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Evaluation of reproductive strategies in captive California yellowtail (Seriola dorsalis) using genetic parentage analyses

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by

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DEDICATION

To my parents, who taught me the value of education, and did everything in their power to ensure I had a great one. Mummy and Diddy, this is for you!

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ABSTRACT

A good understanding of spawning dynamics of species in aquaculture is vital in order to maximize egg production and quality as well as efficient allocation of food and space resources. The breeding program of California yellowtail (CYT; *Seriola dorsalis* previously *Seriola lalandi*) at Hubbs-SeaWorld Research Institute (HSWRI) is set up such that up to 30 wild caught brood fish can broadcast spawn in a group setting, just as CYT reproduce in the wild. The population of brood fish were originally caught offshore from Southern California, and are maintained under ambient sea water and natural lighting conditions. The spawning season at HSWRI lasted from March to September during the 2013 and 2014 study period. Reproductive output from this population of cultured CYT was evaluated through microsatellite-based parentage analyses whereby the percent contribution of offspring was determined across all spawning events over two years.

Methods were first tested to determine the minimum sample size required to accurately describe the parental contributions in this specific group spawning environment. To do this, five spawns were selected from the 2013 season based on spawn volume, which was presumed to represent a high number of female parents thus representing the most complex spawning dynamic. For these five spawning events, there were 19 brood fish present, representing all possible parents. Actual assignment of samples sizes between 47 and over 300 was assessed for each spawn. Except in one instance, the parental contribution from sample size of 47 CYT offspring analyzed per spawn, was not statistically

significantly different than a sample size of over 300 offspring per spawn (average P-value = 0.65). Simulated subsampling via computational bootstrapping, and subsequent statistical analysis, indicated that a sample size of 30 offspring per spawn was adequate to accurately describe the parental contributions. Based on this information, a samples size of 47 was used so that samples from two spawns could be run on a single 96 well plate, including one negative control sample per spawn. This constitutes one of the first studies of sample size quality control for genetic parentage contribution for an aquaculture species.

Offspring were then analyzed from every spawning event (N = 130) over two spawning seasons to characterize spawning events of CYT. Analyzing all spawns during multiple spawning seasons allowed for determination of individual contribution levels, spawn pairings, and analysis of female fecundity. The breeding population consisted of 19 brood fish in 2013, and 37 in 2014; both years were roughly 50: 50 male – female. Brood fish added to the population in 2014 were smaller in terms of mass and total lengths. Spawning events tended to have one female contribute (primary female), with relatively equal paternal contribution during both the spawning events and season, for a possible lottery polygyny spawning system (Nunney, 1993). One female in particular contributed 40% of all offspring during the two years, spawning nearly every 5-6 days during the spawning seasons. The larger females (~21 kg) had an average batch fecundity of ~490,000 eggs per spawn, while smaller females (~8.4 kg) only spawned 35,000 eggs per spawn. Annual and batch spawning totals were

correlated to female mass ($P < 0.0005$). All smaller brood fish spawned fewer times in the season than larger brood fish. This work constitutes the first-ever study of reproductive strategy (lottery polygyny) and parental contribution for a carangid species at the individual-level over several spawning events.

INTRODUCTION TO THESIS

Applications for aquaculture vary from supplying marine organisms for the aquarium trade to wild stock replenishment. However, aquaculture is most commonly used to produce food for human consumption. Large scale commercial aquaculture has been an effective way to generate protein for human consumption since the 1960s, and has grown to account for 42% of global seafood harvested in 2012 (FAO, 2014). Since 2008, the aquaculture industry has provided over 50 million tonnes of seafood each year, increasing by an average annual rate of 3.2%. Of this total, marine finfishes accounted for 5.55 million tonnes in 2012 (FAO, 2014). However, as aquaculture facilities begin to perfect rearing methodologies, new candidate species are being targeted to diversify this growing industry. Candidate species for food aquaculture are usually defined as having fast growth, high quality flesh, high market value, and potential for complete life cycles in culture (Le François *et al.*, 2002).

Much research has been dedicated to achieving optimal growth and production for these species in an aquaculture setting, especially for marine fish. This has led to some aquaculture species being rendered sterile and/or otherwise modified to increase biomass production in culture (Hulata, 2001). Some aquaculture facilities focus on long-term sustainability by utilizing offspring from a pool of brood fish, in contrast to capture-based aquaculture (Ottolenghi, 2008). For all purposes of aquaculture listed above, it is crucial that a scientific understanding of reproduction and life-history is known for each candidate

species, as this will lead to the most effective management for these species in aquaculture.

A promising species for commercial culture in California is *Seriola dorsalis* (previously *S. lalandi*; FAO, USA 2011). This carangid (order: Perciformes) is also known as California yellowtail, yellowtail amberjack, yellowtail kingfish, and hiramasa. Recent publications using genetic data and analysis recommend the old scientific name *Seriola dorsalis* be resurrected for local *Seriola lalandi* found from southern Washington to Mexico (Baxter, 1960; Martinez-Takeshita *et al.*, 2015; Purcell *et al.*, 2015). We recognize these recent publications, but as the global species name was previously *Seriola lalandi*, many of our references and publications about life history refer to *Seriola lalandi*, and not specifically to the species found in the Northeast Pacific. For this work, we use the local species name *Seriola dorsalis*, as well as the local common name California yellowtail (CYT). *Seriola* spp. are high performance pelagic finfishes, defined here as having fast growth and high fecundity, and are found in tropical to sub-tropical globally. Fisheries data from the South Pacific show that in the wild, *S. lalandi* have roughly linear growth, from about 45 cm fork length at one year of age and plateauing at 120 cm fork length at approximately 15 years of age (Stewart *et al.*, 2004). Tag and recapture data of CYT indicate extensive movement in the ocean, from 80 km to 650 km (Baxter, 1960) and more than 2000 km straight line distance from *Seriola lalandi* from the South Pacific (Gillanders *et al.*, 2001).

In Southern California, CYT is a major target of the recreational fishing industry, and worldwide species in this genus are becoming increasingly important for aquaculture (Nakada, 2008; Abbink *et al.*, 2011). Due to its popularity, nutritional benefits, and high market value, the production of cultured *Seriola* has grown recently around the Pacific, including in Japan, Australia and New Zealand, to an industry with a market value worth over one billion USD per year (Poortenaar *et al.*, 2003; Nakada, 2008). Other *Seriola* spp. are already well represented in aquaculture, with the majority of the culture coming out of Japan and South Korea. At about 160,000 tonnes annually, *Seriola quinqueradiata* made up 57% of Japanese farmed finfish in 2003 (Cultured Aquatic Species Information Programme, 2005) and another closely related species, *Seriola rivoliana* has been in commercial production in Hawaii since 2011. Thus far in the United States, only one experimental CYT facility exists, however, high market value coupled with high performance make CYT a prime species for food aquaculture development in the U.S. as well.

CYT in San Diego, California

A unique opportunity exists for scientific research at Hubbs-SeaWorld Research Institute (HSWRI) in San Diego, CA, where CYT are currently being studied for commercial aquaculture potential, including an experimental breeding program. The primary brood fish in this program were collected from the wild off the coast of Southern California, and are housed in San Diego, CA, well within their natural range. The wild-caught CYT are kept in a single large tank, allowing reproductively mature males and females to reproduce freely. This practice

ensures a steady stream of eggs that can be collected during the spawning season and allows for generally "wild-like" or near-natural reproduction conditions. Unfortunately, unlike strip-spawning or hormonal injections, this method of wild-like spawning does not allow for easy parental assignment of offspring. Therefore, the parental contributions to distinct spawning events in this system are not previously known.

Understanding the spawning frequency of each brood fish would be useful for refining aquaculture production by culling non-contributors from the brood fish population or changing sex ratios to potentially improve reproductive output. Individual identification in a population can be done with the use of microsatellite genetic markers, which are inherited sections of genomic DNA that consist of multiple short tandem nucleotide repeats (e.g. CACACACA, GATGATGAT). These repeats can occur anywhere in the genome, but usually occur in non-coding regions for multi-cellular organisms. A region or point on a chromosome is called a locus (plural: loci): and in terms of microsatellites, the different regions where the repeats occur are also called loci (full definitions and explanations in Chistiakov *et al.*, 2006). Loci for microsatellites typically are species- or genus-specific, but due to high mutation rates of these loci, individuals in the population will have unique variations in their nucleotide repeat pattern, called an allele. It is these alleles from multiple microsatellites that allow for individual genetic assessment and relatedness studies, such as parentage analysis.

These alleles are inherited, one each from the male and female parent, and can be used to determine paternity and maternity (Chistiakov *et al.*, 2006;

Mojekwu and Anumudu, 2013). Parentage analysis can then be accomplished by calculating the probability that the offspring share the same alleles at multiple loci with the potential parental pool (in this case, the brood fish), and excluding parents who do not have these same alleles as the offspring. Several microsatellite loci have been identified and published in *Seriola* spp. and are known to be polymorphic in local CYT. These loci have successfully been used to determine subspecies, to find genetic correlations between growth rates and condition factors, and help support the splitting of *Seriola lalandi* into several species based on genetic distance (Miller *et al.*, 2011; Whatmore *et al.*, 2013; Martinez-Takeshita *et al.*, 2015; Purcell *et al.*, 2015).

In the case of HSWRI, the microsatellite genetic markers were used here to determine the percent contribution of each brood fish spawning in the experimental breeding program. In this collaborative project with the Southwest Fisheries Science Center (SWFSC), we aimed to present the first-ever record of individual spawning contribution of CYT over multiple spawning events and seasons. The information gathered from this research on spawning dynamics will allow for enhanced management of the brood fish directly at HSWRI, and also provide valuable information for *Seriola* reproductive dynamics not previously described. Furthermore, with the use of this breeding program and its relatively large output of progeny, it was possible to ground-truth the methods used in this thesis, specifically those related to sub sampling.

Questions and Hypotheses

Due to its ability to reproduce in captivity and a high market value, CYT is a great candidate species for aquaculture. Management improvement and efficient culture of CYT will depend in part on the reproductive contribution of the individual brood fish. At HSWRI these brood fish are allowed to freely reproduce in large spawning events, similar to those seen in the wild. This method allows closure of the wild fish harvesting cycle, by producing the next generation in captivity. However, with this method the only practical way to determine parental contribution is with the use of genetic tools to identify individuals as parents of offspring. Using these tools, it is possible to have a higher level of understanding of wild *Seriola* spp. by examining reproductive strategies that are very difficult to observe directly in the wild. Quantifying contribution will also allow for extrapolated calculations for estimates of fecundity (batch and annual) for each female. This research will also directly affect the management of the fish in this breeding program. Specifically, the goal of my research is to address the following questions by testing the associated hypothesis:

1. How many samples need to be analyzed to accurately understand the patterns of brood fish contribution?

   H_0: No clear patterns in sample size will emerge after analyzing all samples available.
2. What is the contribution of production of offspring of each brood fish in the tank on an annual basis?

H_0: Each brood fish kept in the system will contribute equally to the production of offspring over the course of a season.

3. What is the contribution of production of offspring of each brood fish during each discrete spawning event?

   H_0: Each brood fish kept in the system will contribute equally to the production of offspring during each discrete spawning event.

4. Does female fecundity (batch and/or annual egg production) correlate to: female mass; spawning intervals; environmental factors (water temperature, day length, lunar cycles)?

   H_0: Egg production will not significantly correlate to female mass, spawning intervals or any environmental parameter tested.

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Chapter 1

Methods for genetic parentage assignment of California yellowtail with an emphasis on sample size requirements.

Abstract

Genetic samples were collected from five spawning events of cultured California yellowtail (CYT; *Seriola dorsalis* previously *lalandi*) in 2013. Genetic samples consisted of just-hatched larvae preserved in 100% EtOH. The spawning tank contained 11 male and 8 female fish. These five spawns occurred roughly one month apart throughout the spawning season, and parental contribution was genetically identified using a panel of nine polymorphic microsatellites for *Seriola* spp. The first 47 larvae were initially analyzed for proportional contribution from each brood fish. Subsequently an additional ~300 larvae were analyzed and assigned parentage depending on the number of larvae available. Increasing sample size had no effect on overall proportions of parental contribution observed (average for all spawns $P = 0.65$). For this specific breeding system of CYT a sample size of 47 progeny is adequate to proportion contribution of individual fish to a spawn. Bootstrap resampling of the data using simulated sample sizes ($n = 10 – 300$) revealed that 30 offspring per spawn achieved proportional parental assignment to 95% confidence levels.

