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Title: A study into parental assignment of the communal spawning protogynous hermaphrodite, giant grouper (Epinephelus lanceolatus).

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Keywords: grouper, captive spawning, parentage assignment, microsatellites

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Abstract: Parental contributions of giant grouper to communal spawns in captivity is important for establishing genetic management of the species. In this study, we have followed the spawning dynamics of three males and three females over 6-8 consecutive days, over three time periods. Polymorphic microsatellite markers were validated and utilised to successfully determine parentage in 574 offspring from 20 nights of spawns. Variation of both maternal and paternal contributions between nights in batches of spawns were significant ($P < 0.001$). Most paternal assignments were attributed to one dominant male who initiated each spawning batch, however, all males and females successfully mated over the spawning period. There was a significant ($P < 0.01$) trend towards a polygamous reproductive mode for giant grouper: in two of the three batches of spawns where, on some nights, eggs from all females were fertilised by multiple males. Genetic variation was assessed between parents and offspring. There was a loss of alleles on each spawning night, however, if offspring from a series of consecutive nights were combined, most or all of the genetic variation would be maintained in the F1 generation. This research validates the molecular tools for genetic monitoring of giant grouper and also improves the understanding of spawning dynamics of protogynous hermaphroditic communal spawners over time in an aquaculture setting.

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Conflict of interest

Dear Prof Hulata,

We are pleased to submit for your consideration our paper 'A study into parental assignment of the communal spawning protogynous hermaphrodite, giant grouper (*Epinephelus lanceolatus*)'.

This paper describes a comprehensive investigation of the spawning dynamics of giant grouper over three spawning periods, each of 6-8 days.

The finding of this study show that while the dominant grouper male always initiates the spawning batch, and is primary contributor in the first couple of days, other males, including a recently sex changed male, also contribute to spawns.

This information is important for the understanding of grouper spawning behaviour, it offers important information to grouper growers with respect to the need to collect eggs over consecutive days to ensure the full genetic diversity is captured.

We look forward to your consideration of our manuscript,

Kind regards

David Bright and Abigail Elizur

- Spawning dynamics of captive giant grouper reveals wide spread polygamy within spawning batches
- Multiple giant grouper males contribute to the spawns despite dominant male hierarchy
- Newly sex changed males can successfully contribute to spawning alongside established males

***Statement of Relevance**

The finding of this study show that the dominant giant grouper male initiates spawning and is the primary contributor however other males contribute to spawns.

This contributes to our understanding of grouper spawning behaviour, and offers important information to grouper growers with respect to the need to collect eggs over consecutive days to ensure the full genetic diversity is captured.

Title: A study into parental assignment of the communal spawning protogynous hermaphrodite, giant grouper (*Epinephelus lanceolatus*).

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Abstract:

Parental contributions of giant grouper to communal spawns in captivity is important for establishing genetic management of the species. In this study, we have followed the spawning dynamics of three males and three females over 6-8 consecutive days, over three time periods. Polymorphic microsatellite markers were validated and utilised to successfully determine parentage in 574 offspring from 20 nights of spawns. Variation of both maternal and paternal contributions between nights in batches of spawns were significant ($P < 0.001$). Most paternal assignments were attributed to one dominant male who initiated each spawning batch, however, all males and females successfully mated over the spawning period. There was a significant ($P < 0.01$) trend towards a polygamous reproductive mode for giant grouper: in two of the three batches of spawns where, on some nights, eggs from all females were fertilised by multiple males. Genetic variation was assessed between parents and offspring. There was a loss of alleles on each spawning night, however, if offspring from a series of consecutive nights were combined, most or all of the genetic variation would be maintained in the F_1 generation. This research validates the molecular tools for genetic monitoring of giant grouper and also improves the understanding of spawning dynamics of protogynous hermaphroditic communal spawners over time in an aquaculture setting.

Key words: grouper, captive spawning, parentage assignment, microsatellites

1. Introduction

The giant grouper, *Epinephelus lanceolatus,* of the family Serranidae has been IUCN listed as vulnerable due to overfishing, including destructive cyanide fishing (Halim, 2001; Mak, et al., 2005; Sadovy, 1997). It is one of 159 species across 15 genera that make up the sub family Epinephelinae (Heemstra, Randall, 1993). It is one of the largest reef fish in the world and grows to 2.3 metres, weighs up to 400 kilograms and lives for up to 40 years (Heemstra, Randall, 1993; Zeng, et al., 2008). Giant grouper has a broad distribution, from East Africa to Hawaii, but it has a low population density within this region (Lau, Li, 2000). To date, there have not been any broad scale surveys to accurately assess the size of the population (Yang, et al., 2011). Over the last two decades, along with other grouper species, it has been a target for Southeast Asian and Indian Ocean fishermen, especially for the live reef food fish (LRFF) trade in Hong Kong and mainland China (Johannes, Kile, 2001; Mak, et al., 2005; Muldoon, et al., 2005; Shakeel, Ahmed, 1997; Tew, et al., 2011).

