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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*

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ABSTRACT

Mass spawning fish species pose significant challenges for the design and conduct of effective aquaculture breeding programs. This is because the hatchery manager has limited control over the individual contributions of broodstock within a spawn, and consequently, the number and size of families in the resulting progeny cohorts. While early broodstock contributions have been tracked in many mass-spawning species through early development, it is currently unknown if individuals from poorly represented families are lost by the time of harvest due to differential survival, or if they are still available until the critical moment of selection to contribute as parents to the next generation. In addition, there are no studies based on real harvest data that determine how differential contribution and growth of families seen in mass spawners impact on selection of candidate broodstock. This is particularly important to understand, as mixed families in every cohort require the application of costly DNA genotyping analyses to determine pedigrees. In order to assist in the design of selective breeding strategies for mass spawners, barramundi (*Lates calcarifer*) was used as a model species to evaluate how family survival and growth under skewed contributions may affect the selection of breeding candidates at harvest. In this study, 18 days-post-hatch (dph) larval offspring from three commercial cohorts were firstly genotyped and assigned to their family of origin. Later at harvest (273–469 dph), fish from these cohorts were weighed and genotyped. Results showed that relative family frequencies at harvest were in different proportions than those found in the hatchery phase ($P < 0.05$). However, the majority of families initially identified (124 families) and all 48 broodstock contributing to offspring detected in the hatchery were also detected at the end of the culture cycle (141 families), suggesting that differential family survival did not impact on genetic diversity. Further analyses confirmed no subsequent loss of genetic diversity from 18 dph through to harvest, as measured by effective population size, inbreeding rates, number of alleles, allelic richness, observed and expected heterozygosities and R_{xy} relatedness coefficient. Examination of family contributions and weights at harvest in the three commercial cohorts showed that sampling and 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. Continued sampling after this was ineffective, with each additional 170 fish sampled only returning 1% extra family-specific genetic diversity.

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 behavior,

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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 standardized 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 behavior, 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; 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. Blonk et al., 2009; Borrell et al., 2011; Brown et al., 2005; Fessehaye et al., 2006; Frost et al., 2006; Gold et al., 2010; Herlin et al., 2008). 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 behavior (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; Loughnan et al., 2013; Wang et al., 2008). However, for selective breeding programs, the fate and relative contributions of families at the end of the culture cycle are 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 and 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 minimize 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 optimized parent selection to minimize inbreeding in aquaculture species were mostly conducted under the assumptions of equal family stocking and survival rates across all families (e.g. Hely et al., 2013; Macbeth, 2005; Robinson et al., 2010; Sonesson, 2005). 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. (2010) demonstrated that 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 were 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. Materials and methods

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. Mass spawning of barramundi broodstock, larval rearing, grow out environments and both broodstock and offspring sampling protocols were previously described (Domingos et al., 2013; Loughnan et al., 2013). Briefly, offspring in batches 1 and 2 originated from a group of 12 broodstock (six sires and six dams) spawned four months apart, and offspring in batch 3 originated from a larger group of 33 broodstock (12 dams and 21 sires). Eggs were collected over a single night spawn in each reported batch. 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). Here, to maximize family genetic diversity, all broodstock were checked for reproductive readiness (Liu et al., 2012) and multiple dams bearing

viable eggs (Ø ≥ 400 μm) were hormonally (LHRHa) induced including four dams in batch 1, five dams in batch 2, and ten dams in batch 3. Fish grow out took place in either a commercial intensive indoor recirculation facility in Melbourne (VIC, Australia, batches 1 and 3) or a semi-intensive pond in Innisfail (QLD, Australia, batch 2).

