other studies have focussed on the effect of food deprivation for varying time periods on reproductive performance in fish (Rowe and Thorpe, 1990a; Thorpe *et al.*, 1990; Ali and Wootton, 1999b; Collins and Anderson, 1999). However, the effect of feeding frequency on reproductive performance has received little attention in broodstock nutrition. In particular, consideration of the nutritional effects on male broodstock development has been neglected.

Food deprivation for varying periods and food quantity has shown to affect maturation in some male teleosts. Studies on Atlantic salmon indicated that reduced food intake at a particular time of the year (spring) reduces the number of matured males in the autumn (Rowe and Thorpe, 1990a; Thorpe *et al.*, 1990; Rowe *et al.*, 1991; Reimers *et al.*, 1993). In contrast, a variety of regular patterns of food deprivations and refeeding for short periods was found not to affect the proportion of males maturing in the following reproductive season in Arctic charr (Jobling *et al.*, 1993) or the testis size in male Atlantic cod (Karlsen *et al.*, 1995). These studies suggest that the effects of food deprivation on the proportion of maturing animals may depend on the timing of food deprivation. It is likely that gonad development is suppressed when food deprivation is applied at a certain stage (probably in the initial stages) of gonad development. However, some studies have shown that reduced

feeding or alternate high and low feeding levels did not affect the maturation rate in male fish (Silverstein and Shimma, 1994; Jobling *et al.*, 1993; Karlsen *et al.*, 1995; Bromely *et al.*, 2000). This is probably due to low investment in reproduction in males compared to female animals.

It is well known that sex steroids play an important role in regulating reproductive development (Borg, 1994; Yoshikuni and Nagahama, 1991; details in section 1.4). However, there is very little information on relationships between nutrition, sex hormones and reproduction in fish (Stead *et al.*, 1999). Shearer and Swanson (2000) recently suggested that growth may influence maturation through an interaction between the endocrine systems associated with those two activities. In order to understand these relationships in more detail, an understanding of the effects of nutrition on reproductive endocrinology is required. Thus, the aim of the experiment described in this chapter was to determine the effect of feeding frequency on T and E_2 concentrations in plasma of male barramundi, and to relate this to changes in standard nutritional indices such as growth and tissue composition.

3.2 MATERIALS AND METHODS

3.2.1 Fish and experimental conditions

This experiment was designed as a randomised block design. However, due to uncontrollable events, two size classes of fish were used in this study. Fish reared in the research aquaculture facility of James Cook University were used as the first group, while fish from a commercial farm (NQ Barramundi, QLD, Australia) were used as the second group. The size differences were such that the two size classes of animals responded differently in many cases. Thus, they are largely presented as separate groups in this chapter.

The first group (700 $\pm$ 25g of weight), which are referred to as 'small fish', originated from farmed stock, and were held in the research aquaculture facility at James Cook University for approximately one year. During this period, fish were fed the barramundi commercial diet daily to satiety. The second group of fish (1000 $\pm$ 70 g), which are referred to as 'large fish', were transferred from a commercial farm (NQ Barramundi, Townsville, Australia) and acclimatised to the aquarium system at James Cook University for four weeks prior to commencement of the experiment. These large fish were fed daily to satiety on the same diet as the small fish.

Fish were kept in eight 3500 L indoor fibreglass tanks, according to the experimental conditions described in section 2.1.1. Each tank had 10 individually tagged fish. Fish were held at a constant temperature and photoperiod (section 2.1.1), similar to their spawning environment. Ammonia, nitrite and nitrate levels were measured routinely and were maintained at less than 1.0 $mg.l^{-1}$.

3.2.2 Feeding regime

Four tanks were allocated to each size group, with each tank within a size group receiving a different treatment. Fish were either fed to satiety daily (D), once in 3 days (3D) or once in 7 days (7D) using commercial barramundi pellets (Aquafeed- Brisbane, Australia; 15 mm, 50% - Protein, 12% - Lipid, $18MJ.kg^{-1}$ - Energy) for 24 weeks. The fourth group of fish (St/Rf) was starved for a 12 week period and subsequently refed to satiety daily for 12 weeks. Fish were fed in the morning (0800 - 1000 hr) according to the treatment, and the amount of food given

to each tank (depending on response of fish – section 2.1.2) was recorded throughout the experiment. This was considered as the amount of food consumed for each treatment.

3.2.3 Sampling protocol

At six weekly intervals, fish were anaesthetised and total length and weight were recorded (section 2.1.3 – 2.1.4). Fish were also cannulated (section 2.1.6) whenever possible. A 5 ml blood sample, taken from the caudal vasculature, was transferred to flouride-oxalate tubes to prevent clotting, mixed well, centrifuged at 14,000 g for 10 min and decanted plasma was stored at $^{-}20\ ^{0}C$ for further hormone analysis (section 2.1.5). At week 24 for small fish, and weeks 12 and 24 for large fish, five fish from each tank were killed and gonad, liver and muscle samples were collected as described in section 2.1.7. Samples of muscle and liver were taken for further analysis. Gonads were fixed for 48 h in FACC, dehydrated and embedded in paraffin wax. Five μm sections were stained with haematoxylin and eosin and examined using light microscopy (section 2.5). To maintain stocking density for the large fish, an additional five fish were assigned to each tank at week 12.

Percentage (%) BWG, muscle index, SGR, GSI, HSI and FCE were calculated as in section 2.2. Food consumption was expressed as % $BWt.day^{-1}$. Proximate analysis of muscle, liver and diet samples (moisture, protein, lipid, ash and energy) were performed using the standard methods described in section 2.3. Plasma T and E_2 levels were assayed using Pantex RIA kits (section 2.4). Gonad samples were processed, stained and examined under a binocular microscope (Olympus CH2) to determine the gonadal developmental stage (section 2.5). Sperm quality was determined as described previously (section 2.6).

3.2.4 Statistical analysis

While data for the % BWG, muscle index, GSI, HSI and proximate composition parameters of muscle and liver of the large fish were available at two time points (week 12 and 24), information on the small fish was only available at week 24. At week 24, small and large fish were tested for any differences using randomised block ANOVA. When a non-significant block effect was found, data for small and large fish were pooled. For this reason, parameters for GSI, HSI and liver

composition for small and large fish at week 24 were pooled. Treatment effects for all parameters at different time points (week 12 or 24) were analysed using oneway ANOVA, followed by Sheffe's test for unequal sample sizes (Zar, 1984). An independent t-test was used to detect a time effect within same treatments (eg. Daily feeding group, week 12 and 24). GSI and lipid content of liver were log transformed to achieve homogeneity of variance (Levene's test).

Weight, SGR, E_2 and T data were collected at six weekly intervals. Since initial weight, plasma T and E_2 levels were significantly different between small and large fish (independent t-test), these data for the two size classes were analysed separately.

In small fish, data for weight, SGR and E_2 were analysed using repeated measures ANOVA. However, T levels were not available for all samples, as some plasma samples of small fish were lost during storage. Due to the missing values, repeated measures ANOVA could not be used for the plasma T level of small fish. Similarly, an unbalanced design with large fish precluded the use of repeated measures ANOVA. Consequently, treatment effects at each time point for all parameters (weight, SGR, E_2 and T) were analysed separately using oneway ANOVA, followed by Sheffe's test. For each treatment, time effects between consecutive data points were analysed using a paired t-test or an independent t-test (when n was not equal due to loss of samples). Data for gonadal stages were analysed using log likelihood ratio for small sample sizes (Zar, 1984). Significant effects were assumed at $\alpha = 0.05$. All data were analysed using SPSS version 9.0.

3.3 RESULTS

To aid clarity, data will be presented for small fish, large fish and comparison made between small and large fish. However, since there was no significant block effect, data for GSI, HSI and liver composition of small and large fish at week 24 were pooled and presented as one group. Within each size group, treatment effect and time effect will be presented. St/Rf fish will be referred to as 'Starved' or 'Refed' when referring to starvation or refeeding periods respectively.

3.3.1 Food consumption

Food consumption (% Bwt.day^{-1}) data were obtained only for the small size group (Table 3.1a). Although statistical analysis could not be performed on this data (data available for individual tanks only), the trends are very clear. D, 3D and 7D fish consumed less food during the first six weeks than in the following weeks. Generally, 3D or 7D fish consumed a higher amount of food per meal than D fish. The food consumption of Refed fish was higher than that of D fish which received food continuously. As an additional five fish were assigned to the large fish group at week 12, food consumption data could not be calculated for large fish.

At the beginning of the feeding session, fish generally fed aggressively. Fish in the restricted feeding regimes (3D and 7D) and especially refeeding group fish fed aggressively during feeding compared to fish in daily feeding (D) treatment.

3.3.2 Growth

Weight gain

In both size classes, fish fed daily (D), once every three days (3D) or once in seven days (7D) demonstrated growth throughout the experimental period. St/Rf fish lost weight initially during the period of starvation but then gained weight once feeding was resumed (Fig. 3.1a and b). Fish fed daily had the highest growth, whereas 7D fish showed the lowest growth. However, the body weights of Starved fish and 7D fish at week 12 were not significantly different. In the small size class, 7D fish had

Table 3.1 (a) Food consumption (%BWt.day^{-1}) (mean ± SD of daily measures for 6 week period) and (b) Food conversion efficiency (FCE) of small barramundi fed daily (D), every 3 days (3D), every 7 days (7D) or starved and refed (St/Rf) over a 24 week period. Statistical analysis could not be performed, as these data are available for only one tank each.

(a)

Feeding Regime	Food consumption (%BWt.day^{-1})			
	Wk 0-6	Wk 6-12	Wk 12-18	Wk 18-24
D	0.64 (± 0.42)	1.05 (± 0.63)	1.04 (± 0.37)	0.72 (± 0.32)
3D	1.08 (± 0.70)	2.57 (± 0.68)	2.25 (± 0.63)	2.71 (± 0.77)
7D	1.33 (± 0.79)	2.52 (± 0.53)	2.81 (± 0.32)	2.76 (± 0.18)
St/Rf	Starved	Starved	1.68 (± 0.68)	1.13 (± 0.46)

(b)

Feeding Regime	FCE			
	Wk 0-6	Wk 6-12	Wk 12-18	Wk 18-24
D	0.66	0.53	0.61	0.37
3D	0.51	0.76	0.62	0.63
7D	0.04	0.56	0.37	0.48
St/Rf	starved	Starved	0.83	0.52

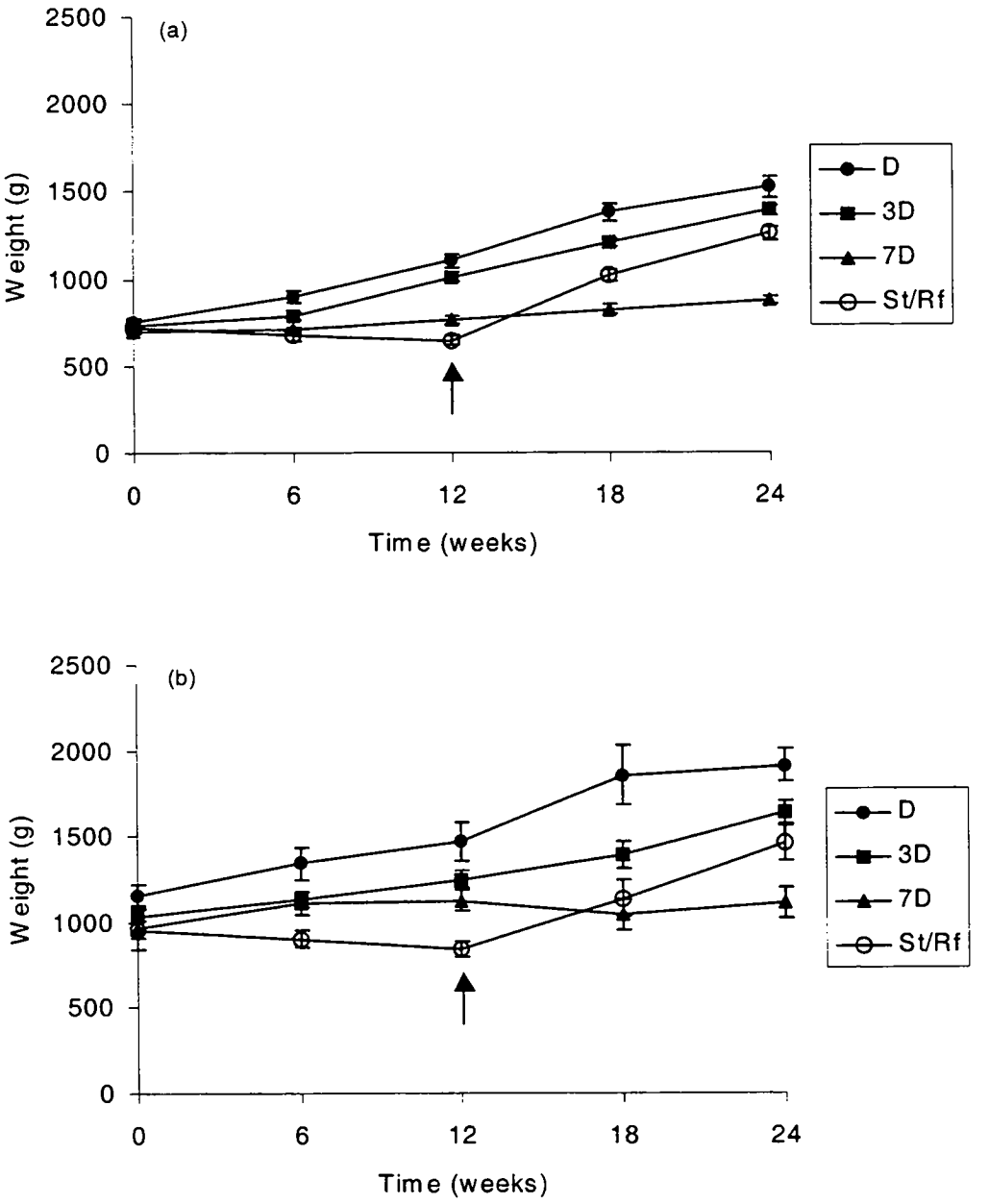

Figure 3. 1 Mean (± SE) body weights of (a) small and (b) large barramundi fed daily (D), every 3 days (3D), every 7 days (7D) or starved and refed (St/Rf) during the 24 week experimental period. The arrow indicates the time of beginning feeding of St/Rf fish.

significantly lower body weights compared to D, 3D and Refed fish at week 24. In the large size class, the body weight of 7D fish at week 24 was not significantly different from that of St/Rf fish. In both size classes, Refed animals showed significant growth after refeeding which was not significantly different from that of animals fed every three days over the whole experimental period. Generally, the small fish had a significantly higher % BWG at the end of the experimental period than large fish receiving the same treatment (Table 3.2a and b; ANOVA; $F= 37.9_{(1,53)}$; $P<0.001$).

Specific Growth Rate

Of the small fish, the D group maintained high SGR for the first 18 weeks. However, after this time SGR was decreased significantly (Fig 3.2a). The low SGR of 3D small fish during the first six weeks increased dramatically over the second six week period before declining over the remaining period. Although, 7D small fish had negligible growth for the first six weeks, it increased significantly after this time. Starved fish showed negative growth during the starvation period. Refed fish showed significant increase in SGR during weeks 12 to 18, which was higher than in all other groups. However, SGR of Refed fish was significantly lower in the final six weeks and reflected D and 3D SGR values.

Large D and 3D fish maintained relatively high SGR during the whole period (Fig 3.2b). 7D fish showed very slow growth up to week 18 after which SGR increased significantly. As with the small fish, Starved large fish showed negative growth, while Refed large fish showed the greatest SGR values, which were maintained for the entire 12 weeks of refeeding. The highest SGR values were found for Refed fish, with SGR values of 1.09 at week 18 and 0.66 at week 18 for small and large fish respectively.

3.3.3 Food Conversion Efficiency

Overall, D and 3D fish showed higher FCE values than 7D fish (Table 3.1b). However, D fish showed low FCE during the last six weeks. 7D fish showed the lowest FCE (0.04) at the beginning of the experiment. In the following six weeks their FCE increased up to 0.56 and maintained a comparatively low level thereafter. Refed fish showed the highest FCE value (0.83) immediately after the start of

Table 3.2 Initial weight, final weight and % body weight gain (%BWG) (mean ± SE) for (a) small and (b) large barramundi fed daily (D), every 3 days (3D), every 7 days (7D) or starved and refed (St/Rf) over a 24 week period . n = sample size. Means with the same superscript in the same column are not significantly different (P>0.05).

(a) Small fish

Treatment	Initial weight (g) (n =10)	Final weight (g) (n = 10)	% BWG (n = 10)
D	754 ± 24 ^{a}	1525 ± 59 ^{c}	102.70 ± 6.53 ^{c}
3D	731 ± 24 ^{a}	1391 ± 28 ^{bc}	91.58 ± 5.88 ^{bc}
7D	701 ± 19 ^{a}	869 ± 22 ^{a}	26.98 ± 3.46 ^{a}
St/Rf	721 ± 28 ^{a}	1258 ± 40 ^{b}	75.08 ± 3.71 ^{b}

(b) Large fish

Treatment	Initial weight (g) (n = 10)	Final weight (g) (n = 5)	% BWG (n = 5)
D	1150 ± 75 ^{a}	1909 ± 97 ^{c}	69.25 ± 21.6 ^{b}
3D	1028 ± 71 ^{a}	1640 ± 70 ^{bc}	53.83 ± 10.18 ^{b}
7D	966 ± 122 ^{a}	1109 ± 91 ^{a}	8.23 ± 2.76 ^{a}
St/Rf	957 ± 54 ^{a}	1457 ± 102 ^{ab}	52.0 ± 5.76 ^{b}

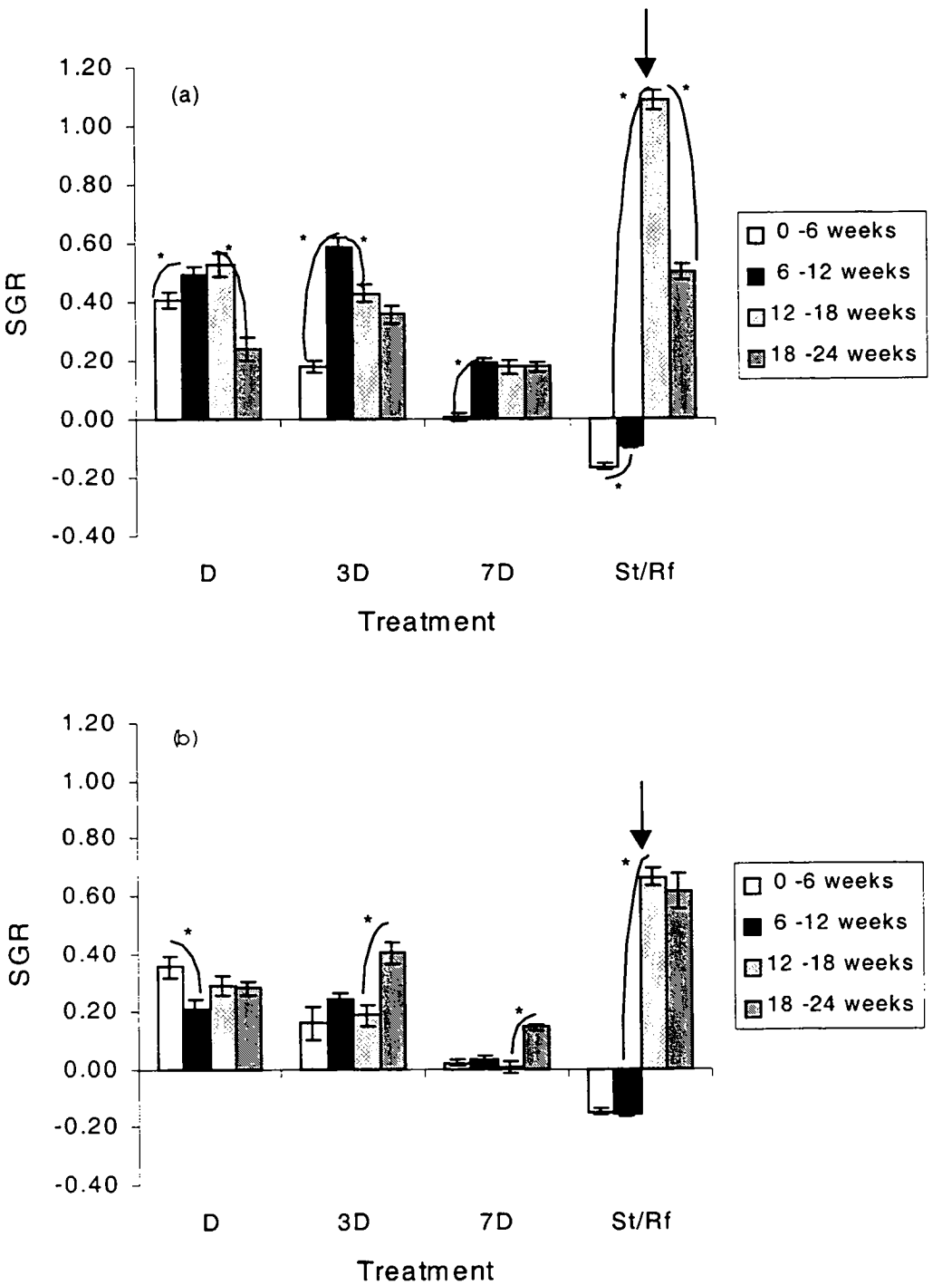

Figure 3.2 Mean (± SE) SGR of (a) small and (b) large barramundi fed daily (D), every 3 days (3D), every 7 days (7D) or starved and refed (St/Rf) during the 24 week experimental period. The arrow indicates the time of beginning feeding of St/Rf fish.

refeeding (weeks 12 – 18). In the following six weeks these values decreased, becoming comparable to those of D and 3D groups.

3.3.4 Muscle index

Small fish had significantly higher muscle weight than large fish in all treatments (Table 3.3) (block design ANOVA; $F=5.51_{(1,34)}$; $P<0.05$). In small fish, muscle index at week 24 was not significantly different between treatments (one way ANOVA; $F=0.65_{(3,16)}$; $P>0.05$). Feeding regime significantly affected the muscle index of large fish at week 12 (one way ANOVA; $F=5.2_{(3,16)}$; $P<0.05$) and week 24 (one way ANOVA $F=3.41_{(3,16)}$; $P<0.05$). Starved large fish at week 12 had a significantly lower muscle index than D or 7D large fish. 7D large fish had the lowest muscle weight (41.8 ± 1.0%) while Refed large fish had the highest (45.4 ± 2.0%) muscle weight at the end of experimental period.

3.3.5 Proximate composition of muscle

All nutrients and energy are expressed as total amount (g.kg wet weight of whole fish^{-1}) and total energy (MJ.kg wet weight of whole fish^{-1}) respectively.

The proximate composition of muscle of small and large fish is given in Tables 3.4a and 3.4b respectively. Since proximate composition of muscle for small and large fish were significantly different at week 24 (Appendix III), these parameters were analysed separately for the two size groups.

Small fish

7D small fish had the highest muscle moisture content (77.3%), while the D group had relatively low muscle moisture (Table 3.4a). Refed small fish had muscle moisture similar to that of 3D fish. Total muscle dry matter, protein and ash of small fish were not significantly different between treatments at week 24. D and 3D fish had significantly higher muscle lipid content than 7D fish. Total muscle energy content of D fish was significantly higher than that of the 7D group at week 24.

Large fish

In the large size class, Starved animals had significantly higher muscle moisture and lower dry matter, protein, ash and energy content compared with all

Table 3.3. Muscle Index (%) (mean ± SE) of small and large barramundi fed daily (D), every 3 days (3D), every 7 days (7D) or starved and refed (St/Rf) over a 24 week period. Means with the same superscript in the same column are not significantly different (P>0.05); means with the same superscript (x,y) in the same row for large fish are not significantly different (P>0.05).

Feeding Regime	Muscle Index		
	Small	Large	
	Wk 24 (n = 5)	Wk 12 (n = 5)	Wk 24 (n = 5)
D	46.2 ± 1.7 ^{a}	45.3 ± 0.8 ^{b}	44.4 ± 0.8 ^{ab}
3D	45.5 ± 1.1 ^{a}	43.8 ± 2.5 ^{ab}	44.5 ± 0.8 ^{ab}
7D	44.5 ± 1.2 ^{a}	45.4 ± 2.6 ^{b,x}	41.8 ± 1.0 ^{a,y}
St/Rf	47.0 ± 1.2 ^{a}	40.9 ± 1.6 ^{a,x}	45.4 ± 2.0 ^{b,y}

Table 3.4(a) Moisture, total dry matter, protein, lipid, ash and total energy (mean ± SE) in muscle of small barramundi fed daily (D), every 3 days (3D), every 7 days (7D) or starved and refed (St/Rf) over a 24 week period. Means with same superscript in the same column are not significantly (P>0.05) different.

Feeding Regime	Muscle Moisture (%)	Total nutrients (g.Kg wet wt of whole fish^{-1})				Total energy (MJ.Kg of whole fish^{-1})
		Muscle dry matter	Muscle protein	Muscle lipid	Muscle Ash	
D	75.3 ± 0.2 ^{a}	114.2 ± 4.5 ^{a}	99.9 ± 3.3 ^{a}	7.8 ± 1.6 ^{b}	7.5 ± 0.4 ^{a}	2.5 ± 0.09 ^{b}
3D	75.8 ± 0.1 ^{ab}	110.4 ± 2.9 ^{a}	97.1 ± 2.5 ^{a}	8.0 ± 0.9 ^{b}	7.3 ± 0.2 ^{a}	2.4 ± 0.08 ^{ab}
7D	77.3 ± 0.1 ^{c}	101.3 ± 3.1 ^{a}	91.1 ± 3.1 ^{a}	3.2 ± 0.2 ^{a}	7.2 ± 0.3 ^{a}	2.1 ± 0.08 ^{a}
St/Rf	76.2 ± 0.1 ^{b}	112.0 ± 2.6 ^{a}	99.2 ± 2.4 ^{a}	5.8 ± 0.5 ^{ab}	7.4 ± 0.2 ^{a}	2.4 ± 0.06 ^{ab}

other treatments at week 12 (Table 3.4b). D, 3D or 7D fish showed no differences in these parameters at week 12. Muscle lipid content of Starved fish was significantly lower than that of D fish (wk 12).

At week 24, Refed large fish had high dry matter or energy contents in muscle, which were not significantly different to those of D or 3D fish. Total ash content of muscle was significantly higher for Refed fish (7.72 ± 0.13) than for all other treatments, and 3D and 7D fish had higher ash content than D fish. Total muscle protein was significantly affected by feeding regime at week 24 (oneway ANOVA; $F=4.09_{(3,15)}$; $P<0.05$); however Scheffe's test was not powerful enough to show the difference. Total muscle lipid at week 24 was not significantly different between feeding regimes.

The percentage of muscle moisture was lower, and total dry matter, protein, ash and energy were significantly higher, in Refed fish than in Starved animals (Table 3.4b). Large D fish had decreased lipid content at week 24. 3D fish showed increased muscle lipid content with time. In 7D fish total dry matter, protein or energy content at week 12 were significantly lower than those at week 24.

The proportion of nutrients (% dry weight) in the muscle of large barramundi at week12 are shown in Table 3.4c. Muscle protein, ash and energy levels were not significantly different between treatments. The muscle lipid content was significantly different between D and 3D treatments.

3.3.6 Hepato Somatic Index

There were no significant differences in HSI values between small and large fish at week 24, so data were pooled (Table 3.5) (Block design ANOVA; $F= 0.325_{(1,34)}$; $P>0.05$). HSI was significantly affected by feeding regime. At week 12, D and 3D fish had significantly higher HSI than Starved fish. At week 24, 7D fish had the lowest HSI which was significantly lower than in 3D or Refed animals. D, 3D or 7D animals did not show differences in HSI with time. Refed fish showed a significant increase in HSI following refeeding, and HSI of Refed fish was approximately three times higher than that of Starved fish.

Table 3.4(b) Moisture, total dry matter, protein, lipid, ash and total energy (mean ± SE) in muscle of large barramundi fed daily (D), every 3 days (3D), every 7 days (7D) or starved and refed (St/Rf) over a 24 week period. Means with same superscript (a,b) in the same column are not significantly different (P>0.05); for each parameter, means with the same superscript (x,y) in the same row are not significantly different (P>0.05)

Feeding Regime	Muscle moisture (%)		Total nutrients (g.Kg wet wt of whole fish^{-1})								Total muscle energy (MJ.Kg wet wt of fish^{-1})	
			Muscle dry matter		Muscle protein		Muscle lipid		Muscle ash			
	12 wk	24 wk	12 wk	24 wk	12 wk	24 wk*	12 wk	24 wk	12 wk	24 wk	12 wk	24 wk
D	76.2 ^{a} ± 0.2	75.8 ^{a} ± 0.2	107.9 ^{b} ± 1.1	107.5 ^{b} ± 1.9	97.5 ^{b} ± 1.0	96.8 ± 2.0	6.9 ^{b,x} ± 0.8	3.8 ^{a,y} ± 0.6	6.8 ^{b} ± 0.4	5.9 ^{a} ± 0.1	2.3 ^{b} ± 0.04	2.3 ^{b} ± 0.03
3D	76.5 ^{a} ± 0.2	76.4 ^{a,b} ± 0.1	102.8 ^{b} ± 2.8	104.8 ^{a,b} ± 2.2	92. 9 ^{b} ± 2.5	94.4 ± 2.0	3.5 ^{a,x} ± 0.2	5.6 ^{a,y} ± 0.7	6.4 ^{b} ± 0.2	7.0 ^{b} ± 0.2	2.2 ^{b} ± 0.07	2.2 ^{ab} ± 0.05
7D	76.4 ^{a} ± 0.2	76.9 ^{b} ± 0.2	106.9 ^{b,x} ± 2.7	96.5 ^{a,y} ± 2.7	96.1 ^{b,x} ± 2.4	87.1 y ± 2.5	5.1 ^{ab} ± 0.5	5.6 ^{a} ± 0.5	6.8 ^{b} ± 0.2	6.7 ^{b} ± 0.1	2.3 ^{b,x} ± 0.05	2.0 ^{a,y} ± 0.06
St/RF	77.6 ^{b,x} ± 0.1	76.5 ^{ab,y} ± 0.2	91.9 ^{a,x} ± 1.5	106.9 ^{b,y} ± 2.1	82.9 ^{a,x} ± 1.2	96.4 y ± 2.3	4.0 ^{a} ± 0.4	4.5 ^{a} ± 0.3	5.3 ^{a,x} ± 0.1	7.7 ^{c,y} ± 0.1	1.9 ^{a,x} ± 0.02	2.3 ^{b,y} ± 0.05

* ANOVA showed significant effect of feeding regime on total muscle protein content of large fish at week 24. However Sheffe's test did not show the difference.

Table 3.4(c) Moisture, protein, lipid and ash (% dry weight) and energy (MJ.100 g of dry tissue^{-1}) (mean ± SE) in muscle of large barramundi fed daily (D), every 3 days (3D), every 7 days (7D) or starved (St) over a 12 week period. Means with same superscript (a,b) in the same column are not significantly different (P>0.05).

Feeding Regime	Nutrient (% dry weight)				Energy (MJ.100g of tissue^{-1})
	Moisture	Protein	Lipid	Ash	
D	76.2 ± 0.2 ^{a}	90.2 ± 0.1^{a}	6.4 ± 0.7 ^{b}	6.3 ± 0.3 ^{a}	20.9 ± 0.1^{a}
3D	76.5 ± 0.2 ^{a}	90.3 ± 0.2 ^{a}	3.4 ± 0.1^{a}	6.2 ± 0.3 ^{a}	21.2 ± 0.2 ^{a}
7D	76.4 ± 0.2 ^{a}	89.9 ± 0.6 ^{a}	4.7 ± 0.5 ^{ab}	6.3 ± 0.2 ^{a}	21.2 ± 0.2 ^{a}
Starved	77.6 ± 0.1 ^{b}	90.3 ± 0.3 ^{a}	4.4 ± 0.4 ^{ab}	5.7 ± 0.1 ^{a}	20.8 ± 0.2 ^{a}

Table 3.5. Hepato Somatic Index (HSI) (mean ± SE) of barramundi fed daily (D), every 3 days (3D), every 7 days (7D) or starved and refed (St/Rf) over a 24 week period. Data for small and large barramundi for week 24 were pooled as there was no significant differences between two groups. n = sample size; means with same superscript (a,b) in the same column are not significantly different (P>0.05); for each parameter, means with same superscript (x,y) in the same row are not significantly different (P>0.05).

Feeding	HSI	
Regime	12 wk (n=5)	24 wk (n=10)
D	$1.36 \pm 0.13^{b,x}$	$1.51 \pm 0.1^{ab,x}$
3D	$1.53 \pm 0.27^{b,x}$	$1.64 \pm 0.04^{b,x}$
7D	$1.05 \pm 0.14^{ab,x}$	$1.30 \pm 0.07^{a,x}$
St/Rf	$0.56 \pm 0.03^{a,x}$	$1.65 \pm 0.06^{b,y}$

3.3.7 Proximate composition of Liver

The proximate composition of liver is given in Table 3.6a. Since all parameters were not significantly different between the small and large fish at week 24, the data were pooled (Appendix III). Generally, all fish at week 24 had higher dry matter and nutrient levels than fish at week 12.

Liver moisture was lower in D fish than in 7D fish at week 12. D fish had higher total liver lipid than 7D or St/Rf fish. Starved fish had a high percentage of lipid (% dry wt) in their liver (Table 3.6b), but as these fish had low HSI values, total amounts of nutrients were lower than in other groups (Table 3.6a). At week 12, total liver protein, ash, dry matter and energy were significantly lower in Starved fish than in D or 3D fish, but not significantly different to those of 7D fish. D fish had significantly higher liver lipid content than 7D or Starved fish.

At week 24, D, 3D and Refed fish had higher dry matter, lipid and energy content than 7D fish. Total liver protein and ash content of Refed fish were higher than those of D fish.

Refeeding of previously starved fish resulted in increased liver dry matter, protein, lipid, ash and energy. Total liver lipid and energy in 3D fish increased significantly from week 12 to week 24. 7D fish had increased protein content at week 24. D fish had no significant changes in their liver proximate composition with time.

3.3.8 Gonado Somatic Index

There were no significant differences in GSI values between the small and large fish at week 24, and the data were pooled (Table 3.7) (Block ANOVA; $F = 1.10_{(1,34)}$; $P>0.05$). No significant differences were observed in GSI between feeding regimes at any particular time. GSI values at week 12 were generally lower than at week 24, with values significantly different for 7D and Starved fish. Refed and 7D fish at week 12 showed significantly higher GSI values than in Starved and 7D fish at week 24. GSI ranged from 0.04 (Starved fish week 12) to 0.13 (3D fish week 24).

Table 3.6a. Liver moisture, total liver dry matter, protein, lipid, ash and energy contents (mean ± SE) of barramundi fed daily (D), every 3 days (3D), every 7 days (7D) or starved and refed (St/Rf) over a 24 week period. Data for small and large fish for week 24 were pooled as there was no significant difference between two groups. Means with same superscript (a,b) in same column are not significantly different (P>0.05); for each parameter, means with the same superscript (x,y) in the same row are not significantly different (P>0.05).

Feeding Regime	Liver moisture (%)		Total nutrients (g.Kg wet wt of whole fish^{-1})								Total energy (MJ.Kg wet wt of whole fish^{-1})	
			Liver dry matter		Liver protein		Liver lipid		Liver ash			
	12 wk	24 wk	12 wk	24 wk	12 wk	24 wk	12 wk	24 wk	12 wk	24 wk	12 wk	24 wk
D	47.9 ^{a}	46.3 ^{a}	7.2 ^{b}	8.1 ^{b}	1.1 ^{b}	1.2 ^{a}	4.5 ^{b}	5.0 ^{b}	0.11 ^{bc}	0.11 ^{a}	0.22 ^{c}	0.23 ^{b}
	± 2.3	± 1.8	± 0.9	± 0.6	± 0.1	± 0.1	± 0.8	± 0.4	± 0.01	± 0.01	± 0.03	± 0.02
3D	54.2 ^{ab}	50.5 ^{ab}	6.9 ^{b}	8.1 ^{b}	1.2 ^{b}	1.4 ^{ab}	3.2 ^{ab,x}	4.7 ^{b,y}	0.14 ^{c}	0.14 ^{ab}	0.18 ^{bc,x}	0.23 ^{b,y}
	+1.8	± 0.9	± 0.9	± 0.2	± 0.1	± .1	± 0.3	± 0.2	± 0.02	± 0.01	± 0.02	± 0.01
7D	59.9 ^{b}	60.1 ^{c}	4.3 ^{ab}	5.3 ^{a}	0.9 ^{ab,x}	1.2 ^{a,y}	2.1 ^{a}	2.4 ^{a}	0.09 ^{ab}	0.12 ^{ab}	0.11 ^{ab}	0.14 ^{a}
	± 1.6	± 1.2	± 0.7	± 0.6	± 0.1	± 0.1	± 0.4	± 0.4	± 0.01	± 0.01	± 0.02	± 0.02
St/Rf	52.6 ^{ab}	53.7 ^{b}	2.7 ^{a,x}	7.7 ^{b,y}	0.6 ^{a,x}	1.6 ^{b,y}	1.8 ^{a,x}	4.2 ^{b,y}	0.05 ^{a,x}	0.14 ^{b,y}	0.08 ^{a,x}	0.21 ^{b,y}
	± 1.6	± 1.2	± 0.2	± 0.4	± 0.1	± 0.1	± 0.1	± 0.4	± 0.01	± 0.01	± 0.01	± 0.02

Table 3.6b Liver lipid (%) (mean ± SE) of large barramundi fed daily (D), every 3 days (3D), every 7 days (7D) or starved (St) over a 12 week period. Means with same superscript (a,b) in same column are not significantly different (P>0.05).

Feeding Regime	Liver lipid (%) 12 wk
D	61.2 ± 3.0 [ab]
3D	47.4 ± 4.6 [a]
7D	47.6 ± 2.6 [a]
St	67.8 ± 1.5 [b]

Table 3.7. Gonado Somatic Index (GSI) (mean ± SE) of barramundi fed daily (D), every 3 days (3D), every 7 days (7D) or starved and refed (St/Rf) over a 24 week period. Data for small and large barramundi for week 24 were pooled as there was no significant differences between two groups. n = sample size; means in the same column are not significantly different (P>0.05); for each treatment, means with different superscript (x,y) in the same row are significantly different (P<0.05).

Feeding	GSI	
Regime	12 wk (n=5)	24 wk (n=10)
D	$0.06 \pm 0.01^{a,x}$	$0.09 \pm 0.01^{a,x}$
3D	$0.07 \pm 0.01^{a,x}$	$0.13 \pm 0.03^{a,x}$
7D	$0.05 \pm 0.01^{a,x}$	$0.10 \pm 0.02^{a,y}$
St/Rf	$0.04 \pm 0.01^{a,x}$	$0.10 \pm 0.01^{a,y}$

3.3.9 Gonadal stages

Gonadal histology confirmed that all fish used in this study were males at the end of the study period. In small fish, no significant differences in gonadal stages at week 24 were found between different treatments (Fig. 3.3a) (Loglikelihood ratio; χ^2 = 2.33; df = 3; P>0.05)05)In large fish, D or 3D fish at week 12 had more advanced (M3) gonads compared to 7D or Starved fish (Fig 3.3b). However, these differences did not differ significantly (Loglikelihood ratio; χ^2 = 7.55; df = 9; P>0.05). Gonadal stages of large fish at week 24 were not significantly different between treatments (Loglikelihood ratio; χ^2 = 15.43; df = 12; P>0.05)

It was noted that most fish were producing sperm continuously, or having repeated development cycles throughout the experimental period. This finding was based on the observation that fish with less advanced gonadal stages had sperm remaining from the previous cycle and the presence of large empty seminiferous tubules (stage 1c).

3.3.10 Sperm quality

Sperm quality data could be obtained only for a few fish (Table 3.8) as cannulation was not done at every sampling date. At week 24, very few fish were observed with milt, and the gonad development stages of these fish corresponded to stage 3. However, the volume of milt was very low (only traces of milt) and microscopical observations showed that most of the sperm were either non-motile or had very low motility rates.