Giant grouper, like many species in the Serranidae family, is an aggregative spawning protogynous hermaphrodite; however, little is known about reproductive sex ratio (number of males to females) at spawning aggregation sites in the wild. A sex ratio of one large male to up to five females has been reported for other aggregative spawners in the family (Rhodes, Sadovy, 2002). This can impact on the genetics of cultured giant grouper in two ways (Liu, et al., 2005). Firstly, it is not possible to determine with confidence who the mother and father of offspring from a communal spawn are without some type of DNA tagging (Wang, et al., 2010). The outcome of lack of parentage assignment in an aquaculture setting is that superior offspring selected for future broodstock may be related, leading to inbreeding. Secondly, only a small number of males may contribute to spawning, thereby reducing the genetic diversity of the offspring, which if released, may have a negative effect on genetic diversity of wild stocks (Allendorf, Phelps, 1980; Hara, Sekino, 2003; Wang, et al., 2010). A recent study of giant grouper aquaculture in Taiwan highlighted the shortage of male broodstock relative to females and claimed

that the majority of giant grouper in the marketplace are derived from inbred broodstock (Kuo, et al., 2014).

Pedigree development and parentage assignment can be achieved using polymorphic DNA microsatellites to establish genotypes of offspring and where available, that of the broodstock (Wang, et al., 2010). Giant grouper is a relatively new aquaculture species and, as such, there has been limited research into the genetic diversity of cultured or wild populations using molecular tools such as DNA microsatellites (Yang, et al., 2011). Microsatellites are one of the most widely used molecular techniques for assessing the genetic variability and pedigree tracing of wild and cultured marine fish species (Antoro, et al., 2006; Chistiakov, et al., 2006; Liu, Cordes, 2004; Perez-Enriquez, et al., 1999; Rhodes, et al., 2003; Schunter, et al., 2011; Wilson, Ferguson, 2002). Microsatellite markers, comparing allele number, heterozygosities and Wright's F-statistics including F_{ST} values, have been used to estimate the genetic diversity within and between populations (including many grouper species) (Antoro, et al., 2006; Hara, Sekino, 2003; King, et al., 2001; Liu, et al., 2005; Perez-Enriquez, et al., 1999; Rhodes, et al., 2003; Rivera, et al., 2010; Schunter, et al., 2011; Wang, et al., 2010; Wang, et al., 2011). Some giant grouper microsatellites (Rodrigues, et al., 2011; Yang, et al., 2011; Zeng, et al., 2008) have been identified but have only been utilised in a few population studies or genetic breeding programs (Kuo, et al., 2014). Kuo, et al. (2014) tested the suitability of microsatellites from other grouper studies and highlighted six loci that would allow for high parentage assignment accuracy.

Giant grouper have spawned in captivity when there are at least two males and multiple females in a tank (Knuckey and Reynolds, unpublished). They spawn over a batch of six to eight nights at a certain time in the lunar cycle. To understand giant grouper captive spawning dynamics we endeavoured to determine the parental contributions in giant grouper communal spawns and how these may vary over a batch of nights in a spawning period.

2. Materials and methods

2.1 Sample collection and DNA extraction

Giant grouper broodstock were maintained at the Finfish Enterprises facility in Cairns, Queensland, Australia. Samples from 34 wild-caught broodstock and 576 eggs from 20 spawns from one spawning tank were collected. The spawning tank consisted of three males and three females which spawned over a four month period. The spawning events were on the 22-29/07/2012, 14-19/09/2012 and 14-21/10/2012 on a lunar cycle (six, six and eight nights per batch). Up to 30 samples were collected and analysed for each spawning night. Fin clip and egg samples were stored in collection tubes containing 70% ethanol. Total genomic DNA was extracted from fin clips using a modified salt (NaCl) extraction method (Lopera-Barrero, et al., 2008). Samples of approximately 20 mg of giant grouper fin clips in Eppendorf micro tubes were mixed with 550 µl of lysis buffer (50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 100 mM NaCl) plus 1% SDS, digested by adding 8 µl of proteinase K, then incubated at 50 °C for two hours during which time tubes were inverted and vortexed at 30 minute intervals. After digestion, 300 µl of 5M NaCl was added then samples were chilled on ice for 10 minutes before being centrifuged for 10 minutes at 13,000 rpm. The supernatant was transferred into new micro tubes and centrifuged for 15 minutes at 13,000 rpm. The supernatant was transferred to a new micro tube, containing 700 µl of ice-cold absolute ethanol and inverted 50 times to precipitate the DNA, then stored at 4 °C overnight. The next day, DNA samples were pelletised in a centrifuge at 13,000 rpm, washed with 700 µl of 70% ethanol and re-suspended in molecular grade water. Egg genomic DNA was extracted using a commercial DNA extraction kit due to very low expected yield from the fertilised eggs (DNeasy 96 Blood & Tissue Kit, QIAGEN).