2.2. Parentage analysis and identification of families

Family pedigrees were reconstructed through DNA parentage analysis. DNA from broodstock, 18 dph larvae and harvested offspring was extracted from fin clips preserved in DMSO-salt solution (Seutin et al., 1991) following a protocol described by Taris et al. (2005) and adapted to 96 well plate formats. A number of previously published *L. calcarifer* microsatellite loci derived from a variety of sources (Wang et al., 2007; Yue et al., 2001, 2002; Zhu et al., 2006a, 2006b) were used in this study. Offspring samples from batches 1 and 2 were genotyped using a PCR multiplex containing a suite of 10 microsatellite markers (*Lca020*, *Lca032*, *Lca040*, *Lca057*, *Lca058*, *Lca064*, *Lca070*, *Lca098*, *Lca287* and *Lca371*). However, due to greater numbers of broodstock and possible family combinations in batch 3, simulations indicated that additional loci were required to achieve high parental assignment rates. Therefore, broodstock and offspring in batch 3 were genotyped using two multiplex suites totalling 17 loci, one containing nine microsatellite loci (*Lca008*, *Lca020*, *Lca021*, *Lca058*, *Lca064*, *Lca069*, *Lca70*, *Lca074* and *Lca098*) as described by Zhu et al. (2006b) and the other containing eight microsatellite loci (*Lca003*, *Lca016*, *Lca040*, *Lca057*, *Lca154*, *Lca178*, *Lca287* and *Lca371*). PCR reactions and cycling conditions, allele sizing (MegaBACE 1000 DNA Analysis Systems, GE Healthcare) and fragment analysis (MegaBACE Fragment Profiler, GE Healthcare) and parentage assignment (95% confidence level) to a parental pair (Cervus 3.0, Kalinowski et al., 2007) followed the methodology described in Domingos et al. (2013).

2.3. 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 (R_{xy} 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 are the mean number of progeny per dam and sire, respectively and V_d and V_s are 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 $P < 0.05$.

2.4. 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 (Table 1). 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 (*ca.* 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.; data available upon request at harvest), 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.

3. Results

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 1). 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 (Fig. 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

Table 1
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. Offspring age given in days post hatch (dph).

	Batch 1		Batch 2		Batch 3	
	Hatchery	Harvest	Hatchery	Harvest	Hatchery	Harvest
	18 dph	345 dph	18 dph	469 dph	18 dph	273 dph
- 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.

any batch (Fig. 1). 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 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 batch 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).

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, Fig. 1(a); families 2, 4, 6 and 7 in batch 2, Fig. 1(b); families 3, 17 and 40 in batch 3, Fig. 1(c)), which caused overall family contributions at harvest to be in statistically different proportions than those previously found in the hatchery (batch 1 $\chi^2 = 125$, d.f. = 6, $P < 0.001$; batch 2 $\chi^2 = 181$, d.f. = 11, $P < 0.001$; batch 3 $\chi^2 = 484$, 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).

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 × 6 dams) could have been generated in batches 1 and 2, and 252 families (21 sires × 12 dams) in batch 3. However, commercial mass spawnings generated a comparatively low number of families, ranging between 19% (batch 1) and 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 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 (batches 1 and 2), ranging from 2.8 to 3.8 when compared to the larger breeding group, which was around 5-fold higher (batch 3, $N_e \sim 15$). 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 *Ar*, 0.04 in *Ho*, 0.06 in *He* and a 0.16 increase in R_{xy} (Table 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).