3.3.11 Plasma hormones

Oestradiol-17β (E_2)

The changes in plasma E_2 during the experimental period for small fish are shown in Table 3.9a. D or 3D groups showed no significant difference in their plasma E_2 level up to week 18, but E_2 levels increased significantly after this time. Plasma E_2 levels of the 7D group were stable during the experimental period. Although, starvation did not influence the plasma E_2 level, this level increased

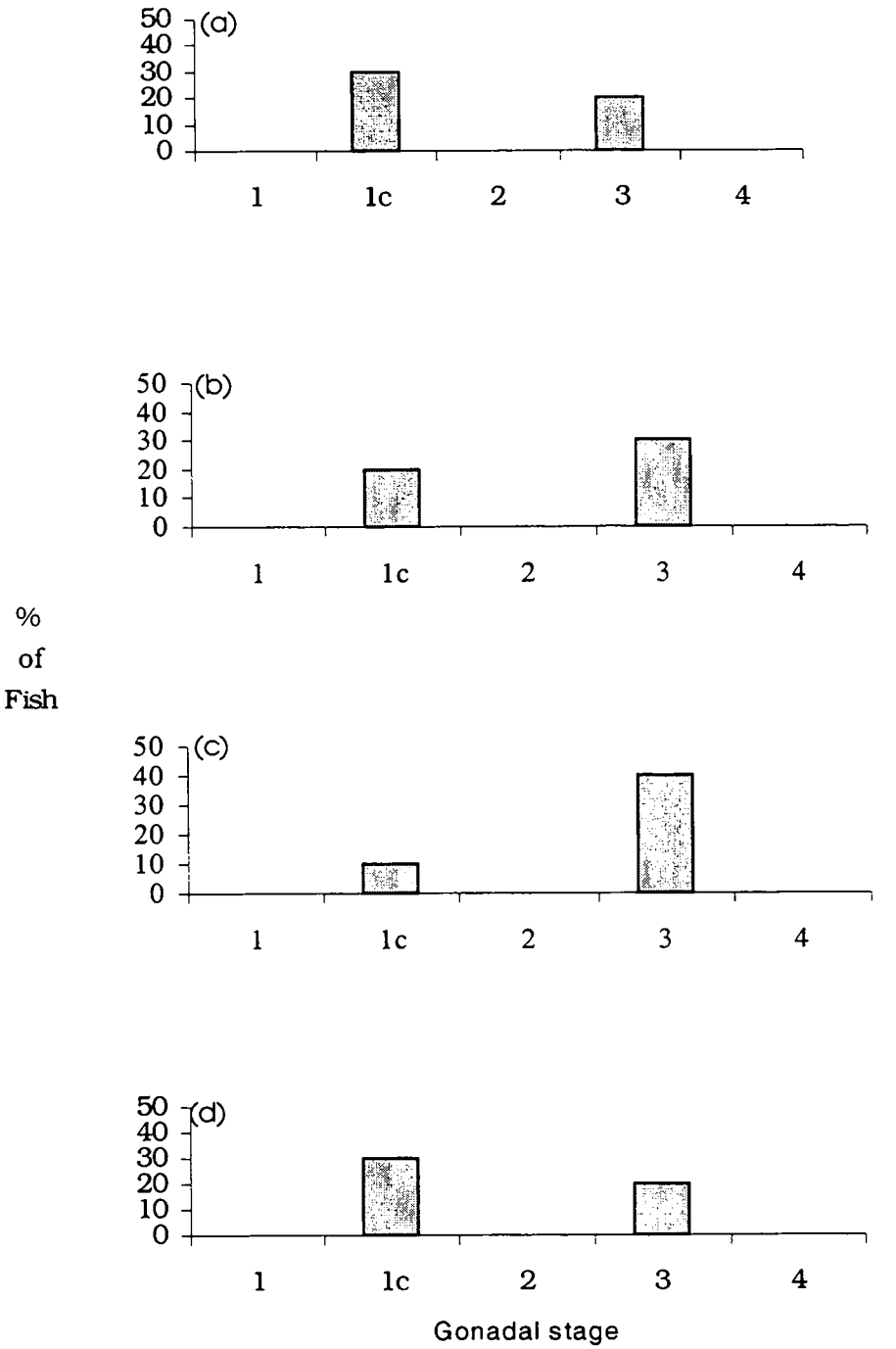

Figure 3. 3a Gonad development stages of small barramundi at week 24 fed (a) daily (D), (b) every 3 days (3D), (c) every 7 days (7D) or (d) starved and refed (St/Rf). Gonadal stages were classified according to Guiguen *et al.* (1994) and details in section 2.5.

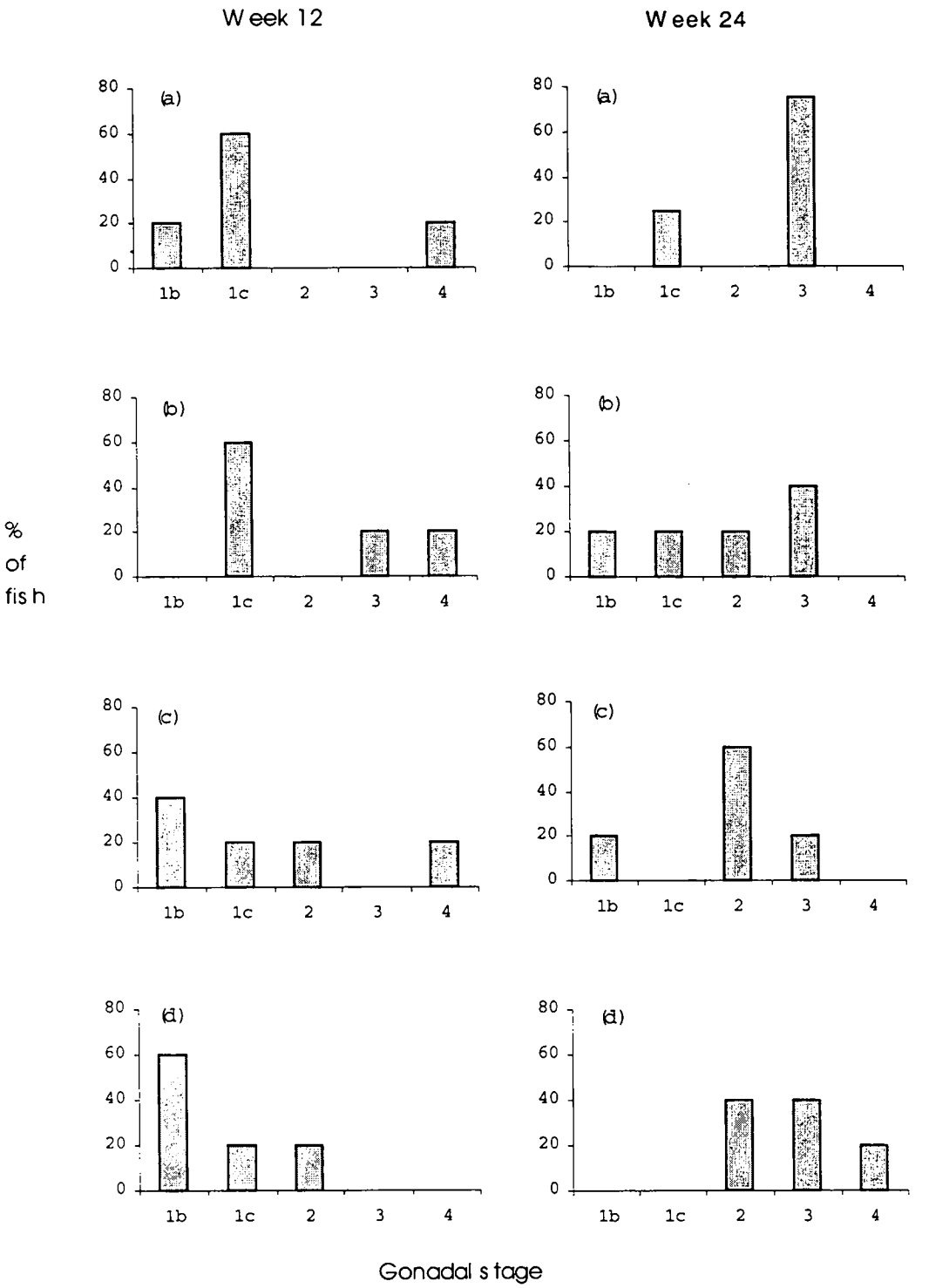

Figure 3.3b Gonad development stages of large barramundi at weeks 12 and 24, fed (a) daily (D), (b) every 3 days (3D), (c) every 7 days (7D) or (d) starved and refed (St/Rf). Gonadal stages classified according to Guiguen *et al.* (1994) and details in section 2.5.

Table 3.8 Number of matured animals which showed presence of milt when cannulated in small and large size groups. Cannulation was not done at every sampling date.

Feeding Regime	Small		Large	
	6 Wk	24 Wk n=10	12 Wk n=10	24 Wk n=5
D	1 #	1	0	1 *
3D		2	0	1
7D		2	0	0
St/Rf		1	0	2

#- cannulation was not done; but one fish from this treatment was observed with expressible milt.
* number of fish in D (large) treatment is 4.

Table 3.9. Plasma Oestradiol-17β concentration (mean ± SE) of (a) small and (b) large barramundi fed daily (D), every 3 days (3D), every 7 days (7D) or starved and refed (St/Rf) over a 24 week period. Means with same superscript (a,b) in the same column are not significantly different (P>0.05); Values indicated by * are significantly different from the preceding value (P>0.05).

(a) small

Feeding	Oestradiol-17β (pg ml^{-1})				
Regime	0Wk	6Wk	12Wk	18Wk	24Wk
D	18.9 ± 2.1 ^{a}	20.5 ± 1.0 ^{b}	19.7 ± 1.3 ^{ab}	22.5 ± 0.8 ^{ab}	37.8 ± 2.0 ^{b,*}
3D	18.1 ± 1.2 ^{a}	19.6 ± 1.0 ^{ab}	22.6 ± 2.0 ^{b}	21.7 ±1.0 ^{a}	40.7 ± 3.2 ^{b,*}
7D	17.8 ± 1.7 ^{a}	19.7 ± 1.2 ^{ab}	22.6 ± 1.7 ^{b}	20.3 ± 0.4 ^{a}	24.9 ± 3.7 ^{a}
St/Rf	16.1 ± 1.5 ^{a}	16.1 ± 0.3 ^{a}	15.6 ± 1.4 ^{a}	26.2 ± 1.7 ^{b,*}	42.4 ± 1.6 ^{b,*}

(b) large

Feeding	Oestradiol-17β (pg ml^{-1})				
Regime	0Wk	6Wk	12Wk	18Wk	24Wk
D	35.4 ± 5.8 ^{a}	35.9 ± 2.3 ^{b}	39.1 ± 2.6 ^{b}	46.8 ± 8.3 ^{a}	23.3 ± 5.5 ^{a}
3D	33.2 ± 4.0 ^{a}	32.9 ± 1.0 ^{b}	34.7 ± 3.5 ^{b}	54.3 ± 8.9 ^{a}	25.7 ± 3.4 ^{a,*}
7D	27.4 ± 2.1 ^{a}	28.8 ± 1.8 ^{ab}	32.4 ± 3.2 ^{b}	38.8 ± 5.1 ^{a}	28.7 ± 2.3 ^{a}
St/Rf	28.8 ± 3.4 ^{a}	22.2 ± 2.4 ^{a}	13.2 ± 2.3 ^{a}	59.8 ± 5.3 ^{a,*}	59.5 ± 6.1 ^{b}

significantly after refeeding. At the end of the experimental period, plasma E_2 levels of the D, 3D and St/Rf groups were significantly higher than that of the 7D group. The mean initial plasma E_2 level of small fish was 17.7 $\pm$ 4.5 pg.ml^{-1}, while the highest mean E_2 level was 42.4 $\pm$ 1.6 pg.ml^{-1} for Refed fish at week 24. In large fish, the level of plasma E_2 showed similar patterns in both D and 3D groups, with the highest E_2 levels at week 18 followed by a significant decrease after this time (Table 3.9b). 7D fish also had increased E_2 levels at week 18. Starvation had a significant negative effect on E_2 levels in circulation. However, with refeeding, E_2 levels increased significantly at week 18, and remained elevated. The highest mean plasma E_2 level was 60 $\pm$ 5.3 pg.ml^{-1} in Refed large fish. The mean initial plasma E_2 level in the large fish was 33.5 $\pm$ 13.7 pg.ml^{-1}, approximately twice that measured in the small fish.

There was a size effect on plasma E_2 concentration (Fig 3.4). Plasma E_2 concentration increased with body size of the small size class (Plasma E_2 (pg.ml^{-1}) = 0.016 * body weight + 7.5129; R^2 = 0.2959; $P<0.05$) irrespective of the feeding regime. However, the same trend was not clear in the large size class.

Testosterone (T)

In D and 3D small fish, plasma T levels increased in the first six weeks and decreased thereafter (Table 3.10). In 7D and Starved fish, T levels decreased significantly in the first six weeks and remained low thereafter. A small but significant increase in plasma T levels was observed in Refed fish. There were no significant differences in plasma T levels at week 24 in the small fish maintained on different feeding regimes. The mean initial plasma T level was 356 $\pm$ 44 pg.ml^{-1}, and the highest observed level of 515 $\pm$ 104 pg.ml^{-1} was found in small D fish at week 6.

In D and 3D large fish, T levels increased, but not significantly, during the 24 week period (Table 3.10). The low plasma T levels of the 7D group increased significantly after week 18. Starvation did not affect T levels in circulation in large fish. Refed fish also had significantly increased plasma T levels compared to the Starved fish. In the large fish, mean initial T level was 129 $\pm$ 17 pg.ml^{-1}. The highest level was 520 $\pm$ 127 pg.ml^{-1} which occurred in Refed fish at week 24.

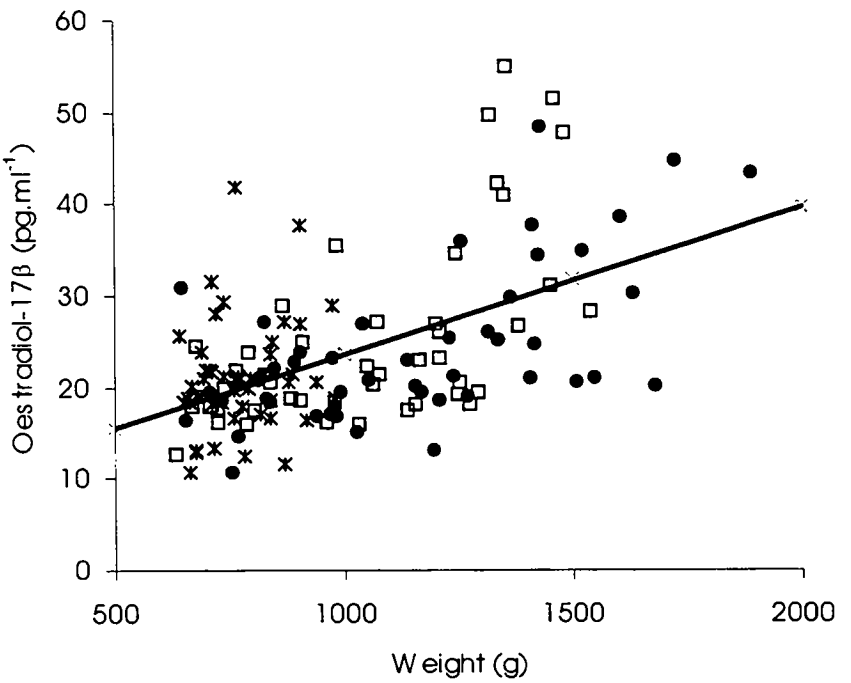

Figure 3.4 Plasma Oestradiol-17β (E_2) ($pg.ml^{-1}$) concentration of small barramundi fed daily (D), every 3 days (3D) or every 7 days (7D). Starved and refed (St/Rf). fish are not included in this graph. (E_2 ($pg.ml^{-1}$) = 0.016 x body weight (g) + 7.5129; n= 149; R^2 = 0.2959; $p<0.05$). (○- D; ▽ - 3D; ▪ - 7D)

Table 3.10. Plasma Testosterone concentration (mean ± SE) of (a) small and (b)large barramundi fed daily (D), every 3 days (3D), every 7 days (7D) or starved and refed (St/Rf) over a 24 week period. Means with same superscript (a,b) in the same column are not significantly different (P>0.05); Values indicated by * are significantly different from the preceding value (P>0.05).

(a) small

Feeding Regime	Testosterone (pg ml $^{-1}$) 0Wk	6Wk	12Wk	18Wk	24Wk
D	356 ± 44 ^{a}	514 ± 104 ^{b}	238 ± 17 ^{ab,*}	168 ± 21 ^{a}	167 ± 23 ^{a}
3D	356 ± 44 ^{a}	460 ± 69 ^{ab}	244 ± 42 ^{b}	214 ± 24 ^{a}	202 ± 53 ^{a}
7D	356 ± 44 ^{a}	1 24 ± 6 ^{ab,*}	98	118 ± 17 ^{a}	105 ± 28 ^{a}
St/Rf	356 ± 44 ^{a}	162 ± 43 ^{a,*}	69 ± 5 ^{a}	218 ± 33 ^{a,*}	252 ± 25 ^{a}

(b) large

Feeding Regime	Testosterone (pg ml $^{-1}$) 0Wk	6Wk	12Wk	18Wk	24Wk
D	171 ± 41 ^{a}	215 ± 32 ^{b}	296 ± 28 ^{c}	331 ± 64 ^{a}	354 ± 92 ^{a}
3D	129 ± 32 ^{a}	167 ± 28 ^{ab}	246 ± 31 ^{bc}	247 ± 56 ^{a}	279 ± 118 ^{a}
7D	101 ± 33 ^{a}	169 ± 25 ^{ab}	169 ± 22 ^{ab}	312 ± 47 ^{a}	443 ± 129 ^{a}
St/Rf	116 ± 29 ^{a}	93 ± 19 ^{a}	115 ± 22 ^{a}	427 ± 106 ^{a*}	520 ± 127 ^{a}

3.4 DISCUSSION

Growth

All aspects of nutrient deposition and growth indicate that D or 3D feeding regimes provided adequate nutrition for growth; the 7D feeding regime provided sufficient nutrients for maintenance; and starvation caused major loss of body condition which was rapidly overcome with refeeding. Similar patterns in weight gain and growth parameters were observed in small and large fish, indicating that the growth of fish was affected by feeding regime in the same way for both size classes. Fish fed daily or once every 3 d showed higher growth during the experimental period, while 7D fish showed clear signs of inadequate food for growth, which was confirmed by low body weight gain and low muscle index. Fish lost weight during starvation, but showed a marked increase in growth with refeeding. Small fish gained significantly greater % BWG, and had better SGR than the large fish. It has been reported that SGR decreased with age in other teleosts (Brett, 1979).

Acclimatisation to feeding regime was apparent in the small fish. 3D or 7D fish showed low SGR for the first six weeks of the experiment, but then appeared to adjust to the feeding regime. Low growth rate during acclimatisation after fish were subjected to a new feeding regime has also been reported in brown trout (Pirhonen and Forsman, 1998). During starvation, animals in both size classes showed negative SGR. However, with refeeding, fish gained body weight, and their SGR was higher than for all other groups at all other times. Both D and 3D fish showed better SGR than 7D or Starved fish over the whole experimental period.

Fish adjusted to the feeding regime by adjusting their food consumption rates to maintain growth. Fish fed daily (D) consumed a lower amount of food per meal (% BWt.day^{-1}) than fish fed once every 3 d. Although 7D fish consumed more food per meal than D or 3D fish, the total food intake per week was less than for the other two groups. Pirhonen and Forsman (1998) also reported that brown trout fed twice a week consume more food than fish fed daily. Although the barramundi in the present study had the opportunity to feed up to satiety, the amount of food that they could consume per meal was limited by their stomach capacity (De Silva and Anderson,

1995). It was clear that after a period of acclimation, the high nutrient intake once in 7 d was sufficient to maintain a low growth rate in these animals.

Refed fish in this study had higher food intake and better conversion efficiency during compensatory growth compared with animals fed continuously. A similar response has been reported for other fish species (Russell and Wootton, 1992; Hayward *et al.*, 1997; Miglavs and Jobling, 1989; Qian *et al.*, 2000). The compensatory growth was accompanied by high rates of food intake (Miglavs and Jobling, 1989), and the restricted-satiation fed fish became hyperphagic compared with the animals fed to satiation throughout the whole experiment. Many animal species adapt to food deprivation and/or reduced levels of energy intake by reducing metabolic expenditure (Miglavs and Jobling, 1989). However, these animals may not re-adapt to high levels of food availability immediately after transfer to better feeding regimes, with low metabolic rates maintained for a short period after they are no longer food-restricted. As a result of low metabolic expenditure and high food intake simultaneously, a large amount of energy is available for growth purposes. This energy surplus ensures rapid growth during this period. St/Rf fish used in this present study seemed to have shown this response during refeeding period. However, total weights were not fully recovered at the end of the refeeding period. Therefore, refeeding fish showed partial compensation in this study. Partial compensation after a period of food restriction occurred when animals display rapid rates of weight gains during refeeding but do not mange to restore full body weight to the same levels as the control individuals (Jobling et al., 1993).

Hepato Somatic Index

The lowest HSI was observed in starved fish at week 12, indicating that they use their liver resources during starvation. However, with refeeding, liver weight increased to become similar to that of fish reared on D or 3D regimes. Significant increases in liver weight during the refeeding phase has also been reported in other fish (Collins and Anderson, 1999; Power *et al.*, 2000). 7D fish also had a relatively low HSI, indicating that the low nutrient intake led to utilisation of liver reserves.

Proximate composition of muscle and liver

The differences in muscle composition between the small and large fish indicate that small fish accumulate more energy in muscle or somatic growth than large fish. The small fish had higher total dry matter, nutrient contents and energy than large fish in similar treatments. This may be a result of the higher growth rate of small fish, allowing for the accumulation of greater energy stores than in large fish.

Similar amounts of muscle nutrients and energy in D and 3D fish indicate that even fish experiencing varying feeding levels can maintain comparable growth by adjusting food intake. This trend was clear in both size classes. The high muscle moisture, and low dry matter, protein, lipid (except large fish) and energy generally observed in 7D fish confirmed the insufficient nutrient intake in this group.

The most remarkable differences in muscle composition were found between Starved and Refed animals. Many authors suggest that both protein and fat are important metabolic fuels during starvation (Doucett *et al.*, 1999; Einen *et al.*, 1998; Booth *et al.*, 1999). Starved fish in this present study used muscle energy for metabolism during starvation. This process is clearly indicated by their weight loss and low total nutrient contents. However, the proportion of nutrients (% dry wt) in the muscle was not affected. Einen *et al.* (1998) observed that long term starvation in large Atlantic salmon corresponded to only marginal changes in relative body composition. However, a shrinkage of the total body mass, as illustrated by weight loss, less fillet and a leaner body shape was observed in starved Atlantic salmon. As observed in the present study, muscle protein and fat contents (%) remained quite stable but a considerable loss of nutrients that was more or less proportional to the weight loss was observed by Einen *et al.* (1998) in Atlantic salmon in response to starvation. Collins and Anderson (1995) also reported that the percentages of protein and lipid did not change with starvation in golden perch (*Macquaria ambigua*) musculature.

Fish fed continuously (D or 3D) or alternatively (St/Rf) for the whole period were able to accumulate a similar amount of total nutrient levels in muscle as indicated by the comparable levels of nutrients or energy observed for all three treatments. Similar findings were observed in other teleosts that starved/refed fish are able to regain their nutrients levels to a level comparable to animals fed

continuously during compensatory growth period (Miglavs and Jobling, 1989; Quinton and Blake, 1990; Collins and Anderson, 1999; Power *et al.*, 2000).

Total liver dry matter, nutrient and energy content of Starved fish were significantly lower than those of fish fed daily or every 3 days for 12 weeks. However, even after starving for a 12 week period, Starved fish were able to maintain similar total liver nutrient levels as observed in fish fed once in 7 days for 12 weeks. It is probable that Starved fish use some proportion of liver nutrients, but mostly liver lipid is stored as a reserve. This is probably due to lipid mobilisation from muscle to different parts of the body and the role of the liver as a centre in distributing energy reserves (Power *et al.*, 2000).

Although the relative amount is comparable, the proportion of nutrients (% dry wt) of Starved fish was different from those of other fish. The amounts of carbohydrate (% dry wt) of liver of D, 3D and 7D fish, calculated by difference, were 21.5, 33.2 and 27.8 % respectively. In Starved fish this was 7.4%. The Starved group had a higher proportion (%) of lipid and a lower proportion of carbohydrate compared with those of the other treatments. This suggests that during starvation, fish used liver carbohydrate and maintained liver lipid in a minimum level. A decrease of liver lipid, glycogen and protein during starvation has been reported in other teleosts (Collins and Anderson, 1995; Power et al., 2000). In golden perch, *M. ambigua,* those nutrients were significantly decreased within the first 60 days and remained unchanged during the rest of the starvation period (Collins and Anderson, 1995). The lipid and protein remaining in the liver of starved fish is presumably the minimum required to maintain cellular function and integrity (Collins and Anderson, 1995). High relative liver lipid levels were also observed in rainbow trout after 48 days of starvation (Jezierska *et al.*, 1982), but the actual amount (total liver lipid) decreased as in the present study. Refed animals were able to regain liver nutrient levels to a point where they were comparable to the levels in D and 3D fish at the end of the experimental period.

In natural environments food availability is not constant and is varied during different periods and fish adapt by adjusting their food consumption to satisfy their energy requirements (Halver, 1989; De Silva and Anderson, 1995). Also, food deprivation for a continuous period is a common phenomena in many environments. Many fish species do not consume food when they are migrating (Rowe and Thorpe,

1990b; Booth *et al.*, 1999) or during drought (Collins and Anderson, 1995). However, fish can adapt to these situations by showing compensatory growth during which they re-gain nutrients when feed is available. The results from the present study show that barramundi also demonstrate this capacity.

Gonado Somatic Index

The GSI of barramundi did not show any significant differences with feeding level. A significant difference in GSI between weeks 12 and 24 was observed in St/Rf fish but a similar change was also observed in 7D fish. A rapid increase in reproductive indices during the re-feeding phase, which is associated with increased growth rates and high food conversion efficiency, was observed in female *M. ambigua* (Collins and Anderson, 1999). The GSI values of barramundi in the present study were very low when compared with other fish species with a mean value of 0.13 ± 0.03 in fish fed every 3 d. However, it is known that male barramundi expend little energy on reproduction, with ripe males having GSI of 0.5 (Moore, 1982).

GSI did not prove to be a useful indicator in this study as the male gonad is so small and the data were only available if the animals were killed. The most useful information would be provided by obtaining GSI data at every sampling date, but this is not practical for this type of study as it would require maintaining a large number (6 fish X 4 sampling days X 4 treatments) of relatively large fish (1-2kg) for a long period.

Gonadal maturity stages

Gonadal histological studies confirmed that all fish in this study were male. Fish in all treatments except the Starved group had maturing gonads, which indicates that even fish which consume low nutrient intake (7D regime) were able to invest some energy into reproductive effort. Artificial conditions such as increased temperature, photoperiod and high salinity which, were comparable to spawning environment, were used throughout the experimental period. Although barramundi used in the present study were smaller than matured animals in natural environments and had small GSI, most fish had maturing gonads (gonads full of sperm and/or few remaining sperm with empty tubules) during this period. Guigen *et al.* (1993) reported that sea bass (barramundi) underwent normal gonadal development

(gametogenesis) in such artificial conditions. The size of male barramundi in cultured broodstocks is about 2-10 kg in Thailand (Maneewong, 1987) and 2-7 Kg in Singapore (Cheong and Yeng, 1987).

Gonadal maturity stages showed a maturation cycle repeated throughout the experimental period. As with GSI, it is difficult to interpret these results, as stage 1 gonadal stage at the end of an experimental period does not necessarily mean that the gonad was at this stage for the whole period. For example, in the small size class, one animal with milt was observed at week six in the daily feeding group. The gonadal maturity stage of this fish was stage 1c (spent and recovering) at the end of the experiment after 18 weeks.

Sperm quality

Sperm had very low motility rates. Although these fish appeared to be mature, very low milt volumes and low motility rates of sperm indicated that they would demonstrate poor performance in breeding. However, sperm quality data were not a good measure in this study as cannulation was not done on every sampling day. In addition, fish which produce sperm in between sampling days could not be counted.

Plasma hormone production

Oestradiol-17β (E_2)

There was both a size effect and a nutritional effect on hormone production in barramundi. It was observed in this study that plasma E_2 level increased with the body size of male barramundi and this may explain why small fish had lower initial plasma E_2 level than large fish. It is also possible that the increase in circulating E_2 in small fish at week 24 was due to a size effect; at this time these fish were all of 1.2 kg or greater. Therefore, the possibility that these small fish attain a threshold size where they can initiate E_2 production cannot be excluded. Although starvation or low nutrient intake did not influence the low E_2 levels in small fish, the relatively high E_2 level in large fish was clearly reduced by starvation. In contrast, E_2 level in golden perch (Collins and Anderson, 1999), or E_2 and 11kT in tilapia (*O. niloticus*) (Toguyeni *et al.*, 1996) were unaffected by starvation. These differences may be due

to species differences or different treatments as Toguyeni *et al.* (1996) measured hormone levels of fish after short term starvation (15 days).

The precise function of E_2 in male teleosts is not very clear; however, in male barramundi this may be related to the natural sex inversion process. It is likely that once fish attain a threshold E_2 level, sex inversion may initiate in those fish.

Testosterone (T)

The role of T as one of the precursors for E_2 (Yoshikuni and Nagahama, 1991) has been well documented. In small fish, it is likely that although T production has started, it is not yet converted to E_2 as indicated by low initial E_2 level. However, it is possible that the high initial E_2 and low T levels in the circulation of large fish compared with the small fish are due to the conversion of T to E_2.

As stated previously, feeding regimes affected the hormone production in small and large fish differently. Thus, in small D and 3D fish, high nutrient intake initially influenced T production, but decreasing levels during the remaining period may suggest that the androgen, T, is subsequently converted to other steroid hormones. Testosterone is also one of the precursors of the androgen, 11 keto-testosterone (11kT) and of the oestrogen, (E_2) (Scott, 1980 a,b). In dietary regimes in which fish were well fed, the decrease in T may be due to an increase in 11kT level as the E_2 level was unchanged. Unfortunately, the 11kT data could not be obtained for this experiment. However, decreasing T levels in 7D and Starved small fish, together with lower E_2 concentration, may suggest insufficient nutrient levels reduce circulating T concentrations. Refeeding provided increased nutrients, as shown by body composition analysis, and significantly increased circulating T which was not different to other treatments at week 24.

In the large size class, D and 3D fish had slightly increasing T levels throughout the experimental period, suggesting that these fish consume sufficient nutrients to maintain T production, and the E_2 level also remained unchanged. However, the decrease of plasma E_2 level at week 24, together with relatively high T level in D and 3D fish, is probably due to conversion of T to other steroids (e.g., 11kT). However as 11kT data were not available, it is difficult to interpret these results. In contrast to small fish, plasma T levels remained unchanged during the first

12 weeks in 7D and Starved fish. It is likely that barramundi can maintain a minimum level of T with low nutrient intake or even with food deprivation and this level is not affected by nutritional status. As observed in small fish, refeeding significantly influenced the plasma T level in the large fish. Plasma T levels observed in this study were comparable to those reported in 3 year old male barramundi (Guigen *et al.*, 1993).

Relationship between nutrition, growth and hormone production

Although small fish store more energy in muscle than large fish, as evidenced by a high muscle index and muscle nutrient levels, the response to the feeding regime in both size classes was similar. Generally, mean body weight gains and SGR in the small fish were higher than those in the large fish, indicating that small fish utilise most of their energy for growth at this stage while large fish utilise less energy for growth but more for reproduction, indicated by the plasma hormone production. Stead *et al.* (1999) found that in early stages of maturation in Atlantic salmon (*Salmo salar*) both males and females had relatively high rates of food consumption and growth, which is associated with the low levels of steroid hormones (E_2 and 11kT). In the later stages, steroid hormone levels rose more rapidly and growth rates decreased in association with inappetence. Tveiten *et al.* (1996) suggested that the high rates of food consumption observed in early maturation are required to achieve sufficient body reserves to support gametogenesis.

The similar patterns of hormone changes and proximate composition of tissues in the D and 3D treatments suggest that although these fish were fed on different feeding regimes, both groups were able to maintain sufficient nutrient levels as indicated by similar body compositions by adjusting their food intake. Both groups were also able to utilise energy in hormone production in a similar pattern. However, these effects were size dependent and this could be due to the different physiological status of fish (Nagahama *et al.*, 1994). D or 3D small fish showed high growth up to week 18, but the decreased growth rate in the last six weeks may be due to investment of energy into reproduction as indicated by increasing plasma E_2 levels occurring at the same time. It may also be due to a negative effect of E_2 on growth as seen in other teleosts. The administration of oestrogens to many fish species caused retarded growth, cessation of feeding and high mortality (Matty and Lone, 1985). In

the present study barramundi showed low food consumption and growth while E_2 was elevated.

The possibility that there are insufficient nutrient levels to maintain T production in the 7D or Starved small fish is confirmed by lower growth and body composition, both reflecting poor nutrition. The increased T level in the last 12 weeks in 7D large fish may be associated with an adaptation to the consumption of fewer nutrients as shown by the increased SGR and food consumption in these fish during this period.

The most significant differences in growth and plasma hormone levels were seen in Refed fish in both size classes. The increased plasma levels of T and E_2 observed in Refed fish indicates that sufficient food availability after starvation stimulates reproductive endocrine activity. This apparent compensatory growth has also been observed in other studies (Miglavs and Jobling, 1989; Russell and Wootton, 1992; Kim and Lovell, 1995; Qian *et al.*, 2000) and it is likely this response occurs in reproductive tissue as well as in somatic tissue. The elevated plasma T and E_2 levels associated with rapid development of gonads in starved/refed fish relative to fed fish were also observed in female golden perch (Collins and Anderson, 1999). However, the increase in T or E_2 level in the small Refed barramundi was not as pronounced as in the large Refed fish in the present study. This may indicate that the small Refed fish invest more energy in growth during the compensatory growth period, while the large Refed fish invest more energy on reproduction. This is also reflected in changes in the SGR and body composition of these fish.

The endocrine mechanisms underlying the differences in growth and maturity are poorly understood (Pirhonen and Forsman, 1998; Hardy, 1999), but it is apparent from the data presented here that there is a size-dependent relationship between nutrition and reproductive hormone levels, providing an indication of such a mechanism.

CHAPTER 4

EFFECT OF DIETARY PROTEIN: ENERGY RATIO ON GROWTH AND PLASMA SEX STEROID HORMONES IN MALE BARRAMUNDI

4.1 INTRODUCTION

A balance between dietary energy and protein is required for efficient utilisation of dietary nutrients. If the amount of dietary energy in relation to dietary protein is low, dietary protein will be used for energy (Lovell, 1984). On the other hand, a dietary excess of energy can limit food consumption which prevents the intake of the necessary amount of protein and other nutrients for maximum growth (Lovell, 1984) since fish adjust their food intake to satisfy their need for energy (Halver, 1989; De Silva and Anderson, 1995).

As discussed in section 1.5.1, the amount of protein in a diet relative to the energy contained in that diet is termed the protein:energy (P:E) ratio. An imbalance in the protein:energy ratio results in wasting of dietary protein or an excess amount of energy aand e production of fatty animals. The optimal dietary protein:energy ratio varies between species, and is also dependant upon the protein source. In the previous chapter, it was shown that restricted feeding influences circulating sex steroids. Since the utilisation of protein and energy in the different processes of maintenance, growth and reproduction may be different, optimal protein:energy ratios for juvenile growth may be different to optimal protein:energy ratios for reproduction. Furthermore, an imbalance in the protein:energy ratio may result in the perturbation of nutrient flow to gonadal products.

In the experiment described in this chapter, constant protein and varying lipid levels were used to vary dietary protein:energy and thus to test the hypothesis that dietary protein:energy ratio affects reproductive function. Specifically, the aim of this study was to determine the effect of protein:energy ratio on growth and plasma sex steroid levels of male barramundi.

4.2 MATERIALS AND METHODS

4.2.1 Fish and experimental conditions

Barramundi of mean weight of 858g (SD ± 100) (range 704 g to 1064 g) obtained from a commercial farm (Barramundi Waters, Innisfail, Queensland, Australia), were transferred to the research aquaculture facility at James Cook University. Fish were held in eight 3500 L tanks according to the conditions described in section 2.1.1. Fish were acclimatised to the aquarium system for four weeks prior to the commencement of experiment.

4.2.2 Experimental diets

Four experimental diets were formulated to obtain varying protein:energy ratios. The diets were prepared on a commercial basis using a Wang extruder by Gatton College, The University of Queensland, Australia. Diets were formulated to be isonitrogenous (50% protein) with variable digestible energy (15, 18, 21 or 24 $MJ.kg^{-1}$) levels using different lipid levels (Table 4.1). Due to difficulties in pelleting high lipid diets, the 21 and 24 $MJ.kg^{-1}$ diets were prepared with less oil and the remaining oil was evenly sprayed to the pellets whilst being tumbled in a concrete mixer. The pellets were ~15mm in diameter.

4.2.3 Experimental design

Following acclimatisation, 10 fish were tagged using colour code T-bar tags for easy identification and assigned to each tank. Fish were maintained under controlled temperature and photoperiod conditions as described in section 2.1.1. A randomised block design was used for the experiment (Table 4.2). Four tanks in each of two systems (blocks) were assigned to each of four diets and fish were fed the respective diet manually once a day in the morning (0800 – 1000 hr) to satiety. The amount of food given to fish/tank was recorded daily throughout the experiment (section 2.1.2) and considered as the amount of food consumed daily for each tank.

Fish fed diets with 15 $MJ.kg^{-1}$, 18 $MJ.kg^{-1}$, 21 $MJ.kg^{-1}$ and 24 $MJ.kg^{-1}$ will be cited as E15, E18, E21 and E24 treatment groups respectively throughout the chapter.

Table 4.1 Composition and proximate analysis of experimental diets with varying levels of energy.

	Dietary energy level ($MJ.kg^{-1}$)			
Ingredient	15	18	21	24
Fishmeal	44.80	44.80	44.80	44.80
Casein	19.20	19.20	19.20	19.20
Corn starch	5.00	5.00	5.00	5.00
Fish oil	4.00	12.00	20.00	27.50
Vitamin mixture	3.00	3.00	3.00	3.00
Mineral mixture	0.50	0.50	0.50	0.50
Cellulose	23.50	15.50	7.50	0.00
Estimated [a]				
Protein (%)	50.74	50.74	50.74	50.74
Digestible Energy ($MJ.kg^{-1}$)	15.40	18.44	21.48	24.33
Measured [b]				
Crude protein (%)	48.8	45.5	48.3	48.8
Crude lipid (%)	8.8	15.8	22.1	27.8
Crude energy ($KJ.g^{-1}$)	19.7	21.8	23.4	25.1
Protein:energy (mg protein.KJ^{-1})	32.5	26.8	24.0	21.6
Calculated Digestible energy [c]	15.02	17.01	20.14	22.53

a- Estimated values based on the diet formulation
b- Actual value measured
c- Digestible energy was calculated based on 23.64 MJ.kg $protein^{-1}$ and 39.54 MJ.kg $lipid^{-1}$ (De Silva and Anderson, 1995)

Table 4.2 Experimental design which describing the assignment of tanks in two blocks. Numbers I - II represent the block number and 1-8 represents the tank number. (E15 = 50% Protein:15 $MJ.kg^{-1}$; E18 = 50% Protein:18 $MJ.kg^{-1}$; E21 = 50% Protein:21 $MJ.kg^{-1}$; E24 = 50% Protein:24 $MJ.kg^{-1}$).

	Treatment/ Tank no.			
Block	E15	E18	E21	E24
I	1	2	3	4
II	5 *	6	7	8 *

* data from these two tanks were excluded from the analysis for the reasons described in section 4.3.2.

4.2.4 Sampling protocol

Fish were anaesthetised, weighed, measured and blood samples were taken at the beginning of the experiment and subsequently at 6 weekly intervals (section 2.1.4). Blood samples were centrifuged and plasma was kept at -20^{0}C until hormonal analysis (section 2.1.5). At the end of the experimental period, five fish from each tank were sacrificed and gonads, liver and muscle samples were collected and stored for further analysis as described in section 2.1.7. In addition, gonad samples from five fish at week 0 were collected and fixed for histological analysis.

Plasma samples were analysed for T, E_2 and 11kT[1] (section 2.4). Proximate composition of diets, muscle and liver samples were performed as described in section 2.3. Gonad samples were processed and examined as described in section 2.5. Food consumption (% Bwt.day^{-1}), FCE, %BWG, SGR, GSI, HSI, muscle and visceral fat index were calculated as in section 2.2. Energy consumption, energy conversion efficiency (ECE) and protein efficiency ratio (PER) were calculated as follows (De Silva and Anderson, 1995).

(a) Energy consumption

$$\text{Energy consumption (kJ.100g fish}^{-1}) = \frac{\text{Energy intake (kJ)}}{\text{Body Weight (g)}} x\,100$$

(b) Energy conversion efficiency (ECE)

$$\text{ECE} = \frac{\text{Wet weight gain (g)}}{\text{Energy intake (kJ)}}$$

Energy intake was determined by the food offered to each tank multiplying by the calculated digestible energy values.

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[1] A method for measuring plasma 11kT in barramundi was established and validated in this laboratory at the end of this study. This method was time consuming and there were time constraints and also due to the failure of fish in tanks 5 and 8 to thrive, only plasma samples from fish from tanks 1-4 (four treatments in one system) were analysed for 11kT.

(b) Protein Efficiency Ratio (PER)

$$PER = \frac{\text{Wet weight gain (g)}}{\text{Dry protein intake (g)}}$$

4.2.5 Statistical analysis

Data from tanks 5 (E15) and 8 (E24) were excluded from the analysis for the reasons described below (section 4.3.2). Some fish from other tanks which lost weight for varying periods (for eg. first 6 weeks, 12 weeks etc.) during the experimental period were also excluded from the analysis. The number of fish used for each analysis is given in Table 4.4.

Data for growth (weight and SGR) and plasma hormone levels were analysed using repeated measures block ANOVA. Means for each treatment at a single time point were analysed using one way ANOVA, followed by Scheffe's test. A paired t-test was used to check the differences between means at different time points with the initial value (Wk 0) within the same treatment and means within the same treatment over successive time points (eg. wks 0 and 6, wks 6 and 12, etc.). Percentage body weight gain, GSI, HSI, visceral fat index, muscle index and proximate analysis data were analysed using a randomised block ANOVA (Univariate analysis) with missing values (SPSS base 8.0- applications guide). Where ANOVA showed a significant effect of treatment, Scheffe's test (for unequal sample sizes) was used to compare the means (Zar, 1984). Data were analysed for homogeneity of variances using Levene's test. Liver dry matter, liver protein and liver energy were analysed using non-parametric analysis (Kruskal-Wallis Test) as transformation of the data to achieve homogeneity of variances was not successful. Statistical significance was considered to occur when $p<0.05$. Data were analysed using the SPSS (version 9.0) statistical package.

4.3. RESULTS

4.3.1 Experimental Fish

Two fish died over the experiment. One fish fed the E24 diet (#4, week 14) died due to disease presumed to be caused by infection during blood sampling and a second fish died after jumping from the tank (# 3, E21 at week 20). After 15 weeks fish in all treatments were treated with fresh water for one week as a preventive method for white spot disease.

4.3.2 Diets

Proximate composition of the diets was between 45.5% and 48.8% protein and 19.7 to 25.1 $MJ.kg^{-1}$ energy (Table 4.1). The protein:energy content of the diet ranged from 21.6 to 32.5 mg protein.KJ^{-1}. The four diets varied in their texture and hardness with consequent palatability problems. It seemed difficult for fish to eat the E15 diet which was hard and being large in size (15mm). To minimise this problem, the pellets were crushed before feeding using a mortar and pestle. The high energy diets were relatively oily and odorous.