All the fish work was done in accordance with the University of the Sunshine Coast animal ethics guide, and approval number AN/A/13/74 and AN/A/13/75.

2.2 Microsatellite markers and genotyping

Forty-eight DNA microsatellite markers were considered from three sources (Rodrigues, et al., 2011; Yang, et al., 2011; Zeng, et al., 2008), of which a shortlist of 30 M-13 labelled microsatellite primer pairs were tested (Schuelke, 2000). Primers were initially tested using touchdown PCR in an attempt to amplify candidate loci from eight genomic DNA samples of giant grouper.

Amplification was conducted using an Eppendorf Thermal Cycler with each sample containing 15 µl: 9 µl of molecular grade water, 1.5 µl of 10x PCR Buffer, 0.3 µl of 0.2 mM dNTPs, 1.125 µl of 1.875 mM $MgCl_2$, 0.3 µl of Taq polymerase, 1 µl of 50 ng genomic DNA template, 0.3 µl of 10 µM of both the forward primer and the fluorescent dye, and 0.6 µl of 10 µM reverse primer. The touchdown PCR conditions were 94 °C for 3 min followed by 20 cycles of 30 sec at 94 °C, 30 sec at 62 °C (decreasing by 0.5 °C per cycle) and 45 sec at 72 °C, followed by 15 cycles of 30 sec at 94 °C, 30 sec at 50 °C, and 45 sec at 72 °C and a final extension of 10 min at 72 °C. PCR products were visualised with ethidium bromide on a 1.5% agarose gel to confirm that satisfactory PCR amplification has occurred and to determine amplification amounts so dilution rates for fragment analysis can be established. Fragment analysis of PCR products was carried out using the AB3500 genotyper with GeneScan LIZ 600 as a size standard (Applied Biosystems). Allele scoring was conducted using the computer program GeneMarker v. 1.95 (SoftGenetics LLC, State College, PA).

2.3 Parentage assignment

Seven primer pairs were selected for parentage assignment. Thirty-four broodstock and 576 offspring DNA were amplified, genotyped and scored following the above protocols. Parentage assignment was conducted using Cervus v. 3.0.3 (Kalinowski, et al., 2007). Allele frequency analysis, simulation of parentage analysis, and parentage analysis (parent pair- sexes known) were conducted with parentage assignment based on relaxed and strict LOD scores of 80% and 95% confidence. Number of alleles (N), homozygosity (Ho), heterozygosity (He), conformity to Hardy-Weinberg equilibrium (HWE) and polymorphic information content (PIC) were also calculated. Genotyping errors including presence of null alleles, stuttering and large-allele dropout were assessed using Micro-checker v. 2.2.3 (Van Oosterhout, et al., 2004).

2.4 Statistical analysis

Statistical analysis was performed on 576 fertilised egg samples collected over three successive spawning batches (20 nights in total). To examine variation in the

contribution of parental breeders when they were assigned over a batch of nights or over the entire spawning period (three spawning batches over 20 nights), log likelihood chi-square test in SAS (SAS Inc., 2009) was used and also reanalysed in SPSS version 22 (Nie, et al., 1975). Further analysis investigated the contribution of sires and dams to total phenotypic variance of the observations (i.e. the proportion of offspring contributed by each pair of parents). In this analysis, the general linear mixed model included the fixed effects of spawning batch (*B*) or nights within a batch. The random terms in the mixed model were sires (*s*) and dam nested within sires (*d*). In mathematical notations, the model was written as:

$$y_{ijkl} = \mu + B_i + s_j + d_k(s) + e_{ijkl} \qquad [1]$$

where y_{ijkl} is the observed number of offspring, the μ is overall mean (constant), and *s* and *d* were as described above.

In addition to the chi-square statistics, multiple comparisons using Tukey test were applied to determine statistical differences in the number of full-sib families and the offspring contributions by parental pairs between spawning batches (or between nights within a batch) and between a sex-changed male compared with other sires in the study. The Tukey test adjusted for the imbalanced data was used to avoid pair-wise family errors, and to examine statistical differences in the total allele number of brood stock parents and offspring in spawning nights within a batch or between batches.

Finally, generalised non-linear model was conducted to examine spawning dynamics of giant groupers communally kept in tanks. For a given spawning night, when a male was observed to mate with only one female, this pair was assigned as monogamy; otherwise, it was called polygamy when a male mated to more than one female (or multiple females). The data were expressed as a binary form (0 for monogamy and 1 for polygamy, respectively). Statistical analysis of the binary traits employed logistic model with a logit link function. Least squares means were back-transformed from the logistic to originally observed proportion scale.

