



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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ABSTRACT

In the establishment of selection programs where foundation broodstock are taken from the wild, or from un-evaluated stocks, there is no prior way to ascertain their genetic merit for growth rate. However, for highly fecund multiple spawning species, selection of broodstock founders could be made more efficient if their estimated breeding values (EBVs) could be determined through progeny testing early in the production cycle. Early progeny testing could allow farmers working with unimproved species to immediately re-spawn best EBV ranked broodstock for stocking grow-out systems, thereby avoiding costs associated with rearing slow-growing families. In this study, we quantified the additive genetic (co)variation of barramundi, *Lates calcarifer*, larval traits which could reveal their parents innate genetic capacity for fast growth. Specifically, we investigated the heritability (h^2) of cellular, biochemical and morphological larval traits (total RNA, total DNA, total protein, RNA/DNA, protein/DNA, the proportion of cells dividing and standard length, L_s) and their genetic correlations (r_g) with two morphological traits at harvest indicative of long-term growth (L_s and wet weight, W). Here, two cohorts originating from partial factorial crosses between 11 dams and 26 sires were sampled at 18 days post hatch (dph) in the hatchery then later at 273–469 dph at harvest. Pedigrees were reconstructed through microsatellite based parentage analyses and genetic parameters estimated through animal models via REML. All larval traits were heritable at 18 dph ($0.19 < h^2 < 0.51$), indicating that their expression is under additive genetic control and therefore that they could have predictive power to estimate parental EBV. This was confirmed by positive and significant r_g for all larval traits (except protein/DNA) and that of fish harvest size ($r_g > 0.60$, $P < 0.01$). In particular, high r_g were found between larval cellular and biochemical traits RNA/DNA, total RNA and the proportion of cells dividing ($0.81 < r_g < 0.88$, $P < 0.001$), indicating that larval families with higher metabolic rates also grew to be the larger and heavier families at harvest. Results showed that genetic differences in growth traits among barramundi broodstock could be determined shortly after spawning by measuring larval indicator traits predictive of long-term genetically determined growth. These larval predictive traits may allow fish breeders working with highly fecund multiple spawners like barramundi to explore the advantages of early progeny testing to expedite the establishment of superior foundation broodstock in breeding programs.

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1. Introduction

Productivity of fish farming is intrinsically linked to survival and growth performance. Fast growing strains developed by aquaculture breeding programs utilize farming input resources like feeds more efficiently and overall significantly reduce production time and costs (Gjedrem et al., 2012). However, the majority of world aquaculture production is based on unimproved stocks, which exhibit high levels of variability in growth and survival among batches. In the establishment of foundation broodstock for hatchery populations or breeding nucleus

of selection programs, there is currently no prior way to ascertain the animal's 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 only realized 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

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evaluation and selection of breeders through the performance of their offspring is the driver of several genetic improvement programs (Norman et al., 2001). For example, progeny testing is the primary source of genetic gain within the Australian dairy industry (ADHS, 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 (Fjalestad, 2005; Gjedrem, 1983). 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 (Butts and Litvak, 2007; Eilertsen et al., 2009; Green and McCormick, 2005; Núñez et al., 2011; Ottesen and Babiak, 2007; Probst et al., 2006; Saillant et al., 2001; Trippel et al., 2005) and reported that heritabilities of diverse traits measured very early in life (e.g. Ma

et al., 2008; Páez et al., 2010; Shimada et al., 2007; Table S1) 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 (Munch et al., 2005; Vandeputte et al., 2002) and wild (Johnson et al., 2010, 2011) fish populations, although genetic links between different ontogenetic stages among marine organisms still remain largely unknown (Marshall and Morgan, 2011).

In fisheries sciences, larval size is undoubtedly the simplest and most studied of all early life history traits (Miller et al., 1988). Larval size, however, may not necessarily be the most predictive trait to disclose long-term determined genetic growth. In fact there is a range of more informative and measurable cellular and biochemical larval traits underlying biological mechanisms driving an organism's increase in body size and which ultimately lead to the expression of observable morphometric characters, such as length and weight. Larval fish increase their body mass through increases in protein biosynthesis (Caldarone, 2005; Houlihan et al., 1988), with the level of protein synthesis largely determined by the availability and transcriptional regulation of RNA (Elser et al., 2000; Henshaw et al., 1971). 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). 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.