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

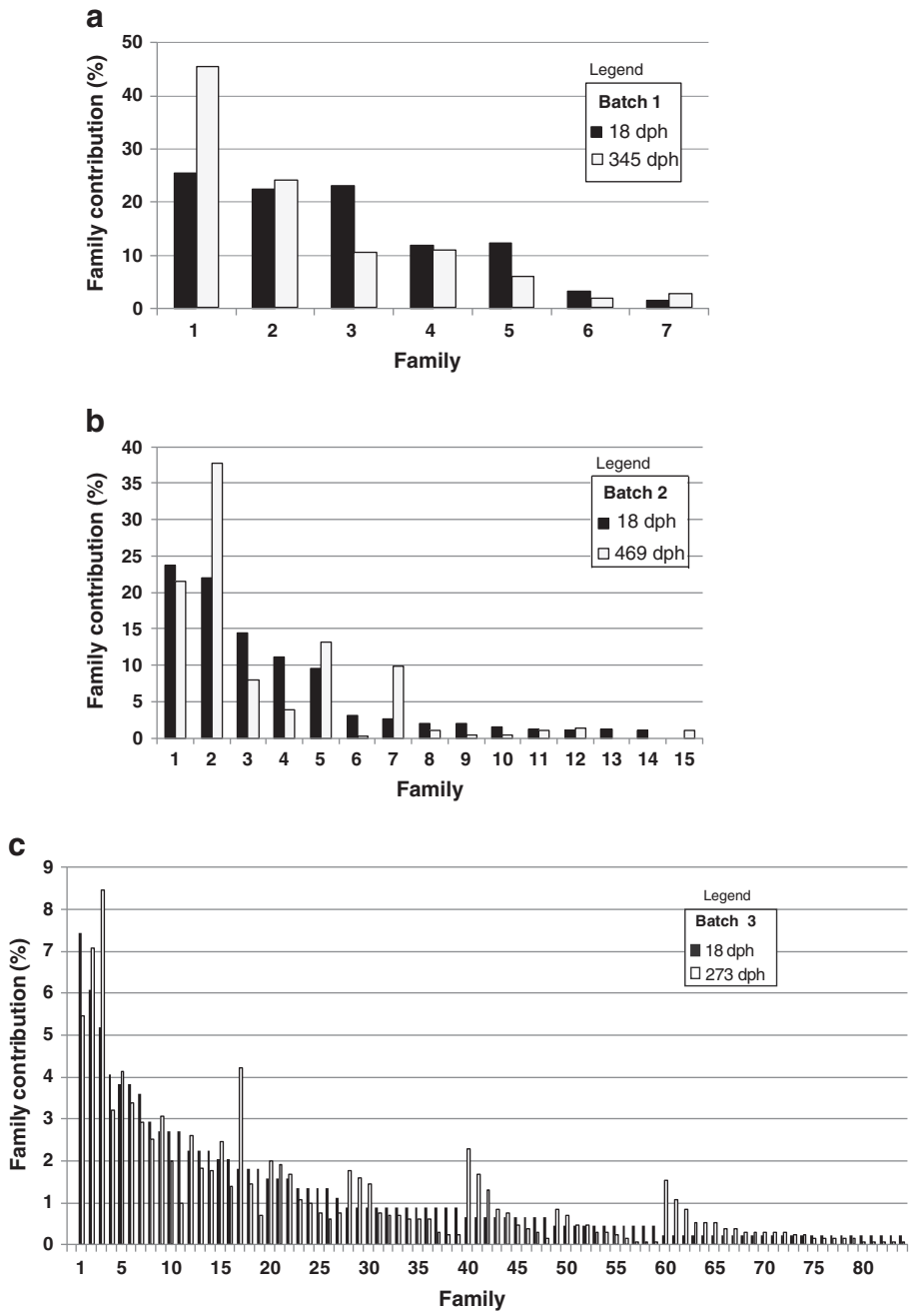

Fig. 1. Relative family contributions of farmed *Lates calcarifer* from hatchery to harvest in (a) batch 1, (b) batch 2 and (c) batch 3. Offspring age reported in days post hatch (dph).

Table 2
Genetic diversity of *Lates calcarifer* broodstock and offspring cohorts sampled at the hatchery and at harvest. Offspring age given in days post hatch (dph).

	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
R_{xy}	0.113	0.254	0.217	0.113	0.458	0.461	0.177	0.198	0.202	0.134	0.303	0.293

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.

