Filling in the gaps impeding the instigation of selective breeding programs in barramundi *Lates calcarifer*: fate of genetic diversity through to harvest, estimation of genetic parameters and early prediction of family growth based on cellular processes

Thesis submitted by

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Experiments within this research were performed in conformity to JCU Animal Ethics Approval Number A1533.

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Abstract

Barramundi (*Lates calcarifer*), also known as Asian seabass, is an emergent aquaculture fish species possessing excellent farming attributes. As a result of its euryhaline life-history, culture of barramundi is extremely flexible and the species displays fast growth in freshwater, brackish, and/or marine environments, where it can be farmed in ponds, cages, or intensive recirculation tank-based systems. Barramundi has been farmed for over 40 years in tropical waters of the Indo-West Pacific region and has recently been introduced into Europe and North America due to increasing consumer demand and high market value, and global production is rapidly increasing. Whereas a wealth of knowledge on the biology and culture of the species have contributed to a developing industry, barramundi farmers still rely on unimproved stocks and often experience inconsistent production as a result of high variability in fish growth and survival between batches. However, for the barramundi industry to meet its full potential in the future it will need to rapidly move towards the instigation of well-informed genetic improvement programs. In order to assist with the design and implementation of a selective breeding program for barramundi, this research aimed to address key knowledge gaps related to the genetic diversity of farmed populations and to the underlying genetic basis of commercially important growth traits. In addition, this research investigated larval progeny testing as a means to rapidly estimate breeding values (EBV) of broodstock currently held at commercial hatcheries, or broodstock to be incorporated into a breeding program.

One significant challenge for genetic studies of farmed barramundi relies on the fact that the species is a strict mass spawner and therefore the number and size of families cannot be fully manipulated by strip spawning and artificial fertilization. Such reproductive behavior has limited the knowledge on the genetic diversity of farmed cohorts, as pedigrees can only be reconstructed through DNA parentage analysis. Therefore, exploring how many families may be produced through mass spawning and, most importantly, retrieved at the end of the production cycle is critical for the development of breeding programs for barramundi. To investigate the fate of genetic diversity over the entire culture cycle, offspring from three commercially produced batches were genotyped with microsatellite markers at 18 days post hatch (dph) and then later at harvest (273-469 dph) for inference of familial relationships.

Results demonstrated that it is possible to produce and retrieve at harvest over 100 families from a single spawning event using large numbers of broodstock (e.g. 33 fish). In addition, genetic diversity within generations was found much more resilient than previously thought. Despite significant changes in relative family frequencies over the culture period ($P < 0.05$), family lines identified at the larval stage were still available for selection by the time of harvest. The stability of several indices to gauge possible changes across the two sampling periods (i.e. effective population sizes, inbreeding rates, number of alleles, allelic richness, observed and expected heterozygosity and R_{xy} relatedness coefficient) also confirmed no subsequent loss of genetic diversity throughout the culture cycle. Therefore, early demographic census would provide an accurate representation of genetic diversity available for selection at harvest. Moreover, a cost-effective genotyping sample scheme targeting to reduce the overall costs of breeding programs of barramundi was also investigated. Here, examination of family contributions and mean family body weights at harvest showed that genotyping the top 1.5% of the population (or 750 individuals > 2.17 S.D. heavier than the population mean) will capture > 75% of family-specific genetic diversity present, whereas continued sampling after this was ineffective. This study suggests that genetic diversity in a barramundi breeding nucleus can be boosted and maintained by using several large breeding groups per generation.

However, before efficient selective breeding programs can be implemented, it is also fundamental to have a comprehensive understanding of key genetic parameters of growth-related traits, such as heritabilities (h^2), genetic correlations (r_g) and genotype by environment interactions (G x E). This knowledge is necessary to predict genetic gains and the efficiency of a breeding program, as well as to determine if genotypes selected under a particular environment will express the same growth performance when reared in a different environment. Heritability estimates for three separate batches measured and genotyped at harvest were consistently high for fish weight (mean W $h^2 \sim 0.40$), standard length (mean *Ls* $h^2 \sim 0.37$) and body depth (mean BD $h^2 \sim 0.40$). As additive genetic effects play a significant role in barramundi body size, selection of heavier (or longer/deeper) fish at harvest is expected to greatly improve fish growth rates. Lower heritability values were found for Fulton's condition factor ($0.00 \leq$ K $h^2 < 0.20$) and body shape ($0.00 \leq$ H $h^2 \leq 0.12$), however, positive genetic correlations with fish weight ($0.36 \leq r_g \leq 0.41$) indicate that selection for heavier fish may also improve fish condition and shape. Also of importance, very high genetic correlations were found for the same growth trait (W, Ls or BD) in fish reared in

either fresh *vs*. sea water cages ($r_g \geq 0.97$), or commercially reared in an intensive tank system *vs*. a semi-intensive pond ($r_g \sim 0.99$). The lack of G x E interactions suggest that the offspring of breeding candidates selected in a breeding nucleus, or in a particular farm, will express superior growth rates in a range of farming conditions which are representative for the barramundi industry. The lack of G x E interactions coupled with high heritability for growth are encouraging outcomes for the wider barramundi industry and the shared investment in a breeding program should return high genetic gains for all stakeholders alike.

Nevertheless, the benefits from a future barramundi breeding program would only be capitalized after a few generations, i.e. a decade or longer. Therefore, alternative means to rapidly ascertain the genetic merit for growth rate of existing broodstock would be highly desirable. In this study, several larval progeny traits strongly associated with the individual growth capacity were investigated as potential early predictors of long-term family growth. High genetic correlations between early and late growth traits would indicate the feasibility of larval progeny testing as a method to infer EBVs of parental broodstock. Firstly, a protocol to measure larval size (*Ls*), total RNA, total DNA, total protein, RNA/DNA, protein/DNA and the proportion of cells dividing within a single larva was developed. Secondly, the heritability of these traits was estimated from a dataset of *ca*. 400 18 dph larvae with known pedigrees. Thirdly, the genetic correlations between heritable larval traits and fish harvest size (weight (W) and *Ls*)) were determined by combining the larval dataset with that of ca. 2000 harvested fish with known pedigrees. All larval traits exhibited moderate to high heritability at 18 dph ($0.19 \leq h^2 \leq 0.51$), indicating that their expression is under additive genetic control and therefore that they could have predictive power to estimate parental EBV for long term growth. This was confirmed by positive genetic correlation between all larval traits (except protein/DNA) and fish harvest W and *Ls* ($r_g \geq 0.60$). In particular, larval RNA/DNA, total RNA and the proportion of cells dividing had the highest predictive power to determine genetic differences in growth potential among barramundi broodstock shortly after spawning ($0.81 \leq r_g \leq 0.88$). Results indicate that testing cellular traits in larval progeny may allow hatchery managers to immediately re-spawn the highest EBV ranked broodstock for stocking grow-out systems, thereby avoiding costs associated with rearing slow-growing families. Furthermore, early progeny testing may allow fish breeders working with long-lived and highly fecund multiple spawners like barramundi to cull introduction of broodstock with inferior genes for growth.

In summary, this research bridges several missing links in the knowledge of genetic diversity and parameters of farmed barramundi necessary for the establishment of a selective breeding program for the species. It has been shown for the first time that despite the mass spawning nature of the species, sufficient genetic diversity can be generated and maintained in a breeding nucleus through to harvest and that growth rates can greatly be improved through selection for increased body weight at harvest. Furthermore, this research unveiled the existence of significant genetic correlations between cellular larval traits and fish harvest size which may be explored as a novel progeny testing tool to fast-track the improvement of fish growth.

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Chapter 1. General Introduction

1.1. The role of aquaculture in human food security

Until recent times, the oceans have been regarded as an inexhaustible source of dietary protein, essential fatty acids and micronutrients for humankind. However, due in large part to unsustainable exploitation and consequent depletion of numerous fishery stocks, annual world fisheries capture has stagnated around 90 million tonnes since the early 1990's (FAO, 2012). At the same time the human population has increased from 5.3 to 7.2 billion people (FAO, 2012). In the early 1990's, the farming of aquatic organisms (aquaculture) only provided a small contribution to total seafood production, with production estimated to be less than 10 million tonnes. Over the last two decades, an increasing understanding of the reproductive biology of finfish, crustaceans and mollusks has allowed aquaculturists to close the complex life cycle of many aquatic species of commercial importance, thereby allowing for a reliable and predictable supply of seedlings for stocking farms. A parallel increase in understanding of physiological and nutritional needs of cultured aquatic species has also allowed for tailoring species-specific feed formulations, and more efficient rearing conditions, which has enabled aquaculture ventures to become highly productive farming systems. With increasing consumer demand for high quality seafood products, global aquaculture production has become the fastest growing food production sector, even surpassing beef production. Aquaculture now accounts for around half of total global seafood supply (Figure 1.1) and plays an important role in human food security (FAO, 2012).

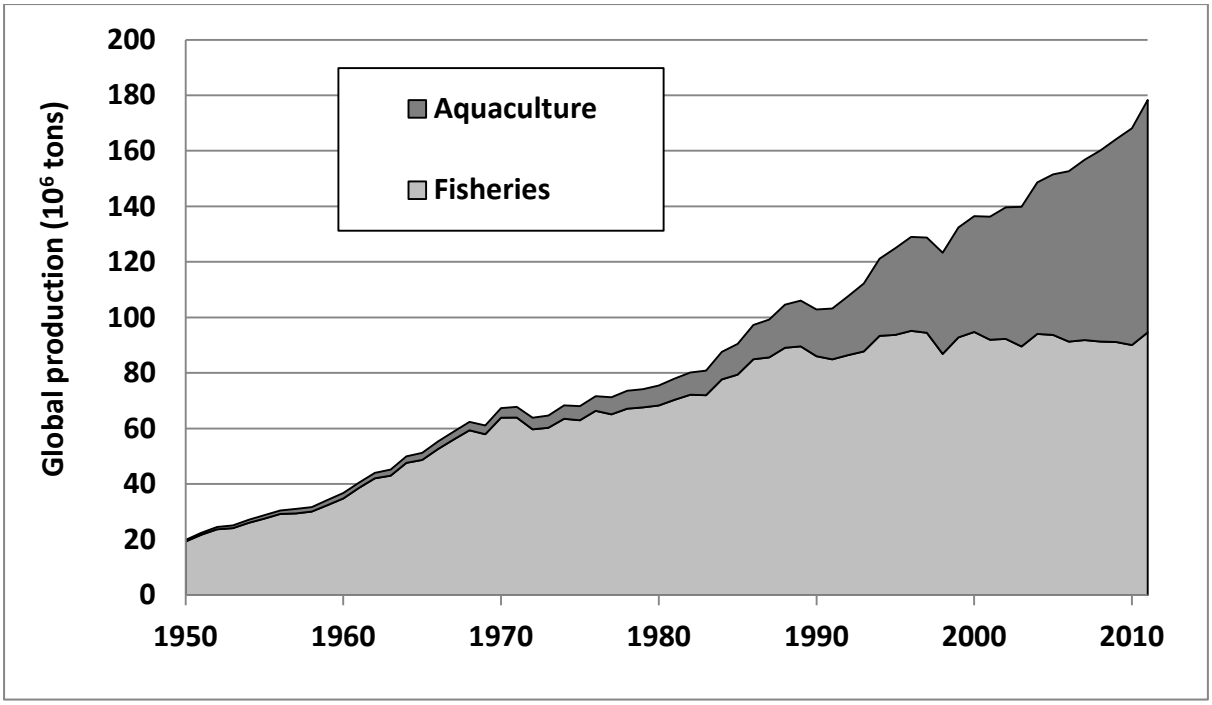


Figure 1.1. Global fisheries and aquaculture production (FAO, 2012).

1.2 New challenges for aquaculture industries

Many established aquaculture industries have now overcome the initial biological impediments for large scale production. However, as with many other fast-growing emerging industries, aquaculture has faced heavy criticism from environmental lobby groups which have brought to public attention cases where aquaculture practices have led to habitat destruction, water pollution, indiscriminate catch and stocking of juveniles from the wild, introduction of exotic species and excessive use of fish oil and meal in feeds (Pillay, 2004). As sound environmental practice translates into healthier and more productive crops, researchers and producers together have continuously strived to develop and adopt more sustainable approaches to enable aquaculture's long-term viability. Initiatives to advance environmentally and socially responsible aquaculture spring from industry-based organizations which advocate best management practices and certify members abiding to strict codes of conduct (Boyd et al., 2002). Nevertheless, aquaculture industries currently face

the challenge of economic sustainability. Increased costs for compliance with stricter environmental laws, greater risks of disease outbreaks and fierce international competition under the current globalized economy are associated with large scale production. The adoption of optimized husbandry practices has become a pre-requisite for business to survive in the present economic climate. However, many aquaculture industries still rely on larvae produced from wild caught or unimproved broodstock, where high levels of variability in production performance and uncertainty of long-term growth and survival are commonly observed. In cases where aquaculture production costs and/or productivity cannot be further improved through best management practices, the use of genetically superior stocks offers a cost-effective alternative to ensure financial viability.

1.3 Sustainable aquaculture through the use of genetically improved stocks

The Norwegian Atlantic salmon (*Salmon salar*) and rainbow trout (*Oncorhynchus mykiss*) industries pioneered with the implementation of the first industrialized aquaculture selective breeding programs in 1975 (Gjedrem, 2010). Due to the broad genetic diversity found in wild stocks, short generation intervals, extremely high fecundities, high phenotypic variances and moderate heritabilities of commercially important traits, such as fish weight, the Norwegian programs achieved encouraging responses to selection of 14% per generation. These pioneering programs demonstrated that selection for high growth rates in fish actually yielded 4-5 fold faster genetic gains than that obtained by selective breeding of terrestrial livestock (Gjerde and Korsvoll, 1999). In addition, significant correlated responses in feed conversion efficiency, protein- and energy-retention, also indicated that salmon selected for faster growth better utilized feed resources compared to unselected animals (Thodesen et al., 1999). As the productivity of fish farming is intrinsically linked to growth performance, and feeds represent the largest operating costs of most fish farms (Tacon and Metian, 2008), fast growing strains

that more efficiently utilize input resources, reduce wastage and offer significantly reduced production time and costs are highly desired (Gjedrem et al., 2012).

The genetic improvement of salmonids demonstrated for the first time that more sustainable and profitable aquaculture farming operations could be achieved through the implementation of large scale family-based selective breeding programs (Gjedrem, 2010). The success of salmonid selective breeding programs prompted the development of improvement programs for other aquatic species including the Nile tilapia (*Oreochromis niloticus*), with the Genetic Improvement of Farmed Tilapia (GIFT) project in 1989 (Eknath and Hulata, 2009), and a selective breeding program for the Pacific white shrimp (*Penaeus vannamei*) started in 1993 (Fjalestad et al., 1997). The outcomes from these initial programs showed that selective breeding is the only genetic approach to offer the opportunity for permanent, continuous and cumulative genetic gains over generations. In contrast, other technically feasible methods to genetically improve productivity (e.g. hybridization and cross-breeding, chromosome manipulation, sex control and transgenesis, the latter made sterile through triploidization for GMO containment) only offer a "once-off" benefit, as the gains made are usually not transmissible beyond the generation they are applied to (Lind et al., 2012). The large economic benefit of selective breeding to aquaculture has now led to the implementation of over 100 programs worldwide (Neira, 2010; Rye et al., 2010). Despite the large number of programs the vast majority (~ 75%) target the continuous improvement of those three groups for which selective breeding was first demonstrated, with 42 programs for salmonids, 28 for tilapia and seven for shrimp (Neira, 2010; Rye et al., 2010). Emerging industries working with unimproved species must compete for market share with these larger more developed industries. Gjedrem et al. (2012) estimated that over 90% of world aquaculture production in 2010 was still derived from wild caught, or unimproved, domesticated broodstock. The use of

genetically improved stocks is a key factor for aquaculture of existing large scale industries and will also be vital for rapid and sustainable development of emerging industries.

1.4 Barramundi, *Lates calcarifer*, an emergent aquaculture fish species

Barramundi, *Lates calcarifer* (also known as Asian seabass), is a highly valued and popular aquaculture finfish species widely distributed throughout the Indo-West Pacific region, for which production is rapidly increasing worldwide (Figure 1.2). Barramundi is a catadromous species, having a high tolerance to culture in fresh, brackish, or marine farming conditions that can vary from extensive or super-intensive pond, tank or cage-based culture systems. In addition, biological characteristics such as high fecundity (females spawn an average of 300,000 eggs per Kg of body weight) and fast growth rates (reaching up to 2 Kg on farm in 12 months) together with good market acceptance have led to the rapid development of an emerging barramundi aquaculture industry (Grey, 1987; Rimmer and Russel, 1998; Garcia, 1990; Schipp et al., 2007). Barramundi's hardiness and suitability for farming in diverse environments, not matched by any other aquaculture fish species, may also justify greater levels of production and has led to recent interest in farming in temperate regions far beyond the species natural distribution (Walsh, 2011). Barramundi aquaculture commenced in Thailand during the 1970s and rapidly spread throughout Southeast Asia and Australia, although global production (69,116 tons in 2011) remains primarily based on unimproved farmed stocks (FAO, 2012). Despite the numerous positive farming attributes, the FAO has long recognized that one major area of research and development (R&D) need, but to date little effort, is in genetic selection programs targeting faster growth and disease resistance for barramundi (FAO, 2014).

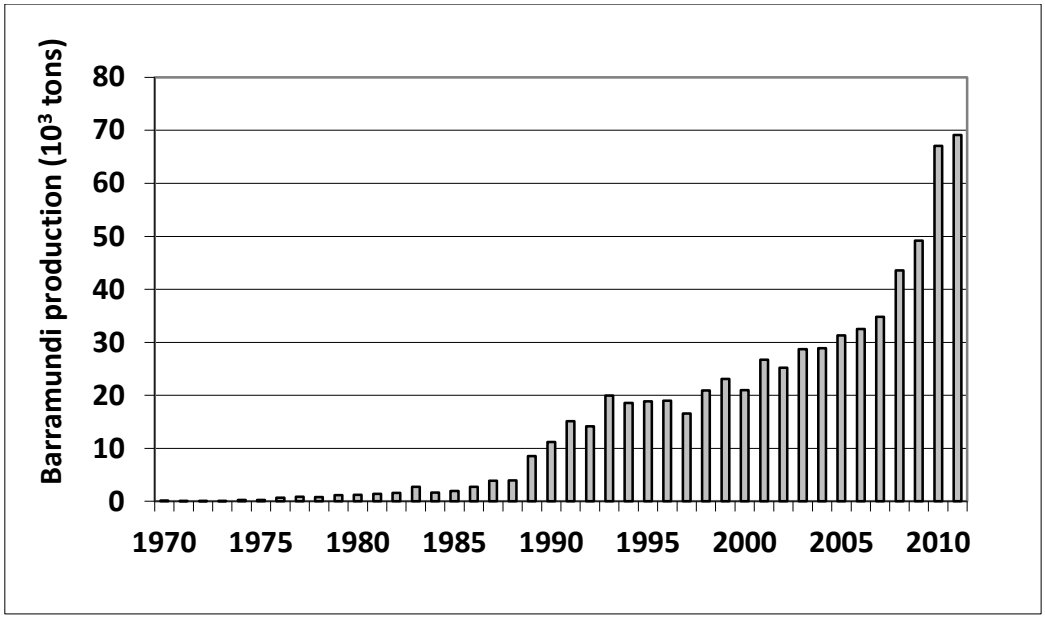


Figure 1.2. Growth of global aquaculture production of barramundi *Lates calcarifer* (FAO, 2012).

1.5 Considerations for implementing selective breeding programs for barramundi

The development of genetically superior strains of fish through effective and sustainable selective breeding programs is a complex task. An important requirement is the maintenance of genetic diversity. This is a key biological requisite for population resilience and productivity and is a vital primary consideration for selective breeding programs to deliver permanent and continuous genetic gains (Ponzoni et al., 2011). However, due to the high fecundity of aquatic species and the logistics of maintaining large breeding stocks, aquaculturists frequently produce large populations from just a few individuals. As a consequence, the domestication process itself has been shown to be a bottleneck for genetic diversity and effective population sizes (*Ne*) of many aquaculture species including Atlantic salmon (Norris et al., 1999), Black Tiger shrimp (Dixon et al., 2008), Humpback grouper (Na-Nakorn et al., 2010), haddock (Trippel et al., 2009), pearl oysters (Lind et al., 2009), gilthead seabream (Brown et al., 2005), red sea bream (Hamasaki et al., 2010), and also barramundi (Yue et al., 2009). Moreover, selection of progeny as broodstock for the next generation without accounting for the relatedness among individuals has been associated with

the genetic degeneration of captive stocks through inbreeding depression and genetic drift, hampering the ability of populations to respond to selection (Lind et al., 2012).

To avoid the pitfalls associated with reduction of effective population sizes (N_e), i.e. loss of genetic diversity and inbreeding depression, breeding programs generally start from a foundation population with a broad genetic base formed by crossing genetically diverse wild and farmed strains (Eknath et al., 1993; Wang et al., 2002; Gjedrem, 2010). Moreover, family based programs, such as those first developed for salmonids are now considered the standard for aquaculture species because they allow for high selection intensities (and thus high selection responses) with minimum inbreeding (Gjedrem, 2010). Modern family based breeding programs commonly utilize combined selection methods (e.g. individual, between and within-family selection) based on quantitative genetic theory, which take into account trait performance from both individuals and their relatives (e.g. full and half-sib and unrelated families, parent-offspring, etc.), as well as non-genetic source information (maternal and common environmental effects including sex, batch, farm environment, tank or pond effects) to estimate individual breeding values (EBV). However, as the number of test families must be large, these programs require considerable investments from a supporting production sector (Gjedrem et al., 2012). Therefore, before barramundi selective breeding programs can be implemented, it is fundamental to have a clear understanding of the genetic resources available (Robinson and Jerry, 2009). In fact, there is a comprehensive knowledge of its biology and culture (Jerry, 2013) and recently numerous genomic resources have become available (Xia et al., 2010; Wang et al., 2011; Yue, 2013; Zhu et al., 2010). However, there is still limited knowledge of the genetic basis of traits of commercial importance, as the only estimates are limited to 3 month-old juveniles (Wang et al., 2008). One of the first steps taken by the Norwegian programs was the estimation of the phenotypic and genetic parameters of

economically important traits, which are known for over 40 years (Gjedrem, 2010). This information is fundamental because the magnitude of heritabilities, genetic correlations and genotype by environment interactions (G x E) of traits to be improved dictates the most effective type of breeding design for any given breeding program (Falconer and Mackay, 1996).

Furthermore, in order to exploit the full potential of selective breeding, there needs to be an understanding of how the environment influences the overall realization of the phenotype and whether selection decisions under one set of environmental conditions will be correlated with similar genetic gains when progeny are reared under disparate environments. When G x E interactions are considerable, a breeding program will need to be tailored to account for each of the different environments in which the animals are to be commercially produced. Addressing this knowledge gap is particularly important for barramundi given the broad range of production systems used for cultured of this species. In addition, barramundi is now farmed far outside its Southeast Asian and Australian natural distribution range, and conditions for grow out in a selective breeding nucleus are likely to differ markedly from those on farm.

Barramundi is a protandrous hermaphrodite (i.e. fish first mature as males than change into females later in life) and reproduces by mass spawning. Currently, aquaculturists do not have complete control over sexual maturation, differentiation and reproduction in captivity and this lack of reproductive control presents challenges for the selective breeding of the species (Robinson and Jerry, 2009; Robinson et al., 2010). The ideal generation of families in balanced, full factorial crosses through strip spawning and artificial fertilization for performance testing, as suggested by Macbeth and Palmer (2011) and based on a previous

successful report (Palmer et al., 1993), is yet to be adopted by the barramundi aquaculture industry. Because strip spawning is difficult to perform, current breeding practices in Australia still rely on mass spawning using one or two females and four or more males in ~20,000 l tanks (Macbeth et al., 2002). Although mass spawning results in the production of several maternal and paternal full- and half-sib families, this random mating scheme has prevented hatcheries from being able to control variability in the genetic contribution of brooders to the next generation. Because the relative contribution from each brooder to the total number of offspring is unknown, and selected fish for the next generation may be the offspring of a limited number of parents, the practice of mass spawning in a breeding nucleus has been discouraged (Gjerde, 2005).

Pedigree tracking is a basic prerequisite for any breeding program and fundamental for determining an individual's EBV (Gjedrem and Baranski, 2009). Classically, aquaculture breeding programs have maintained pedigree information by rearing individual families (generated through strip spawning or single pair matings) in separate tanks until animals are large enough for physical tagging and communal grow out. However, as previously mentioned, this practice is not possible for barramundi. In fact, knowledge of the relationship among barramundi individuals was only recently facilitated with the development of a number of DNA-based microsatellite markers (e.g. Yue et al., 2001, 2002, 2009; Sim and Othman, 2005; Wang et al., 2007; Zhu et al., 2006a, 2006b), which enabled pedigrees to be reconstructed through parentage assignment software (Frost et al., 2006; Wang et al., 2008; Zhu et al., 2010; Loughnan et al., 2013; Liu et al., 2012). These studies highlighted that mass spawned cohorts were produced by a few dominant broodstock, thus bearing low levels of family-specific genetic diversity, which is complicating factor to selective breeding of

barramundi due to risks of inbreeding depression (Kincaid, 1983; Su et al., 1996; Bentsen and Olesen, 2002).

Application of DNA parentage analysis in past studies of barramundi reproductive success and broodstock contributions have, however, only focused on the initial rearing periods up to 90 days post hatch (dph). These earlier studies did not address fundamental questions for the establishment of breeding programs, such as levels of family-specific genetic diversity, as well as differences in family metrics or genetic parameters *at harvest* for traits of primary importance (such as growth). Additionally, many questions still remain to be answered related to the consequences of mass spawning for the development of effective genetic management and improvement strategies for cultured barramundi. For instance, is there differential family survival from hatchery to harvest and how does it impact on the available genetic diversity for selection? How does family-specific genetic diversity compare between mass spawnings involving few, or large numbers of broodstock? Are there expected differences in parental contribution between males and females for optimizing sex ratios among broodstock? How many of the best performing individuals should be genotyped at harvest and selected as parents for the next generation in order to capture all the available families present in a cohort? What are the heritabilities, genetic correlations and G x E interactions for harvest growth traits? To date, such important questions have not yet been answered for barramundi and this knowledge gap has certainly prevented the design of an efficient breeding program for the species.

Moreover, considering that the results of breeding programs are only realised after a few selected generations, which may take a decade or longer, it is reasonable to conclude that the achievement of economic sustainability through the use of genetically superior stocks is still

a long way away, not only for barramundi, but for many important aquaculture industries. While such programs are considered and/or implemented, alternative short-term genetic strategies to achieve production gains would be highly desirable. Ultimately, genetic improvement programs target the selection of broodstock with superior genetic merit (i.e. fish with higher estimated breeding values, EBV) for the traits of commercial interest. While recent advances of genome mapping and quantitative trait loci (QTL) analyses have been conducted in barramundi to assist in marked-assisted selection (reviewed by Yue, 2013), it is still uncertain if QTLs associated with growth traits are family specific or universal, i.e. published QTLs need further validation. Alternatively, a simpler solution to evaluate the genetic merit of broodstock would be through progeny testing (Macbeth and Palmer, 2011). Like most fishes, barramundi reproduce throughout their adult lives (salmonids excepted, as high mortality rates are expected after spawning). In Australian hatcheries, due to the large size and high value of female broodstock, it is not an uncommon practice that the same broodfish is repeatedly spawned over several years, first as a male, then again as a female after sexual inversion. Even though the same broodfish are often repeatedly spawned, rarely is the decision used to choose spawners based on performance records of progeny, more often focus is merely weighted towards whether the broodfish was a good contributor to larvae numbers in the hatchery. Therefore, the development of methods to assess EBV of existing broodstock currently held at commercial hatcheries would allow hatchery managers to identify and thereafter produce grow out cohorts from only those EBV best ranked broodstock. Use of best EBV broodstock would mitigate the negative environmental and economic impacts associated with farming of poor performing, genetically inferior families. Furthermore, if progeny were tested early in the production cycle to reveal parents with superior genes for growth, such a method of broodstock selection could enable the improvement of fish growth rates as soon as the subsequent spawning cycle and potentially

lead to immediate increases in aquaculture productivity. Therefore, the development of early progeny testing techniques which disclose at larval stages those barramundi broodstock passing on to progeny greater genetic potential for long term growth would be highly advantageous for industries working with species like barramundi, which lack traditional breeding programs.

1.6 Thesis aims and structure

This thesis aimed to address key knowledge gaps of the genetic diversity of farmed barramundi cohorts over the culture cycle and on the genetic parameters of harvest growth traits and provides information that is fundamental for the development of effective strategies for selective breeding programs. In addition, this thesis aimed to advance understanding of the genetics underpinning the large phenotypic variability seen in early developmental stages by assessing multiple cellular and molecular processes fundamentally linked to growth. Lastly, this thesis aimed to assess the use of several larval traits as an early progeny testing tool which could allow for a more immediate realization of increases in aquaculture productivity and profitability through estimation of broodstock EBV, without having to rear progeny through to harvest. The structure of thesis data *Chapters 2* to *5* is as follows:

Chapter 2 tracked the retention of genetic diversity in three commercial batches of barramundi all the way from the hatchery to harvest. This study is unique as it is the first to estimate individual broodstock and family contribution/distributions in a mass spawning fish (i.e. barramundi) through the entire production cycle and explores how uneven family contributions and differential family survival may affect the selection of unrelated breeding candidates at harvest. Moreover, based on a phenotypic and genetic dataset comprised of 3651 DNA assigned progeny, this study assessed the genotyping effort, i.e. the number of

fish that would have to be sampled and genotyped under a walk-back selection program to capture family-specific genetic diversity at the critical point of selection. The genotyping sample scheme identified here may be used to reduce the overall costs of breeding programs of barramundi and possibly other mass spawners. Chapter 2 was published in the journal *Aquaculture* (Domingos et al., 2014a) and is presented herein with minor modifications (http://www.sciencedirect.com/science/article/pii/S0044848614000076).

Chapter 3 investigated for the first time how additive genetic variability in barramundi affects economically important traits at harvest and how families perform relative to each other over the culture cycle in similar, or disparate environments. Specifically, the heritabilities and genetic correlations of barramundi body weight, standard length, body depth, Fulton's condition factor and body shape index were estimated at 62 dph and, more importantly, at harvest (273-469 dph). Furthermore, this chapter looked for evidence of genotype by environment (G x E) interactions in barramundi reared in fresh water *vs.* sea water cages and in a semi-intensive pond *vs.* intensive tank system. Chapter 3 was published in the journal Aquaculture (Domingos et al., 2013) and is presented herein with minor modifications (http://dx.doi.org/10.1016/j.aquaculture.2013.03.029).