The development of sensitive fluorometric and cytometric techniques has allowed fish biologists to measure the rate of such cellular metabolic processes early on in development. In particular, 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 Chicharo and Chicharo, 2008; Ferron and Leggett, 1994; Perez-Dominguez and Dahm, 2011). More recently, flow cytometric (FCM) cell cycle analyses have also proved to be a powerful tool to directly assess growth of fish larvae by quantifying the relative proportion of cells which are actively dividing, i.e. cells found in the S (DNA synthesis phase) and G₂-M (gap 2 and mitosis) replicative phases of the cell cycle (Bromhead et al., 2000; Domingos et al., 2012; González-Quirós et al., 2007; Porter and Bailey, 2011; Theilacker and Shen, 2001). To date, all of 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 the potential for a correlated genetic response with long-term growth performance. Based on significant parental effects (Høie et al., 1999) and moderate heritability (Bang et al., 2006) found for Atlantic herring (*Clupea harengus*) larval RNA/DNA at hatch ($h^2 = 0.31$), study authors have 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 (Bang et al., 2006; Høie et al., 1999). 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 nutrient availability is not a limiting factor. 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 (Wang et al., 2011; Xia et al., 2010; 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, Domingos et al., 2013). 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 (L_s), 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 L_s 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.

2. Materials and methods

2.1. Experimental fish

Two commercially cultured barramundi cohorts (referred to as batches 1 and 2 hereafter) were produced through mass spawnings at two different hatcheries located in Queensland, Australia. Details on barramundi broodstock sampling, spawning induction, egg collection and larval and juvenile rearing have been previously described (Domingos et al., 2013, experiments 1 and 2). Briefly, there were 12 dams and 21 sires in batch 1 and six dams and six sires in batch 2 present in the tank at spawning, all of which were fin clipped (~0.5 cm²) for later DNA parentage analyses (Section 2.4). Upon hatching, larvae were sent to a third hatchery for culture following intensive clear water finfish larviculture protocols (Schipp et al., 2007) until 26 days post hatch (dph), when fish were sent for grow out in two commercial farms, an intensive indoor recirculating facility in Victoria, Australia (referred to as Tank; fish from both batches 1 and 2) and a semi-intensive grow out pond in Queensland (referred to as Pond; only fish from batch 2). Salinity during spawning, hatching and larval culture was 30 ppt until 18 dph and progressive lowered to 2 ppt until 26 dph. In both farms, fingerlings were acclimated to fresh water rearing conditions. Fish husbandry followed commercial protocols at the discretion of each farm's individual management. Fish farmed in Tank were subjected to periodic grading into different size classes to avoid cannibalism until an average weight of 250 g, and smaller size classes were eventually culled, although the exact grading and culling schedule were not disclosed. Offspring sampling took place at 18 dph in the hatchery (larvae) and then at 273–469 dph in the farms prior to harvest. All harvested fish were of a size consistent with young adult males, as barramundi are protandrous hermaphrodites.

2.2. Larval sampling at hatchery

Preliminary trials indicated that 18 dph prior to juvenile metamorphosis was a suitable age for sampling, as at this age barramundi larvae have enough body mass for the subsequent series of cellular and molecular analyses described below and had not begun to cannibalize which may have introduced an adverse environmental component to measurement of larval trait (Domingos, J.A., unpublished data). A total of 463 (batch 1) and 387 (batch 2) 18 dph larvae were sampled in the hatchery before the first daily feed so that gut contents did not interfere with posterior analyses. Sampled larvae were transported live to the 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 Corporation). Larval L_s measurements were made *a posteriori* with Image-J 1.43 software (National Institutes of Health) from photographs. Larvae were then fin clipped (~2 mm²) for DNA parentage analyses and the fin clip and remaining larvae placed into separate 48 well microtiter plates covered with strip caps (Axygen). After fin-clipping the larvae were immediately frozen by placing plates afloat onto liquid nitrogen for 30 min before being stored at –80 °C (batch 1), or were preserved in 150 µl of RNAlater (Ambion) per well and kept at 2 °C overnight before being stored at –80 °C (batch 2). Plates containing fin clips were immediately processed for DNA parentage analyses.