Introduction

Determining appropriate sample size is an important consideration in scientific planning and sample design. With advancement in mathematics, determining sample sizes needed from an affected populated has become relatively easy for parametric tests. Power analyses were designed to ensure that the proper number of subsampled individuals (n) from a total population (N) are analyzed to have the power (P-value) to detect significant structuring with the population. These types of power analyses can either generate the number of individuals needed to subsample, or if subsampling values are already set, the statistical power of the test (e.g. t-test, ANOVA). For more complicated analyses that require nonparametric testing, statistical programs are available that can capture the complexity for subsampling.

In practical use, it is often desirable to keep sample sizes to a minimum. This may be due to a range of factors, including time limitations in the field or lab, cost of equipment, or rarity of specimens available for study. In molecular genetic studies, cost of reagents and time associated with lab work are often the primary drivers to optimize sample sizes, though availability of samples may also be a factor. Population genetics studies utilizing microsatellite markers typically conclude that 90-95% of the genetic diversity of a population is described after analyzing 30 individuals, with little information being gained as sample size increases (Lu-Na and De-Xing, 2004; Miyamoto *et al.*, 2008; Hale *et al.*, 2012). Simulation studies of stable populations have suggested that subsampling at least 480 individuals is needed to correctly assess census populations over 10,000

(Tallmon *et al.*, 2010). The need to justify the sample size is desirable for studies of parentage in broadcast spawning fishes due to the large number of samples available for analysis (e.g. eggs and larvae) as well as the number of potential parents within the spawning event.

While these studies do focus on genetic markers, the motivation behind them differs from genetic studies of parentage. Rather, the investigators focus on detection of all alleles present in a population. In studies that have focused on microsatellites used to determine parentage, subsampling values have ranged from 60 – 810 with no mention of how sample size was determined (e.g. Estoup *et al.*, 1998; Vandeputte *et al.*, 2004; Hauser *et al.*, 2011; Gruenthal and Drawbridge 2012; Liu *et al.*, 2012), rather, great emphasis has thus far been put into calculating numbers of loci or alleles necessary for correct parental assignment (e.g. Estoup *et al.*, 1998; Bernatchez and Duchesne, 2000; Dakin and Avise, 2004; Liu *et al.*, 2004; Briñez *et al.*, 2011).

The use of animals in an aquaculture setting is an ideal test area for the evaluation of the necessary sampling scheme to quantify patterns of parentage. Using microsatellites as a genetic marker for determining parentage, confidence in subsampling was calculated using the *Seriola dorsalis*, previously *Seriola lalandi*, experimental breeding system at Hubbs-SeaWorld Research Institute in San Diego, California (Purcell *et al.*, 2015). Hereafter *Seriola dorsalis* may be referred to as California yellowtail (CYT). This closed population ensured that 100% of potential mothers and fathers present and identifiable for the 2013 spawning season. The aim of this study was to take a more in-depth examination

at how the number of offspring analyzed predicts actual parental contribution to a single spawning event. Determination of optimal sample size for analyzing parentage in a is needed due to the sheer number of available samples per spawn as well as the number of spawns produced per season. These parentage assignments can ultimately be used to fine-tune broodstock selection and husbandry practices for CYT.

Materials and Methods

Sample collection

All CYT broodstock were housed in a 140 m^3 fiberglass tank at Hubbs-SeaWorld Research Institute (HSWRI), fed by recirculating ambient seawater and exposed to natural light cycles (Stuart and Drawbridge, 2013). Seawater was filtered and recirculated at a rate to allow for 3-6 turnovers per day with feedings 3-5 times a week. Prior to introduction to the tank, broodstock were individually pit-tagged, genetically sampled, and sexed. Eight females and 11 males were present in the breeding population in 2013 at the time of this study. Annually, all brood fish were also measured for mass (kg), total length (mm) and standard length (mm). Prior to this study, fin-clips (5-10 mm) were taken from each brood fish for future genetic analysis and stored separately in 100% non-denatured ethyl-alcohol (EtOH) until processing.

During the spawning season (March – September), 500 µm mesh egg-traps were placed in a collection basin (sump), through which seawater was pumped before sterilization and drainage. The mesh traps were checked every morning for the presence of eggs. In the event of a spawn, all eggs were collected and enumerated volumetrically in 10 L graduated cylinders. Volumes were converted to total eggs using average CYT egg density of ~500 eggs / ml. A subsample of approximately 3 ml of fertilized (floating) eggs was grown to hatching from each spawning event. During the 2013 spawning season there were 69 spawns from which at least 50 zero-days-post hatch (dph) yolk-sac larvae

(YSL) offspring were collected for genetic analysis. YSL were euthanized in an overdose of MS-222 treated seawater and stored in 100% non-denatured EtOH until processing.

At the end of the spawning season, all specimens were transported to the National Oceanic and Atmospheric Administration Southwest Fisheries Science Center (NOAA SWFSC) in La Jolla, California for genetic analyses. Fin-clips from potential parents and all YSL were genotyped using *Seriola*-specific microsatellite markers (Table 1). After an initial subset of 47 YSL (one-half 96-well plate including a no template control) were analyzed from each spawn (methods below), five of these spawns were identified as having multiple female parents. Several hundred YSL collected from each of these five spawns were used to examine sample size requirements for accurate parental proportional assignments and were used to explore the statistical validity of the genetic methods of parentage in greater detail. These spawns, occurred on April 29, May 17, June 21, July 26, and August 26, 2013.

DNA extraction and PCR amplification

DNA was extracted from an approximately 3 x 1 mm clip of fin tissue for broodstock or from entire YSL in 0.2ml 8-well strip tubes (one individual per well). Only whole YSL were chosen; no DNA was extracted from partial or damaged YSL to ensure that each YSL was genotyped only once. One to two no template negative controls were included per 96 wells (milli-Q water, EMD Millipore, Darmstadt, Germany). All glass-wear and dissection equipment was

carefully washed before and between handling each fish and nitrile gloves were worn to prevent cross-contamination. Extractions were performed by pipetting 150 µl of a 10% (w/v) Chelex resin solution (Bio Rad Laboratories Inc., Berkeley, CA) into each well, incubating the mixtures at 60 °C for 20 minutes, and boiling at 103 °C for 25 minutes. Supernatant was used directly for polymerase chain reaction (PCR) amplification of microsatellite regions. Upon completion of genotyping, all DNA extractions were stored at -80 °C.

Previously published microsatellite loci (Table 1) were chosen based on ongoing research at the SWFSC, which identified a panel of nine loci that were diverse enough to distinguish parentage in the larvae among brood fish parents at HSWRI (Purcell and Hyde, unpublished data). Loci were pooled for multiplex PCR in the following combinations: 1) Sequ 38; 2) Sequ 77, Sdu gA3D, Sdu 46 and Sdu 4; and 3) Sequ320, Sequ230, Sdu 10 and Sdn 06. DNA amplification were completed in 11 µl volumes containing 10X PCR Buffer (Appendix 1), 2 mM each dNTP (Bioline, Taunton, MA; Appendix 1), bovine serum albumin (BSA, Appendix 1), 10 mM primer pairs, 5-6 units of *Taq* polymerase (New England Biolabs, Ipswich, MA), and milli-Q water, and 1 µl sample DNA. Forward primers for all PCRs were marked with fluorescent labels for genotyping via fragment analysis (Integrated DNA Technologies, Coralville, IA). DNA extraction and PCR set up were all completed in a clean, pre-PCR lab, before being moved to a PCR laboratory for amplification and further processing.

All three PCRs had the following conditions: denaturation at 94 °C for 4 minutes, followed by 35 cycles of 94 °C for 30 s, annealing between 53-59 °C for

35 s, and elongation at 72 °C for 30 s, and a final extension for 5 minutes at 72 °C, followed by a 4 °C hold until further analysis. The annealing temperature was 53 °C for PCR 1, 55 °C for PCR 2, and 59 °C for PCR 3 (all information for PCRs in Appendix 2).

Genotyping

The three PCRs were further multiplexed for genotyping, combining PCRs 1 and 2, with product from PCR 3 run separately. PCR product was diluted 1:25 in milli-Q water for use in further laboratory processes. The first dilution plate contained 23 µl water with 1 µl each from PCR 1 and 2. The second dilution plate contained 24 µl water and 1 µl from PCR 3. In a clean 1.5 ml tube, 12 µl of GeneScan 500 ROX size standard was added to 1000 µl HiDi Formamide (Applied Biosystems, Waltham, MA). Nine µl of this HiDi/ROX mix, and 1 µl of diluted DNA products were added to each well in a clean 96-well semi-skirted plates. Semi-skirted plates were covered with septa silicon sealing mats (Applied Biosystems), vortexed briefly and spun down. DNA was denatured at 95 °C for 5 minutes followed by 8 °C for 5 minutes. Immediately after denaturing, semi-skirted plates were loaded into plate-holders, and placed into 3730 DNA Analyzer with 50 cm capillary array filled with Pop-7 polymer for genotyping via fragment analysis (Applied Biosystems).

Post fragment analysis, visualization and sizing of microsatellites was done using GeneMapper v4.0 (Applied Biosystems). Microsatellites were analyzed using the Microsatellite Default Analysis Method, CYT panel of

microsatellites (Table 1), red size standard dye, and the size calling curve for the ROX 500 size standard modified to incorporate only the following standard peaks (in bp): 75, 100, 139, 150, 160, 200, 300, 340, 350, 400, 490, 500. Alleles were scored automatically and visually confirmed; to ensure replication in scoring and minimize errors, all plates were scored by the author, and were viewed multiple times for correction.

Scored peaks were then exported as a text files, and transferred to Microsoft Excel 2010 for rounding and standardization. Microsatellite standardization was conducted by graphing loci individually to linear best-fit lines. The resulting equation was applied to alleles from that locus, and then rounded to the nearest integer to create a size reference bin. This ensured all scored alleles were properly binned when inter-allele distance differed from expected repeat distance (e.g. 2 or 4 bp repeats) due to variation in fragment mobility due to sequence composition.

Converted allele calls were used for parentage assignment, using exclusion (Jones and Arden, 2003) algorithms on Cervus v3.0.6 software (Kalinowski *et al.*, 2007, www.fieldgenetics.com). Allele frequency analysis was completed to assess Hardy-Weinberg Equilibrium (HWE), using a minimum expected frequency of 5, and Bonferonni correction to evaluate significance of HWE. Simulations of parentage were assessed for 10,000 offspring, using three increasing methods of stringency for the simulation. For the first pass, the confidence was calculated using LOD with 80% relaxed confidence levels, 95% strict confidence levels, and minimum typed loci set to 5; the second pass only changed the minimum typed

loci to 6; and in the third pass the minimum typed loci was changed to 6 and the relaxed confidence level was changed to 90%. For known parentage assessments, proportions of parents sampled was set to 1.0. Using these settings, 100% of offspring were able to be assigned for all simulations at the 95% confidence level. Parentage assignment was completed using the allele frequency file, and one of the simulations, to assign parents with either broodstock sexes unknown or known, respectively. Parentage files were compared to ensure 100% agreement between all assignment methods. In events where assignment did not agree, further analysis was completed using the most conservative method of assignment.

Data analysis

Mass (kg) and total length of brood fish were first tested for normality using a Shapiro-Wilks test. This was done in R version 3.0.2. Both masses and lengths were normally distributed (P-values = 0.19 – 0.91) for the 2013 spawning season.

A theoretical sample size was calculated (e.g. power analysis) to determine how many YSL should be analyzed for statistical significance. This was completed using a sample size calculator aimed at allelic contribution. Using a freely available online sample size calculator from AusVet Animal Health Services (http://epitools.ausvet.com.au/content.php?page=1Proportion), inputs for genetic parameters were conservatively chosen. Estimated proportion was set to 0.5 (e.g. equal allelic contribution from each brood fish), confidence level set to

95%, and desired precision of estimate set to 0.05 (e.g. P = 0.05). The sample size calculator also assumed an infinite population (e.g. unlimited YSL samples). Using these values in the sample-size calculator, the sample size needed to estimate true proportions of allelic contribution was n = 385.