Chapter 4 described the development of analytical methods to measure multiple cellular and biochemical traits strongly associated with growth rates in barramundi larvae, which reveal genetically superior fast growing families for selective breeding programs. Specifically, protocols were developed to measure (i) the percentage of cells actively dividing through flow-cytometric (FCM) cell cycle analysis, (ii) RNA/DNA ratios and (iii) protein/DNA ratios through quantitation of total nucleic acid fluorescence and total protein absorbance. Early larval traits such as RNA/DNA have traditionally been used in fisheries science as indices of

larval growth rates and nutritional condition, however, they have never been used for barramundi and previous studies have not successfully combined the analysis of multiple such traits in a single larva. This chapter also investigated the phenotypic correlations between each one of these cellular and biochemical indices (i-iii) and larval growth rates for the first time under commercial aquaculture conditions. Part of this chapter was published in the Journal of Fish Biology (Domingos et al., 2012) (http://dx.doi.org/10.1111/j.1095-8649.2012.03278.x).

Chapter 5 explored the potential for the larval traits previously investigated in *Chapter 4* to identify in early life stages those families with genes for fast or slow growth. Here, the heritabilities of seven larval traits (total RNA, total DNA, total protein, RNA/DNA, protein/DNA, the proportion of cells dividing and standard length) and their genetic correlations with economically important harvest growth traits (fish weight and length) were estimated. Furthermore, the relationship between broodstock EBVs assessed from offspring traits in the distinct (hatchery and harvest) stages were analysed to explore the feasibility of early progeny testing through the use of larval indicator traits as a selection method for rapid pre-screening and identification of broodstock with the best growth characteristics. Chapter 5 was published in the journal Aquaculture (Domingos et al., 2014b) and is presented herein with minor modifications (http://dx.doi.org/10.1016/j.aquaculture.2014.02.037).

Chapter 2. Fate of genetic diversity within and between generations and implications for DNA parentage analysis in selective breeding of mass spawners: a case study of commercially farmed barramundi, *Lates calcarifer*

2.1. Introduction

The maintenance of genetic diversity is a key biological requisite for population resilience and productivity and is a vital consideration for selective breeding programs to deliver permanent and continuous genetic gains. Accordingly, successful genetic improvement strategies must carefully factor the number of selected breeders and their genetic relationships to avoid pitfalls associated with inbreeding depression and its counterproductive consequences to the productivity of captive stocks (Gjedrem and Baranski, 2009). In aquaculture species, it has been suggested that selective breeding of a minimum of 50 unrelated broodstock pairs is necessary to maintain inbreeding at acceptable levels and ensure continuous genetic gains (Bentsen and Olesen, 2002). Selection of such broodstock numbers may be readily attainable in species where the reproductive biology is easy to manipulate, allowing the number of broodstock contributing to the cohort and the size of families to be controlled through single pair spawns, and/or strip spawning. This allows for very practical and effective breeding designs, as partial or complete factorial mating schemes with even family contributions to progeny cohorts under evaluation can be controlled. However, in species with strict natural mass spawning behaviour, where artificial striping is difficult to perform and where several sexually mature males (sires) and females (dams) simultaneously release their gametes in the same tank for the production of offspring, the generation of high family diversity and standardised family sizes prove to be far more challenging (Brown, 2003). Consequently, instigation of selection programs for mass spawning fish species lags

behind those species where family numbers and sizes can be easily manipulated (e.g. Neira, 2010; Rye et al., 2010), primarily due to concerns about the ability to generate sufficient numbers of genetically diverse families from which breeding candidates can be selected (Gjerde, 2005).

As the conduct of mass spawning in fish largely relies on natural reproductive mating behaviour, the hatchery manager often has limited control over the contribution of each individual breeder and thus the number of families generated in each progeny cohort. Families with variable and unknown numbers of offspring are randomly generated and mixed from the egg stage in a communal environment. In this case, progeny pedigrees and individual parental contributions can only be reconstructed through DNA parentage analyses (Martínez and Fernández, 2009); a costly exercise which relies on genetic resources often not available for many aquaculture species. For those species where genetic markers are available, DNA parentage analyses have invariably shown that mass spawning generates highly skewed parental contributions, where a few dominant breeders produce the majority of offspring (e.g. Brown et al., 2005; Frost et al., 2006; Fessehaye et al., 2006; Herlin et al., 2008; Blonk et al., 2009; Gold et al., 2010; Borrell et al., 2011). Such skewed levels of reproductive success and the absence of control over the individual parental contribution to the progeny cohort are considered to be a major bottleneck to the development of selection programs in fish species with this type of mating behaviour (Robinson and Jerry, 2009; Robinson et al., 2010).

In addition to difficulties in producing sufficient families and controlling for differential family contributions, another limitation for selective breeding of many mass spawning species is a lack of knowledge on the fate of family-specific genetic diversity over the entire culture period. For example, in the case of mass spawning barramundi (*Lates calcarifer*), an

unimproved, but high-valued aquaculture species, information on the fate of broodstock contribution and family distributions within cohorts is only known until 90 days post hatch (dph), a time when culture practices thought to impact on the available genetic diversity, such as grading and culling of slow growers, are heavily applied (Frost et al., 2006; Wang et al., 2008; Loughnan et al., 2013). However, for selective breeding programs, the fate and relative contributions of families at the end of the culture cycle is more important than that observed during initial rearing periods because selection of breeding candidates is performed at later ages closer to harvest (e.g. between 1-2 years of age in barramundi). In most mass spawners, and certainly for barramundi, how initial family compositions within cohorts change from hatchery to harvest is currently unknown.

Given the difficulties in producing large numbers of equally represented families the approach of walk-back selection has been proposed as a way to minimise the risks of inbreeding depression and achieve high genetic gains (Robinson and Jerry, 2009; Robinson et al., 2010). Walk-back selective breeding programs apply DNA fingerprinting for high-intensity, within-family selection, as a way to manage inbreeding, while obtaining high responses to selection (Doyle and Herbinger, 1994). Only the best performing individuals from each family are kept as breeding candidates. This is accomplished by a two stage selection approach. Firstly, a number of potential breeders are selected based on their phenotypic value, with the heaviest fish at harvest physically tagged and separately held until being genotyped for parentage analysis. Secondly, when pedigree information becomes available for these candidates only unrelated fish, or a limited number of individuals from each family, are kept as new broodstock (Doyle and Herbinger, 1994, 1995; Sonesson, 2005). Under the scenario of walk-back selection, skewed parental contributions generated by mass spawning and possible differential family survival pose a significant challenge to selective

breeding (Robinson and Jerry, 2009; Robinson et al., 2010). Unequal family sizes (often coupled with differential family growth) dictates that many more fish, or batches of fish, will need to be genotyped until sufficient numbers of unrelated breeding candidates are selected to keep inbreeding at acceptable levels (Bentsen and Olesen, 2002; Sonesson, 2005). The problem here is that the majority of stochastic simulations investigating optimised parent selection to minimise inbreeding in aquaculture species were mostly conducted under the assumptions of equal family stocking and survival rates across all families (e.g. Sonesson, 2005; Macbeth, 2005; Robinson et al., 2010; Hely et al., 2013). This is rarely the case in aquaculture populations and is especially so for mass spawning species. In the only simulation study that has taken into account skewed family contributions, Blonk et al. (2010a) demonstrated there was a compromise between inbreeding rates and response to selection for mass spawners when 5-10% of the best performing animals in the cohort were genotyped under specific simulated conditions (i.e. population of 8000 selection candidates; nucleus size of 200-300 broodstock and heritability of 0.2). However, simulated parameters such as heritability are species and population specific and results are likely to vary according to the actual number of surviving families available for selection at harvest and according to the real extent found in differences among family sizes and growth rates. Thus, results obtained under previous simulated conditions may not necessarily reflect the demographics of commercial mass spawning populations. To date, there have been no real studies investigating how uneven contribution of mass spawners and differential family survival through to harvest affect the selection of breeding candidates under a walk-back selection approach.

The objective of this study was to provide important information for the development of selective breeding strategies for mass spawners, using barramundi as a model. Efforts were

made in two Australian commercial hatcheries to maximize the broodstock contribution to three progeny cohorts. Firstly, genotyping and DNA parentage analysis was used to determine the number of families produced and identified at both the hatchery, and then later at harvest, in order to assess the fate of genetic diversity over the entire culture period. Secondly, the relationship between genotyping effort and genetic diversity identified at harvest was investigated to identify the most cost-effective genotyping strategy. The proportion of breeding candidates (i.e. heaviest animals) necessary to genotype in order to select individuals from all available families under commercial mass spawning and grow-out culture conditions was determined.

2.2. Materials and Methods

2.2.1. Experimental populations and fish sampling

Three commercial batches (cohorts) of mass spawned barramundi were sampled at 18 dph (larvae) at the hatchery, then later at harvest (*ca.* 500 g wet weight; 273-469 dph), to investigate changes in relative family contributions over time and the number of surviving families which could provide breeders to the next generation. Parents consisted of both locally wild caught and farmed fish, although the exact origin of each broodstock is unknown. Preceding each spawning, all broodfish present in the spawning tank were first anesthetised with 40 ppm of AQUI-S® (AQUI-S), PIT-tag identified, fin clipped for later DNA parentage analyses and assessed for spawning condition. Dams were cannulated using a plastic tube (1.2 mm internal diameter) and oocytes examined under a light microscope and those bearing loose, spherical and greater than 400 µm oocytes, were induced with a single intramuscular injection of luteinizing hormone release hormone analogue (LHRHa) at a dosage of 50 µg/Kg, which included four dams in batch 1, five dams in batch 2, and ten dams

in batch 3. More than 50% of males freely expressed milt by gentle pressure on the abdomen and no males were hormonally induced.

Because of the high fecundity of the species, i.e. a 10 kg mature female spawns an average of 3 million eggs (Garcia, 1990), hatcheries in Australia usually use only one or two dams and four or more sires (Macbeth et al., 2002). Numbers of broodstock present in the spawning tanks and number and age of offspring genotyped in each sampling period are summarised in Table 2.1. Spawning occurred in the broodstock holding tanks around dusk during the second and third nights after injection. Buoyant eggs from mixed families (i.e. with the least possible degree of environmental bias among families) were collected by skimming the water surface, eggs that sunk were discarded. Fertilization rates for the first nights spawn were generally low (< 50%) and only the eggs from the second night (fertilization rates > 85%) were kept for all three batches.

Table 2.1. *Lates calcarifer* broodstock census size and number of offspring sampled in the hatcheries and at harvest. Offspring age is reported in days post hatch (dph).

Number of	Batch 1	Batch 2	Batch 3
- broodstock, N_{tank}	12	12	33
- dams, $N_{d\ tank}$	6	6	12
- sires, $N_{s\ tank}$	6	6	21
- offspring sampled at hatchery (age)	550 (18 dph)	400 (18 dph)	472 (18 dph)
- offspring sampled at harvest (age)	470 (345 dph)	431 (469 dph)	1428 (273 dph)

Larval rearing followed intensive clear water marine finfish larviculture protocols, which included periodic grading after 18 dph to avoid cannibalism (Dhert et al., 1992; Schipp et al., 2007), until 26 dph (approximate fish size range at 26 dph: 6 – 18 mm standard length and

0.05 – 0.20 g wet weight), when offspring were sent to farms. Fish grow out took place in either a commercial intensive indoor recirculation facility in Melbourne (VIC, Australia) where grading and culling of slow growers was periodically done until fish reached 200g (batches 1 and 3), or a semi-intensive pond in Innisfail (QLD, Australia, batch 2) where no grading or culling was performed after stocking. Larvae sampled at 18 dph (just prior to first grading) were transported to a laboratory, euthanized with 0.5 ml/l of 2-phenoxyethanol, placed onto a glass slide next to a ruler and photographed under an Olympus® SZ61 stereo microscope connected to an Olympus® DP25 digital camera (Olympus) for posterior morphometric analysis (as described in Chapter 5), and fin clipped (~ 2 mm^2) for DNA parentage analyses. The larval fin clips and remaining larval body were placed into separate 48 well microtitre plates covered with strip caps (Axygen). Larvae were preserved by either adding 150 µl of RNA*later* (Ambion) per well and keeping plates at 2 ^{0}C overnight before storing at – 80 ^{0}C, or by placing plates afloat onto liquid nitrogen for 30 min before being stored at – 80 ^{0}C. Plates containing larval fin clips were immediately processed for DNA parentage analyses. Sampling at harvest consisted of anesthetising fish (AQUI-S), fin clipping for DNA analysis, weighing fish for wet weight (W) with an electronic scale (to nearest 1 g) and photographing fish next to a ruler and an individual label tag identifier with a digital camera mounted on a camera stand for posterior morphometric analysis (as described in *Chapter 3*).

2.2.2 DNA extractions

Broodstock and harvested offspring fin tissues collected throughout this study were preserved into individually labelled microfuge tubes containing DMSO-salt solution (20% DMSO, 0.25 M disodium-EDTA and NaCl to saturation at pH 8) (Seutin et al., 1991) until DNA was extracted, whereas larval DNA was extracted from fresh tissue. A small subsample of fin

tissues (*ca.* 1 mm^2) from barramundi broodstock and juvenile offspring was used for DNA extraction. DNA was extracted by placing individual tissues into individual wells of 96 well microtitre plates containing 100 µl of Tween®-20 lysate buffer (670 mM Tris-HCl pH 8.0, 166 mM Ammonium sulphate, 0.2% v/v Tween®-20, 0.2% v/v IGEPAL® CA-630) and 5 µl of 20 mg/ml Proteinase K. Plates were incubated at 55 ºC for 4 h followed by 95 ºC for 20 min (modified from Taris et al., 2005). Plates were then centrifuged at 930 *g* for 5 min and stored at -20 ºC overnight. The supernatant (0.5 µl) of the tissue crude lysate was used as a genomic DNA (gDNA) template for PCR within 48 h.

2.2.3 PCR amplification of microsatellites

A number of microsatellites markers have become available for barramundi parentage assignment and population genetics studies in the last decade (Yue et al., 2001, 2002, 2009; Sim and Othman, 2005; Frost et al., 2006; Wang et al., 2008; Zhu et al., 2006a, 2009, 2010, Loughnan et al., 2013). From those, a single PCR multiplex suite of 10 microsatellites (*Lca020*, *Lca032*, *Lca040*, *Lca057*, *Lca058*, *Lca064*, *Lca070*, *Lca098*, *Lca287*, *Lca371*) was developed based on simulations of assignment rates for the particular group of brooders in the spawning tank for batches 1 and 2. Forward and reverse primers for each multiplex were first combined into a 10x primer mix (1-3 µM of each primer, adjusted according to product fluorescence intensities). The PCR reagents consisted of 1 µl of 10x primer mix (1-3 µM of each primer), 0.2 µl of dNTPs, 0.2 µl of Phire® Hot Start II DNA Polymerase (Thermo Scientific), 2 µl of 5x Phire® Reaction Buffer, 6.1 µl of water and 0.5 µl of gDNA crude lysate. PCR cycling conditions were 98 ºC for 1 min (initial denaturation) followed by 34 cycles of 98 ºC for 5 s (denaturation), 60 ºC for 5 s (annealing), 72 ºC for 15 s (extension) and 72 ºC for 1 min (final extension). However, due to greater number of broodstock and possible family combinations in batch 3, simulations indicated that additional loci were required to

achieve high parental assignment rates. Therefore, for batch 3, seventeen microsatellite markers were used to genotype broodfish and offspring in two multiplex PCR suites of eight (*Lca003*, *Lca016*, *Lca040*, *Lca057*, *Lca154*, *Lca178*, *Lca287*, *Lca371*) and nine (*Lca008*, *Lca020*, *Lca021*, *Lca058*, *Lca064*, *Lca069*, *Lca070*, *Lca074*, *Lca098;* these as multiplexed by Zhu et al., 2006b) microsatellite markers. PCR reagents consisted of 1 µl of 10x primer mix, 5 µl of 2x Type-it® PCR Buffer (Qiagen), 3.5 µl of water and 0.5 µl of crude lysate gDNA (10 µl reaction). Microsatellite amplification was performed on a C1000 Thermal Cycler (Bio-Rad) using the following cycling conditions: 95 °C for 5 min (initial denaturation) followed by 10 cycles of 95 °C for 30 s (denaturation), 57 °C for 90 s (annealing) and 72 °C for 30 s, then 20 cycles of 95 °C for 30 s (denaturation), 55 °C for 90 s (annealing) and 72 °C for 30 s (extension), then a final step of 60 °C for 30 min.

The success of PCR amplification was checked by visualisation on a 1.5% TBE agarose gel containing GelGreen® (Biotium Inc.), where 2 µl of PCR products were loaded and electrophoresed for 25 min at 80 V and 400 mA. The remaining PCR products were diluted with 12 µl of water and desalted through a Sephadex® G-50 Fine (GE Healthcare) filtration spin column (3 min at 930 *g*).

2.2.4 Genotyping and parentage analysis

PCR products were genotyped on a MegaBACE® 1000 DNA Analysis System (GE Healthcare) at the Genetics Analysis Facility of James Cook University (Townsville, Australia). Sizing of the various PCR products was achieved by utilising 5' fluorochrome labelling of the forward primer (FAM, HEX or TET) to visualise the product and compare to the MegaBACE® ET400-R size standard run concurrently with the sample (batches 1 and 2) or MegaBACE ET500-R size standard (batch 3). Fragment analysis was carried out using the

MegaBACE® software Fragment Profiler®. Genotyping was considered successful when scoring of PCR products provided size information for at least seven markers per sample. Parentage assignment of genotyped offspring was performed based on a likelihood approach using Cervus 3.0 (Kalinowski et al., 2007). Prior to parentage assignment, Cervus 3.0 firstly utilizes broodstock allele frequencies for simulating parents of known sex and returns the expected assignment likelihood based on the number of loci genotyped and numbers of parents from each sex. Accordingly, the following parameters were utilized for simulations: 100% of loci typed, the allowance of 1% error rate for scoring genotypes and 10,000 offspring simulated. Samples with less than seven markers genotyped (due to PCR or genotyping errors) were not tested for parentage analysis, as *a priori* simulations of offspring assignment to a parental pair indicated reduced levels of assignment rates below seven microsatellite markers. Parentage assignment was considered successful and samples retained for further analysis when offspring were assigned with $\geq$ 95% confidence to a parental pair. Further information on the properties of microsatellites used may be found in

2.2.5 Statistical analysis

Several indices to gauge genetic variation within broodstock and offspring sampled at the hatchery and later at harvest were used to assess for potential losses of genetic diversity over time. Analyses included the total number of alleles (*k*), mean number of alleles per locus (k_{locus}), observed heterozygosity (*Ho*) and expected heterozygosity (*He*), total number of families and broodstock contributing in each sampling period (Cervus 3.0, Kalinowski, et al. 2007); allelic richness (*Ar*) (FSTAT 2.9.3, Goudet, 2001) and relatedness estimates (*Rxy* Queller and Goodnight (1989) relatedness estimator) (Coancestry 1.0.1.2, Wang, 2011). To obtain unbiased R_{xy} estimates within each group (i.e. broodstock, larval and harvested cohorts), an allele frequency file based on 400 wild *L. calcarifer* individuals sampled from

the same genetic stock as hatchery broodstock was used to produce the reference, randomly mating population (Jerry and Smith-Keune, 2013). The effective population size (N_e) was also calculated, as it is considered the primary variable of importance for monitoring the genetic variation present in a population and predicting the impact of management practices on the loss of genetic variability (Lande and Barrowclough, 1987). The N_e demographical approach of Lande and Barrowclough (1987) was used as this algorithm accounts for uneven sex ratio by factoring in the effective number of dams (N_{ed}) and sires (N_{es}) in each sampling period as

$$N_e = 4 / [(1/ N_{ed}) + (1/N_{es})] \quad (1)$$

$$N_{ed} = (N_d V_d - 1) / [K_d + (V_d/K_d) - 1] \quad (2)$$

$$N_{es} = (N_s V_s - 1) / [K_s + (V_s/ K_s) - 1] \quad (3)$$

where K_d and K_s is the mean number of progeny per dam and sire, respectively and V_d and V_s is the variance in contribution for dams and sires. Additionally, the rate of inbreeding (ΔF) was calculated as $\Delta F = 1/2(N_e)$ (Falconer and Mackay, 1996).

Differences in relative family contributions over time (i.e. differential family survival from hatchery to harvest) were assessed with Pearson's 2-sided chi-square tests (SPSS 19.0, IBM). Results were considered significant at $P< 0.05$.

2.2.6. Quantifying genotyping efforts under skewed family contribution

Cohorts with skewed family contributions demand greater genotyping efforts than cohorts with even numbers of offspring per family, increasing genotyping costs of breeding programs (Sonesson, 2005). However, the magnitude of this difference has not yet been established

under commercial mass spawning conditions. To quantify the extent of additional sampling needed to capture all available familial genetic diversity, a comparison was made between a hypothetical cohort with equal sizes of families and a real mass spawned cohort with skewed family contributions. The parentage assignment dataset of batch 3 at harvest, comprising 1400 offspring from one of 121 parental pairs (i.e. families) was used as the real mass spawned cohort (Results section, Table 2.2). A simulated dataset of a batch with even family contributions was artificially generated in Microsoft Office Excel, also comprising 1400 individuals, but equally distributed to 121 families (i.e. being 69 families with 12 offspring each and 52 families with 11 offspring each). For each dataset, groups in multiples of 24 individuals (as genotyping is usually performed in sub-multiples of 96-well plate format) were randomly taken 50 times and the total number of families in each group counted until all 121 families could be identified.

Furthermore, as in walk-back selection only the best performing (e.g. heaviest) fish from each family are retained as broodstock, it is of interest to gauge the required genotyping effort considering fish weight and family of origin data under commercial mass spawning and grow out conditions. To provide sampling baselines for future barramundi breeding programs, genotyping efforts to capture the heaviest fish from each family were therefore quantified as (i) proportion of the population selected for genotyping based on harvest weight, (ii) number of heaviest fish and (iii) cut-off values in units of standard deviation (S.D.) from the population mean. Firstly, each harvested population (*c.a.* 50,000 individuals) were reconstructed *in silico* using Microsoft Office Excel based on the real phenotypic and genetic datasets acquired for each batch (20 replicates per batch). Family sizes were based on the relative proportion of family contributions and progeny weight distributions were reconstructed based on family metrics (mean family weight ± S.D), assumed normally

distributed. Each individual was given a family identifier and then pooled to reconstitute the harvested population. To increase statistical confidence of family metrics, only families where DNA parentage analyses returned assignments for five or more offspring were included in the population modeling (i.e. seven, nine and 66 families of batches 1, 2 and 3 respectively). Then, each reconstructed population was ranked by harvest weight and multiples of 24 sub-samples of the heaviest individuals were taken until at least one fish from all families present in each batch was identified.

2.3. Results

2.3.1. Fate of family contributions from hatchery to harvest

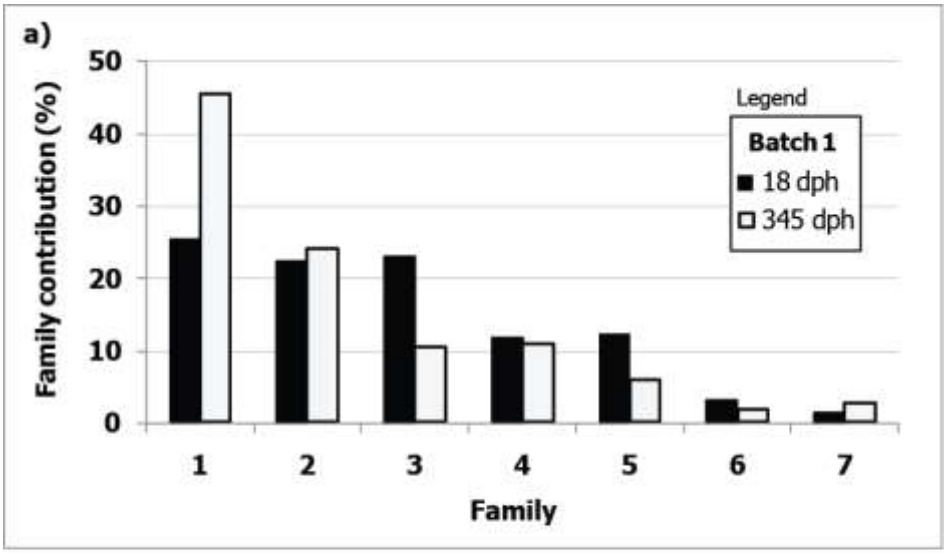
A total of 3651 offspring were DNA pedigreed in the hatchery (1391 larvae) and at harvest (2260 fish) in the three mass spawning events monitored in this study (Table 2.2).

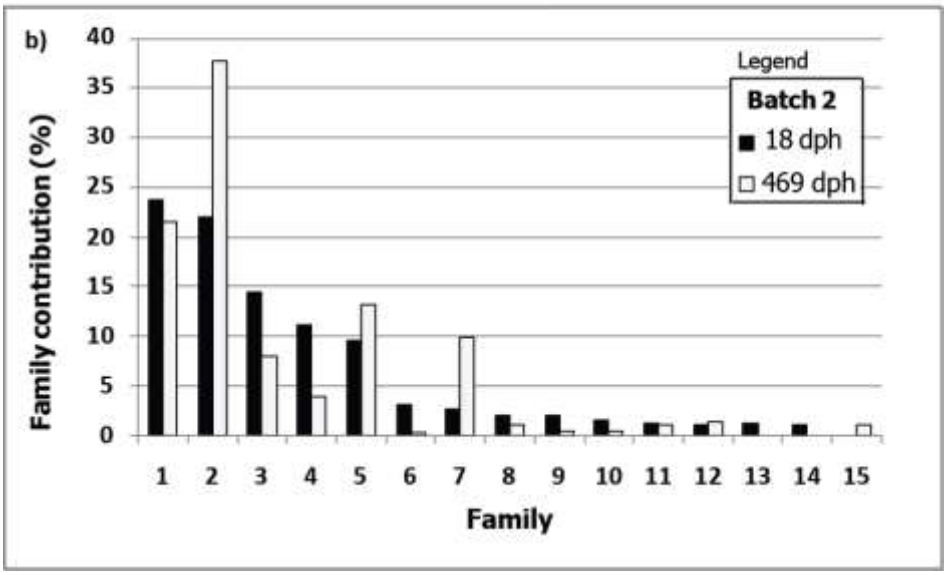
Overall, the offspring assignment rate to a parental pair was 97.3% (95% confidence level). Family contributions were highly skewed, with a few families dominating the majority of the cohort, especially in batches 1 and 2 (Figure 2.1). Nevertheless, the large majority of families identified earlier in the hatchery were also found later at harvest and no major family drop-out was observed for any batch (Figure 2.1).

Table 2.2. Numbers of *Lates calcarifer* offspring samples assigned to a parental pair, families and broodstock in the hatchery and at harvest in three mass spawning events.

		Batch 1		Batch 2		Batch 3	
		Hatchery	Harvest	Hatchery	Harvest	Hatchery	Harvest
- assigned offspring (genotyping success)		541 (98.4%)	445 (94.7%)	387 (96.7%)	415 (96.3%)	463 (98.1%)	1400 (98.0%)
- families (unique)		7 (0)	7 (0)	14 (2)	13 (1)	103 (19)	121 (37)
- dams	$N_{d\ tank}$	6	6	6	6	12	12
	N_d	2	2	3	3	8	8
	N_{ed}	0.9	0.9	1.2	1.0	5.2	5.9
	$N_{ed} / N_{d\ tank}$	0.15	0.15	0.20	0.17	0.43	0.49
- sires	$N_{s\ tank}$	6	6	6	6	21	21
	N_s	6	6	6	6	21	21
	N_{es}	4.9	3.2	4.6	3.8	12.5	10.8
	$N_{es} / N_{s\ tank}$	0.82	0.53	0.77	0.63	0.60	0.51
- broodstock	N_{tank}	12	12	12	12	33	33
	N	9	9	10	10	29	29
	N_e	3.0	2.8	3.8	3.2	14.7	15.2
	N_e / N_{tank}	0.25	0.23	0.31	0.27	0.45	0.46
ΔF		16.8%	17.9%	13.1%	15.5%	3.4%	3.3%

$N_{d\ tank}$, $N_{s\ tank}$, N_{tank} = number of dams, sires and broodstock present in the spawning tank.
N_d , N_s , N = number of dams, sires and broodstock which parented offspring.
N_{ed} , N_{es} , N_e = effective number of dams, sires and broodstock.
ΔF = inbreeding coefficient


a)
Family contribution (%)
50
40
30
20
10
0
1
2
3
4
5
6
7
Family
Legend
Batch 1
18 dph
345 dph


b)
Family contribution (%)
40
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Family
Legend
Batch 2
18 dph
469 dph

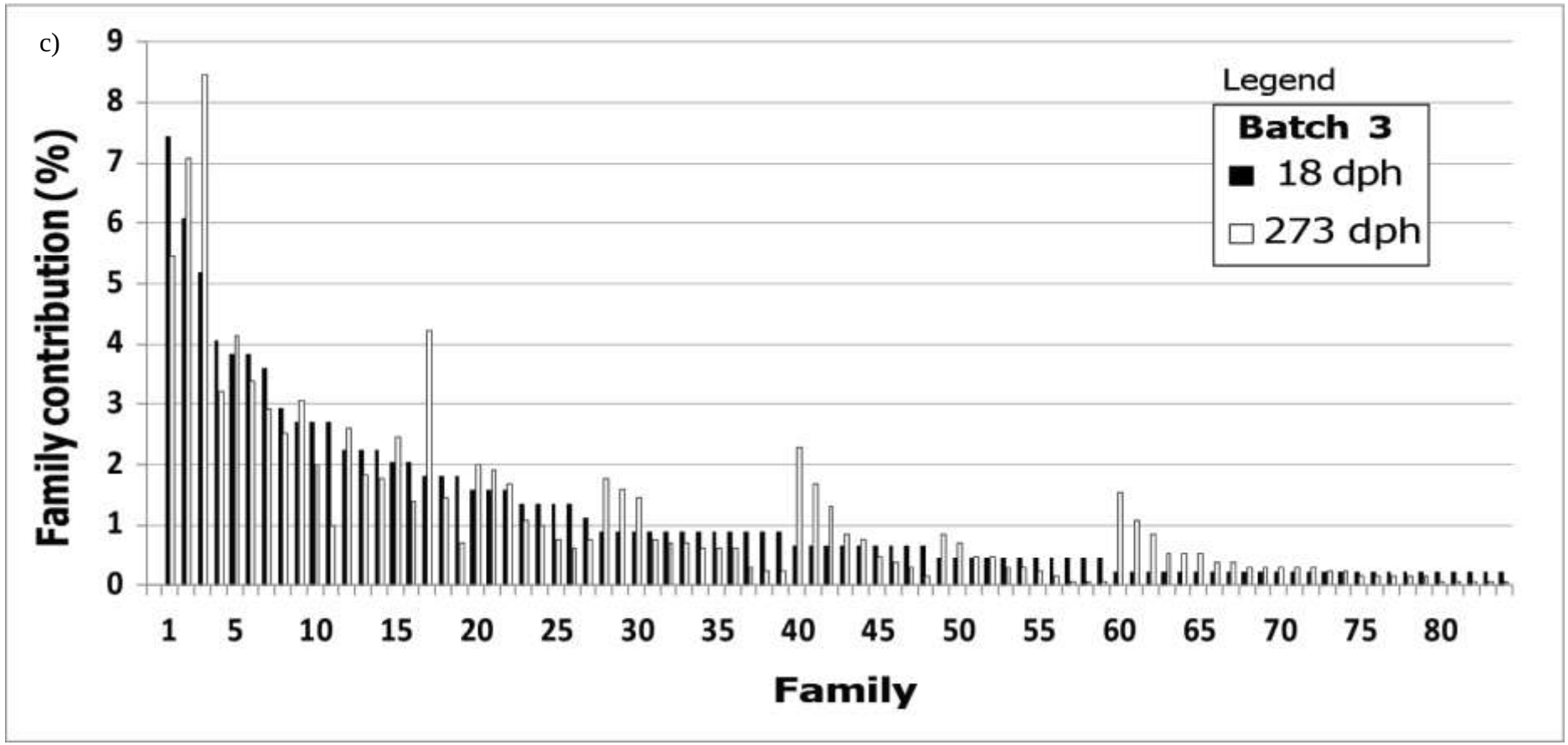


Figure 2.1. Relative family contributions of farmed *Lates calcarifer* from hatchery to harvest in (a) batch, (b) batch 2 and (c) batch 3.Offspring age reported in days post hatch (dph).