3. Results

3.1 Microsatellite suitability

Eight polymorphic microsatellite loci were identified as suitable based on reliability of PCR amplification and allele scoring of 34 wild broodstock (Table 1). Seven were selected for parentage analysis based on variation of parental genotypes (ELMS007 excluded based on low allele number and deviation from HWE due to homozygote excess). There was no evidence for null alleles, scoring errors due to stuttering or large-allele dropout (Van Oosterhout, et al., 2004). The number of alleles ranged from 6 to 14 and the levels of heterozygosity and polymorphism information content (PIC) were high (Table 1).

3.2 Parentage assignment

Parentage analysis (parent pair- sexes known) was performed on 576 offspring. Parental assignment rates for the three batches of spawns were 100%, 100% and 99% respectively at a strict confidence level of 95% with 574 individuals accurately assigned parent pairs. They are the progeny of three dams and three sires across spawning nights and batches. Two individuals were excluded from assignment due to typing at less than five of the seven loci.

3.3 Variation in parental contributions

All six of the broodstock were assigned to offspring in batch 1 (offspring, n=155). The number of contributing parents ranged from two, to five on any given night (Figure 1.a, b). The largest male (NFC01) was the dominant male spawner, siring the majority of the offspring in the batch. Total maternal contributions were more even than male contributions. Initially, a pair of parents spawned and as the spawning batch progressed, more parents were contributing to a peak of five parents per night.

The analysis of spawning batch 2 consisted of 179 offspring. Five of the six parents (two males, three females) were assigned parentage (Figure 1.c, d). The range of parental contributions on a given night within this batch was the same as for batch 1. The general trend of the number of parental contributions over the duration

of the spawning batch was similar to batch 1, as the number of contributing individuals increased to four or five toward the middle and end of the batch of nights.

Batch 3 (offspring, analysed n=240) ran for eight nights (Figure 1.e, f). Four of the six parents contributed to the spawning batch (two males, two females). The dominant male (NFC01) spawned first but contributions generally decreased as the batch progressed, concluding with only 17% offspring assigned to this male on the final night. The smaller, recently sex-changed male spawned on most of the nights in this batch and dominated the paternal assignment by the conclusion of this batch of nights.

The analysis of variance (Table 2) shows significant differences in parental contribution to half-sib and full-sib families between spawning batches or between nights within a batch. That is, the contribution of sires and dams differed between batches and between nights in each batch ($P < 0.001$). Over the entire spawning period (20 nights over three spawning batches), the effects of sire or dam nested within sire, on the paternal and maternal families, were also significant ($P < 0.05$ for sire and $P < 0.001$ for dam).

The number of full-sibling families per spawning night was calculated based on parentage assignment (Figure 2). There was a maximum of nine theoretical full-sibling families based on the number of broodstock in the spawning tank. The number of full-sibling families in the offspring was initially low, but rose as the spawning batch progressed. The middle to latter phase of each spawning batch showed the highest number of identified full-sibling families per night. The increase in full-sibling family numbers across nights in the batches were statistically significant ($P <0.01$). This suggests that the spawning batch was initiated by one spawning pair that lead to polygamous mass spawning events as the spawning batches progressed and concluded.

3.4 Genetic variation between parents and offspring

Allele number was calculated for parents, three batches of offspring and spawning-nights within batches for seven microsatellites. Allele numbers on nights within batches were generally lower than total allele number of batches , $P < 0.01$ (Figure 3a). Total allele numbers of offspring in the three spawning batches were the same or slightly lower than the total allele number of the possible broodstock, P =

0.564 (Figure 3b). There was no significant difference in total allele number between parents and offspring of the three spawning batches (P = 0.498). Variation in allele number (data not shown) between nights within batches was evident, with some nights showing a high loss of alleles, (e.g. N_a = 2.6 in offspring of night 1 in batch 1 *vs.* N_a = 4.7 in parents), but the loss was not significantly different especially in the second and third batches (P = 0.194 to 0.393).

3.5 Giant grouper spawning dynamics- monogamy vs. polygamy

Analysis of communally housed giant grouper spawning dynamics showed that polygamous mating (one male mated to multiple females or vice versa) was predominant in each nightly spawning event (Table 3). There were significant differences in relative proportion of monogamous and polygamous matings in the first two spawning batches (P < 0.001), but not in the third batch (P > 0.05). Two males and two females spawned across the eight nights of batch three. The dominant male, NFC01, predominately spawned with female NFC20, and as their contributions dropped, NFC09 and NFC19 increased. Across the three spawning batches, there was a significant difference between polygamous compared to monogamous mating events (P < 0.001).