Feeding habits

In the acclimation period, fish were fed commercial barramundi pellets. All fish consumed this diet well. However, most fish did not immediately accept the experimental diets probably due to the different textures, taste or smell. Although some fish started to eat, others did not eat for varying periods. In some cases fish took the pellets into the mouth but immediately rejected them. It was noted that if one fish in the tank accepted the experimental diet, then the others would start to follow this behaviour. Fish established different feeding habits in different tanks and there were hierarchies present within tanks. This was observed when feeding fish and the more dominant fish always stayed at the upper level of water and consumed more than other fish. Generally, it was noted that all fish increased their food consumption after 15 weeks, perhaps due to the fresh water change during this period.

Although the amount of food offered (depending on fish response) to tank 5 was similar to those to tank 1, fish in tank 5 fed the E15 diet had very low FCE values during

first 12 weeks (-0.10 (wk 0-6) and 0.03 (wk 6-12)) meant these fish actually consumed less food during that period. Low FCE values may be due to the difficulties in measuring correct amount of food given to the fish, as E15 diet was crushed before feeding. Also, the tank system design prevented collection of uneaten food and this difficulty may confound FCE or PER values. However, care was taken to give the pellets on fish response only and up to satiety. Although fish in tank 5 started to eat well after 14 weeks, as it was observed from the previous experiment (chapter 3) that after a period of low food intake fish will show compensatory growth which will affect their hormone levels, these data were excluded from the analysis. In addition, high HSI and liver lipid levels of these fish (tank # 5) compared to the fish fed the same diet but in the replicate tank (# 1) indicate an effect of feeding behaviour on overall nutrition.

Similarly fish in tank 8 (E24 diet) were excluded from the analysis since these fish did not eat at all for the first 12 weeks. All fish stayed at the bottom of the tank during the feeding session and did not consume food at all. However, they started to eat after 12 weeks and the fresh water change during this period appeared to influence their feeding habits. However, to avoid the confounding effects of compensatory growth, these fish were also excluded from the analysis. In contrast, fish in tank 4 fed the high energy diet (E24) started to eat well from the beginning. That, fish in one tank (# 4) started to eat well but fish in the other tank (# 8) rejected the same food, indicating the problem lay with individual groups of fish rather than the feed *per se*.

Food consumption

Food offered is an indicator of feed consumption but is not precise. Generally, fish fed on E15, E18 and E21 consumed less food in the first 12 weeks. However, it appears that after week 12 all fish increased food consumption (Table 4.3a). Overall food consumption ranged from 0 % - 1.6 % BWt.day^{-1}. Low energy groups tend to consume more food than high energy group but this could not be tested statistically. Mean daily food consumption for the last 6 weeks were 0.95, 0.92, 0.84 and 0.72 (%BWt.day^{-1}) for E15, E18, E21 and E24 groups respectively (Table 4.3a).

Mean daily energy consumption (KJ.100 g wet weight of fish^{-1}) showed E15 and E21 treatments had lower energy intake during the first 12 weeks which increased in the last 12 weeks (Table 4.3b). Fish fed the highest energy (E24) diet had high

Table 4.3a Food consumption (% Bwt.day^{-1}) (mean and SD of daily measurements for six weekly periods) of barramundi fed diets with different protein:energy ratios. (E15 = 50% Protein:15 MJ.kg^{-1}; E18 = 50% Protein:18 MJ.kg^{-1}; E21 = 50% Protein:21 MJ.kg^{-1}; E24 = 50% Protein:24 MJ.kg^{-1}). * Mean value for two tanks.

Diet	Mean Food consumption (% BWt.day^{-1})			
	0-6 wk	6-12 wk	12-18 wk	18-24wk
E15	0.34 ± 0.27	0.41 ± 0.32	0.73 ± 0.41	0.95 ± 0.37
E18 *	0.50 ± 0.41	0.56 ± 0.41	0.77 ± 0.48	0.92 ± 0.34
E21 *	0.14 ± 0.21	0.40 ± 0.38	0.77 ± 0.48	0.84 ± 0.38
E24	0.67 ± 0.51	0.43 ± 0.41	0.52 ± 0.47	0.74 ± 0.33

Table 4.3b Mean energy consumption (intake) (for six weekly periods) of barramundi fed diets with different protein:energy ratios. (E15 = 50% Protein:15 MJ.kg^{-1}; E18 = 50% Protein:18 MJ.kg^{-1}; E21 = 50% Protein:21 MJ.kg^{-1}; E24 = 50% Protein:24 MJ.kg^{-1}). * mean and range for two tanks.

Diet	Mean energy consumption (KJ.100 g wet weight of fish^{-1})			
	0-6 wk	6-12 wk	12-18 wk	18-24wk
E15	5.17	6.23	10.9	14.2
E18 *	8.58 (8.0 – 9.1)	9.49 (7.9 – 11.0)	13.1 (12.6 – 13.5)	15.6 (15.0 – 16.3)
E21 *	2.87 (1.1 – 4.6)	8.14 (7.9 – 8.3)	15.5 (15.3 – 15.7)	16.9 (16.0 – 17.8)
E24	15.1	9.62	10.7	16.3

Energy consumption was calculated based on calculated digestible energy level (KJ.g^{-1}).

energy consumption during the first 6 weeks. However, energy consumption decreased during the second 6 weeks but increased again during the last 6 weeks. Energy consumption for last 6 weeks were 14.2, 15.6, 16.9 and 16.3 KJ.100g of fish^{-1} for the E15, E18, E21 and E24 groups respectively.

These data were not analysed statistically, as food consumption and energy consumption data were available only for a group of fish (each tank) but not for the individual fish. In addition, these data only give a general idea about each group as food consumed by individual fish may be varied largely within the same tank.

Feeding pattern

Daily food consumption of fish showed peaks and troughs in all treatments. Mean weekly food consumption (% Bwt.day^{-1}) increased with time in all treatments (Fig 4.1). Apart from the period where the fish were acclimatising to diet, low weekly food consumption corresponded to uncontrollable disturbances during the experimental period such as low temperatures or system water changes (wk 15).

4.3.3 Growth

Fish in all treatments showed low growth during the first 12 weeks compared to the remaining period (Fig 4.2). Growth of fish at different time points were not significantly affected by dietary energy level. Mean body weights at week 24 were 1552 ± 98, 1714 ± 50, 1549 ± 45, and 1654 ± 65 g for E15, E18, E21 and E24 diets respectively (Table 4.4). Percentage body weight gains (% BWG) for the whole period were not significantly different between treatments which ranging from 84.6 – 94.7% (Table 4.4).

Repeated measures ANOVA showed that SGR increased with time ($F=81.45_{(2.2, 99.1)}$; $P<0.05$) and there was significant interaction between dietary energy level and time (repeated ANOVA- $F=3.97_{(6.6,99.1)}$; $P<0.05$). Comparisons between means within treatment showed that fish in E15 and the E18 treatments had low SGR up to week 12 and SGR increased significantly after this time (Fig 4.3). The E21 group had low SGR to week 6 which increased significantly to week 12 and again to week 18 and remained high thereafter. SGR of fish fed on the highest energy diet (E24)

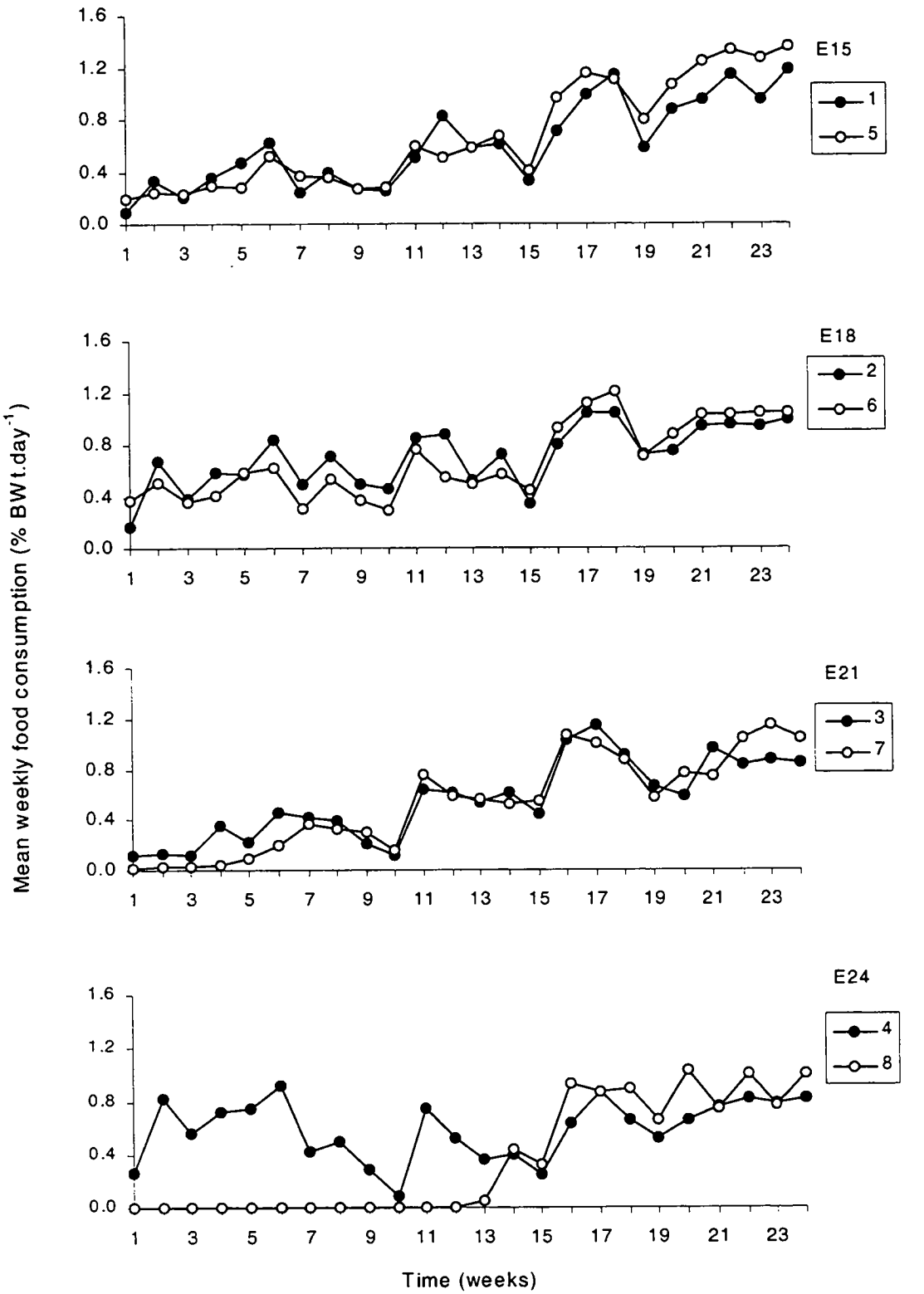


Figure 4. 1 Mean weekly food consumption (% Bwt.day^{-1}) of barramundi fed diets with different protein:energy ratios over a 24 week period. (E15 = 50% Protein:15 MJ.kg^{-1}; E18 = 50% Protein:18 MJ.kg^{-1}; E21 = 50% Protein:21 MJ.kg^{-1}; E24 = 50% Protein:24 MJ.kg^{-1}). Tank numbers in each block represented by 1-8.

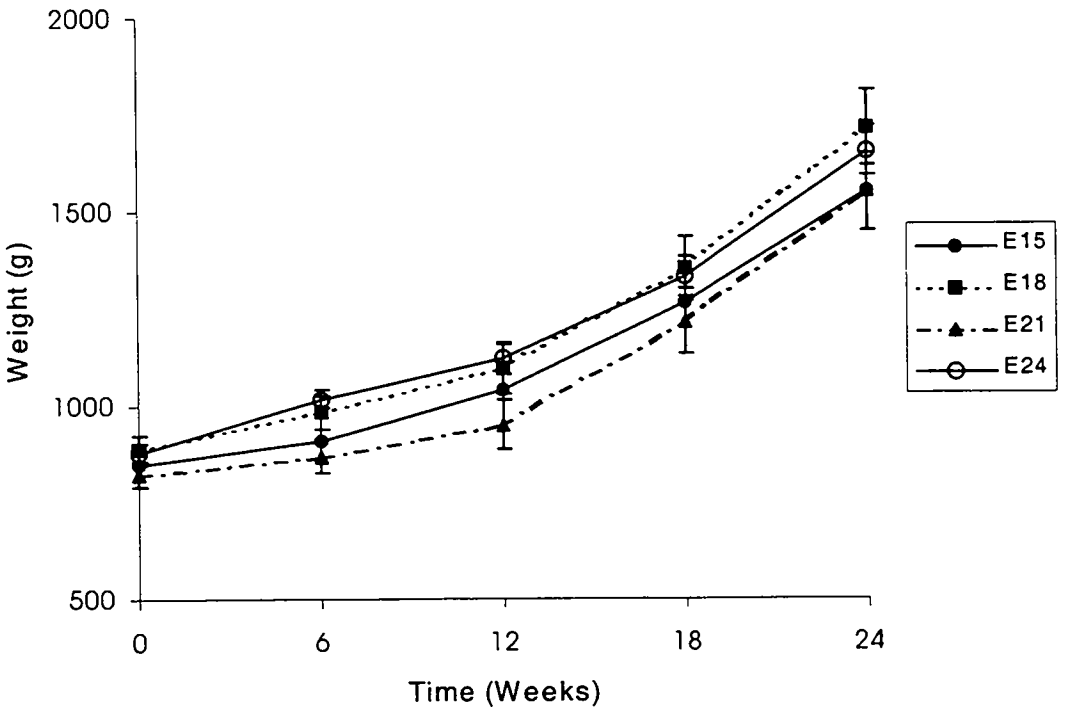


Figure 4. 2 Mean (± SE) body weights of barramundi fed diets with different proten:energy ratios. (E15 = 50% Protein:15 MJ.kg^{-1}; E18 = 50% Protein:18 MJ.kg^{-1}; E21 = 50% Protein:21 MJ.kg^{-1}; E24 = 50% Protein:24 MJ.kg^{-1}).

Table 4.4 Initial weight, final weight and % body weight gain (%BWG) (Mean ± SE) of barramundi fed different diets with varying protein:energy ratios. (E15 = 50% Protein:15 $MJ.kg^{-1}$; E18 = 50% Protein:18 $MJ.kg^{-1}$; E21 = 50% Protein:21 $MJ.kg^{-1}$; E24 = 50% Protein:24 $MJ.kg^{-1}$). n = number of fish. Means with same superscript in the same column are not significantly different (P>0.05).

Diet	n	Initial weight (g)	Final weight (g)	% BWG
E15	7	845 ± 34 [a]	1552 ± 98 [a]	84.6 ± 10.6 [a]
E18	18	887 ±27 [a]	1714 ± 50 [a]	94.7 ± 5.9 [a]
E21	16	823 ±20 [a]	1549 ± 45 [a]	88.1 ± 3.0 [a]
E24	9	876 ±29 [a]	1654 ± 65 [a]	88.0 ± 8.4 [a]

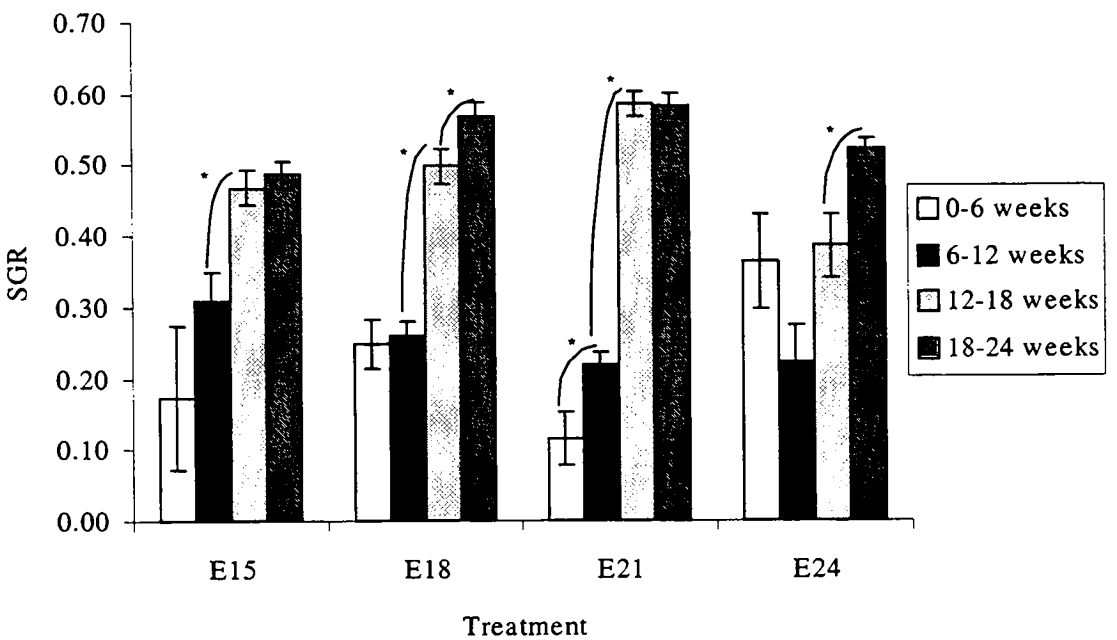


Figure 4. 3 Mean (± SE) Specific Growth Rate (SGR) of barramundi fed diets with different protein:energy ratios. (E15 = 50% Protein:15 MJ.kg^{-1}; E18 = 50% Protein:18 MJ.kg^{-1}; E21 = 50% Protein:21 MJ.kg^{-1}; E24 = 50% Protein:24 MJ.kg^{-1}).* indicates a significant difference by paired t-test between adjacent values. The legend shows the period of time to which each column refers.

fluctuated during the experiment but significant changes occurred between weeks 18 and 24.

As the feeding was considered to be reliable for only the last 12 weeks, the relationships between SGR and protein:energy ratio were determined from the data for this period (Fig 4.3a). Multiple regression of SGR during the last 12 weeks vs protein:energy ratio gave the relationship:

$$SGR = -0.0031X^2 + 0.1688X - 1.7198 \ (r^2 = 0.6165; F = 0.80; df = 1; sig = 0.619)$$

Where X = dietary protein:energy ratio.
This indicated an optimal protein:energy ratio of 27.2 mg protein.kJ $^{-1}$ for growth.

4.3.4 Food Conversion Efficiency, Protein Efficiency Ratio and Energy Conversion Efficiency

FCE, PER and ECE showed similar patterns (Table 4.5a,b,c). In the E15 group low FCE, PER and ECE were observed during the first 6 weeks but increased during remaining period. All parameters were relatively low during the first 12 weeks in the E18, E21 and E24 groups and higher values were observed during the last 12 weeks. Generally, the E15 group had lower values than the other groups.

FCE

The E15 group had very low FCE during the first 6 weeks which increased during the remaining period (Table 4.5a). The E18, E21 and E24 groups had relatively low FCE during the first 12 weeks, which improved during the last 12 weeks. FCE for the last 6 weeks were 0.61, 0.71, 0.69 and 0.80 for the E15, E18, E21 and E24 treatments respectively.

As for SGR, only the data from last 12 weeks was considered reliable (Fig 4.3a). Multiple regression of FCE during the last 12 weeks vs protein:energy ratio gave the relationship:

$$FCE = -0.0012X^2 + 0.0423X - 0.4247 \ (r^2 = 1.0; F = 287505; df = 1; Sig = 0.001)$$

Where X = dietary protein:energy ratio.

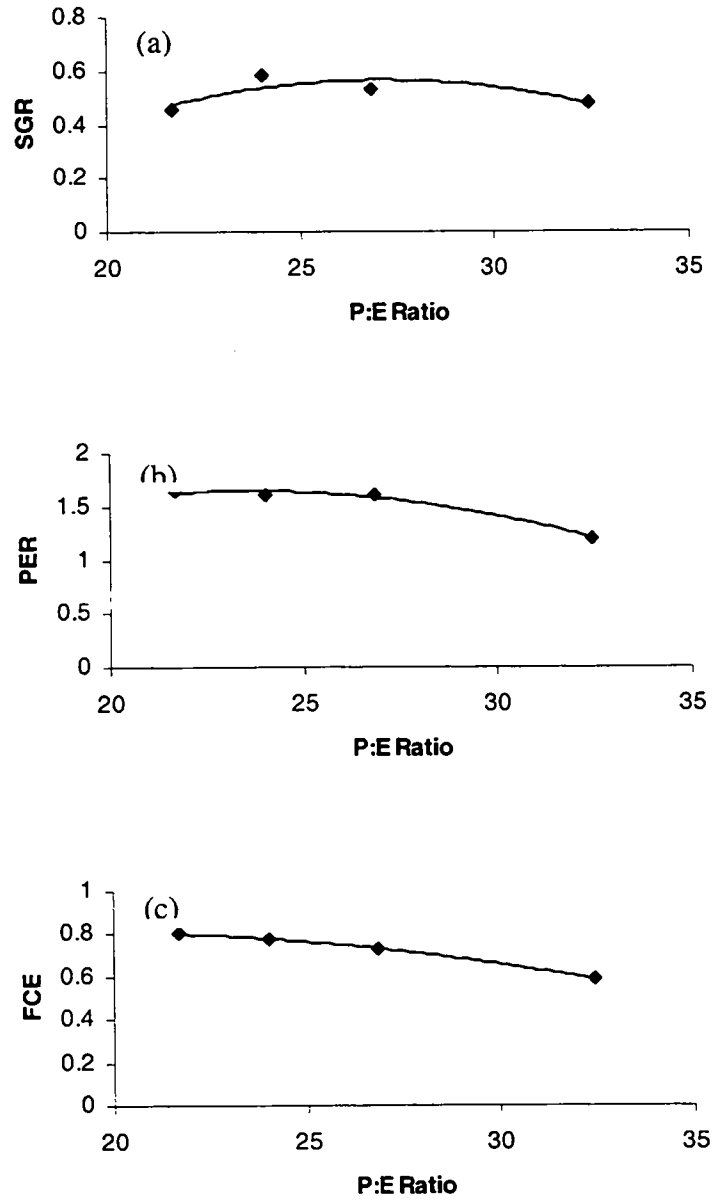


Figure 4.3a. The relationships between (a) specific growth rate (SGR), (b) protein efficiency ratio (PER) and (c) food conversion efficiency (FCE) and dietary protein:energy ratio (P:E ratio) for the last 12 weeks of the experimental period. The multiple regressions for the above relationships are indicated in the relevant text.

Table 4.5a Food conversion efficiency (FCE) for 6 weekly periods for barramundi fed diets with different protein:energy ratios. (E15 = 50% Protein:15 MJ.kg^{-1}; E18 = 50% Protein:18 MJ.kg^{-1}; E21 = 50% Protein:21 MJ.kg^{-1}; E24 = 50% Protein:24 MJ.kg^{-1}). * mean and range for two tanks.

Diet	FCE			
	0-6 wk	6-12 wk	12-18 wk	18-24wk
E15	0.13	0.48	0.56	0.61
E18*	0.45 (0.37 – 0.52)	0.44 (0.36 – 0.53)	0.75 (0.69 – 0.82)	0.71 (0.66 – 0.76)
E21*	0.62 (0.40 – 0.84)	0.40 (0.34 – 0.47)	0.78 (0.72 – 0.84)	0.77 (0.75 – 0.78)
E24	0.57	0.56	0.80	0.80

Table 4.5b Protein efficiency ratio (PER) for 6 weekly periods for barramundi fed diets with different protein:energy ratios. (E15 = 50% Protein:15 MJ.kg^{-1}; E18 = 50% Protein:18 MJ.kg^{-1}; E21 = 50% Protein:21 MJ.kg^{-1}; E24 = 50% Protein:24 MJ.kg^{-1}). * mean and range for two tanks.

Diet	PER			
	0-6 wk	6-12 wk	12-18 wk	18-24wk
E15	0.26	0.99	1.14	1.26
E18*	0.98 (0.82 – 1.14)	0.98 (0.79 – 1.16)	1.66 (1.55 – 1.88)	1.56 (1.45 – 1.66)
E21*	1.28 (0.82 – 1.73)	0.84 (0.70 – 0.97)	1.62 (1.50 – 1.75)	1.59 (1.56 – 1.62)
E24	1.17	1.16	1.64	1.64

Table 4.5c. Energy conversion efficiency (ECE) for 6 weekly periods for barramundi fed diets with different protein:energy ratios. (E15 = 50% Protein:15 MJ.kg^{-1}; E18 = 50% Protein:18 MJ.kg^{-1}; E21 = 50% Protein:21 MJ.kg^{-1}; E24 = 50% Protein:24 MJ.kg^{-1}). * mean and range for two tanks.

Diet	ECE			
	0-6 wk	6-12 wk	12-18 wk	18-24wk
E15	0.008	0.032	0.037	0.041
E18*	0.026	0.026	0.044	0.042
	(0.02 – 0.03)	(0.02 – 0.03)	(0.04 – 0.05)	(0.04 – 0.05)
E21*	0.031	0.020	0.039	0.038
	(0.02 – 0.04)	(0.02 – 0.02)	(0.04 – 0.04)	(0.04 – 0.04)
E24	0.025	0.025	0.035	0.036

ECE (g.KJ^{-1}) = wet weight gain (g)/ Energy offered (kJ); Digestible energy was used to calculate ECE.

This indicated an optimal dietary protein:energy ratio of 17.6 mg protein.kJ $^{-1}$ for food conversion.

PER

During the first 6 weeks, PER was 0.2 in the E15 group but increased up to 1.0 at week 12, and remained high during the remaining period (Table 4.5b). In the E18 groups, PER was lower during the first 12 weeks than the last 12 weeks. The high initial PER decreased after 6 weeks but increased again and remained high in the E21 group. Multiple regression of FCE during the last 12 weeks vs protein:energy ratio (Fig 4.3a) gave the relationship:

$$PER = -0.0054\ X^2 + 0.2556\ X - 1.3542\ (r^2 = 0.984;\ F = 30.89;\ df = 1;\ sig = 0.126)$$

Where X = dietary protein:energy ratio.
This indicated an optimal dietary protein:energy ratio for protein retention of 23.6 mg protein.kJ $^{-1}$.

ECE

The E15 group showed low ECE (0.008 g.kJ^{-1}) during the first 6 weeks which increased during the remaining period (Table 4.5c). The E18 and E24 groups had low ECE during the first 12 weeks but it increased thereafter. The E21 group had relatively high ECE during the experimental period except for the second 6 weeks. ECE for first 12 weeks were 0.025 g.kJ^{-1} which increased to 0.035 g.kJ^{-1} during the second 12 weeks. Generally E15 and E18 groups had higher ECE during last 6 weeks than E21 and E24 treatments.

4.3.5 Visceral Fat Index and Muscle Index

The visceral fat content was significantly affected by the dietary energy level (Table 4.6). The E15 group had the lowest visceral fat content (3.6 ± 0.6) which was significantly different from the E24 group (5.3 ± 0.4). Muscle index showed no relationship to dietary energy level (Table 4.6).

Table 4.6 Muscle index, visceral fat index, Gonado Somatic Index (GSI) and Hepato Somatic Index (HSI) (mean ± SE) of barramundi fed different diets with varying protein:energy ratios. (E15 = 50% Protein:15 $MJ.kg^{-1}$; E18 = 50% Protein:18 $MJ.kg^{-1}$; E21 = 50% Protein:21 $MJ.kg^{-1}$; E24 = 50% Protein:24 $MJ.kg^{-1}$). Means with same superscript in the same column are not significantly different (P>0.05).

Diet	n	Muscle Index (%)	Visceral fat Index (%)	GSI	HSI
E15	5	44.6 ± 2.9^{a}	3.6 ± 0.6^{a}	0.05 ± 0.01^{a}	1.19 ± 0.10^{a}
E18	10	43.1 ± 1.0^{a}	4.4 ± 0.2^{ab}	0.05 ± 0.01^{a}	1.25 ± 0.03^{a}
E21	10	40.3 ± 1.1^{a}	4.9 ± 0.4^{ab}	0.06 ± 0.01^{ab}	1.22 ± 0.05^{a}
E24	5	43.9 ± 2.2^{a}	5.3 ± 0.4^{b}	0.08 ± 0.01^{b}	1.36 ± 0.03^{a}

4.3.6 Hepato Somatic Index

HSI values did not vary significantly with increasing dietary energy (Table 4.6). However, there was a general trend for HSI to increase with dietary energy level. The highest mean HSI value (1.36 ± 0.03) corresponded to E24 fish and the lowest (1.19 ± 0.1) was recorded from fish in the E15 treatment.

4.3.7 Proximate composition

Muscle

All nutrients were expressed as total nutrients (g.Kg of whole body weight of $fish^{-1}$) and energy as total energy (MJ.Kg of whole body weight of $fish^{-1}$) or as relative amounts (% wet weight basis (g.100g of wet $tissue^{-1}$) or MJ.kg $tissue^{-1}$).

Total muscle lipid significantly increased with dietary energy level (Table 4.7a), with the E24 group having the highest muscle lipid content (12.2 g.Kg of whole body weight of $fish^{-1}$), twice that of other fish which were not different. Total dry matter, protein, ash and energy were not significantly different between treatments. However, there was a general tendency for muscle dry matter, protein and energy to decrease with increasing dietary energy from 15 $MJ.kg^{-1}$ to 21 $MJ.kg^{-1}$. Total muscle carbohydrate (calculated) increased with decreasing dietary lipid level. Fish fed E15, E18 and E21 diets had significantly greater carbohydrate than fish fed the highest energy diet (E24).

Percentage proximate composition data showed that muscle lipid, ash and energy were significantly affected by dietary energy level (Table 4.7b). Percentage muscle lipid and energy (MJ.kg of $tissue^{-1}$) increased with dietary energy level with the E24 group having the highest % lipid content and energy content. Percentage muscle carbohydrate significantly increased with decreasing dietary lipid level. Fish fed the E15 diet had the lowest muscle ash (%) which was significantly different from all other three treatments.

Liver

Total liver dry matter, lipid and energy were significantly affected by dietary energy level (Table 4.8a). Liver dry matter and energy significantly increased with

Table 4.7a Total moisture, dry matter, protein, lipid, ash and total energy (mean ± SE) in muscle of barramundi fed on different protein:energy ratios. (E15 = 50% Protein:15 MJ.kg^{-1}; E18 = 50% Protein:18 MJ.kg^{-1}; E21 = 50% Protein:21 MJ.kg^{-1}; E24 = 50% Protein:24 MJ.kg^{-1}). Means with same superscript in the same column are not significantly different (P> 0.05).

Diet	Total nutrients (g.Kg wet wt of whole fish^{-1})						Total energy (MJ.Kg of whole fish^{-1})
	Total Muscle Moisture	Total Muscle Dry matter	Total Muscle Protein	Total Muscle Lipid	Total Muscle Ash	Total Muscle carbohydrate (calculated)	
E15	338.9 ± 18.2^{a}	106.7 ± 5.5^{a}	88.6 ± 4.3^{a}	5.2 ± 0.4^{a}	4.8 ± 0.1^{a}	8.2 ± 1.4^{b}	22.2 ± 0.11^{a}
E18	327.4 ± 7.3^{a}	103.6 ± 2.5^{a}	85.7 ± 1.8^{a}	6.4 ± 0.3^{a}	5.8 ± 0.2^{a}	5.7 ± 0.8^{b}	21.8 ± 0.48^{a}
E21	311.5 ± 4.0^{a}	99.7 ± 1.5^{a}	81.4 ± 1.4^{a}	6.3 ± 0.4^{a}	5.5 ± 0.2^{a}	6.5 ± 0.5^{b}	21.3 ± 0.37^{a}
E24	331.9 ± 16.8^{a}	107.4 ± 5.5^{a}	87.4 ± 4.4^{a}	12.2 ± 1.0^{b}	5.7 ± 0.3^{a}	1.9 ± 0.2^{a}	23.1 ± 0.12^{a}

Table 4.7b Percentage moisture, dry matter, protein, lipid, ash and energy (mean ± SE) in muscle of barramundi fed diets with different protein:energy ratios. (E15 = 50% Protein:15 $MJ.kg^{-1}$; E18 = 50% Protein:18 $MJ.kg^{-1}$; E21 = 50% Protein:21 $MJ.kg^{-1}$; E24 = 50% Protein:24 $MJ.kg^{-1}$). Means with same superscript in the same column are not significantly different (P> 0.05).

Diet	g.100g of wet $tissue^{-1}$ (% wet wt)					Energy
	Muscle Moisture	Muscle Protein	Muscle Lipid	Muscle Ash	Carbohydrate (calculated)	(MJ.Kg of wet $tissue^{-1}$)
E15	76.1 ± 0.1^{a}	19.9 ± 0.2^{a}	1.2 ± 0.1^{a}	1.1 ± 0.1^{a}	1.8 ± 0.2^{b}	4.9 ± 0.1^{a}
E18	75.9 ± 0.1^{a}	19.9 ± 0.1^{a}	1.5 ± 0.1^{a}	1.4 ± 0.1^{b}	1.3 ± 0.2^{b}	5.1 ± 0.1^{ab}
E21	75.8 ± 0.1^{a}	19.8 ± 0.1^{a}	1.5 ± 0.1^{a}	1.4 ± 0.1^{b}	1.6 ± 0.1^{b}	5.2 ± 0.1^{ab}
E24	75.6 ± 0.2^{a}	19.9 ± 0.1^{a}	2.8 ± 0.2^{b}	1.3 ± 0.1^{b}	0.5 ± 01^{a}	5.3 ± 0.1^{b}

Table 4.8a Total moisture, dry matter, protein, lipid, ash and total energy (mean ± SE) in liver of barramundi fed diets with different protein:energy ratios. (E15 = 50% Protein:15 $MJ.kg^{-1}$; E18 = 50% Protein:18 $MJ.kg^{-1}$; E21 = 50% Protein:21 $MJ.kg^{-1}$; E24 = 50% Protein:24 $MJ.kg^{-1}$). Means with same superscript in the same column are not significantly different (P> 0.05). * dietary energy level significantly affected the parameter (non- parametric; Kruskal-Wallis test); # -dietary energy level did not affect the parameter (non- parametric; Kruskal-Wallis test).

Diet	Total nutrients (g.Kg wet wt of whole $fish^{-1}$)						Total energy *
	Total Liver Moisture	Total Liver Dry matter *	Total Liver Protein #	Total Liver Lipid	Total Liver Ash	Liver Cabohydrate (calculated)	(MJ.Kg of whole $fish^{-1}$)
E15	5.9 ± 0.3^{a}	6.1 ± 0.7	1.04 ± 0.07	3.64 ± 0.55^{a}	0.09 ± 0.06^{a}	1.28 ± 0.2^{a}	1.85 ± 0.24
E18	5.9 ± 0.2^{a}	6.6 ± 0.2	1.02 ± 0.02	4.04 ± 0.15^{ab}	0.10 ± 0.01^{a}	1.41 ± 0.1^{a}	2.03 ± 0.07
E21	5.8 ± 0.1^{a}	6.8 ± 0.2	1.10 ± 0.01	4.35 ± 0.20^{ab}	0.10 ± 0.01^{a}	1.2 ± 0.1^{a}	2.10 ± 0.06
E24	5.8 ± 0.4^{a}	7.9 ± 0.4	1.05 ± 0.03	5.05 ± 0.25^{b}	0.10 ± 0.01^{a}	1.5 ± 0.1^{a}	2.37 ± 0.07

Table 4.8b Percentage moisture, dry matter, protein, lipid, ash and energy (mean ± SE) in liver of barramundi fed diets with different protein:energy ratios. (E15 = 50% Protein:15 MJ.kg^{-1}; E18 = 50% Protein:18 MJ.kg^{-1}; E21 = 50% Protein:21 MJ.kg^{-1}; E24 = 50% Protein:24 MJ.kg^{-1}). Means with same superscript in the same column are not significantly different (P> 0.05).

Diet	g.100g of wet tissue^{-1} (% wet wt)					Energy
	Liver Moisture	Liver Protein	Liver Lipid	Liver Ash	Carbohydrate (calculated)	(MJ.Kg of wet tissue^{-1})
E15	49.7 ± 1.9 ^{b}	8.8 ± 0.3 ^{b}	30.0 ± 2.1 ^{a}	0.7 ± 0.1^{a}	10.7 ± 0.8 ^{a}	15.4 ± 0.7 ^{a}
E18	47.7 ± 0.8 ^{ab}	8.2 ± 0.1 ^{ab}	32.3 ± 1.0 ^{ab}	0.8 ± 0.1 ^{a}	11.2 ± 0.4 ^{a}	16.2 ± 0.3 ^{ab}
E21	46.3 ± 0.6 ^{ab}	8.7 ± 0.2 ^{b}	34.3 ± 1.1 ^{ab}	0.8 ± 0.1 ^{a}	9.8 ± 0.4 ^{a}	16.6 ± 0.2 ^{ab}
E24	42.3 ± 2.7 ^{a}	7.7 ± 0.2 ^{a}	37.1 ± 1.6 ^{b}	0.7 ± 0.1 ^{a}	11.0 ± 0.7 ^{a}	17.4 ± 0.4 ^{b}

dietary energy level. Fish fed the E15 diet had the lowest lipid content (3.64 ± 0.55) which was significantly different from fish fed the high energy (E24) diet. Total liver moisture, protein, ash and carbohydrate were not significantly affected by dietary energy level.

Percentage composition of liver showed that % moisture, % protein, % lipid and energy (MJ.kg of tissue^{-1}) were significantly affected by dietary energy level (Table 4.8b). Fish fed the E15 diet had the highest moisture, protein and lowest lipid and energy content which were significantly different from fish fed the E24 diet. Percentage carbohydrate or ash was not significantly different between treatments.

4.3.8 Gonado Somatic Index

GSI values of barramundi were significantly affected by dietary energy level with GSI increasing significantly with increasing dietary energy level (Table 4.6). Fish fed the E24 diet had the highest mean GSI (0.08 ± 0.01) which was significantly higher than those of fish fed on lower energy diets (E15 and E18).

4.3.9 Gonadal stages

At the start of the experiment (week 0) fish had varying gonad developmental stages. Two fish were found with stage 4 (spent) gonads and one of each at stage 1 (immature), 2 and 3. All fish were male at the end of the experiment and had varying gonadal stages (Fig 4.4). Gonadal stages were not significantly different between treatments (Likelihood ratio, F = 16.81, df = 9, P = 0.052). Gonads of fish in the E15 treatment showed only early developmental stages compared to the other treatments. Gonia cells were tightly packed in these gonads (Fig 4.5a). In the other three treatments (E18, E21 and E24) gonads of fish showed a range of different levels of developmental stages including stage 1b, 2 and 3 (Fig 4.5). Gonads identified as stage 1b clearly showed the further development of gonia cells with presence of clusters of spermatocytes and spermatids while other cells were mostly in the gonia stage (Fig 4.5b). However these stage 1b gonads are clearly different from the gonadal stages observed in E15 treatment which had tightly packed gonia cells without showing any further development.

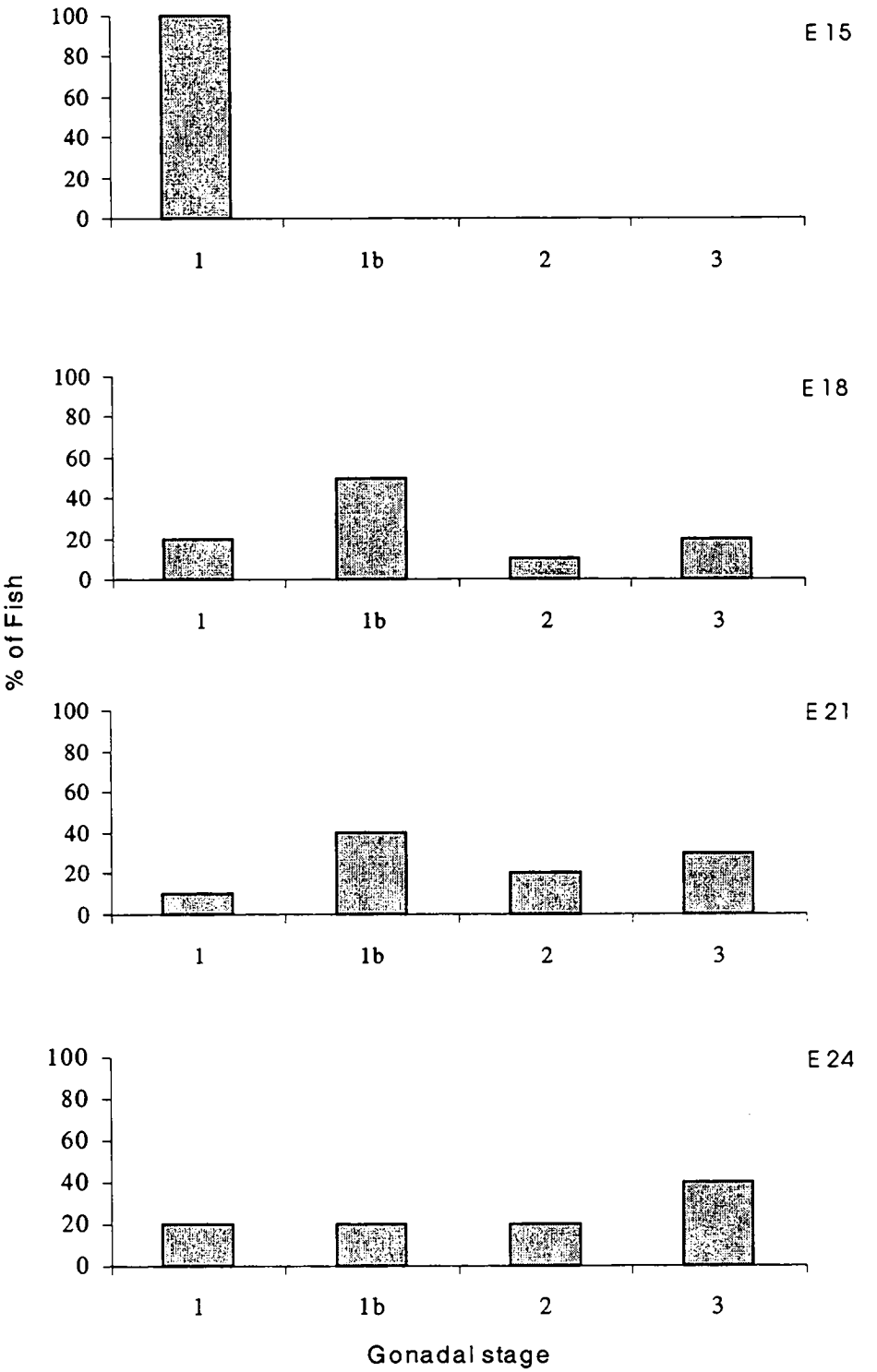


Figure 4. 4 Gonadal stage of barramundi fed diets with different protein:energy ratios over a 24 week period. (E15 = 50% Protein:15 MJ.kg^{-1}; E18 = 50% Protein:18 MJ.kg^{-1}; E21 = 50% Protein:21 MJ.kg^{-1}; E24 = 50% Protein:24 MJ.kg^{-1}).Gonadal stages are categorised as according to Guiguen *et al.* (1994) and as in section 2.5.