For instance, all seven families in batch 1 initially identified in the hatchery were also found at harvest. In batches 2 and 3, individuals belonging to unique families were found in both sampling periods (Table 2.1), most likely due to the high variance in family contributions in these cohorts and the low probability to sample individuals from least represented families. In addition, all 48 broodstock parenting offspring at the hatcheries were also found to parent offspring at harvest in all of three batches examined. Despite the long period between sampling periods within cohorts (*ca.* one year), the effective number of broodstock (N_e) reduced only slightly in batches 1 (3.0 to 2.8) and batch 2 (3.8 to 3.2), and even increased slightly in batch 3 (14.7 to 15.2), likely due to a greater sampling effort at harvest. Inversely, inbreeding rates increased from hatchery to harvest slightly from 16.7% to 17.9% in batch 1, from 13.5% to 15.6% in batch 2 and decreased slightly in batch 3 from 3.4% to 3.3%. Likewise, as revealed by insignificant changes of N_e between larval and harvested cohorts, comparative analyses of multiple indices based on allelic data also indicated that once larval cohorts reached 18 dph there were no significant changes in genetic diversity within cohorts over the culture period, as assessed one year later at harvest (Table 2.3).

Table 2.3. Genetic diversity of *Lates calcarifer* broodstock and offspring cohorts sampled at the hatchery and at harvest. Offspring age given in days post hatch (dph). *k* = number of alleles, k_{locus} = mean number of alleles per locus, *Ar* = allele richness, *Ho* = observed heterozygosity, *He* = expected heterozygozyty, R_{xy} = relatedness coefficient.

	Batch 1			Batch 2			Batch 3			Overall average		
	Broodstock	Hatchery	Harvest	Broodstock	Hatchery	Harvest	Broodstock	Hatchery	Harvest	Broodstock	Hatchery	Harvest
	-	18 dph	345 dph	-	18 dph	469 dph	-	18 dph	273 dph	-	18 dph	362 dph
k	42	41	40	42	36	35	73	68	69	52.3	48.3	48.0
k_{locus}	4.2	4.1	4.0	4.2	3.6	3.5	4.3	4.0	4.1	4.2	3.9	3.9
Ar	4.12	3.41	3.38	4.12	3.03	3.01	3.94	3.48	3.62	4.06	3.31	3.34
Ho	0.684	0.686	0.673	0.684	0.546	0.610	0.515	0.518	0.505	0.628	0.583	0.596
He	0.648	0.599	0.613	0.648	0.512	0.523	0.509	0.501	0.496	0.602	0.537	0.544
Rxy	0.113	0.254	0.217	0.113	0.458	0.461	0.177	0.198	0.202	0.134	0.303	0.293

Differences were observed in survival rates for several families within each batch. The relative contribution of particular families either increased, or decreased substantially over time (e.g. families 1, 3 and 5 in batch 1, Figure 2.1 (a); families 2, 4, 6 and 7 in batch 2, Figure 2.1 (b); families 3, 17 and 40 in batch 3, Figure 2.1 (c)), which caused overall family contributions at harvest to be in statistically different proportions than those previously found in the hatchery (batch 1 χ^2 = 125, d.f. = 6, P< 0.001; batch 2 χ^2 = 181, d.f. = 11, P< 0.001; batch 3 χ^2 = 484, d.f. = 83, P< 0.001). Notwithstanding, differential family survival did not lead to elimination of families. Furthermore, differences in family survival rates were not significant enough to erode genetic diversity from 18 dph through to harvest, as values of all genetic diversity indices remained stable over the culture period (Table 2.2).

2.3.2. Generation of families through mass spawning and broodstock reproductive success

If strip spawning and artificial fertilization were possible, a total of 36 families (6 sires x 6 dams) could have been generated in batches 1 and 2, and 252 families (21 sires x 12 dams) in batch 3. However, commercial mass spawnings generated a comparatively low number of families, ranging between 19% (batch 1) to 48% (batch 3) of all possible families. Family numbers were significantly higher in batch 3, which comprised the largest broodstock group (up to 140 families), when compared to batches 1 and 2, where only seven to 15 families were generated, respectively.

All sires present in the spawning tanks fathered offspring, whereas only 33 to 67% of dams mothered progeny (Table 2.1). Parental contributions were highly skewed, most notably for dams. In mating groups involving lower numbers of potential breeders, a single dam mothered an average of 96.8% of the cohort in batch 1 (N_{ed} = 0.9) and 87.4% of the cohort in batch 2 (N_{ed} = 1.1), despite the fact that four and five out of six dams, respectively, bore

viable eggs and had been hormonally induced. As a result, dams had the lowest ratios between effective parental contribution and census size ($N_{ed}/N_{d\ tank}$), averaging 0.15, 0.18 and 0.46 in batches 1, 2 and 3, respectively. Sires had higher $N_{es}/N_{s\ tank}$ ratios than dams, averaging 0.67 (batch 1), 0.70 (batch 2) and 0.55 (batch 3). Additionally, when the unequal sex ratios were also taken into account to assess overall population sizes, N_e was very low for the smaller breeding group (batch 1 and 2), ranging from 2.8 and 3.8 when compared to the larger breeding group, which was around 5-fold higher (batch 3, N_e ~ 15). Even so, in all batches including batch 3, only a small proportion of all available broodstock effectively contributed to offspring. The ratio between broodstock effective contribution and census size (N_e/N_{tank}) was on average 0.24 in batch 1, 0.29 in batch 2 and 0.45 in batch 3. As a consequence, average inbreeding rates were very high for batch 1 (17.3%) and batch 2 (14.5%), and high for batch 3 (3.3%). As revealed by N_e / N_{tank}, only part of the genetic diversity of all broodstock available in the tanks was transferred to offspring, with overall reductions of 8.0% in k, 7.1% in k_{locus}, 18.2% in A_r, 0.04 in H_o, 0.06 in H_e and a 0.16 increase in R_{xy} (Table 2.2). Again, the use of greater numbers of broodstock in batch 3 was shown to soften the loss of genetic variability from parents to offspring. For instance, R_{xy} relatedness values between generations (i.e. broodstock to offspring) increased significantly in batch 1 (from 0.113 to ~0.236) and batch 2 (from 0.113 to ~0.460), whereas this index increased just slightly in batch 3 (from 0.177 to ~0.200) (Table 2.2).

2.3.3. Skewed family contributions require greater genotyping efforts

Based on the largest dataset from this study (batch 3, harvest), a cohort with skewed family contributions required more than twice as many samples to detect all the available genetic diversity than a similar simulated cohort having an even number of individuals per family (Figure 2.2). For example, with 192 samples genotyped (i.e. equivalent to two 96-well

plates), only 54% of all families could be identified from this mass spawning, whereas 78% of all families would have been identified if family representation was even. With an increased genotyping effort of 600 samples, only 80% of all mass spawned families could be identified, compared to 100% of families present in an evenly distributed cohort at this level of genotyping effort. To identify all 121 families from this mass spawning, simulations suggested that 1392 genotyped samples were in fact needed.

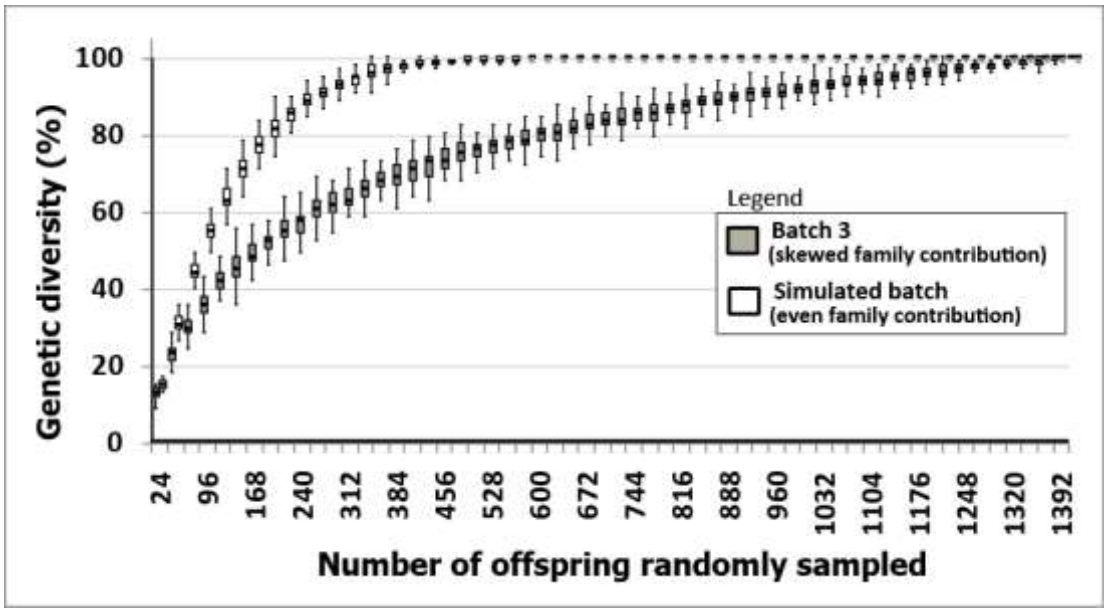


Figure 2.2. Comparison between the genotyping efforts of a randomly sampled simulated cohort with even family contribution and a randomly sampled true mass spawned cohort with skewed family contribution (parentage dataset of Batch 3 at harvest). Both cohorts contained 121 families (100% of family-specific genetic diversity). Box plots represent 50 replicates per sampling size.

2.3.4. How many of the heaviest fish need to be genotyped to capture all family diversity?

Mean fish weights at harvest were similar among batches *ca.* 500 g (mean (g) ± S.D./c.v. = 531 ± 143/27.1%; 485 ± 128/26.5%; 495 ± 125/25.3 %, for batches 1, 2 and 3 respectively), but age at harvest varied from 273 to 469 dph, due to different environmental conditions and rearing management protocols. Based on the relative family contributions and family metrics

(mean family weight ± S.D.) at harvest, the weight distribution curves of the original populations were reconstructed *in silico* and individuals ranked by harvest weight. Due to skewed family contributions, the family-specific genetic diversity captured according to the number of genotyped fish followed a logarithmic function (Figure 2.3).

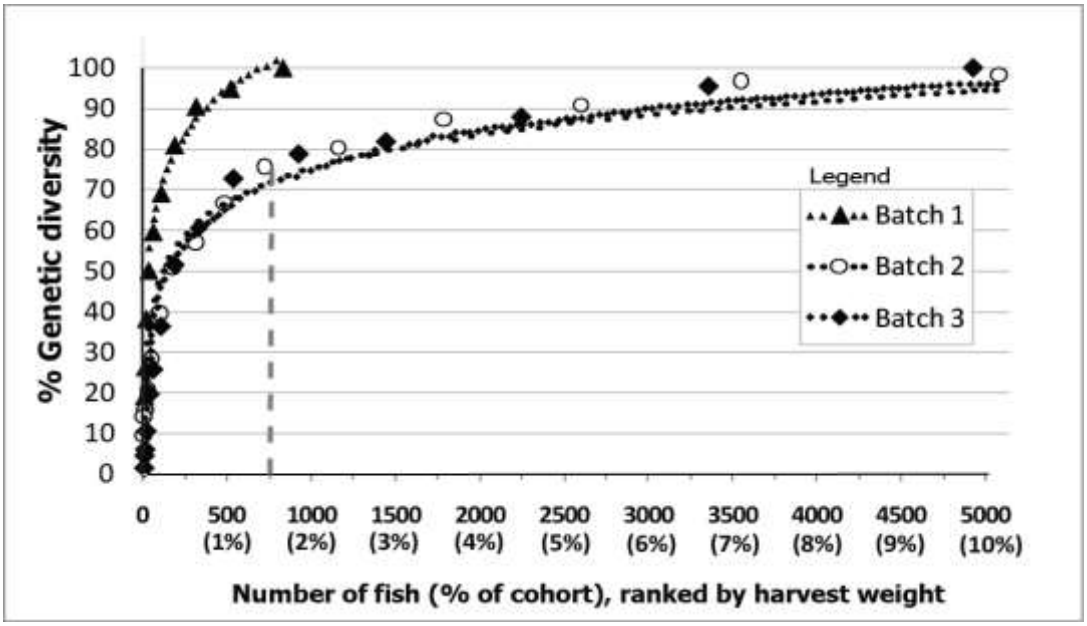


Figure 2.3. Number of heaviest *Lates calcarifer* at harvest (also reported in % of cohort and sampling threshold in harvest weight standard deviations, S.D.) needed to be genotyped in batches 1 to 3 to include offspring from all families. Dashed line corresponds to the top 1.5% of the population (~ 750 heaviest fish or individuals ≥ 2.17 S.D. heavier than the population mean), which included 75% or more of the family-specific genetic diversity present in the three batches. 100% of family-specific genetic diversity represents seven, nine and 66 families with five or more assigned offspring in batches 1, 2 and 3, respectively.

The genotyping efforts required to capture the heaviest individual from all families varied among batches. A lower sample size was needed for batch 1, which included all fish heavier than +2.13 S.D. from the population mean (~ 1.6% top heaviest). For batches 2 and 3, all fish heavier than +1.28 S.D. from the mean (~ top 10% heaviest) would have to be genotyped, despite the discrepancy in the absolute number of families between these batches (> 7-fold

difference). In practice, *ca.* 5000 of the heaviest fish would need to be genotyped to capture the entire available genetic diversity. As additional breeding candidates were genotyped up to the heaviest 1.5% of the population (or 750 breeding candidates heavier than +2.17 S.D. of the population mean), there was a sharp initial increase in the proportion of genetic diversity captured, which included around 75% of all families. However, beyond 1.5 % of the population a much greater genotyping effort was needed to detect individuals from the remaining 25% of families. In other terms, in the first 1.5% of the heaviest fish in each cohort, each 10 additional fish genotyped identified a further 1% of the family-specific genetic diversity available. However, beyond 1.5%, *ca.*170 more fish were needed for genotyping to yield an additional 1% of the genetic diversity available.

2.4. Discussion

2.4.1. Genetic diversity from hatchery to harvest

Interestingly in this study, the majority of families and all 48 broodstock contributing to offspring initially identified at 18 dph were also detected at harvest in all three commercial batches of barramundi examined. Moreover, even when the highly differential parental contributions, both between and within, gender were taken into account, there was no reduction in N_e over the culture period in the largest batch 3 and only a small reduction in batch 1 (absolute N_e reduction of 0.2) and batch 2 (absolute N_e reduction of 0.6), which for practical breeding purposes can be considered insignificant. N_e has been used as an index of how aquaculture operations are capturing and maintaining genetic diversity, as N_e changes can predict the impacts of and the long-term success of genetic selection programs (Lind et al., 2010). Not surprisingly, genetic analyses based on genotypic data (Table 2.2) mirrored trends revealed by N_e. Families with low starting contributions at the hatcheries were expected to be eliminated during culture, with a resulting loss of allelic diversity available for

selection at harvest. However, this was not the case in practice. In batches 1 and 3 around 80% of the hatchery population was culled, sold as young juveniles, or lost due to mortalities throughout the production cycle (P. Harrison, Mainstream Aquaculture Pty Ltd., *pers. comm.*), and yet no major family drop-out was observed. Results are surprising taking into account that there was differential family survival over the culture period. Differential family survival overtime is not uncommon and has also been reported for various aquaculture species, such as salmon (Herbinger et al., 1999), pearl oysters (Lind et al., 2009) and prawns (Jerry et al., 2006). However, once families were produced and offspring reached 18 dph, stochastic events which led to differential family survival from hatchery to harvest were not significant enough to erode genetic diversity in the barramundi harvested cohorts genotyped here.

A previous smaller scale study of barramundi broodstock contribution warned of potential loss of genetic diversity due to husbandry practices such as mass spawning a small number of breeders, size grading and culling of slow growers, reporting that effective population size decreased from 4.5 to 4.3 and from 2.7 to 2.4 in two commercial hatcheries in the short time span of 2 dph to 27 dph (Frost et al., 2006). This study suggested that greater losses could happen up until harvest. More recently, a similar study investigating the effects of broodstock contribution after mass spawning and size grading of barramundi during early culture detected no significant changes in any of the indexes used to gauge genetic diversity from 1 to 90 dph (Loughnan et al., 2013). However, the contradictory results from these previous studies were based on larvae and young juveniles (e.g. at 90 dph barramundi weighs around 10g) and on small sample sizes (e.g. 276 fish sampled at 90 dph by Loughnan et al., 2013). Therefore, no information was previously available on the fate of genetic diversity within cohorts until the critical moment of selection (harvest). Here, results based on the much

longer rearing period from 18 dph to harvest on large sample sizes of three commercial batches provided strong evidence that despite differential family survival, genetic diversity was retained within cohorts over the culture cycle. Therefore, grow out rearing practices did not represent a bottleneck for genetic variability within generations, as was the case between generations (parent-offspring).

2.4.2. Bottlenecks and strategies to improve genetic diversity of mass spawners

Results showed that the realization of adequate N_e and generation of a sufficiently large number of families at the start of a batch is the main bottleneck to achieving and maintaining high levels of genetic diversity for selective breeding programs of commercially important mass spawners like barramundi. Here, attempts to boost genetic diversity through the hormonal induction of several ripe dams within a single tank still yielded low N_e and high rates of inbreeding. Highly variable levels of parental contributions leading to reductions in levels of genetic diversity from broodstock to offspring are in close agreement to those obtained for other barramundi studies (Frost et al., 2006; Wang et al., 2008, Liu et al., 2012; Loughnan et al., 2013), as well as those reported for other commercially important mass spawners, such as gilthead seabream (*Sparus aurata*) (Brown et al., 2005; Borrell et al., 2011) and Atlantic cod (*Gadus mohua*) (Herlin et al., 2008). Thus, the development of successful strategies to maintain acceptable levels of genetic diversity in a breeding nucleus is a main challenge to selective improvement of commercially important mass spawners. For aquaculture breeding schemes, Bentsen and Olesen (2002) recommended the selection of 50 broodstock pairs to keep inbreeding levels below 1% per generation. Even within a successful large mass spawn, such as in batch 3, which reached the highest genetic diversity reported within the literature to date for a single barramundi cohort, the inbreeding rates were *ca.* 3.3%.

Nevertheless, one positive implication for selective breeding of mass spawners is that results obtained in this study are in many aspects similar, or even superior, to those obtained from artificially fertilized cohorts of European sea bass (*Dicentrarchus labrax*), where eggs from different families were mixed after fertilization (Novel et al., 2013). In this study, high differences in parental contributions from a complete factorial mating among five dams and 30 sires also resulted in a reduction of genetic diversity between generations, and likewise it was found for barramundi here, no significant bottlenecks were identified within generations (Novel et al., 2013). From 35 broodstock with a mean number of alleles corrected by sample size (*An*) of 15.25, larval (40 dph) *An* decreased to 12.0 but not significantly further over the production cycle (4-7 months juveniles *An* = 11.9; 2 year adults *An* = 11.3) (Novel et al., 2013). Despite Novel et al. (2013)'s claim that 120 growth-selected F1 adults (based on coancestry matrices from ten microsatellite markers) could be a good broodstock to establish a breeding program for European sea bass, caution should be exerted by relying only in one batch per generation. Even with the important resource of artificial fertilization, it is important to note that F1 selected adults in Novel et al. (2013) originated from only five maternal families and N_e of F1 was critically low (N_e = 10.5).

For mass spawners, such as barramundi, it is not possible to equalize family sizes at larval or juvenile stages before communal rearing, a practice shown to retain larger N_e at later stages (Lind et al., 2010; Garber et al., 2010). Therefore, to retrieve selection candidates at harvest from sufficient numbers of unrelated families in order to avoid inbreeding depression, breeding programs must account for spawning (and selecting from) several batches per generation. Alternatively, it has been suggested that broodstock could be spawned in several isolated small groups per tank (e.g. one female and two to three males) and equal quantities

of larvae pooled for rearing (Robinson et al., 2010). The advantages of this practice, however promising for a future breeding nucleus, have not yet been reported for barramundi. In any case, sampling schemes that minimise costly genotyping efforts are therefore highly desirable.

In this study, important trends which may assist future barramundi broodstock management were observed. Relative contributions among available sires were on average 2.4 times greater than the contributions of available dams. In batches 1 and 2, one dam parented the majority of the progeny. Likewise, Liu et al. (2012) reported that females had more variable success in spawning than males for captive stocks of Asian populations of barramundi, especially when breeders were not carefully checked for reproductive status. Contrarily, greater paternal contributions may be expected. All sires present in the three batches monitored in the present study fathered offspring. Among other factors, a possible justification for differential levels in contribution among broodstock, especially dams, may be due to differential spawning timings and lack of synchronization in the release of gametes among different dams and sires. Such trends in expected reproductive performance between genders can be further exploited in mating schemes. However, it is important to note that no single rule applies for mass spawners, as expected gender performance is most likely a species-specific reproductive characteristic (e.g. Brown et al., 2005; Herlin et al., 2008).

2.4.3. Skewed family contributions requires greater genotyping efforts

The selection, tagging and genotyping of the best performing fish are all integral parts of modern sustainable breeding programs with the basic objective to improve growth rates, while controlling for inbreeding. Sampling efforts to retrieve all available genetic diversity from a cohort with skewed family contributions was double that of a cohort with even family

contributions, as individuals from families with lower contributions were less likely to be sampled than individuals from the highly represented families. Therefore, skewed family contributions indeed impact on genotyping efforts and increase sampling costs (Sonesson, 2005). Nevertheless, in breeding programs applying walk-back selection, only the best performing fish are genotyped. Here, the similarity in genotyping efforts to identify individuals from all families between batches 2 (9 families modelled) and 3 (66 families modelled) indicated that the number of samples needed may not necessarily be related to the total number of families. More likely, it is likely that differential growth rates among families may have a significant impact upon the number of genotyped samples necessary to capture all the genetic diversity. This measure of the between-family group variance over the total variance, also known as phenotypic intra-class correlation (Falconer and Mackay, 1996), has been shown to significantly impact on the rates of inbreeding (Macbeth, 2005). This is because in cohorts with significant differences among family-specific growth rates, greater sampling efforts are needed to include the heaviest individuals from poor growing families. For instance, simulations based on mean and variance weights of 30 families of the Kuruma prawn, *Penaeus japonicus*, revealed that not all families would be captured unless 4000 of the heaviest prawns (out of 6000 originally collected) were genotyped (Jerry et al., 2006).

2.4.4. Trade-offs between sampling effort and genetic diversity may reduce genotyping costs and increase genetic gains

The simulations performed in the present study, based on real phenotypic and genetic data, indicated that a genotyping effort representing around 10% of the population (*ca.* 5000 fish or all individuals heavier than 1.28 S.D. of population mean) would be necessary to capture the best performing individuals from all families. In practice, such numbers may be both impractical and cost prohibitive, especially if breeding candidates from several batches over

the breeding season are genotyped. Furthermore, as additional lighter breeding candidates belonging to slower growing families are genotyped, EBV of future broodstock would decrease, lowering selection intensity and the response to selection. The achievement of high genetic gains with low inbreeding rates, while factoring for genotyping efforts and associated costs, has been proven feasible under stochastic simulations where ideal numbers of broodstock equally contribute to offspring (e.g. Sonesson, 2005). However, equal family sizes are obviously not applicable for barramundi and other mass spawning species. Considering species with mass spawning behaviour, but utilizing entirely simulated parameters, Blonk et al. (2010a) reported that genotyping 5% to 10% of a population of 8000 offspring (i.e. 400 to 800 fish) from a breeding nucleus of 200 to 300 fish would reach a compromise between inbreeding rates and response to selection. In our study, under realised commercial mass spawning conditions, a trade-off between genotyping effort and the proportion of genetic diversity captured in barramundi was found to be around 1.5% of the heaviest fish in the population (*ca.*750 candidate breeders or fish heavier than 2.17 S.D. of the population mean). Genotyping the top 1.5% would not only significantly reduce sampling effort, but would also provide a combined selection strategy which maximizes selection intensity by combining within and between family selections, as simulations indicated that the best performing individuals belonging to *ca.* 75% of families would be present. Due to the necessity to genotype several batches for the selection of sufficient numbers of breeding candidates, the genotyping sample scheme identified here may contribute to minimising the overall costs of breeding programs for mass spawners such as barramundi and speed progress towards selective improvement of commercially important aquaculture species.

In conclusion, results from this study have shown that it is possible to generate high levels of genetic variability in mass spawned cohorts by mating a large number of broodstock and

paying particular attention to conditioning and maturation of females, as recently also observed for *L. calcarifer* Asian strains (Liu et al., 2012). Other positive implications for a barramundi breeding program were that family-specific genetic diversity was retained from 18 dph to harvest (273-469 dph) and that genotyping costs may be reduced by sampling the top 1.5% of the population.

Chapter 3. Heritability of harvest growth traits and genotype–environment interactions in barramundi, *Lates calcarifer*

3.1 . Introduction

Efforts to improve commercially important farmed traits in barramundi through selective breeding are currently underway in Asia (Yue et al., 2009) and being considered in Australia (Robinson and Jerry, 2009). Quantifying the amount of genetic variation determining the phenotypic expression of commercially important traits (e.g. heritability and genetic correlations) is fundamental for the design of barramundi selective breeding programs (Robinson and Jerry, 2009). To date, the impact of different rearing environments on the realisation of genetic potential in barramundi has not been investigated. Such knowledge, however, is of particular importance as barramundi is farmed under a broad range of culture systems. If G x E interactions are considerable, breeding programs will need to be tailored to account for each of the different environments in which the animals are to be commercially produced (Gjedrem and Olesen, 2005).

Heritability estimates for weight, length and condition factor for juveniles (~ 18 g and 10 cm length) at 90 days post hatch (dph) have been estimated for Asian barramundi stocks by Wang et al. (2008). Estimation of genetic parameters for early growth traits is important, because selecting future breeding candidates at an early age would decrease fish maintenance costs in a breeding nucleus. In addition, size grading and culling of smaller fish (i.e. a form of early mass selection) is a common procedure in commercial barramundi culture to avoid cannibalism and production losses and is usually first undertaken around the third week post-hatch (Schipp et al., 2007). Early size grading significantly impacts genetic diversity of

barramundi (Frost et al., 2006), therefore these current culture practices (i.e. grading and culling) are also likely to impact on barramundi genetic parameters by the end of the culture cycle. To date, little is known about how additive genetic variability in barramundi affects economically important traits at harvest size (i.e. closer to sexual maturity, when breeding candidates are generally selected) and how families perform relative to each other over the culture cycle in similar or disparate environments. Therefore, in this chapter the heritability of barramundi traits at 62 dph and at harvest (273 - 469 dph) and genetic correlations for body weight, standard length, body depth, Fulton's condition factor and body shape index at harvest were estimated. Furthermore, evidence for G x E interactions in barramundi family-performance reared in fresh water *vs.* sea water cages and in a semi-intensive pond *vs.* intensive tank system was investigated.

3.2. Material and methods

Methods regarding mass spawning of broodstock, larval rearing, fish sampling, genotyping and DNA parentage analysis protocols were previously described in *Chapter 2*. In the present *Chapter 3*, three barramundi cohorts were analyzed, referred to here as "experiments" (outlined in detail below). Experiment 1 refers to fish from *Chapter 2 batch 3*; experiment 2 refers to fish from *Chapter 2 batch 2* and also included a second harvested progeny cohort which had been sent to tank grow out conditions; and experiment 3 refers to an additional mass spawned cohort which had not been previously described.

3.2.1 Experimental groups, grow out environments and fish sampling

The number of dams and sires present in tanks at spawning, the offspring rearing environment and type of analysis performed in each experiment are shown in Table 3.1. Fish sampling at harvest were previously described in *Chapter 2*. In addition to fish weight (W),

further morphometric measurements collected included standard length (*Ls*) and body depth (BD), using Image-J software (ImageJ) from photographs. Fulton's condition factor (K) was calculated for each fish as $K = 10^6 W/Ls^3$. A body shape index (H) based on the ratio between BD and *Ls* was also calculated as H = 10 BD/Ls, as some niche markets in Australia prefer deeper barramundi with higher BD to *Ls* ratios (Paul Harrison, Mainstream Aquaculture Ltd., *pers. comm*.) (Figure 3.1).

Table 3.1. Barramundi (*Lates calcarifer*) offspring rearing conditions, number of dams and sires present in the spawning tank and type of analysis performed in each experiment. Tank = commercial intensive tank culture, Pond = semi-intensive pond culture, Cage = cages within 2000-L tanks in controlled environment, FW = fresh water, SW = sea water, h^2 = heritability, r_g = genetic correlation between traits, r_p = phenotypic correlation between traits, G x E = genotype by environment interactions.