Effective population for females (Ne_f), males (Ne_m), and both males and females (N_e) were calculated for each spawning event and season using Microsoft Excel 2010 and the following equations from Gold *et al.* (2008):

$$N_e = \frac{4 * Ne_f * Ne_m}{(Ne_f + Ne_m)}$$

Where,

$$Ne_{f,m} = \frac{1}{\sum_{k=1}^{n_{f,m}} q_k^2}$$

Where,

$n_{f,m,e}$ = number of spawning females, males, or both

q = proportion of contribution of each female or male

Values for q were calculated using proportion of offspring genotyped from each spawn. For methods testing, this calculation was done twice, first with the initial 47 YSL analyzed, then with the additional 278 – 330 YSL analyzed.

Binomial tests were conducted between values obtained for proportions of initial parental contribution (n = 47 YSL) and proportions of final parental contribution (n = 325 – 377 YSL). For binomial tests, proportions from initial

YSL were compared to “true” proportions, with “true” (in quotes) being defined as the proportion seen from the grand total of YSL genetic samples examined from each spawn. Each of the spawning events had 100,000s of eggs, analysis of all larvae would be cost and time prohibitive. However, analyzing every genetic sample stored was the closest to getting statistically true results, and thus put into quotes. In order from earliest to latest spawn date, the total numbers of YSL analyzed were: n = 357, 355, 366, 377, and 325. These totals represent the entire amount of genetic samples stored in EtOH, and were the spawns which contained the most preserved YSL from the year.

After parentage assignment, the data were bootstrapped using R software for simulated sample sizes. Each spawn was split into male and female parent data files, and these data files were bootstrapped 10,000 times, with final n-values set to: 10, 20, 30, 40, 47, 60, 100, 150, 200, 250, and 300. From the bootstrapped data, averages and 95% confidence intervals were calculated for both males and females. Binomial tests were conducted to determine whether parental contribution levels of resampled pools were different from “true” proportions.

Table 1: Microsatellite primer pairs, GenBank accession numbers, relevant publications, proper annealing temperatures, and fragment lengths for multiplexed PCR of CYT at HSWRI.

Table 1 -- *Seriola* spp. microsatellite primer pairs used

Locus	Forward (F)	Reverse (R)	Accession number	Annealing temperature	Publication	Approx. fragment length (bp)
Sequ38	CCATTACAATTTGTCTCTC	CTTATCAACACACGAGCG	AB098518	53 °C	Ohara *et al.*, 2003	100-145
Sequ77	CCTACACATGCACATGAA	CAAGGCTGATACGTCATG	AB098519	55 °C	Ohara *et al.*, 2003	135-190
Sdu gA3D	CTCAACATGAGAGGCAACG	GCATGGCTTCATGGGAAGG	DQ435602	55 °C	Porta *et al.*, 2009	140-180
Sdu 46	GCAGTGTGAGCCATACATTAC	CTACAGGACAAAAGCCATT	DQ883580	55 °C	Renshaw *et al.*, 2007	220-260
Sdu 4	GGAAATAGTTTGGATCACGCTGG	GGATGCTCAGTGAAGTTGTGC	DQ468084	55 °C	Renshaw *et al.*, 2006	270-310
Sequ320	GACAGGGTAAGAAACGAAAC	GACAATGACCAAAGCTGCC	AB508215	59 °C	Ohara *et al.*, 2005	90-140
Sequ230	CTCCAGAAACGCCACATAAC	AAGCAAACCGCACAAGTAGG	AB508202	59 °C	Ohara *et al.*, 2005	150-165
Sdu 10	CCAAGTCCTCCGCTACTACCAT	CCTTGTGGATGACCTGTTTG	DQ468090	59 °C	Renshaw *et al.*, 2006	250-310
Sdn 06	GGGTTTGTGCTGTGAGTG	TCCGTCTGTCTTTTCCTGT	N/A	59 °C	Nugroho & Taniguchi, 1999	300-330

Results

Spawns for sample size validation were chosen based on the number of archived samples, as well as the requirement that more than one female contributed to the spawning event based on preliminary analysis of 47 YSL. These spawns occurred on April 29, May 17, June 21, July 26, and August 26. All microsatellite loci were in HWE. Using a freely available sample size calculator, sub sampling of all five spawns should have been n = 385 offspring, to obtain a P-value = 0.05.

The proportional contribution from each brood fish is consistent among all five spawns whether sample sizes of 47 or 300 analyzed (Figure 1). Indeed, the proportions between the first 47 YSL (first one-half of a 96-well plate processed; half-plate) were not significantly different from the maximum total sample size, except for one instance, indicated by the "*" in Figure 1, which corresponds to the bolded value in Table 3. In this instance, one male brood fish with pit tag number 083-070-054, contributed 17% to the first 47 YSL, but only 7.3% out of the total, resulting in P = 0.019. Overall, proportions from females were closer to "true" values, than from males, with primary and secondary females clearly identified among the first 47 samples. This is also seen with P-values from contributing females at or near 1.000, and females with no contribution in both the first 47 YSL and the "true" value indicated as N/A (Table 3).

For the spawn that occurred on April 29th, the primary female contributor was 061-363-804 at 60.3% YSL, followed by female 083-103-352 at 39.2% YSL.

Two other females contributed less than 1% (Figure 2). This is consistent with the calculated effective population of females (Ne_f) of 1.82 from the first 47 YSL, and 1.93 from the total sample (Table 2). These percentages of female contribution were observed at all levels of simulated sample size, via bootstrapping, however at lower levels of simulated sample size 95% confidence intervals (CI) were much larger than at higher levels. For example, for the primary female in this spawn, the 95% CI at the lowest level of simulated sample size, n = 10 YSL, was ± ~0.3, but decreases to ± ~0.02 at the highest simulated sample size, n = 300 (Table 4). As the simulated sample size increase for each spawn, the 95% CI bars become closer to the mean, showing a truer representation of the "true" proportions (Figures 2-6, all listed in Appendices 3 and 4).

From the spawn on May 17, female 083-026-876 and 083-103-352 each contributed 49.8% of the YSL to the spawning event (Figure 3), agreeing with Ne_f = 2.0 (Table 2). Female 061-375-363 contributed almost every YSL from the June 21 spawning event, at 94.7% (Figure 4), and similarly female 061-363-804 was prominent for the July 26 spawning event at 99.4% (Figure 5). These two spawns also had an Ne_f of ~1 (Table 2). Female 083-027-609 is the primary female spawning 76.3% of the YSL, and female 061-363-804 contributing a secondary amount with 23.4% of the YSL from August 26 (Figure 6). All other females shown in figures did contribute to the spawning event but at much lower levels, in some cases, less than 1% of the spawning event. All male or female brood fish not individually graphed had zero contribution to the spawn.

From each spawn analyzed, no male brood fish contributed more than 25% of the YSL to a spawn, and the mean contribution from each male to a spawning event was ~10% (Figure 2-6), or roughly equal contribution from the participating male brood fish. This results in a calculated male contribution for each spawn being 3.65 – 8.28 (Table 2). All spawning events appear to have a primary female, or in one case, two females that contributed nearly equal proportions (May 17, 2013, Figure 3). The remaining analyzed spawns all had one female that contributed > 70% of the YSL (Figure 4-6), with two spawns having one female contribute ~100% of the YSL from that spawning event (Figure 4-5). While each spawn seemed to have a primary female contributor, at each spawning event it was a different female who took on this role.

All simulated sample sizes quickly found the "true" mean, and had decreasing 95% CI as the simulated sample size becomes closer to the total number of YSL analyzed. Calculated census and effective population numbers also quickly find "true" values, with the first 47 YSL effective populations nearly matching "true" effective populations (Table 2). A complete list of means and 95% CI from all brood fish, at each spawn and each simulated sample size, are available in Appendix 3.

Figure 1: Frequency of contribution from first 47 yolk-sac larvae (YSL) assigned parentage (black), and then total processed (gray) from each spawn processed. Pit tag numbers before the vertical line indicate female brood fish, those after indicate male brood fish. The X-axis represents brood fish pit-tag number. Each graph represents different spawn date, and all light gray values were calculated from different totals (N). A) Spawn date from April 29 with N = 357, B) spawn date from May 17 with N = 355, C) spawn date from June 21 with N = 366, D) spawn date from July 26 with N = 377, E) spawn date from August 26 where N = 325.

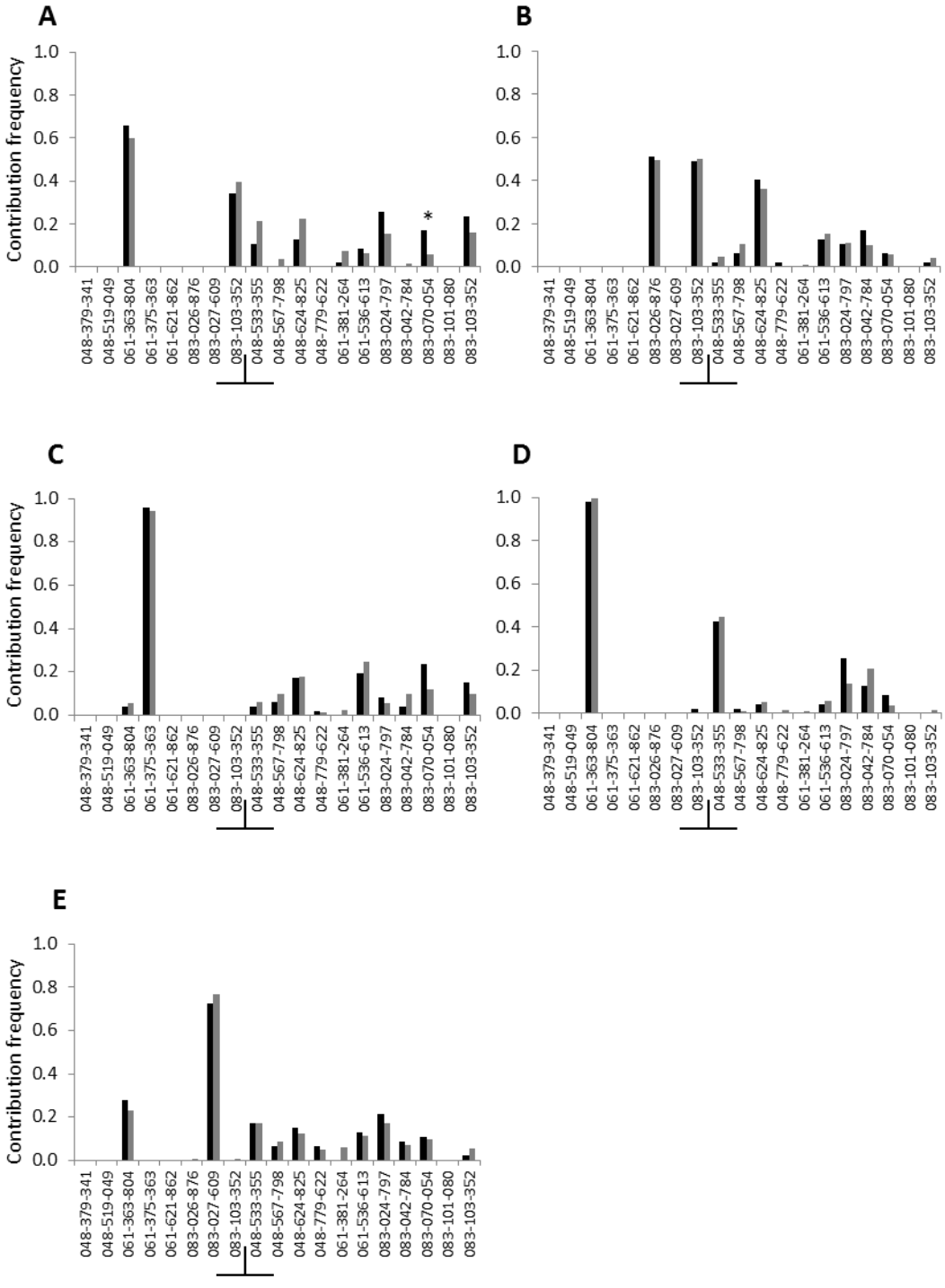
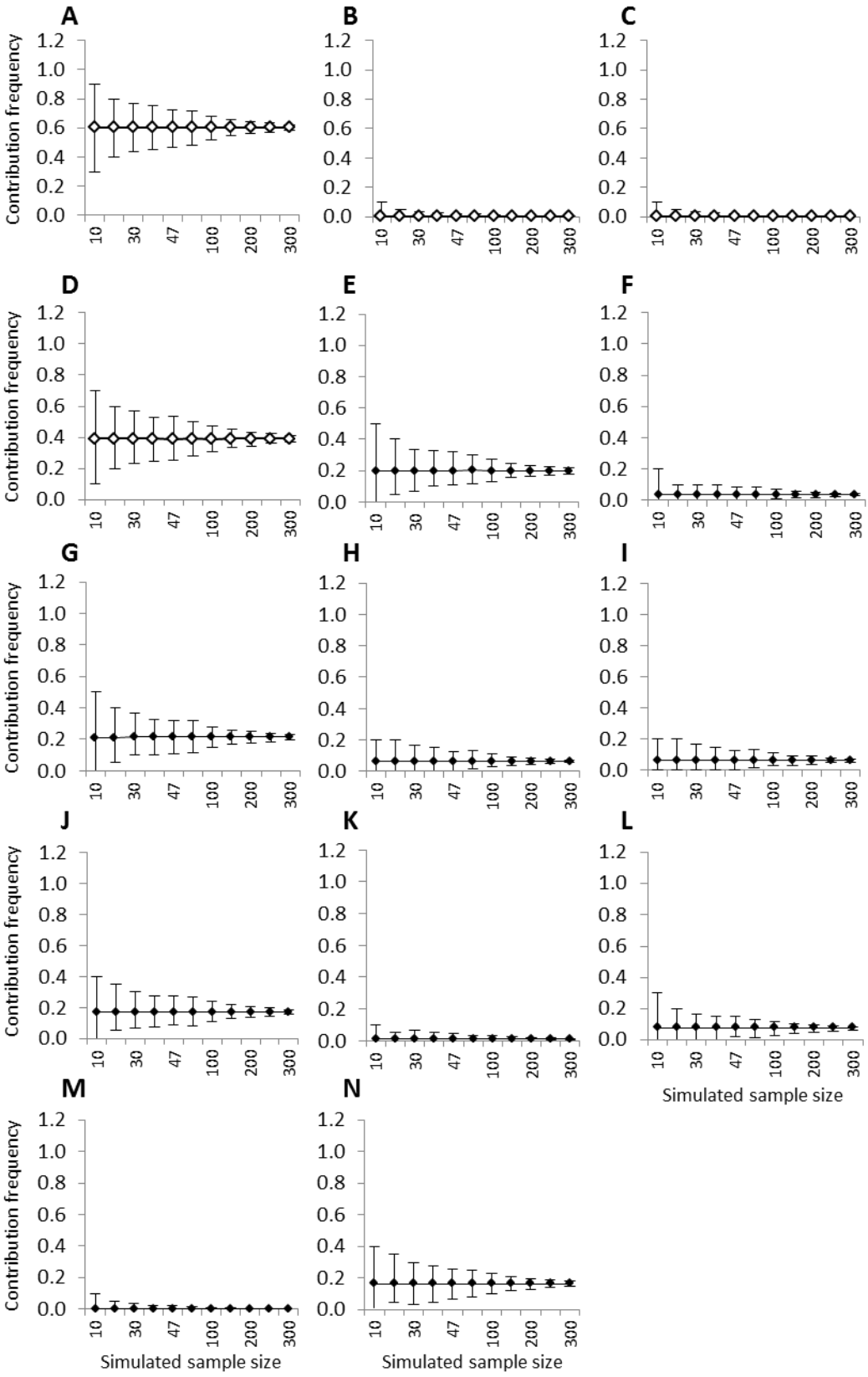