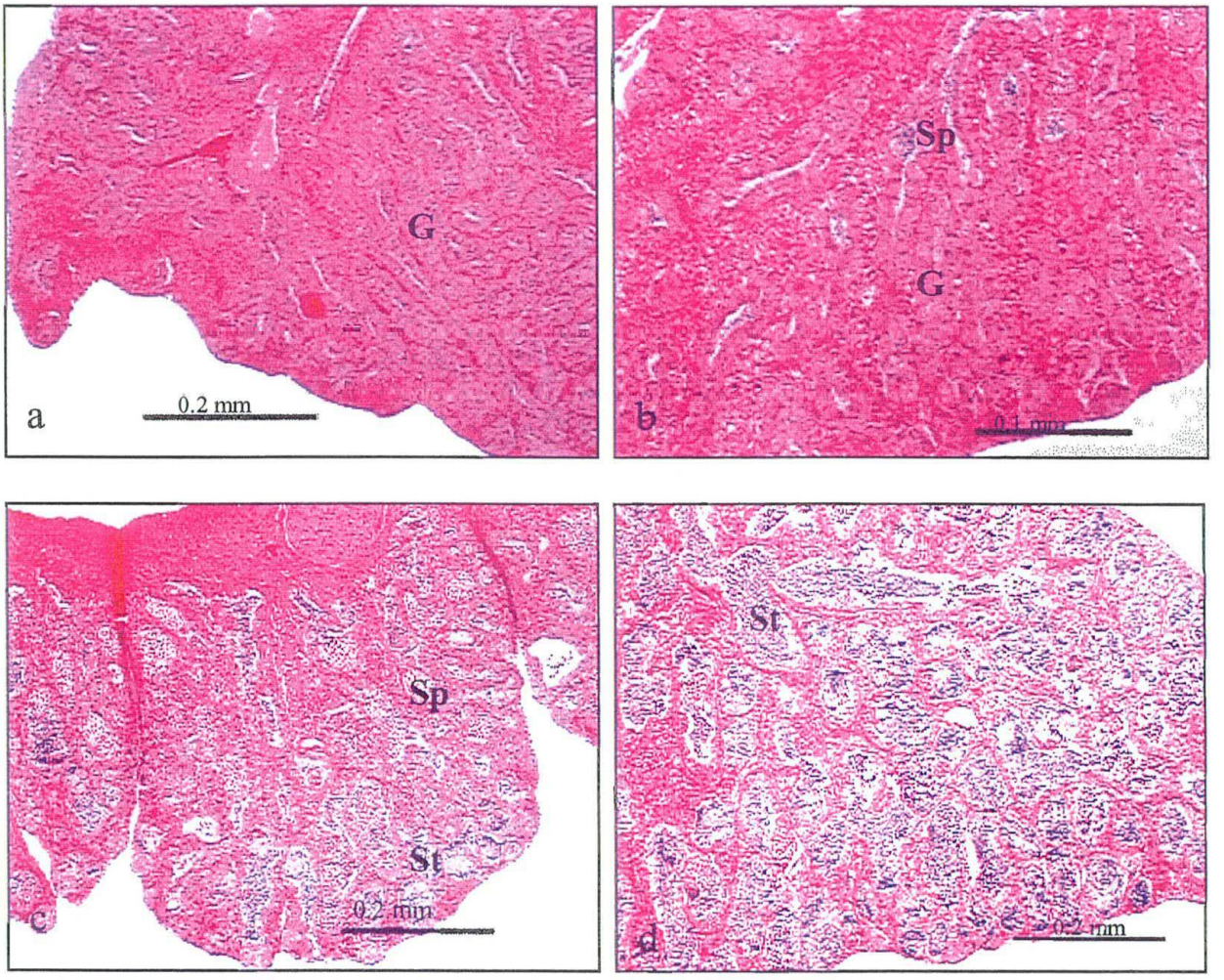
Figure 4.5 Transverse sections of gonads showing different testicular maturation stages.

(a) Stage 1a testes showing tightly packed gonia cells (G) without further development

(b) Stage1b testes showing gonia cells and the presence of spermatogonia (Sp)

(c) Stage 2 testes showing spermatogenesis stage. Sp - Spermatogonia; St – Spermatids

(d) Stage 3 testes showing relatively larger proportion of spermatids (St) than other tissues


G
a
0.2 mm
Sp
G
b
0.1 mm
Sp
St
c
0.2 mm
St
d
0.2 mm

Gonads classified as stage 2 and 3 (Fig. 4.5c,d) show continuous development of gametocytes. For example, fish with stage 2 gonads had gonia cells, different spermatocyte stages and a small amount of sprematids. Stage 3 gonads had gonia cells, spermatocytes and a high amount of spermatids.

4.3.10 Oestradiol-17β

Repeated measures ANOVA showed that there was a time effect on plasma E_2 concentrations ($F=2.99_{(3.3,149.5)}$; $P<0.05$) but dietary energy level had no significant effect (repeated measures ANOVA; $F=1.83_{(3,45)}$; $P>0.05$). There were no significant differences found between treatments at any time point (Table 4.9). E_2 levels at week 24 were generally lower than the previous time point in all treatments but significant decreases were found only for E18 and E24 groups.

4.3.11 Testosterone

Repeated measures ANOVA showed that plasma T level significantly decreased with time ($F=4.06_{(2.55,114.9)}$; $P<0.05$). With the exception of week 0, there was no significant effect of dietary energy level on plasma T concentration (ANOVA- $F=1.79_{(7.6,114.9)}$; $P>0.05$) (Table 4.10). Fish fed the E15 diet had a significantly higher initial T level than other groups, which led to a significant difference between initial T levels. However, the initial high T level decreased significantly at week 6 and remained stable thereafter in E15 group. Fish fed the E18 diet also had significantly decreased plasma T level at week 6, which decreased again at week 18. In both E15 and E18 groups, plasma T levels at week 6, 12, 18 and 24 were significantly lower than the initial levels. In E21 and E24 groups plasma T level declined during the experimental period. In E21 group, plasma T level at weeks 12, 18 and 24 were significantly lower than the initial level. The plasma T level at week 24 of fish fed on the high energy diet (E24) was significantly lower than the initial T level.

4.3.12 11keto Testosterone

Plasma 11kT level did not show any particular trend with time (Table 4.11) (Repeated measures ANOVA- $F = 2.02_{(4,112)}$; $P>0.05$). A significant interaction of dietary energy level and time on plasma 11kT level was observed (interaction between time and diet; Repeated measures ANOVA - $F = 3.819_{(12,112)}$; $P<0.05$). One

Table 4.9 Plasma Oestradiol-17β (E_2) concentrations of barramundi fed diets with different protein:energy ratios. (E15 = 50% Protein:15 MJ.kg^{-1}; E18 = 50% Protein:18 MJ.kg^{-1}; E21 = 50% Protein:21 MJ.kg^{-1}; E24 = 50% Protein:24 MJ.kg^{-1}). Means in the same column are not significantly different (P> 0.05). * indicates significant difference (P<0.05) with the preceding value; # indicates significant difference with the initial value (week 0).

Diet	n	Oestrdiol-17β (pg.ml^{-1})				
		Wk 0	Wk 6	Wk 12	Wk18	Wk24
E15	7	33 ± 5	21 ± 3	26 ± 2	28 ± 2	23 ± 2
E18	18	29 ± 2	24 ± 2	32 ± 2 *	28 ± 1	23 ± 2 *
E21	16	27 ± 3	30 ± 2	31 ± 2	29 ± 2	26 ± 2
E24	9	31 ± 2	30 ± 2	29 ± 2	33 ± 2	22 ± 2 *,#

Table 4.10 Plasma Testosterone (T) concentrations of barramundi fed diets with different protein:energy ratios. (E15 = 50% Protein:15 MJ.kg^{-1}; E18 = 50% Protein:18 MJ.kg^{-1}; E21 = 50% Protein:21 MJ.kg^{-1}; E24 = 50% Protein:24 MJ.kg^{-1}). Means with same superscript in the same column are not significantly different (P> 0.05). * indicates significant difference with the preceding value; # indicates significant difference with the initial value (week 0).

Diet	n	Testosterone (pg.ml^{-1})				
		Wk 0	Wk 6	Wk 12	Wk18	Wk24
E15	7	219 ± 9 ^{b}	111 ± 22 ^{a,*}	88 ± 12 ^{a}	100 ± 10 ^{a}	93 ± 9 ^{a}
E18	18	158 ± 14 ^{a}	97 ± 11 ^{a,*}	114 ± 10 $^{a,\#}$	81 ± 7 $^{a,*,\#}$	99 ± 9 $^{a,\#}$
E21	16	139 ± 13 ^{a}	120 ± 11 ^{a}	96 ± 7 $^{a,\#}$	84 ± 7 $^{a,\#}$	78 ± 8 $^{a,\#}$
E24	10	123 ± 19 ^{a}	115 ± 10 ^{a}	86 ± 5 ^{a,*}	75 ± 7 ^{a}	66 ± 6 $^{a,\#}$

Table 4.11 Plasma 11keto Testosterone (11kT) concentrations of barramundi fed diets with different protein:energy ratios over a 24 week period. (E15 = 50% Protein:15 $MJ.kg^{-1}$; E18 = 50% Protein:18 $MJ.kg^{-1}$; E21 = 50% Protein:21 $MJ.kg^{-1}$; E24 = 50% Protein:24 $MJ.kg^{-1}$). Means with different superscripts in the same column are significantly different ($P<0.05$). * indicates significant difference ($P<0.05$) with the preceding value; # indicates significant difference with the initial value (week 0).

Diet	n	11keto Testosterone ($pg.ml^{-1}$)				
		Wk 0	Wk 6	Wk 12	Wk 18	Wk 24
E15	7	475 ± 55^{a}	470 ± 64^{a}	328 ± 82^{a}	464 ± 85^{a}	425 ± 83^{a}
E18	9	251 ± 48^{a}	305 ± 43^{a}	$663 \pm 91^{b,*,\#}$	$438 \pm 46^{a,*,\#}$	$464 \pm 62^{a,\#}$
E21	8	363 ± 62^{a}	366 ± 50^{a}	439 ± 28^{ab}	443 ± 65^{a}	$305 \pm 68^{a,*}$
E24	10	361 ± 49^{a}	483 ± 47^{a}	$328 \pm 55^{b,*}$	471 ± 50^{a}	$301 \pm 58^{a,*}$

way ANOVA showed that this was due to 11kT levels at week 12 being significantly different between treatments, with the E18 group having the highest 11kT concentration. However, 11kT level did not show any significant differences between treatments at any other time point.

No effect of time on plasma 11kT concentration was detected in the E15 treatment during the 24 week period. In the E18 treatment, 11kT level increased significantly at week 12 and then decreased significantly at week 18 and remained stable. In this group, 11kT level at weeks 12, 18 and 24 were significantly higher than the initial level. In the E21 group, a significantly lower 11kT level was found at week 24, however none of the 11kT levels were significantly different from the initial value. In the E24 group, 11kT levels at weeks 12 and 24 were significantly lower than those of the preceding time point.

4.4 DISCUSSION

Limitations of this study

Some difficulties, which are likely to have affected the results, were experienced in this study. Firstly, it was very difficult to get fish to eat the experimental diets at the beginning of the experiment. For example the lowest energy diet (E15) was very hard and the high energy diets were very oily and odorous. Consequently, the pellets were varied in texture, hardness and smell which was related to the energy content of diet, which means that any effect due to those characters could not be eliminated.

Secondly, some uncontrollable disturbances were experienced during the study period which appeared to affect the food consumption or feeding behaviour of fish. For example, during week 10, fish were fed by another person and food given to the tanks was very low which resulted in low food consumption during that period. System water changes (fresh water) were done as a preventive method to white spot disease during week 15 and 16 which could also affected the feeding habits of the fish. During week 18, the low ambient temperatures (25 ^{0}C) meant it was difficult to maintain the system temperature. Sampling was programmed during this time and both these factors probably affected the food consumption of fish.

The acclimation period observed in this study was much longer than in the previous study (chapter 3). In the previous study (chapter 3) fish were fed the commercial barramundi diet before the start of the experiment and the same feed was used in the study; only the feeding regime was changed during the experimental period. In the present study, the experimental diets were very different to the commercial diet in terms of hardness, texture and smell with associated palatability problems. Generally, low food consumption, growth and SGR were observed during the first 6-12 weeks and it can be assumed this was the acclimation period to the experimental diets. Fish appeared to be adjusted to the new diets after 12 weeks and this can be clearly seen from food consumption data, especially in groups of fish fed low energy diets. High food consumption during the last 12 weeks in the E15 treatment, in spite of the hardness of pellets indicates that fish adapted to these variations.

Food consumption was measured as feed offered to the fish but not the actual ingestion of fish. It was difficult to measure accurate food consumption, as large tanks meant any uneaten food could not be collected from the system. This may have resulted in lower than actual values for FCE and PER values. This is a common problem in studies of this type. Regost *et al.* (2001) reported that although similar FCE was observed in brown trout fed diets with different fat levels, it was probably due to food losses as it was difficult to record accurate feed intake in cage culture conditions.

Feeding habits of individual fish in tanks also appeared to vary with hierarchies present within the same tank. While some fish were feeding well and maintaining good growth, other fish in the same tank grew little or lost weight. That growth can be affected by social interactions between individuals has also been described for Arctic Charr (Jobling, 1983). Although food offered was not a limiting factor, feeding response and presumably growth of certain fish can be inhibited by the presence of larger individuals (Jobling, 1983).

Growth

Barramundi reared on different protein:energy ratios showed comparable growth at the end of the experiment while showing different food consumption, feed efficiency and protein efficiency patterns. Fish fed the low energy levels tended to eat more food, while fish fed the high energy levels consumed less food but improved their FCE and PER. However, energy consumption seems to be comparable between treatments. Body composition analysis showed that fish tended to store more fat with increasing dietary lipid level which is evidenced by high muscle and liver lipid levels and visceral fat content. Although fish had a low SGR to week 12, probably due to adaptation to the new diets during this period, SGR between weeks 12 and 24 were comparable to the values (0.20 – 0.53) found in the previous chapter (3).

Fish tended to increase their food consumption with decreasing dietary energy level. Although this trend is not clear in the first 12 weeks, the food consumption data for the last 6 weeks showed that the fish in the E15 treatment consumed 0.95 % Bwt.day^{-1} while the fish in the E24 treatment consumed only 0.74 % Bwt.day^{-1}. The ability of fish to compensate for low food quality or quantity by eating more food

(hyperphagic) seems to be common. For example, sunshine bass fry fed a low energy diet had significantly higher feed intake and protein intake, while energy intake was not different to fish fed the high energy diets (Keembiyehetti and Wilson, 1998). Bream fingerlings fed low energy levels also showed a better feeding rate than fish fed on high energy levels (Haiqing and Xiqin, 1994). Male Arctic charr fed on restricted feeding levels eat more food per meal than fish on a regular feeding level (Jobling, 1983). Similar results were observed in the previous chapter. Barramundi fed less frequently (once in 3 days or 7 days) consumed more food per meal than fish fed daily.

Slow gastrointestinal passage time with energy dense diets (Jobling, 1983; De Silva and Anderson, 1995) may be associated with the reduced food consumption of barramundi fed the high energy diets, as seen in other teleosts (Keembiyehetti and Wilson, 1998). In some other teleosts, for eg: salmon species, it has been observed that high body lipid contents also have an inhibitory effect on appetite and therefore reduce consumption (Silverstein *et al.*, 1999; Shearer and Swanson, 2000).

Feed conversion and protein efficiency improved with increasing dietary energy level in the present study. It is likely that fish fed the E15 diet consumed more food to maintain their energy requirement. Consequently, they utilised protein to meet their energy requirements which results in low (poor) FCE and PER. Although the high energy group consumed less food, they probably used non-protein energy sources for energy requirements. Protein can then be used for growth resulting in improved FCE and PER. Catacutan and Coloso (1995) also reported that juvenile barramundi (1.5 g) fed diets with constant protein (50%) and 15% lipid showed better growth, PER and FCE than those fed 5% or 10% lipid diets. This trend of increasing PER and FCE with increasing dietary energy level appears to common in teleosts. For example, increasing dietary energy from 11.72 $MJ.kg^{-1}$ to 16.74 $MJ.kg^{-1}$ significantly improved FCE and PER but low weight gain was observed in the high energy group compared to the low energy group in sunshine bass (Keembiyehetti and Wilson, 1998). In another study, Dias *et al.* (1998) observed that an increase in dietary energy led to an overall improvement in growth performance in European sea bass as evidenced by better growth and higher food conversion and protein efficiency. El-Dahhar and

Lovell (1995) in *O. mossambicus* and Hillestad *et al.* (1998) in Atlantic salmon also shown that PER increased with increasing dietary energy level.

Barramundi receiving low energy diets consumed more food to maintain their energy requirements and consequently all fish had comparable body weight/growth at the end of the experimental period. Similar trends have been observed in other teleosts. Male Arctic charr reared at different food quality levels (high, medium protein using inclusion of carbohydrate) but fed to satiety also showed similar growth rates (Svedang, 1991). Brown trout fry fed on different rations had similar growth but FCE was lower in high rations (Arzel *et al.*, 1998). Yellow perch juveniles reared on diets with constant protein and varying energy levels also showed similar weight gains (Ramseyer and Garling, 1998). However yellow perch were fed on constant ration (3%) and it was not clear whether fish consumed all the feed provided or not. These findings indicate that animals eat to satisfy their energy requirements (De Silva and Anderson, 1995) by showing varying food consumption, and changed FCE and PER, and consequently they can show comparable growth rates.

The optimum protein:energy ratio observed for growth for barramundi in this study was 27.2 mg protein.kJ^{-1}. The optimal protein:energy ratios reported for finfish range from 17- 28.7 mg protein.kJ^{-1} for different species (De Silva and Anderson, 1995). However, optimal P:E ratios for food conversion and protein retention for barramundi in this study were 17.6 and 23.6 mg protein.kJ^{-1} respectively. The optimal P:E ratios will vary significantly between species and according to the digestibility and amino acid composition of the protein source (De Silva and Anderson, 1995).

Although the dietary energy level did not significantly affect the growth of fish or muscle weight, visceral fat content was significantly affected by dietary energy level in the present study. Fish fed the highest energy diet (E24) had the highest visceral fat content and the lowest dietary energy group (E15) had the lowest visceral fat content. HSI also tended to increase with dietary lipid level. It seems apparent that barramundi store excess energy in visceral and liver tissue. Increasing visceral fat content and liver weight with increasing dietary energy level has also been observed in other teleosts including juvenile striped sea bass (Nematipour *et al.*, 1992), sunshine bass (Keembiyehetti and Wilson, 1998) and brown trout (Regost *et al.*, 2001).

Proximate composition

Proximate composition analysis revealed that muscle and liver total lipid levels increased with increasing dietary energy level or decreasing dietary P:E ratio. This is consistent with the findings of other teleosts that body lipid content is directly related to dietary energy intake (Sargent *et al.*, 1989; Nematipour *et al.*, 1992; review Shearer, 1994; Dias *et al.*, 1998; Keembiyehetti and Wilson, 1998; Regost *et al.*, 2001).

There was a general tendency for decreased muscle moisture, dry matter and protein with increasing dietary energy from 15MJ.kg^{-1} to 21MJ.kg^{-1} in the present study. Similar results were reported for juvenile barramundi (Catacutun and Coloso, 1995). However, in some teleosts, whole body but not muscle composition was affected by dietary P:E ratio. Whole body protein level decreased while whole body lipid and dry matter increased with dietary energy level in hybrid striped sea bass (Nematipour *et al.*, 1992) and in red drum (Daniels and Robinson, 1986). This is not a universal observation, however, as some teleost species showed no differences in whole body compositions in relation to dietary P:E ratio (Haiqing and Xiqin, 1994; Dias *et al.*, 1998; Ramseyer and Garling, 1998).

There appears to be a shift of type of energy stores with increasing dietary energy level. Muscle carbohydrate (calculated) increased with decreasing dietary lipid level which is evidenced by fish fed the low energy (E15) diet having the lowest lipid but the highest carbohydrate content in tissues. In contrast, fish fed the highest energy diet showed high lipid contents but low carbohydrate contents in tissues.

Although total muscle energy content did not show any significant differences between treatments, proportional energy content as MJ.kg of tissue^{-1} showed that fish fed on the highest energy diet had high energy content per unit of muscle tissue. Percent muscle lipid and ash also increased with dietary energy level and % carbohydrate decreased with the dietary energy level in this study.

Dietary energy level significantly affected the liver composition. Total liver dry matter, lipid and energy significantly increased with dietary energy level. The fish in the highest energy group (E24) had the highest dry matter, lipid and energy content compared with those in the other three groups. Although the amount of liver nutrient

is relatively small compared to the muscle nutrient content, it may significantly contribute to the gonadal maturation as the liver plays an important role in distributing nutrients to other tissues (Shelkh-Eldin *et al.* 1996). In contrast, liver lipid or moisture content is not affected by dietary energy levels in some teleosts (Regost *et al.*, 2001).

Percentage composition data showed that liver moisture and protein decreased and liver lipid and energy increased with dietary energy level. However, a comparison of total nutrients shows that this was due to the change in lipid content with dietary energy level while protein and moisture content did not change significantly. This is an example where percentage (%) composition data must be interpreted carefully as it does not compare the total amount of tissue but changes in the proportion of lipid which then result in changes in the proportion of other components.

Effect on reproduction

Although GSI increased with dietary energy level, it is difficult to draw conclusions from this data due to the very small gonad sizes. Gonads were very thin strap like structures and it is difficult to separate fat bodies from the gonad making GSI difficult to determine accurately.

Gonadal histological analysis confirmed that all fish were male at the end of the experimental period. Gonads of fish fed the low energy diet (E15) were at an early developmental stage (M1) with tightly packed gonia cells. These gonads did not show evidence of any further development as observed in other treatments. However, fish in other treatments had maturing gonads which showed continuous development of gonia cells and appearance of spermatocytes and spermatids.

Histological observations of the gonads of five individuals at week 0 revealed two fish at stage 4 (M4) and one of each at stage 2 (M2), 3 (M3) and immature and it can be assumed that fish were undergoing natural gonadal developmental cycles before starting the experiments. Suppressed gonadal development (stage 1a – gonad with gonia cells without further development) observed in animals fed the lowest energy diet (E15) may be due to the fish turning off reproduction with either low dietary energy or lipid. It is known that energy is required for maintenance, growth and reproduction of animals (De Silva and Anderson, 1995). The energy requirements of basal metabolism are first to be satisfied and the remaining energy can then be

devoted to other purposes. Fish fed the low energy diet will utilise protein as their energy source leaving a shortage of nutrients for growth and storage. This is supported by the fact that these fish had low muscle and liver lipid levels and visceral fat index. However, these fish had increased their total energy intake to match that of fish feeding a higher energy diets. Thus, an alternative explanation is that the reduced level of gonadal development may be due to an insufficient lipid supply. In either case, from this observation it can be hypothesised, that the energy stored or consumed as lipid may be an important factor for inducing gonadal maturation.

It has been observed in other teleosts that body lipid levels influenced the incidence of maturation. Shearer and Swanson (2000) found that higher body fat levels one-year prior to maturation were associated with higher rates of male maturity in chinook salmon. Body fat levels also seem to have an influence on male maturation in Atlantic salmon. Total lipid started to increase in maturing fish at an earlier stage than in non-maturing fish and maturing fish had significantly more mesenteric fat than non-maturing fish (Rowe *et al.*, 1991). It was proposed that fat stores affect the induction of gonadal development in salmon (Rowe and Thorpe, 1990b). In the present study, barramundi fed the high energy diets had higher visceral fat content, muscle and lipid content than fish fed the low energy diets and also had more advanced gonadal stages. This suggests there may be a similar role of fat stores in maturation of barramundi as that suggested for salmon by Rowe and Thorpe (1990b).

Dietary energy level did not significantly affect the plasma hormone levels in male barramundi in the present study. The changes in plasma E_2 level are relatively small and did not show differences between treatments. The plasma E_2 levels are comparable to the values found in the previous experiment (range 18 – 40 $pg.ml^{-1}$; Chapter 3) suggesting that the levels observed are the minimum range of E_2 maintained in male barramundi plasma. Low plasma E_2 values in male barramundi compared to those of females were reported previously by Guiguen *et al.* (1993). However, the E_2 levels (25 – 100 $pg.ml^{-1}$) reported bt Guiguen *et al.* (1993) are somewhat different to those from this present study probably due to the limited sensitivity of the assay used by those authors.

Initial plasma T levels were highly variable within individual fish. This is probably due to natural variation of plasma hormone levels associated with varying

gonadal development stages. Similar findings were observed for barramundi in this laboratory (T level ranged from 46 – 510 $pg.ml^{-1}$) (Anderson, pers comm.). Guigen *et al.* (1995) also reported that plasma T level was highly variable in male barramundi. The physiological reason for the observed decrease in plasma T level in all groups with time is not clear. However, as gonad sizes were comparatively small at the end of the study, it seems likely that low T concentration is related to the small gonad sizes.

Plasma 11kT levels fluctuated in barramundi in the present study and showed no clear trend. Guigen *et al.* (1994) also reported that plasma 11kT fluctuated during the reproductive cycle but the differences were not associated with different testicular maturation stages. This is due to the ongoing gametogenetic activity of male sea bass. Barramundi in the present study seem to have continuous gametogenic development and therefore these findings are consistent with the results of Guigen *et al.* (1994). High levels of variation of plasma 11kT level in individuals was also reported in other teleosts (Cuisset *et al.*, 1994). However, the changes in plasma 11kT level in relation to dietary energy level in the present study is not clear. Gonad stages were determined only at the end of the experiment and there is no information about the gonad development stages of the fish during the 6 month period. Again it is likely that 11kT concentrations vary as a function of the continuous development of gametocytes observed here and by others (Guigen *et al.*, 1994).

In the present study it is very clear that gonads of fish fed the lowest energy diet (E15) had suppressed gonad development indicating that this diet was inadequate to support reproduction. Although the fish fed the other three energy levels (E18, E21 and E24) showed continuous development of gametocytes, these fish had very small gonads. It appeared that fish had directed most of their energy into somatic growth. This is evidenced by comparatively high SGR values observed. Although these fish had very small gonads they had not completely switched off reproduction with some ongoing gamogenetic activity occurring.

Therefore it seems that either protein:energy ratios and/or protein and energy levels used in the present study did not influence reproductive activity in these fish. As barramundi is a carnivorous fish and trash fish is used to feed broodstock, a high protein level (50%) was maintained in these diets. The aim of this study was to investigate the effect of dietary protein:energy level, thus constant protein and

different energy levels used. It has been shown for other teleosts that an optimum protein level, as well as optimum energy level is required for maximum growth of fish. In addition protein:energy ratio is also important in partitioning energy and utilising energy efficiently, as imbalance in ratio will waste the nutrients. However, that fish appeared to switch from reproduction to growth is difficult to explain.

Although the physiological mechanisms by which maturity is triggered are not clear (Silverstein *et al.*, 1999), some studies suggest that a threshold size, growth rate or energy storage must be surpassed during critical periods of gonad development to ensure maturation (Rowe and Thorpe, 1990b; Silverstein *et al.*, 1994; 1999; Shearer and Swanson, 2000). If growth in certain periods of the year (spring) is inadequate, maturation is switched off physiologically in male Atlantic salmon (Rowe and Thorpe, 1990b) as fish cannot acquire sufficient reserves for spawning. Rowe *et al.* (1991) also proposed a physiological model for the control of maturation in male Atlantic salmon. They observed that prematuration peaks of cortisol, T and oestradiol coincide with the growth of fat reserves in maturing Atlantic salmon. A major site of aromatisation in addition to brain may be fat stores. Thus, fat levels exert control over maturation by limiting the extent of aromatase activity and hence the production of oestrogens needed to stimulate production of the appropriate GtH.

In the present study, the barramundi fed the highest energy diet had apparently ample fat storage. Thus either the model of Rowe *et al.* (1991) does not fit this data or there are some other conditions required. In this present study, fish were in artificial conditions (high temperature, salinity and constant photoperiod) but in the study of Rowe *et al.* (1991) fish were in their natural conditions with changing photoperiods, temperature, feeding cycles etc., and displayed a natural lipid/ energy mobilisation patterns during the reproductive cycle. In the present study fish were held in spawning conditions for 6 months, which differs from their natural habitat. However, these conditions have resulted in animals becoming mature in our laboratory and elsewhere (T. Anderson, personal communication). Constant photoperiod in commercial hatcheries results in animals mantaining spawning condition and being able to be spawned at least once per month (QDPI, personal observation). However, no information about the effect of this practice on sex hormones is available.

The continuous gonadal developmental cycles of barramundi in captivity as noted in this and the previous chapter (chapter 3) make interpretation of histological observations and effects of P:E ratios difficult to interpret. It seems as in the availability of food described in chapter 3 that extreme cases of high P:E ratio or low energy diets inhibit gonadal maturation. Further conclusions of the effect of protein and energy on gonadal development are difficult to make.

CHAPTER 5

EFFECT OF DIETARY FATTY ACIDS ON GROWTH AND PLASMA SEX STEROID HORMONES OF MALE BARRAMUNDI.

5.1 INTRODUCTION

Animals mainly require dietary lipids for two purposes: for metabolic energy and for incorporation into polar lipids which are required for the formation of cell membranes (Sargent *et al.*, 1993). Body lipids stored in adipose tissue are mainly composed of triglycerols and are utilised as a source of fatty acids to provide energy. The lipids found in cell membranes are mainly composed of polar lipids and cholesterol, and are much more stable than adipose tissue, reflecting its critical role in maintaining cell structure and cell function (Sargent *et al.*, 1993). Liver is the main site of fatty acid synthesis in teleosts (Henderson and Sargent, 1985).

Although unable to synthesise n-3 and n-6 fatty acids *de novo*, it is generally thought that fresh water fish are capable of converting C18 polyunsaturated fatty acids (PUFA) of both n-3 and n-6 series to their highly unsaturated homologous (longer and biologically active) fatty acids (C20 and C22) (March, 1993). For example, 18:2 n-6 (Linoleic acid) can be elongated to 20:4 n-6 (Arachidonic acid; AA) or 22:5 n-6, and 18:3 n-3 (Linolenic acid) can be elongated to 20:5 n-3 (Eicosapentaenoic acid; EPA) and 22:6 n-3 (Docosahexaenoic acid; DHA). Marine fish however, are thought to be incapable of these conversions, and therefore require preformed highly unsaturated fatty acids (HUFA). This is due to their lack of delta-5 desaturase, which is responsible for the conversion of C18 into HUFA (Sargent *et al.*, 1993; Kanazawa, 1985b).

In fish, DHA (22:6 n-3), EPA (20:5 n-3) and AA (20:4 n-6) are all involved in maintaining cell membrane structure and function (Sargent *et al.*, 1999), with DHA and EPA being present at higher concentrations than AA in tissues (Sargent *et al.*, 1997; 1999). The high n-3 HUFA contents in tissue lead to a higher dietary requirement for n-3 than n-6 HUFA by fish. As a result, n-3 HUFA has received much more emphasis than n-6 HUFA in fish nutrition studies in the last few decades (Bell *et al.*, 1995; Sargent *et al.*, 1999).

Several studies have shown that marine finfish require n-3 HUFA such as EPA (20:5 n-3) and DHA (22:6 n-3) for normal growth. Navarro *et al.* (1988) showed that a deficiency of dietary n-3 HUFA resulted in low survival in sea bass larvae, while Furuita *et al.* (1999) suggested that diets rich in n-3 HUFA increased growth and

survival of larvae in Japanese flounder. High incorporation of EPA (20:5 n-3) into the gall bladder, swim bladder, liver and the pyloric caeca were observed in larval fish (reviewed by Kanazawa, 1985b) suggesting that EPA (20:5 n-3) is a constituent of cellular membranes of these tissues. DHA (22:6 n-3) which was found in high levels (29 - 37%) in juvenile and young skipjack tuna (Tanabe *et al.*, 1999), is conserved during starvation in *Dentex dentea* larvae (Mourente *et al.*, 1999). As high levels of DHA (22:6 n-3) are found in the retina and the brain of teleosts (Castell, 1979), it appears to be crucial for visual and neural system function in embryonic and larval stages (Sargent, 1995). It has also been shown that AA (20:4 n-6) is essential in the growth and development of fish larvae (Bell *et al.*, 1997; Furuita *et al.*, 1998) and is an important constituent in eggs and larval tissues (Furutia *et al.*, 2000). Chou and Shiau (1999) have revealed that both n-3 and n-6 fatty acids are required for maximum growth of juvenile hybrid tilapia. Furthermore, several studies have demonstrated the importance of PUFA in the embryonic and early development of larvae in many teleosts (Fernandez-Palacios *et al.*, 1995; 1997; Gallacher *et al.*, 1998).

In addition to a role in regulating membrane permeability (March, 1993), a major function of the essential fatty acids present in the phospholipids of cell membranes is as a source of the free HUFA. These HUFA are immediate precursors of eicosanoids which are biologically active compounds in animals (Lands *et al.*, 1977). Eicosanoids are responsible for regulating a wide range of physiological functions, including osmo- regulation, cardiovascular function, neural control and functioning of reproductive systems (Sargent *et al.*, 1993).

Prostaglandins, one of the eicosanoid compounds, play an important role in vertebrate reproduction (Stacey and Gotez, 1982). Prostaglandins are not stored preformed but as precursors. These precursors are incorporated in membrane lipids and are reported to be present in testes, ovaries and blood in fish (Stacey and Goetz, 1982). Prostaglandins which are synthesised from the three principle precursors, ETA (20:3 n-6), AA (20:4 n-6) and EPA (20:5 n-3), are known as PG1, PG2 and PG3 respectively. The ETA (20:3 n-6) and AA (20:4 n-6) have the optimum chain length and double bond position from the methyl end for formation of eicosanoids (Lands *et al.*, 1977). In teleosts, it has been shown that AA (20:4 n-6) is a better precursor for prostaglandins than EPA (20:5 n-3) (Henderson *et al.*, 1985). PG2 is biologically

more active than PG1 or PG3 (Lands *et al.*, 1977). Nevertheless, EPA (20:5 n-3) competitively inhibits the formation of eicosanoids from AA (20:4 n-6). In addition, eicosanoids derived from EPA (20:5 n-3) competitively interfere with the action of eicosanoids formed from AA (20:4 n-6) (Sargent *et al.*, 1999). Consequently, EPA (20:5 n-3) and PG3 act as inhibitors for production of PG2 from AA (Lands *et al.*, 1977; Henderson *et al.*, 1985; Wade *et al.*, 1994). Therefore, all these fatty acids are important in regulating prostaglandin production. Prostaglandins have been shown to stimulate GtH secretion, follicular rupture and ovulation in fish, and to induce female spawning behaviour and to act as a pheromone (Lam, 1982; Stacey and Gotez, 1982; Villars *et al.*, 1985).

In addition to their role as a source of energy, dietary lipids are believed to be important determinants for gonadal development, and consequently, influence spawning performance of broodstock. Lipid depletion in tissues during gonad development has been observed in many teleosts (Cerda *et al.*, 1995; Singh and Singh, 1990; Lund *et al.*, 2000). Various studies have shown that n-3 deficient broodstock diets result in lower fecundity, especially low larval viability and survival in a variety of species (Watanabe *et al.*, 1984; Kanazawa, 1985a,b; Fernandez-Palacios *et al.*, 1995; Navas *et al.*, 1995; 1997; 1998; Rodriguez *et al.*, 1998; Bruce *et al.*, 1999; Furuita *et al.*, 2000). The production of vitellogenin, a precursor of oocyte yolk protein, also requires a supply of long chain fatty acids (March, 1993). Furthermore, since steroidogenesis is dependant on the adequate supply of cholesterol, lipids can influence the hormonal function of the gonads, (Nagahama, 1983).

Dietary lipids and fatty acids have received much attention in teleost broodstock nutrition studies in last few decades. Interestingly, most studies were focussed on female broodstock performance and/or egg and larval quality and development. As a result, less research has been done on the effects of dietary lipids, particularly fatty acids, on male broodstock performance or fertility. It is only very recently that attention has been given to male broodstock development. Recent studies showed the importance of AA (20:4 n-6) in male broodstock nutrition. Bell *et al.* (1996) reported that the fatty acids found in the spermatozoa of wild European sea bass (*Dicentrachus labrax*) and the broodstock fed 'trash fish' had higher levels of AA than those from the broodstock fed a commercial diet containing vegetable oil.

Although these authors did not observe spawning performance of male broodstock, they suggested that eicosanoid metabolism, and consequently reproductive function, could be affected by altered fatty acid levels.

Asturiano *et al.* (2001) compared the reproductive performance of male *D. labrax* fed on wet diet (trash fish) with those of fish fed two commercial diets enriched with PUFA. This wet diet was previously shown to be the most beneficial for female reproductive performance. Compared to the male fish fed a wet diet, animals fed HUFA enriched commercial diets displayed longer spermiating periods, higher total milt volumes and spermatozoa densities, and higher % of spermiating males. Although initial fertilisation rates were not different between groups, significantly higher % survival rates of larvae were observed with eggs fertilised with milt from fish fed commercial diets. These authors suggested that a reduction in n-3 HUFA and an increase in AA, which resulted in an altered n-3 to n-6 ratio, may improve male reproductive performance in *D. labrax*. Although the high EPA and n-3 to n-6 ratio in the wet diet might have resulted in a better reproductive performance in females, Asturiano *et al.* (2001) suggested that dietary PUFA requirements of male fish may be different.

Effect of fatty acids on steroids

There is some evidence that the dietary fatty acid profile altered the plasma sex steroid levels in some teleosts. Navas *et al.* (1998) found that female *D. labrax* broodstock fed on formulated diets, with relatively low levels of AA (20:4 n-6), DHA:EPA and AA:EPA compared to a natural diet, produced eggs with low viability and hatchability. Plasma E_2 and GtH II levels were also different in these experimental groups compared to broodstock fed the natural diet, which produced good quality larvae with high viability and hatching rates. In a separate study, Cerda *et al.* (1995) had previously demonstrated that sea bass broodstock fed n-3 deficient diets had reduced fecundity and viability, together with lower levels of T and E_2. These fish also showed altered patterns of plasma lipids and increased presence of atretic oocytes when compared to the females fed a natural diet. In a more recent study, Acharia *et al.* (2000) reported that female cat fish (*Clarias batrarchus*) broodstock fed diets enriched with 18:3 n-3 had elevated T and E2 levels together with increased GSI. Fish fed the 18:2 n-6 diet however, showed the low levels of plasma T and E_2 associated

with decreased GSI. These studies demonstrate that dietary fatty acid composition may affect the reproductive performance by altering sex hormone levels.

In vitro studies have shown that fatty acids are involved in modulating gonadal steroidogenesis in teleosts. Van der Kraak and Chang (1990) demonstrated that AA (20:4 n-6) stimulates T production in preovulatory goldfish ovarian follicles. Differential actions of PUFAs on basal and GtH stimulated T production have also been demonstrated in gold fish and in rainbow trout ovarian follicles (Van Der kraak, 1990; Mercure and Van Der Kraak, 1995). In both species, AA (20:4 n-6) stimulated T production, but EPA (20:5 n-3) and DHA (22:6 n-3) inhibited T production. These effects are also observed in testis. Wade and Van der Kraak (1993) and Wade *et al.* (1994) demonstrated that AA (20:4 n-6) stimulate T production in the gold fish testis, whereas n-3 fatty acids, particularly EPA (20:5 n-3), may function as inhibitory regulators of steroid production in this tissue. These studies also indicated that AA stimulates T production through conversion to prostaglandins.

From this data, it is apparent that dietary fatty acids play an important role in regulation of reproductive function. Therefore, the objective of this study was to investigate the effect of different dietary fatty acids on growth, and plasma hormone levels which is expected to be a measure of gonadal development, in male barramundi.

5.2 MATERIALS AND METHODS

5.2.1 Fish and experimental conditions:

The barramundi used in this study were obtained from a commercial farm (Bluewater Barramundi, Mourilyan Harbour, Queensland, Australia) in December 1999 and were held in eight 3500 L tanks according to the conditions described in section 2.2.

During the acclimation period, white spot disease was observed in the system twice (in early January and February 2000), and some fish died as a result. As a treatment for white spot disease, fish were transferred to fresh water, where they were kept for at least one week each time. During that period, fish were also treated with 50 ppm formalin for one hour every two days for four days. Due to this infection, fish were kept in the system for 10 weeks before commencement of the experiment. During the experimental period, white spot disease occurred in the system during week 6, and fish were held in fresh water for five days.

5.2.2 Experimental diets:

Using varying fatty acid sources, four experimental diets were formulated (Table 5.1) to be isonitrogenous (50% protein) and isocalorific (21 $MJ.kg^{-1}$). In the previous experiment, dietary energy of 24 $MJ.kg^{-1}$ gave the highest GSI values. However, difficulties in formulating a diet with 24 $MJ.kg^{-1}$ energy level using the arachidonic acid source led to 21$MJ.kg^{-1}$ dietary energy level being used for the final formulation. AquaGrow-AA contained some protein so the proportion of fishmeal and casein in this diet was reduced accordingly.

Flaxseed oil (Linseed oil) (Biogenic Health Foods, Australia), soybean oil (Meadow Lea Foods Ltd, NSW, 2020, Australia), fish oil (Ridley's Agriproducts, Brisbane, Qld, Australia) and AquaGrow-AA (Martek Biosciences Coporation, Columbia, Maryland, USA) were used as different fatty acid sources. These oils provided high amounts of short chain n-3, short chain n-6, long chain n-3 and long chain n-6 fatty acid respectively. Olive oil was used to achieve the desired energy level in the AA diet. Throughout the chapter, the diets/treatments will be referred to as LIN, SOY, FISH and AA, according to their respective fatty acid source.

Table 5.1 Composition ($g.100g^{-1}$) and proximate analysis of experimental diets with varying sources of fatty acids. (LIN = Linseed oil; SOY = Soybean oil; FISH = Fish oil and AA = Arachidonic acid).