Experiment / spawn	1	2	3
Rearing systems	Tank	Tank *vs.* Pond	Cage FW *vs.* SW
Number of dams	12	6	10
Number of sires	21	6	13
Analysis	h^2 / r_g / r_p	h^2 / GxE	h^2 / GxE

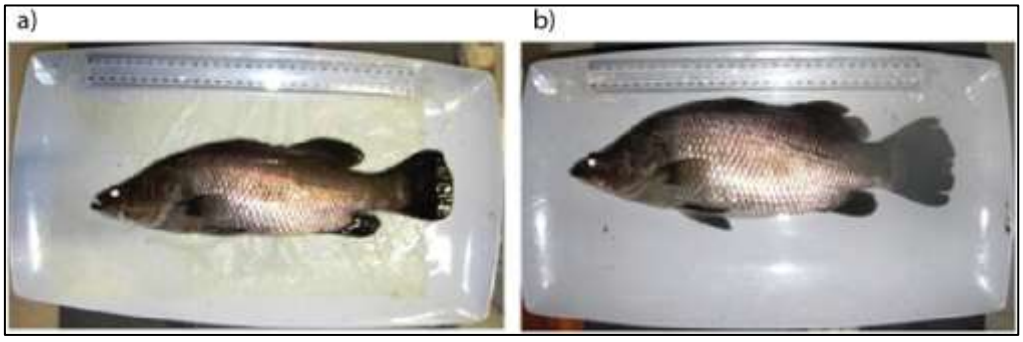


Figure 3.1. Illustrative examples of barramundi *Lates calcarifer* with extreme differences in body shape index H (10 BD/Ls): a) a shallow fish for its length (H = 3.04) and b) a deep fish for its length (H = 3.91).

3.2.1.1 Experiment 1- Genetic parameters at harvest size

Offspring for experiment 1 (spawn 1: Table 3.1) were reared in tanks at an intensive (50-100 kg fish/m^3) indoor recirculation grow out facility in Melbourne (VIC, Australia, Farm 1) and harvested at 273 dph. At Farm 1 fish were maintained in fresh water with oxygen concentrations > 5 mg/l and temperature 28 ± 2 ºC and fed at least twice daily. Fish were periodically sorted into large, medium and small size categories (grading). Smaller size classes were occasionally discarded (i.e. culling of slow growers) until fish reached an average weight of 200 g. Grading was performed mechanically based on the criterion body width; here fish were passed through a grate of various width. Fish were sampled from two different rearing tanks at harvest, tank 1 with medium and larger fish sizes and tank 2 with medium and smaller sizes as a result of earlier grading (weight distributions of tank 2 completely overlapped weight distributions of tank 1 at time of sampling and variances between tanks at harvest were homogeneous). Data from experiment 1 were used to estimate heritability and genetic and phenotypic correlation between traits at harvest.

3.2.1.2 Experiment 2 – Performance in tank vs. pond environments

Offspring for experiment 2 (spawn 2: Table 3.1) were sent to two different commercial farm environments; Farm 1 (described above) and Farm 2, a semi-intensive pond for grow out in Innisfail (QLD, Australia). At Farm 2, barramundi were stocked and maintained in one 1,000 m^2 earthen pond, however, further information on the culture conditions (water quality and feeding parameters) were not provided. Fish sampling in both farms was also performed at harvest following the same protocol as in experiment 1. All fish were immature males at harvest (as barramundi are protandrous hermaphrodites). Husbandry protocols and harvest date were at the discretion of each farm's management and varied between farms. At Farm 1, fish grading followed the same procedures described in experiment 1, with the exception that

for the last half of the culture all fish were stocked and reared in a single tank until harvest. Fish at Farm 2 were stocked into the pond without any grading or culling. At Farm 1, fish were harvested at 343 dph, whereas fish reared on Farm 2 (pond) were harvested at 469 dph. Data from experiment 2 were used to estimate heritabilities in each environment and G x E interactions between tank *vs.* pond culture.

3.2.1.3 Experiment 3 – Juvenile performance in fresh vs. sea water environments

Offspring for experiment 3 (spawn 3: Table 3.1) were also reared in different environments, fresh water (FW: 0 ppt) *vs.* sea water (SW: 33 ± 1.5 ppt) cage culture. Here, fish were graded into three size classes, although only animals in the small (7.6 ± 0.1 mm) and large (15.7 ± 0.2 mm) size classes were stocked separately into two floating cages (85 fish per cage) within three replicate FW and three replicate SW 2000-l recirculating tanks at the Marine and Aquaculture Research Facility Unit (MARFU), James Cook University (two salinity treatments × two grade sizes × three replicates). Tank flow rate was maintained at 20 l/min, temperature at 28 ± 1 °C, dissolved oxygen above 5 mg/l, total ammonia below 1 mg/l and all parameters monitored daily throughout this experiment. Fish were fed once daily to satiation on 0.3 to 1.2 mm pellets of a commercial marine fish diet (Ridleys Aquafeeds) and tanks were also siphoned and cleaned daily. All animals were sampled at 62 dph, when fish became too large for the cages. Sampling was performed as previously described, however, wet weight was measured with a more sensitive electronic scale (to nearest 0.1 g) and *Ls* and BD measured with electronic callipers (to nearest 0.1 mm). At final sampling, 97% of weight distributions of small fish overlapped weight distribution of larger fish, and more than 75% of size (*Ls*) range distribution between the two grades overlapped. Data from experiment 3 were used to estimate heritability at 62 dph and G x E interactions between fresh *vs.* sea water cage culture.

3.2.2 Statistical analysis

Statistical analysis of phenotypic data was performed with SPSS Statistics 19.0 (IBM). Data was first assessed for normality and homogeneity of variances (Kolmogorov–Smirnov and Levene's test, respectively, $P > 0.05$) and phenotypic outliers were excluded. In addition, to increase statistical confidence on observed family metrics and estimates of genetic parameters, families with four or less assigned offspring were excluded from the datasets. Overall, the data approximated a normal distribution and Ln-transformation of slightly skewed datasets yielded similar results to those obtained when the data was analysed in its original scale. Therefore, most analyses were performed using the original scale. However, variances of growth traits between fish reared in pond *vs.* tank (experiment 2), and between fish from the small and large size classes (experiment 3), were heterogeneous and required standardization when those groups (considered as fixed effects) were analysed within the same models (i.e. to calculate (co)variances for combined h^2 and G x E interactions). The phenotypes were therefore standardised between rearing environments, or size classes, by scaling the observed values by the standard deviation in the level of each rearing environment or size class, as suggested by Hill (1984) (also see Bentsen et al., 2012). Statistical differences for growth traits in experiment 2 (pond *vs.* tank) were not assessed due to large differences in husbandry techniques and age at harvest. Differences in growth traits between FW and SW (experiment 3) at the end of the experiment (62 dph) were analysed with a mixed model ANOVA, where salinity and initial grade size were fitted as fixed effects, whereas replicate tanks nested within salinity were fitted as a random effect. Results were considered significant at $P < 0.05$.

Genetic analyses were performed with linear mixed models using the software ASReml 3.0 (VSNi) (Gilmour et al., 2009). For each experiment, restricted maximum likelihood (REML)

methods were used to decompose phenotypic variance into genetic and environmental components fitting an animal model (Henderson, 1984; Wilson et al., 2010):

$$y = X\beta + Zu + e \quad (1)$$

where y is the vector of observations (or standardized phenotype) of each trait (W, Ls, BD, K and H), β is the vector of the fixed effects, u is the vector of the random animal additive genetic effects, e is the vector of the random residual effects, X and Z are incidence matrices that relate observations to the respective effects. Bivariate models similar to univariate models (1) were used to obtain covariance components for different fish traits or for the same trait recorded in different test environments. In the genetic models, replicate tanks were considered as fixed effects in experiment 1 and 3 (two and three levels, respectively) and initial grade size in experiment 3 (two levels). Salinity in experiment 3 was not significant and excluded from the models. Preliminary analyses including dam as a random effect indicated that non-genetic maternal effects were not significant for any traits and therefore the dam component was excluded from the models.

Heritabilities of traits were estimated as $\sigma^2_A/(\sigma^2_A + \sigma^2_e)$, where σ^2_A and σ^2_e were the variances attributed to additive genetic and residual error effects respectively. The genetic correlations (r_g) between traits at harvest (experiment 1), or the genetic correlations (r_g) between the same trait in different test environments (experiments 2 (pond *vs.* tank) and 3 (FW *vs.* SW cages)) treated as different traits (Falconer, 1952) were calculated as $r_g = \sigma_{A1A2} / (\sqrt{(\sigma^2_{A1})}\ \sqrt{(\sigma^2_{A2})})$, where A_1A_2 is the estimated additive genetic covariance component between the two traits, or the same trait in each environment. In the G x E interactions analyses, the residual covariance was set to zero, as only one observation per trait was taken for each individual and the

covariance matrix was constrained to be positive definite. Phenotypic correlations (r_p) between traits at harvest (experiment 1, measured on the same animal) were also calculated using ASReml 3.0.

3.3. Results

3.3.1 Genotyping, parentage assignment and reproductive success

A total of 3110 barramundi offspring were genotyped in this study across the three experiments (Table 3.2). Genotyping success based on fish with seven or more microsatellite markers amplified was 99.3% on average (ranging between 97.7 - 99.8%) and progeny assignment to a parental pair (95% confidence) was 98.6% on average (98.2 - 99.4%) for the three experiments (Table 3.2). In general, male brooders present in the spawning tanks were more likely to contribute as parents (95%, 100% and 46% contributing as parents in experiments 1, 2 and 3, respectively) than female brooders (67%, 50% and 33%). Mass spawning from experiment 1 produced the greatest genetic diversity with 121 families detected by genotyping at harvest, 66 of which had five or more offspring assigned to a parental pair.

Random mass spawnings generated 48.0%, 47.2% and 30.8% of all possible crosses between available male and female brooders in experiments 1, 2 and 3, respectively. However, due to highly skewed family contributions, only 26.2%, 25.0% and 8.5% of all possible family crosses were taken into account for the data analyses in experiments 1, 2 and 3 respectively, as many families present with fewer than five progeny represented in the genotyped sample were excluded from the datasets. The offspring excluded from further analysis and belonging to these poorly represented families (and a few phenotypic outliers) represented 7.3% of total assigned offspring and 45.5% of all families detected.

Table 3.2. Number of dams, sires, families and offspring of *Lates calcarifer* in each mass spawn / experiment. Tank = commercial intensive tank culture, Pond = commercial semi-intensive pond culture, Cage = cages within 2000-L tanks in controlled environment, FW = fresh water, SW = sea water.

Experiment	1	2		3	
Dams[1]	8 (12)	3 (6)		3 (10)	
Sires[1]	20 (21)	6 (6)		6 (13)	
System	Tank	Tank	Pond	Cage FW	Cage SW
Families[2]	66 (121)	9 (17)	9 (13)	11 (40)	11 (40)
Offspring:					
Genotyped	1428	301	431	491	459
Tested[3]	1426	297	421	488	457
	(99.8%)	(98.7%)	(97.7%)	(99.4%)	(99.6%)
Assigned[4]	1400	293	415	485	452
	(98.2%)	(98.7%)	(98.6%)	(99.4%)	(98.9%)
Analysed[5]	1275	275	403	451	418
	(91.1%)	(93.9%)	(97.1%)	(93.0%)	(92.5%)

[1] parents contributing with five or more assigned offspring, total candidate parents in spawning tank in brackets.
[2] families represented in dataset with five or more assigned offspring; total families represented in brackets.
[3] offspring with seven or more loci amplified.
[4] assignment of progeny to a parent pair with 95% confidence.
[5] number of progeny analysed from families with five or more assigned offspring and after phenotypic outliers removed.

3.3.2 Phenotypic data

Mean phenotypic data for growth related traits in each experiment are provided in Table 3.3. In experiment 2, fish from intensive tanks were harvested earlier due to superior performance for all growth traits compared to fish from the semi-intensive pond, growth differences were due to differential husbandry management techniques and varying environmental conditions in each farming system. In experiment 3, there was no statistical difference in early growth of cage-reared barramundi for most traits in either fresh or sea water ($P > 0.05$), with the exception of Fulton's condition factor, which was higher for fish reared in fresh water (mixed model ANOVA; $F(1, 824) = 19.1$; $P < 0.05$).

Table 3.3. Phenotypic data (observed mean ± s.d.) of *Lates calcarifer* measured for growth traits from each experiment. Tank = commercial intensive tank culture, Pond = semi-intensive pond culture, Cage = cages within 2000-L tanks in controlled environment, dph = days post hatch, FW = fresh water, SW = sea water, W = wet weight, *Ls* = standard length, BD = body depth, K = Fulton's condition factor, H = body shape index (10 BD/Ls).

Experiment	1	2		3	
System	Tank	Tank	Pond	Cage FW	Cage SW
Age (dph)	273	343	469	62	62
W (g)	495.0 ± 118.2	785.6 ± 227.4	485.0 ± 128.9	8.6 ± 2.8	8.2 ± 3.1
Ls (mm)	276.2 ± 22.1	308.8 ± 25.1	295.0 ± 25.9	67.3 ± 7.9	67.1 ± 8.3
BD (mm)	94.0 ± 8.5	109.9 ± 12.0	89.3 ± 8.0	21.9 ± 2.7	22.0 ± 3.0
K	2.30 ± 0.9	2.6 ± 0.3	1.85 ± 0.2	2.72 ± 0.2	2.55 ± 0.2
H	3.40 ± 0.1	3.6 ± 0.2	3.03 ± 0.1	3.26 ± 0.2	3.27 ± 0.2

3.3.3 Genetic analysis

High heritability estimates for barramundi growth related traits W, *Ls* and BD were found at harvest (Table 3.4, experiments 1 and 2), ranging from h^2 *Ls* = 0.31 ± 0.21 (tank, experiment 2) to h^2 *Ls* = 0.43 ± 0.22 (pond, experiment 2). Estimates for these traits at harvest were similar between experiments and environments. However, data from experiment 1 provided more accurate estimates (i.e. h^2 ~ 0.41 ± 0.11) due to greater number of families and offspring analysed. Heritability estimates for W, *Ls* and BD were lower for younger fish than for older fish, ranging from h^2 BD = 0.13 ± 0.09 (Cage combined, experiment 3) to h^2 *Ls* = 0.32 ± 0.16 (Cage FW, experiment 3). Overall, when data from groups reared in different environments were combined, heritability estimates were slightly lower or tended to be in the lower range of groups analysed separately. Heritability estimates for the morphometric ratios K and H were lower than those of W, *Ls* and BD for all age groups, even being nil for some datasets (pond, experiment 2), implying that the environmental component plays a major role in the phenotypic expression of barramundi condition factor and body shape.

Table 3.4. Heritability estimates (h^2 ± s.e.) for *Lates calcarifer* growth traits measured in different environments and ages. Dph = days post hatch, *Ls* = standard length, W = body weight, BD = body depth, K = Fulton's condition index, H = body shape index (10 BD/Ls).

Experiment	1	2			3		
System	Tank	Tank	Pond	Combined	Cage FW	Cage SW	Combined
Age (dph)	273	343	469	-	62	62	62
W	0.42 ± 0.11	0.40 ± 0.23	0.41 ± 0.22	0.37 ± 0.18	0.23 ± 0.13	0.21 ± 0.11	0.21 ± 0.11
Ls	0.42 ± 0.11	0.31 ± 0.21	0.43 ± 0.22	0.32 ± 0.17	0.32 ± 0.16	0.25 ± 0.12	0.25 ± 0.14
BD	0.39 ± 0.11	0.42 ± 0.24	0.42 ± 0.22	0.36 ± 0.18	0.18 ± 0.11	0.14 ± 0.09	0.13 ± 0.09
K	0.20 ± 0.07	0.17 ± 0.12	0.00 ± 0.00	0.10 ± 0.08	0.26 ± 0.17	0.15 ± 0.12	0.13 ± 0.10
H	0.12 ± 0.05	0.11 ± 0.08	0.00 ± 0.00	0.16 ± 0.11	0.01 ± 0.04	0.17 ± 0.15	0.09 ± 0.07

Mean family weights at harvest under intensive tank rearing (experiment 1) were found to be highly variable (Figure 3.2 (a)). Among the 66 families with the highest contribution to the cohort (i.e. with at least 5 offspring assigned), the fastest growing family (F01, 628 ± 37 g) was 75% heavier than the family with slowest growth (F66, 359 ± 51 g) at harvest. Contribution of families to the cohort under random mass spawning conditions was uneven and highly skewed (Figure 3.2 (b)). Interestingly, the family with the second slowest growth had the greatest contribution to the cohort (F65, 7.9%). Growth performance differed markedly with parent of origin (Figure 3.3 (a)). Variability in harvest weight among maternal half-sib families ranged from 433 ± 8 g (D08) to 566 ± 13 g (D01) and among paternal half-sib families ranged from 395 ± 7 g (S20) to 565 ± 8 g (S01). Parental contribution ranged from 1.7% (D03) to 22.6% (D06) for dams and from 0.6% (S10) to 16.1% (S20) for sires (Figure 3.3 (b)). The same uneven and skewed family and parental contribution pattern was observed for experiments 2 and 3 (data not shown).

Phenotypic correlations among traits measured at harvest reflected estimated genetic correlations (Table 3.5). Genetic correlations, however, were higher than phenotypic correlations indicating the influence of environmental noise on the expression of phenotypes. Very strong phenotypic and genetic correlations for barramundi growth related traits W, *Ls* and BD were found (0.91 to 0.99), with larger and deeper fish being heavier than smaller and shallower fish. Fulton's condition factor (K) and body shape index (H) were also strongly correlated (r_p = 0.67, r_g = 0.70) demonstrating that barramundi with higher depth to length ratios are somewhat bulkier (i.e. heavier for their length or in good nutritional condition). Correlations between the latter indexes (K and H) and growth related traits were positive, although very weak for Ls, weak for W and weak to moderate for BD; however, larger errors

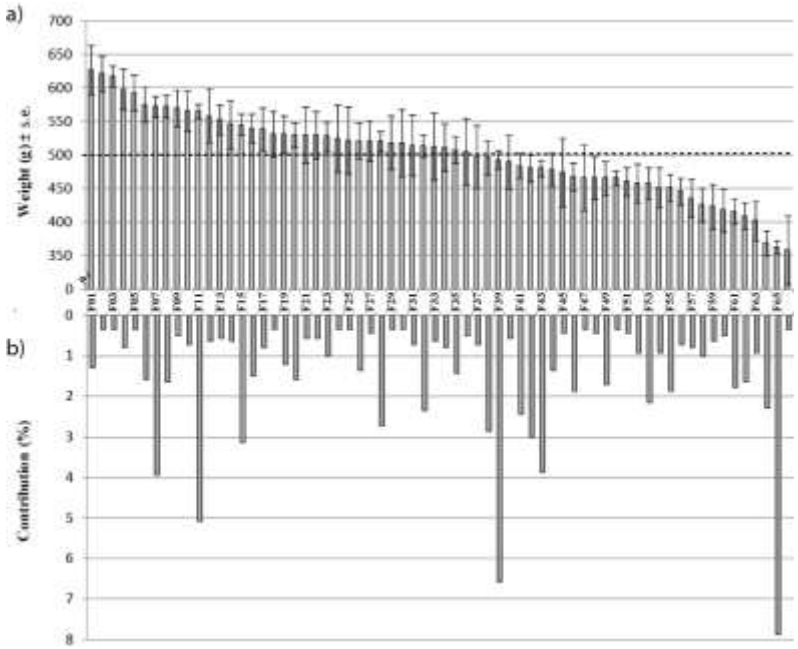


Figure 3.2. Mean (± s.e.) (a) harvest weights and (b) overall contribution to progeny represented in harvest of the 66 most represented *Lates calcarifer* families (with at least five offspring assigned) commercially reared in intensive tanks (273 dph) in experiment 1. Dashed line represents mean harvest weight (502 ± 7 g) of the 66 most represented families.

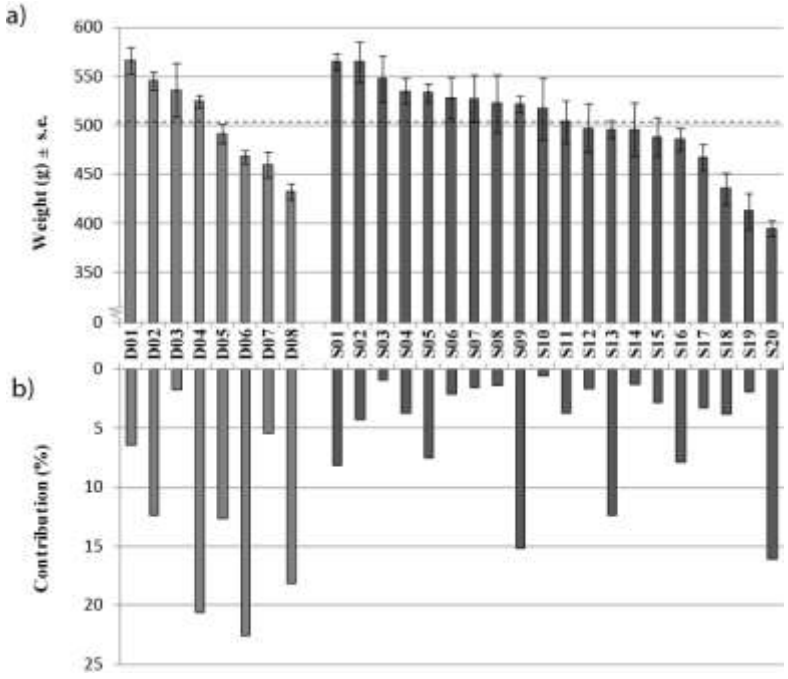


Figure 3.3. Mean (± s.e.) (a) harvest weights and (b) overall contribution of the offspring of dams (D01 to D08) and sires (S01 to S20) of *Lates calcarifer* commercially reared in intensive tanks (273 dph) in experiment 1. Dashed line represents mean harvest weight (503 ± 9 g) of offspring from the twenty eight parents.

were found in estimates of genetic correlations involving K and H, possibly due to lower precision when estimating these traits.

Table 3.5. Genetic (below diagonal) and phenotypic (above diagonal) correlations (± s.e.) between *Lates calcarifer* harvest growth traits when progeny were reared commercially in intensive tanks (273 days post hatch) (experiment 1). W = wet weight, *Ls* = standard length, BD = body depth, K = Fulton's condition factor, H = body shape index (10 BD/Ls).

Trait	W	Ls	BD	K	H
W	-	0.96 ± 0.00	0.95 ± 0.00	0.32 ± 0.04	0.26 ± 0.03
Ls	0.98 ± 0.01	-	0.91 ± 0.01	0.07 ± 0.04	0.08 ± 0.04
BD	0.99 ± 0.00	0.97 ± 0.02	-	0.34 ± 0.04	0.61 ± 0.07
K	0.36 ± 0.22	0.18 ± 0.24	0.38 ± 0.22	-	0.67 ± 0.12
H	0.41 ± 0.23	0.27 ± 0.25	0.88 ± 0.08	0.70 ± 0.30	-

High positive genetic correlations ($r_g \geq 0.97$) between the same growth traits (i.e. W, *Ls* and BD) were found for barramundi reared at both different salinities at 62 dph (experiment 3: fresh *vs.* sea water) and under different farming systems at harvest (343-469 dph) (experiment 2: intensive tank culture *vs.* semi-intensive pond), indicating the absence of strong family-specific genotype by environment interactions in growth performance (Table 3.6). However, these results should be interpreted with caution due to the limited number of families analysed (~ 10) and the relatively high standard error estimates (r_g s.e. = 0.14 - 0.50). Genetic correlations for Fulton's condition factor (and likewise for body shape index) and growth traits for the alternate environments tested were also high ($r_g \geq$ 0.87), but less reliable due to large errors associated with those estimates (r_g s.e. = 0.40 - 0.77), possibly due to the weak heritable basis for those traits. Although there was a large discrepancy in performance for all

phenotypic traits between barramundi farmed in tank *vs.* pond (Table 3.3), estimates of genetic correlation were high, implying that under the specific conditions of this study, the genetic merit of barramundi families influenced growth performance regardless of the farming environment in which they were reared.

Table 3.6. Genotype by environment ($r_g \pm$ s.e.) estimates for *Lates calcarifer* traits measured in fish at harvest in intensive tanks (343 dph) *vs.* a semi-intensive pond (469 dph) (experiment 2) and in fish reared in fresh water (FW) *vs.* sea water (SW) cages at 62 days post hatch (dph) (experiment 3) and W = wet weight, *Ls* = standard length, BD = body depth, K = Fulton's condition factor, H = body shape index (10 BD/Ls).

Experiment	2	3
System	Tank *vs.* Pond	Cages FW *vs.* SW
W	0.99 ± 0.23	0.98 ± 0.26
Ls	0.99 ± 0.18	0.99 ± 0.14
BD	0.99 ± 0.24	0.97 ± 0.28
K	0.87 ± 0.52	0.93 ± 0.40
H	0.98 ± 0.77	0.89 ± 0.51

3.4. Discussion

3.4.1 Implications of genetic parameter estimates for selective breeding

The contribution of additive genetic variability underpinning the phenotypic expression of commercially important traits is fundamental for the design of efficient breeding programs for barramundi. Before DNA based markers became available, Chandra et al. (2000) attempted to estimate genetic parameters for early growth traits in barramundi, arbitrarily

assuming single pair matings out of four spawning events. However, due to inaccurate pedigree information, Chandra et al. (2000)'s heritability estimates for body length ranged from 0.15 ± 0.16 (at 21 dph) to 0.96 ± 0.42 (at 80 dph), which is not informative for a parameter that ranges from 0 to 1. In this chapter, the heritability and associated genetic correlations of growth traits in Australian barramundi when measured at commercial harvest sizes is reported for the first time. Heritability estimates for barramundi growth related traits at 62 dph (combined h^2 W = 0.21 ± 0.11; h^2 *Ls* = 0.25 ± 0.14) were in the same range as those reported for Asian stocks at 90 dph by Wang et al. (2008) (h^2 W ~ 0.23 ± 0.17; h^2 *Ls* ~ 0.27 ± 0.17), but were much higher at harvest size (e.g. experiment 1, at 273 dph: h^2 W = 0.42 ± 0.11; h^2 *Ls* = 0.42 ± 0.11). Heritability estimates were more precise (i.e. lower standard errors) for experiment 1 due to a more robust study design (i.e. greater number of samples and families) when compared to experiments 2 and 3. Nevertheless, heritability estimates of W, *Ls* and BD at harvest were similarly high across experiments 1 and 2 (Table 3.4). Thus, additive genetic variance is a significant component affecting the growth performance of barramundi, especially as fish reach market size.

A positive implication for barramundi selective breeding is that higher genetic gains would be expected per generation than those previously predicted with simulation modelling, based on the assumption of lower heritability for growth rate (e.g. h^2 = 0.25 as used in models by Macbeth et al., 2002; Robinson et al., 2010; Macbeth and Palmer, 2011). Similar heritability estimates (also using a simple animal model) for body weight and length have been found for the European seabass (*Dicentrarchus labrax*) farmed to mean weight of 338-487 g under a range of environments (h^2 W = 0.38 ± 0.14 ~ 0.44 ± 0.14; h^2 *Ls* = 0.27 ± 0.11 ~ 0.41 ± 0.15) (Dupont-Nivet et al., 2008). Heritability estimates for body weight in rainbow trout (*Oncorhynchus mykiss*) and European sea bass have also been shown to increase with age (Su

et al., 1996; Saillant et al., 2006). Wang et al. (2008) suggested that barramundi future breeding candidates could be selected at 90 dph for later growth. However, as shown for other species, heritability estimates for weight (and all other traits) in barramundi increased with age, either due to reduced environmental (and error) effects on phenotypes overtime, or due to longer periods for higher accumulated expression of additive genetic effects. Therefore, even if initial selection early in the production cycle might reduce fish maintenance costs for a breeding nucleus, greater accuracy in selecting the best genotypes, and higher genetic gain, would be achieved by selecting older, faster growing juveniles as future breeding candidates. The cost-benefit of operation of a selective breeding program using different ages of selection needs to be evaluated.

Heritability estimates for K and H, however, were lower than those for W, *Ls* and BD, being zero for the pond environment, which highlights the low additive genetic and high environmental effects accounting for the total phenotypic variation for these body ratio traits in this environment. Low and imprecise heritability estimates for Fulton's condition factor have also been found for Asian stocks of barramundi (Wang et al., 2008) and for rainbow trout (Gunnes and Gjedrem, 1981). In general, H was the trait with the lowest heritability, which implies that body shape would hardly be improved through a breeding program. Nonetheless, as phenotypic and genetic correlations between K and W, and H and W were positive (Table 3.5), selection for heavier fish alone might lead to minor improvements in K and H over time (Gjedrem and Thodesen, 2005). To a larger extent, selection of heavier fish should also lead to indirect selection of longer and deeper fish (and vice-versa) due to *Ls* and BD high heritability, standard deviation and genetic correlation with W (Gjedrem and Thodesen, 2005).

In barramundi culture, grading and culling of slow growers are common commercial practices which take place in the first few months of culture. While the genetic parameters of commercially reared fish were quantified at harvest (experiments 1 and 2), the impacts of grading and culling on the genetic parameters were not assessed in this study. Grading procedures were shown to result in underestimation of heritabilities of W and *Ls* of the common sole (*Solea solea*) under commercial culture conditions, especially when the size range of classes comprised less than 65% of the total size range, which are more likely to occur when fish are recently graded into several classes (Blonk et al., 2010b). This was not the case of fish in tanks of experiment 1 which had homogeneous variances at harvest, as sufficient time elapsed after previous grading for size/weight distributions to significantly overlap. In addition, the use of standardized phenotypes to correct for heterogeneous variances between specific groups (e.g. fish graded at the beginning of experiment 3 and fish from different environments in experiment 2) resulted in similar heritabilities and genetic correlations as those found when models were run with observed phenotypes (data not shown). Therefore, it is unlikely that sampling from different tanks would significantly bias the estimation of genetic parameters.

3.4.2 Absence of G x E interactions

In this chapter, within two broodstock cohorts from the Queensland coast, G x E interactions were insignificant for the environments and growth traits compared. Genetic correlations for the same growth traits (W, *Ls* and BD) were positive and high (r_g close to unity) in both experiment 2 (comparing semi-intensive pond farming in the tropics *vs.* intensive tank farming under artificial heated water conditions) and experiment 3 (a replicated and fully controlled laboratory experiment comparing fresh *vs.* sea water cage culture) (Table 3.6), therefore the ranking of families in terms of estimated breeding values was similar in both

environments tested. Slightly lower estimates of genetic correlations between environments and high standard errors were found for Fulton's condition factor (K) and body shape index (H) (Table 3.6). Sae-Lim et al. (2010) showed that estimates of genetic correlations for the same trait in different environments are biased downward for traits with low heritability (~0.1) and when family sizes are small (~10), which is the case for K and H in experiments 2 and 3. Therefore, the inability to properly estimate G x E interactions for K and H is likely to be due to their low heritability (which could not be estimated for the pond environment).