2.3. Fish sampling at the farms

Fish age at harvest varied between batches and farms. In batch 1 fish age was 273 dph from tanks and in batch 2 fish age was 345 dph from a single tank and 469 dph from the pond. A total of 1428 fish were sampled from batch 1 (1230 from tank A and 198 from tank B) and 732 fish were sampled from batch 2 (301 from one tank and 431 from the pond). Sampling consisted of placing fish anesthetized with AQUI-S (Aqui-S New Zealand) onto an electronic scale positioned below a camera stand. Fish wet weight (W) was recorded to the nearest 1 g and a photograph taken next to a ruler for later image measurements of L_s , as described for larvae. A fin clip was taken and preserved in 1.2 ml of DMSO-salt solution (20% DMSO, 0.25 M disodium-EDTA and NaCl to saturation at pH 8; Seutin et al., 1991) for DNA parentage analysis.

2.4. Identification of families

Barramundi families present in the hatchery and at harvest were identified through retrospective DNA parentage analysis. In batch 1, two multiplex suites containing 8 and 9 microsatellite markers were used due to greater numbers of broodstock in the spawning tank and greater possibility of family combinations. In batch 2 only one multiplex suite of 10 microsatellite markers was sufficiently powerful to reconstruct pedigrees (Zhu et al., 2010). Detailed methodology of DNA extraction, PCR reagents and cycling conditions, microsatellite multiplex combinations, genotyping, parentage assignment and quality controls is described elsewhere (Domingos et al., 2013). To increase statistical confidence for the quantitative genetic analyses (Section 2.6), only larvae belonging to families containing five or more assigned individuals were subjected to biochemical and cellular analyses (Section 2.5). 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. Differential family survival was observed from 18 dph until harvest; however, there was no major family drop out and genetic diversity was retained within batches over the production cycle (Domingos et al., 2014).

2.5. Larval cell cycle analysis and quantification of total RNA, DNA and protein

Individual whole larval homogenates were used for quantification of total RNA, DNA, protein and FCM cycle analysis. The FCM protocol employed in this study for estimating the proportion of cells in the S and G₂-M phases of the cell cycle, hereafter referred to as the proportion of cells dividing, has been described in detail in Domingos et al. (2012). Briefly, larvae were defrosted on ice and cut into five to seven pieces and hand homogenized with a Tenbroek tissue grinder (Kimble-Chase) in ice-cold sucrose-citrate buffer (SCB: 250 mM sucrose, 40 mM trisodium citrate dihydrate, 5% dimethyl sulphoxide, pH 7.6; Vindeløv et al., 1983). The volume of sucrose-citrate buffer (µm) per larvae, critical for the recovery of clean nuclei and reproducible FCM results, was standardized to larval size (mm) (SCB vol. = 2.8 Ls³; Domingos et al., 2012). Nuclei DNA was stained by mixing 100 µl of larval homogenate to 900 µl of ice cold propidium iodide (PI) solution (3.4 mM trisodium citrate dihydrate, 9.65 mM NaCl, 0.03% IGEPAAL CA-630, 0.015 mM PI; Ormerod, 2000). Data from a minimum of 8000 nuclei per larva were collected in a BD LSRFortessa Cell Analyser (BD Biosciences) and DNA content histograms deconvoluted with FlowJo software (Tree Star) cell cycle platform.

Following larval homogenization, total RNA and total DNA were immediately quantified in separate microplates using two nucleic acid fluorescence-specific assay kits, Quant-iTTM RNA and Quant-iTTM 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-iTTM 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 previous trials confirmed the reliability and reproducibility of the method, as suggested by Caldarone et al. (2001). These trials intended to determine (i) the ratio between the volume of larval homogenate to Quant-iTTM working solutions so that fluorescence readings were set in the linear range of the respective RNA and DNA standard curves; (ii) the coefficient of variation (c.v.) 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) the absence of homogenate auto-fluorescence (i.e. homogenate fluorescence was not detected when no fluorophore was added to the Quant-iTTM working solution).

Quantification of individual larval total protein was also determined in a 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 VersaMaxTM ELISA microplate reader (Molecular Devices).

Further quantitative genetic analyses on larval traits (see Section 2.6) included larval size and crude larval biochemical constituents (i.e. total RNA, DNA and protein), and also the ratios between RNA/DNA and Prot/DNA, as they provide a more standardized metabolic index among individuals (Ferron and Leggett, 1994).

2.6. 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). 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 1) 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 1 and batch 2), 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 (Eq. (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 $\sigma_a^2/(\sigma_a^2 + \sigma_e^2)$ where σ_a^2 and σ_e^2 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 $r_g =$

$\sigma_{a1a2}/\sqrt{(\sigma_{a1}^2)}\sqrt{(\sigma_{a2}^2)}$, 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 trait 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.