	Diet			
Ingredient	LIN	SOY	FISH	AA
Fishmeal [a]	42.0	42.0	42.0	40.6
Casein [b]	18.0	18.0	18.0	17.4
Corn starch [c]	3.5	3.5	3.5	1.0
Fatty acid source [d]	20.0	20.0	20.0	23.4
Olive oil [e]	--	--	--	0.8
Vitamin mixture [f]	3.0	3.0	3.0	3.0
Mineral mixture [g]	0.5	0.5	0.5	0.5
Cellulose [h]	10.0	10.0	10.0	3.1
Gelatine [i]	3.0	3.0	3.0	3.0
Estimated				
Protein ($g.100g^{-1}$)	50.2	50.2	50.2	50.6
Digestible Energy ($MJ.kg^{-1}$)	21.0	21.0	21.0	21.2
Measured				
Crude protein ($g.100g^{-1}$)	48.66	49.23	49.13	48.61
Crude lipid ($g.100g^{-1}$)	19.89	23.25	20.12	19.06

a- Fishmeal –(Ridleys Aqua Products, Brisbane, QLD, Australia)
b- Casein –(Malanda, Australia)
c- Corn starch – (Anglosouce, QLD, Australia)
d- Linseed oil, Soybean oil, Fish oil and AquaGrow-AA were used as fatty acid source in LIN, SOY, FISH and AA diets respectively.
e- Olive oil- (Meadow Lea foods Ltd, Australia)
f- Vitamin mixture- (Rabor custom mix, DPI northern fisheries, Australia)
g- Mineral mixture- (DPI ARI Fish mineral premix, Rhone-Poulenc, Animal Nutrient Pty. Ltd., Queensland, Australia)
h- Cellulose – (Hahnflock, Hahn & Co, Germany)
i- Gelatine- (Farmhouse Kitchen foods Pty. Ltd, QLD 4073, Australia)

The diets were prepared in the laboratory at James Cook University, Australia. Corn starch was autoclaved at 121°C for 30 minutes to improve digestibility prior to mixing with ingredients. All dry ingredients were mixed for ~30 min using a Hobart mixer (Hobart corporation, Troy, Ohio, USA) before adding the oil. Water was added (~40%) to the homogeneous mixture to allow pelleting. The mixture extruded through a Hobart #12 chopper attachment with 3/8 inch die hole diameter. The pellets were then dried at 50 °C for ~ 14 h and stored at –20 °C in sealed plastic bags until use.

Pellets were of a smaller size (~5mm) than the commercial barramundi pellets on which the fish were fed prior to the start of the experiment. Experimental diets were generally similar in texture and in hardness (easily broken into pieces), and fish adapted easily to the experimental diets.

5.2.3 Experimental design

Following acclimatisation, six fish, tagged using colour T-bar tags for easy identification, were assigned to each tank. Fish were held at a constant temperature and photoperiod (section 2.1.1), similar to their spawning environment. A randomised block design was used in this experiment. Four tanks in each of two blocks were assigned to one of four diets, and fish were fed the respective diet manually once a day in the morning (0800 - 1000 hr) to satiation. The amount of food given to each tank (depending on fish response) was recorded daily throughout the experiment and this was considered the amount of food consumed for each treatment. The experiment was terminated at 18 weeks.

Ammonia, nitrate and nitrite levels were measured using standard test kits (Aquarium phamaceuticals, Inc. USA) and were maintained at less than 1.0 $mg.l^{-1}$.

5.2.4 Sampling

At six weekly intervals, fish were anaesthetised, and total length (cm) and weight (g) recorded (section 2.1.4). A 5 ml blood sample, taken from caudal vasculature, was transferred to fluoride-oxalate tubes to prevent clotting, mixed well and kept on ice for up to 2 h. Blood was centrifuged at 14,000 g for 10 min, and plasma decanted into 1.5 ml microtubes and stored at –80 ^{0}C until assay (section 2.1.5). At the end of the experimental period, all fish were anaesthetised and killed,

and gonads, liver and muscle samples were collected and weighed as described in section 2.1.7. Samples of muscle and liver were taken for proximate analysis. Gonads were fixed in FACC for histological analysis (Section 2.1.7). Five μm sections were stained with haematoxylin and eosin and examined using light microscope (section 2.5).

Growth, % BWG, SGR, HSI and GSI were determined using formulae described in section 2.2. Food consumption (%) and FCE were also determined as in section 2.2. Proximate composition of diets was performed as described in section 2.3. Plasma samples were analysed for T, E_2 and 11 kT according to the methods described in section 2.4.

5.2.5 Fatty acid analysis

Lipid was determined by a modification of the method of Folch *et al.* (1957) as previously described (section 2.3.4) with some further few alterations. The organic layer was filtered through a pipette filter (composed of glass wool and Na_2SO_4) into a preweighed screw cap tube, and then rinsed with 2 ml of $CHCl_3$. The extracts were dried under a stream of N_2 to prevent oxidation of fatty acids.

The extracted lipid was redissolved in 2 ml of hexane (HPLC Grade, Sigma) and proceeded for methylation. Five to 15 mg of lipid were used for methylation. The required volume of hexane containing the correct amount of lipid was transferred to 5 ml Reactivials (Pierce, Illinois 61105, USA) and made up to 2 ml of hexane. Two ml of boron trifluroide (7% in methanol) was added to vials, which were capped and sealed with Teflon tape after being flushed with N_2. The mixture was incubated in a block heater for 1 h at 100 ^{0}C and then allowed to cool on the bench for 1 h. The top organic layer was removed to a screw cap tube and kept aside under N_2. The bottom layer was re-extracted twice with 2 ml of hexane, and the extracts were pooled in the screw cap tube. Two ml of double distilled water was added to the hexane extract. After being vortexed, the bottom layer (water) was removed using a pasteur pipette. The remaining hexane layer was dried with Na_2SO_4 (anhydrous) (~ 0.5 g) and was removed to another preweighed screw cap tube. The hexane extraction was dried under a stream of N_2. The tube was reweighed and extracted fatty acid methyl esters (FAME) were re-dissolved in 2 ml hexane and kept at -80 ^{0}C until analysed.

GC method

Analysis of FAME was carried out using a GC-17A (Shimadzu corporation, Kyoto, Japan) gas chromatograph, equipped with a flame ionisation detector. The FAME were separated on an Omegawax 320 capillary column (Supelco 24152, 30m x 0.32mm x 0.25μm film thickness). The injector and detector were maintained at 250 ^{0}C and 260 ^{0}C respectively. Column oven temperature was maintained at 185 ^{0}C for 10 min, increased to 230 ^{0}C at 3 ^{0}C .min^{-1}, and held at 230 ^{0}C for 10 min. Helium carrier gas flow was maintained at 83 KPa ratio with a split of 1:5. Data were collected and integrated using the Glass GC10 software package (Shimadzu).

Fatty acid methyl esters (FAME) were identified by comparing them to the external standards (Supelco 37 component FAME mix, Supelco, Bellefonte, PA) previously calibrated by GCMS. Fatty acid quantities are reported as a percentage of total fatty acids measured.

5.2.6 Statistical analysis

Data for growth parameters and plasma T and 11kT levels were analysed using repeated measures block ANOVA (General linear method-repeated measures). Means for each treatment at a single time point were analysed using one way ANOVA followed by Sheffe's test. A paired t-test was used to check the differences between means at different time points either with the initial value (week 0) or with the adjacent value within the same treatment. The data for plasma E_2 levels were only available at week 18. Unfortunately, the research laboratory was contaminated with E_2 and the plasma samples from week 0 to week 12 were also contaminated. Therefore, data for E_2 levels for these samples were excluded from the analysis. However, the plasma samples at week 18 were processed in another laboratory and used in the analysis. E_2, % BWG, GSI, HSI and % lipid data were analysed using one way ANOVA and Scheffe's test (Zar, 1985). Homogeneity of variances was tested using Levene's test. Data for transitional and female fish were excluded from the analysis. Gonadal stages were analysed using loglikelihood ratio for small sample size (Zar, 1984).

Principle component analysis (PCA) using a correlation matrix was performed on the fatty acid data to summarise the differences among treatments and to determine

the most important fatty acids which are contributed to the differences. The general practise of performing ANOVA on fatty acid profiles leads to an increased chance of finding a significant result (Type I error) (Sheikh-Eldin *et al.*, 1996), and this tendency is avoided or minimised by performing PCA. Principle component analysis using a correlation matrix also allows the importance of relative changes in individual fatty acids to be identified rather than the analysis being driven by those fatty acids that are present in relatively large amounts. Thus, a change in an individual fatty acid of 10 times is found to be important even though the fatty acid is present at a low percent of the total while a doubling a fatty acid that is present at 20% of the total may not be.

All analyses were performed using SPSS version 9.0 statistical package. Statistical significance was considered significant at the 0.05 level.

5.3 RESULTS

5.3.1 Diets

The diets were similar in texture and fish in all treatments adapted easily to the experimental diets.

5.3.2 Food consumption

Mean food consumption and FCE did not vary between treatments (Table 5.2a,b). However, statistical analysis could not be performed on this data as data were available for each tank and not for individual fish.

5.3.3 Growth

Growth of fish was affected by diet (Fig 5.1). Fish in all treatments gained weight significantly throughout the experiment (repeated measures ANOVA; $617.6_{(1.2,50)}$; $P<0.05$). There was a significant interaction of time and dietary treatment, reflecting the fact that the body weight of fish varied significantly between treatments over time (repeated measures ANOVA; $8.09_{(3.6,50)}$; $P<0.005$). One way ANOVA showed that the mean body weights of fish were not significantly different between treatments during the first 12 weeks, but were significantly different in the last 12 weeks (Table 5.3a). Fish fed the LIN diet were significantly smaller in weight than fish in AA treatment, but not different to fish fed the SOY or FISH treatments during the last 12 weeks (Table 5.3a). Fish fed the SOY or FISH diet showed intermediate growth by the end of the experimental period.

The % BWG of fish fed the LIN diet was significantly lower than that of fish fed the FISH or AA diet, but not different to that of fish fed the SOY diet (Table 5.3a) (one way ANOVA; $F=8.84_{(3,42)}$; $P<0.05$). The % BWG of fish fed the other three diets (SOY, FISH and AA) were not significantly different from each other.

Growth rate of fish varied significantly throughout the experiment (Fig 5.2) (repeated measures ANOVA, $20.55_{(1.3,51)}$, $P<0.05$), and a significant interaction between time and dietary treatment on SGR was observed (repeated measures ANOVA, $2.89_{(3.9,51)}$, $P<0.005$). In the first 12 weeks, SGR of fish in the LIN treatment was significantly lower than that of fish in the AA treatment. SOY and FISH

Table 5.2a Food consumption (mean ± SD of daily measures for six weekly perods) of barramundi fed diets with different fatty acid sources. (LIN = Linseed oil; SOY = Soybean oil; FISH = Fish oil and AA = Arachidonic acid).

Diet	Mean Food consumption (% BWt.day^{-1})		
	0-6 wk	6-12 wk	12-18 wk
LIN	0.60 ± 0.30	0.61 ± 0.26	0.68 ± 0.27
SOY	0.69 ± 0.35	0.58 ± 0.26	0.67 ± 0.23
FISH	0.72 ± 0.35	0.69 ± 0.32	0.61 ± 0.21
AA	0.86 ± 0.45	0.71 ± 0.38	0.65 ± 0.26

Table 5.2b Food conversion efficiency (FCE) for barramundi fed diets with different fatty acid sources. (LIN = Linseed oil; SOY = Soybean oil; FISH = Fish oil; AA = Arachidonic acid). These values are mean and range for two tanks.

Diet	FCE		
	0-6 wk	6-12 wk	12-18 wk
LIN	0.52	0.57	0.49
	(0.39 – 0.65)	(0.48 – 0.67)	(0.47 – 0.51)
SOY	0.60	0.67	0.55
	(0.54 – 0.66)	(0.63 – 0.72)	(0.52 – 0.57)
FISH	0.72	0.77	0.64
	(0.70 – 0.73)	(0.76 – 0.77)	(0.62 – 0.66)
AA	0.71	0.78	0.55
	(0.66 – 0.75)	(0.75 – 0.81)	(0.54 – 0.57)

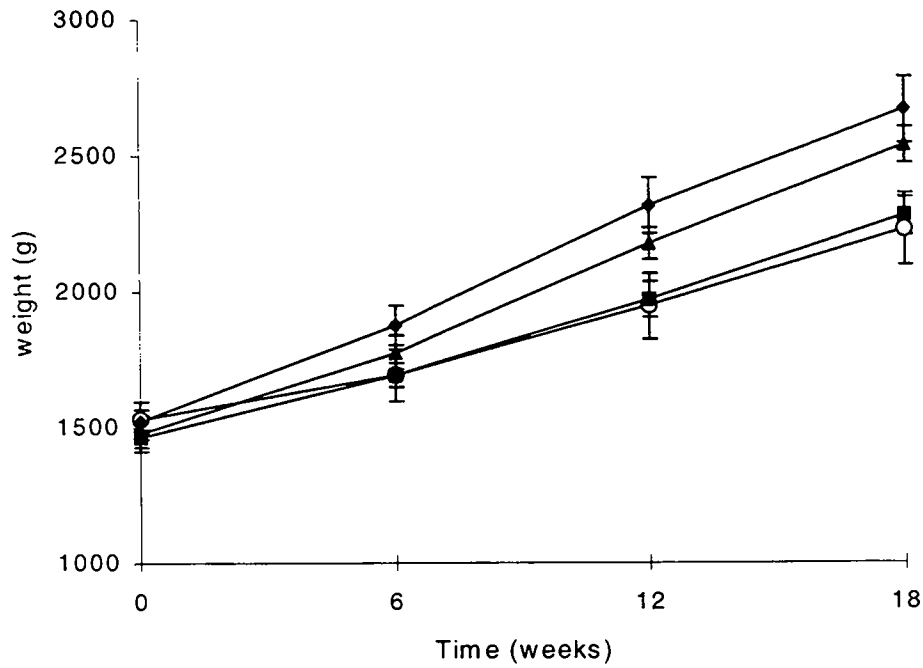


Figure 5.1 Mean (± SE) body weight of barramundi fed diets with varying fatty acid sources. (○ - LIN (Linseed oil); ■ - SOY (Soybean oil); ▲- FISH (Fish oil); ◆ - AA (Arachidonic acid)).

Table 5.3a Initial weight, final weight, body weight gain (% BWG), Hepato Somatic Index (HSI), Gonado Somatic Index (GSI) and lipid content (%) of tissues (mean ± SE) of male barramundi fed diets with varying fatty acid sources (LIN = Linseed oil; SOY = Soybean oil; FISH = Fish oil and AA = Arachidonic acid). n = sample size; Means with the same superscript in any column are not significantly different (P>0.05).

Diet	n	Initial weight (g)	Final weight (g)	% BWG	HSI	GSI	% Lipid	
							Liver	Gonad
LIN	10	1528 ± 71^{a}	2226 ± 131^{a}	45.2 ± 4.3^{a}	1.25 ± 0.05^{a}	0.02 ± 0.01^{a}	32.9 ± 3.4^{a}	10.0 ± 4.2^{a}
SOY	12	$1461 + 32^{a}$	2276 ± 72^{ab}	55.8 ± 4.0^{ab}	1.41 ± 0.07^{ab}	0.02 ± 0.01^{a}	33.8 ± 6.4^{a}	6.4 ± 2.2^{a}
FISH	11	$1476 + 65^{a}$	2538 ± 64^{ab}	73.7 ± 5.2^{b}	1.56 ± 0.06^{b}	0.03 ± 0.01^{a}	34.2 ± 1.2^{a}	7.2 ± 2.4^{a}
AA	12	$1520 + 44^{a}$	2667 ± 121^{b}	75.1 ± 5.3^{b}	1.49 ± 0.06^{ab}	0.02 ± 0.01^{a}	30.1 ± 3.6^{a}	11.5 ± 2.5^{a}

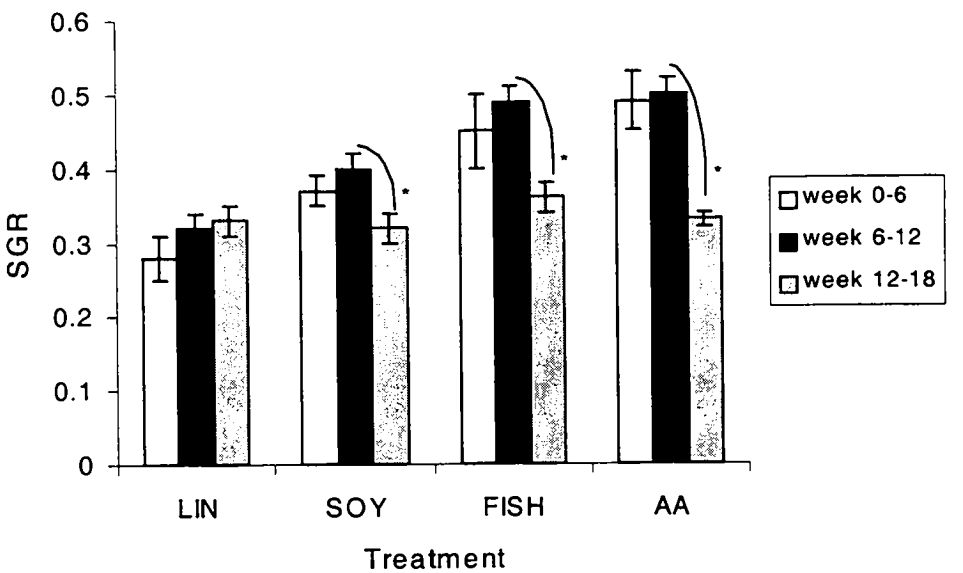


Figure 5. 2 Specific Growth Rate (SGR) (mean ± SE) of barramundi fed diets with different fatty acid sources. (LIN = Linseed oil; SOY = Soybean oil; FISH = Fish oil; AA = Arachidonic acid). * indicates a significant difference by paired t-test between adjacent values. The legend shows the period of which each column refers.

treatments showed intermediate SGR values. In the last six weeks, SGR of SOY, FISH and AA fish were significantly reduced, and the SGRs were comparable in all four treatments. Comparison within the same treatment showed that SGR over time did not vary significantly in fish in the LIN treatment.

5.3.4 Hepato Somatic Index

Dietary treatment significantly influenced the HSI (Table 5.3a) (one way ANOVA; $F= 4.224_{(3,40)}$; $P<0.05$). Fish fed the LIN diet had the lowest mean HSI value, which was significantly different from those of fish fed the FISH diet. Fish fed the SOY and AA diet had intermediate HSI values. HSI for transitional and female fish are shown in Table 5.3b.

5.3.5 Gonado Somatic Index

The GSI of male fish was not significantly different between treatments at week 18 (Table 5.3a) (one way ANOVA; $F= 0.969_{(3,41)}$; $P>0.05$). The GSI for transitional and female fish are presented in Table 5.3b.

5.3.6 Histology

Gonadal histology showed that two fish from the LIN treatment had transitional gonads (T4 stage) (Fig 5.3a) and one fish from the FISH treatment was female (Fig 5.3b). Other fish showed significantly different testicular developmental stages ranging from stage 1 to stage 4 with diet (Fig 5.3 c-f; Fig 5.4) (Loglikelihood ratio; $\chi^2 = 28.8$; $df = 15$; $P<0.05$).

5.3.7 Lipid content

Percentage lipid content of the liver or the gonad of male fish was not significantly different between treatments (Table 5.3a) (one way ANOVA; $F= 0.812_{(3,17)}$; $P>0.05$ and $F= 1.462_{(3,20)}$; $P>0.05$ for the liver and the gonad respectively).

Table 5.3b Final body weight, gonad weight, Goando Somatic Index (GSI), Hepato Somatic Index (HSI) and % lipid of tissues of transitional and female barramundi. (LIN = Linseed oil; FISH = Fish oil).

Treatment	Gonadal Stage	Final body Wt (g)	Gonad Wt (g)	GSI	HSI	% Lipid	
						Liver	Gonad
LIN	*Transitional*	3033	1.05	0.04	1.83	46.4	8.9
	Transitional	1744	2.19	0.13	1.48	28.6	6.9
FISH	*Female*	2840	1.60	0.05	1.70	36.9	5.0

Figure 5.3 Transverse sections of gonads showing different gonadal development stages.

(a) Transitional gonad (stage T4) showing more than 50% of ovarian tissue and no traces of male tissue. (O - Oocytes; C – connective tissue)

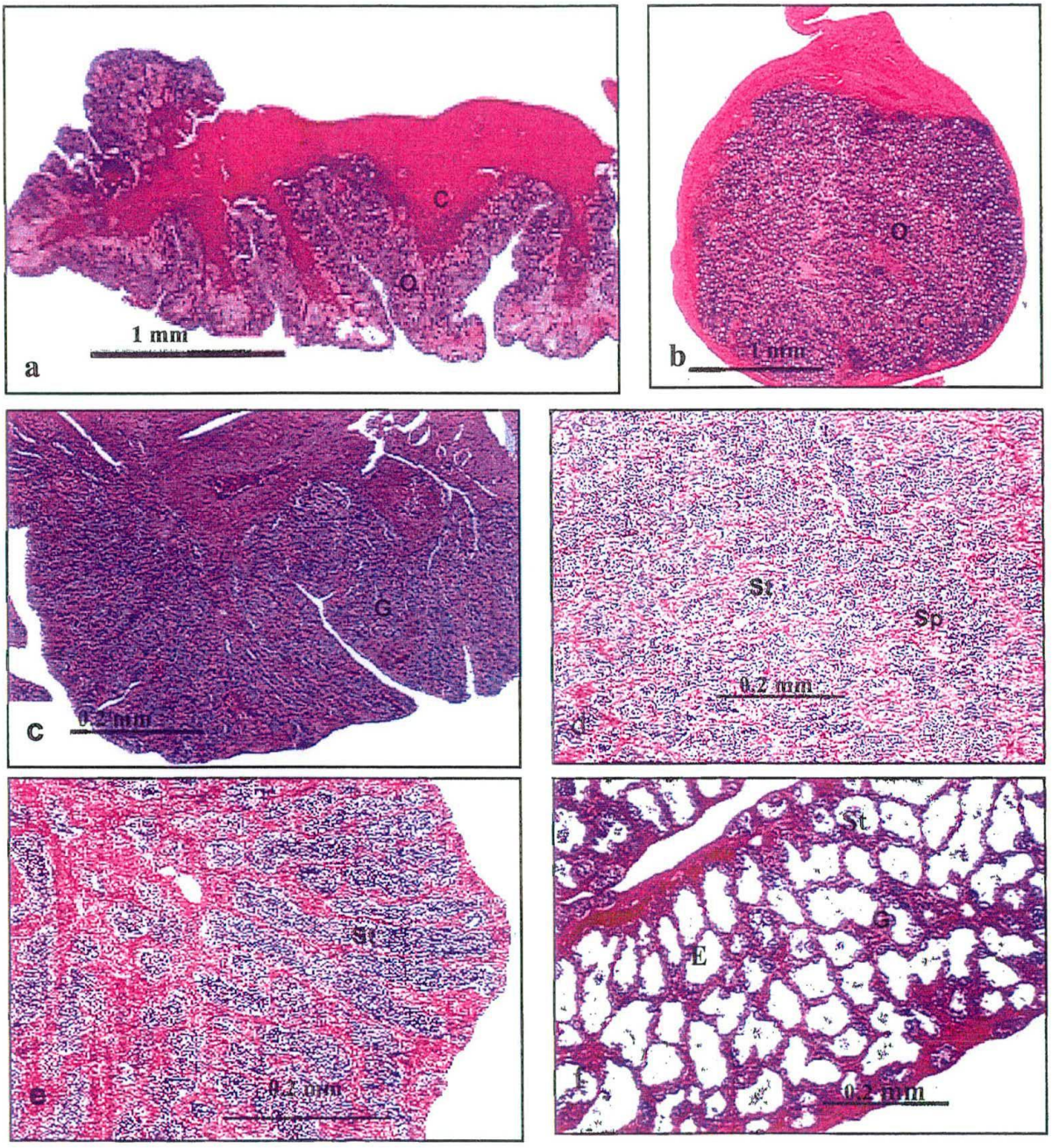
(b) Ovary showing previtellogenic oocytes (O) arranged in within ovarian lamellae.

(c) Stage 1 testis showing predominance of gonia (G).

(d) Stage 2 testis showing spermatogenesis stage. Spermatocytes (Sp) and spermatids (St) predominate.

(e) Stage 3 testes with relatively larger proportion of spermatids (St) than other tissue

(f) Stage 4 testis. Most of the testicular lobules were devoid of spermatozoa, but some gonia (G) and spermatids (St) remained. (E-Empty lobules).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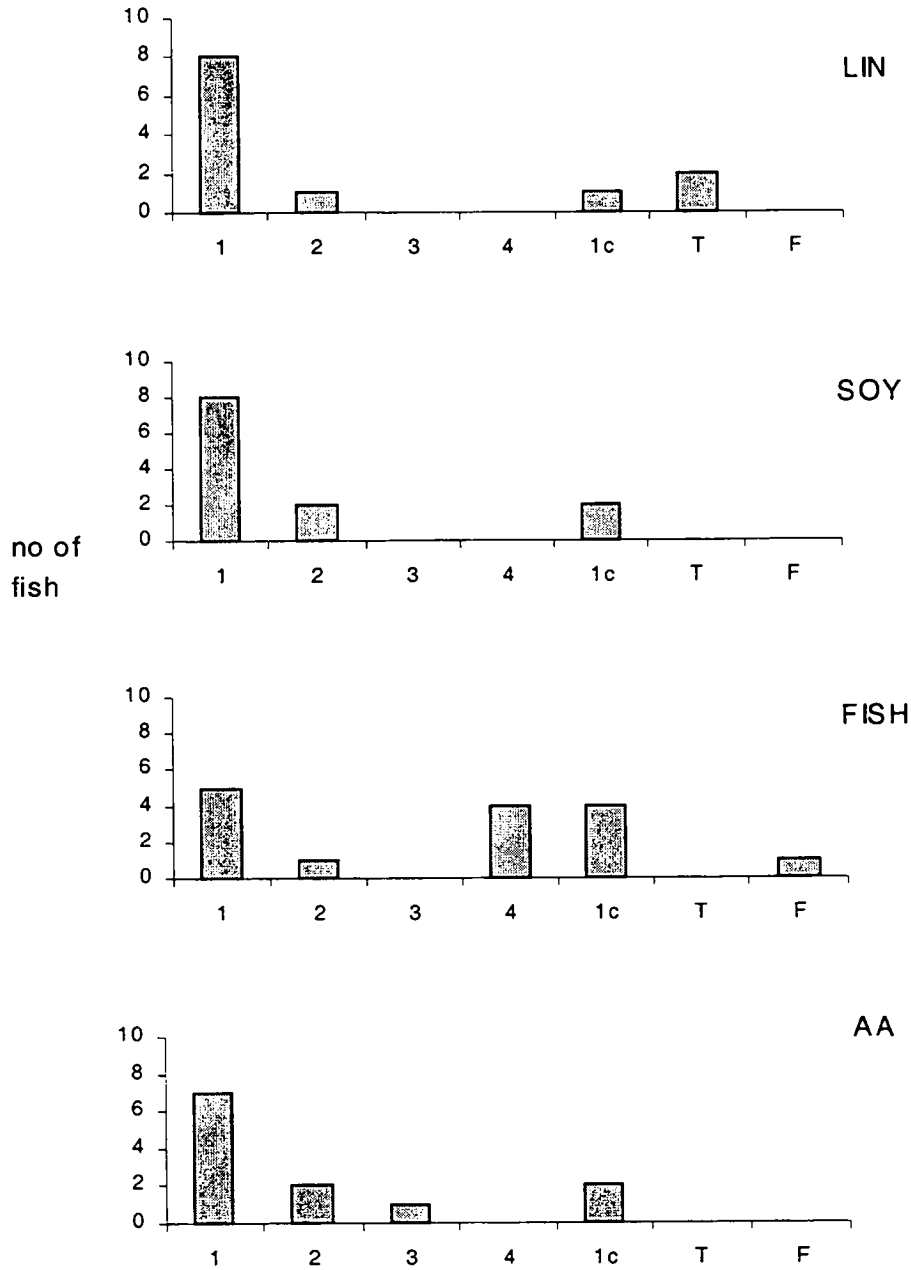

Figure 5. 4 Gonadal stages of barramundi fed diets with different fatty acid sources at week 18. Gonadal stages are classified according to Guiguen *et al.* (1993) and Table 1.1. (1-4 male gonadal stages; T = transitional; F = female; details are in section 2.5).

5.3.8 Fatty acids

Diets

The fatty acid composition (% of total fatty acids) of the diets is given in Table 5.4. The dietary fatty acid composition was significantly correlated with the fatty acid composition of the gonads and the livers of fish in all cases (Table 5.5).

As expected, n-3 and n-6 fatty acid levels were different in the four diets. The LIN diet contained a high amount (51.32 ± 1.05%) of short chain n-3 fatty acids (largely 18:3 n-3) while SOY diet had a high amount (47.54 ± 0.76%) of short chain n-6 fatty acids (largely 18:2 n-6). The long chain n-3 fatty acids were high in the FISH diet with the amount of 20:5 n-3 and 22:6 n-3 was 14.02 ± 0.68% and 17.84 ± 1.52% respectively. Arachidonic acid (20:4 n-6; long chain n-6 fatty acid) comprised 20.56 ± 0.39% of the total fatty acids in the AA diet. In addition, the fatty acids 16:0 and 18:1 n-9 were present in high amounts in all of the diets, making up 7.5 - 18.9 % and 15.2 - 30.0% of the total respectively.

Total saturates and monoenes varied between diets. The fatty acids 16:0 and 18:0 were the predominant saturates, except for in the FISH diet, which also contained 6.47% of 14:0. The predominant monoene was 18:1 n-9, although again the FISH diet had 5 - 7% each of 16:1 n-7, 20:1 and 22:1 n-9. The FISH diet had the highest percentages of total saturates and monoenes (29.68 ± 0.11% and 35.18 ± 0.31% respectively), while the LIN diet had the lowest proportion of these fatty acids.

The highest total n-3 fatty acids were found in the LIN diet (56.16 ± 1.09%), with the fatty acid 18:3 n-3 being the main contributor. The FISH diet also had a higher amount of total n-3 (33.68 ± 2.19%) than the SOY and the AA treatments. The n-3 to n-6 ratio varied between diets. The FISH diet had a higher ratio (11.19) than the LIN diet, which was in turn higher (3.98) than both SOY and AA diets (0.25).

Principle component analysis clearly separated the FISH diet from the other three diets along Axis 1 which described 52% of the total variance (Fig 5.5). The parameters contributing to the difference are in order: 22:1 n-9, 20:1, 20:5 n-3, 20:3 n-3, 15:0, n-3 to n-6 ratio, 16:1 n-7, 22:6 n-3, 14:0, 17:0 and 17:1 in a positive direction and 18:0 in a negative direction. Axis 2 described 29% of the total variance, and

Table 5.4 Fatty acid composition (% of total fatty acids) (mean ± SE) of experimental diets. (LIN = Linseed oil; SOY = Soybean oil; FISH = Fish oil and AA = Arachidonic acid). nd = not detected.

Fatty acid	LIN	SOY	FISH	AA
14:0	0.89 ± 0.05	0.98 ± 0.04	6.47 ± 0.05	2.02 ± 0.09
15:0	0.02 ± 0.02	0.31 ± 0.02	0.58 ± 0.01	0.06 ± 0.03
16:0	7.57 ± 0.24	11.74 ± 0.21	18.89 ± 0.18	15.02 ± 0.26
17:0	0.07 ± 0.01	0.11 ± 0.01	0.36 ± 0.01	0.19 ± 0.01
18:0	4.08 ± 0.23	3.60 ± 0.10	2.82 ± 0.03	5.71 ± 0.01
20:0	0.14 ± 0.01	0.32 ± 0.01	0.22 ± 0.01	0.52 ± 0.01
22:0	0.09 ± 0.01	0.33 ± 0.01	0.10 ± 0.04	0.71 ± 0.03
24:0	0.08 ± 0.01	0.12 ± 0.01	0.11 ± 0.00	0.61 ± 0.03
16:1 n-7	1.03 ± 0.01	0.75 ± 0.38	7.09 ± 0.06	1.96 ± 0.03
17:1	0.18 ± 0.01	0.18 ± 0.07	0.35 ± 0.01	0.07 ± 0.00
18:1 n-9	15.27 ± 0.68	21.13 ± 0.62	15.49 ± 0.05	30.08 ± 0.08
20:1	0.39 ± 0.04	0.52 ± 0.01	5.07 ± 0.09	0.61 ± 0.01
22:1 n-9	0.05 ± 0.01	0.07 ± 0.01	7.03 ± 0.28	0.07 ± 0.01
20:2 n-4	nd	0.04 ± 0.01	0.35 ± 0.01	0.31 ± 0.02
n-3 series				
18:3 n-3	51.32 ± 1.05	7.41 ± 0.17	1.66 ± 0.02	0.38 ± 0.02
20:3 n-3	0.03 ± 0.01	nd	0.15 ± 0.02	nd
20:5 n-3	2.95 ± 0.03	3.06 ± 0.03	14.02 ± 0.68	3.81 ± 0.09
22:6 n-3	1.86 ± 0.08	1.62 ± 0.26	17.84 ± 1.52	4.09 ± 0.16
n-6 series				
18:2 n-6	13.96 ± 0.01	47.54 ± 0.76	2.06 ± 0.07	10.48 ± 0.12
18:3 n-6	0.05 ± 0.03	0.37 ± 0.35	0.15 ± 0.03	1.44 ± 0.01
20:3 n-6	0.02 ± 0.01	nd	0.05 ± 0.01	1.28 ± 0.11
20:4 n-6	0.07 ± 0.01	0.07 ± 0.01	0.75 ± 0.03	20.56 ± 0.39
Totals				
Saturates	12.95 ± 0.48	17.29 ± 0.35	29.68 ± 0.11	24.85 ± 0.24
Monoenes	16.91 ± 0.71	22.66 ± 0.48	35.18 ± 0.31	32.80 ± 0.06
n-3	56.16 ± 1.09	12.10 ± 0.32	33.68 ± 2.19	8.28 ± 0.26
n-6	14.09 ± 0.02	47.98 ± 0.58	3.01 ± 0.07	33.76 ± 0.38
n-3:n-6	3.98 ± 0.08	0.25 ± 0.01	11.19 ± 0.81	0.25 ± 0.01

Table 5.5 Pearson correlation coefficients for comparisons between the fatty acid profiles of the diet and of the tissues from barramundi fed on different fatty acid levels. (LIN = Linseed oil; SOY = Soybean oil; FISH = Fish oil and AA = Arachidonic acid). All correlations are significant at $P<0.01$.

	Treatment			
Tissue	LIN	SOY	FISH	AA
Liver	0.886	0.943	0.973	0.926
Gonad	0.951	0.957	0.974	0.989

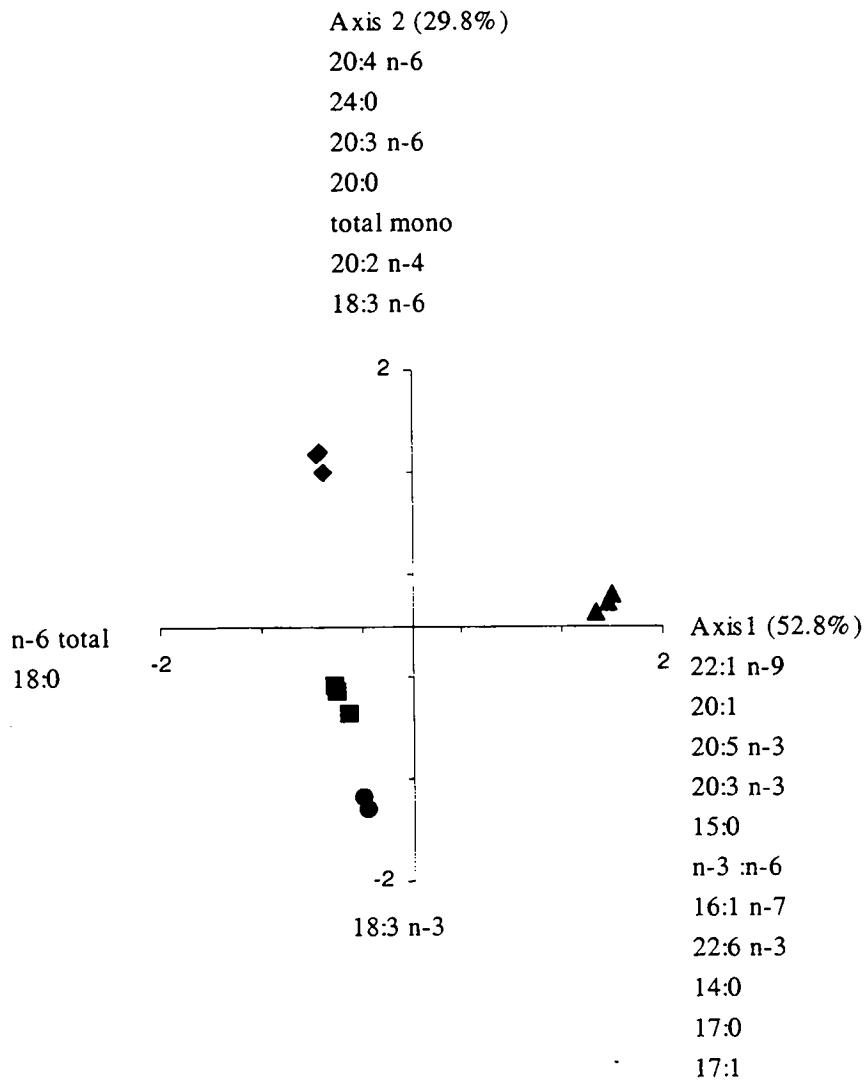

Figure 5. 5 Plot of the result of the principle component analysis of the fatty acids of the diets. The amount of variance attributable to each axis and the fatty acids providing the major contribution to the derivation of the axes are indicated on each axis. (● - LIN; ■ - SOY; ▲- FISH; ♦ - AA)

Table 5.6 Fatty acid composition (% of total fatty acids) (mean ± SE) of liver of barramundi fed different diets. (LIN = Linseed oil; SOY = Soybean oil; FISH = Fish oil; AA = Arachidonic acid). nd = not detected.

Fatty acid	LIN	SOY	FISH	AA
14:0	1.07 ± 0.12	0.95 ± 0.04	2.90 ± 0.09	1.31 ± 0.09
15:0	0.07 ± 0.01	0.07 ± 0.01	0.28 ± 0.01	0.07 ± 0.01
16:0	13.84 ± 1.02	15.42 ± 1.00	24.93 ± 0.64	25.57 ± 0.16
17:0	0.13 ± 0.01	0.17 ± 0.01	0.28 ± 0.01	0.17 ± 0.01
18:0	7.41 ± 0.33	7.09 ± 0.65	5.08 ± 0.23	8.96 ± 0.51
20:0	0.16 ± 0.01	0.25 ± 0.01	0.18 ± 0.01	0.41 ± 0.01
22:0	0.09 ± 0.01	0.20 ± 0.01	0.07 ± 0.01	0.47 ± 0.02
24:0	0.05 ± 0.01	0.05 ± 0.01	nd	0.60 ± 0.02
16:1 n-7	1.56 ± 0.09	1.36 ± 0.14	5.79 ± 0.03	2.99 ± 0.29
17:1	0.10 ± 0.01	0.09 ± 0.02	0.39 ± 0.01	0.12 ± 0.01
18:1 n-9	24.70 ± 0.49	26.80 ± 0.98	21.32 ± 0.22	32.30 ± 0.42
20:1	0.75 ± 0.05	0.83 ± 0.02	4.40 ± 0.08	0.93 ± 0.04
22:1 n-9	0.07 ± 0.01	0.08 ± 0.01	0.79 ± 0.02	0.08 ± 0.01
20:2 n-4	0.24 ± 0.01	0.53 ± 0.04	0.26 ± 0.01	0.22 ± 0.02
n-3 series				
18:3 n-3	28.70 ± 1.09	2.80 ± 0.28	0.59 ± 0.03	0.12 ± 0.02
20:3 n-3	0.79 ± 0.04	0.09 ± 0.01	0.10 ± 0.01	nd
20:5 n-3	2.42 ± 0.15	1.88 ± 0.15	9.24 ± 0.22	1.25 ± 0.18
22:6 n-3	3.16 ± 0.39	2.79 ± 0.24	20.64 ± 0.69	3.58 ± 0.35
n-6 series				
18:2 n-6	12.84 ± 0.60	31.51 ± 1.81	1.40 ± 0.11	4.69 ± 0.42
18:3 n-6	1.50 ± 0.11	5.69 ± 0.58	0.34 ± 0.01	1.34 ± 0.01
20:3 n-6	0.19 ± 0.01	1.06 ± 0.11	0.15 ± 0.01	1.32 ± 0.09
20:4 n-6	0.17 ± 0.02	0.31 ± 0.04	0.80 ± 0.02	13.48 ± 0.91
Totals				
Saturates	22.81 ± 1.45	24.21 ± 1.64	33.74 ± 0.75	37.57 ± 1.40
Monoens	27.18 ± 0.55	29.16 ± 1.08	32.75 ± 0.31	36.43 ± 0.63
n-3	35.06 ± 1.29	7.55 ± 0.64	30.56 ± 0.88	4.94 ± 0.49
n-6	14.70 ± 0.69	38.58 ± 2.13	2.69 ± 0.09	20.82 ± 1.38
n-3:n-6	2.39 ± 0.05	0.20 ± 0.01	11.40 ± 0.41	0.24 ± 0.01

separated the AA diet and FISH diet from the other two diets. This variation is associated with 20:4 n-6, 24:0, 20:3 n-6, 20:0, total monoenes, 20:2 n-4 and 18:3 n-6 in a positive direction and 18:3 n-3 in the negative direction.