Results suggest that barramundi breeding programs to improve growth rates may be able to service a wide range of culture systems without the requirement for extensive on-farm testing. However, due to the lack of full control over barramundi reproduction, which would enable balanced factorial mating for the generation of large number of families, both G x E interactions study designs were not optimum due to the low number of families analysed (~10). Based on simulation studies, Sae-Lim et al. (2010) warned that under such scenarios r_g may be under or overestimated, therefore results should be interpreted with caution. In addition, although fish from experiment 3 had an average 56-fold increase in their initial weight, they were only cultured in separate rearing environments for a short period (from 26 to 62 dph), sharing a previous communal environment for 48% of their lifetime. In European seabass, G x E interactions were small when weight was considered (Dupont-Nivet et al., 2008), but large when growth rate was considered (Dupont-Nivet et al., 2010), in an experiment where fish were raised under a communal environment for 14 months, tagged around 35 g and measured again around 400 g (a 11.4-fold increase in initial weight) after another 12 months in separate rearing environments. Therefore, further studies on barramundi G x E interactions using more robust study designs (i.e. longer duration and greater number of families) are warranted to confirm the present findings.

Genotype by environment interactions for aquaculture species have shown differences in the magnitude of interaction depending on the type of study, cultured species and extent of environmental differences (Gjedrem, 2005). Considerable differences in the ranking of genotypes in different environments have been shown for some aquaculture species under different conditions (e.g. re-ranking was significant for one out of the six environments tested for rainbow trout by Sylven et al., 1991), however for most aquaculture species, negligible G x E interactions for growth have been found (as reviewed by Gjedrem, 2005; Gjedrem and Baranski, 2009; Gjedrem et al., 2012). In the case of Nile tilapia, *Oreochromis niloticus*, a number of strains, originally derived from the GIFT Tilapia strain, are grown throughout Asia (e.g. China, India, Vietnam, Thailand, Malaysia, Philippines), South and Central America (e.g. Mexico and Brazil) and North America (USA), mostly in pond or tank environments (Eknath and Hulata, 2009). Joint analyses of data from pond and cage farming of tilapia in Malaysia and available literature for several aquaculture species tested in a range of environments has led to the general conclusion that separate selection and testing programs to improve growth related traits (e.g. body weight) were not necessary (Nguyen and Ponzoni, 2006). More recently, however, a comprehensive study involving six generations of the GIFT strain in the Philippines found high genetic correlations (r_g ~ 0.89) between harvest body weights across a wide range of pond environments, but significant G x E interactions were detected when pond environments were compared with intensive cage culture (Bentsen et al., 2012). Such results confirm that large datasets obtained over multiple generations are needed for accurate estimations of genetic parameters. Like tilapia, barramundi is a robust and versatile fish species in which production is increasing worldwide. This can be attributed in part to its euryhaline physiology, its ability to cope with stocking densities up to 100 kg/m^3 (as in Farm 1, experiments 1 and 2) and high growth efficiency over different salinity

conditions (experiment 3) and a wide range of temperatures (Katersky and Carter, 2007). This ability to tolerate a wide range of farm conditions, and absence of G x E interactions found in the diverse environments tested in this preliminary study, suggest that the benefits from a single barramundi selective breeding program could be broadly distributed and have a large impact on the efficiency and sustainability of production for this species around the world.

In conclusion, additive genetic variation is a significant component in barramundi growth traits at harvest. Analyses demonstrated that the majority of growth traits exhibit high heritability and positive genetic correlations, warranting improvement through selection for increased body weight at harvest. Lack of genotype by environment effects highlight that family genetic merit is relatively consistent in barramundi when individuals are reared under disparate environmental and culture systems. Results from the present chapter suggest that a breeding program should be able to improve growth rates for a wide range of farming systems without the need to develop environment-specific selected strains, however, further studies involving larger number of families and founder strains are warranted.

Chapter 4. A combined flow-cytometric and nucleic acid based protocol for assessing growth rate of hatchery-reared barramundi, *Lates calcarifer*, larvae

4.1. Introduction

A thorough understanding of the genetic effects driving the variability in growth rates among individuals is fundamental for effective aquaculture selective breeding programs. Fish breeders frequently exploit the disparities of genetically determined growth trajectories in weight as a means to select superior families and individuals when developing improved farmed strains (Gjedrem, 1983). In practice, differences among fish growth rates are measured at harvest, but in fact growth differences may be realized within early life history stages leading to the variability in larval sizes routinely observed in fish hatcheries. Such variability in larval growth is seen both among and within batches subjected to communal rearing conditions, where water quality parameters, food quality and quantity are maintained as close as possible to the species' optimum physiological requirements. However, the genetic effects underpinning larval growth variability are still poorly understood and to date the use of larval traits to disclose genetic differences among families have never been investigated, or exploited, by fish breeders.

Several traits can be used to study growth potential of early life stages. In the fisheries sciences, larval size is undoubtedly the simplest and most studied of all early life history traits, as many of the factors critical to larval growth, survival and recruitment in the wild are size-dependent (Miller et al., 1998). Larval size, however, may not necessarily be the most predictive trait to disclose determined genetic growth over the longer term, as size is a consequence of many physiological processes. In fact, there is a range of measurable larval traits

underlying cellular mechanisms which lead to the expression of morphometric characters and are the ultimate drivers for an organism's increase in body size that could also be exploited for the study of early growth rates. For instance, larval fish increase their body mass through increases in protein biosynthesis (Houlihan et al., 1988; Caldarone, 2005), with the level of protein synthesis largely determined by the availability and transcriptional regulation of RNA (Henshaw et al., 1971; Elser et al., 2000). Unlike that of its precursor template DNA, which remains relatively stable in an organism over time, RNA levels fluctuate dramatically depending on the metabolic activity and growth rate of cells (McNamara et al., 1999), especially during early life stages when mass-specific metabolism is at its highest (Johnston, 2006). Thus the level of RNA and protein synthesis to background DNA may reflect the growth rate of an organism. In addition, post embryonic growth in teleosts is largely manifested through cell division (hyperplasia) and growth of muscle tissue, where myoblasts from a proliferating population of myogenic progenitor cells fuse with muscle fibers as fiber diameter and length increase (Johnston, 2006). Therefore, faster growing larvae are expected to exhibit higher RNA:DNA ratios (RNA/DNA) and higher rates of cell division than slow growing larvae.

Recently, the development of sensitive fluorometric and cytometric techniques has allowed fish biologists to measure the rate of such cellular metabolic processes early in development. In particular, RNA:DNA ratio (RNA/DNA) and protein:DNA ratio (Prot/DNA) have been extensively validated as indirect metabolic indices indicative of nutritional condition and growth potential for a broad range of fish species (reviewed by Ferron and Leggett, 1994; Chícharo and Chícharo, 2008; Perez-Dominguez and Dahm, 2011). More recently, several studies have shown that flow cytometric (FCM) cell cycle analyses can also be a powerful tool to directly assess growth of fish larvae by estimating the relative proportion of cells which are actively dividing (Theilacker and Shen, 1993, 2001; Bromhead et al., 2000;

González-Quirós et al., 2007; Porter and Bailey, 2011). FCM DNA analysis offers the precision to rapidly quantify the DNA content of tens of thousands of cells and nuclei and accurately estimate the proportion of cells in different phases of the cell cycle G_0/G_1 (gap 0, non-dividing phase: gap 1, pre-replicative phase), S (DNA synthesis phase) and G_2/M (gap 2, chromosomes are duplicated and cell prepares for mitosis–mitosis, duplicated chromosomes are separated and mother cell divides into two identical daughter cells) (Ormerod, 2000). Cells in G_2/M have double the DNA content (4*N*) of cells in G_0/G_1 phase (2*N*), and cells in S phase have an intermediate DNA content ($2N < S < 4N$). FCM DNA analysis demonstrated that larvae with good nutritional condition and fast growth rates had higher proportions of cells in the S and G_2/M replicative phases of the cell cycle than malnourished larvae. To date these studies have focused on the phenotypic correlations of larval growth and their corresponding cellular and metabolic responses to environmental conditions (e.g. food availability, temperature, etc.), without addressing their potential correlated genetic response with long-term growth performance.

As for any quantitative trait, the genetic component underpinning the phenotypic variation in larval growth traits can be estimated provided the relatedness between individuals within a population is known and can be accurately measured for the larval traits of interest. Fish hatcheries offer the ideal conditions for quantitative genetics studies on larval traits, as pedigrees are easily traceable and any potential bias from non-genetic effects can be kept to a minimum by communally rearing larvae from many families in well controlled environmental conditions. However promising as potential early predictors of long-term genetically determined growth, RNA/DNA, Prot/DNA and FCM cell cycle analysis have never been simultaneously analysed within a single larvae, or reported in larval barramundi. Therefore, there are no available protocols with which to explore the phenotypic and genetic correlations between *L. calcarifer*

larval cellular and biochemical traits and growth rates. Extensive literature and commercial kits are available for high throughput quantification of nucleic acids, and these can be readily applied to homogenates of whole larval fish and small invertebrates using fluorescence microplate readers (e.g. Caldarone et al., 2001; McGinty et al., 2008). Nevertheless, the paucity of appropriate protocols to prepare the essential single cell suspensions required for FCM (Ormerod, 2000), from small, but complex, aquatic organisms such as fish larvae (comprised of fibrous and hard tissues including muscle, bones and scales) has precluded a broader application of FCM in aquatic sciences. To overcome the challenge of sample preparation, the few FCM studies on fish larvae have adopted a careful and tedious micro-dissection step to isolate individual larval tissues. For instance, brain cell division rates in larval fishes have been correlated with growth rates when growth rates were artificially manipulated through physiologically stressful treatments, such as starvation or unfavourable temperatures (Theilacker and Shen, 1993, 2001; Bromhead et al., 2000; González-Quirós et al., 2007; Porter and Bailey, 2011). Compared to other fish tissues, brain is relatively easy to dissociate with minimal mechanical disruption providing appropriate single cell suspensions. Evidence suggests, however, that fish brain may be one of the last tissues to be affected by nutritional deprivation and may not be a sensitive tissue to estimate short-term growth differences (Mustafa and Mittal, 1982; Theilacker and Shen, 2001; Drew et al., 2008). Indeed, early attempts to use FCM cell-cycle analysis of brain cells as a growth index for barramundi larvae reared under commercial hatchery conditions (i.e. neither food nor temperature limited) indicated no differences in the proportions of brain cells dividing between fast and slow growing fish (Domingos, unpublished data). Whereas FCM based on brain cells of fish larvae may be useful for fisheries research in detecting large differences in growth due to nutritional condition of wild larvae, this tissue alone is unlikely to be sensitive enough to detect more subtle growth differences in larvae reared under commercial

aquaculture conditions. Alternatively, FCM cell-cycle analysis of larval muscle for assessing physiological condition has also been reported for a single species, the walleye pollock *Theragra chalcogramma* (Theilacker and Shen, 2001; Porter and Bailey, 2011). However, numerous methodological problems for obtaining nuclei from larval muscle of acceptable quality for FCM (G_0/G_1 coefficient of variation (c.v.) <6%) have been reported for other fish species, including sea bass *Dicentrarchus labrax* (Catalán *et al*., 2007) and larval Atlantic cod *Gadus morhua* (González-Quirós *et al*., 2007). Likewise, in larval *L. calcarifer* muscle, the methods proposed by Theilacker and Shen (2001), which involved releasing cell nuclei by passing the muscle tissue through a 25 gauge needle, resulted in high debris and low nuclei yields making it unsuitable for FCM cell cycle analysis (Domingos, unpublished data).

Practical difficulties in micro-dissecting larval fish tissues, such as contamination with adjacent tissues, a physical reduction of an already small sample available, and the unsatisfactory results previously trialled with *L. calcarifer* larval brain and muscle tissues necessitated the development of a homogenization and nuclei extraction method from whole fish larvae that could be used to determine both the rate of cell division using FCM and other cellular and biochemical indices which are strongly associated with growth rates within a single larvae. This thesis chapter describes the development and validation of such analytical methods. Specifically, a robust sample preparation protocol and data acquisition for FCM cell-cycle analysis using whole barramundi larvae is described. In addition, protocols to quantitate nucleic acids and protein content from barramundi whole larvae homogenates which allow for measurements of cellular and biochemical growth related traits (e.g. RNA/DNA, Prot/DNA) in a single larva were also developed. Moreover, the relationship between each cellular and biochemical index and barramundi larval growth rates was assessed in larvae reared under both stressed and ideal environmental conditions. These

analytical methods will later be applied to hatchery populations of known pedigree to elucidate the extent to which genetics underpin variation in growth related traits in early life history stages and investigated as potential early indicator traits (i.e. predictive traits) of long-term family growth performance (thesis *Chapter 5*).

4.2. Material and Methods

4.2.1. Larval samples

Lates calcarifer larvae used in all experiments were sampled from a commercial fish hatchery located in Townsville, Australia (Mainstream Aquaculture Ltd. Pty). Larval rearing followed intensive clear water marine finfish larviculture protocols (i.e. high culture densities, oxygen diffusion and use of rotifers, artemia and commercial diets), which included periodic grading after 18 dph to avoid cannibalism (Dhert et al., 1992; Schipp et al., 2007). Barramundi larval growth rates under commercial hatchery conditions were firstly assessed to determine an ideal sampling age to conduct further analysis. For that 16 to 80 larvae were sampled and measured (*Ls*) from 1 to 17 dph (mean = 47 larvae/day), 419 larvae were sampled at 18 dph just prior to the first larval grading and a final sample of 428 larvae was taken at the end of the hatchery run at 26 dph from four different tanks, each containing a particular grade size. Sampled larvae were transported to a laboratory, euthanized with 0.5 ml/l of 2-phenoxyethanol and subsequently placed onto a glass slide and photographed under an Olympus® SZ61 stereo microscope connected to an Olympus® DP25 digital camera (Olympus). The software ImageJ 1.43 (National Institutes of Health) was used to measure larvae standard length (*Ls*) defined as the distance from the tip of snout to the end of the caudal peduncle.

4.2.2. Reagents

All reagents used were molecular biology grade (Sigma-Aldrich). Solutions and reagent concentrations for FCM analysis are given in Table 4.1. Except for the sheath fluid, solutions were kept ice-cold at all times. Larval nucleic acids and protein were quantified using commercial kits (section below).

Table 4.1. Solutions, reagents (Sigma-Aldrich) and concentrations used to extract nuclei of *Lates calcarifer* for flow cytometric analysis.

Solution	Reagent	Concentration
Sucrose-citrate Buffer [1]	Sucrose	250 mM
(pH adjusted to 7.6)	Trisodium citrate dihydrate	40 mM
	Dimethyl sulphoxide (DMSO)	5%
RNase A solution	Ribonuclease A in nuclease free water	1 mg ml^{-1}
(frozen in 1 ml aliquots)		
PI-detergent solution [2]*	Trisodium citrate dihydrate	3.4 mM
(frozen in 15 ml aliquots	NaCl	9.65 mM
wrapped in aluminium foil)	IGEPAL® CA-630	0.03%
	Propidium iodide	0.015 mM
Sheath fluid (6x)	Sucrose	150 mM
(sheath fluid container filled	Trisodium citrate dihydrate	42 mM
with 1 l of 6x sheath fluid	NaCl	52 mM
and 5 l of distilled water)	DMSO	3%
	IGEPAL® CA-630	0.162%

[1] from Vindeløv et al. (1983)
[2] from Ormerod (2000) modified from Petersen (1985)
*immediately before use 150 µl of RNase solution was added in 15 ml of PI-detergent solution.

4.2.3. Preparation of control samples for FCM cell cycle analysis

Lates calcarifer blood was used to adjust FCM settings and quality control (outlined below) as it provides a practical suspension of non-cycling (G_0 stage) nucleated single cells. Here approximately 300 µl of blood was drawn from the caudal vein of a single anesthetised 200 g juvenile *L. calcarifer* with a 1 ml syringe and a heparinised 19-gauge needle. Ten µl aliquots of blood were then transferred into 1.5 ml tubes filled with 1 ml of PBS. Tubes were inverted five times and centrifuged at 800 *g* for 5 min. The supernatant was poured off, cells resuspended in 1 ml of 70% ethanol and stored in the fridge for up to 6 months. Cells to be used as controls were centrifuged at 2,300 *g* for 5 min., resuspended in 1 ml PBS, then pelleted again and resuspended in 1 ml of ice cold PI-detergent solution (Table 4.1). Tubes were wrapped in aluminium foil and blood cells subjected to the same nuclei extraction procedures as experimental samples.

4.2.4. Preparation of whole larval samples

In each experiment, all sampled larvae were hatched on the same day and subjected to the same environmental conditions (i.e. tank water quality and food availability), except for the fasting experiment (outlined below). Larval age, given in days post-hatch (dph) varied for each experiment. Before homogenization, larvae had their gut microdissected and removed (intestine, liver, swim bladder and visceral fat) and were rinsed with PBS to remove excess blood, a procedure which minimised debris and nuclei contamination from prey organisms. When larvae were sampled from hatchery tanks in the morning before the first daily feed, removal of the empty gut was unnecessary. In addition to using fresh larvae, two alternative preservation methods were trialled including freezing larvae immediately at -80 ^{0}C or at -20 ^{0}C in RNA*later*® solution (Ambion). To assess the feasibility of using individual fish tissues such as brain, liver, fin and muscle tissue, these organs were also dissected from a 90 dph

juvenile *L. calcarifer*, separately kept in 15 ml tubes containing ice cold PBS and trialled following the protocol developed for whole larvae.

4.2.5. Larval homogenization

Individual larvae (16-30 dph, depending on the experiment) were cut into 5-7 pieces with scissors, then hand homogenized until no pieces could be seen (10-20 strokes) with a Tenbroek tissue grinder (15 ml capacity, frosted-glass vessel and pestle, clearance 0.076 – 0.127 mm) in ice-cold sucrose-citrate buffer (Table 4.1). The following empirically determined formulas based on the buffer volume (µl) per amount of tissue, measured either as larval size (*Ls* in mm) or wet mass (g), provided consistent recovery of clean nuclei for FCM analysis: Buffer volume = 2.8 Ls^3 or 140 wet mass. The volume of sucrose-citrate buffer per fish/tissue was critical for reproducible FCM results. Lower buffer volumes increased the amount of debris, increased the coefficient of variation (c.v.) and shifted mean G_0/G_1 peaks from that of the control, indicating improper staining. Higher buffer volumes increased FCM sample acquisition times, reducing sample throughput.

4.2.6. Nuclei extraction and DNA staining for FCM cell cycle analysis

Following homogenization, 100 µl of homogenate was transferred with a broad ended pipette tip into a 1.5 ml tube containing 900 µl of ice cold Propidium Iodide (PI) detergent solution (Table 4.1) for nuclear extraction and DNA staining. PI is a fluorochrome that binds stoichiometrically to DNA; therefore the fluorescence intensity of each nucleus is proportional to its DNA content. The high density homogenate had to be immediately mixed with this solution by gently pipetting up and down a few times, otherwise cells clumped together impairing proper nuclei release and staining. Tubes were wrapped in aluminium foil and placed on an Intelli-Mixer RM-2 (ELMI) at 2.8 x 10^{-4} *g* in the fridge at 2-4 ^{0}C for 3 h.

Higher *g*, or longer (i.e. overnight) mixing, reduced the yields of nuclei in S-phase, probably due to the fragility (or absence) of the nuclear membrane in advanced mitotic cells. Standardization of mixing temperature, time, and speed was critical when samples were extracted on different days for comparability. Nuclei were then filtered with a 60 µm nylon mesh into a FACS tube kept on ice in the dark and samples read within 2 h.

4.2.7. Flow cytometric settings and quality control

Flow cytometric data were collected with a BD LSRFortessa™ Cell Analyser and FACSDiva™ Software v6.0 (BD Biosciences). The cytometer baseline performance and day-to-day performance checks were conducted using BD Cytometer Setup and Tracking Beads™ (BD Biosciences) with a sheath fluid a solution of 90% sucrose-citrate buffer and 10% PI-detergent solution without PI to equalize sample and sheath fluid refractive index (Table 4.1). This reduced the c.v. of G_0/G_1 of DNA histograms and prevented changes in dye binding efficiency due to differences in ionic strength (Vindeløv *et al.*, 1983). Cellular aggregates or debris, as well as doublets (i.e. two or more single nuclei stuck together), which may be confounded with a cell in G_2/M stage (4*N*) are inevitable noises registered by the cytometer and need to be removed from further analysis. To ensure the collection of clean DNA content from 10,000 to 20,000 single nuclei per sample, a cytometer worksheet (FACS Diva™) was set up and used during data sample acquisition. This standard worksheet contained the three graphs which enabled the noises to be sequentially excluded: a) Forward Scatter (FSC) *vs.* Side Scatter (SSC) logarithmic dot-plot to depict nuclei size and shape (Figure 4.1(a)); gated to exclude larger aggregates), b) PI-Pulse Area (PI-A) *vs.* PI-Pulse Height (PI-H) linear dot-plot which enabled the discrimination of single nuclei from (Figure 4.1(b)); gated to remove doublets) and c) a PI-A histogram, which represents the frequency of nuclear DNA content (Figure 4.1(c)).

Lates calcarifer blood was run before every batch of samples was acquired to optimize critical instrument settings required for reliable analysis of DNA histograms. Barramundi nucleated erythrocytes provided a convenient standard sample to adjust the cytometer FSC and SSC laser voltages so that nuclei were positioned on a detectable scale, and to adjust PI voltage to place fluorescence of G_0/G_1 peak around channel 50,000 ((Figure 4.1(c)). Further, blood was used as a quality control (QC) to verify consistency of the staining procedure and FCM resolution (c.v. of G_0/G_1 peaks from blood histograms lower than 3% confirms instrument precision) and linearity (ratio of doublets to singlets mean channel position between 1.95 and 2.05 confirms instrument alignment).

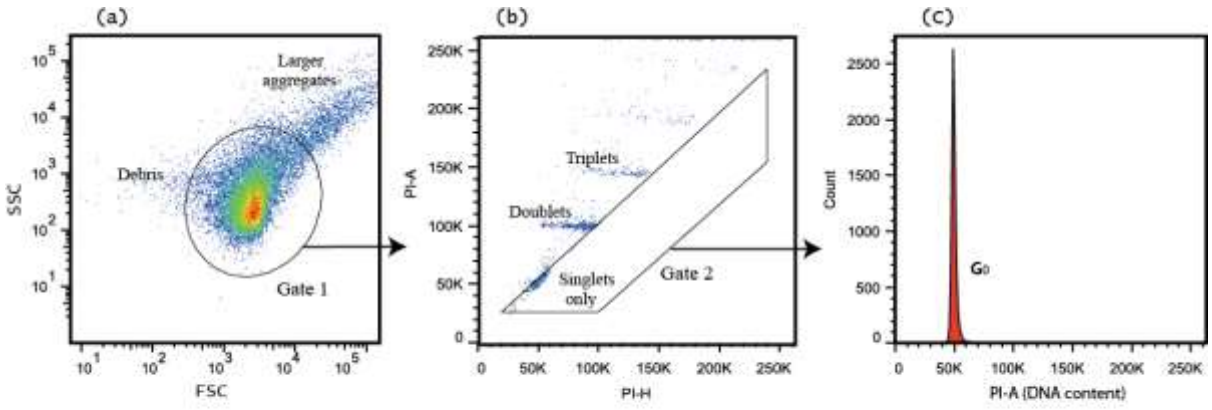


Figure 4.1. Basic cytometer set up to eliminate noise (cell debris, aggregates and doublets) through serial gating for obtaining single nuclei: flow-cytometry plots generated using FACSDiva™ (BD Biosciences) to visualize data acquisition of propidium iodide (PI)-stained blood cells nuclei of *Lates calcarifer* (control sample): (a) forward scatter (FSC) *vs.* side scatter (SSC) dot-plot in log scale, (b) PI-area (PI-A) *vs.* PI-height (PI-H) dot-plot in linear scale and (c) PI-A (DNA content) histogram in linear scale. Blood cells are non-cycling; therefore, lack S (DNA synthesis) and gap 2: mitosis (G_2/M) phases.

4.2.8. Cell cycle analysis

Sample files were exported to FlowJo software (Tree Star) and PI-Area histograms deconvoluted with the Cell Cycle Platform. The Watson Pragmatic (G_0/G_1 c.v. = 2.78 ± 0.36 %; G_2/M c.v. = 2.81 ± 0.62 %) was chosen instead of the Dean/Jet/Fox (G_0/G_1 c.v. = 2.94 ± 0.38 %; G_2/M c.v. = 12.81 ± 21.76 %) mathematical model, as the first provided lower and more consistent coefficient of variation (fresh whole larvae, n = 75). The sum of the percentages of nuclei in the replicative stages of the cell cycle, namely S (or DNA Synthesis phase), G_2 (or Gap 2 where cell continues to grows and prepares to divide) and M (or Mitosis where cell divides into two daughter cells), as measured by FCM, are hereafter referred to as "cells dividing". The concept of using S+G_2+M phases of the cell cycle as a means to assess larval nutritional condition was first suggested and validated by Theilacker and Shen (2001).

4.2.9. Quantification of total RNA, DNA and protein from larval homogenates

The methods for quantification of total nucleic acids and protein were developed adopting the same SCB homogenisation buffer necessary for FCM cell cycle analysis in order to allow for multiple cellular and biochemical measurements from a single larva. Total RNA and total DNA were immediately quantified after larval homogenisation in separate microplates using two nucleic acid fluorescence-specific assay kits, Quant-iT™ RNA and Quant-iT™ PicoGreen® dsDNA (Invitrogen), as suggested in McGinty et al. (2008). Standards provided in the kits (*E. coli* rRNA and calf thymus DNA) were serially diluted with SCB for the construction of a seven point standard curve ($R^2 \geq 0.98$). Each microplate contained triplicates of standards and larval homogenate samples (5 µl of standards or larval homogenates and 50 µl of Quant-iT™ working solution per well). RNA- and DNA-dye complexes were excited at 644 nm and 510 nm, respectively, and fluorescences were measured at 673 nm and 527 nm, respectively, in a MJR DNA engine fitted with a Chromo 4

detector (Bio-Rad). The above fluorometric protocols to estimate RNA and DNA contents of whole barramundi larvae homogenized in SCB were adopted after a series of optimization trials confirming the reliability and reproducibility of the methods, as suggested by Caldarone et al. (2001). These trials intended to determine (i) the ratio between the volume of larval homogenate to Quant-iT^{TM} working solutions so that fluorescence readings set in the linear range of the respective RNA and DNA standard curves; (ii) the coefficient of variation of standards and larval homogenate fluorescence readings (c.v. values averaging 6% could be routinely achieved through vortexing of sample homogenate and accurate pipetting, thus ensuring reproducibility); (iii) the recovery rates of control homogenates spiked and non-spiked with RNA and DNA standards, which were above 96%, indicating no problems with fluorescence quenching, and; (iv) absence of homogenate auto-fluorescence (i.e. homogenate fluorescence was not detected when no fluorophore added to Quant-iT^{TM} working solution).

Quantification of individual larval total protein was also determined in similar microplate format with a colorimetric Bradford assay (Bradford, 1976) using the Protein Quantification Kit-Rapid (Fluka® Analytical, Sigma-Aldrich). Bovine serum albumin (BSA) standard provided in the kit was serially diluted with SCB for the construction of standard curves ($R^2 \geq 0.98$). Each microplate contained triplicates of standards and larval homogenate samples (10 µl of standards or samples and 150 µl of Coomassie Brilliant Blue G per well). Absorbance was read at 595 nm in a VersaMax^{TM} ELISA microplate reader (Molecular Devices).

4.2.10 Relationship between larval growth rates and cellular and biochemical indices

Three experiments were performed to validate the application of the FCM cell cycle analysis, RNA/DNA and Prot/DNA protocols to studies of growth in the early life history of barramundi. The first two experiments focused only on FCM cell cycle analysis, whereas the

last experiment explores the phenotypic relationships between larval growth rate, cell cycle analysis, RNA/DNA and Prot/DNA.

4.2.10.1 Experiment 1 - Cell cycle analysis of nutritionally stressed larva

Here the effect of fasting on *L. calcarifer* larvae cell division rate was evaluated against a well fed control group. Larvae had been previously graded for size homogeneity to minimize initial size (and growth rate) differences and were fasted for 2 days (26 and 27th dph). Larvae were acclimated to a 50 l aquarium at 28 ^{0}C and fed three times a day with a commercial marine fish larvae diet for 2 days (28th and 29th dph). On the 30th dph half of the larvae (n = 80) were randomly transferred to an identical aquarium. In this experiment, representative sampled larvae were also weighed (to the nearest 0.1 mg) on an AAA250LE analytical balance (Adam Equipment). The larvae from the first aquarium (12.67 ± 0.53 mm; 40.4 ± 5.2 mg) were fed until satiation four times a day (FED), whilst larvae in the 2nd aquarium (12.76 ± 0.38 mm; 40.5 ± 3.5 mg) received no food (FASTED) for 6 days. FCM cell cycle analysis in 10 randomly sampled fresh larvae from each treatment at days 0, 2, 4 and 6 after commencement of experiment was performed.

4.2.10.2 Experiment 2 - Cell cycle analysis of larvae from different size grades

To assess the sensitivity of FCM cell cycle analysis to detect differences in larvae growing at different rates, cell division rates were quantified in a group of 36 larvae (16 dph) graded into four size classes (S - 6.27 ± 0.13 mm; M - 7.65 ± 0.10 mm; L – 8.61 ± 0.19 mm; and XL – 9.44 ± 0.27 mm). Each grade thus comprised of nine fish, which were pooled in three groups of three larvae, homogenized and analysed together.

4.2.10.3 Experiment 3 - Cell cycle analysis, RNA/DNA and Prot/DNA of ungraded larvae

In this experiment cell cycle analysis, RNA/DNA and Prot/DNA were evaluated on individual larva under normal commercial culture conditions; however, analysis was performed before commercial size grading practices had been applied. Samples comprised of 80 ungraded 18 dph *L. calcarifer* larvae randomly sampled from a hatchery tank exhibiting large size variability among individuals (size range 5.81 to 12.05 mm *Ls*, c.v. = 15.8 %), which had been measured and then frozen in liquid nitrogen and preserved at – 80 ^{0}C for three months. Absolute growth rate in length (G_L; mm/day) of individual larva was calculated through the formula: $G_L = (Ls_t - Ls_0)\ t^{-1}$, where Ls_t and Ls_0 represent standard length at sampling and mean length at hatch, respectively, and t represents time in dph. Total larval RNA and DNA were immediately quantified after homogenization in SCB, followed by quantification of larval protein and FCM cell cycle analysis. The relationships between larval size and its biochemical content and between larval G_L and cells dividing, RNA/DNA and Prot/DNA were further investigated.