3.6 Sex changing individuals' reproductive success

Parentage assignment identified that a smaller, recently sex-changed male (NFC09) is able to contribute to communal spawns and sired 18% of the 574 offspring across the three batches (Figures 1a, c, e). The contribution of NFC09 to the total number of offspring collected from the three spawning batches was lower than the dominant male (P = 0.029).

4. Discussion

This study examined the spawning dynamics of the giant grouper in a tank over a period of four months consisting of 20 spawning events over three batches. Previously published microsatellites were tested and eight were deemed suitable for parentage assignment (Rodrigues, et al., 2011; Yang, et al., 2011; Zeng, et al., 2008). Observed and expected heterozygosity for the eight loci, tested on 34 wild giant grouper, were similar to reported values, however, there was an increase in number of alleles identified at most loci, indicating greater variability in the Australian

population than the individuals used to initially test the microsatellites (Yang, et al., 2011; Zeng, et al., 2008). Seven microsatellite loci were identified and utilised to successfully assign parentage at a strict confidence level ($P<0.05$) for 99% of the 576 giant grouper offspring. Seven loci is an acceptable number for parentage assignment when the allele number, heterozygosity and polymorphic information content is high (Sefc, Koblmüller, 2009). Genetic differences between the six broodstock in the spawning tank in this trial were sufficient to attain very high parentage assignment rates.

In all three batches of spawns the largest male (NFC01) was the dominant spawning male. He was the sole or primary sire for the first night or two. As the nights progressed, other males, including the recently sex changed male, successfully contributed to the spawn. In the first two batches, this occurred on nights three, five and six and on most nights in batch two, siring nearly 50% on the final night. In spawning batch three, the decrease in NFC01 contribution was more gradual, and by the end of the eight nights of spawns, only 17% of offspring were assigned to him. This suggests that as the spawning batch progresses he tires and becomes a less active breeder. This allows less dominant males to successfully contribute to the spawn and sire offspring. Maternal contribution was markedly different from the male dynamic. Generally only one female, yet not the same one in each spawning batch, would spawn on the first night. Towards the middle and end of the batch, all three females (except in batch three) would spawn on the same night with multiple males fertilising the eggs. This indicates that the spawning behaviour of the females, unlike the males, shows no clear dominance hierarchy. The male and female reproductive strategies varied and both sire and dam contributions significantly differed between nights within batches ($P < 0.001$). Parentage assignment of three spawns from the white grouper, *E. aeneus*, in a batch of spawns, showed a similar trend to this study, where one parent pair was assigned on the first reported spawn and four individuals paired and spawned in varying combinations across the next two spawning events (Dor, et al., 2014). Not all of the broodstock in the white grouper batch spawn were identified as spawning contributors, but this may be due to small offspring sample size (n= 21, 22 and 9) and number of spawning events (n=3) sampled in the batch of spawns. In a separate study, parentage assignment of communally spawning Humpback grouper (*Cromileptes altivelis*) was conducted on offspring (n=120) that were collected over a series of 10

nights in a batch and showed that seven of 20 potential broodstock contributed to the batch of spawns (Na-Nakorn, et al., 2010). Their findings differs to what was found in this study as only one-third of the potential parents contributed to the batch of 10 spawns. However, offspring were sampled at four months of age, which may have skewed the observed parental contribution towards broodstock that successfully spawned in the initial period of the spawning batch due to larvae and juvenile cannibalism mortalities. Groupers, and giant grouper in particular, are highly susceptible to intra-cohort cannibalism (Hseu, Huang, 2014; Hseu, et al., 2004; Hseu, et al., 2007), therefore, pooling of grouper eggs over 10 nights of spawns may have led to many offspring from latter spawns in the batch succumbing to cannibalism and subsequent parental contribution drop-out.

Using allele number as an indicator of genetic variation, captive-bred seven-band grouper (*Epinephelus septemfasciatus*) were found to have lower average allele number than wild parental broodstock (An, et al., 2014a; An, et al., 2014b) which was attributed to a combination of grouper spawning traits (communal spawning and skewed sex ratio) and hatchery practices. In the present study we found no significant difference in allele number between parents and batches of spawns but a large drop in allele number from parent to offspring on isolated spawning nights. Parental allele number is largely maintained in all batches of spawns (Figure 3) which supports the parental assignment results, showing most or all parents may not contribute on any given night, but most contribute in a batch of nights.