Liver

When comparing treatments, the fatty acid profile of livers (Table 5.6) clearly reflected the fatty acid profiles of the broodstock diet (Table 5.5). There was no difference between fatty acid profiles of livers in transitional or female fish with that of male fish (Fig 5.6). Fish fed the LIN diet had a predominance of total n-3 fatty acids in the liver, which were mainly composed of short chain n-3 (18:3 n-3, 28.7%) fatty acids (Table 5.6). The livers of fish fed the SOY diet largely contained the short chain n-6 fatty acid (18:2 n-6) which made up 31.5% of total fatty acids. Animals fed the FISH diet predominantly contained long chain n-3 fatty acid, 22:6 n-3 and 20:5 n-3, in the livers, which were 20.6 % and 9.2 % of the total fatty acids respectively. The fatty acid 20:4 n-6 was high in the livers of fish fed the AA diet, comprising 13.5 ± 0.9 % of the total fatty acids.

The predominant monoene in the livers of all fish was 18:1 n-9, which made up between 21.3 – 25.6%, while the saturate 16:0 made up between 13.8 to 25.6 % of total liver fatty acids (Table 5.6). The saturated fatty acid 18:0 was also found in greater amounts (5.08 – 8.96%) in the liver when compared to other fatty acids. The proportion of saturates and monoenes were higher in the livers of fish fed on the FISH and the AA diets. Total n-3 and n-6 fatty acids levels varied between treatments, and reflected the fatty acid levels of the diets. The differences in total n-3 and n-6 resulted in varying n-3 to n-6 ratios in the livers of fish on different treatments. The n-3:n-6 ratio of livers ranged from 0.20 to 11.4.

Principal component analysis clearly separated the fatty acid composition of the livers of the animals in the FISH treatment from those of the fish in the other three treatments (Fig 5.6). Axis 1 described 50% of the total variation. The parameters contributing to this separation were in order: n-3:n-6 ratio, 20:5 n-3, 22:6 n-3, 22:1 n-9, 15:0, 20:1, 17:1, 14:0 and 16:1 n-7. These parameters were greater in the livers of animals on the FISH treatment than those of animals in the other three treatments. The livers of animals in LIN, SOY and AA treatments had higher total n-6 fatty acids.

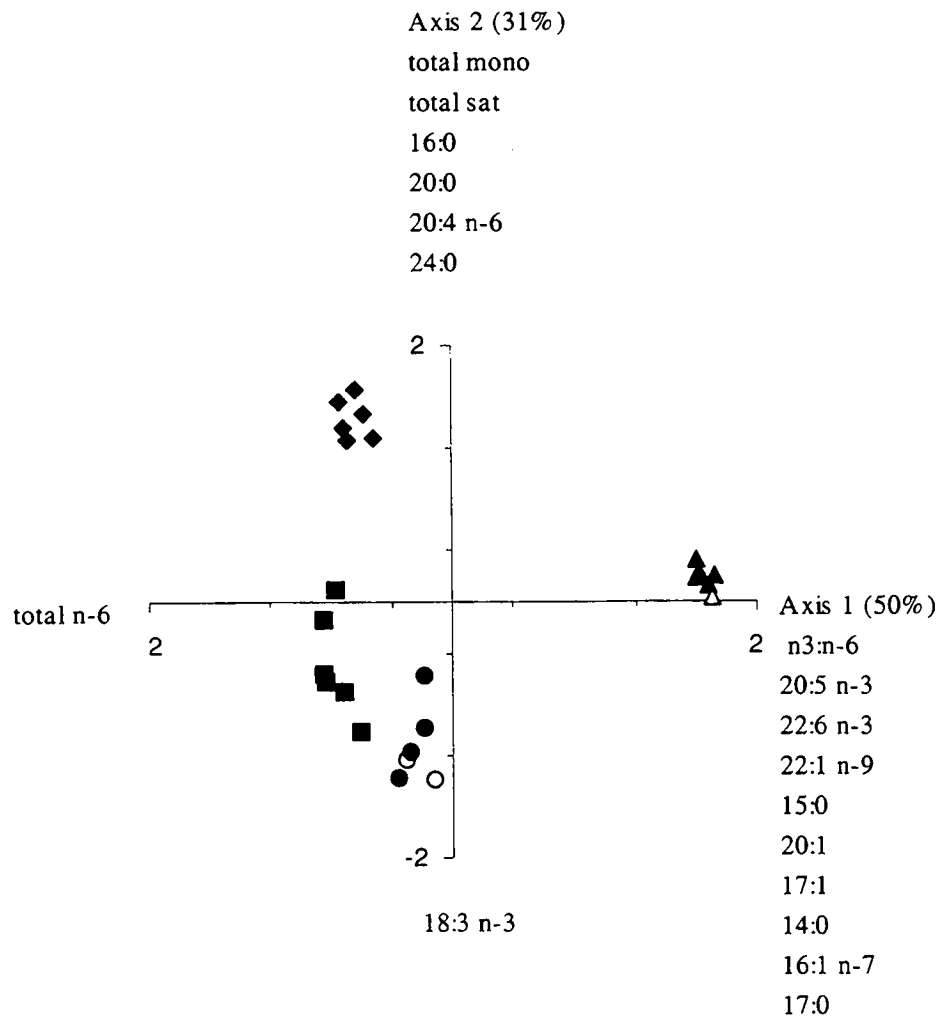

Figure 5. 6 Plot of the result of the principle component analysis of the fatty acids of the livers of barramundi fed on diets containing different fatty acids. The amount of variance attributable to each axis and the fatty acids providing the major contribution to the derivation of the axes are indicated on each axis. (● - LIN; ■ - SOY; ▲- FISH; ◆ - AA). Open symbols indicate transitional or female fish. ○ -LIN transitional; Δ - FISH female).

Axis 2, which accounted for 31% of the total variation, separated the livers of fish on the AA diet from those of fish on the other three treatments (Fig 5.6). The parameters contributing to this separation were in order: total monoenes, total saturates, 16:0, 20:0, 20:4 n-6 and 24:0 in the positive direction, and 18:3 n-3 in the negative direction.

Gonads

When comparing treatments, the fatty acid profile of gonads (Table 5.7) also correlated with the fatty acid profile of the diets (Table 5.5). The fatty acid profiles of the gonads from the transitional and female fish were not different to those of the male fish in the same treatment (Fig 5.7). As expected, n-3 and n-6 fatty acid contents were different in treatments and reflected the dietary fatty acid levels. The gonads of fish in LIN, SOY, FISH and AA treatments had high amounts of short chain n-3 (18:3 n-3), short chain n-6 (18:2n-6), long chain n-3 (22:6 n-3 & 20:5 n-3) and long chain n-6 (20:4 n-6) respectively. The fatty acids 18:1 n-9 (17.6 – 27.9%) and 16:0 (12.4 – 21.2%) were present in high amounts in the gonads of fish in all treatments. When compared to other fatty acids, the saturate 18:0 was also found in comparatively higher amounts (5.7 – 7.6%) in gonads in all treatments.

The gonads of animals fed the FISH diet had the highest amount of total saturates, while those of animals fed the LIN diet had the lowest amount of total saturates (Table 5.7). Total monoenes were highest in gonads of fish fed the AA diet, and intermediate levels were found in those of animals fed the FISH diet. Total n-3 was highest in the gonads of fish on the LIN treatment, and lowest in the gonads of those on the AA treatment. The n-3 to n-6 ratio varied from 0.35 to 5.97.

Principal component analysis separated the gonads of animals fed the FISH diet along axis 1, accounting for 46.8% of the total variation (Fig 5.7). The parameters which contributed to the separation were, in order: n-3 to n-6 ratio, 20:5 n-3, 20:1, 15:0, 14:0, 22:1 n-9, 22:6 n-3 and 16:1 n-7. Total n-6 and 18:3 n-6 were greater in the gonads of animals from the other three treatments. The parameters contributing to the separation along axis 2 were 16:0, total saturates and total monoenes in the positive direction and 18:3 n-3, 20:3 n-3 and total n-3 in the negative direction. The gonads of fish in the AA treatment had the highest scores, while the gonads of animals fed the

Table 5.7 Fatty acid composition (% of total fatty acids) (mean ± SE) of gonad of barramundi fed different diets. (LIN = Linseed oil; SOY = Soybean oil; FISH = Fish oil; AA = Arachidonic acid). nd = not detected.

Fatty acid	LIN	SOY	FISH	AA
14:0	2.20 ± 0.13	2.03 ± 0.18	4.74 ± 0.23	2.34 ± 0.10
15:0	0.19 ± 0.02	0.11 ± 0.05	0.49 ± 0.01	0.15 ± 0.02
16:0	12.40 ± 0.49	14.91 ± 0.48	21.24 ± 0.29	18.11 ± 0.31
17:0	0.22 ± 0.02	0.25 ± 0.03	0.36 ± 0.01	0.23 ± 0.03
18:0	6.34 ± 0.45	7.59 ± 1.12	5.73 ± 0.51	6.84 ± 0.17
20:0	0.20 ± 0.02	0.29 ± 0.04	0.14 ± 0.04	0.31 ± 0.06
22:0	0.12 ± 0.01	0.26 ± 0.04	0.10 ± 0.01	0.42 ± 0.07
24:0	0.08 ± 0.01	0.14 ± 0.03	0.02 ± 0.01	0.35 ± 0.02
16:1 n-7	2.23 ± 0.13	1.89 ± 0.25	6.01 ± 0.45	2.89 ± 0.13
17:1	0.39 ± 0.09	0.37 ± 0.05	0.41 ± 0.03	0.32 ± 0.08
18:1 n-9	18.13 ± 0.14	20.28 ± 0.92	17.64 ± 0.31	27.88 ± 0.74
20:1	1.24 ± 0.10	1.24 ± 0.15	3.95 ± 0.21	1.04 ± 0.12
22:1 n-9	0.13 ± 0.01	0.13 ± 0.01	0.49 ± 0.05	0.12 ± 0.01
20:2 n-4	0.16 ± 0.01	0.40 ± 0.06	0.30 ± 0.09	0.24 ± 0.03
n-3 series				
18:3 n-3	32.25 ± 2.45	4.45 ± 0.55	1.26 ± 0.05	0.37 ± 0.03
20:3 n-3	0.45 ± 0.01	0.05 ± 0.02	0.09 ± 0.02	nd
20:5 n-3	4.97 ± 0.50	4.58 ± 0.34	11.27 ± 0.25	3.47 ± 0.15
22:6 n-3	5.43 ± 0.87	6.85 ± 1.06	19.23 ± 0.66	6.28 ± 0.48
n-6 series				
18:2 n-6	11.56 ± 0.36	30.87 ± 2.08	2.95 ± 0.25	8.36 ± 0.35
18:3 n-6	0.37 ± 0.02	0.94 ± 0.13	0.14 ± 0.03	1.20 ± 0.05
20:3 n-6	0.15 ± 0.02	0.57 ± 0.12	0.13 ± 0.01	1.15 ± 0.04
20:4 n-6	0.74 ± 0.20	1.76 ± 0.47	2.51 ± 0.45	17.85 ± 0.47
Totals				
Saturates	21.78 ± 1.07	25.62 ± 1.68	32.92 ± 0.57	28.82 ± 0.16
Monoens	22.14 ± 0.92	23.91 ± 1.12	28.61 ± 0.97	32.31 ± 0.61
n-3	43.09 ± 3.22	15.91 ± 0.86	31.83 ± 0.66	10.12 ± 0.65
n-6	12.82 ± 0.16	34.12 ± 1.58	5.74 ± 0.65	28.62 ± 0.51
n-3:n-6	3.36 ± 0.06	0.48 ± 0.05	5.97 ± 0.64	0.35 ± 0.03

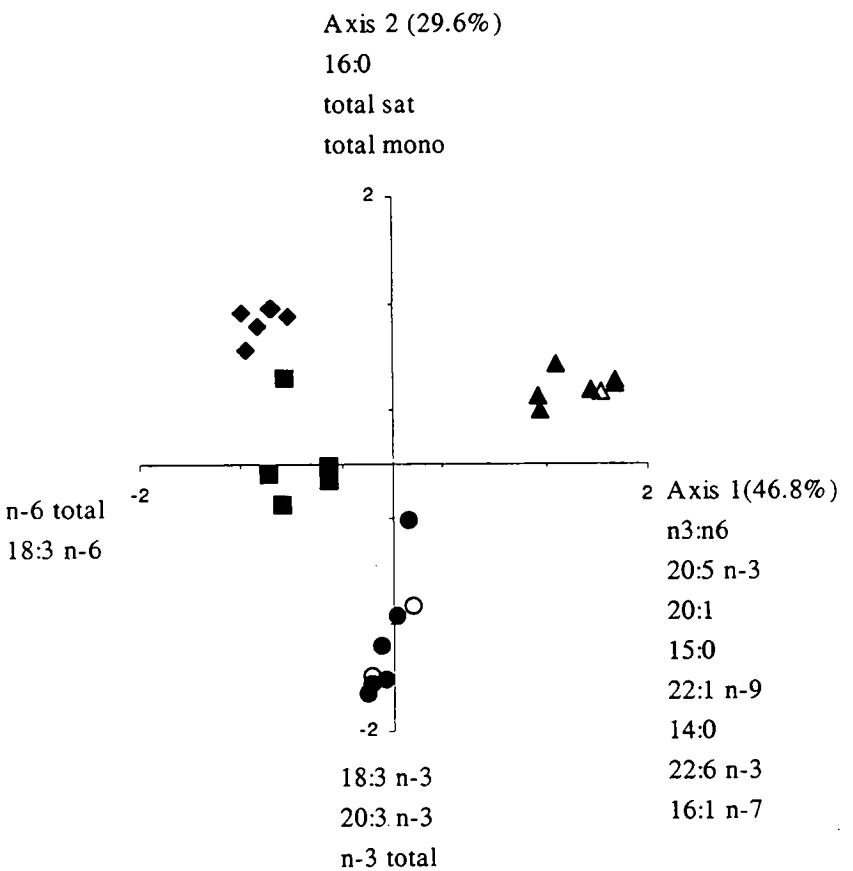

Figure 5. 7 Plot of the result of the principle component analysis of the fatty acids of the gonads of barramundi fed on diets containing different fatty acids. The amount of variance attributable to each axis and the fatty acids providing the major contribution to the derivation of the axes are indicated on each axis. (● - LIN; ■ - SOY; ▲- FISH; ♦ - AA). Open symbols indicate transitional or female fish. ○ -LIN transitional; Δ - FISH female).

LIN diet had the lowest scores in this axis. The gonads of fish fed the FISH and the SOY diets had the intermediate scores on axis 2 (Fig 5.7).

Difference between diet and tissues

To further investigate the variation in fatty acid profiles between tissues and diet in each treatment, additional PCAs were carried out separately on the diet and the tissue fatty acid profiles from each treatment. Within each treatment, the diet and the two tissues were separated from each other by their fatty acid profiles.

The fatty acid consistently separated diets and livers was 18:1 n-9. This fatty acid was always higher in the liver when compared to the dietary composition. Except in the FISH diet where they were present in high amounts, 20:3 n-3 and 20:2 n-4 were also higher in livers of fish compared to the diet in the other three treatments.

The fatty acids which consistently separated livers and gonads in all four treatments were 20:4 n-6 and 20:5 n-3. The gonads had higher concentrations of these fatty acids than the livers. In addition, 22:6 n-3 was also higher in the gonads than in the livers of fish on the LIN, SOY and AA treatments. In the FISH treatment however, both tissues contained a high amount of this fatty acid.

LIN treatment

In general, 18:3 n-3 fatty acid was decreased in livers and gonads compared with the LIN diet (Table 5.8). The fatty acids 20:5 n-3 and 22:6 n-3 were ~1.6 times and ~3 times higher respectively in the gonads than in the diet. The level of 20:5 n-3 did not vary in the liver compared to the diet, but the amount of 22:6 n-3 was ~1.7 times higher than that of the diet. The fatty acid 20:4 n-6 (Arachidonic acid), which was found in very low amounts in the diet, was increased in the gonads tenfold (Table 5.8). Overall, total saturates and monoenes were higher in the livers and the gonads than in the LIN diet. However, total n-3 levels were lower in the liver and the gonads than in the diet. Total n-6 and n-3 to n-6 ratio did not vary between the diet and the tissues.

The principle component analysis summarises the data for the LIN treatment. The first two axes account for 48.7% and 34.7% of the total variation (Fig 5.8). The parameters 17:0, 20:0, 22:6 n-3, 20:4 n-6, 22:1 n-9, 16:1 n-7, 20:1, 20:5 n-3, 14:0,

Table 5.8 Fatty acid composition (mean ± SE) (% of total fatty acids) of diet and tissues of barramundi fed the LIN diet. nd = not detected.

Fatty acid	LIN diet	Liver	Gonad
14:0	0.89 ± 0.05	1.07 ± 0.12	2.20 ± 0.13
15:0	0.02 ± 0.02	0.07 ± 0.01	0.19 ± 0.02
16:0	7.57 ± 0.24	13.84 ± 1.02	12.40 ± 0.49
17:0	0.07 ± 0.01	0.13 ± 0.01	0.22 ± 0.02
18:0	4.08 ± 0.23	7.41 ± 0.33	6.34 ± 0.45
20:0	0.14 ± 0.01	0.16 ± 0.01	0.20 ± 0.02
22:0	0.09 ± 0.01	0.09 ± 0.01	0.12 ± 0.01
24:0	0.08 ± 0.01	0.05 ± 0.01	0.08 ± 0.01
16:1 n-7	1.03 ± 0.01	1.56 ± 0.09	2.23 ± 0.13
17:1	0.18 ± 0.01	0.10 ± 0.01	0.39 ± 0.09
18:1 n-9	15.27 ± 0.68	24.70 ± 0.49	18.13 ± 0.14
20:1	0.39 ± 0.04	0.75 ± 0.05	1.24 ± 0.10
22:1 n-9	0.05 ± 0.01	0.07 ± 0.01	0.13 ± 0.01
20:2 n-4	nd	0.24 ± 0.01	0.16 ± 0.01
n-3 series			
18:3 n-3	51.32 ± 1.05	28.70 ± 1.09	32.25 ± 2.45
20:3 n-3	0.03 ± 0.01	0.79 ± 0.04	0.45 ± 0.01
20:5 n-3	2.95 ± 0.03	2.42 ± 0.15	4.97 ± 0.50
22:6 n-3	1.86 ± 0.08	3.16 ± 0.39	5.43 ± 0.87
n-6 series			
18:2 n-6	13.96 ± 0.01	12.84 ± 0.60	11.56 ± 0.36
18:3 n-6	0.05 ± 0.03	1.50 ± 0.11	0.37 ± 0.02
20:3 n-6	0.02 ± 0.01	0.19 ± 0.01	0.15 ± 0.02
20:4 n-6	0.07 ± 0.01	0.17 ± 0.02	0.74 ± 0.20
Totals			
Saturates	12.95 ± 0.48	22.81 ± 1.45	21.78 ± 1.07
Monoens	16.91 ± 0.71	27.18 ± 0.55	22.14 ± 0.92
n-3	56.16 ± 1.09	35.06 ± 1.29	43.09 ± 3.22
n-6	14.09 ± 0.02	14.70 ± 0.69	12.82 ± 0.16
n-3:n-6	3.98 ± 0.08	2.39 ± 0.05	3.36 ± 0.06

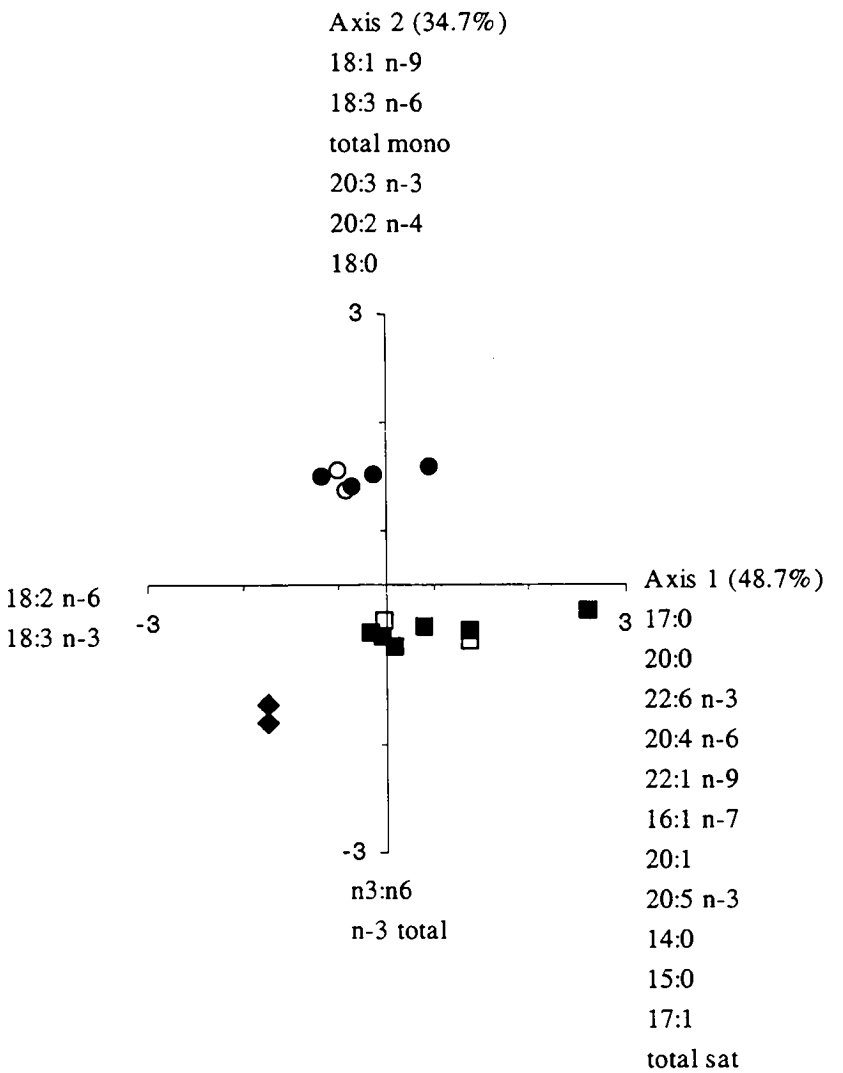

Figure 5. 8 Plot of the result of the principle component analysis of the fatty acids of the gonads and livers of barramundi fed diet containing linseed oil. The amount of variance attributable to each axis and the fatty acids providing the major contribution to the derivation of the axes are indicated on each axis. (● - Liver; ■ - Gonad; ▲ - LIN diet; ○ - liver (transitional fish); Δ - gonad (transitional fish)).

15:0, 17:1, and total saturates contributed in that order to axis 1 in a positive direction, while two fatty acids, 18:3 n-6 and 18:3 n-3, contributed in a negative direction. The livers of fish fed the LIN diet had higher amount of 18:1 n-9, 18:3 n-6, total monoenes, 20:3 n-3, 20:2 n-4 and 18:0 than in the gonads and the LIN diet, and contributed to the variation on axis 2. The gonads and the diet had a higher n-3:n-6 ratio and total n-3 than in the liver.

SOY treatment

Compared to the diet, total saturates, 16:0 and 18:0 increased in both tissues (Table 5.9). Total monoenes increased in the livers compared to the diet which is due to the increase of 18:1 n-9 in the liver. The fatty acid 18:3 n-3 decreased in the liver and the gonad. The 20:5 n-3 decreased in the livers, but increased in the gonads when compared to the diet. The fatty acid 22:6 n-3 was four times higher in the gonads than in the diet. In general, 18:2 n-6 decreased in both tissues compared to the diet, while 18:3 n-6, 20:3 n-6 and 20:4 n-6 increased in both tissues (Table 5.9). Although the dietary 20:4 n-6 amount was very low (0.07%), it was increased four fold (0.31%) and 25 fold (1.76%) in the livers and the gonads respectively. While total saturates were higher in the gonads and the livers than in the diet composition, total n-6 levels in tissues comparatively decreased.

Based on these variations, PCA separated the diet and the tissues (Fig 5.9). Axis 1 contributed to 41% of the total variation and separated the gonads from the diet and the liver. The parameters n-3:n-6, 22:6n-3, 20:4 n-6, 22:1 n-9, 17:0, 20:5 n-3, 14:0, total n-3, 20:1 and 17:1 contributed to the separation in that order in a positive direction, and total n-6 in a negative direction. The variation accounted for axis 2 was 33.5%, and separated the diet from the tissues. The contributing parameters were 20:3 n-6, 20:2 n-4, 16:0, total monoenes, 20:3 n-3, 18:1 n-9 and total saturates in a positive direction, and 18:3 n-3 and 18:2 n-6 in a negative direction.

FISH treatment

The fatty acid 16:0 showed a slight increase in both tissues compared to the diet (Table 5.10). A twofold increase of the saturate 18:0 was found in both tissues. Although, total monoenes slightly decreased in both tissues, 18:1 n-9 was increased in

Table 5.9 Fatty acid composition (mean ± SE) (% of total fatty acids) of diet and tissues of barramundi fed the SOY diet. nd = not detected.

Fatty acid	SOY diet	Liver	Gonad
14:0	0.98 ± 0.04	0.95 ± 0.04	2.03 ± 0.18
15:0	0.31 ± 0.02	0.07 ± 0.01	0.11 ± 0.05
16:0	11.74 ± 0.21	15.42 ± 1.00	14.91 ± 0.48
17:0	0.11 ± 0.01	0.17 ± 0.01	0.25 ± 0.03
18:0	3.60 ± 0.10	7.09 ± 0.65	7.59 ± 1.12
20:0	0.32 ± 0.01	0.25 ± 0.01	0.29 ± 0.04
22:0	0.33 ± 0.01	0.20 ± 0.01	0.26 ± 0.04
24:0	0.12 ± 0.01	0.05 ± 0.01	0.14 ± 0.03
16:1 n-7	0.75 ± 0.38	1.36 ± 0.14	1.89 ± 0.25
17:1	0.18 ± 0.07	0.09 ± 0.02	0.37 ± 0.05
18:1 n-9	21.13 ± 0.62	26.80 ± 0.98	20.28 ± 0.92
20:1	0.52 ± 0.01	0.83 ± 0.02	1.24 ± 0.15
22:1 n-9	0.07 ± 0.01	0.08 ± 0.01	0.13 ± 0.01
20:2 n-4	0.04 ± 0.01	0.53 ± 0.04	0.40 ± 0.06
n-3 series			
18:3 n-3	7.41 ± 0.17	2.80 ± 0.28	4.45 ± 0.55
20:3 n-3	nd	0.09 ± 0.01	0.05 ± 0.02
20:5 n-3	3.06 ± 0.03	1.88 ± 0.15	4.58 ± 0.34
22:6 n-3	1.62 ± 0.26	2.79 ± 0.24	6.85 ± 1.06
n-6 series			
18:2 n-6	47.54 ± 0.76	31.51 ± 1.81	30.87 ± 2.08
18:3 n-6	0.37 ± 0.35	5.69 ± 0.58	0.94 ± 0.13
20:3 n-6	nd	1.06 ± 0.11	0.57 ± 0.12
20:4 n-6	0.07 ± 0.01	0.31 ± 0.04	1.76 ± 0.47
Totals			
Saturates	17.29 ± 0.35	24.21 ± 1.64	25.62 ± 1.68
Monoens	22.66 ± 0.48	29.16 ± 1.08	23.91 ± 1.12
n-3	12.10 ± 0.32	7.55 ± 0.64	15.91 ± 0.86
n-6	47.98 ± 0.58	38.58 ± 2.13	34.12 ± 1.58
n-3:n-6	0.25 ± 0.01	0.20 ± 0.01	0.48 ± 0.05

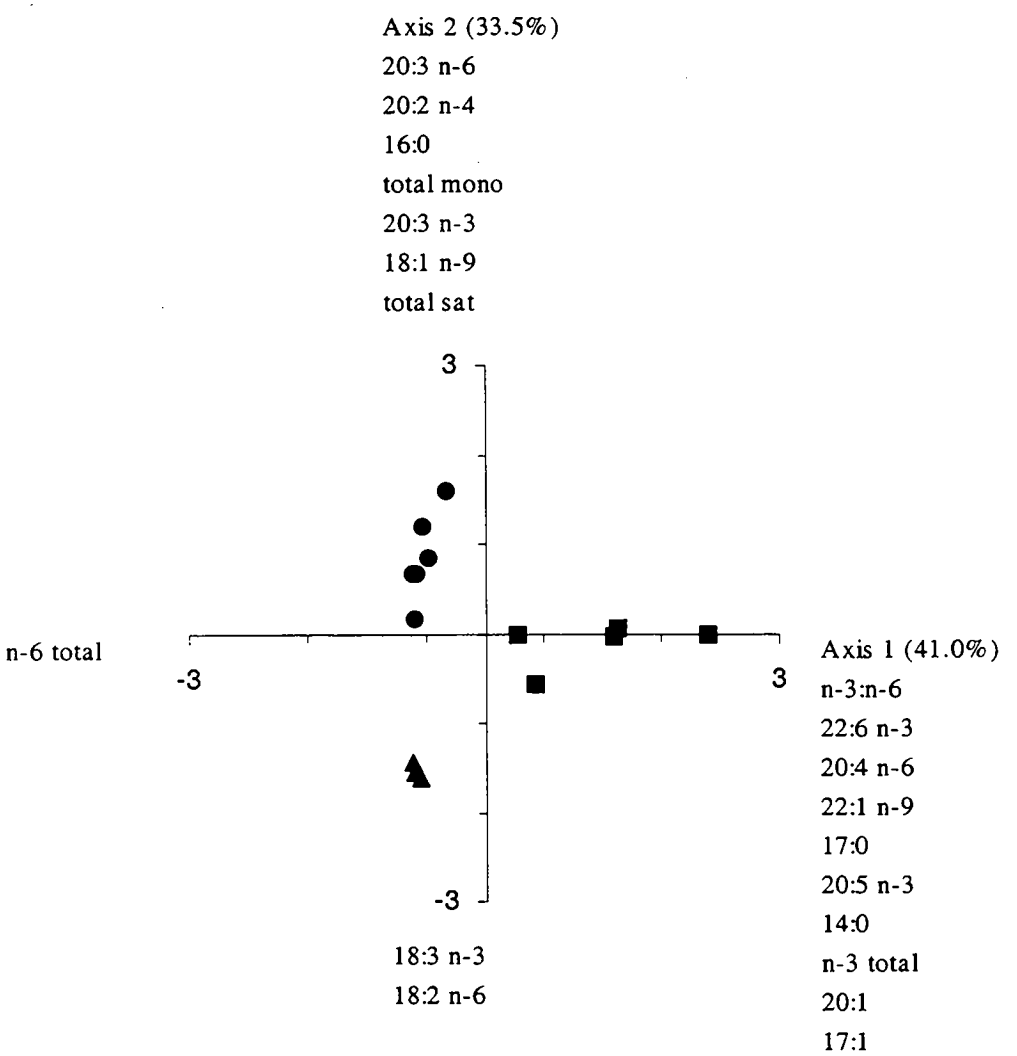

Figure 5. 9 Plot of the result of the principle component analysis of the fatty acids of the gonads and livers of barramundi fed diet containing soybean oil. The amount of variance attributable to each axis and the fatty acids providing the major contribution to the derivation of the axes are indicated on each axis. (● - Liver; ■ - Gonad; ▲ - SOY diet).

Table 5.10 Fatty acid composition (mean ± SE) (% of total fatty acids) of diet and tissues of barramundi fed the FISH diet. nd = not detected.

Fatty acid	FISH diet	Liver	Gonad
14:0	6.47 ± 0.05	2.90 ± 0.09	4.74 ± 0.23
15:0	0.58 ± 0.01	0.28 ± 0.01	0.49 ± 0.01
16:0	18.89 ± 0.18	24.93 ± 0.64	21.24 ± 0.29
18:0	2.82 ± 0.03	5.08 ± 0.23	5.73 ± 0.51
20:0	0.22 ± 0.01	0.18 ± 0.01	0.14 ± 0.04
22:0	0.10 ± 0.04	0.07 ± 0.01	0.10 ± 0.01
24:0	0.11 ± 0.00	nd	0.02 ± 0.01
16:1 n-7	7.09 ± 0.06	5.79 ± 0.03	6.01 ± 0.45
17:1	0.35 ± 0.01	0.39 ± 0.01	0.41 ± 0.03
18:1 n-9	15.49 ± 0.05	21.32 ± 0.22	17.64 ± 0.31
20:1	5.07 ± 0.09	4.40 ± 0.08	3.95 ± 0.21
22:1 n-9	7.03 ± 0.28	0.79 ± 0.02	0.49 ± 0.05
20:2 n-4	0.35 ± 0.01	0.26 ± 0.01	0.30 ± 0.09
n-3 series			
18:3 n-3	1.66 ± 0.02	0.59 ± 0.03	1.26 ± 0.05
20:3 n-3	0.15 ± 0.02	0.10 ± 0.01	0.09 ± 0.02
20:5 n-3	14.02 ± 0.68	9.24 ± 0.22	11.27 ± 0.25
22:6 n-3	17.84 ± 1.52	20.64 ± 0.69	19.23 ± 0.66
n-6 series			
18:2 n-6	2.06 ± 0.07	1.40 ± 0.11	2.95 ± 0.25
18:3 n-6	0.15 ± 0.03	0.34 ± 0.01	0.14 ± 0.03
20:3 n-6	0.05 ± 0.01	0.15 ± 0.01	0.13 ± 0.01
20:4 n-6	0.75 ± 0.03	0.80 ± 0.02	2.51 ± 0.45
Totals			
Saturates	29.68 ± 0.11	33.74 ± 0.75	32.92 ± 0.57
Monoens	35.18 ± 0.31	32.75 ± 0.31	28.61 ± 0.97
n-3	33.68 ± 2.19	30.56 ± 0.88	31.83 ± 0.66
n-6	3.01 ± 0.07	2.69 ± 0.09	5.74 ± 0.65
n-3:n-6	11.19 ± 0.81	11.40 ± 0.41	5.97 ± 0.64

the liver and the gonads. The fatty acid 22:1 n-9 was very low in the liver and the gonads compared to the diet.

The long chain n-3 fatty acids were found in high amounts in both the diet and the tissues. The proportion of 20:4 n-6 in the gonads was three times higher than in the liver or the diet, which had very low amounts of that fatty acid. While total n-6 levels increased, n-3 to n-6 ratio decreased in the gonads compared to the diet and the liver composition. PCA separated the diet from the livers on axis 1, describing 42.6% of the total variation (Fig 5.10). However, the fatty acid profiles of gonads were in between those two groups. The parameters associated with this variation were 14:0, 18:3 n-3, 15:0, 20:5 n-3, 20:2 n-4, 22:1 n-9, 24:0 and 16:1 n-7 in the positive direction, and 16:0, 18:1 n-9, 20:3 n-6, total saturates and 18:3 n-6 in the negative direction. Axis 2 separated gonads from diet and liver, and accounted for 31 % of the total variation. The major parameters which contributed are, in order, total n-6, 20:4 n-6, 18:2 n-6, 17:0 and 18:0 in the positive direction, and total nonoenes, n-3:n-6 and 20:1 in the negative direction.

AA treatment

In the AA treatment, the fatty acid 16:0, and therefore total saturates increased in the livers compared to the diet (Table 5.11). Total monoenes were also elevated in the liver compared to the diet. In general, 18:2 n-6, 20:4 n-6 and total n-6 were decreased in the liver compared to the diet, but these fatty acids were higher in the gonads. The fatty acid 20:5 n-3 was lower in the liver compared to the diet and the gonads, but 22:6 n-3 increased in the gonads when compared to the diet. The gonad had a higher amount of 22:6 n-3 compared to the liver and the diet. Total n-3 was lower in the liver but higher in the gonad than in the diet. The fatty acid 20:3 n-3 was not found in the diet or any tissue.

PCA separated the diet and the gonads from the liver on axis 1, describing 50.7% of the total variation (Fig 5.11). The major parameters were in order, total n-3, 18:3 n-3, 14:0. 20:5 n-3, 22:6 n-3, n-3 to n-6 ratio, 20:4 n-6, total n-6 and 18:2 n-6 in the positive direction and 18:1 n-9, total monoenes, 16:0, total saturates and 18:0 in the negative direction. Liver and gonads were separated from the diet on axis 2 based

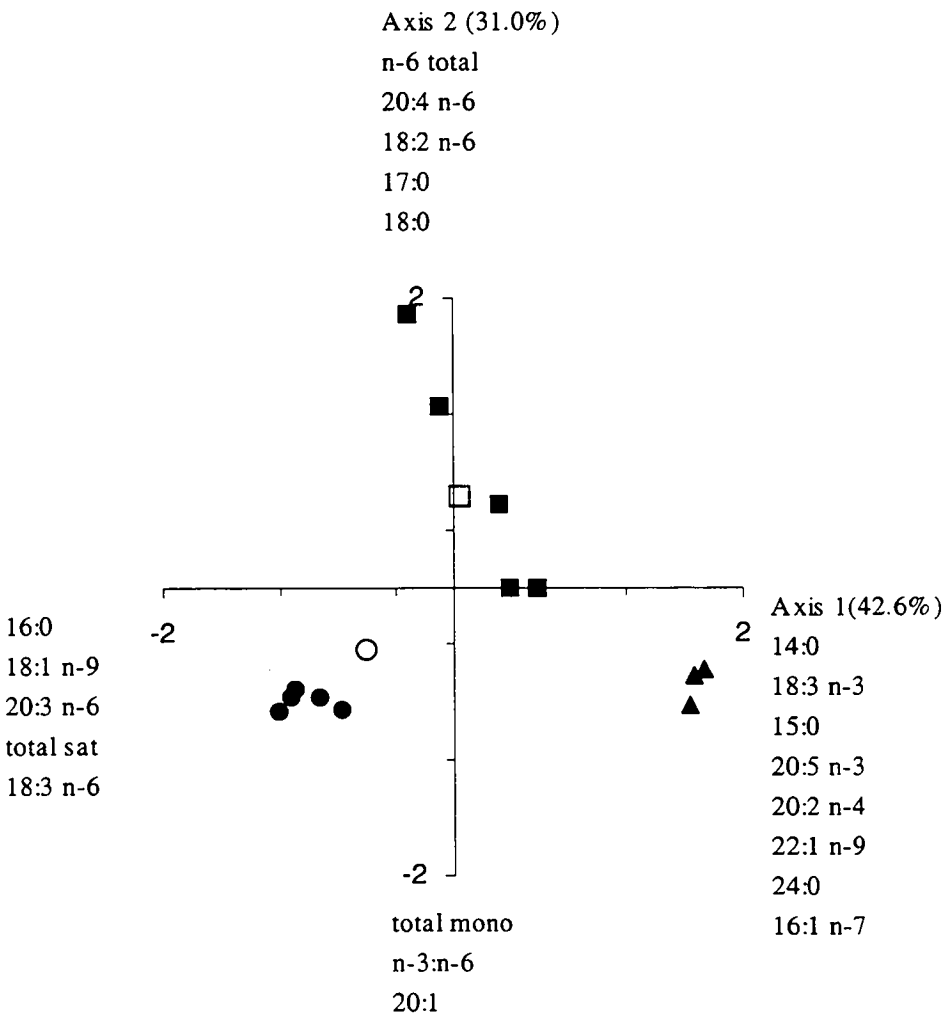

Figure 5. 10 Plot of the result of the principle component analysis of the fatty acids of the gonads and livers of barramundi fed diet containing fish oil. The amount of variance attributable to each axis and the fatty acids providing the major contribution to the derivation of the axes are indicated on each axis. (● - Liver; ■ - Gonad; ▲ - FISH diet; ○ - liver (female fish); Δ - gonad (female fish)).

Table 5.11 Fatty acid composition (mean ± SE) (% of total fatty acids) of diet and tissues of barramundi fed the AA diet. nd = not detected.

Fatty acid	AA diet	Liver	Gonad
14:0	2.02 ± 0.09	1.31 ± 0.09	2.34 ± 0.10
15:0	0.06 ± 0.03	0.07 ± 0.01	0.15 ± 0.02
16:0	15.02 ± 0.26	25.57 ± 0.16	18.11 ± 0.31
17:0	0.19 ± 0.01	0.17 ± 0.01	0.23 ± 0.03
18:0	5.71 ± 0.01	8.96 ± 0.51	6.84 ± 0.17
20:0	0.52 ± 0.01	0.41 ± 0.01	0.31 ± 0.06
22:0	0.71 ± 0.03	0.47 ± 0.02	0.42 ± 0.07
24:0	0.61 ± 0.03	0.60 ± 0.02	0.35 ± 0.02
16:1 n-7	1.96 ± 0.03	2.99 ± 0.29	2.89 ± 0.13
17:1	0.07 ± 0.00	0.12 ± 0.01	0.32 ± 0.08
18:1 n-9	30.08 ± 0.08	32.30 ± 0.42	27.88 ± 0.74
20:1	0.61 ± 0.01	0.93 ± 0.04	1.04 ± 0.12
22:1 n-9	0.07 ± 0.01	0.08 ± 0.01	0.12 ± 0.01
20:2 n-4	0.31 ± 0.02	0.22 ± 0.02	0.24 ± 0.03
n-3 series			
18:3 n-3	0.38 ± 0.02	0.12 ± 0.02	0.37 ± 0.03
20:3 n-3	nd	nd	nd
20:5 n-3	3.81 ± 0.09	1.25 ± 0.18	3.47 ± 0.15
22:6 n-3	4.09 ± 0.16	3.58 ± 0.35	6.28 ± 0.48
n-6 series			
18:2 n-6	10.48 ± 0.12	4.69 ± 0.42	8.36 ± 0.35
18:3 n-6	1.44 ± 0.01	1.34 ± 0.01	1.20 ± 0.05
20:3 n-6	1.28 ± 0.11	1.32 ± 0.09	1.15 ± 0.04
20:4 n-6	20.56 ± 0.39	13.48 ± 0.91	17.85 ± 0.47
Totals			
Saturates	24.85 ± 0.24	37.57 ± 1.40	28.82 ± 0.16
Monoens	32.80 ± 0.06	36.43 ± 0.63	32.31 ± 0.61
n-3	8.28 ± 0.26	4.94 ± 0.49	10.12 ± 0.65
n-6	33.76 ± 0.38	20.82 ± 1.38	28.62 ± 0.51
n-3:n-6	0.25 ± 0.01	0.24 ± 0.01	0.35 ± 0.03

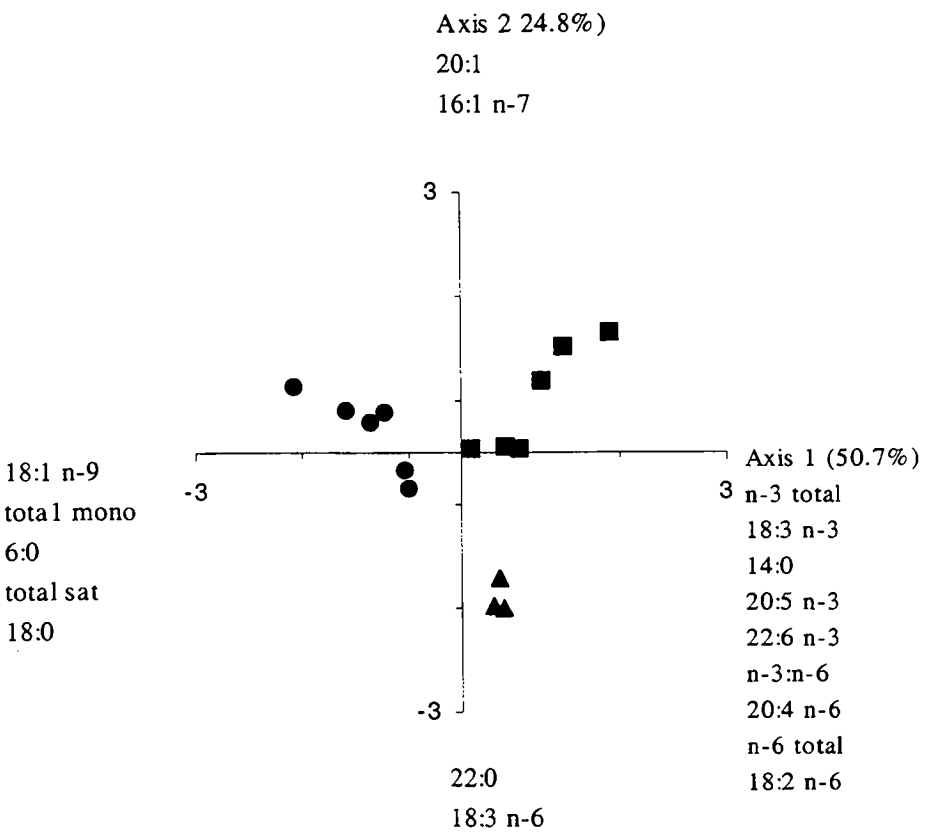

Figure 5. 11 Plot of the result of the principle component analysis of the fatty acids of the gonads and livers of barramundi fed diet containing Arachidonic acid. The amount of variance attributable to each axis and the fatty acids providing the major contribution to the derivation of the axes are indicated on each axis. (● - Liver; ■ - Gonad; ▲ - AA diet).