4.2.11. Statistical analysis

Statistical analysis was performed with SPSS Statistics 19.0 (IBM). Data were first assessed for normality and homogeneity of variances (Kolmogorov-Smirnov and Levene's test, respectively, $P > 0.05$) with no transformations required. The respective statistical tests conducted for each experiment (i.e. ANOVA's, Pearson product-moment correlation and linear regression) are presented in the results section. Tuckey's HSD test was applied to resolve significant differences among groups (results were considered significant at $P < 0.05$). A non-parametric Mann Whitney U test was also utilised in comparisons of larval *Ls* between 18 and 26 dph because in this comparison variances were not homogeneous. Reported and graphical values represent mean ± s.e.

4.3 Results

4.3.1 Sampling age, larval homogenization trials and FCM data quality

Larval measurement at the hatchery indicated that 18 dph was the optimum sampling period under the larviculture conditions employed in the hatchery. This was because at this age larvae attained a size large enough (8.86 ± 0.08 mm) for the subsequent series of cellular and molecular analyses described below and exhibited marked size variability (range = 5.30 – 13.15 mm; c.v. = 18.4%) without yet suffering the effects of agonistic interactions (e.g. aggressive competitive and/or cannibalistic behaviour) that could affect normal larval development and bias future genetic analysis (Figure 4.2). At the end of the larviculture stage (26 dph) the different size grades obtained presented even greater size variation (small grade, S = 9.05 ± 0.08 mm; medium grade, M = 12.19 ± 0.13 mm; large grade, L = 16.83 ± 0.12 mm; extra large grade, XL = 23.83 ± 0.28 mm). Slow growing larvae from the S grade, even when separated from more dominant and aggressive larger larvae, presented stunted development with insignificant growth in length compared to the whole population size at 18 dph ($Ls_{18\ dph}$ = 8.86 ± 0.08) (Mann-Whitney U Test, U = 18161, z = -1.48, $P > 0.05$).

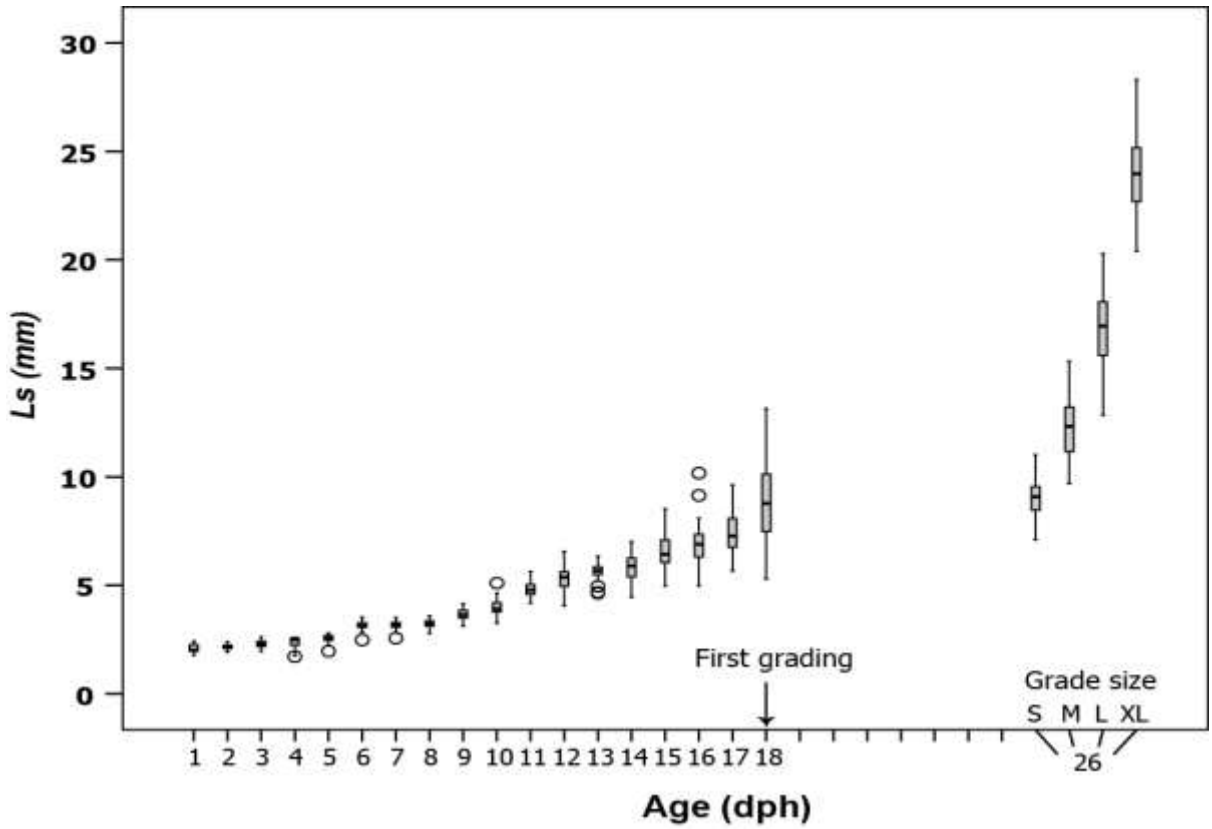


Figure 4.2. Growth curve of commercially reared *Lates calcarifer* larvae (*n* = 1660). Box plots represent 95% standard length (*Ls*) data, mid-lines represent *Ls* median, whiskers represent maximum and minimum *Ls* values and circles represent outliers. Larval age reported in days post hatch (dph). Sampling at 18 dph occurred just prior to first larval grading. Final sampling at the end of the hatchery period (26 dph) was taken from four tanks each containing larvae from different grade size (arbitrarily referred to as S, M, L and XL).

In extensive preliminary trials a variety of protocols to produce single-cell suspensions with whole *L. calcarifer* larvae were tested. These involved both mechanical dissociation (i.e. mincing with surgical blades, sieving through stainless steel mesh, pipetting or syringe and needle pass) and enzymatic digestion (i.e. collagenase, trypsin) in PBS prior to cell permeabilization (ethanol or non-ionic detergents) and PI staining. Approaches where a centrifugation step was performed resulted in irreversible cell clumping with too few nuclei. With the exception of brain tissue, which can be easily dissociated by simple pipetting, all DNA histograms from centrifugation protocols resulted in low and indistinguishable G_0/G_1

peaks. However, the use of a Tenbroek tissue grinder for homogenizing whole larvae in cold isotonic sucrose-citrate buffer ensured a complete and homogeneous dissociation into fine particles without any loss of tissue or nuclei damage. Initially, a final nuclear extraction and staining of the homogenate was attempted, following Vindeløv's *et al.* (1983) established protocol for fine needle aspiration of mammalian (mice and human) normal tissues and tumours. However, G_0/G_1 peaks were too broad for appropriate deconvolution of DNA histograms (Figure 4.3). Mixing the homogenate in PI-detergent solution at 2-4 ^{0}C (Petersen, 1985) for 3 h was the only method tested that yielded consistent DNA histograms with low c.v. of G_0/G_1 for different *L. calcarifer* tissues (c.v. < 4%) and either fresh (c.v. = 2.78 ± 0.36 %), frozen (c.v. = 3.2 ± 0.16 %) or RNA*later*® (c.v. = 4.86 ± 0.36 %) preserved fish larvae (Figure 4.4).

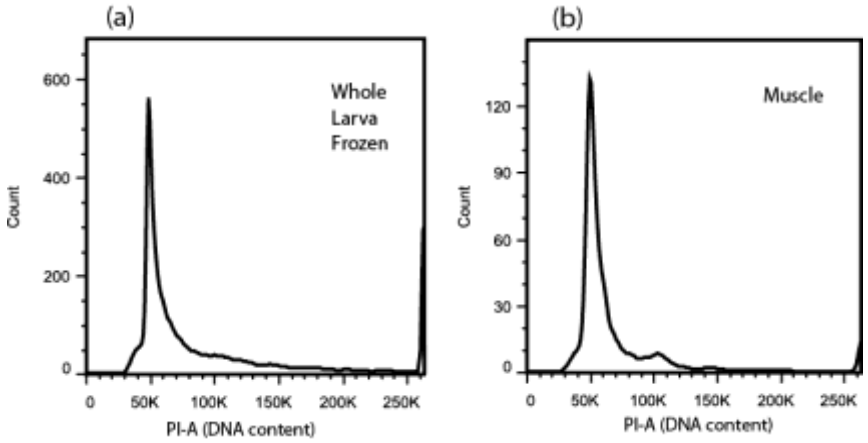


Figure 4.3. Propidium-iodide area (PI-A; DNA content) frequency distributions of *Lates calcarifer* with poor resolution to be deconvoluted for cell-cycle analysis from samples prepared with Vindeløv *et al*. (1983) detergent–trypsin method: (a) whole larva frozen, indistinguishable G_2/M peak and high number of counts above G_2/M and (b) muscle, broad G_0/G_1 peak (c.v. >10%) and low nuclei yields.

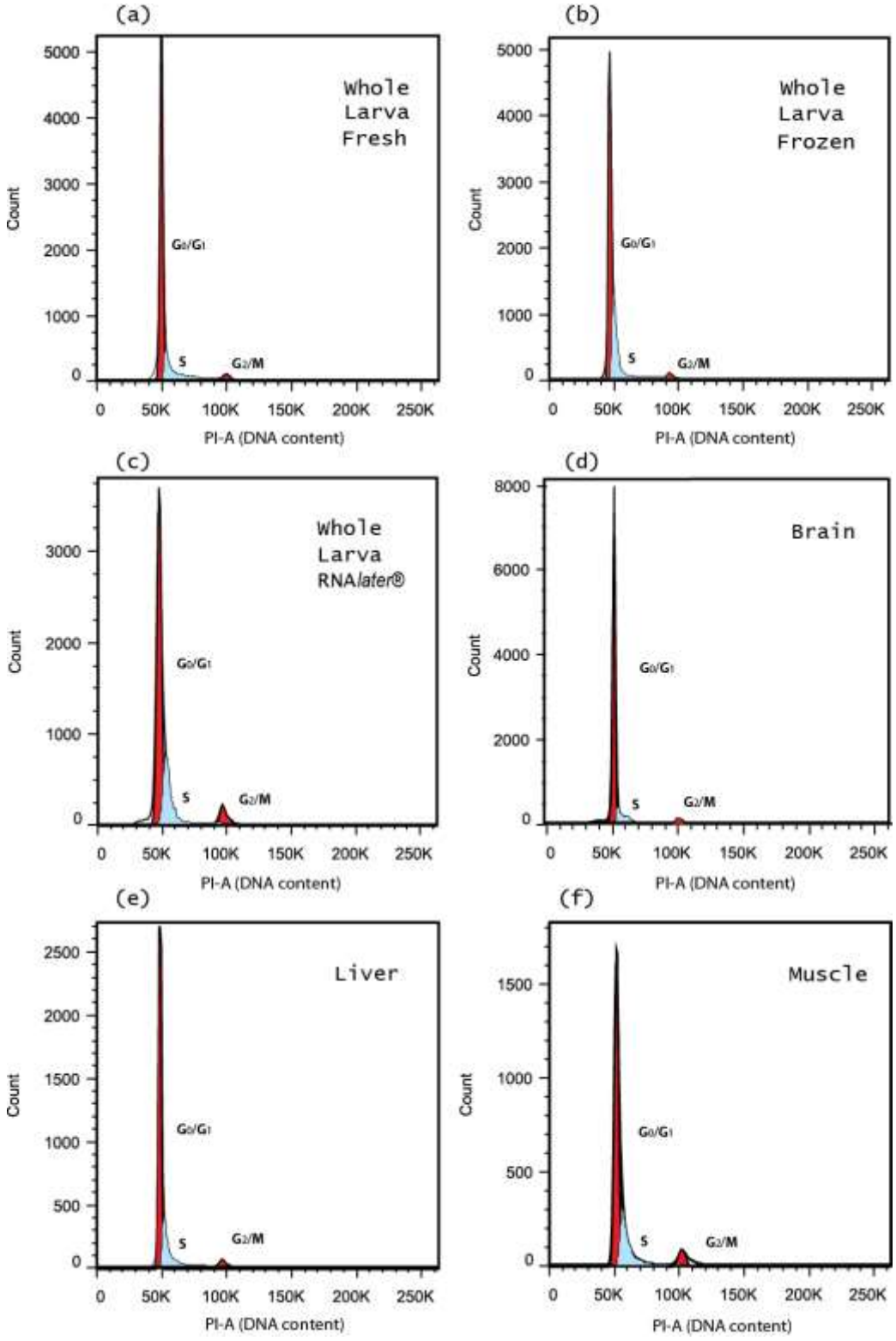


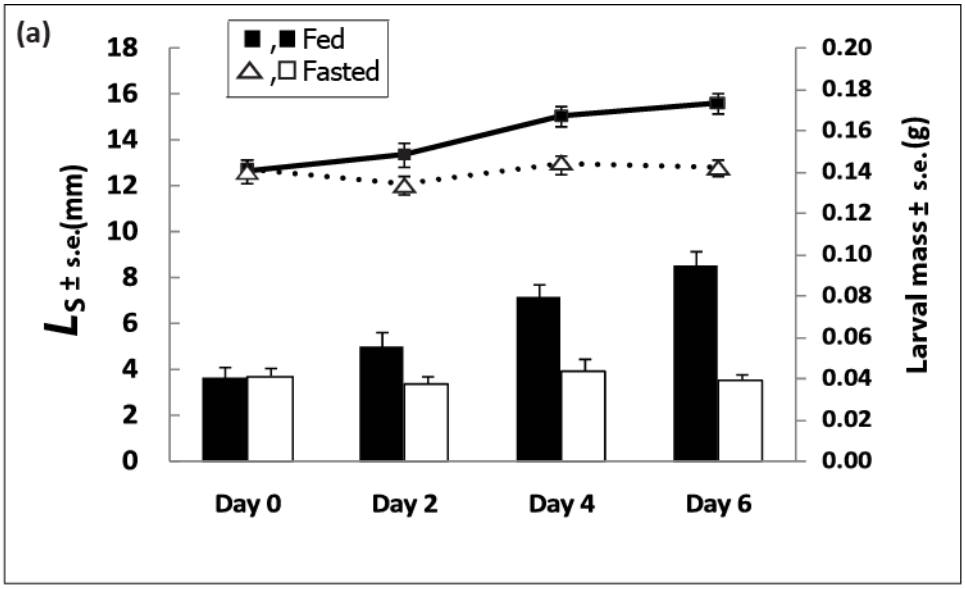
Figure 4.4. Propidium-iodide area (PI-A; DNA content) frequency distributions of (a)–(c) whole larvae (a) fresh, (b) frozen and (c) RNAlater preserved and individual fresh tissues (d) brain, (e) liver and (f) muscle of Lates calcarifer deconvoluted by FlowJo cell-cycle platform (Tree Star) into the three major phases (G_0/G_1, S, G_2/M) of the cell cycle as indicated.

4.3.2.1. Experiment 1 - Cell cycle analysis of nutritionally stressed larva

This experiment was performed to evaluate the effects of short term starvation on cell division rates of *L. calcarifer* larvae. Cell division at day 0 was not significantly different between Fed and Fasted groups (independent samples t-test: t (14) = 0.23; $P > 0.05$ (two-tailed)). Fasted larvae exhibited no change in length or mass during the six day period, whereas fed larvae grew 23.1 % in size and 133 % in mass over the same period. Differences in length and mass between the two groups became evident after day 4 of fasting (Figure 4.5 (a)). There was a statistically significant interaction effect between fasting and fasting period on cell division (Two-way ANOVA, F (2,48) = 4.1; $P < 0.05$), with differences between treatments noticed after day 4 (Figure 4.5 (b)). Similar to growth, there was no difference in cell division rates for the fasted group (One-way ANOVA, F (3,31) = 0.54; $P > 0.05$) throughout the experiment (22.8 ± 1.1 %), however, the fed group exhibited an increase in the percentage of cells dividing in day 4 (27.5 ± 1.3 %) and day 6 of the trial (28.1 ± 1.3 %) (One-way ANOVA, F (3,31) = 10.3; $P < 0.05$) relative to day 0.

4.3.2.2. Experiment 2 - Cell cycle analysis of larvae from different size grades

Here the rate of cell division in *L. calcarifer* larvae sampled from four size-graded hatchery tanks was evaluated. There was a statistically significant difference in the percentage of cells dividing among the different grade sizes (one-way ANOVA; F (3,8) = 13.1, $P < 0.05$). The smallest (S) and the largest (XL) size classes of larvae graded due to their respectively slower and faster growth rates exhibited statistically lower (S: 16.1 ± 0.9 %) and higher percentages (XL: 23.2 ± 0.8 %) of cells dividing compared to the middle groups (M: 19.7 ± 0.3 % and L: 20.1 ± 1.1 %) (Figure 4.6).



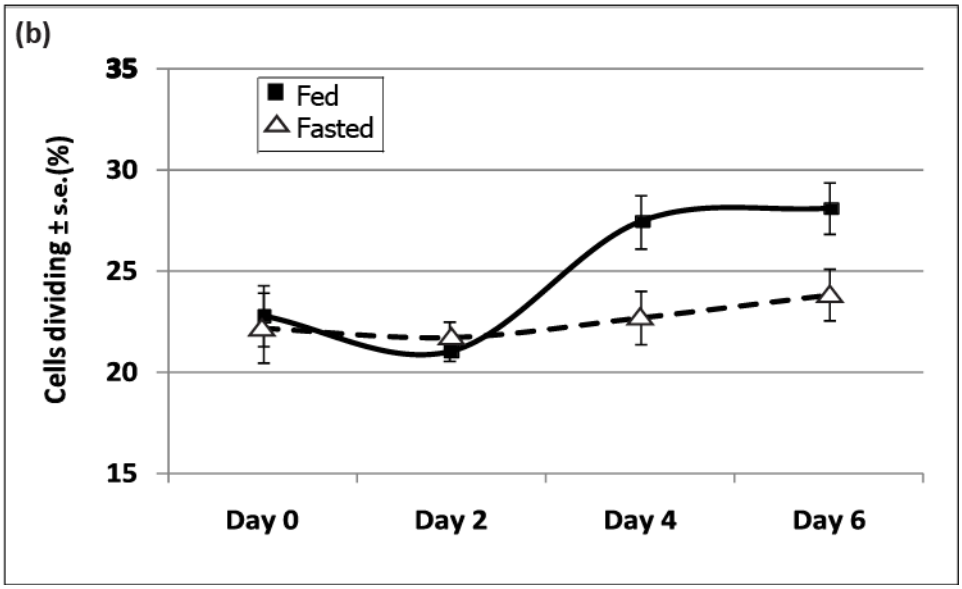


Figure 4.5. Fed and fasted 30 days post-hatch *Lates calcarifer* larvae over a 6 day period: (a) growth in standard length (*Ls*, lines) and mass (g, bars) and (b) respective percentages of cells dividing. Values are means ± s.e.

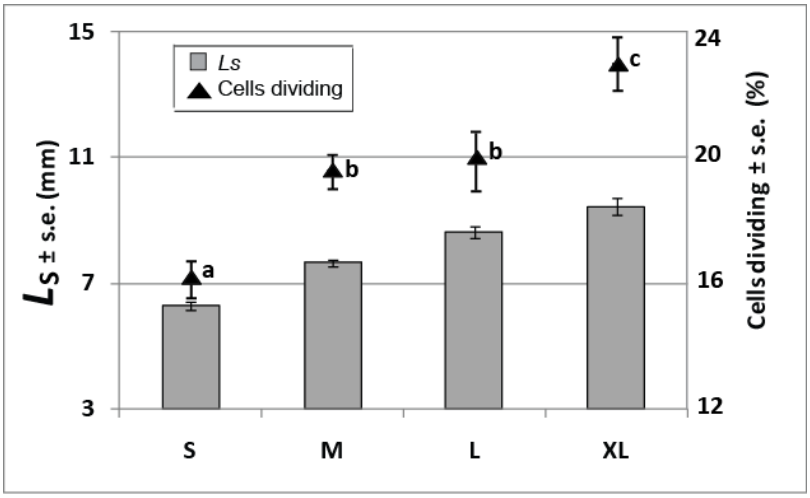


Figure 4.6. *Lates calcarifer* larvae reared under commercial culture conditions: (a) 16 dph larvae graded in four standard length (*L*s) classes (S, M, L and XL) and their respective percentages of cells dividing. Values are mean ± s.e.. Lower case letters denote statistical differences (one-way ANOVA, P <0.05).

4.3.2.3. Experiment 3 - Cell cycle analysis, RNA/DNA and Prot/DNA of ungraded larvae

As expected, *L. calcarifer* larvae sampled from a hatchery tank at 18 dph exhibited large differences in G_L (0.24 - 0.61 mm/day), total RNA (6.2 – 35.6 ng), total DNA (3.9 – 14.0 ng), total protein (178.0 – 1257 ng), RNA/DNA (1.54 – 2.42), Prot/DNA (33.5 – 87.7 ng) and cells dividing (10.3 - 30.3%). Larval nucleic acid and protein contents were exponentially correlated with larval size (i.e. RNA = 0.0138 $Ls^{3.54}$, R^2 = 0.84; DNA = 0.0283 $Ls^{2.81}$, R^2 = 0.83; Prot = 0.355 $Ls^{3.69}$, R^2 = 0.74) (Figure 4.7). In general, fast growing larvae had higher percentages of cells dividing, RNA/DNA and Prot/DNA than slow growing larvae (Figure 4.8). Highly significant positive correlations were found between larval growth and cell division rates and between larval growth and RNA/DNA (respective Pearson product-moment correlation coefficient; r = 0.67 and r = 0.69, n = 80, P < 0.001), whereas a moderate correlation was found between larval growth and Prot/DNA (r = 0.46, P < 0.01). The standard linear regressions of cells dividing, RNA/DNA and Prot/DNA to predict growth rate explained 21 to 45% of the variability seen in larval growth rates (i.e. G_L = 0.013 (% Cells

dividing) + 0.166 (R^2 = 0.45; F (1,76) = 63.3, P < 0.001); G_L = 0.134 (RNA/DNA) + 0.096 (R^2 = 0.45; F (1,77) = 62.6, P < 0.001) and G_L = 0.001 (Prot/DNA) + 0.299 (R^2 = 0.21; F (1,76) = 19.7, P < 0.001).

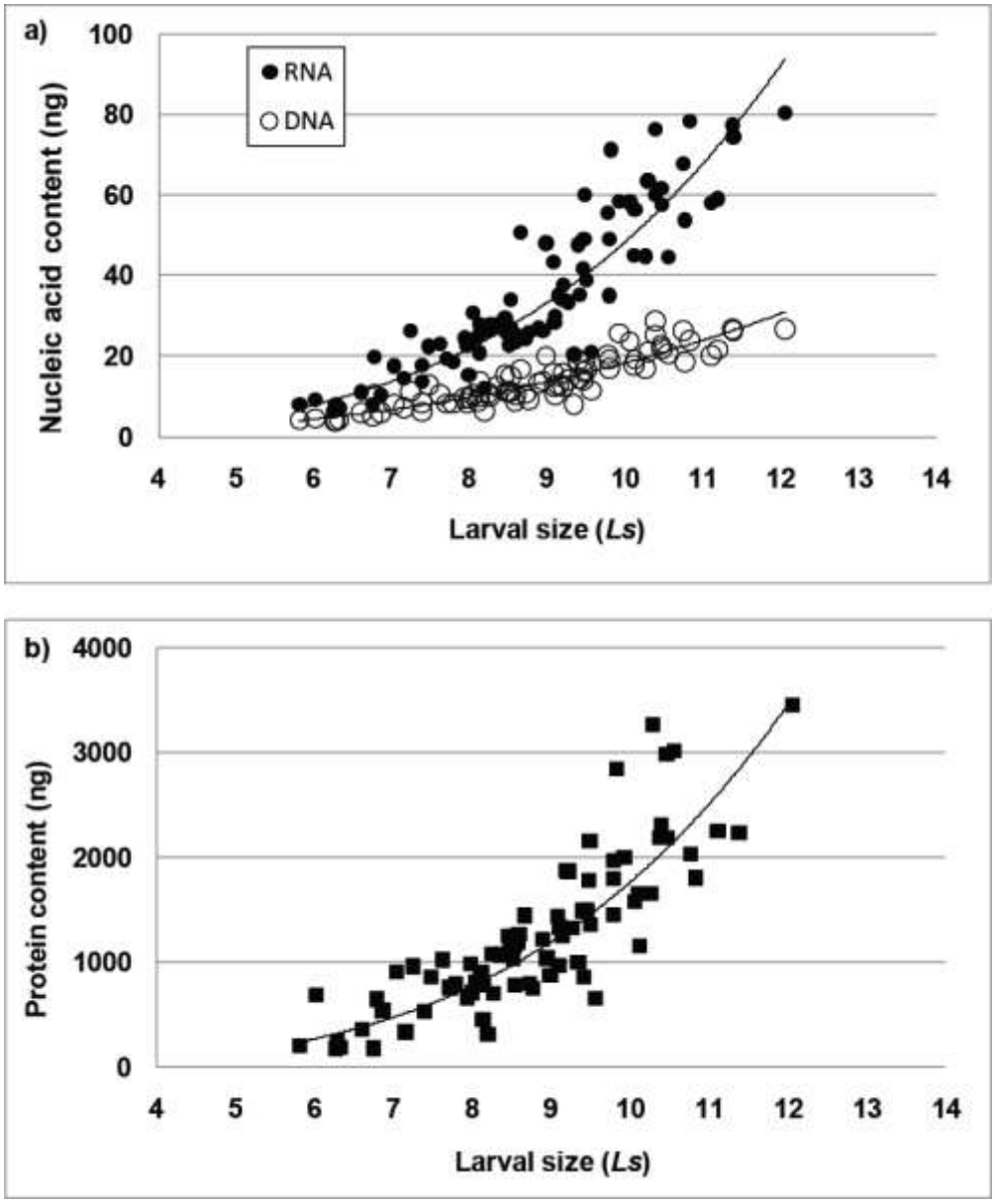


Figure 4.7. Relationship between 18 dph *Lates calcarifer* standard length (*Ls*) and (a) total nucleic acid content and (b) total protein content.

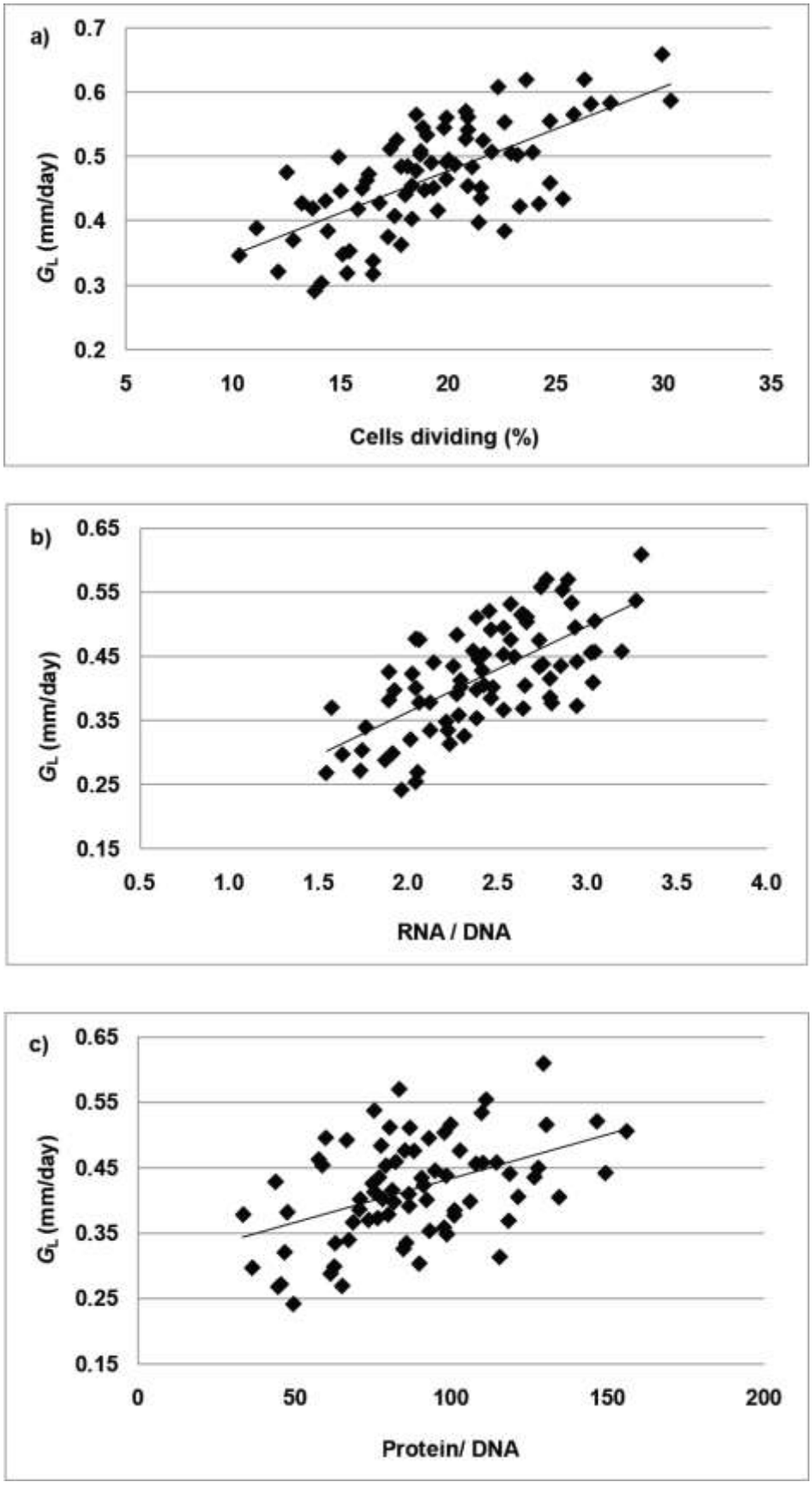


Figure 4.8. Relationship between 18 dph *Lates calcarifer* absolute growth rate (G_L) and (a) percentages of cells dividing, (b) RNA/DNA and (c) Protein/DNA.

4.4. Discussion

In this chapter, multiple cellular and biochemical analytical methods to measure metabolic processes related to growth within a single fish (barramundi) larva were developed. The employment of such techniques, associated with pedigree information, will enable the assessment of the additive genetic (co)variance of a range of larval traits which could disclose genetically determined growth potential *(Chapter 5*).