Sampling and genotyping eggs or larvae may give an inflated level of parental contributions as parents with low rates of offspring assignment may drop out over time as larval mortality occurs or size grading is implemented to lessen cannibalism and slower growing larval cohorts are discarded (Frost, et al., 2006). An analysis of survival of larval sea bass, *Dicentrarchus labrax,* found that although there was substantial variation in survival rates between families through the larval and nursery rearing stages, all families retained representative offspring up to 116 DPH (Garcia de Leon, et al., 1998). Recently a study of Barramundi broodstock contribution to mass spawning events in captivity assessed allelic richness of offspring from 1 to 90 DPH and found negligible differences (Loughnan, et al., 2013). These finding suggests that the genetic variation identified in offspring across batches of spawns of

giant grouper may be maintained through to grow-out, however a longitudinal study of parental contributions over larval rearing would be necessary to verify this.

Studies of parental assignment in Barramundi found that only half the male broodstock successfully contributed to any one spawn, (Frost, et al., 2006), highlighting the risks of collecting eggs from only one night in commercial hatcheries. Parentage assignment of the European Anchovy showed that only 15% of breeders were effectively contributing to a spawn (Borrell, et al., 2011), and in Japanese flounder, just 57% of parents were confirmed as contributors to a spawn (Hara, Sekino, 2003). These levels of parental contributions are similar to what we have observed when assigning parentage on single nights within batches of spawns in giant grouper, where as few as one pair of broodstock spawned. Extending the parental assignment across a batch of spawns, rather than one night, identified most or all potential broodstock contributed. Our findings suggest that selecting eggs for grow-out farms or for re-stocking the wild from one night in a batch may limit the capture of parental contribution and subsequent genetic diversity of the offspring. Retention of genetic diversity is very important for the aquaculture industry as it maintains a broader base to conduct genetic selection for stock enhancement and also may impart hardiness to environmental fluctuations and disease tolerance to future generations (Kuo, et al., 2014).

A secondary finding of this research relates to the protogynous hermaphroditic characteristic of grouper biology. In our study, an individual grouper that had recently undergone transition to male in captivity was added to the spawning tank. It successfully participated in spawns with multiple dams on some spawning nights across all three spawning batches. Male grouper are aggressive and territorial so a smaller and younger male can be attacked by the larger male in a confined spawning tank (Reynolds and Knuckey, personal communication). With an acknowledged shortage of males (Kuo, et al., 2014) leading to potential inbreeding, identifying whether recently sex-changed males can successfully contribute to a spawn is very important as not only can we increase the genetic variation in our F_1 and subsequent generations, we can also justify artificially sex-changing the largest females in a breeding population to males to increase the potential number of full-sibling families. The timing of natural sex-change in giant grouper has not been reported, however in other similarly long-lived groupers, sex change to males can take up to 17 years (Tan, Tan, 1974). Inducing sex change in female broodstock will dramatically cut

down generation time for enhanced genetic selection but unless the smaller males successfully contribute to spawns, there is no reason to undergo this process. The successful spawning of the recently sex-changed male identified in this study opens the door to this approach. Another important consideration of sex-changing broodstock is that the largest females in a cohort will change gender, which may result in recently sex-changed males spawning with full- or half-sibling sisters of the same cohort. This adds another potential risk for inbreeding. It is possible that this has already occurred in Taiwan (Kuo, et al., 2014) and further supports the need for accurate genetic monitoring of giant grouper and other hermaphroditic fish species with molecular markers.

5. Conclusion

Successful parentage assignment of giant grouper has been conducted for the first time. Seven microsatellites have been utilised to achieve 99% parentage assignment of 576 offspring to genotyped potential parents. Parental contributions in communal spawns in captivity significantly varied between spawning nights, and within and between batches. However, when allele number of parents and batches of offspring were analysed, genetic variation was largely maintained as giant grouper dams and sires both exhibited a significant tendency towards polygamous mating. The importance of this finding is that it will enable better genetic management of stocking regimes by modifying egg collection from single nights to batches. Selecting eggs from a single night at the start of a batch of spawns could result in a loss of genetic diversity and low parental contribution, however, if eggs are selected from multiple nights in the middle or end of a batch, most parental genetic variation should be maintained. A potential negative aspect of stocking grow-out facilities with eggs from multiple spawns is a greater likelihood of cannibalism which can be costly, however this can be managed through multiple larval rearing tanks.

Furthermore, a recently sex-changed male successfully contributed to communal spawns, which means that while there is a dominance hierarchy in the male population, younger males may be added to a breeding population and actively contribute to the spawning. Females that exhibit preferred traits, such as faster growth or desirable colour for market, can be sex-changed and successfully spawn which will assist in selective breeding of the species.

This study provides the tools and a greater understanding of giant grouper captive spawning dynamics over batches of spawns.

Acknowledgements

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Figure and Table legends

Table 1. Selected microsatellite loci for parentage assignment.