A
B
C
D
E
Contribution frequency
1.0
0.8
0.6
0.4
0.2
0.0
*
048-379-341
048-519-049
061-363-804
061-375-363
061-621-862
083-026-876
083-027-609
083-103-352
048-533-355
048-567-798
048-624-825
048-779-622
061-381-264
061-536-613
083-024-797
083-042-784
083-070-054
083-101-080
083-103-352

Figure 2: Mean frequency of contribution to spawn, from 10,000 bootstrap replicates at various simulated sample sizes, for CYT spawn occurring on April 29. Error bars represent plus/minus 95% confidence intervals of the mean. Simulated sample sizes were n = 10, 20, 30, 40, 47, 60, 100, 150, 200, and 300. All broodstock that contributed were represented, with empty squares representing females (A-D) and filled black squares representing males (E-N). Graphs each represent one brood fish by pit-tag number, with A) 061-363-804, B) 061-621-862, C) 083-027-609, D) 083-103-352, E) 048-533-355, F) 048-567-798, G) 048-624-825, H) 061-381-264, I) 061-536-613, J) 083-024-797, K) 083-042-784, L) 083-070-054, M) 083-101-080, and N) 083-103-352.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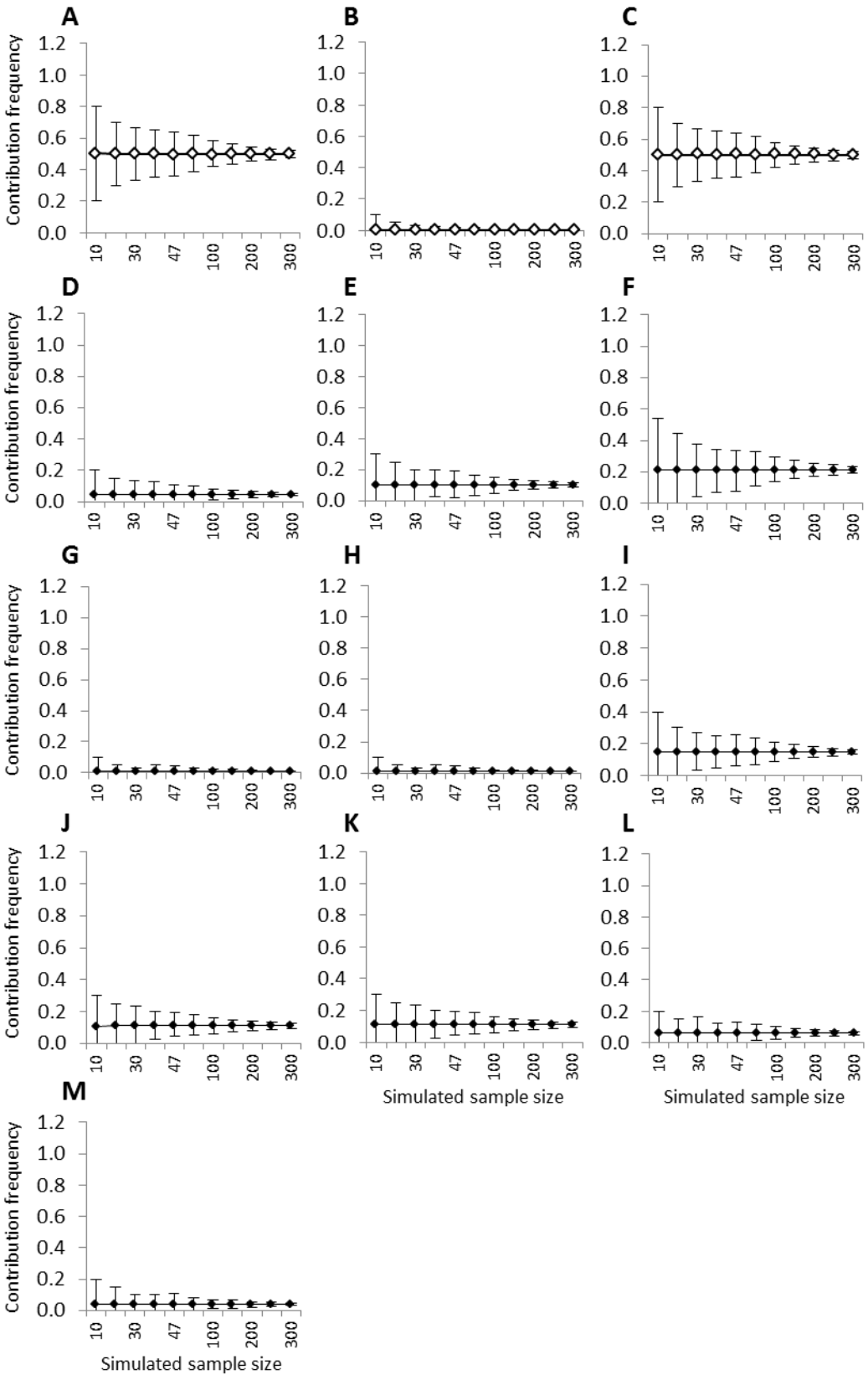
N
Contribution frequency
0.0
0.2
0.4
0.6
0.8
1.0
1.2
10
30
47
100
200
300
Simulated sample size

Figure 3: Mean frequency of contribution to spawn, from 10,000 bootstrap replicates at various simulated sample sizes, for CYT spawn occurring on May 17. Error bars represent plus/minus 95% confidence intervals of the mean. Simulated sample sizes were n = 10, 20, 30, 40, 47, 60, 100, 150, 200, and 300. All broodstock that contributed were represented, with empty squares representing females (A-C) and filled black squares representing males (D-M). Graphs each represent one brood fish by pit-tag number, with A) 083-026-876, B) 083-027-609, C) 083-103-352, D) 048-533-355, E) 048-567-798, F) 048-624-825, G) 048-779-622, H) 061-381-264, I) 061-536-613, J) 083-024-797, K) 083-042-784, L) 083-070-054, and M) 083-103-352.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Contribution frequency
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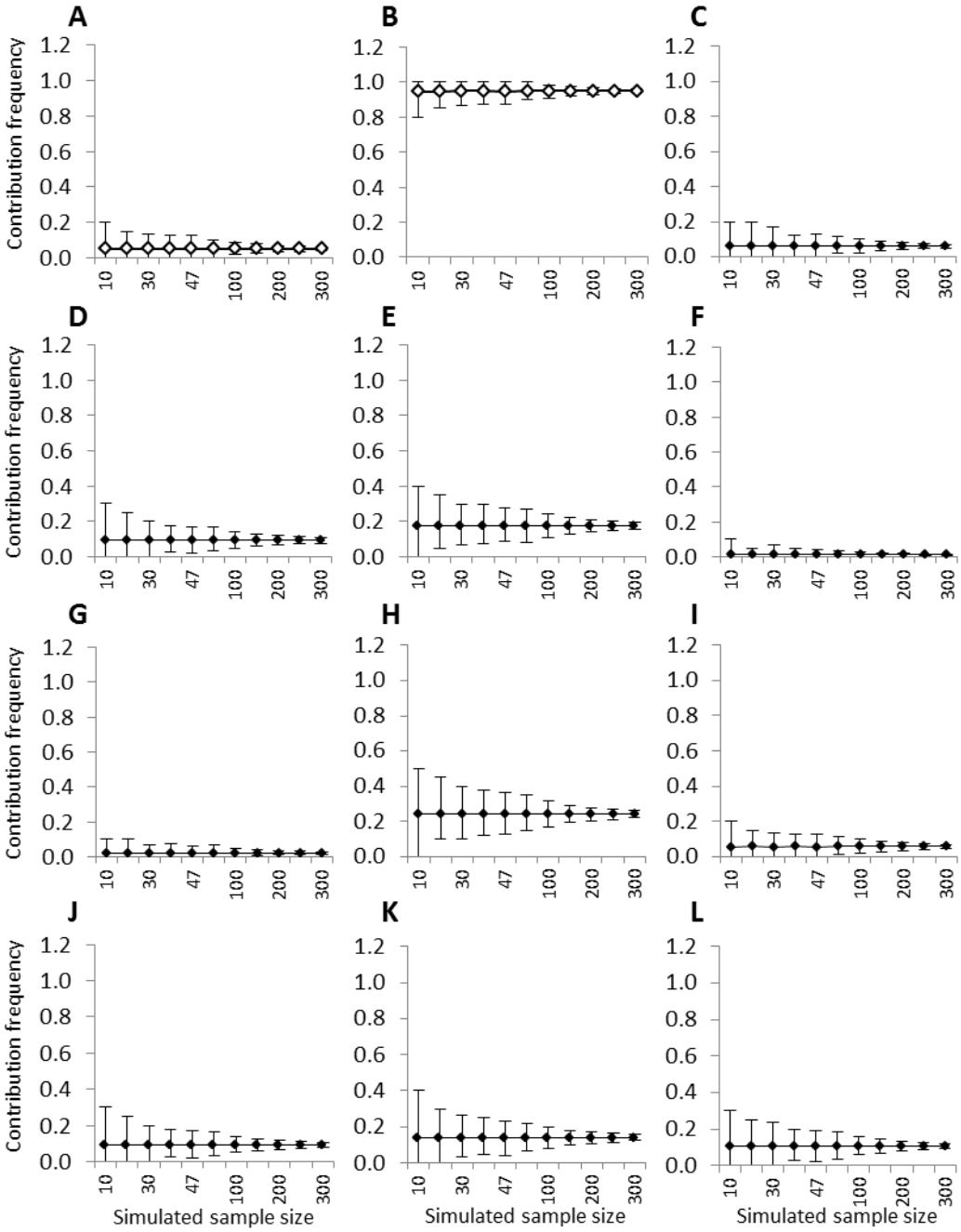
1.0
0.8
0.6
0.4
0.2
0.0
10
30
47
100
200
300
Simulated sample size