Protein synthesis and cellular division are among some of the most fundamental biological processes underlying growth and robust methodologies for the study of such processes in larval fish are highly desirable. Studies of larval growth rates in fish are of importance in aquaculture and fisheries in addressing future food security and examining impacts of human activities on marine habitat. Importantly, multiple experiments applying these methods revealed that these processes were strongly associated with larval growth. Primarily, this chapter reports a practical protocol to prepare single nuclei suspensions from whole fish at early life stages to reliably obtain high quality frequency histograms of DNA content through flow cytometry (FCM). To date, the challenge of sample preparation for FCM cell cycle analysis from whole fish larvae (comprised of fibrous and hard tissues including muscle, bones and scales) has precluded a broader application of FCM for the study of fish larval growth and development. Furthermore, it was demonstrated here for the first time that it is feasible to study the relationship between the growth rates of individual fish larvae under commercial aquaculture conditions and the overall rate of cell division, RNA/DNA and Prot/DNA.

The paucity of adequate protocols for FCM cell cycle analysis for fish larvae have forced the few studies conducted either to discard up to 70% of data (González-Quirós et al., 2007;

Catalán et al., 2007), or to increase the G_0/G_1 c.v. cut-off up to 9% (Porter and Bailey, 2011). DNA histograms with a G_0/G_1 c.v. below 6%, like those obtained in the present study, are considered acceptable for the reliable estimation of the percentages of nuclei within G_0/G_1, S and G_2/M (Darzynkiewicz, 2010). From fresh material, a coefficient of variation in the order of 3% or less should be routinely obtained (Ormerod et al., 1998). When performed with individual tissues such as fin, brain, muscle, and liver the newly described protocol presented herein consistently produced high quality DNA histograms (Figure 4.4; c.v. from tissues below 4%), indicating that the protocol is not tissue-specific and nuclei from whole larvae can be analysed. In addition, larvae preserved at -80 ^{0}C and at -20 ^{0}C in RNA*later*® presented slightly higher G_0/G_1 c.v. than fresh larvae, however, within acceptable ranges for cell cycle analysis, demonstrating that it is feasible to collect and store samples aboard research vessels, or at aquaculture facilities, for later analysis. The ability to assess cell division ratios from whole larvae precludes the careful and tedious micro-dissection step normally required to isolate individual larval tissues and avoids a further reduction in sample size, thus allowing for a higher sample throughput. Metabolic activity and cell division rates are likely to be tissue-specific. However, the use of homogenates from whole fish larva to indirectly assess larval nutritional condition through RNA:DNA ratios has been widely validated in the last few decades (Bulow, 1970; Buckley, 1984; Ferron and Leggett, 1994; Chícharo and Chícharo, 2008). Likewise, results obtained in the present study demonstrated that FCM cell cycle analysis of whole larvae can also be assessed to estimate larval growth.

A suspension of single particles is the essential element of successful and robust FCM (Ormerod, 2000). In FCM cell cycle analyses, the quality of sample preparation and staining is as important in the precision of measurements as the design of the fluidic, optical, and electronic components of the instrument itself (Carter and Ormerod, 2000). Due to the

complex nature and heterogeneity of tissues and dominance of long multinucleated skeletal myocytes (sometimes greater than 1 mm) in whole fish larvae, isolation of nuclei was mandatory for this purpose, despite the fact that information from a number of mitotic cells lacking a nuclear envelope might be lost with this method (Darzynkiewicz, 2010). 1. The use of tissue grinders with tight-fitting pestles and cold sucrose solutions have a long history of use in the isolation of intact nuclei from rat tissues such as liver (Schneider, 1948; Wilbur and Anderson, 1951) and skeletal muscle (Edelman et al., 1965). Although not mentioned in protocols of sample preparation for FCM DNA analysis (Vindeløv et al., 1983; Petersen, 1985; Ormerod, 2000; Gomez et al., 2001; El-Naggar and Vielh, 2004; Heinlein et al., 2010), the use of tissue grinders has been reported for DNA analysis of human tumours and cell lines (Piwnicka et al., 1983; Oud et al., 1986; Remvikos et al., 1988). A high density sucrose solution for tissue dissociation reportedly aids to split gap and tight junctions responsible for cell clusters while inclusion of sodium citrate aids to loosen intercellular bonds by sequestrating cations (Ca^{2+} and Mg^{2+}) responsible for cell surface and intercellular structural matrix integrity (Pallavicini, 1987). These factors abovementioned may have significantly contributed for the reproducible FCM results obtained herein for a variety of tissues, fresh and preserved.

Standardizing the volume of sucrose-citrate buffer per larval size (where the cubic relation of larval length is highly correlated with larval mass) during homogenization, and the volume of homogenate mixed with the PI-detergent solution, were critical for consistently comparable DNA histograms among samples. By empirically determining the optimal buffer to tissue ratio, the ratio between nuclei number and PI concentration was maintained roughly constant among samples (staining equilibrium), enabling G_0/G_1 peaks to fall consistently into the same PI-area channels as blood controls. This critical improvement to the protocol precludes the

need for tedious nuclei counting and dilution steps. There is a consensus among clinical pathologists that a minimum of 10,000 cells (or nuclei) should be recorded for representative FCM DNA analysis (Ormerod et al., 1998) and indeed consistent results from replicates of the same larval homogenate in previous trials demonstrated that nuclei discarded in preparation steps are in the same proportions as those analyzed.

Recent nutritional history and food availability may play an important role in regulating hyperplasia and hypertrophy in *L. calcarifer*. Cell division rates in well fed, fast growing fish in experiment 1 only differed from the fasted group after the 4^{th} day. The length of this lag period in cell division rates between groups could have been influenced by the larval nutritional status prior to commencement of the experiment, as all animals had been previously fasted for 48 h. Interestingly, a constant and relatively high baseline cell division rate (22.8 ± 1.1 %) was observed for fasted fish, instead of a decline, even when there was no growth throughout the 6 day period. Previous studies on starved juvenile *G. morhua* indicated that starved fish were able to maintain a constant level of protein synthesis whereas well-fed, fast growing fish displayed greater protein synthesis accompanied by increased levels of efficiency of the retention of synthesized protein (Houlihan et al., 1988), a similar trend to what was observed in the present study for cell division rates of fed and fasted barramundi larvae. An interesting future direction for research in early life history of fishes would be to examine the association between FCM cell cycle analysis and the histological assessment of hyperplasia and hypertrophy in the phenomenon of compensatory growth in fish (Ali et al., 2003).

Lates calcarifer larvae reared under commercial culture conditions presented extensive variability in growth rates, especially after the second week of culture. Data obtained in the

present chapter suggest that fast growing *L. calcarifer* larvae have higher metabolic rates and an overall hyperplasia advantage compared with slow growing larvae, as significantly higher numbers of cells dividing were observed among fish with higher growth rates and fish belonging to larger size grades. Greater rates of cell division in larger larvae might explain the increasingly greater differences in sizes during larval culture, which compels fish culturists to grade fish every 2-3 days to avoid cannibalism and production losses (Schipp et al., 2007). Strong and highly significant correlations were found between growth rates, cell division and RNA/DNA (r = 0.67), which accounted for 45% of the variability in growth of *L. calcarifer* larvae reared under optimum physiological requirements. Most studies validating the correlation between RNA/DNA (or cells dividing) and larval growth have employed physiological stressful treatments as unfavourable rearing temperatures, or different feeding levels (Ferron and Leggett, 1994). However, when individual nucleic acid determination (and likewise FCM cell cycle analysis) has been performed on well fed larvae reared under identical conditions, results commonly reveal a large and often unexplained inter–individual variability in the RNA content and in the RNA/DNA (Ferron and Leggett, 1994, and references therein). Based on the strength and significance of correlations found between barramundi rates of cell division and RNA/DNA and growth rates under standardized environmental conditions, it is reasonable to conclude that the combined flow-cytometric and nucleic acid based protocols presented in this chapter are sensitive enough to be applied in practical studies, such as the evaluation of different hatchery protocols or, as investigated later in *Chapter 5*, to assess genetically determined growth potential.

Complex genotype-environment interactions are known to regulate the balance between the increase in cell number (hyperplasia) and volume (hypertrophy) and cell death (apoptosis) which dictates an organism's increase in body mass (growth). The causes of high individual

variability found in cellular and biochemical indices within fish larvae is still poorly understood, and it is surprising that genetic effects, known to significantly impact on fish growth rates, have never been accounted for in previous models. It is important to consider that absolute growth rate in length (G_L; mm/day) is the end result of various previous early life history events which led to the size attained at 18 dph (i.e. rate of embryonic development and relative hatching time, early swimming and foraging ability, intra-specific competition, etc.). In a sense, measurements provided by indices such as RNA/DNA and the proportion of cells dividing depict current and on-going fundamental metabolic processes which will impact on the rates of protein synthesis and tissue growth. Therefore, biochemical and cellular traits may be more informative than larval size to forecast the likelihood of augmentation in larval mass and length affecting future larval growth.

In conclusion, the methods developed in this chapter for the simultaneous assessment of FCM cell cycle analysis, RNA/DNA and Prot/DNA coupled with other physiological, nutritional, environmental or genetics variables will enable fish biologists and aquaculturists to achieve a better understanding of how cell division rates and biochemical indices influence fish growth in natural and artificial conditions. For instance, the ability of such larval traits to predict either short or long term future fish growth trajectories, however, has never been established to date and will be examined in the last chapter of this thesis (*Chapter 5*).

Chapter 5. Early prediction of long-term family growth performance based on cellular processes – a tool to expedite the establishment of superior foundation broodstock in breeding programs

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5.1. Introduction

In the establishment of selection programs where foundation broodstock are collected directly from the wild, or from unevaluated stocks, there is no prior way to ascertain their genetic merit for growth rate. Classically, broodstock are spawned and progeny reared for a long time, often to harvest sizes, before individuals are measured and estimates of breeding values (EBV) of the animal and families can be determined as a basis for selection. Permanent and continuous genetic gains are often realized only after a few selected generations, usually in the order of a decade. Thus, the development of genetically superior farmed stocks is still a long way away for most aquaculture industries, especially those working with species with long generation intervals (e.g. 2-4 years).

To date, breeding programs in aquaculture have not yet exploited the enormous potential of progeny testing for the selection of genetically superior broodstock. In established terrestrial livestock, however, the evaluation and selection of breeders through the performance of their offspring is the driver of several genetic improvement programs. For example, progeny testing is the primary source of genetic gain within the Australian dairy industry (ADHIS, 2012). In fact, for traits with moderate heritability (h^2), as is the case for growth-related traits (e.g. harvest weight, length) in aquatic species (Gjedrem and Olesen, 2005), progeny records are considered the ultimate source of information for the calculation of an animal's EBV (Bourdon, 2000). This is because progeny testing is the most accurate method to estimate the

breeding value of an animal (e.g. more accurate than individual, within-family, sib-selection or combined selection approaches), provided records from a number of progeny from half-sib families are taken (Gjerde, 1991, 2005). Nevertheless, large scale family-based breeding programs, first developed for salmonids (Gjedrem, 2010) and now established as the industry standard (Gjedrem, 2012), generally spawn each breeder only once, missing out on the opportunity to generate and promptly disseminate consecutive cohorts of fast growing fish from those highly (EBV) ranked breeders to grow-out farms. For fishes like salmonids this approach is used because high mortality rates are expected after spawning, which in most cases impedes the generation of a second progeny cohort from the same breeders (Gjerde, 2005). For a large number of highly fecund multiple spawning species which reproduce for longer periods (e.g. months to years) there is, however, obvious potential for progeny testing. For aquaculture industries working with such species, breeding programs using foundation stock from progeny tested broodstock can theoretically yield faster rates of genetic gain than contemporary breeding programs (Macbeth and Palmer, 2011). Therefore, the use of progeny tested breeders could allow for a more immediate realization of increases in aquaculture productivity and profitability.

One shortcoming of progeny testing in aquaculture has been attributed to longer generation intervals, possibly doubled that of traditional schemes, because selection of broodstock is not performed until offspring have been measured (Gjedrem, 1983; Fjalestad, 2005). This is particularly true if progeny are tested after individual growth trajectories have been confidently established (e.g. one or two year old progeny) and progeny are sexually mature. However, this principle does not apply if progeny testing was performed very early in the production cycle and the best (EBV) ranked broodstock immediately re-spawned to produce the cohorts to be stocked by farmers. This could be achieved by exploring potential genetic

correlations between economically important traits (e.g. fish harvest weight) and traits measured at larval stages. When an inherited trait expressed early in life is influenced by the same set of genes which also affect a trait later in life, such early and late traits are genetically correlated, that is, there is correlation between breeding values for those traits, even when there may be no observable phenotypic links between them (Falconer and Mackay, 1996). Therefore, if a larval trait is heritable and highly genetically correlated with a harvest growth trait, the progeny larval records could be used to determine parental EBVs, allowing for pre-screening and identification of broodstock with the best growth characteristics. This method of selection could enable the improvement of fish growth rates as soon as the subsequent spawning cycle and potentially lead to immediate increases in productivity. For biologically suited species, early progeny testing could prove an important additional tool for traditional breeding programs and expedite the establishment of superior foundation broodstock.

Increasing evidence suggests that fish larval traits are under the influence of a strong genetic component which could reveal their parents innate genetic capacity (EBV) for fast growth. Numerous studies have shown that family origin and/or paternal (i.e. purely genetic) effects play a significant role in fish growth as early as embryonic and larval stages (Saillant et al., 2001; Green and McCormick, 2005; Trippel et al., 2005; Probst et al., 2006; Ottesen and Babiak, 2007; Butts and Litvak, 2007; Eilertsen et al., 2009; Núñez et al., 2011) and reported that heritability of diverse traits measured very early in life (Table 5.1) share similar moderate magnitudes as those reported for adults (Gjedrem and Olesen, 2005). Most importantly, positive genetic correlations between adult breeder size and offspring early life history traits (e.g. larval size, weight gain, swimming performance and survival) have been demonstrated in both captive and wild fish populations (Vandeputte et al., 2002; Munch et

al., 2005; Johnson et al., 2010, 2011), although genetic links between different ontogenetic stages among marine organisms still remain largely unknown (Marshall and Morgan, 2011).

Table 5.1. Summary of heritability (h^2) estimates of teleost larval traits. W = wet weight, *Ls* = standard length, *Lt* = total length, Swim = swimming performance, RNA/DNA = RNA:DNA ratio, °C days = degree – days, dph = days post hatch, dpf = days post fertilization.

Trait	h^2	Fish age	Species	Study
Ls	0.65	0 dph [b]	Atlantic herring *Clupea harengus*	Bang et al. (2006)
Ls	0.14 ± 0.06 [a]	0-50 dph	Japanese flounder *Paralichthys olivaceus*	Shimada et al. (2007)
Ls	0.29 ± 0.09	0 dph [b]	Bicolour damselfish *Stegastes partitus*	Johnson et al. (2010)
Lt	0.24 ± 0.06	41-57 dpf	Red drum *Sciaenops ocellatus*	Ma et al. (2008)
Lt	0.26 ± 0.17	0 dpf [b]	Atlantic salmon *Salmo salar*	Páez et al. (2010)
Lt	0.22 ± 0.13	unreported (emergence)	Atlantic salmon *Salmo salar*	Páez et al. (2010)
W	0.28 ± 0.13	786 °C days	Brown trout *Salmo trutta*	Vandeputte et al. (2002)
W	0.22 ± 0.06	41-57 dpf	Red drum *Sciaenops ocellatus*	Ma et al. (2008)
Swim	0.20 ± 0.06	0 dph [b]	Bicolour damselfish *Stegastes partitus*	Johnson et al. (2010)
RNA/DNA	0.31	0 dph [b]	Atlantic herring *Clupea harengus*	Bang et al. (2006)

[a] average value; [b] 0 dph = hatching day.

Based on significant parental effects (Høie et al., 1999) and moderate heritability found for Atlantic herring (*Clupea harengus)* larval RNA/DNA at hatch (h^2 = 0.31; Bang et al., 2006), it has been suggested that the genetically determined expression of growth-related processes early in life could also persist in later life stages, affecting overall fish growth trajectories (Høie et al., 1999; Bang et al., 2006). Although this hypothesis has never been tested in fish, selection experiments for higher RNA/DNA content in mammary glands of female mice over 13 generations in mice resulted in significant increases in mammary gland weight and body weight of mothers at second parturition (Sung, 1970). Genetic differences between fish individuals leading to disparities in growth rate are well known (Gjedrem, 1997). If the kinetics of these processes is enacted from early development it is conceivable that they could predict the long-term genetic growth potential of progeny and/or their parents. Therefore, fish that are increasing body mass due to their innate genetic capacity for fast growth should produce similar high trends in cellular and biochemical predictors as seen when temperature, or nutrients availability, are not limiting factors. Through carefully designed mating strategies, where confounding environmental effects are kept constant among individuals it should be possible to examine the intraclass correlation and expression of larval traits within and between families and establish the reliability of these parameters to predict growth potential due to genotype.

Barramundi is a typical example of a highly valued aquaculture species in which production is currently based on unimproved stocks, despite a comprehensive knowledge of its biology and culture (Jerry, 2013) and recent availability of numerous genomic resources (Xia et al., 2010; Wang et al., 2011; Yue, 2013; Zhu et al., 2010). The species is ideally suited for progeny testing (Macbeth and Palmer, 2011) due to their high fecundity (millions of eggs per spawn, Garcia, 1990), longevity (> 10 years, Davis and Kirkwood, 1984) and high heritability for growth traits (h^2 harvest weight ~ 0.4, *Chapter 4*). In addition, because

barramundi is a protandrous hermaphrodite (i.e. initially mature as males then invert to females), sire progeny testing could allow for very high selection intensities of future dams. In this study, the hypotheses that larval cellular and metabolic parameters are heritable and may predict long-term family growth in barramundi were tested. Firstly, the genetic contribution to trait variation (i.e. heritability) of a range of morphological, biochemical and cellular larval traits (namely standard length (*Ls*), total RNA, total DNA, total protein, RNA/DNA, Prot/DNA and the rate of cellular division) were estimated through an animal model. Secondly, the genetic correlations between these larval traits and two morphological traits indicative of long-term growth at harvest (namely *Ls* and wet weight (W)) were calculated. Finally, to assess the feasibility of early progeny testing as a broodstock selection tool, the relationship between broodstock EBVs for early and late traits were further investigated.

5.2. Materials and methods

Methods regarding mass spawning of broodstock, fish culture conditions, sampling, genotyping and DNA parentage analysis protocols were previously described in *Chapters 2* and 3. In the present *Chapter 5*, phenotypic and genetic data were analysed from both 18 dph larvae and 273 - 469 dph harvested offspring of the two most successful mass spawning events, in terms of genetic diversity (i.e. number of families produced), namely *Chapter 2* batches 2 and 3. Briefly, there were six dams and six sires in batch 2 and 12 dams and 21 sires in batch 3 present in the tank at spawning and offspring grow out took place in two commercial farms, an intensive indoor recirculating facility in Victoria, Australia (referred to as Tank; fish from both batches 2 and 3) and a semi-intensive grow out pond in Queensland (referred to as Pond; only fish from batch 2). Larval *Ls* measurements, cell cycle analysis and quantification of total RNA, DNA and protein followed the protocols previously described in

Chapters 2 and *4*, whereas protocols for morphological measurements (*Ls* and W) of fish at harvest were described in *Chapters 2* and *3*.

5.2.1. Statistical analysis

SPSS Statistics 19.0 (IBM) was used to assess phenotypic data for homogeneity of variances and normality (Levene's test and Kolmogorov–Smirnov, respectively, $P > 0.05$) and calculate linear regression statistics (below). To increase statistical confidence for the quantitative genetic analyses, only larvae belonging to families containing five or more assigned individuals were subjected to biochemical and cellular analyses. For families with larger contributions, only the first 24 genotyped larvae were used. Likewise, at harvest, only data from fish belonging to families with 5 or more offspring were used for the quantitative genetic analyses. Natural log transformation was applied for larval size and crude biochemical constituents because observed values did not conform to normality. In addition, variances of harvested fish W and *Ls* between Tank and Pond environments (batch 2) were not homogeneous and therefore all observed harvest data were scaled by the standard deviation in the level of each environment prior to genetic analysis (Hill, 1984), although genetic parameters obtained from normalized harvest data were nearly identical to those obtained with raw data. Estimates of (co)variance components, heritabilities (h^2) and genetic correlations (r_g) between traits were estimated by restricted maximum likelihood (REML) procedures in ASReml 3.0 (Gilmour et al., 2009) fitting an animal model:

$$y = X\beta + Zu + e \quad (1)$$

where y is the vector of (normalized) observations for each trait, β is the vector of the fixed effects batch (two levels, batch 2 and batch 3), u is the vector of the random animal additive

effects and *e* is the vector of random residual effects. *X* and *Z* are design matrices that relate recorded (normalized) observations to respective fixed and direct genetic effects. Bivariate models similar to univariate models (1) were used to obtain covariance components between larval and adult traits. These bivariate models also included two additional fixed effects present in harvest data, farming system (two levels, Tank and Pond) and tank of origin (two levels, tank A and tank B). The residual covariance was set to zero in bivariate models, as only one observation per trait was taken for each individual and the covariance matrix was constrained to be positive definite. When fixed effects were statistically not significant (conditional Wald F-tests, $P > 0.05$), these were removed from the models.

Heritability of larval traits at 18 dph was estimated from univariate models as $\sigma^2_a / (\sigma^2_a + \sigma^2_e)$ where σ^2_a and σ^2_e were variances attributed to additive genetic and residual error effects respectively. To test whether the heritability of a larval trait was significantly different from zero, a likelihood ratio test was calculated as twice the absolute differences in log-likelihoods of the final model and that of a model excluding the random animal additive effects, where the P value was estimated from a χ^2 distribution with one degree of freedom (Wilson et al., 2010). Likewise, to test whether non-genetic maternal effects were significant, likelihood ratio tests were performed comparing the univariate models with similar models where maternal identity (dam) was fitted as an additional random effect (Wilson et al., 2010).

The genetic correlations (r_g) between larval traits and adult size (*Ls*, W) at harvest were calculated from the bivariate models as $r_g = \sigma_{a1a2} / ((\sqrt{(\sigma^2_{a1})}\ \sqrt{(\ \sigma^2_{a2})})$, where a_1a_2 is the estimated additive genetic covariance component between the two traits. To test whether the genetic correlations were significantly different from zero, likelihood ratio tests were also performed between the actual model and a model constraining the genetic covariance to zero

(Wilson et al., 2010). Likewise, to test the significance of possible non-genetic maternal covariance between larval and adult traits, the log-likelihood for the final model was compared to a model which included maternal identity (dam) as an additional random effect.

Whenever significant genetic correlations were found between early and late life history growth traits, broodstock EBVs estimated for the different larval traits were plotted against EBVs estimated for harvest weight (W) to assess and illustrate the feasibility of early progeny testing. Broodstock EBVs for each trait were obtained from ASReml 3.0 (Gilmour et al., 2009) and standardized into z-scores (mean of 0 and standard deviation of 1) to facilitate the visual comparison among plots and their linear regression statistics. The coefficients of determination (R^2 values) were used to assess the strength of the covariation between broodstock EBVs and the model fit accuracy, whereas the standardized coefficient β (slope) values were used to assess which larval traits EBVs (independent variable, x) had greater effects (i.e. expected change) on harvest weight EBV (dependent variable, y). Thus, regressions with greater β and R^2 values were expected to reflect the most useful and reliable larval traits for early progeny testing in barramundi.

5.3. Results

The two batches combined provided a dataset for the genetic analyses comprising 36 and 77 families with five or more offspring evaluated at the hatchery and at the farms, respectively. In each batch, larval and adult families were connected by at least one parent, as the animal model makes use of information of all types of relationships within complex, unbalanced pedigrees (Wilson et al., 2010). A greater number of families were identified at harvest on the farms due to greater sampling and genotyping efforts. In all, 850 larvae were measured for *Ls*, *c.* 400 larvae were measured for cellular biochemical traits and *c.* 2000 young males were

measured at harvest. Sample size and summary statistics for observed phenotypic data input in uni and bivariate models are found in Table 5.2. Batch 2 larvae attained a larger size at 18 dph than those in batch 1, which reflected greater measurements of total DNA, RNA and protein observed in the second batch. In both batches, coefficients of variation (CV) of larval biochemical contents were greater than CV of other (morphological or ratio) traits, indicating large individual variability of larval RNA, DNA and protein contents. Despite the absolute differences in larval size and biochemical content between the two batches, the individual variability of RNA/DNA, Prot/DNA and Cells dividing were similar between batch 1 and batch 2 (Table 5.2).

5.3.1. Heritability of larval traits

All barramundi larval traits measured at 18 dph were heritable ($P < 0.001$). Additive genetic effects accounted for a high proportion of the phenotypic variance of larval RNA/DNA, Prot/DNA and proportion of cells dividing during early development ($0.47 < h^2 < 0.51$), a moderate to high proportion of the phenotypic variance of total nucleic acid content ($0.28 < h^2 < 0.36$) and a low to moderate proportion of the phenotypic variance of larval size and total protein ($h^2 \sim 0.20$) (Table 5.3). Therefore, parental and family origin did influence the variability observed in larval size and to a greater extent to larval biochemical traits and cellular processes associated with larval growth. The non-genetic maternal effects were non-significant ($P > 0.05$), indicating that the effects of any initial nutritional advantages provisioned by the dams (e.g. yolk quality and quantity) were short lived, with the effects dissipated by the time larvae reached 18 dph. Accordingly, non-genetic maternal effects were therefore excluded from the models.

Table 5.2. *Lates calcarifer* larval size and biochemical traits and adult harvest weight (observed mean ± s.e. and coefficient of variation, CV). N = number of observations. *Ls* = standard length, DNA = total larval DNA, RNA = total larval RNA Prot = total larval protein, RNA/DNA = RNA:DNA ratio, Prot/DNA = Protein/DNA ratio, Cells dividing = % of nuclei in the S and G2/M phases of the cell cycle, Weight = wet weight at harvest, dph = days post hatch.

		All data			Batch 1			Batch 2		
Trait	dph	N	mean ± s.e.	CV	N	mean ± s.e.	CV	N	mean ± s.e.	CV
Ls (mm)	18	850	8.08 ± 0.07	25.3	387	7.17 ± 0.06	16.5	463	8.86 ± 0.10	24.3
DNA (µg/larva)	18	395	10.34 ± 0.36	69.2	160	5.12 ± 0.18	44.5	235	13.89 ± 0.47	51.9
RNA (µg/larva)	18	394	26.08 ± 1.06	80.7	160	12.5 ± 0.56	56.7	234	35.38 ± 1.46	63.1
Prot (µg/larva)	18	435	869.4 ± 35.0	84.0	160	389.8 ± 14.4	46.7	234	1281.3 ± 50.0	59.7
RNA/DNA	18	383	2.39 ± 0.3	24.6	160	2.41 ± 0.05	26.2	223	2.37 ± 0.04	25.2
Prot/DNA	18	389	81.4 ± 1.25	30.3	159	71.4 ± 1.6	28.3	230	88.3 ± 1.6	27.5
Cells dividing (%)	18	374	19.2 ± 0.3	30.2	136	19.2 ± 0.4	24.3	238	19.2 ± 0.3	24.1
Weight (g)	273-469	1951	533.8 ± 4.0	33.1	275[*1]	785.6 ± 13.3	28.1	1103[*3]	507.9 ± 3.4	22.2
					403[*2]	485.0 ± 6.2	25.7	172[*4]	397.8 ± 7.7	25.4
Ls (mm)	273-469	1951	284.9 ± 0.6	9.3	275[*1]	308.8 ± 1.5	8.1	1103[*3]	279.2 ± 0.6	7.1
					403[*2]	295.0 ± 1.3	8.8	172[*4]	256.9 ± 1.6	8.2

[*] Age at harvest/rearing conditions were: [1] 343 dph/tank; [2] 469 dph/pond; [3] 273 dph/tank A; [4] 273 dph/tank B.

Table 5.3. Heritability estimates (h^2 ± s.e.) of *Lates calcarifer* larval traits and significance of batch as fixed effect in the model. *Ls* = standard length, DNA = total larval DNA, RNA = total larval RNA Prot = total larval protein, RNA/DNA = RNA:DNA ratio, Prot/DNA = Protein/DNA ratio, Cells dividing = % of nuclei in the S and G_2/M phases of the cell cycle.

Larval trait	h^2 ± s.e.
Ls	0.20 ± 0.09 [b]
Prot	0.19 ± 0.09 [b]
DNA	0.28 ± 0.11 [b]
RNA	0.36 ± 0.13 [b]
RNA/DNA	0.51 ± 0.15
Prot/DNA	0.49 ± 0.15
Cells dividing	0.47 ± 0.14

[b] significant batch effects ($P < 0.001$)

5.3.2. Genetic correlations between larval traits and size at harvest

All genetic correlations between traits across the two different life stages were significant and positive, except between larval Prot/DNA and adult size, which was not significantly different from zero (Table 5.4). Similar correlations were found between larval traits and either adult *Ls* or W. In particular, high genetic correlations were found for larval total RNA, RNA/DNA, the proportion of cells dividing and that of harvest size ($0.81 \leq r_g \leq 0.88$; $P < 0.001$). Also of importance, moderate genetic correlations were found for larval *Ls*, total DNA and total protein and harvest size ($0.60 \leq r_g \leq 0.66$; $P < 0.01$). Highly significant genetic correlations between larval and harvest traits indicated that barramundi families with larger larvae and larvae possessing greater metabolic capacities were also more likely to be heavier and longer at harvest than families with smaller larvae and with lower metabolic capacities.

Table 5.4. Genetic correlations (r_g ± s.e.) between *Lates calcarifer* larval traits at 18 days post hatch (dph) and adult weight (W) and standard length (*Ls*) at harvest (273-469 dph) and significance of fixed effects of batch, farm and tank in the model. *Ls* = standard length, DNA = total larval DNA, RNA = total larval RNA Prot = total larval protein, R/D = RNA:DNA ratio, Prot/D = Protein/DNA ratio, Cells dividing = % of nuclei in the S and G_2/M phases of the cell cycle.

	Genetic correlations (r_g)	
	Harvest traits	
	W	Ls
Larval traits		
Ls	0.65 ± 0.20 **	0.65 ± 0.20 **
DNA	0.60 ± 0.22 **	0.67 ± 0.21 **
RNA	0.83 ± 0.15 ***	0.86 ± 0.14 ***
Prot	0.63 ± 0.22 **	0.64 ± 0.21 **
RNA/DNA	0.88 ± 0.10 ***	0.84 ± 0.11 ***
Prot/DNA	0.15 ± 0.25 *ns*	0.22 ± 0.24 *ns*
Cells dividing	0.81 ± 0.12 ***	0.81 ± 0.12 ***

*** $P < 0.001$; ** $P < 0.01$; *ns* = not significant.

5.3.3. Association between broodstock EBVs for larval and adult traits

As expected from positive genetically correlated traits, linear regression statistics revealed that parental harvest weight EBVs could be predicted from larval trait EBVs. Broodstock harvest weight EBVs tended to be positive (or negative) when larval traits EBVs were positive (or negative), that is, broodstock EBVs were mainly distributed in quadrants I (+, +) and III (-, -) of the plots (Figure 5.1). In particular, larval RNA, RNA/DNA and proportion of cells dividing EBVs most influenced harvest weight EBV ($0.71 < \beta < 0.74$; $P < 0.001$), explaining more than half of the variability seen in harvest weight EBV ($0.51 < R^2 < 0.55$). Also of statistical significance, in decreasing order of predictive values and model fit accuracies were the traits larval DNA ($\beta = 0.65$; $R^2 = 0.42$; $P < 0.001$), larval *Ls* ($\beta = 0.54$; $R^2 = 0.29$; $P < 0.05$) and larval Prot ($\beta = 0.50$; $R^2 = 0.25$; $P < 0.05$) (Figure 5.1). The range of *L.*

calcarifer broodstock EBVs and accuracies of estimates (s.e.) for each trait are provided in Table 5.5.