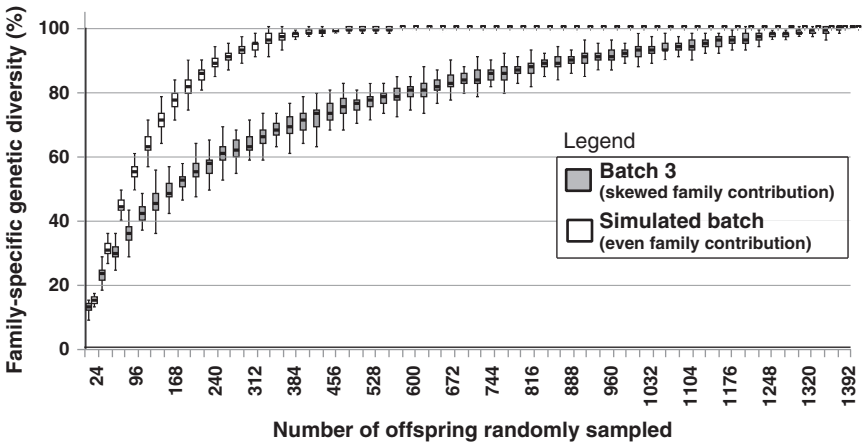

Fig. 2. Comparison between the genotyping efforts of a randomly sampled simulated cohort with even family contribution and a randomly sampled true mass spawned cohort with skewed family contribution (parentage dataset of batch 3 at harvest). Both cohorts contained 121 families (100% of family-specific genetic diversity). Box plots represent 50 replicates per sampling size.

(Fig. 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.

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 (Fig. 3). 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 1.5% of the population (or 750 breeding candidates heavier than +2.17 S.D. of the population mean), there was a

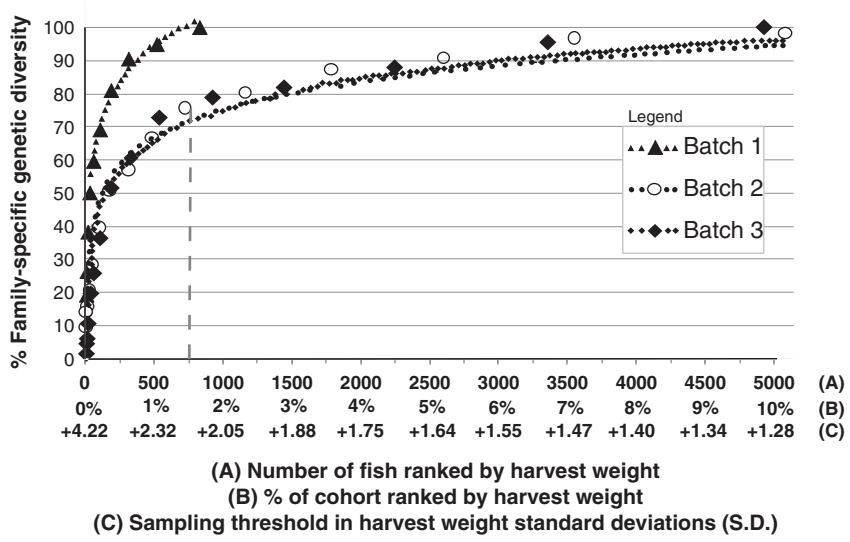

Fig. 3. Number of heaviest *Lates calcarifer* at harvest (also reported in % of cohort and sampling threshold in harvest weight standard deviations, S.D.) needed to be genotyped in batches 1 to 3 to include offspring from all families. Dashed line corresponds to the top 1.5% of the population (~750 heaviest 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.

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 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.

4. Discussion

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) 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 10 g) 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).

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; Liu et al., 2012; Loughnan et al., 2013; Wang et al., 2008), as well as those reported for other commercially important mass spawners, such as gilthead seabream (*Sparus aurata*) (Borrell et al., 2011; Brown et al., 2005) 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%.

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 (Garber et al., 2010; Lind 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 future breeding nucleus, have not yet been reported for barramundi. In any case, sampling schemes that minimize 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).

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 were 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 modeled) and 3 (66 families modeled) indicated that the number of samples needed may not necessarily be related to the total number of families. More likely, it is possible that differential growth rates among families may also 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).

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 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 behavior, but utilizing entirely simulated parameters, Blonk et al. (2010) 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 realized 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 minimizing the overall costs of breeding programs for mass spawners such as barramundi and speed progress towards selective improvement of commercially important aquaculture species.

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