Figure 4: Mean frequency of contribution to spawn, from 10,000 bootstrap replicates at various simulated sample sizes, for CYT spawn occurring on June 21. Error bars represent plus/minus 95% confidence intervals of the mean. Simulated sample sizes were n = 10, 20, 30, 40, 47, 60, 100, 150, 200, and 300. All broodstock that contributed were represented, with empty squares representing females (A-B) and filled black squares representing males (C-L). Graphs each represent one brood fish by pit-tag number, with A) 061-363-804, B) 061-375-363, C) 048-533-355, D) 048-567-789, E) 048-624-825, F) 048-779-622, G) 061-381-264, H) 061-536-613, I) 083-024-797, J) 083-042-784, K) 083-070-054, and L) 083-103-352.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J
K
L
Contribution frequency
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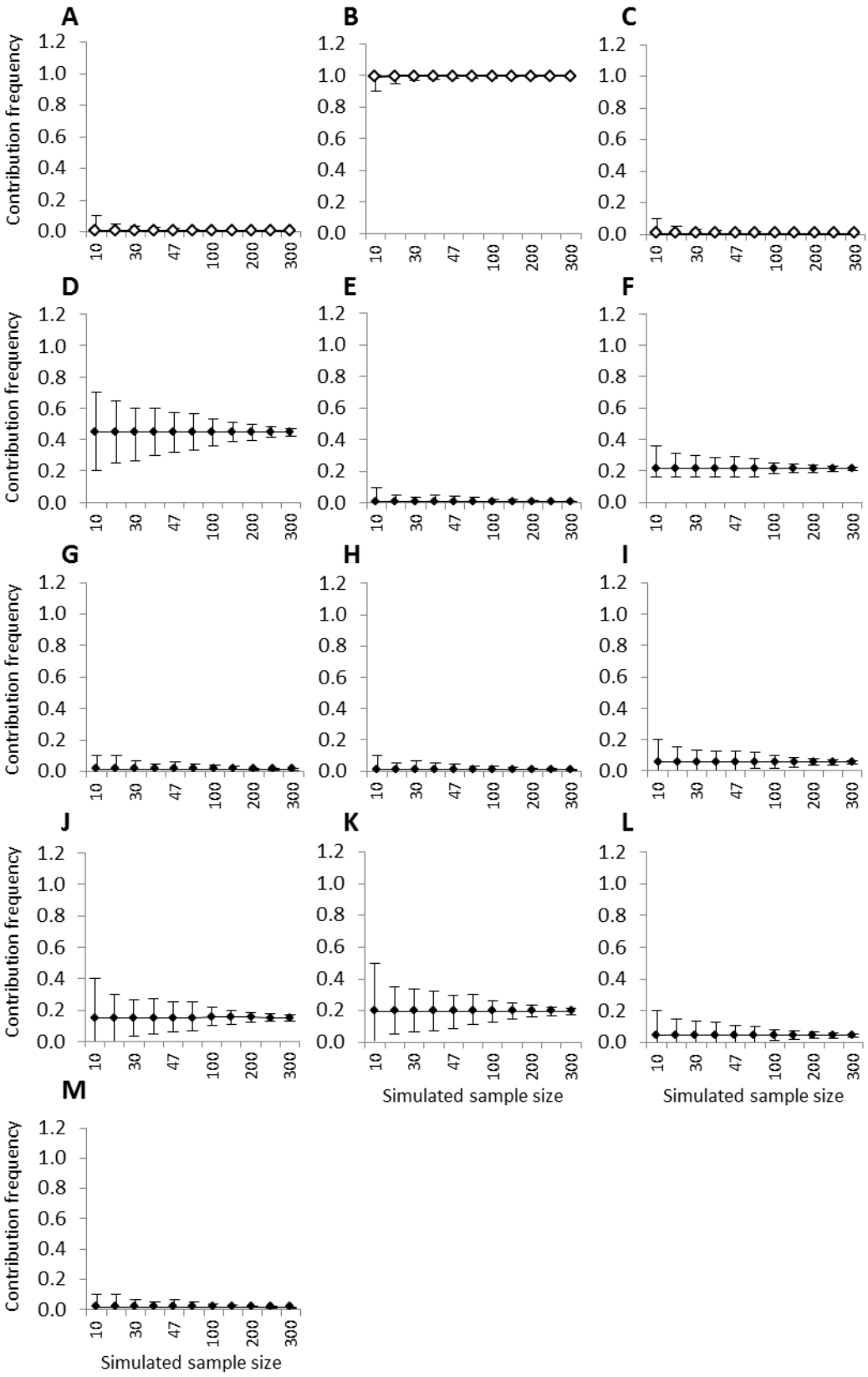
1.0
0.8
0.6
0.4
0.2
0.0
10
30
47
100
200
300
Simulated sample size

Figure 5: Mean frequency of contribution to spawn, from 10,000 bootstrap replicates at various simulated sample sizes, for CYT spawn occurring on July 26. Error bars represent plus/minus 95% confidence intervals of the mean. Simulated sample sizes were n = 10, 20, 30, 40, 47, 60, 100, 150, 200, and 300. All broodstock that contributed were represented, with empty squares representing females (A-C) and filled black squares representing males (D-M). Graphs each represent one brood fish by pit-tag number, with A) 048-379-341, B) 061-363-804, C) 083-103-352, D) 048-533-355, E) 048-567-798, F) 048-624-825, G) 048-779-622 H) 061-381-264, I) 061-536-613, J) 083-024-797, K) 083-042-784, L) 083-070-054, and M) 083-101-080.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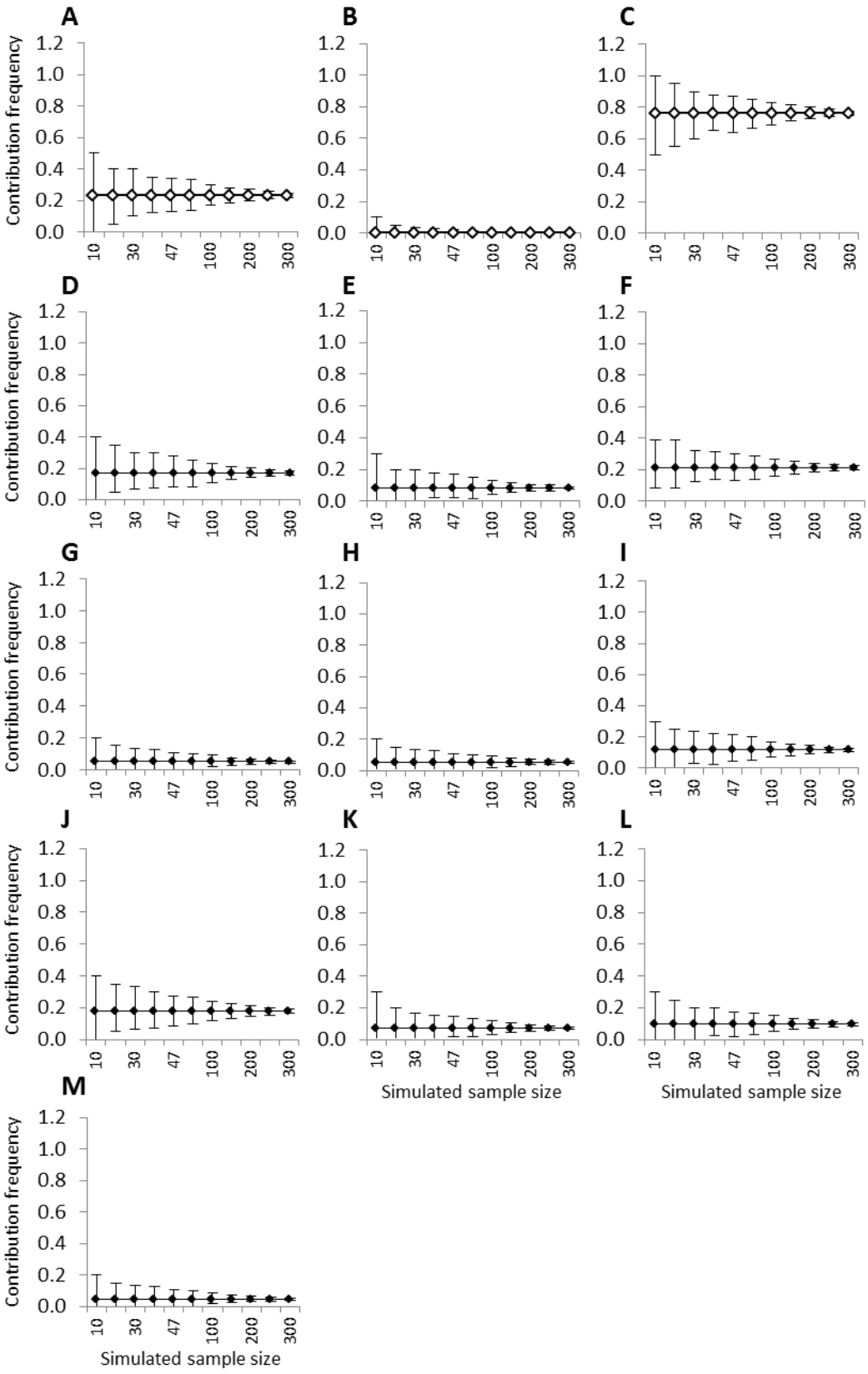
J
K
L
M
Contribution frequency
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10
30
47
100
200
300
Simulated sample size

Figure 6: Mean frequency of contribution to spawn, from 10,000 bootstrap replicates at various simulated sample sizes, for CYT spawn occurring on August 26. Error bars represent plus/minus 95% confidence intervals of the mean. Simulated sample sizes were n = 10, 20, 30, 40, 47, 60, 100, 150, 200, and 300. All broodstock that contributed are represented, with empty squares representing females (A-C) and filled black squares representing males (D-M). Graphs each represent one brood fish by pit-tag number, with A) 061-363-804, B) 083-026-876, C) 083-027-609, D) 048-533-355, E) 048-567-798, F) 048-624-825, G) 048-779-622, H) 061-381-264, I) 061-536-613, J) 083-024-797, K) 083-042-784, L) 083-070-054, and M) 083-103-352.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Contribution frequency
0.0
0.2
0.4
0.6
0.8
1.0
1.2
10
30
47
100
200
300
Simulated sample size

Table 2: Calculated census (*n*) and effective (*Ne*) population from each spawn during the 2013 spawning season, for female brood fish (*f*), male brood fish (*m*), and both (N_e). Calculations were made from the first 47 YSL analyzed, and with grand totals of YSL from each spawn (N ≥ 325 YSL).