3. Results

The two batches combined provided a dataset for the genetic analyses comprising 36 families evaluated at the hatchery (25 in batch 1 and 11 in batch 2) and 77 families evaluated at the farms (66 in batch 1 and 11 in batch 2), respectively. Families with less than five assigned offspring were not evaluated. 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 L_s , 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 1. 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 1).

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 the 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 2). 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

Table 2

Heritability estimates ($h^2 \pm$ s.e.) of *Lates calcarifer* larval traits and significance of batch as fixed effect in the model. L_s = 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.

Larval trait	$h^2 \pm$ s.e.
L_s	0.20 ± 0.09^a
Prot	0.19 ± 0.09^a
DNA	0.28 ± 0.11^a
RNA	0.36 ± 0.13^a
RNA/DNA	0.51 ± 0.15
Prot/DNA	0.49 ± 0.15
Cells dividing	0.47 ± 0.14

^a Significant batch effects ($P < 0.001$).

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.

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 3). Similar correlations were found between larval traits and either adult L_s 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 ($0.81 < r_g < 0.88$; $P < 0.001$). Also of importance, moderate genetic correlations were found for larval L_s , total DNA and total protein and harvest size ($0.60 < r_g < 0.66$; $P < 0.01$). 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.

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 trait EBVs were positive (or negative), that is, broodstock EBVs (Z-scored for comparison purposes) were mainly distributed in quadrants I (+, +) and III (–, –) of the plots (Fig. 1). In particular, larval RNA, RNA/DNA and the

Table 1
Lates calcarifer larval size and biochemical traits and adult harvest weight (observed mean \pm s.e. and coefficient of variation, CV). N = number of observations. L_s = 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.

Trait	dph	All data			Batch 1			Batch 2		
		N	Mean \pm s.e.	CV	N	Mean \pm s.e.	CV	N	Mean \pm s.e.	CV
L_s (mm)	18	850	8.08 ± 0.07	25.3	387	7.17 ± 0.06	16.5	463	8.86 ± 0.10	24.3
DNA ($\mu\text{g}/\text{larva}$)	18	395	10.34 ± 0.36	69.2	160	5.12 ± 0.18	44.5	235	13.89 ± 0.47	51.9
RNA ($\mu\text{g}/\text{larva}$)	18	394	26.08 ± 1.06	80.7	160	12.5 ± 0.56	56.7	234	35.38 ± 1.46	63.1
Prot ($\mu\text{g}/\text{larva}$)	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
L_s (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 3

Genetic correlations ($r_g \pm$ s.e.) between *Lates calcarifer* larval traits at 18 days post hatch (dph) and adult weight (W) and standard length (L_s) at harvest (273–469 dph) and significance of fixed effects of batch, farm and tank in the model. L_s = 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 G2/M phases of the cell cycle.

Larval traits	Genetic correlations (r_g)	
	Harvest traits W	L_s
L_s	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 <i>ns</i>	0.22 ± 0.24 <i>ns</i>
Cells dividing	0.81 ± 0.12***	0.81 ± 0.12***

ns = not significant.

*** $P < 0.001$.

** $P < 0.01$.

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 a 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 L_s ($\beta = 0.54$; $R^2 = 0.29$; $P < 0.05$) and larval Prot ($\beta = 0.50$; $R^2 = 0.25$; $P < 0.05$) (Fig. 1). The range of *L. calcarifer* broodstock EBVs and accuracies of estimates (s.e.) for each trait are provided in Table 2S.

4. Discussion

Results show 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 microsatellite 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 a 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 S1). A second important requisite is that the larval trait must be genetically correlated with the trait of commercial interest, here harvest size (W and L_s) 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 (Fig. 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 have 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 the 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 (Darzynkiewicz et al., 1979). Thus, total larval RNA and RNA/DNA (standardized RNA index), and the 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).