Table 5.5. Mean and range (maximum and minimum values) of *Lates calcarifer* broodstock estimated breeding values (EBV) and standard errors (s.e.) based on progeny testing (calculated on raw data and expressed on original units). *Ls* = standard length, DNA = total larval DNA, RNA = total larval RNA Prot = total larval protein, RNA/DNA = RNA:DNA ratio, Prot/DNA = Protein/DNA ratio, Cells dividing = % of nuclei in the S and G2/M phases of the cell cycle, Weight = wet weight at harvest, dph = days post hatch.

		Broodstock EBV ± s.e.	
Offspring trait/age	dph	means	range
Ls (mm)	18	0.00 ± 0.48	(-0.72 / 1.04 ± 0.35 / 0.61)
DNA (µg/larva)	18	0.00 ± 2.60	(-4.40 / 6.34 ± 1.94 / 3.20)
RNA (µg/larva)	18	0.00 ± 7.08	(-8.75/13.55 ± 5.37 / 8.68)
Prot (µg/larva)	18	0.00 ± 197	(-281 / 338 ± 159 / 228)
RNA/DNA	18	0.00 ± 0.37	(-0.62 / 0.76 ± 0.24 / 0.50)
Prot/DNA	18	0.00 ± 13.5	(-28.5 / 30.3 ± 9.0 / 18.2)
Cells dividing (%)	18	0.00 ± 2.48	(-4.43 / 5.21 ± 1.63 / 3.35)
Weight (g)	273-469	0.00 ± 43.5	(-133.9 / 184.5 ± 26.4 / 75.0)
Ls (mm)	273-469	0.00 ± 7.45	(-27.1 / 25.6 ± 4.5 / 13.2)

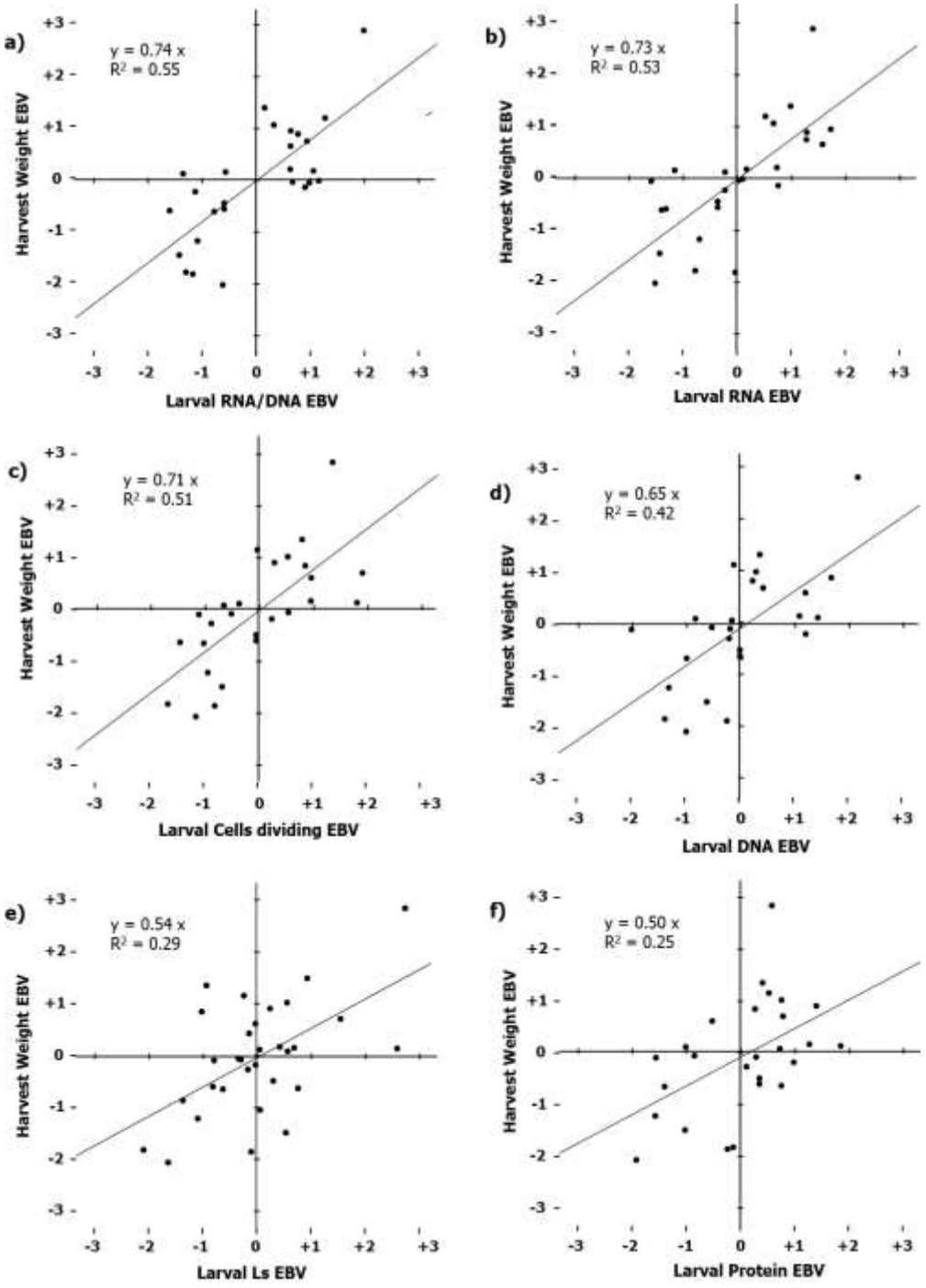

Figure 5.1. Scatterplots of barramundi *Lates calcarifer* broodstock harvest weight estimated breeding value (EBV) (y-axis) against larval traits EBV (x-axes) (a) larval RNA/DNA = RNA:DNA ratio, (b) larval RNA = total larval RNA, (c) larval Cells dividing = % of nuclei in the S and G_2/M phases of the cell cycle, (d) larval DNA = total larval DNA, (e) Larval *Ls* = standard length, and (f) larval Prot = total larval protein. For comparative purposes EBVs were z-scored standardized.

5.4. Discussion

Results showed that growth-related genetic differences among unimproved barramundi broodstock could be determined by measuring larval indicator traits positively correlated to harvest weight. Therefore fish farmers working with highly fecund, long lived multiple spawners, such as barramundi, could take advantage of early progeny testing and pre-screen their broodstock based on measurement of larval biochemical/molecular traits and only retain those for subsequent breeding with high predicted EBV. Productivity and more sustainable allocation of farming resources could be promptly increased by rearing offspring from superior families in subsequent spawning events. In addition, early progeny testing could further increase the genetic gains if incorporated into traditional family based breeding programs (Macbeth and Palmer, 2011), for instance as a tool to expedite the establishment of superior foundation broodstock. The ability to farm genetically superior, fast growing fish is a critical factor to the productivity, profitability and sustainability of aquaculture operations (Gjedrem et al., 2012). As improved stocks are currently limited to a few species for which production accounts for less than 10% of global aquaculture (Gjedrem et al., 2012), early progeny testing might assist industries working with unimproved species.

Early progeny testing as performed in the present study is relatively simple and rapid to implement (i.e. in barramundi around ten weeks from spawning), provided genetic resources such as microsatellites markers are available for reconstruction of familial pedigrees. Measurements of larval cellular and biochemical traits are affordable (i.e. cost of chemicals < US$ 2 trait/sample); however, like genotyping, there is a need of specialized molecular genetics laboratory and personnel. Heritability is a primary requisite for the trait to be useful as an indicator trait predictive of genetically determined growth, from which parental EBV can be calculated. Here, all seven barramundi growth-related larval traits presented relatively

moderate heritabilities at 18 dph, with h^2 values within the range also found for other larval teleosts (Table 5.1). A second important requisite is that the larval trait must be genetically correlated with the trait of commercial interest, here harvest size (W and *Ls*) was chosen, as it is usually the primary trait of economic importance. Further investigation revealed that six larval traits were moderately to highly genetically correlated with harvest size and therefore potential good predictors of long-term growth. Scatterplots (Figure 5.1) of broodstock EBV assessed at the two sampling periods illustrated that if broodstock were indirectly selected for high larval trait EBVs (e.g. individuals with larval EBVs > +1) and bred in a subsequent spawn, the following progeny cohort would likely be on average heavier at harvest than progeny from the previous batch where all broodstock participated. Alternatively, low ranked broodstock (e.g. individuals with larval EBVs < -1) could be replaced by new progeny tested broodstock before further farming costs were realized. In theory, the expected response to selection in harvest weight is given by the regression on harvest weight EBV on larval trait EBV (Falconer and Mackay, 1996). Therefore, if a hatchery manager were to breed his 20% top ranked broodstock based on progeny larval trait EBVs, the following progeny cohort would be 5.7% (Prot EBV) to 11.4% (RNA/DNA EBV) heavier at harvest, compared to 12% heavier if selection were based on progeny harvest weight EBV, however, assessed one year later. As expected, higher R^2 and β values were obtained for larval traits with higher genetic correlations with harvest weight, reflecting the most useful predictive traits. Scatterplots were primarily drawn here to illustrate how the properties of genetic correlated traits can be exploited as an early progeny testing tool. It is important to stress that progeny testing will achieve higher selection accuracies (and thus have a greater impact on productivity) when the heritability of the trait and number of progeny tested from multiple matings is high (e.g. accuracy ~ 80% with four mates, full-sib family size of ten and h^2 = 0.30) (Bourdon 2000; Gjerde, 2005). An interesting direction for future research would be to determine the rate of

genetic progress and realized genetic parameters for early progeny testing schemes based on selection experiments.

Larval metabolic traits were more sensitive and better able to predict future growth trajectories than larval size. This is probably because larval RNA synthesis and cellular division rates are fundamental biological processes underlying the phenotypic expression of morphological traits, such as larval and adult size. In addition, such metabolic traits had the highest heritabilities in barramundi larvae. Although the exact genetic mechanisms regulating the development of muscle fibers in teleosts has not been fully elucidated, changes in the number of myogenic progenitor cells (i.e. cells responsible for post-embryonic growth in teleosts) formed in the embryo were shown to produce persistent effects on growth in adult stages (Johnston, 2006). Most notably, three biochemical and cellular traits indicative of the larval capacity to undergo rapid protein biosynthesis and cellular proliferation (RNA/DNA, total RNA and proportion of cells dividing) were strongly correlated ($r_g > 0.81$; $P < 0.001$) with harvest size. The similar r_g values obtained for these distinct metabolic larval traits is not surprising, as it has been demonstrated that actively dividing cells transverse the S phase of the cell cycle at rates proportional to the quantity of stainable RNA, and cells with high RNA content complete DNA replication five times faster than cells with low RNA content (Darzinkiewicz et al., 1979). Thus, RNA/DNA, total RNA and proportion of cells dividing are in fact distinct measurements of coupled metabolic processes which lead to increases in fish body mass (Theilacker and Shen, 2001). Larval Prot/DNA, however, was not a good predictor trait of growth and therefore would not be suited to discriminate broodstock potential to generate fast growing offspring. “The absence of genetic correlation between larval Prot/DNA and fish harvest weight (and length) might be due to a limited capacity of this trait to reveal significant changes in future growth trajectory of the individual. For

instance, in Atlantic cod subjected to high feeding levels, only about 40% of the protein synthesized was retained as growth and even under starving conditions relatively high basal levels of protein synthesis were observed (Houlihan et al., 1988). Therefore, it is possible that cells may be more resilient to gross changes in their protein content (as evaluated by Prot/DNA) than to changes in their RNA content (as evaluated by RNA/DNA). As such, Prot/DNA would possibly be a less labile and sensitive index to infer or predict the capacity of the organism to grow.

The high genetic correlations between early and late growth-related traits in barramundi are in fact not unexpected and the idea to explore the properties of such genetic correlations for genetic improvement is not new. Wang et al. (2008) suggested that barramundi could be selected at 90 dph due to positive phenotypic correlations between barramundi weight at 90 dph and 289 dph (r = 0.601). However, studies on genetic correlations at very early stages are surprisingly rare and limited to Munch et al. (2005) and Johnson et al. (2011), which reported genetic correlations between broodstock *Ls* and offspring *Ls* at hatch (r_g ~ 0.2). Vandeputte et al. (2002) suggested that selection at much earlier stages could be applied for cultured fish, based on observations that larval progeny of a brown trout strain selected for fast growth had higher developmental rates than those of an unselected control line. However, for genetic gains to be significant high selection pressures on early stages would have to be applied (Vandeputte et al., 2002). Such correlations are in fact well known and explored by farmers through the practice of fish grading and culling of smaller size classes, as practical experience shows that runts seldom "catch up" to achieve economically sustainable growth rates (Paul Harrison, Mainstream Aquaculture Pty. Ltd, *pers. comm.*). The high positive genetic correlations observed here between larval and adult growth traits not only corroborate fish

farmer's empirical knowledge, but also highlight an opportunity for broodstock selection through early progeny testing.

Important family-specific differences in larval metabolic rates and juvenile growth potential had been previously reported in two studies where RNA/DNA of Atlantic cod (*Gadus morhua*) families up to 10 weeks of age was continually monitored (Clemmesen et al., 2003) and growth followed up to nine months (Paulsen et al., 2009). Unfortunately, the additive genetic (co)variances were not decomposed in these studies and the important genetic links between larval RNA/DNA ratios and late growth had never been established before. Contrary to studies on larval metabolic traits, genetic studies on late juvenile and adult morphological traits are not uncommon and tend to report high and positive genetic correlations between closer ages which become progressively lower between more distant ages (e.g. Gjerde et al., 1994; Su et al., 2002; Saillant et al., 2006). The weakening of genetic correlations over time is particularly noticeable between periods of reduced growth such as after the onset of sexual maturation and is generally attributed to mechanisms of compensatory growth (Riska et al., 1984). For some species, like barramundi in this study, correlations are still high after a long rearing period; however, no information is available for older (e.g. 2-3 years) or bigger barramundi (e.g. 2-3 kg). In rainbow trout *Salmo gairdneri*, r_g estimates for weight between 4-8 week old fry and 1-year old yearlings were high ($0.61 \leq r_g \leq 0.92$) (McKay et al., 1986) and moderate between 1-year old presmolts and 3.5 year old Atlantic salmon *Salmo salar* ($r_g = 0.59 \pm 0.34$) (Gjerde et al., 1994). Therefore, factors such as the larval traits of choice and developmental stage, coupled with species-specific growth patterns, later age at sampling, possible behavioural (e.g. size-induced aggressiveness, competition, cannibalism) and puberty effects are important variables likely to affect the strength of genetic correlations

between early and late traits and hence should be carefully considered by the breeder interested in early progeny testing.

In conclusion, high genetic correlations between barramundi larval traits (in particular larval RNA, RNA/DNA and the proportion of cells dividing) and fish size at harvest indicate the feasibility of early progeny testing as a rapid means of evaluation and selection of genetically superior broodstock. When applied it may allow farmers to produce progeny from superior broodstock in the absence of a selection program, or be incorporated into existing selection programs for early evaluation of estimates of family breeding values.

Chapter 6. General discussion

Aquaculture currently plays an important role in human food security, as approximately half of today's available seafood now comes from aquatic farming systems. However, the long-term sustainability of the aquaculture sector critically depends on the development and use of genetically improved stocks (Rye et al., 2010). By growing fish that have been genetically improved with selective breeding over several generations, it has been possible to increase the efficiency of production per unit farm area and per total input resources (feed, labour, etc.), thus reducing wastage and greatly improving the economic value of fish farming (Gjedrem et al., 2012). Nevertheless, aquaculture breeding programs to date have focused on the improvement of a few species, mainly salmonids, tilapia and shrimp and less than 10% of global aquaculture production is based on improved stocks (Neira, 2010; Rye et al., 2010; Gjedrem et al., 2012). Therefore, there is still enormous potential for selective breeding programs to improve commercially important traits, such as growth rate, in many important species such as barramundi.

The Australian barramundi industry has long appreciated the potential for improving the sustainability and profitability of production through the implementation of a selective breeding program (Robinson and Jerry, 2009). However, the existence of gaps in knowledge of key genetic parameters has been considered one serious constraint for development of an appropriate genetic improvement strategy for the species (Robinson and Jerry, 2009). This study aimed not only to bridge this gap to expedite the establishment of a breeding program for the Australian barramundi *Lates calcarifer*, but also to provide the industry with an alternative and more immediate solution to improve production. Nevertheless, due to the

technical complexities involved in successfully running a breeding program, the small-scale nature of Australian barramundi farms, and the need for commitment to a long-term investment plan, it is unlikely that a sustainable barramundi breeding program will be successful in Australia without an industry-wide coordinated effort. Now that several knowledge gaps impeding the instigation of selective breeding programs in barramundi have been filled, the future of selective breeding of the species depends on the industry's desire to consolidate such programs.

6.1 Understanding magnitude and importance of the family-specific genetic diversity of farmed cohorts and the genetic basis of harvest growth traits for the implementation of efficient barramundi breeding programs.

Barramundi is an emergent aquaculture fish species due to its popularity as a food fish, rapid growth rate, and wide environmental tolerances, for which production levels are increasing worldwide. For these reasons, barramundi selective breeding programs are currently being considered in Australia and have recently been implemented in Asia (Yue et al., 2009; Robinson and Jerry, 2009). However, barramundi's protandry and strict mass spawning reproductive biology differs completely from the fish species which have been improved to date. Such reproductive characteristics pose considerable challenges for the attainment of sufficient genetic diversity (Robinson et al., 2010), in addition to the need of molecular parentage analysis for reconstruction of pedigrees (Liu et al., 2012).

At the commencement of this thesis, key knowledge gaps seriously restrained any attempt to properly develop efficient genetic management and improvement strategies for barramundi. The present research increased our understanding on the genetics in several areas relevant to

the implementation of a selective breeding program for barramundi. The knowledge generated from the studies reported in *Chapters 2* and *3* is of utmost importance for the design and implementation of a breeding program for barramundi. Without knowing how many families can be produced under different mass spawning conditions and, most importantly, how many families initially identified at the hatchery in fact survive until the critical moment of selection later in the production cycle, it is impossible to plan optimum mating schemes which will ensure the attainment of sufficient genetic diversity for the next generation to maintain inbreeding rates at acceptable levels and thus allow for the long-term sustainability of a breeding program. Specifically, *Chapter 2* tracked the relative contributions of families from hatchery to harvest for the first time in a mass spawning fish in three commercial batches all the way from the hatchery to harvest and used phenotypic and genetic data to simulate the genotyping effort that would be required to capture family-specific genetic diversity present within cultured populations.

One positive outcome for selective breeding of the species is that changes in relative family frequencies over the culture cycle, also known as differential family survival, were not significant enough to erode family-specific genetic diversity, despite previous studies on early stages suggesting that differential family survival could significantly reduce family-level genetic diversity in this species (Frost et al., 2006). Therefore, it can be concluded that an early census (~ 18 dph) of *Ne* should provide a reliable indication of expected *Ne* at the end of the culture cycle at the moment of selection, and rapidly confirm broodstock reproductive success and expected family numbers available for selection at harvest. In addition, the study conducted in *Chapter 2* is the first for a mass spawning species with skewed family distributions to use real data (as opposed to simulations) to explore the number of fish that would have to be selected to capture family-specific genetic diversity.

The genotyping sampling scheme identified here showed that sampling the top 1.5% of the population, or individuals 2.17 S.D. heavier than the population mean, was required to capture at least 75% of family-specific genetic diversity, and revealed that it would not be cost-effective to genotype more individuals to capture the remaining 25% of families. Due to the observed trade-offs between genotyping effort and the available genetic diversity adoption of the sampling/genotyping scheme reported here could significantly reduce the overall costs of a barramundi breeding program.

Furthermore, results from *Chapter 2* demonstrated that it is possible to boost the genetic diversity of barramundi farmed cohorts by mass spawning a relatively large number of broodstock (33 fish were used) to generate a large number of families (up to 121 families were identified at harvest). Nevertheless, results highlighted that a single spawn, even using large numbers of broodstock, would not be sufficient to reach adequate effective population sizes (N_e) and keep inbreeding rates at acceptable levels. Therefore, it is recommended that each generation developed within a breeding nucleus should be comprised of offspring originated from multiple and diverse spawnings. Moreover, it was consistently shown across three commercial batches of barramundi fingerlings that all available males fathered offspring; whereas females were less consistent. These differences in gender reproductive performance are consistent with those also reported for an Asian strain of barramundi (Liu et al., 2012), indicating that such behaviour under hatchery conditions is likely to be expected for the species. This information may allow hatchery managers of a future breeding nucleus to place greater emphasis on female reproductive conditioning and devise better mating strategies (e.g. manipulate male to female sex ratios) to increase the contribution of a greater diversity of females.

Chapter 3 investigated the genetic basis of barramundi harvest growth traits. This information is important because without knowledge of the magnitude of additive (heritable) genetic components determining barramundi size at harvest, it is impossible to predict if and how selection will impact on increasing the proportion of desirable phenotypes within the population. Therefore, without knowing the heritability of traits to be improved, it would be impossible to accurately inform the supporting industry on the expected outcomes to be achieved by any breeding program, such as the responses to selection, the long-term genetic gains and consequently the return on investment. In addition, without knowing how families perform relative to each other over the culture cycle in multiple environments and, of practical importance, if the offspring from broodstock grown and selected at particular environments (e.g. at a breeding nucleus or a farm with a particular environment) will also display superior performance when grown in different environments (G x E interactions), it is also not possible to assure industry that the benefits of the breeding program will in fact benefit all stakeholders.

The results from *Chapter 3* have encouraging implications for the instigation of a selective breeding program for barramundi. The analyses here demonstrated that the majority of harvest growth traits exhibit high heritability and positive genetic correlations, warranting improvement through selection for increased body weight at harvest. The heritability estimates obtained for harvest weight were consistently high and similar (h^2 W ~ 0.4 for three commercial batches comprising a total of 1953 offspring from 84 families), therefore high genetic gains should be expected. Heritability for harvest growth traits found in *Chapter 3* provide a more robust value for modelling breeding programs for barramundi than the previously available estimates of Wang et al. (2008) for 90 dph juvenile (h^2 W ~ 0.2). In addition, it should be noted that while body shape (H) may also be slightly improved through

indirect selection of heavier fish (r_g between W and H ~ 0.4), the low (and even nil for pond environment) heritability values found for H (h^2 H < 0.1) suggest that fish should not be directly selected on body shape as a primary trait.

Finally, the lack of genotype by environment effects observed under different salinities (e.g. fresh *vs*. sea water) and farming systems (e.g. semi-intensive pond *vs*. intensive tank systems) highlight that realization of family genetic merit is relatively consistent in barramundi when individuals are reared under disparate environmental and culture systems. These results suggest that a single breeding program should be able to improve growth rates for a wide range of farming systems without the need to develop environment-specific selected strains. Although further research investigating the performance of greater number of families and strains from different populations (other than that of the QLD coast) are warranted, the genetic correlations for growth traits across environments were close to unity. These results strongly suggest that barramundi farmers in Australia who utilize different environments and production systems may be confident to invest in and capitalize from a single centralized breeding program.

6.2 Larval cellular and biochemical traits as a fast track method to increase genetic gains and optimize production through early prediction of growth.

Despite the great potential identified for the improvement of growth rates in farmed barramundi, as of the conclusion of this thesis in January 2014, no integrated selective breeding program has yet been established for this species in Australia. Considering that it takes at least a couple of selected generations for the development of superior farmed strains, it is reasonable to foresee that the first positive impacts on production levels generated by the

implementation of a traditional family-based breeding program for barramundi would only be achieved within the time frame of at least a decade. Therefore, until the benefits of a well-coordinated breeding program are disseminated to the industry, farmers have no options to deal with the environmental and economic consequences of farming genetically inferior fish. While the effectiveness and long-term sustainability of selective breeding programs have been demonstrated for many aquatic species, there are currently no "easy and quick" methods to evaluate the innate genetic potential (also referred to as the estimated breeding value, EBV) for fast growth of individual broodstock. Hatchery managers therefore have no means to know which fish amongst their existing broodstock are more likely to generate fast growing offspring. This knowledge would be extremely valuable when selecting foundation breeding program spawners, as differences in harvest weight of up to 75% (359 ± 51 *vs*. 628 ± 37 g) were found herein between the heaviest and the lightest barramundi families (*Chapter 3*). Based on these results, it can be concluded that a significant proportion of the current variability seen in production performance is attributed to the unique (and unknown) genetic makeup of broodstock parenting each commercial batch.

Due to the current lack of alternatives to fast track the genetic improvement of barramundi farmed stocks, the second part of this study (*Chapters 4* and *5*) developed and then explored a relatively simple and rapid identification method to identify barramundi broodstock with superior genes for growth through an early progeny testing approach. This approach was based on the hypothesis that differences in genetically determined growth could be determined at early life stages using cellular-based predictors. As a primary requisite, early progeny testing involved the simultaneous spawning of a number of brood fish to be tested. As is usual practice with barramundi, mass spawning was employed and evaluated. However, wherever the biology of the species allows, artificial fertilization and pooling of eggs (or

newly hatched larvae) from multiple families may be employed. Importantly, larvae must be reared communally to minimize potential environmental effects (e.g. tank or age effects) which could be confounded with true genetic effects. Early progeny testing was then performed just a few weeks after hatching, focused on larval stages. Under the commercial hatchery conditions employed in this study, barramundi larval sampling was standardized at 18 dph. The methodology for early progeny testing *per se* combined for the first time the use of three key techniques. Firstly, each larva was DNA genotyped and parentage analysis conducted to obtain the population pedigree. Secondly, a range of larval traits hypothesized to be associated to the individual's innate growth capacity were measured by employing sensitive quantification techniques. The third and last technique for early progeny testing employed the use of modern quantitative genetics theory (i.e. the use of linear mixed models using restricted maximum likelihood methods) to untangle the additive genetic variance from the larval pedigree and phenotypic data and generate individual broodstock EBV.

The research undertaken throughout this thesis was only possible because of the involvement of two commercial hatcheries, which agreed to condition and induce to spawn an unusually large number of barramundi broodstock (12 to 33 fish). Without mass spawning performed on such a large scale, it would not have been possible to obtain sufficient genetic diversity for the estimation of genetic parameters of growth traits. Morphological, cellular and biochemical larval growth-related traits (i.e. *Ls*, total RNA, DNA and protein, RNA/DNA, Prot/DNA and cells dividing) traditionally used by fish biologists to investigate larval nutritional conditioning and instantaneous growth rates were therefore chosen as potential early predictor traits indicative of long term growth for early progeny testing. However, it was first necessary to devise a robust sample preparation protocol which allowed multiple quantitative analytical procedures to be performed on a single fish larva. In addition, it was

also important to validate the relationship between larval growth rates and the potential predictor trait values not only under stressed conditions (i.e. fasted *vs*. fed larvae), but also under commercial aquaculture rearing conditions, where larvae are communally reared in a nearly optimum nutritional and physiological environment. The protocols developed in *Chapter 4* thus allowed for a range of phenotypic measurements to be obtained from a single individual. These protocols were a fundamental pre-requisite for the estimation of heritabilities and for the assessment of the genetic correlations between larval and adult traits undertaken in *Chapter 5*.

Encouragingly, a moderate to large proportion of the phenotypic variance observed in larval traits was attributed to additive genetic effects (i.e. moderate to high h^2), which indicated that larval genetic makeup and family of origin have strong influences on larval growth capacity. However, to validate if such parental influences seen in larval stages persist over the culture period until harvest, it was necessary to assess the significance and strength of genetic correlations between larval and harvest traits. In this sense, the study undertaken in *Chapter 5* is unique as it is the first to identify and report positive and highly significant genetic correlations ($0.60 \leq r_g \leq 0.88$) between a range of larval and adult traits in an aquatic organism. To our knowledge, the magnitude of genetic correlations obtained between some of the early and late growth traits are the highest reported in the animal breeding literature (e.g. r_g between larval RNA/DNA and fish harvest weight = 0.88 ± 0.10). Of practical importance, results obtained in *Chapter 5* validated the usefulness of early progeny testing as a novel tool to rapidly identify broodstock with superior growth potential and fast track the improvement of aquaculture production. High genetic correlations between early and late growth traits imply high correlations between individual EBVs (Falconer and Mackay, 1996). Therefore, in accordance to quantitative genetics theory, it is expected that if broodstock were

indirectly selected for high larval trait EBV and bred in a subsequent spawning cycle, the following progeny cohort would likely be on average heavier at harvest than progeny from the previous batch where all broodstock participated. For barramundi farmers holding large numbers of broodstock, and possibly other fish farmers working with long lived multiple spawning species, larval testing may allow a rapid means of ranking fish in accordance to their EBV for growth. Such larval testing combined with subsequent breeding of best EBV ranked broodstock should lead to immediate increases in productivity and profitability of farming operations without the need to wait for a selective breeding nucleus to be established.

The high genetic correlations between early and late life stages found in *Chapter 5* may have significant implications in several areas of knowledge. As previously mentioned, it may assist farmers to quickly identify superior brood fish through early progeny testing or selection at very early stages, for the improvement of productivity and achievement of more sustainable crops. For fisheries sciences and management, such high genetic correlations imply that size selective harvest may have a greater impact on larval development and recruitment than previously thought, as recently suggested by Johnson et al. (2011). The removal of bigger or faster growing fish from natural populations may not only result in cohorts of smaller larvae and/or increase period fish stays at the most vulnerable (larval) stages, affecting population replenishment, but also possibly reduce overall population size at age and biomass. Finally, for ecology and evolutionary studies, results may contribute to deepen our understanding of how distinct traits seen in different ontogenetic stages may co-evolve. Genetic correlations between larval and adult traits imply that selection upon a unique trait during a specific life stage may indirectly impact on the evolution of another trait expressed during a distinct life stage (e.g. larval RNA and adult size). As the genetic links between different ontogenetic

stages have rarely been reported for aquatic organisms (Marshall and Morgan, 2011), genetic correlations found in *Chapter 5* may encourage further studies to investigate trait evolution.

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