Table 2: Censes and effective population of five spawns from 2013

Date mm/ dd	n_f 47	n_f Total	Ne_f 47	Ne_f Total	n_m 47	n_m Total	Ne_m 47	Ne_m Total	n_e 47	n_e Total	N_e 47	N_e Total
04/29	2.00	4.00	1.82	1.93	7.00	10.00	5.43	6.38	9.00	14.00	5.44	5.93
05/17	2.00	3.00	2.00	2.01	9.00	10.00	4.36	5.00	11.00	13.00	5.48	5.74
06/21	2.00	2.00	1.09	1.11	9.00	10.00	6.33	6.89	11.00	12.00	3.72	3.82
07/26	2.00	3.00	1.04	1.01	7.00	10.00	3.65	3.71	9.00	13.00	3.25	3.18
08/26	2.00	3.00	1.67	1.57	9.00	10.00	7.15	8.28	11.00	13.00	5.41	5.28

Table 3: P-values from all binomial tests between frequencies of first 47 YSL assigned, and total frequencies. Frequencies calculated from totals (N) were used as “true frequencies” from the spawn, and values from first 47 were measured against this value to discern how correct this sample-size predicted parentage from spawn. Solid bars beside pit tag numbers indicates female brood fish, dashed bar indicates male brood fish. Frequency from totals for each spawn were calculated from different total YSL, in order left-to-right, N = 357; 355; 366; 377; and 325. Cells containing “N/A” indicate a frequency of zero from first 47, and zero from totals. Bolded values indicate statistically significance differences ($P < 0.05$).

Table 3: P-values from each spawn date and brood fish

	Spawn date (mmdd20yy)				
Pit-tag number	04/29/13	05/17/13	06/21/13	07/26/13	08/26/13
048-379-341	N/A	N/A	N/A	1.000	N/A
048-519-049	N/A	N/A	N/A	N/A	N/A
061-363-804	0.551	N/A	1.000	0.221	0.492
061-375-363	N/A	N/A	1.000	N/A	N/A
061-621-862	1.000	N/A	N/A	N/A	N/A
083-026-876	N/A	0.885	N/A	N/A	1.000
083-027-609	1.000	1.000	N/A	N/A	0.496
083-103-352	0.552	1.000	N/A	1.000	N/A
048-533-355	0.142	0.724	1.000	0.884	1.000
048-567-798	0.410	0.626	0.799	0.313	1.000
048-624-825	0.210	0.651	1.000	1.000	0.658
048-779-622	N/A	0.329	0.403	1.000	0.503
061-381-264	0.374	1.000	0.628	1.000	0.178
061-536-613	0.124	0.640	0.498	1.000	0.819
083-024-797	0.123	1.000	0.345	0.065	0.566
083-042-784	1.000	0.237	0.318	0.275	0.775
083-070-054	**0.019**	0.757	0.087	0.161	0.806
083-101-080	1.000	N/A	N/A	N/A	N/A
083-103-352	0.235	1.000	0.341	1.000	0.730

Discussion

For this CYT spawning system consisting of 11 male and 8 female brood fish, 47 YSL samples was enough to understand patterns of parental contribution and a convenient number to analyze in a 96 well format. When analyzing simulated sample sizes, 30 YSL was sufficient to get the mean ± 95% CI to not differ significantly differ from "true" proportions as determined by sampling more than 300 YSL. These sample numbers are in the same range as other studies that focus on sample collection for population genetics (Hale *et al.*, 2012). It is important to note that while computer simulation suggests that a sample size of 30 is adequate, one initial value at n = 47 YSL was significantly different than "true" values (Table 3). However, with a Bonferonni correction, this P-value is no longer significant, as it is $P > 0.01$.

For practical use, and for more conservative statistical analysis, n = 47 YSL offspring was chosen for this study. This is a convenient number for genetic work since, with the addition of a negative control, two groups (e.g. spawns) fit into a standard 96-well plate. Initial proportional contribution was not statistically significantly different between n = 47 and N > 300 YSL, in all cases but one (Table 3). These subsample numbers of n = 30 – 47 YSL, are an order of magnitude smaller than a predicted power analysis, which was n = 385 YSL. With the addition of 300 samples assigned, little information is gained in terms of proportional contribution of parentage, at the cost of time and laboratory expenses.

Additionally, if resources are limiting, or for pilot studies, reasonably accurate average parental contribution proportions were seen in as few as 10 offspring (Figures 2-6). However, broader conclusions would be limited, since 95% CI when n = 10 YSL were extreme, and outside the "true" proportion. The fact that average proportions of parental contribution in as few as n = 10 YSL being reasonably accurate could be due to the low number of potential parents (N = 19). As relatively few females contribute per spawning event (lottery polygyny, Nunney, 1993), 10 YSL was enough sub sampling to quickly predict the primary female, but not to understand how males were contributing. However, for any statistical power in parental proportional contribution, $n \geq 30$ offspring is necessary.

As this system is closed to the wild, even rare alleles will be detected, which is extremely important for population genetic studies (Crandall *et al.*, 2000) or for studies regarding evolutionary migration (Slatkin, 1985). This is not an issue for parentage presented here, as all potential parents were first genotyped at loci for this study and rare alleles actually ensure offspring to be assigned parentage. This study did not address potential parents not genetically identified, or a more fluid movement of parental pairs, and inferences from this study become difficult to apply to different breeding systems or where more potential pairs are present.

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Chapter 2

Reproduction of wild-caught California yellowtail (*Seriola dorsalis*)

in an aquaculture setting with implications for broodstock management.

Abstract

Microsatellite markers were used to determine parental contribution from a captive population of California yellowtail (CYT, *Seriola dorsalis* previously *lalandi*) over two spawning seasons. Parental contribution from these brood fish would have otherwise been unknown without genetic tools. Forty-seven yolk-sac larvae offspring were assigned to parents for all spawning events in 2013 and 2014. In 2013 there were 11 males and 8 females in the population weighing 20 kg on average. In 2014 18 smaller fish of 7.5 kg average were added to the pool bringing the population up to 19 males and 18 females. In most spawning events, one of the larger females produced most of the eggs, at or near 100%. After a female spawned, she did not reproduce again for another ~11 – 19 days. The average, batch fecundity of larger females was approximately 500,000 eggs per spawn while for smaller females it was approximately 35,000 eggs per spawn. Female mass and egg production, both batch and annual fecundity, were positively correlated ($\rho > 0.739$) in the 2014 spawning season only. In 2014, smaller female brood fish only contributed ~10% to the annual egg production. For both years, approximately six males would participate in each spawning event, with no clear primary contributing male fish in this breeding system.

Introduction

Understanding reproduction is a vital component of sustainable aquaculture management. Fish make for good model organisms for studying reproduction, as they utilize a wide variety of reproductive strategies (reviewed in Gonçalves-de-Freitas *et al.*, 2009). In spawning systems where parental contribution is unclear, genetic tools such as microsatellites can be used to determine who is breeding with whom. Microsatellites have previously been used to determine contribution of brood fish in captivity, notably in turbot (*Scophtalmus maximus*), trout (*Oncorhynchus mykiss*), flounder (*Paralichthys olivaceus*), common carp (*Cyprinus carpio*), red drum (*Scaiaenops ocellatus*), tilapia (*Oreochromis* spp.) and white seabass (*Atractoscion nobilis*) (Estoup *et al.*, 1998; Hara and Sekino, 2003; Vandeputte *et al.*, 2004; Gold *et al.*, 2010; Briñez *et al.*, 2011; Gruenthal and Drawbridge, 2012). For studies of red drum and white seabass, genetic analysis was used to determine which brood fish were contributing over multiple spawning events, while the flounder were only assessed at one point during the spawning season. In the case of common carp, brood fish were selected for fast weight gain in a breeding program (Vandeputte *et al.*, 2004). For aquaculture, parentage analysis (SNPs or microsatellites) help ensure a controlled breeding program while limiting conflicts from inbreeding (Vandeputte and Haffray, 2014). These methods can also aide in the selection of F_1 populations that can become the next generation of aquaculture brood fish and thereby reducing wild capture to supplement aquaculture production.

Reproductive studies of pelagic fishes, including mackerels, jacks and tuna species, show that many are iteroparous asynchronous batch-spawners (Murua and Saborido-Rey, 2003). An up and coming candidate species for aquaculture is the pelagic finfish California yellowtail (FAO, USA 2011). Until very recently, this species was globally known as *Seriola lalandi*, but recent genetic data suggest the original name of *Seriola dorsalis* be resurrected for the species found in the Northeast Pacific (Baxter, 1960; Martinez-Takeshita *et al.*, 2015; Purcell *et al.*, 2015) and will refer to the species studied in this paper as the local common name, California yellowtail (CYT). Much like other pelagic finfishes, *Seriola dorsalis* are aggregate, broadcast spawning fish; releasing gametes multiple times in synchronized events during warmer summer months (Baxter, 1960; Sumida *et al.*, 1985; Poortenaar *et al.*, 2001; Sala *et al.*, 2003).

From laboratory research, *Seriola* spp. spawned only when water temperature was above 17 °C, and courtship behavior has been described as males "nipping" or "nudging" ripe females (Moran *et al.*, 2007; Stuart and Drawbridge, 2013). Assessment of wild *Seriola lalandi* from the Southwest Pacific confirm median size at reproductive maturity of between 834 and 944 mm fork length for females and 450-750 mm fork length for males (Gillanders *et al.*, 1999; Poortenaar *et al.*, 2001). Average standard lengths of the *Seriola dorsalis* ranged from 350 – 970 mm (Baxter, 1960). However, little is known about individual spawning dynamics of CYT. Due to large aggregation sizes and the broadcast of gametes at times of breeding, details of reproductive parental contribution in the wild is very difficult to ascertain.

Reproductive strategies in CYT have not been characterized in detail, and much information about reproduction is still unknown. Reproductive strategy here refers to the basic reproductive systems that a fish species exhibits, including monogamous breeding pairs (one male and one female), polygyny (one male with multiple female sexual partners), or lottery polygyny (males competing equally for a single female, Nunney, 1993) systems. This term can also refer to the period or cyclicality of reproduction. Fecundity is a measurement of a species' ability to reproduce, typically used in regard to female egg production. Fecundity is measured by quantifying egg production, and it has been well described in fish species that older, larger females tend to be more fecund and produce higher quality gametes due to the more mature development of the ovaries (Hixon *et al.*, 2013). In species that can reproduce multiple times over a spawning season, distinction is made between batch and annual fecundity. The former is a measurement of eggs per female produced during one spawning event, and the latter is a measurement of total eggs per female produced during one year.

As detailed above, wild *Seriola* spp. broadcast spawn freely during the warmer months (Baxter, 1960; Sumida *et al.*, 1985; Poortenaar *et al.*, 2001; Sala *et al.*, 2003). This same breeding system is set up for a captive group of CYT brood fish at the Hubbs-SeaWorld Research Institute (HSWRI) experimental breeding program, in San Diego, CA. At HSWRI, the CYT are bred to investigate the feasibility of culturing this species for food, so larval and juvenile stages are studied extensively (Stuart and Drawbridge, 2013). Wild-caught CYT brood fish are kept in a single large tank, allowing reproductively mature males and females

to reproduce freely. This practice ensures a steady stream of eggs that can be collected throughout the spawning season and allows for the most "wild-like" or near-natural reproduction conditions available. Unfortunately, unlike strip-spawning or hormonal injections, this method of wild-like spawning does not allow for easy parental assignment to offspring. Therefore, it is typically unknown which brood fish are contributing to distinct spawning events in this system. Knowing this information would be valuable in order to help maximize the egg production from the system, as well as to potentially link quality metrics of the offspring to the parents.

For the breeding system at HSWRI, previously published *Seriola* microsatellite markers were used to determine parentage of offspring. These microsatellite loci have been used to investigate population connectivity of *Seriola* spp. in the wild, refine the taxonomy of the *Seriola lalandi* species complex, and to find genetic correlations between growth rates and condition factors (Miller *et al.*, 2011; Whatmore *et al.*, 2013; Martinez-Takeshita *et al.*, 2015; Purcell *et al.*, 2015). Knowing the egg production output of individual brood fish is useful for refining aquaculture production because non-contributors can be culled from the brood fish population or sex ratios can be changed to fit reproductive strategy. Additionally, linking correlates of egg quality to individual parents can increase the efficiency of the culture system.

By tracking parental contribution over two spawning seasons, the first detailed description of CYT reproductive strategy was described here. This study enhances our understanding of the spawning dynamics of a species that is

otherwise not well understood. It is known that CYT are aggregate breeders with large spawning events, but this study provides an individual-level analysis of spawning frequency of male and female brood fish in a captive population. The genetic data generated from this study provides information on female fecundity and spawning intervals, information that has yet been characterized in this species. This characterization of each spawning event will directly impact management of these fish in a culture setting, and may represent a proxy of spawning capacities of CYT in the wild.