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), who reported genetic correlations between broodstock L_s and offspring L_s at hatch ($r_g \sim 0.2$). 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

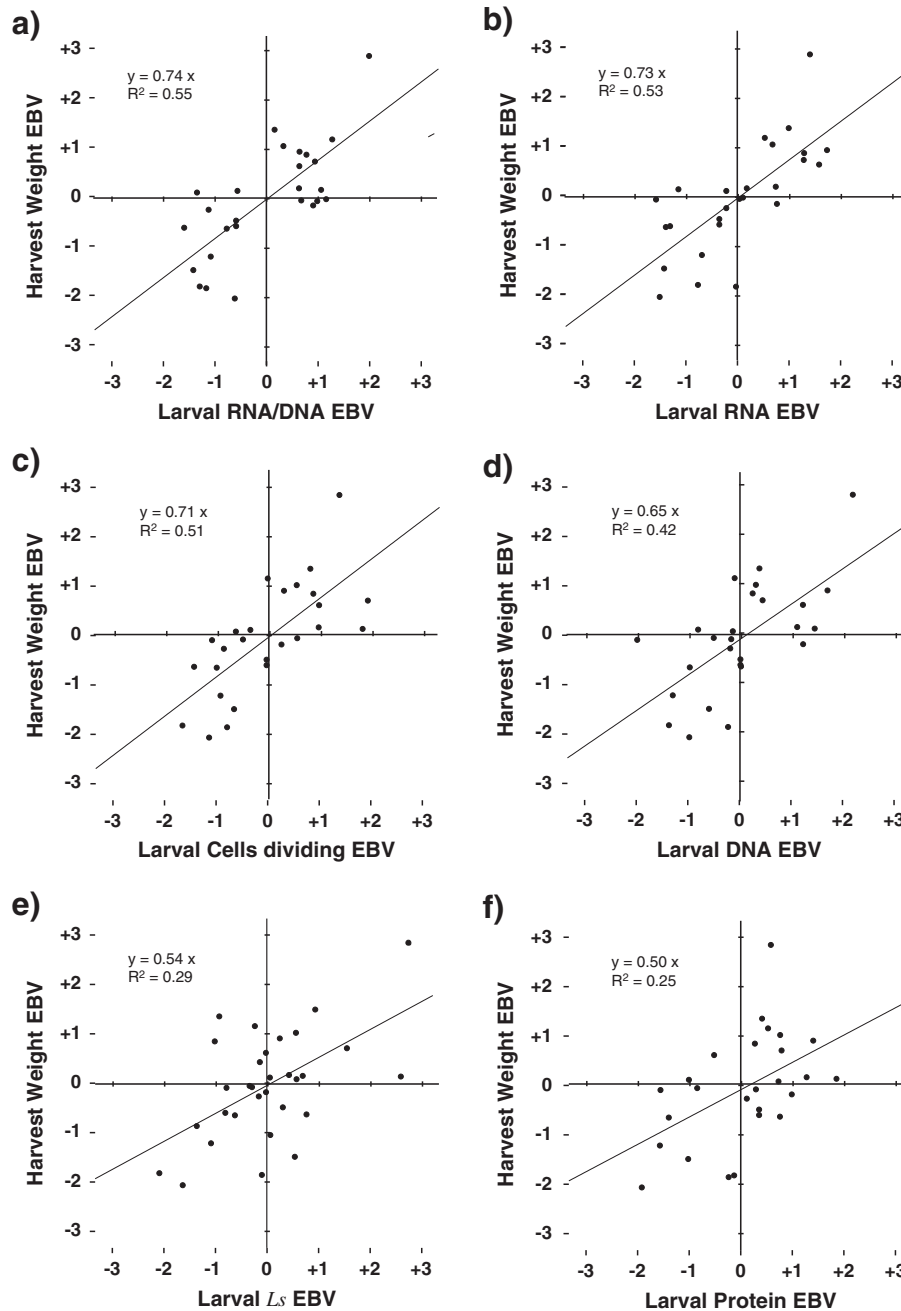


Fig. 1. Scatterplots of barramundi *Lates calcarifer* broodstock harvest weight estimate breeding value (EBV) (y-axis) against larval trait EBV (x-axes) (a) larval RNA/DNA = RNA:DNA ratio, (b) larval RNA = total larval RNA, (c) larval cells dividing = % of nuclei in the S and G2/M phases of the cell cycle, (d) larval DNA = total larval DNA, (e) larval L_s = standard length, and (f) larval Prot = total larval protein. For comparative purposes EBVs were z-score standardized.

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; Saillant et al., 2006; Su et al., 2002). 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- and 8-week old fry and 1-year old yearlings were high ($0.61 < r_g < 0.92$) (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 and possible behavioral (e.g. size-induced aggressiveness, competition, cannibalism) and puberty effects (e.g. growth energy diverted into gonadal maturation) 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.aquaculture.2014.02.037>.

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