Table 2: Effects of spawning batches and nights within each batch on number of paternal and maternal families

Table 3: Spawning dynamics (monogamy *vs.* polygamy) in giant grouper

Figure 1: Paternal (NFC01, NFC02 and NFC03) and maternal (NFC17, NFC19 and NFC20) assignment rate (percentage) for giant grouper offspring on different nights of spawns in three batches: (a) n=155, Chi-square test ($\chi^2_{1,10}$ = 90.8, P< 0.001); (b) n=155, Chi-square test ($\chi^2_{1,10}$ = 140.5, P< 0.001); (c) n=179, Chi-square test ($\chi^2_{1,5}$ = 49.6, P< 0.001); (d) n=179, Chi-square test ($\chi^2_{1,10}$ = 89.3, P< 0.001); (e) n=240, Chi-square test ($\chi^2_{1,7}$ = 88.3, P< 0.001); (f) n=240, Chi-square test ($\chi^2_{1,7}$ = 125.1, P< 0.001).

Figure 2: The increase in number of full-sibling families identified per night in three batches of serial spawning ($\chi^2_{1,18}$ = 65.1, P< 0.001).

Figure 3: a. Comparison of allele number between broodstock, offspring in a batch of spawning nights and a night (P < 0.05); b. total allele numbers identified for seven microsatellite loci for parents and offspring of the three spawning batches (n=580, P > 0.05).

Table(s)

Table 1

Locus	Primer sequences (5'-3')	Size	TM	N	Ho	He	PIC	HWE
ELMS007	F: TGTAAAACGACGGCCAGTTTTGCCTTTCCTAGACTTAT	325–341	50	2	0.301	0.477	0.362	*
	R: CATCACATGATTCCTTTCTAT							
ELMS009	F: TTCCACAGCAATTAGCAGCA	257–300	56	9	0.710	0.732	0.785	NS
	R: TTTCCTCCCACAGTCCAAAG							
ELMS019	F: TCAGCAAGCACTTTTTGGAC	377–385	56	6	0.696	0.669	0.598	NS
	R: TGCTTCCTTCAGTGCATCAG							
An2	F: TGCCCCTCCGACAACTAATA	226-250	61	14	0.826	0.723	0.783	NS
	R: AACGGGACTTGTGGTTTTTG							
An4	F: TGTAAAACGACGGCCAGTGCTCGAAGATGAGCTGGAAG	192-210	60	13	0.721	0.810	0.785	NS
	R: AAGGTGCTGCTCCTGCTTT							
An8	F: TGTAAAACGACGGCCAGTACCATGCATAAATGCCCACT	148-162	60	11	0.556	0.557	0.766	NS
	R: GCTCTCTGTCTCGCAAGGAT							
An25	F: TGTAAAACGACGGCCAGTTCTGTGCTGATGCCGACTAC	146-200	58	12	0.825	0.868	0.848	NS
	R: CCGTGTTTGCACACTCTCTG							
An31	F: TGTAAAACGACGGCCAGTTCATGTGTGCAAACGCTGTA	184-218	61	9	0.785	0.768	0.748	NS
	R: CAACATGGCCGAAACCTAAT							

TM, annealing temperature (°C); N, allele number; HO, observed heterozygosity; HE, expected heterozygosity; PIC, polymorphism information content; HWE, Hardy–Weinberg equilibrium; *, significant (P < 0.05),NS, not significant.

Table 2

Relationship	Effect	Degree of freedom	F-statistic value	Probability
Paternal families	Spawning batch	2	32.8	<0.0001
	Night (Batch)	17	15.0	<0.0001
Maternal families	Spawning batch	2	57.9	<0.0001
	Night (Batch)	17	15.6	<0.0001

Table 3

Spawning batch	Monogamy (%)	Polygamy (%)	Significant probability*
1	18.8	81.3	0.0124
2	11.1	88.9	0.0010
3	37.5	62.5	0.3172
All	22.0	78.0	0.0001

* The significant probability of the difference between monogamy and polygamy.

Figure(s)