Materials and Methods

Brood fish were collected from the wild between 2001 and 2004, and have since been maintained in a 140 m^3 breeding tank, under natural day length and water temperature (Stuart and Drawbridge, 2013). Genetic samples (fin clips) were collected from all individuals within the brood fish population. Water temperatures were collected directly from brood fish tank, and lunar cycles and day length data were gathered online from www.timeanddate.com.

In 2013, the broodstock population was composed of 11 males and 8 females. The population yielded 69 spawning events between March 29th and September 30th. From this spawning year, at least 47 zero-day post-hatch (dph) yolk-sac larvae (YSL) were collected from 66 spawns, 9-13 YSL were collected from two spawns, and one spawn had zero larvae collected due to eggs not hatching. In total, 3170 YSL from 2013 were assigned to parental pair brood fish. In 2014, 18 younger animals originally collected in 2012 were added to the broodstock population, resulting in 18 females and 19 males. One male and one female were also culled from the system for health reasons, for a final total of 17 females and 18 males in the tank for the 2014 season. The 2014 spawning season resulted in 62 unique spawning events with at least 47 YSL being collected from 58 of these spawning events. In two spawning events 32-38 YSL were collected, and no larvae were collected from two other spawning events because the eggs did not hatch. A total of 2789 YSL were assigned to brood fish from the 2014 spawning season.

It should be noted that previous simulation and statistical calculations suggested an optimal subsample of $n \geq 30$ YSL per spawn were necessary to make accurate proportional assignment of CYT parents in the HSWRI breeding system (Smith *et al.*, in prep). For this project, 47 YSL were targeted for analysis from every spawning event during 2013 and 2014. In the event a spawn had less than 47 YSL samples preserved, all available YSL were analyzed. The YSL were obtained by hatching a subsample of eggs and storing them in 100% non-denatured ethanol for genetic analysis.

All primer pairs, PCR conditions, genotyping protocols, and analysis of published microsatellites are discussed in detail in Chapter 1 and Purcell *et al.*, 2015. Briefly, DNA was extracted using a 10% (w/v) Chelex resin solution (Bio Rad Laboratories Inc., Berkeley, CA), and amplification of 9 microsatellite loci (Table 1) were multiplexed into three PCRs (Appendix 2): 1) Sequ 38; 2) Sequ 77, Sdu gA3D, Sdu 46 and Sdu 4; and 3) Sequ320, Sequ230, Sdu 10 and Sdn 06. Forward primers for all PCRs were fluorescently labeled for genotyping via fragment analysis. All three PCRs had the following conditions: denaturation at 94 °C for 4 minutes, followed by 35 cycles of 94 °C for 30 s, annealing between 53-59 °C for 35 s, and elongation at 72 °C for 30 s, and a final extension for 5 minutes at 72 °C, followed by a 4 °C hold until further analysis (complete information for PCRs in Chapter 1 and Appendix 2).

For fragment analysis and genotyping, PCRs 1 and 2 were combined with PCR 3 run separately. PCR product was diluted 1:25 in milli-Q water (EMD Millipore, Darmstadt, Germany) for fragment analysis. Fragment analysis was

completed using a 1:84 dilution of GeneScan 500 ROX size standard in HiDi Formamide (Applied Biosystems, Waltham, MA). Nine µl of this HiDi/ROX mix, and 1 µl of diluted DNA products were added to each well in a clean 96-well semi-skirted plate, denatured, and placed into 3730 DNA Analyzer for genotyping via fragment analysis (Applied Biosystems). Microsatellites were scored using GeneMapper v4.0 (Applied Biosystems). Scored allele peaks were exported to Microsoft Excel 2010 for rounding and standardization. Converted allele calls were used for parentage assignment using Cervus v3.0.6 (Kalinowski *et al.*, 2007, www.fieldgenetics.com). Both years yielded 100% assignment of known parental pairs.

Female fecundity, female spawning intervals, effective and census populations (From Gold *et al.*, 2008, presented in Chapter 1), and proportional contribution of each spawn were calculated using Microsoft Excel 2010. Heterozygotes observed and expected, as well as Hardy-Weinberg Equilibrium were calculated in Cervus 3.0.6. Statistical analyses were completed using R v3.0.2 software. Proportional contribution from each individual from each spawn was compared to theoretical equal proportions using a non-parametric binomial test. This tested to see if each brood fish contributed equally or disproportionately. For example, for all eight female brood fish available in 2013, actual proportions of offspring contribution were compared to the theoretical value of each female contributing equally, or each female contributing 1/8 of offspring. This was completed for both years for number of males and females. Shapiro-Wilks tests were performed to evaluate the normality of fish masses. Statistical differences

among fish masses were tested using a two-tailed t-test. Correlations regarding egg totals and environmental factors, and female masses and batch/annual fecundity were tested using Spearman's rank correlation coefficient tests.

Table 1: Microsatellite primer pairs, GenBank accession numbers, relevant publications, proper annealing temperatures, and fragment lengths for multiplexed PCR of CYT at HSWRI.

Table 1 -- *Seriola* spp. microsatellite primer pairs used

Locus	Forward (F)	Reverse (R)	Accession number	Annealing temperature	Publication	Approx. fragment length (bp)
Sequ38	CCATTACAATTTGTCTCTC	CTTATCAACACACGAGCG	AB098518	53 °C	Ohara *et al.*, 2003	100-145
Sequ77	CCTACACATGCACATGAA	CAAGGCTGATACGTCATG	AB098519	55 °C	Ohara *et al.*, 2003	135-190
Sdu gA3D	CTCAACATGAGAGGCAACG	GCATGGCTTCATGGGAAGG	DQ435602	55 °C	Porta *et al.*, 2009	140-180
Sdu 46	GCAGTGTGAGCCATACATTAC	CTACAGGACAAAAGCCATT	DQ883580	55 °C	Renshaw *et al.*, 2007	220-260
Sdu 4	GGAAATAGTTTGGATCACGCTGG	GGATGCTCAGTGAAGTTGTGC	DQ468084	55 °C	Renshaw *et al.*, 2006	270-310
Sequ320	GACAGGGTAAGAAACGAAAC	GACAATGACCAAAGCTGCC	AB508215	59 °C	Ohara *et al.*, 2005	90-140
Sequ230	CTCCAGAAACGCCACATAAC	AAGCAAACCGCACAAGTAGG	AB508202	59 °C	Ohara *et al.*, 2005	150-165
Sdu 10	CCAAGTCCTCCGCTACTACCAT	CCTTGTGGATGACCTGTTTG	DQ468090	59 °C	Renshaw *et al.*, 2006	250-310
Sdn 06	GGGTTTGTGCTGTGAGTG	TCCGTCTGTCTTTTCCTGT	N/A	59 °C	Nugroho & Taniguchi, 1999	300-330

Results

A total of 3170 YSL were assigned to parental pairs during the 2013 spawning season, and 2789 YSL were assigned from the 2014 spawning season, with 100% assignment. All microsatellite loci were in Hardy-Weinberg Equilibrium for 2013 and 2014, with allelic variation of 7 – 22 alleles per locus (Table 2). Fish masses from 2013 and 2014 were normally distributed (P = 0.11 – 0.91). Spawns occurred approximately every 6 days, and were usually average 900,000 – 1.6 million total eggs (standard deviation: 700,000 – 1 million eggs; Figure 1). In 2013, the largest spawn was on April 22 with 3.65 million spawned eggs from three females. For the 2014 spawning season, the largest spawn was from May 12, with 3 million total eggs being spawned by three females. No significant correlation was found between spawning occurrence and environmental parameters (water temperature, lunar cycle, or day length) for the 2013 (P = 0.5539 – 0.9276) or the 2014 (P = 0.1457 – 0.8794) spawning season. During these two spawning seasons, female 083-026-609 had the highest contribution of offspring, with >1000 YSL assignment, or ~40% of the total fertilized eggs during these years (Figure 2), an estimated 23 – 27 million eggs in total from this one female.

Brood fish contributions during 2013 and 2014

During both spawning seasons multiple males contributed to each spawn in roughly equal proportions to each other; typically 2 – 15 (average: 9, standard deviation: 1.9) males contributed to a spawn. Of the larger males, male 083-101-080, consistently contributed far less than the other males. The older brood fish

demonstrated proportional contribution of paternity in visually similar patterns across both spawning seasons (Figure 2). Overall, the younger brood fish that were added at the end of 2013 (i.e. wild-caught fish from 2012) did not participate at similar levels of the older broodstock. During 2014, only 9.6% of the offspring were produced by younger females, and 14.6% of offspring from younger males. The effective population for males (Ne_m) during the 2013 spawning season was 8.63, and the effective population for males and females (N_e) for 2013 was 12.46 (Table 3). During 2014, Ne_m was 9.79 and N_e was 13.24 (Table 4). With the exception of female 057-815-612 and male 057-790-030, all other newly added fish had < 100 YSL assigned over the entire spawning season.

The male contribution to offspring was more equal than female contribution in 2013 when analyzed by individual spawning event (Figure 6). This was supported statistically ($P > 0.05$), as the male distribution did not differ significantly from equal contribution. This is visualized by heat map colors being various shades of lighter grays indicating that males contributed on average 9.03% of offspring per spawn (Figure 6). On average 5.87 males (range = 1.67-12.37), contributed during each spawn in 2013 (Table 3). Spawning patterns for older brood fish males were similar in both the 2013 and 2014 spawning seasons, however, the younger males that were added to the 2014 brood fish population showed lower overall participation per spawn (Figure 8). In 2014 on average 6.24 males (range 2.77-9.73) participated in spawn events in 2014 (Table 4), with newly added males not participating in 30-93% of the spawning events as illustrated by the white colored cells (0% contribution) in Figure 8. During the

2014 spawning season, male number 083-042-784 was culled from the system on August 1 so it was no longer a possible sire after this date (Figure 8). For both males and females, during the 2014 season only, values of 0% contribution did not differ significantly from equal contribution ($P > 0.05$; blank cells in Figures 7 – 8). The numbers of contributing females and males, and the weighted value as a calculated effective population, are presented for each spawn, and entire spawning season, in Tables 3 and 4.

Overall contribution from female 083-027-609 was highest in terms of proportion of YSL assigned, and when standardized to total number of eggs during the spawning year for both 2013 and 2014, at ~40% (Figure 3). This female was also the most consistent in spawn interval timing, with her mean and median spawning interval being 5.2 and 5 days, respectively, in 2013. Female 061-621-862 had a mean and median spawning interval of 11.4 and 11 days, and female 083-103-352 was 6.8 and 7 days. This agrees with the most common female frequency of spawning interval and median spawning interval being 5 – 10 days, during 2013 (Figure 4, Table 5). All other female spawning mean and median intervals differed by >4 days. These three females also participated in the most spawning events - 32 for female 083-027-609, 16 for female 061-621-862, and 20 for female 083-103-352. The effective population for females (Ne_f) for the entire 2013 spawning season was 4.87 (Table 2), which is ~3 less than the total number of female fish in the system.

During the 2014 spawning season, female 083-027-609 spawned 28 times at an average spawning interval of 6.3 days. Female 083-026-876 was the second

highest contributing female with 14.8% of YSL assigned, with 13 spawns at an average interval of 6 days. However, spawning intervals for most females during the 2014 season were closer together than observed in 2013, typically 1 – 5 days (Figure 4). The mean and median spawning intervals for females during 2014 were the same at ~19 days (Table 5). The differences between mean and median female spawning interval timing from 2014 were more varied than the 2013 year. For the 2014 spawning season, three females did not contribute to any of the spawns, all being from the younger brood fish added that year. These were females 057-816-535, 057-798-784, and 008-362-851 (Figure 3). Despite 10 new females being added to the system, the observed effective female population size (Ne_f) from 2014 (~5.0) was similar to that of 2013 (Tables 3 and 4). During this year, female 061-621-862 only had 10.6% of the total YSL assigned, but in terms of total number of eggs was the second highest contributing female, at 20.1% total eggs. Out of the newly added fish, the female that contributed the most in terms of proportion of YSL and total eggs was female 057-815-612, at 8.2% and 2.7% respectively. Three of the newly added females did not contribute any offspring throughout the entire season.