Table 5.12 Plasma Oestradiol-17β concentrations (mean ± SE) at week 18 of male barramundi fed diets with varying fatty acid sources. (LIN = Linseed oil; SOY = Soybean oil; FISH = Fish oil; AA = Arachidonic acid). n = Sample size; Means are not significantly different (P> 0.05).

Diet	n	Oestradiol-17β ($pg.ml^{-1}$) Wk18
LIN	8	32.0 ± 2.7
SOY	12	37.7 ± 2.9
FISH	11	33.2 ± 2.9
AA	12	36.3 ± 2.5

on 20:1 and 16:1 n-7 fatty acids in the positive direction and 22:0 and 18:3 n-6 in the negative direction. Axis 2 contributed to 24.8% of the total variation.

5.3.9 Plasma hormone levels

Oestradiol-17β (E_2)

Data for plasma E_2 levels were only available at week 18 (Table 5.12). Plasma E_2 levels were not significantly different between treatments (one way ANOVA; $F = 0.844_{(3.40)}$; $P>0.05$). Plasma E_2 levels of transitional and female fish were relatively higher compared to those of male fish (Table 5.13).

Testosterone (T)

Fish in all four treatments showed a similar pattern of plasma T changes during the experimental period (Table 5.14). Repeated measures analysis showed that plasma T levels significantly varied with time (repeated measures ANOVA; $F = 53.9_{(2.42,103.0)}$; $P<0.05$). There was also a significant interaction between time and dietary treatment (repeated measures ANOVA; $F = 2.92_{(7.26,103.0)}$; $P<0.05$). One way ANOVA showed that this variation is due to plasma T concentrations at week 12 being significantly different between treatments. However, plasma T levels at week 0, 6 or 18 were not significantly different between treatments.

In all four treatments plasma T level significantly decreased at week 6 and then increased significantly at week 12. However, plasma T concentrations significantly decreased at the last sampling date (week 18) in all but the FISH treatment.

11keto Testosterone (11kT)

Plasma 11kT level showed a similar pattern in all four treatments (Table 5.15), decreasing significantly with time (repeated measures ANOVA, $F = 30.6_{(1.82,69.3)}$, $P<0.05$), but showing no difference between treatments (repeated measures ANOVA, $F = 1.27_{(5.47,69.3)}$, $P>0.05$).

Comparison between means within the same treatment showed that fish on the LIN diet had significantly lower plasma 11kT concentration at week 12 than at week 6, at week 12 and 18 than at week 0. In the SOY treatment, plasma T concentration.

Table 5.13 Plasma Oestradiol-17β (E_2), Testosterone (T) and 11keto-Testosterone (11kT) levels of transitional and female fish. (LIN = Linseed oil; FISH = Fish oil).

Treatment/ Gonad stage		Wk 0	Wk 6	Wk 12	Wk18
(a) *Oestradiol-17β*					
LIN	*Transitional(T4)*	No data available			73.5 **
	Transitional (T4)				59.8 **
FISH	*Female*				59.9 **
(b) *Testosterone*					
LIN	*Transitional (T4)*	128	54	150	55
	Transitional (T4)	50	63	157	72
FISH	*Female*	118	85	119	69
(c) *11keto testosterone*					
LIN	*Transitional (T4)*	468	347	727	68 *
	Transitional (T4)	379	331	268	95 *
FISH	*Female*	616	556	280	119 *

*11kT values at week 18 are relatively lower than values from male fish (range 169- 2640 $pg.ml^{-1}$ except in one male fish (LIN treatment) which had low plasma 11kT level of 122 $pg.ml^{-1}$).

** E_2 values are relatively higher than those from male fish (range 16-53 $pg.ml^{-1}$)

Table 5.14 Plasma Testosterone (T) concentrations (mean ± SE) of barramundi fed diets containing different fatty acids. (LIN = Linseed oil; SOY = Soybean oil; FISH = Fish oil; AA = Arachidonic acid). # indicates significant difference (P<0.05) with the initial value. # indicates significant difference (P<0.05) with the initial value (wk 0). * indicates significant difference (P<0.05) with the preceding value. Means with the same superscript in any column are not significantly different (P> 0.05).

Diet	Testosterone ($pg.ml^{-1}$)			
	Wk 0	Wk 6	Wk 12	Wk18
LIN	155 ± 11	82 ± 8 $^{\#}$	172 ± 10 ^{*,ab}	93 ± 13 $^{\#,*}$
SOY	162 ± 12	84 ± 6 $^{\#}$	197 ± 12 ^{*,b}	68 ± 8 $^{\#,*}$
FISH	182 ± 22	98 ± 9 $^{\#}$	145 ± 5 ^{*,a}	118 ± 21
AA	192 ± 18	106 ± 9 $^{\#}$	178 ± 9 ^{*,ab}	66 ± 9 $^{\#,*}$

Table 5.15 Plasma 11keto Testosterone (11kT) concentrations of barramundi fed diets containing different fatty acids. (LIN = Linseed oil; SOY = Soybean oil; FISH = Fish oil; AA = Arachidonic acid). Means in any column are not significantly different (P> 0.05). # indicates significant difference (P<0.05) with the initial value (Wk 0). * indicates significant difference (P<0.05) with the preceding value.

Diet	11keto Testosterone ($pg.ml^{-1}$)			
	Wk 0	Wk 6	Wk 12	Wk18
LIN	946 ± 97	770 ± 77	398 ± 45 $^{\#,*}$	487 ± 72 $^{\#}$
SOY	1362 ± 184	626 ± 75 $^{\#}$	484 ± 50 $^{\#}$	382 ± 56 $^{\#}$
FISH	1077 ± 260	860 ± 116	530 ± 99 $^{\#,*}$	435 ± 35 $^{\#}$
AA	1024 ± 179	741 ± 111	504 ± 70 $^{\#,*}$	429 ± 61 $^{\#}$

significantly decreased at week 6, and was maintained at a low level. In the FISH and AA treatments, plasma 11kT concentration significantly decreased at week 12. Values at week 12 and 18 were significantly lower than at week 0.

Plasma 11kT concentrations found in transitional and female fish were relatively lower than those of male fish (Table 5.13).

5.4 DISCUSSION

A number of major findings were observed in this study.

These are: Growth of barramundi was influenced by dietary fatty acid profile; Dietary fatty acid profile was highly correlated with the tissue fatty acid profile; Gonads preferentially accumulate n-3 and n-6 HUFA, particularly 20:5 n-3, 22:6 n-3, and 20:4 n-6; and the dietary fatty acid profile used in the present study did not influence plasma reproductive steroid hormone concentrations.

The growth of barramundi in this study was significantly affected by dietary fatty acid level. Fish fed the LIN diet had a lower body weight at the end of the experiment, indicated by low SGR and %BWG throughout. Fish fed the AA diet had the highest growth, while fish fed the SOY and FISH diets had intermediate growth. Mean SGR values ranged between 0.28 and 0.49. These were comparable to those found in previous chapters (0.24 - 0.53 for Daily feeding group in chapter 3 and 0.11-0.58, chapter 4).

Generally, all fish exhibited good growth, and no fatty acid deficiency symptoms were apparent during the study period. Therefore, it can be assumed that all diets provided sufficient fatty acid requirements for growth of adult barramundi, albeit at different rates.

There is no information available on the particular dietary fatty acid requirements of barramundi broodstock in the literature and there is scarce information on those of other species. Furuita *et al.* (2000) reported that Japanese flounder male broodstock fed diets with different n-3 HUFA levels (0.4 – 2.1% of the diet) showed no significant differences in growth. However, growth of females was improved with elevated levels of dietary n-3 HUFA. Similarly, no differences were found in growth of male European sea bass (*D. labrax*) broodstock fed diets with different levels of EPA (20:5 n-3) and AA (20:4 n-6) (Asturiano *et al.*, 2001). Similar to the present study, Acharia *et al.* (2000) reported that female cat fish (*Clarias batrarchus*) broodstock fed a diet with 18:2 n-6 exhibited higher growth than fish fed a diet rich in 18:3 n-3. However, many studies did not pay attention to the effect of dietary fatty acids on broodstock growth, but concentrated on the reproductive performance (Fernandez-Palacios *et al.*, 1995; Bell *et al.*, 1996; 1997; Navas *et al.*,

1997; 1998; Rodriguez *et al.*, 1998; Bruce *et al.*, 1999). These studies did not mention dietary fatty acid requirements of the broodstock they studied.

The importance of HUFA as a dietary component of barramundi larvae has been reported previously. Dhert *et al.* (1990) have shown that newly hatched barramundi larvae fed on n-3 HUFA (20:5 n-3 and 22:3 n-6) enriched artemia had superior physiological conditions, which were reflected by low mortality rates. Those fed on non-enriched artemia did not achieve metamorphosis and died of a nutritional deficiency. Furthermore, it has been reported that the dietary n-3 HUFA requirements of barramundi fingerlings is 1.0 to 1.7 % of the diet (Boonyaratpalin, 1997). However, there is no other information available on the fatty acid requirements of barramundi.

Previous studies showed that marine fish species require n-3 HUFA, mainly EPA (20:5 n-3) and DHA (22:6 n-3), for normal growth and survival. This need is mainly due to the important structural role of those fatty acids as membrane phospholipid components (Sargent *et al.*, 1989), and the inability of marine fish to synthesise EPA and DHA from linolenic acid (18:2 n-3) (Sargent *et al.*, 1989; March, 1993; Steffens, 1997).

Although numerous studies have reported on the effect of fatty acids on the growth of larval fish in other teleosts, the results are often controversial due to the different species studied and the different levels and sources of fatty acids incorporated in the diets. For example, it has been reported that milk fish fry fed diets with different fatty acid sources constotuting 1% of the diet (18:2 n-6, 18:3 n-3, 20:4 n-6 or n-3 HUFA (60% 20:5 n-3 + 40% 22:6 n-3)) showed no differences in growth, food conversion, SGR and survival (Alava and Kanazawa, 1996). However, studies on channel catfish showed that high levels of dietary 18:2 n-6 or 18:3 n-3 (10% of safflower or linseed oil) reduced growth, whereas saturated, mono saturated and HUFA (fish oil) enhanced growth (Stickney and Andrews, 1972; Murray *et al.*, 1977). Ibeas *et al.* (1996) reported that gilthead seabream juveniles fed a diet containing 1% dietary n-3 HUFA (20:5 n-3 and 22:6 n-3) had the highest growth and feed conversion compared to fish fed diets with 0.1% or 1.5% of n-3 HUFA. In another study, gilthead sea bream larvae fed diets containing 1% AA (20:4 n-6) showed better growth than fish fed on diets containing 0.1% AA (Bessonart *et al.*, 1999). It has also been reported that excess dietary n-3 HUFA depresses growth in

several fish species (Watanabe, 1982). For example, high percentages of n-3 HUFA (>5%) depressed growth in red drum larvae, while low levels (<5%) of these fatty acids improved the growth (Brinkmeyer and Holy, 1998). Results from these studies demonstrate that an optimum dietary fatty acid level is required for maximum growth and survival of larval fish. However, varying nutrient requirements due to different physiological status of larval and adult fish, make it difficult to compare the dietary fatty acid requirements between these two stages.

A key feature of these studies is a recognition of the difficulty in establishing the absolute requirements of these important fatty acids for fish. This is due to the fact that not only the requirement for each fatty acid be determined, but also the relative proportions of DHA to EPA, AA to EPA and n-3 to n-6 ratio should be satisfied. For instance, Sargent *et al.* (1999) discussed the importance of the dietary ratio EPA:AA in determining eicosanoid actions, and the difficulties in achieving dietary balances of those fatty acids practically.

In addition, differences in fatty acid requirements of males and females has also been reported in some teleosts. For example, male and female hybrid red tilapia fed the same diet showed differences in fatty acid profiles of muscle and liver (De Silva *et al.*, 1997). In the present study, fatty acid profiles of tissues of male and transitional or female barramundi were not different from each other.

Tissue Fatty Acids

When comparing treatments, it was apparent that the fatty acid composition of the gonads and the livers of barramundi in the present study were affected by the fatty acid profiles of the diet. The predominant fatty acids in the diets were all present in comparatively high amounts in both tissues. This is similar to the finding that dietary fatty acid compositions influence the patterns of tissue fatty acid levels (Henderson and Sargent, 1985; reviewed by Steffens, 1999), which has been observed both in wild and cultured environments (Labbe *et al.*, 1993; Sheikh-Eldin *et al.*, 1996; Salhi *et al.*, 1997; Ahlgren *et al.*, 1999; Roy *et al.*, 1999; Sargent *et al.*, 1999; Grun *et al.*, 1999; Czesny *et al.*, 2000),

In the present study, the fatty acids 16:0 and 18:1 n-9 were abundant in both tissues in fish in all treatments and were present in high amounts in all diets. Similar

results were found for other teleosts. Major fatty acids found in rainbow trout spermatozoa were 16:0 and 18:1 (Pustowka *et al.*, 2000), while in sea bass the major fatty acids were 16:0, 18:0, 18:1 n-7 and 18:1 n-9 (Bell *et al.*, 1996). Drokin (1993) examined fatty acid profiles of sperm from several marine and freshwater fish species and reported that 16:0, 18:0, 18:1 and 20:1 were the most abundant saturates and monoenes in sperm. Many studies reported that 16:0 and 18:1 n-9 are the main sources of energy in the tissues, and are the primary fatty acids selectively incorporated into membrane phospholipids (Ibeas *et al.*, 1996; Navas *et al.*, 1997; Rodriguez *et al.*, 1998). In addition, 18:1 n-9 is the major end product of the *de novo* synthesis (Rodriguez *et al.*, 1998).

It appears that the most available fatty acid source is incorporated into the tissues. However, the amount or proportion of incorporation differed between treatments. Tissues of fish fed the LIN and the SOY diets contained very high amounts of short chain n-3 and n-6 fatty acids respectively compared to the other two treatments, but the proportion of these fatty acids decreased considerably in both tissues in comparison to their respective diet. In contrast, both tissues of fish fed FISH or AA diets were comprised of high amounts of long chain n-3 or n-6 fatty acids and the proportions were unchanged in comparison to FISH or AA diets.

It is apparent that fish prefer to accumulate long chain fatty acids in the liver and the gonads at the expense of short chain fatty acid content. In fish from the LIN treatment, the level of 18:3 n-3 was decreased compared to the diet (but higher compared to other treatments), while the levels of 20:5 n-3 and 22:6 n-3 were increased in concentration in the gonads. The concentration of fatty acid 20:4 n-6 also increased 10 fold in the gonads. In the livers of these fish, 22:6 n-3, 18:3 n-6 and 20:3 n-6 fatty acids increased in concentration in comparison with the LIN diet. In fish on the SOY treatment, the levels of short chain n-6 fatty acid (18:2 n-6) decreased in both tissues compared to the diet, while 18:3 n-6 and 20:3 n-6 levels increased in concentration in the liver and 20:4 n-6 accumulated (25 fold) in the gonads. In addition, while there was less 18:3 n-3 in both tissues, long chain n-3 fatty acids (20:5 n-3 and 22:6 n-3) accumulated in the gonads.

In the FISH treatment, long chain n-3 fatty acids were readily available in the diet and there was no considerable change in the proportion of these fatty acids in the

gonads. However, these fish apparently accumulated 18:2 n-6 and 20:4 n-6 in their gonads. This accumulation resulted in considerable change (~50% decrease) in n3: n-6 ratio in the gonads of fish fed the FISH diet. In the AA treatment, 20:4 n-6 was readily available in the diet. Although, there was less 20:4 n-6 in the liver than in the diet, fish preferentially accumulated this fatty acid in their gonad. In addition, the amount of 18:2 n-6 was lower in the liver but greater in the gonads. The long chain n-3 fatty acids (20:5 n-3 and 22:6 n-3) were accumulated in the gonads when compared to the liver.

When comparing tissues from all treatments, gonads generally contained higher proportions of 20:5 n-3, 22:6 n-3, 20:4 n-6, and therefore total n-3, than in livers. Asturiano *et al.* (2001) reported that irrespective of the dietary fatty acid profile, 22:6 n-3 and 20:4 n-6 increased in the milt of European sea bass fed different diets. Drokin (1990) has also shown that 18:2 n-6, 20:4 n-6, 20:5 n-3 and 22:6 n-3 are the most abundant polyunsaturates in the sperm of both marine and fresh water fish. Therefore, accumulation of these fatty acids in reproductive tissues seems to be common in teleosts.

From these results, it seems that although the fatty acid profile of tissues mainly depends on the dietary fatty acid profile, fish preferentially accumulate 20:4 n-6, 20:5 n-3 and 22:6 n-3 in their gonads. This accumulation is especially apparent when short chain fatty acids were predominant in the diet. The accumulation of long chain fatty acids may occur due to elongation and desaturation of fatty acids in the tissues or through preferential hydrolysis of short chain fatty acids (Sheikh-Eldin *et al.*, 1996). When preformed dietary long chain fatty acids are available, fish appear to accumule those fatty acids in their gonads.

The liver has a major role in fatty acid metabolism, acting as a centre of distribution of nutrients to other tissues (Collins and Anderson, 1995). In liver tissue, 18:1 n-9 increased in concentration compared to the respective diet. The 18:1n-9 has been identified as the predominant fatty acid in the liver of other teleosts (Sheikh-Eldin *et al.*, 1996), and as the major end product *of de novo* synthesis (Rodriguez *et al.*, 1998). Saturates and monoenes were higher than to long chain n-3 and n-6 fatty acids in the liver. In the n-6 fatty acid series, 18:2 n-6 tended to decrease, while 18:3 n-6 and 20:3 n-6 tended to increase in the liver. The further desaturated fatty acid,

20:4 n-6, increased in concentration in the gonads. This indicates that 20:4 n-6 is either being synthesised by elongation and desaturation in the tissues, or being spared from hydrolysis (Sheikh-Eldin *et al.*, 1996) and accumulated in the gonads.In all treatments except the FISH treatment, where long chain n-3 fatty acids were already high in content, there were more 20:5 n-3 and 22:6 n-3 in the gonads than in the livers. Both tissues had higher concentrations of those fatty acids than in diets. In the LIN and the SOY treatment where high concentrations of dietary 18:3 n-3 were available, both tissues had lesser amount of this fatty acid compared to the respective diet. The decrease of short chain fatty acids and increase in long chian fatty acids again indicates that 20:5 n-3 and 22:6 n-3 are being synthesised, possibly by elongation and desaturation of 18:3 n-3, or protected from hydrolysis or perhaps a combination of both (Sheikh-Eldin *et al.*, 1996).

The importance of EFAs on reproduction has been reported for some cultured fish species. However, these studies were mainly focussed on females. The dietary intake of EFAs, especially long chain PUFA of the n-3 series, has a profound effect on ovarian and embryonic development (Navas *et al.*, 1997). N-3 HUFA deficiency in diets resulted in lower spawning quality, fecundity, viability and hatchability of eggs in many teleosts (Fernandez-Palacios *et al.*, 1995; Navas *et al.*, 1995; 1997; Rodriguez *et al.*, 1998; Furuita *et al.*, 2000). An increase of n-3 HUFA levels in the broodstock diet improved the % of normal larvae and survival in Japanese flounder (Furuita *et al.*, 2000) and in gilthead seabream (Fernandez-Palacios *et al.*, 1997). Sheikh-Ekdin *et al.* (1996) reported that tank reared Macquarie perch had lower levels of n-6 and higher n-3 to n-6 ratio in oocytes compared to wild fish. These authors suggested the differences in fatty acid profiles may be responsible for preventing the completion of the maturation cycle and ovulation of tank reared fish.

Excess n-3 HUFA in the broodstock diet was also shown to be negatively correlated with egg and larval viability. Fernandez-Palacios *et al.* (1995) reported that larval survival was reduced when broodstock gilthead sea bream were fed with excess n-3 HUFA (>1.6%). Bell *et al.* (1997) discussed the importance of establishing the best ratios of DHA/EPA/AA in formulated diets for broodstock when attempting to improve egg and larval viability. Morehead *et al.* (2001) observed that eggs of striped trumpeter broodstock fed the commercial pellets had relatively low DHA/EPA and n-

3:n-6 ratios when compared to those of broodstock fed chopped fish. These authors suggested that the commercial diet may be inferior for larval growth and survival.

Few studies have investigated the dietary fatty acid influence on male broodstock performance. Leray and Pelletier (1985) reported that the eggs and sperm of rainbow trout fed diets either enriched with n-3 fatty acids or deficient in n-3 but rich in n-6, also had increased amounts of the respective fatty acid. Fatty acid profile of sperm of rainbow trout fed corn oil or fish oil diets clearly reflected dietary fatty acid profile (Labbe *et al.*, 1993). Although most available fatty acids were incorporated into the sperm, this incorporation did not alter sperm fertilising ability (Labbe *et al.*, 1993). In contrast, Pustowka *et al.* (2000) reported the influence of the dietary fatty acid profile on the fertilising capacity of rainbow trout spermatozoa during cryopreservation. Dietary fatty acids affected the fatty acid profile of sperm and consequently influenced the plasma membrane integrity and fertility of rainbow trout. Spermatozoa from fish fed diets low in PUFA but high in saturates appeared to have increased resistance to cryopreservation damage, consequently produced higher % of eyed embryo than those from fish fed high n-3 or high n-6.

These results indicate that optimum fatty acid levels (particularly HUFA) are needed for better spawning performance and quality of broodstock. Conversely, deficiency or excess of these fatty acids is unfavourable for spawning performance or quality. However, as described previously, absolute amounts of these fatty acids are difficult to determine, as not only the amount but also the relative proportions are important in regulating these effects.

Gonado Somatic Index

The gonads of fish in all treatments were very small and GSI values did not differ. GSI were low (mean 0.02; range 0.015-0.034) when compared to those (mean 0.06; range 0.03-0.12) from chapter 4. Interestingly, even though the gonads were relatively small, continuous development occurred in these tissues and fish had various gonad stages (1-4) at the end of the experiment. However, it is apparent that gonad development of male barramundi varied largely within the same treatment group (although fish originated from the same stock and were nearly the same size and

were reared in the same conditions) and it is therefore not predictable. This has been previously observed in barramundi in this laboratory. (Anderson, pers. comm.)

Fatty acids and hormone levels

There were no significant relationships found between plasma hormone levels and dietary fatty acids levels in the present study. Plasma E_2 data are only available for week 18 and where it did not vary between treatments. Plasma E_2 levels of male barramundi in the present study were comparable to the values found in the previous chapters (18 - 40 $pg.ml^{-1}$ in chapter 3; 21 - 33 $pg.ml^{-1}$ in chapter 4). This may be the minimal level or range of plasma E_2 levels in male barramundi plasma. Plasma E_2 levels of transitional or female barramundi were slightly, but clearly, higher (59 - 74 $pg.ml^{-1}$) than the levels found in male plasma. The comparatively low plasma levels of E_2 in transitional and female fish may be related to the comparatively smaller sizes of gonads observed in the present study. Female barramundi have plasma E_2 concentrations up to 300 $pg.ml^{-1}$ (Anderson, pers. comm.)

Plasma T levels showed similar patterns, with decreasing T levels at weeks 6 and 18 and increasing T levels at week 12 in all treatments. The physiological reason for this pattern is difficult to explain. However, as all fish had similar changes in plasma hormone levels at the same time, these patterns indicate that they experienced similar environmental or other factors which influenced hormone production. There were no differences in plasma T concentrations in male and transitional or female fish. As it is well known that T is a precursor of E_2 and 11kT in teleosts (Matty, 1985; Gower, 1988), and high levels of T can be found both in male and female fish (section 1.4.3.1), it is not surprising that plasma T levels did not vary between male, transitional and female fish.

Plasma 11kT concentrations clearly declined with time in all treatments. Nevertheless there is no relationship between dietary fatty acid levels and plasma 11kT levels. The plasma 11kT levels of transitional and female fish were clearly lower than those of male fish. Guiguen *et al.* (1993) reported higher levels of plasma 11kT in male barramundi compared to the transitional and female fish. The low levels of plasma 11kT in females were reported in many other teleosts (review Borg, 1994; section 1.4.3.1).

Dietary fatty acid levels and reproduction

There is some evidence that dietary fatty acids influence plasma steroid levels in other teleosts and consequently affect reproductive performance. Cerda *et al.* (1995) reported that European sea bass (*D. labrax*) broodstock fed a commercial diet containing low PUFA and total n-3 fatty acids compared to the natural diet had lower levels of plasma T and E_2 levels. The fish fed the commercial diet had reduced fecundity and egg viability. Navas *et al.* (1998) has shown European sea bass broodstock fed a natural diet that contained a higher AA content and higher DHA:EPA and AA:EPA ratio displayed low plasma E_2 levels together with better egg quality. In contrast, broodstock fed the commercial diets containing low DHA:EPA and AA:EPA ratios had low egg quality associated with altered plasma E_2 and GtH II levels (Navas *et al.*, 1998).

Some authors have reported the effect of short chain fatty acids on plasma steroid levels. Female catfish, *C. batrarchus*, broodstock fed a diet enriched with linseed oil (18:3 n-3) had elevated GSI and plasma T and E_2 levels (Acharia *et al.*, 2000). Fish fed a diet enriched with sunflower oil (18:2 n-6) had decreased plasma T levels and GSI, however their E_2 levels were not different to those of fish fed the basal diet (Acharia *et al.*, 2000). These authors concluded that 18:3 n-3 PUFA has a stimulatory role in steroid production. However, it is well known that fresh water fish have an ability to elongate and desaturate short chain PUFA into long chain C20 and C22 HUFA. These HUFA involved in synthesis of prostaglandin, which in turn may influence reproduction. Although these authors did not discuss this associated factor, there is a strong possibility that long chain fatty acids rather than short chain fatty acids influence the steroid production.

Although, the importance of n-3 HUFA in reproduction in female teleosts has been well established, very little information is available on the affect of these fatty acids on male reproductive performance in teleosts. There is evidence that dietary PUFA requirements of male and female broodstock may be different at least in some teleost species. Asturiano *et al.* (2001) compared the reproductive performance of male sea bass fed on a wet diet (trash fish), which had previously proved to be the most beneficial for female reproductive performance, with that of fish fed two commercial diets enriched with HUFA. Fish fed the HUFA enriched diets displayed

longer spermiating periods, and higher milt volumes, spermatozoa densities and % of spermiating males than those fed on the wet diet. Higher survival rates of larvae were observed with eggs fertilised from milt of fish fed on commercial diets. These authors suggested that a reduction in n-3 HUFA and an increase in AA which resulted in an altered n-3 to n-6 ratio may improve male reproductive performance in European sea bass. Although, high EPA and n-3 to n-6 ratio in the wet diet might have resulted in a better reproductive performance in females, it seems from their study that dietary PUFA requirements of male fish are different.

Steroidogenesis and eicosanoids are essential for testicular function. *In vitro* studies suggest the involvement of HUFA in testis function in male teleosts. Wade and Van der Kraak (1993) and Wade *et al.* (1994) showed that AA (20:4 n-6) stimulates testosterone production, while n-3 HUFA (20:5 n-3 and 22:6 n-3) attenuates GtH–stimulated steroid production and acts as an inhibitory regulator in gold fish testis *in vitro*. AA (20:4 n-6) induced prostaglandin production, while DHA (22:6 n-3) reduced prostaglandin production in European sea bass testis *in vitro* (Asturiano *et al.*, 2000). In addition, human chronic gonadotropin (HCG) enhanced AA-induced prostaglandin production, but suppressed EPA-induced prostaglandin production. From these results, it is clear that AA stimulates T production *in vitro* through conversion to prostaglandins. Booth *et al.* (1999) reported a decline in plasma levels of AA during development of testis in Atlantic male salmon, and suggested this may be correlated with the role of AA in prostaglandin synthesis.

The role of PUFA as precursors of prostaglandin in fish has been well established. Mercure and Van Der Kraak (1995) had previously demonstrated that AA stimulates testosterone production of ovarian follicles of gold fish *in vitro*, but inhibits T production at higher doses. In contrast EPA and DHA inhibited T production. Generally, there is no information on the importance of prostaglandins in conversion of T to 11kT in teleosts. Even though the role of PUFAs and eicosanoids in testicular and ovarian physiology of marine teleosts is not clear (Asturiano *et al.*, 2000), the results from those studies have demonstrated that PUFAs affect the endocrine control of reproduction through multiple mechanisms (Van Der Kraak and Biddiscombe, 1999).

There is also some evidence that free fatty acids influence endocrine function by affecting the binding of steroid hormones to proteins in the plasma and/or target tissues. In many species, reproductive steroids are bound to a sex steroid binding hormone (SSBP) and transported in the plasma (Laidley and Thomas, 1994; Hobby and Pankhurst, 2000). The presence of SSBP helps to protect steroids from rapid metabolic degradation and regulate the amount of steroid available to target tissues (Van Der Kraak and Biddiscombe, 1999). In gold fish, PUFA such as AA (20:4 n-6), EPA (20:5 n-3) and DHA (22:6 n-3), strongly inhibit the binding of E_2 to the SSBP and therefore reduce the affinity of SSBP (Van Der Kraak and Biddiscombe, 1999). This inhibition of binding will result in greater rates of degradation of sex steroids in animals with high levels of AA, EPA and DHA.

In the present study, dietary fatty acid levels did not show any relationship to the plasma hormone levels of fish. In all fish, plasma hormone levels showed similar patterns with time with the major male steroid 11kT, declining over the course of the experiment. The fatty acid levels of the diets used in the present study are extreme and are likely to contain more than the optimum fatty acid level. Therefore, high levels of fatty acids may inhibit prostaglandin production and in turn negatively affected the T production as suggested by Mercure and Van der Kraak (1995). Greater rates of degradation associated with reduced affinity for SSBP (Van der Kraak and Biddiscombe, 1999) may further result in the reduction in 11kT observed. As discussed by Hobby and Pankhurst (1997), the concentration of T measured is the function of rates of synthesis and degradation. Thus the fluctuation in T concentration observed at week 12 may be the result of decreased 11kT synthesis perhaps under the influence of a reduction in prostaglandin synthesis.

CHAPTER 6
GENERAL DISCUSSION

6.1 Introduction

Nutrition of fish is known to impact upon reproduction. This has been shown to be manifest by changes in time to reproduction and in various parameters related to spawning performance (Watanabe, 1985; Kanazawa, 1985a,b; Springate *et al.*, 1985; Shepard and Bromage, 1988; De Silva and Anderson, 1995). Food ration affects fecundity, egg size and the ability to achieve maturation. Favourable feeding conditions induce early maturation and reducing available food delays maturation (Scott, 1962; Bagnel, 1969, Wootton, 1972; Springate and Bromage, 1985; Springate *et al.*, 1985; Ali and Wootton, 1999a,b) and reduces fecundity (Scott, 1962; Bagnel, 1969, Ali and Wootton, 1999a,b). However, in some teleosts, unfavourable feeding conditions have induced maturation (Lowe-McConnell, 1982; Radampola, 1990; Svedang, 1991). The latter circumstance is probably an adaptation to harsh and changing environments which occur during their evolution (Lowe-McConnell, 1982).

The composition of the broodstock diet is also believed to have an influence on the reproduction and egg quality of several species. The roles of individual dietary components such as protein, lipid (in particularly fatty acids), vitamins and pigments have received attention in fish broodstock nutrition studies. Optimum nutrient levels as well as ratios between particular micronutrients, especially fatty acids, affect spawning performance in fish. Dietary protein level and quality affect oocyte development and consequently fecundity and egg and larval quality (Santiago *et al.*, 1983; 1985; 1988; De Silva and Radampola, 1990; Gunasekera *et al.*, 1995; Gunasekera and Lam 1997). Low dietary protein negatively affects ovarian recrudescence, spawning performance and egg quality (Santiago *et al.*, 1983; 1985; 1988; Cerda *et al.*, 1994; De Silva and Radampola, 1990; Gunasekera *et al.*, 1995; Gunasekera and Lim, 1997). Particular long chain HUFA deficient diets also negatively affect egg and larval quality in several teleosts (Watanabe, 1982; Cerda *et al.*, 1995; Navas *et al.*, 1997; 1998; Rodriguiez *et al.*, 1998; Bruce *et al.*, 1999; Furuita *et al.*, 2000).

Much of the work discussed above has been performed on females, with little work on males reported. Although sperm quality is a critical factor for egg hatchability/survival, male broodstock performance and/or sperm quality has been neglected in those studies. The few studies evaluating male broodstock nutrition

examined the effects of dietary fatty acid profile, lipid or food deprivation on spawning performance. Of those that have done so, contradictory results prevent general conclusions. Some studies have shown that reproductive investment in males is relatively small compared with that in females. It is proposed that dietary treatments do not influence maturation of males compared to females as a consequence of this reduced investment (Jobling *et al.*, 1993; Silverstein and Shimma, 1994; Karlsen *et al.*, 1995; Cerda *et al.*, 1994; Bromely *et al.*, 2000). On the other hand, food deprivation during particular periods of the reproductive cycle prevented maturation in male fish (Rowe and Thorpe, 1990a,b; Thorpe *et al.*, 1990; Reimers *et al.*, 1993) and dietary fatty acid profile influenced reproduction in male European sea bass (Asturiano *et al.* 2001). Again in apparent contradiction, low food quality induced maturation in male Arctic charr (Svedang, 1991).

All of the above studies discussed the effect of nutrition on reproductive output of animals. Although it is known that nutritional factors are involved in reproductive development, very few studies exist of the physiological mechanisms by which this occurs in fish (Watanabe and Miki, 1991). Development of the gonads in fish is under the control of the brain-pituitary-gonadal axis. GnRH produced by the hypothalamus act on pituitary cells which release gonadotropins into the circulation (Zohar *et al.*, 1989). The actions of FSH and LH are affected through stimulation of the production of gonadal steroids, including T, E_2 and 11kT (Fostier *et al.*, 1983). Increased plasma sex hormone levels are associated with gametogenesis and gonadal development in a variety of teleosts including barramundi (Guiguen *et al.*, 1993).

There is some evidence that food intake and endocrine function are closely related in fish and consequently, endocrine systems are sensitive to alterations in nutrient intake (MacKennzie *et al.*, 1998). Alterations in dietary quality, quantity and time of feeding have been shown to influence metabolic hormones, thyroid hormones and growth hormones (MacKennzie *et al.*, 1998). Although manipulation of diet composition or feeding regime is commonly performed in aquaculture, endocrine function is rarely evaluated in fish nutrition studies.

Standard barramundi hatchery practice in Australia involves one female to many male broodstock, largely because male barramundi performance is unpredictable (Anderson, pers. comm.). There is no common feeding practice for

barramundi broodstock in commercial hatcheries. It is hypothesized that nutrition will impact on reproductive performance through changes in concentrations of sex steroids. Thus, the aim of the present study was to investigate the effect of nutritional factors on reproductive performance of male barramundi in captivity. The effect of three major nutritional regimes; feeding frequency (food deprivation), dietary protein:energy ratio and dietary fatty acid profile was investigated. The effect of nutritional factors on plasma steroid concentrations and standard nutritional indices such as growth and body composition were evaluated.

6.2 Effects on growth and body composition

The nutritional treatments applied in this study led to predictable outcomes on growth and body composition.

Starvation resulted in loss of body weight and tissue nutrients, while nutrients were regained and compensatory growth occurred during the refeeding period. Low feeding frequency (every 7 d) resulted in reduced or no growth in fish, whilst fish fed more frequently (daily or every 3 d) showed higher growth. These findings were also reflected in improved body composition of fish fed more frequently.

Varying protein:energy ratios by varying dietary lipid resulted in comparable growth as a result of increased consumption of low energy diets, but body composition showed that high dietary energy led to greater storage of fat.

Dietary fatty acid profile affected the growth of the fish. Fish fed a diet with short chain n-3 fatty acids derived largely from linseed oil had lower growth than those of other treatments. Fish fed short chain n-6 and long chain n-3 fatty acids had intermediate growth and fish fed long chain n-6 had the highest growth. Dietary fatty acids profiles significantly affected the tissue fatty acid profile with tissue fatty acid profile highly correlated with the dietary fatty acid profile.

6.3 Effect on gonadal development

Some feeding regimes impacted significantly on gonadal development. In general, gonads were cycling with a range of stages of development including development of gonia cells and appearance of spermatocytes and spermatids apparent in any particular population (treatment group) of fish. However, a larger number of

testes from barramundi that were starved were at stage 1 (M1) compared with those of fish in other treatments. Similarly, gonads of fish fed the lowest energy diet were at an early developmental stage (M1) with tightly packed gonia cells. These gonads did not show evidence of any further development as observed in other treatments. Different dietary fatty acids had no apparent effect on the stage of gonadal development.

Despite the evidence of ongoing gametogenesis, the sizes of gonads observed in this study were relatively small. No information is available on GSI data for male barramundi in other captive or hatchery conditions. Moore (1982) reported that male barramundi in wild have GSI up to 0.5% (ripe males). GSI of 1.11% (2.7 kg fish) and 0.6% (2.5 kg fish) have been observed in our laboratory. These fish had running-ripe gonads. However, other fish held in the same tank under the same conditions had relatively small gonads and low GSI values (0.03 – 0.17 for 2.1 and 2.9 kg fish respectively). Thus, gonadal development in captive male barramundi appears to be highly variable.

6.4 Effects on reproductive hormones

Some clear effects of dietary treatment on reproductive hormones were observed. The effects were most clear in cases of extreme treatments.

Feeding regime affected the hormone production in small and large fish differently. Although starvation or low nutrient intake did not influence the low E_2 levels in small fish, the relatively higher E_2 level in larger fish was clearly reduced by starvation. In contrast, relatively high T level of small fish was reduced by starvation but low T level was not influenced by starvation in large fish. It may be that barramundi can maintain minimum plasma hormone concentrations with low nutrient intake or even with food deprivation and this level is not affected by nutritional status. However, in the less extreme treatments, the effects were not as clear. Fish in D or 3D regimes increased plasma T level although not significantly. Plasma E_2 level significantly decreased at week 18. In each of these treatments plasma hormone changes were similar. On the other hand, a period of refeeding after starvation clearly influenced the concentration of plasma steroid hormones in both size groups. Collins and Anderson (1999) have shown that reproductive development and plasma sex steroid levels of golden perch, respond to an increase in available food after a period

of starvation. This is probably an adaptation to fluctuating and unpredictable environmental conditions.

Dietary energy level did not significantly affect the plasma hormone levels in male barramundi. Plasma E_2 was relatively low throughout and did not show differences between treatments but plasma T level reduced with time. Interestingly, although gonad histology showed that the low energy diet suppressed gonad development while fish receiving any of the other three diets had gonads with normal development, the hormone levels were not different between treatments. Gonad sizes were comparatively small at the end of the study and it seems possible that the low T concentration is related to the small gonad sizes. However, in a group of animals killed at the beginning of experiment 2 (week 0), small gonads were found in fish with high T levels. Plasma 11kT did not show any particular trend with dietary P:E ratio, even in the lowest energy diet.

Dietary fatty acid profile did not affect the circulating concentration of plasma T or E_2. Plasma E_2 level was low at week 18 in male fish and plasma T level showed similar changes in all treatments. Plasma 11 kT level clearly declined with time and it is suggested that this may have been in response to the high dietary fatty acid levels negatively affecting hormone production. For example *in vitro* studies have shown that AA (20:4 n-6) at some level is known to induce T production, but DHA and EPA negatively affect on hormone production (Van der Kraak and Chang, 1990, Wade and Van der Kraak, 1993; Wade *et al.*, 1994). Alternatively, that high dietary fatty acid levels used in this study may have inhibited plasma T production.

6.5 Size effects

The fish used in this study were about the size of those transferred into commercial hatcheries and so the experiments reflected commercial practice. In all three experiments described here, gonad sizes were relatively small. Also common in commercial practice is poor male performance (Hogan *et al.*, 1987). For example, male barramundi captured from the wild in the main breeding season at Cairns had traces of milt but no fish produced any useable quantities (Hogan *et al.*, 1987). In addition, captive brood males were usually 'dry' and produced only 1-2 ml of milt when first handled but nothing at all a few hours later (Hogan *et al.*, 1987). Therefore

it seems, that small gonad size may be one possible reason for poor performance of barramundi in hatcheries. Similar difficulties in achieving sperm production in captive male common carp have been reported (Rothbard, 1981 cited in Stacey *et al.*, 1994).