Figure 1

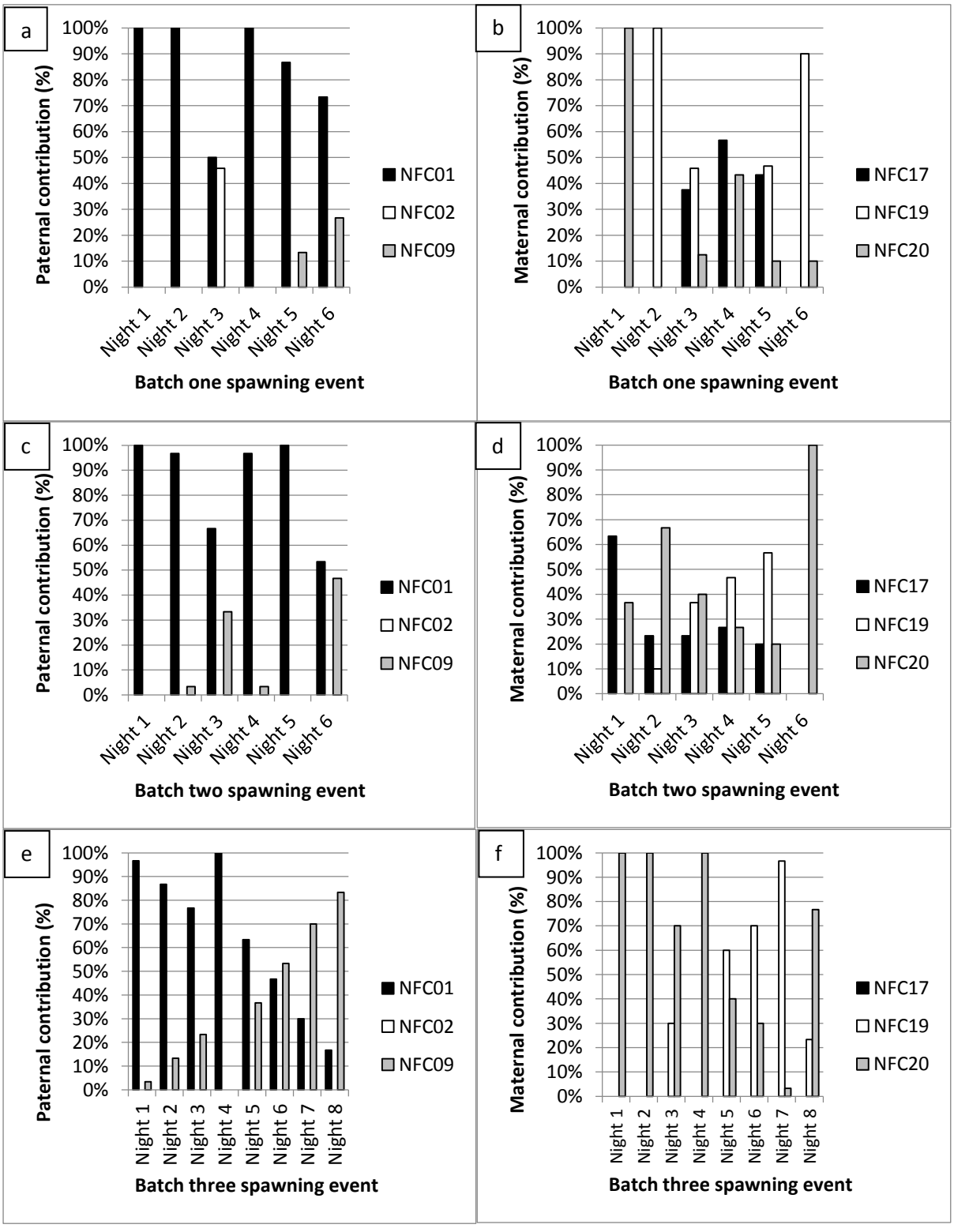

Figure 2

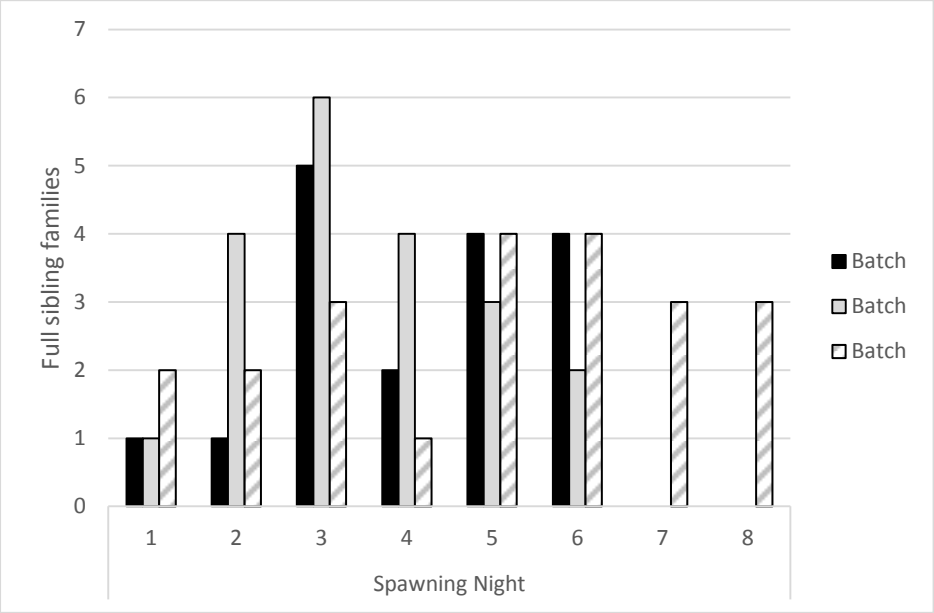

Figure 3