Small gonad size may be related to the early stage of maturation expected of fish of this size. In both these experiments and in commercial practice, fish were selected to be male. Since barramundi are not sexually dimorphic, it is not possible to identify male, transitional and female fish without sacrificing the fish. Thus in order to be sure that fish were male, it is necessary that smaller fish sizes be used. The impact of using fish that are too small was most clearly apparent in the first experiment (chapter 3) where the smaller group of fish appeared to using energy for growth rather than for reproduction.

In all experiments, ongoing gametogenetic activity was observed in the less extreme treatments. This implies that the regulatory mechanisms for maintaining the cycling continued even though it appeared that these fish were directing most of their energy into growth rather than gonadal development. Since the major sites of sex steroid hormone production in teleosts are gonads (Billard *et al.*, 1982; Yoshikuni and Nagahama, 1991), it is possible that reduced or low plasma hormone levels observed in these studies may be related to the small gonad size. However, small gonads were found in fish with high T levels at the start of experiment 2. A number of other factors influence plasma sex steroids including development stage (Borg, 1994; Nagahama *et al.*, 1994), the balance between synthesis and degradation and metabolism of hormones (Hobby and Pankhurst, 1997), and stress (Pankhurst and Van der Kraak, 1997). Stress has also been shown as a critical factor that negatively influenced reproduction in many teleosts (Pankhurst and Van der Kraak, 1997). Although no stress parameters were measured in the present study, some barramundi of similar size reared in same conditions in our laboratory had high cortisol levels in the plasma. However, no reason was apparent for the high cortisol level. Therefore it may be that barramundi used in the present study were stressed and this negatively impacted on reproduction. Numerous stressors are possible in such captive conditions such as six weekly sampling, water quality changes, disease problems, and uncontrolled disturbances (eg., electricity failures, disturbances by people etc.) which may have impacted on reproduction of this fish.

In addition, factors affecting secretion of GnRH and GtH from the brain are also critical in regulating plasma hormone levels (Shepard and Bromage, 1988). Therefore, reduced hormone production may be associated with any of several factors unable to be measured in this study.

A further confounding factor in these studies is the nutritional history of the animals. Rowe and Thorpe, (1990), Thorpe *et al.* (1990b), Reimers *et al.* (1993) and Rowe *et al.*, (1991) found that the nutritional history of Atlantic salmon affected reproductive development over a long period. The current studies were conducted over 4.5 to 6 months and were clearly able to modify body composition in predictable ways but with limited clear outcomes on reproductive development. However, our understanding of nutritional effects on reproduction is so poor that the possibility of effects of long-term nutritional history cannot be discounted.

6.6 Which parameters?

Difficulties are experienced in studies of this type in identifying appropriate parameters to measure as indices of reproductive function. Most studies report proportion of males matured as judged by presence of milt (Cerda *et al.*, 1994; Bromely *et al.*, 2000), GSI (Rowe and Thorpe, 1990b; Svedang, 1991; Karlsen *et al.*, 1995; Ma *et al.*, 1998), visual inspection of gonad (Jobling *et al.* 1993; Silverstein and Shimma, 1994) or even scanning (Reimers *et al.* 1993). However, none of these parameters really represent the state of male reproductive maturity since they do not mention sperm quality data such as milt volume, sperm concentration and motility or fertilisation rate. For example, presence of milt does not give any idea about quality and quantity of milt. Such parameters were determined and reported by Asturiano *et al.* (2001). Further, the parameters mentioned above are the outcomes of reproductive development and provide no information about the mechanisms by which it is regulated.

An additional problem of this type of experiment is to find a method to evaluate parameters over time. Measurement of components in the circulation can be undertaken over a long period with repeated sampling, without having to sacrifice the animal. Providing this has no impact on the physiology of the system being evaluated, this will give a greater chance of evaluating a response of an individual to a treatment.

This is particularly important for studies of broodstock where animals are large and expensive and so regular sacrifice is undesirable. So although GSI and gonad histology may give a more easily interpreted indication of gonadal development, this cannot be achieved without killing experimental animals. Although perhaps difficult to interpret, one would expect that plasma hormone levels will give an indication about gonad development, as it is well established that plasma sex hormones are related to gonad development in barramundi (Guiguen *et al.*, 1993). Therefore, evaluating blood samples from individuals for a long period should provide an understanding of the impact of treatments on gonad development. However, in this study there appeared to be little direct relationship between the levels of hormone measured and gonadal development when the animals were sacrificed. As experienced by others (Hogan, 1987), difficulties in obtaining milt samples from barramundi also limited the use of more direct, non-sacrificial methods of evaluating gonadal development.

Although, plasma hormone changes during natural reproductive cycle of barramundi were available, no information on plasma hormone levels was available for barramundi reared in controlled conditions. Natural hormone cycles of barramundi appear comparable to other teleosts (Guiguen *et al.*, 1993). However, these observations were made when fish were in increasing photoperiod and temperature. In the present study, fish were in controlled conditions of high temperature, long photoperiod and high salinity for a long period. These conditions intentionally mimic those required for spawning. However, unpredictable male performance and the lack of synchronisation of male development under these conditions indicate that fish may become confused in these environments. As a consequence, they show different responses, thereby confounding the observations in any other than extreme conditions such as starvation or very low energy diets.

Other studies of the effects of nutrition on male (and female) reproductive development have investigated animals held under natural conditions (Rowe and Thorpe, 1990b; Svedang, 1991; Jobling *et al.*, 1993; Reimers *et al.*, 1993; Thorpe *et al.*, 1993; Cerda *et al.*, 1994; Silverstein and Shimma, 1994; Karlsen *et al.*, 1995; Bromley *et al.*, 2000; Asturiano *et al.*, 2001). However, very few studies, which were on females, revealed the impact of nutritional factors on reproductive hormones. In

these few studies, either plasma hormones were maximal at a different time or concentration with different dietary treatments (Cerda *et al.*, 1995; Navas *et al.*, 1997; 1998; Pereira *et al.*, 1998) allowing clear conclusions to be drawn.

6.7 General conclusion and future work

This study has contributed to our understanding in two important ways. There is very little information on the role of nutrition in the reproductive development of male teleosts and no other information for barramundi. Secondly, the attempt to focus on the mechanisms by which nutrition can influence reproduction is rare.

It is apparent from this study that extreme cases of nutrition are detrimental to male barramundi reproductive development. Starvation or low energy (15 $MJ.kg^{-1}$) resulted in impaired reproductive development. Alternatively, refeeding after food deprivation clearly enhanced plasma hormone concentrations. Under less extreme conditions, there appeared to be little effect. This matches some of the data in the literature which indicates that male fish expend less energy on reproduction and so are less affected by moderate changes in nutritional conditions (Wootton, 1990; Jobling 1993; Silverstein and Shimma 1994; Karlsen *et al.*, 1995; Damsgard *et al.*, 1999; Bromley *et al.*, 2000). However, conclusions regarding the effects of the moderate treatments are constrained. Since, the conditions under which these experiments were conducted appeared to be not ideal for reproductive development of male barramundi, with small gonad sizes observed even in the presence of gametogenesis.

Thus, this study must be considered a preliminary investigation. It does nevertheless provide a significant platform for future work regarding the effects of nutrition on male barramundi broodstock development and teleost reproduction in general. Work remains to be done investigating gross nutritional parameters of barramundi such as protein requirement and feeding level which will complement the studies of feeding frequency and energy level described in chapters 3 and 4.

This study also raises questions regarding the physiological mechanisms whereby nutrition and reproduction interact. The role of fatty acid nutrition in prostaglandin production and action, particularly at the whole animal level, is an exciting new area for investigation. Similarly, the interactions between nutrition,

reproductive steroids and other hormones is poorly considered in the literature and requires much additional work to achieve a complete understanding.

Future work will face similar constraints as the present study. The ability to deliver diets with equivalent characteristics such as palatability and smell while having different levels of protein and lipid and different fatty acids and vitamins will impact on the ease with which experimental outcomes can be interpreted. Similarly, provision of suitable conditions for reproduction, particularly in those species such as barramundi where broodstock are large, and the physiological impacts of using controlled environments to ensure year round reproduction, will provide challenges. It will be necessary to meet these challenges, however, if we are to gain a complete understanding of nutritional effects on reproduction and to provide our industries with information that will allow them to maintain commercially viable and profitable production.

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APPENDIX I

The recipes for buffer solutions and other reagents used throughout the study.

Formaldehyde Acetic acid calcium chloride (FACC) solution (for histology)	Formaldehyde (37-40%) 10 ml Galcial Acetic Acid 50 ml Calcium chloride dihydrate 1.3 g The solution made up to 100 ml using tap water.
Potassium Phosphate Buffer (pH 7.4)	0.04 M K_2HPO_4 0.01 M KH_2PO_4)
Steroid Assay Buffer (SAB) (pH 7.4)	0.08 M K_2HPO_4.3H2O 0.02 M KH_2PO_4 0.15 M NaCl 1 mM Ethylenediamine tetraacetic acid (EDTA) 0.15 mM Sodium Azide 0.1 %Bovine Serum Albumin)
Wash buffer	16 mM K_2HPO4.3H2O 3.9 mM KH_2PO4 30.8 mM NaCl 0.05% Polyoxyethylenesorbitan monolaurate (Tween-20)
Ellman reagent	0.8 M K_2HPO_4.3H2O 0.2 M KH_2PO_4 1.54 M NaCl 27 mM 5,5-dithiobis (2 nitrobenzic acid) (DTNB) 34.5 mM Acetyl thiocholine iodide) Store at – 20 ^{0}C as a concentrated solution and dilute 0.5 ml of aliquot to 20 ml with double distilled water just prior to use as the solution is light sensitive.

APPENDIX II

1701 BERKELEY STREET/ SANTA MONICA, CA 90404
PANTEX
1-800-421-6529 or (310) 828-7423
FAX (310) 829-9032

PANTEX

direct ^{125} I

ESTRADIOL

Catalog no. 174M

07/97

TABLE OF CONTENTS

ASSAY BACKGROUND AND USE

Pantex estradiol (E2) ^{125}I kits measure levels of **E2, principal estrogenic hormone,** in serum. Levels are used in diagnosis and therapy of infertility. Estradiol penetrates cell walls, binds to receptors which attach to nuclear elements to mediate estrogen activity (21). Uterus, breast, vagina, urethra, hypothalamus, pituitary, liver, skin (known targets) contain specific E2 receptors (1). Ovary, placenta, adrenal (prepubertal, postmenopausal), testis are sources. Only 1-2% is free in blood, the rest is bound to sex steroid hormone binding globulin (SHBG). Estradiol is rapidly oxidized to estrone (E1). Estrone is conjugated to form estrone sulfate, or oxido-reduced to estriol, etc. Kidneys excrete hydroxylated forms conjugated with glucuronide or sulfate. Estrogenic activities of free forms vary with tissue: Urogenital estrogen receptors do not bind estrone (2); estrone does not affect hypothalamus-pituitary (3), but induces more liver protein than E2. Estradiol is the major tissue hormone, estrone is a prohormone (2).

Failure of the old follicle to produce E2 causes menses which begin the **menstrual cycle.** Then, when a new follicle develops, Estradiol production measured in serum reflects its stage of development. **Estradiol peaks before ovulation** and feeds back positively to the hypothalamus-pituitary. LH surges produce ovulation. Estradiol drops after a broad lower luteal peak, or rises if pregnancy occurs. Synthetic estrogen produces precocious puberty, E2 < 20 pg/mL. (Adult E2 levels are found in other types of precocious puberty: true from, normal FSH; estrogen-secreting tumor, low FSH.) Oral contraceptives produce E2 levels <50 pg/mL. **Elevated E2** is often found in adult or prepubertal **males** with gynecomastia. HCG-secreting testis, liver or adrenal tumors or liver cirrhosis can increase E2 (4-7).

One of six couples is infertile. The causes are (in order of frequency): tubal dysfunction, male factors, anovulation. **Treatment is** usually **superovulation or IVF-ET** (except for bromocriptine treatment of hyperprolactinemia, and artificial insemination in cases of aspermia). Successes of **in vitro fertilization (with embryo transfer)** have popularized IVF-ET Clinics. **IVF-ET includes** superovulation; laparoscopy, aspiration of oocytes from follicles; in vitro fertilization; embryo transfer to uterus. Embryo implantation is the least efficient step, so several embryos are used. Gonadotropins recruit and develop multiple follicles (superovulation) followed by hCG or spontaneous LH surge. **Use of human menopausal gonadotropin (hMG)** was introduced in 1962, long before the era of oocyte removal and in vitro fertilization, to treat **anovulation** (symptom, amenorrhea; caused by hypothalamic/pituitary disorders (HA), polycystic ovarian syndrome (PCO), hyperprolactinemia) (8).

Superovulation is possible if functioning ovarian tissue exists. Clomiphene citrate (**CC**) is commonly tried first; **hMG** (followed by hCG), most powerful; human urine follicle stimulating hormone (hU-FSH), in trial; gonadotropin releasing hormone (GnRH), experimental. All are usable in IVF-ET and anovulation; CC is the most widely used (9). (Sera from patients taking 150 mg CC/day were assayed using Pantex 174 direct E2 kits) (10). **With CC** or CC plus hMG test LH every 4 hours to detect surges (to time laparoscopy before ovulation or to cancel cycles). **With hMG** schedule laparoscopy 34 to 36 hours after hCG. Estradiol levels per follicle distinguish nonsurge patients with 196 pg/mL per follicle from surge patients with 331 (11).

Crucial times to stop hMG and to give hCG were based on changes of cervical mucus and on urine estrogen. **Times are now based on serum E2** to measure maturity and quality of follicles, and on ultrasound to measure number and diameter of follicles. Changes of E2 may mean more than absolute values: falling E2 cycles correlate with decreased fertilization and cleavage; rising cycles correlate with increased fertilization (12).

Complications of gonadotherapy are **multiple births** (21 to 34%) and **ovarian hyperstimulation** (HS), an iatrogenic condition seen only after gonadotherapy. Risk of multiple birth with superovulation is minimized if hCG is withheld in cycles with large numbers of mature follicles (8, 13-15). Severe HS (ovary > 10 cm) includes ascites, pleural effusion, oliguria, hemoconcentration hypotension, azotemia, electrolyte imbalance, weight gain > 10 lbs., thromboembolism (death is preventable using excision). HS is rare with CC alone (8, 13). Estradiol assays are preferable to ultrasound as predictors of HS (14, 15). **To prevent hyperstimulation withhold HCG if E2 levels exceed critical values.** To limit severe HS to <1% (<5% in pregnancy cycles) **serum E2 must be <2417** pg/mL in patients with hypothalamic amenorrhea, **<3778** in PCO patients (15). To prevent all severe HS cases in CC nonresponders E2 must be **<950;** and pregnancy rates drop. (Results of in-house extraction E2 assays at the University of Wisconsin and PANTEX 174 kit assays correlated well.)

REAGENTS SUPPLIED

Store kits at 2 to 8°C. Reagents are color coded (see FLOW SHEET), contain antibacterial. Volumes can be aliquoted into **100 tubes.** Reagents containing **human serum** are indicated; **see PRECAUTIONS** Use only reagents supplied with this kit. Do not interchange reagents with different lot numbers. Expiration dates are printed on the labels. In order of use:

1. **Estradiol calibrators** 1 mL of 0 calibrator; 0.5 mL of 10, 30, 100, 300, 1000, 3000 pg/mL in **human serum.**
2. **^{125}I-estradiol tracer** 10 mL in pH 7.4 buffer. The number of µCi are given on the label or on lot data supplied.
3. **first antiserum** 50 mL. Rabbit-produced antiestradiol.

3a. **NSB buffer** 5.0 mL. pH 7.4 buffer.

4. **second antiserum** 50 mL. Goat-produced. Mix gently just before use.

MATERIALS NEEDED BUT NOT SUPPLIED

1. centrifuge [rpm equivalent to RCFXG (relative centrifugal force x gravity):

$$rpm_{desired} = RCF_{specified} \quad (rpm/RCF)_{centrif.\ as\ used}$$

laboratory supply representatives or catalogs have rpm/RCF data on centrifuges as used.]

2. plastic / glass disposable tubes, 10 /12 x 75 mm
3. devices or pipets to dispense 50, 100, 500 µL
4. test tube racks
5. vortex mixer
6. logit-log graph paper
7. gamma counter
8. 37°C waterbath

PROCEDURE FLOW SHEET

	(1)	(2)	(3)	3a	(4)	5	6	7
	cal or sample	**tracer**	**first antiserum**	Mix, leave at 37° C. for 90 minutes.	**second antiserum**	Mix, leave at room temp. for 10 minutes.	Centrifuge, remove supernatants.	Count tubes.
color	none	yel.	blue		red			
TC**	0 µL	100 µL	0 µL		0 µL			
NSB	50*	100	***		500			
cal (pg/mL)								
0	50	100	500		500			
10	50	100	500		500			
30	50	100	500		500			
100	50	100	500		500			
300	50	100	500		500			
1000	50	100	500		500			
3000	50	100	500		500			
control	50	100	500		500			
sample	50	100	500		500			
mixed color	none	yel.	green		brown			

*use 0 cal **remain yellow ***500 µL NSB buffer

PROCEDURE

Label tubes: calibrators (cal), controls, samples, nonspecific binding (NSB), total count (TC). Pipet reagents at room temperature after mixing. Colors after reagent additions are noted in FLOW SHEET.

1. **Pipet 50 µL of calibrators, samples and controls into tubes. Add zero calibrator to NSB tubes.**
2. **Pipet 100 µL of tracer into tubes.**
3. **Add 500 µL of first antiserum to tubes (NSB buffer to NSB tubes; none to total count tubes).**

3a. **Mix tubes. Leave tubes at 37°C for 90 minutes.**

4. **Add 500 µL of second antiserum to tubes (none to total count tubes).**
5. **Mix, leave tubes at room temperature 10 minutes.**
6. **Centrifuge tubes at 2500 RCFxG 15 minutes or 3000 RCFxG 10 minutes. See materials. Aspirate and discard supernatant immediately. Do not disturb precipitates. If decanting, absorb liquid on tube lips with tissue.**
7. **Count tubes: precipitates of calibrators, samples, controls, NSB; total count tubes.**

Low ligand concentrations will be calculated if some liquid is left in tubes since unbound radioactivity in it increases values of B/Bo

DILUTION PROCEDURE

Dilute high patient samples with the zero standard supplied in the kit or human steroid stripped serum can be used.

ASSAY PRINCIPLES

Estradiol calibrators and fixed amounts of radiolabeled estradiol (tracer) compete for binding sites in fixed volumes of antiserum. Estradiol (ligand) from sera or calibrators reduces tracer binding by first antiserum. The **basis of radioimmunoassay** is the quantitative relation between ligand concentration and proportion of tracer bound to antiserum. Second antiserum precipitate is counted after supernatant removal. Binding values of calibrators are plotted vs. concentrations. The best straight line is drawn through points. Control or sample bindings interpolated on the line mark estradiol concentrations.

DATA TABLE

	duplicate CPM		mean CPM	net CPM	%B/Bo	E2 pg/mL
NSB	1483	1425	1454			
cal (pg/mL)						
0	21982	23230	22606	21152		
10	20506	21126	20816	19362	91.5	
30	17914	18037	17976	16522	78.1	
100	13207	13152	13179	11726	55.4	
300	6844	7086	6965	5511	26.1	
1000	3570	3866	3718	2264	10.7	
3000	2341	2492	2417	962	4.5	
control	18320	18027	18174	16720	79.0	29.0
control	4248	4601	4424	2971	14.0	761
sample	13940	14049	13994	12540	59.3	78.1
TC	42321	41922	42122			

CALCULATION

To illustrate data handling, quality control data shown in the **Date Table** are plotted in **Figure 1.** Binding ratios are calculated from net CPM values.
Net CPM = mean CPM - NSB CPM.

1. Calculate %B/Bo of calibrators (bindings relative to 0 calibrator).

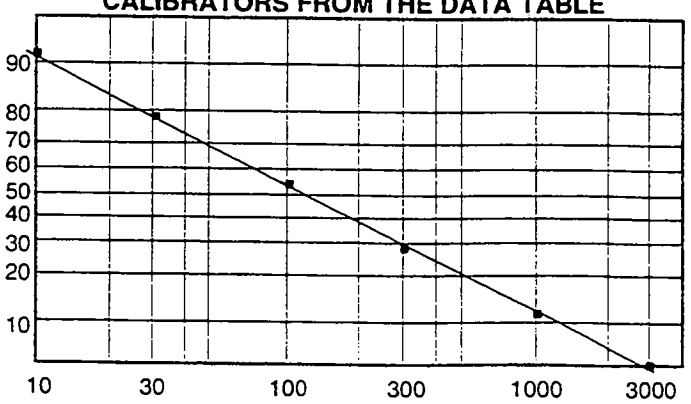
$$\%B/Bo = \frac{\text{net CPM of calibrator}}{\text{net CPM of 0 calibrator}} \times 100$$

2. Plot on logit-log graph paper points relating calculated %B/Bo values and calibrator concentrations (16). Draw the best line through these points.
3. Calculate %B/Bo values of samples.

$$\%B/Bo = \frac{\text{net CPM of sample}}{\text{net CPM of 0 calibrator}} \times 100$$

4. Interpolate %B/Bo values on line. Project points to the X-axis. Intersections mark estradiol concentrations.

FIGURE 1 - LOGIT LOG PLOT OF ESTRADIOL CALIBRATORS FROM THE DATA TABLE



ordinate: percent bound
abscissa: concentration of ESTRADIOL (pg/mL)

SAMPLES

Allow to clot. Separate sera promptly. EDTA plasma gives equivalent values. Sera are stable 1 week at <28°C, 1 month at <4°C, 1 year in frost-free freezers, 3 years in non-defrosting freezers

PERFORMANCE CHARACTERISTICS

1. **Sensitivity** The lowest detectable weight of estradiol is 0.5 pg: equivalent to10 pg/mL in serum.

2. **Dilution study** Sera and dilutions with 0 calibrator were assayed using Pantex 174M E2 kits:

initial	2 fold	4 fold	8 fold	recovery
2282 pg/mL	1066	572	271	96%
1777	901	416	195	94
979	510	243	125	103

3. **Recovery** Estradiol was added to patient sera. Pre-, post-addition estradiol assay using Pantex 174 M kits:

serum	initial	added	found	expected	recov.
A	110 pg/mL	142	228	252	90.5 %
		500	633	610	103.8
B	19	142	161	161	100.0
		500	514	519	99.0
C	103	142	234	245	95.5
		273	371	376	98.7
		500	596	603	98.8
D	0	59	64	59	109.0
		142	133	142	94.0
		273	262	273	96.0
		500	476	500	95.2

4. **Precision and reproducibility** Means, standard deviations (S) in pg/mL, coefficients of variation (CV) from 90 simultaneous estradiol assays and from 12 routine quality control estradiol assays of serum pools.

intra-assay

	pool 1	pool 2	pool 3
mean ± S	49.92 ± 2.11	128 ± 6.02	415.08 ± 16.04
CV	4.2 %	4.7 %	3.9 %

interassay

	pool 4	pool 5	pool 6
mean ± S	53.21 ± 3.76	130.25 ± 5.31	405.38 ± 16.62
CV	7.1 %	4.1 %	4.1%

5. **Comparison with other assays** 23 serum samples were assayed for estradiol using the Pantex 174M kit and a commercial double antibody estradiol kit (COM). Correlation coefficient of results was 0.9942.

	COM	PANTEX 174M
mean	575 pg/mL	561
range	13-2421	11-2033

Equation of the linear regression:
$E2\ conc_{174M} = 0.968 \times E2\ conc_{COM} + 11.8$

REFERENCE VALUES

		serum E2	(pg/mL)	ref
female				
prepubertal		4	- 12	17
postmenopausal or castrate		5	- 18	17
ovulating:	early follicular	30	- 100	17
	late follicular	100	- 400	17
	luteal phase	50	- 150	17
	pregnant		to 35000	17
	on oral contraceptives		<50	18,19
hMG treatment				
effective range: >1000; 300-400 pg / mL / follicle				20
acceptable stim: <2400 in HA; <3700 in PCO				15
male				
prepubertal		2	- 8	17
adult		10	- 60	17

Each laboratory should analyze samples from normal subjects to establish normal ranges.

ANTISERUM CROSS REACTIVITIES

Ratios of concentrations of unlabeled estradiol over compound X that displace 50% of labeled estradiol from antiserum:

cross reactivity of X = 100 (conc. E2)/(conc. X)

compound X	relative activity (%)
β-estradiol	100
estrone	5.60
ethynylestradiol	2.63
α-estradiol	1.90
estriol	0.68

Cholesterol, corticosterone, danazol, desoxycorticosterone, 11-desoxycortisol, DHEA-S, dihydrotestosterone, 17α.-hydroxypregnenolone, 17α-hydroxyprogesterone, pregnenolone, pregnenolone sulfate, progesterone, testosterone have values <0.01%.

OOPHORECTOMY & HYSTERECTOMY PATIENT STUDY USING ESTRADIOL 174M

E2 pg/mL	E2 pg/mL	E2 pg/mL mean	
280.0	259.0	269.5	3 days before surgery (follicular phase)
21.0	26.7	23.8	8 hrs post surgery
7.0	6.6	6.8	1st day post surgery
1.5	4.9	3.2	2nd day post surgery
9.6	15.7	12.6	3rd day post surgery
6.6	9.9	8.2	4th day post surgery
4.0	1.0	1.6	5th day post surgery
7.2	4.2	5.6	6th day post surgery
8.9	11.2	10.0	7th day post surgery
6.6	6.4	6.5	8th day post surgery
134.0	134.0	134.0	14th day post surgery, (1.25 mg Estrogen /day) & 2.5 mg Testosterone was given

PRECAUTIONS

1. Only physicians, clinical labs, hospitals may acquire, possess and use radioactive material in these kits and only for **in vitro assays,**not for administration to man or animal. Use and transfer are regulated and licensed by the U.S. Nuclear Regulatory Commission or by the states it permits to do so.
2. Compare contents and packing list. If there is breakage/shortage, notify Pantex immediately.
3. Although radiation exposures from these small amounts of radioactivity is negligible, designate storage areas >10 feet from work areas.
4. Keep radioactive areas exceptionally clean.
5. Persons under 18 must not handle radioactive material or enter storage or use areas.
6. **Do not smoke, eat or drink while doing assays.**
7. Do not pipet radioactive solutions by mouth.
8. **Wash hands after handling radioactive reagent.**
9. Dispose of soluble radioactive waste in the sewer. Dilute it with large amounts of water
10. Use disposable gloves to blot **radioactive spills** with absorbent. Wash surfaces with detergent. Dispose of wastes in sewer. Cut gloves and dispose of them as ordinary solid waste.
11. Treat **human sera** as potentially infectious; do not pipet by mouth. Check REAGENTS SUPPLIED to see if any reagent in this kit contains human serum. Units of serum used in reagents have been tested and found negative for AIDS antibody and hepatitis B surface antigen, but negative results do not guarantee absence of virus.
12. **WARNING.** Reagents contain **sodium azide** which can react with copper or lead plumbing to form explosive metal azides. Flush down with large volumes of water to prevent azide buildup.

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PANTEX

direct ^{125} I

ESTRADIOL

Catalog no. 174M

07/97

PANTEX
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TESTOSTERONE

direct ^{125} I

Catalog no. 135

07/97

TABLE OF CONTENTS

ASSAY BACKGROUND AND USE

Pantex testosterone (T) ^{125}I kits are used to measure **levels of T**, the very potent androgen, in **hirsutism** and **hypogonadism.** Testosterone is secreted by testes, ovaries and adrenals. Luteinizing hormone (LH) controls testosterone secretion by testicles and ovaries. ACTH and unidentified pituitary factors control adrenal androgen secretion. Enzymes in liver, principal site of metabolism of T, oxidize, hydroxylate and conjugate T. Testosterone metabolites are excreted in urine.

Free Testosterone penetrates cell membranes and binds to a specific receptor protein which in turn penetrates nuclei where T is enzymatically transformed to other potent androgens, according to the theory of androgen action. Testosterone bound to sex steroid hormone binding globulin (SHBG) does not provide T to most tissues since SHBG-bound T dissociates too slowly while passing through capillaries. A portion of albumin-bound T dissociates rapidly enough (1,2).

Testosterone levels are relatively low before puberty but for brief highs **in males** in the 12th week of gestation and just after birth. Serum T increases sharply in males at puberty, then remains relatively constant, except for diurnal variation. There is an early morning peak, then a 25% decrease to the evening minimum. Slow, progressive decreases begin in the forties. Serum T increases **in females** at puberty with cyclic patterns of 1-2 day elevations at midcycle (3). Testosterone production and metabolism decrease proportionally after the menopause, so pre-and postmenopausal T levels are similar (4).

Apparent **hypogonadism in males** is confirmed by **low serum T levels.** High serum LH indicates primary hypogonadism; low LH, secondary. Complete evaluation of hypogonadism includes the **Leydig cell function test:** T assay before and after stimulation with hCG. Increase of T levels during exogenous testosterone therapy proves absorption (5).

Hirsutism, a type of excessive hairiness, motivates women to see their doctors who usually order T assays. Monitoring of T levels during ovarian and/or adrenal suppression determines both the **source of T** and **effectiveness of therapy.** For example, elevated serum T values before and after adrenal suppression prove ovarian androgenization.

Male fetuses can be identified using T assays of amniotic fluid (7). Differentiation is most certain in midgestation when serum T peaks in the male fetus (8). It is not possible to identify male fetuses using T assays of maternal serum (9).

Kinds of testosterone assays include **total testosterone** (direct, extraction, coated tube) and **free testosterone. TOTAL TESTOSTERONE** in plasma **includes** free T and T bound to: SHBG, albumin, corticosteroid binding globulin (CBG). Mean percent of each in **normal females:** 1.4, 55, 43, <0.5; in **normal males:** 2.7, 32, 65, <0.1 (10). Solvents break the protein binding in extraction assays; blocking agents release T from protein in direct assays. **FREE T assay** has the advantage that FREE T concentrations are the testosterone concentrations in equilibrium with testosterone receptors in the organs. Non-SHBG-bound testo normals fall between total T and free T: female, 10-25%; male, 20-70% (2,11).

REAGENTS SUPPLIED

Store kits at 2 to 8°C. Reagents are color coded (See FLOW SHEET), contain antibacterials. Volumes can be aliquoted into **100 tubes.** Use only reagents supplied with the kit. Do not interchange reagents with different lot number. Expiration dates are printed on the labels. In order of use:

1. **Testosterone calibrators** 0 calibrator (1 mL); of 0.1, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, 25.6 ng/mL (0.5 mL) of testosterone in pH 7.4 purified protein solution.

2. **^{125}I-testosterone tracer** 10 mL in pH 7.4 buffer. Number of µCi is printed on the label or on lot data.

3. **first antiserum** 20 mL. Rabbit-produced anti-testosterone using testosterone-19-carboxy-methylether-BSA.

3a. **NSB buffer** 3 mL. pH 7.4 barbital buffer.

4. **second antiserum** 50 mL. Goat-produced. Mix just before use.

MATERIALS NEEDED BUT NOT SUPPLED

1. Centrifuge [rpm equivalent to RCF x G (relative centrifugal force x gravity):

$$rpm_{desired} = RCF_{specified}(rpm/RCF)_{centrif.\ as\ used}$$

Laboratory supply representatives or catalogs have rpm/RCF data on centrifuges as used.]
2. glass disposable test tubes, 10/12x75mm
3. 20, 100, 200, 500 µL pipettors
4. test tube racks
5. vortex mixer
6. gamma counter
7. logit-log graph paper
8. 37°C waterbath

PROCEDURE FLOW SHEET

	(1)	(2)	(3)	3a	(4)	5	6	7
	cal or sample	**tracer**	**first antiserum**		**second antiserum**			
color	none	yel.	blue	Mix, leave tubes for 30 minutes at 37°C.	red	Mix, leave at room temp. for 10 minutes.	Centrifuge, remove supernatants.	Count tubes.
TC**	0 µL	100 µL	0 µL		0 µL			
NSB	20*	100	***		500			
cal ng/mL								
0	20	100	200		500			
0.1	20	100	200		500			
0.4	20	100	200		500			
0.8	20	100	200		500			
1.6	20	100	200		500			
3.2	20	100	200		500			
6.4	20	100	200		500			
12.8	20	100	200		500			
25.6	20	100	200		500			
control	20	100	200		500			
control	20	100	200		500			
sample	20	100	200		500			
mixed color	none	yel.	green		brown			

* use 0 cal **remain yellow *** 200 µL NSB buffer

PROCEDURE

Label tubes: calibrators (cal), controls, samples, nonspecific binding (NSB), total count (TC). Pipet reagents at room temperature after mixing. Colors after reagent additions are noted in the PROCEDURE FLOW SHEET.

1. **Pipet 20 µL of calibrators, samples and controls into tubes. Add zero calibrator to NSB tubes.**
2. **Pipet 100 µL of tracer into tubes.**
3. **Add 200 µL of first antiserum to tubes; NSB buffer to NSB tubes; none to total count tubes.**

3a. **Mix tubes. Leave tubes at 37°C for 30 minutes.**

4. **Add 500 µL of second antiserum to tubes (none to total count tubes).**
5. **Mix, leave tubes at room temperature 10 minutes.**
6. **Centrifuge tubes at 2500 RCFxG 15 minutes or 3000 RCFxG 10 minutes. See materials. Aspirate and discard supernatant immediately. Do not disturb precipitates. If decanting, absorb liquid on tube lips with tissue.**
7. **Count tubes: precipitates of calibrators, sample, controls, NSB; total count tubes.**

Low ligand concentrations will be calculated if some liquid is left in tubes since unbound radioactivity in it increases values of B/Bo.

SAMPLES

Allow to clot. Separated sera are stable at room temperature. (to 28°C) for 7 days, at refrigerator temperature. (2-8°C) for 1 month. Freeze samples for later use. Plasma can also be used.

DATA TABLE

	duplicate CPM		mean CPM	net CPM	%B/Bo	T ng/n
NSB	1914	2072	1993			
calibrator ng/mL						
0	23590	23356	23473	21480		
0.1	22494	22418	22456	20463	95.3	
0.4	21117	21032	21074	19082	88.8	
0.8	19406	19789	19598	17604	82.0	
1.6	18324	17813	18068	16076	74.8	
3.2	14637	15031	14834	12841	59.8	
6.4	12712	12667	12690	10696	49.8	
12.8	10098	10127	10112	8120	37.8	
25.6	7854	7870	7862	5869	27.3	
control	21897	21759	21828	19835	92.3	0.2
control	9876	9297	9586	7594	35.4	14.7
sample	14041	14080	14061	12068	56.2	4.5
TC	47399	48438	47918			

CALCULATION

To illustrate data handling, quality control data shown in th **Data Table** are plotted in **Figure 1.** Binding ratios are calculate from net CPM values. Net CPM = mean CPM - NSB CPM.

1. Calculate %B/Bo of calibrators (bindings relative to calibrator).

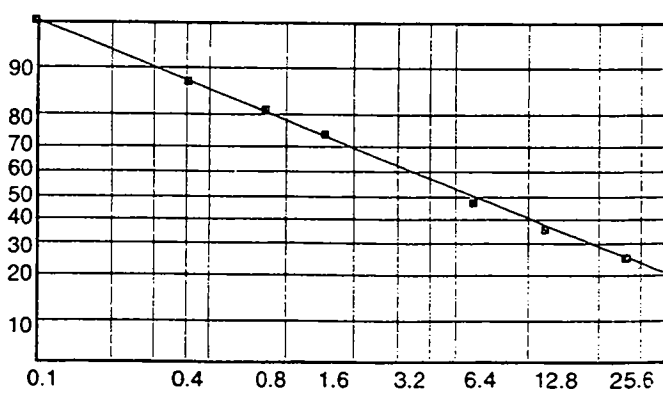
$$\%B/Bo = \frac{\text{net CPM of calibrator}}{\text{net CPM of 0 calibrator}} \times 100$$

2. Plot on logit-log graph paper points relating calculated %B Bo values and calibrator concentrations (12). Draw th best line through these points.
3. Calculate %B/Bo values of samples.

$$\%B/Bo = \frac{\text{net CPM of sample}}{\text{net CPM of 0 calibrator}} \times 100$$

4. Interpolate %B/Bo values on line. Project points to the X axis. Intersections mark testosterone concentrations.

FIG. 1 - LOGIT-LOG PLOT OF TESTOSTERONE CALIBRATORS FROM THE DATA TABLE



ordinate: percent bound
abscissa: concentration of TESTOSTERONE (ng/mL)

ASSAY PRINCIPLES

Testosterone calibrators and fixed amounts of radiolabeled testosterone (tracer) compete for binding sites in fixed volumes of antiserum. Testosterone (ligand) from sera or calibrators reduces tracer binding by first antiserum. The **basis of radioimmunoassay** is the quantitative relation between ligand concentration and proportion of tracer bound to antiserum. Second antiserum precipitates the complex of ligand bound to first antiserum. Precipitate is counted after supernatant removal. Binding values of calibrators are plotted vs. concentrations. The best straight line is drawn between points. Control or sample bindings interpolated on the line mark testosterone concentrations.

REFERENCE VALUES

age (years)	normal serum T ranges (ng/mL)		ref
	male	female	
0-10	<0.04 - 0.37	<0.1 - 0.4	14
10-16	<0.1 - 7.6	0.2 - 0.95	14
16-20	2.6 - 7.3	0.4 - 0.95	14
20-25	3.4 - 11.2	0.4 - 0.95	14
adult	3-12	0.25-0.85	13
pregnant		up to 4x normal	13

Each laboratory should analyze samples from normal subjects to establish normal ranges.

ANTISERUM CROSS REACTIVITIES

Ratios of concentrations of unlabeled testosterone over compound X that displace 59% of labeled testosterone from antiserum:

cross reactivity of X = 100 (conc. T) / (conc. X)

compound X	relative activity
testosterone	100.0%
5α-dihydrotestosterone	6.9
androsterone	0.52
Anadrol	0.15

Aldosterone, androstenedione, cholesterol, corticosterone, cortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, desoxycorticosterone, 11-desoxycortisol, α-estradiol, ß-estradiol, estriol, estrone, etiocholanolone, 17-hydroxypregnenolone, 17α-hydroxyprogesterone, 20-hydroxyprogesterone, pregnenolone, pregnenolone sulfate, progesterone have values under 0.1%.

Durabolin (nandrolone) causes interference: Pantex 005 or 035 kits can be used for testo assay of samples containing Durabolin.

PERFORMANCE CHARACTERISTICS

1. **Sensitivity** The lowest detectable weight of testosterone is 2 pg: equivalent to 0.1 ng/mL in serum.

2. **Precision and reproducibility** Means, standard deviation (S) in ng/mL, coefficient of variation (CV) from 60 T assays and 20 routine quality control testosterone assays of serum pools:

	pool 1	pool 2	pool 3
intra-assay			
mean ± S	0.40 ± 0.42	1.7 ± .17	17.6 ± 1.39
CV	10.5%	10.0%	7.9%
Interassay			
mean ± S	0.41 ± 0.49	1.7 ± .18	17.4 ± 1.41
CV	12.0%	10.6%	8.1%

3. **Recovery** Purified human serum with testosterone added gave testosterone "found" in ng/mL using Pantex 135 kits:

added	found	net	recovery
0	0.50	0	
0.625	1.16	0.66	106
1.25	1.78	1.28	102
2.5	2.98	2.48	99
5.0	5.2	4.7	94
10.0	10.0	9.5	95
			mean 99.2

PRECAUTIONS

1. Only physicians, clinical labs, hospitals may acquire, possess and use radioactive material in these kits and only for **in vitro assays,** not for administration to man or animal. Use and transfer are regulated and licensed by the U.S. Nuclear Regulatory Commission or by the states it permits to do so.
2. Compare contents and packing list. If there is breakage/ shortage, notify Pantex immediately.
3. Although radiation exposures from these small amounts of radioactivity is negligible, designate storage areas > 10 feet from work areas.
4. Keep radioactive areas exceptionally clean.
5. Persons under 18 must not handle radioactive material or enter storage or use areas.
6. **Do not smoke, eat or drink while doing assays.**
7. Do not pipet radioactive solutions by mouth.
8. **Wash hands after handling radioactive reagent.**
9. Dispose of soluble radioactive waste in the sewer. Dilute it with large amounts of water.
10. Use disposable gloves to blot **radioactive spills** with absorbent. Wash surfaces with detergent. Dispose of wastes in sewer. Cut gloves and dispose of them as ordinary solid waste.
11. Treat **human sera** as potentially infectious; do not pipet by mouth. Check REAGENTS SUPPLIED to see if any reagent in this kit contains human serum. Units of serum used in reagents have been tested and found negative for AIDS antibody and hepatitis B surface antigen, but negative results do not guarantee absence of virus.
12. **WARNING.** Reagents contain **sodium azide** which can react with copper or lead plumbing to form explosive metal azides. Flush down with large volumes of water to prevent azide buildup.

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APPENDIX III

ANOVA tables used in the chapter 4.

(1) Randomized block ANOVA to check block effect between small and large fish at week 24.

Proximate composition - Muscle

Parameter	df	F	P	Sig.
% moisture	1,33	3.99	.049	P<0.05
Total Dry matter	1,33	6.75	.014	P<0.05
Total Protein	1,33	3.257	.08	ns
Total Lipid	1,33	3.806	.06	ns
Total Ash	1,33	4.73	.037	P<0.05
Total Energy	1,33	13.55	.001	P<0.05

As some parameters were significantly different between small and large fish, separate tests were performed for small and large fish.

(2) Randomized block ANOVA to check block effect between small and large fish at week 24.

Proximate composition of Liver

Parameter	df	F	P	Sig.
% moisture	1,33	2.684	.111	ns
Total Dry matter	1,33	.031	.861	ns
Total Protein	1,33	.139	.712	ns
Total Lipid	1,33	.175	.679	ns
Total Ash	1,33	.010	.921	ns
Total Energy	1,33	.048	.827	ns

As all liver parameters were not significantly different between small and large fish at week24, poled data were analysed using ONEWAY ANOVA.