Analysis of individual spawning events, as opposed to annual contribution, indicated a primary female per spawn for the 2013 and 2014 spawning season (Figures 6 and 7). This is shown with colors for the female brood fish being mostly either 100% contribution (black) or 0% contribution (white). This agrees with the calculated Ne_f for a spawning event, being on average 1.37 females per spawn in 2013 (Tables 3 and 5) and 1.21 in 2014

(Tables 4 and 5). Values at or near 100% (Figures 6 and 7) were also significantly different than equal contribution (***; $P < 0.0001$) for both spawning seasons. During 2014, female 061-363-804 was culled from the system on July 18 (Figure 7).

Female mass and fecundity

Mean mass of the older females was 20.75 kg (range 14.6 – 27.2 kg). The mean mass of the newly added females from the 2014 spawning was 8.44 kg (range 4.28 – 13.54 kg; Figure 5). The older females did not significantly increase their mass over the two years ($P = 0.45 – 0.66$). The smaller females added in 2014 all increased significantly in mass ($P < 0.0001$). Annual and batch fecundity was not significantly correlated to female mass for the 2013 spawning year ($P = 0.50 – 0.62$). Each female from 2013 spawned on average 420,000 eggs/female kg/year during the season, for an annual total of approximately 8.2 million eggs per female. Female masses and both annual and batch fecundity were significantly, and positively correlated during the 2014 spawning year (respectively, $\rho = 0.753 – 0.739$; $P = 0.0003 – 0.00045$). Annual fecundity of the older brood fish from 2014 was 330,000 eggs/female kg/year, but only 23,000 eggs/female kg/year for the newly added females. This resulted in each older female contributing a total of 6.8 million eggs in 2014 and only a total of 200,000 eggs per female from the smaller females. Female batch fecundity from 2013 and 2014 did not correlate with days between spawning events (spawning intervals; $P > 0.251$).

When analyzed by spawning event, proportional contribution can be extrapolated into batch fecundity. Batch fecundity per female from 2013 is on average 460,000 eggs/spawn, and from 2014 on average 250,000 eggs/spawn (Table 4). However, when the 2014 year was broken down into older females (~20 kg) vs. newly added females (~8 kg), batch fecundity is on average 520,000 and 35,000 eggs, respectively. These values were calculated using only data available from when females spawned (e.g. ignoring days when individual females did not participate).

Figure 1: Total number of eggs volumetrically calculated from each spawn throughout the 2013 (A) and 2014 (B) spawning season. Dates of spawning season are along the X-axis. Overlaid on top of spawn volume, are day length (dashed line) and water temperature (solid line), both values are represented on the secondary Y-axis. Circles represent lunar cycles, both new moon (white/empty) and full moon (black).

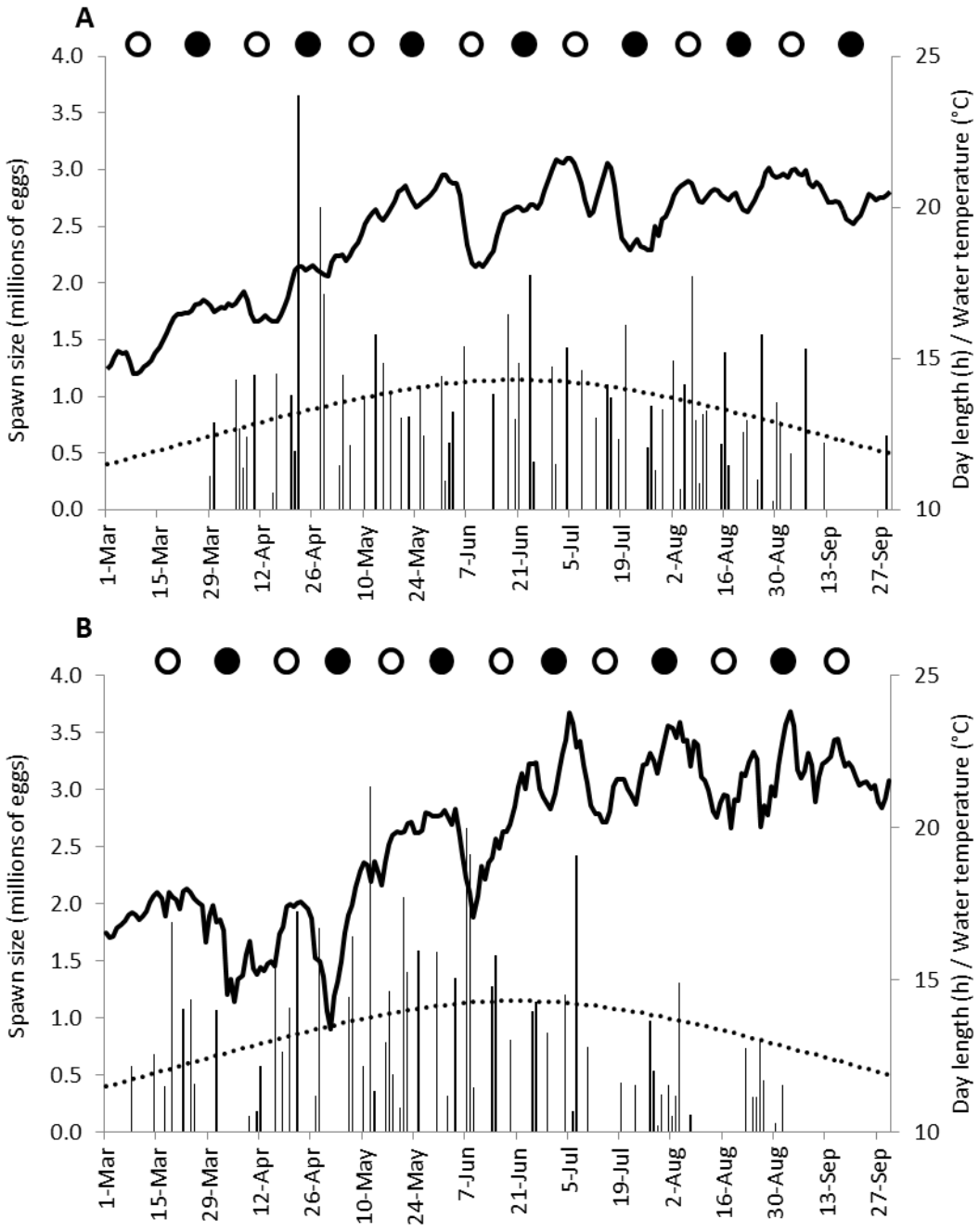

A
B
Spawn size (millions of eggs)
Day length (h) / Water temperature (°C)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
10
15
20
25
1-Mar
15-Mar
29-Mar
12-Apr
26-Apr
10-May
24-May
7-Jun
21-Jun
5-Jul
19-Jul
2-Aug
16-Aug
30-Aug
13-Sep
27-Sep

Figure 2: Total number of YSL assigned to each broodstock CYT, from the 2013 (A), and the 2014 (B) spawning season. All brood fish are listed by pit-tag number, with females in black and males in gray. For the 2014 spawning season, additional brood fish were added to the tank. In 2013, there was a total 3170 YSL assigned, and 2789 YSL from 2014.

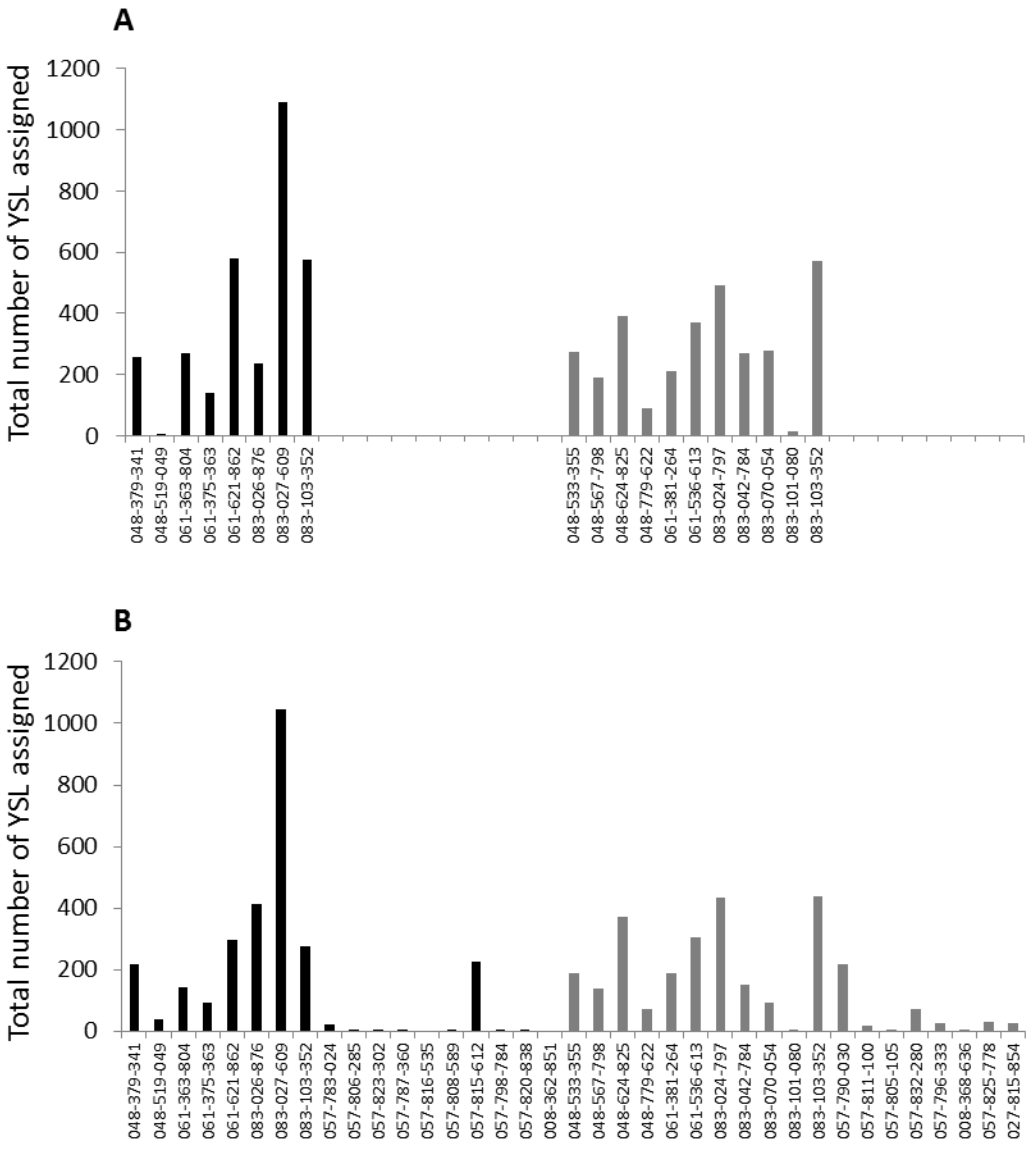

A
Total number of YSL assigned
1200
1000
800
600
400
200
0
048-379-341
048-519-049
061-363-804
061-375-363
061-621-862
083-026-876
083-027-609
083-103-352
048-533-355
048-567-798
048-624-825
048-779-622
061-381-264
061-536-613
083-024-797
083-042-784
083-070-054
083-101-080
083-103-352
B
Total number of YSL assigned
1200
1000
800
600
400
200
0
048-379-341
048-519-049
061-363-804
061-375-363
061-621-862
083-026-876
083-027-609
083-103-352
057-783-024
057-806-285
057-823-302
057-787-360
057-816-535
057-808-589
057-815-612
057-798-784
057-820-838
008-362-851
048-533-355
048-567-798
048-624-825
048-779-622
061-381-264
061-536-613
083-024-797
083-042-784
083-070-054
083-101-080
083-103-352
057-790-030
057-811-100
057-805-105
057-832-280
057-796-333
008-368-636
057-825-778
027-815-854

Figure 3: Proportions of n = 47 YSL (gray) and percentage from total eggs (black) assigned to each potential broodstock female, from the 2013 (A) and the 2014 (B) spawning season. Differences between proportions attributed to uneven egg totals from each spawning event. All fish are listed by pit-tag number.

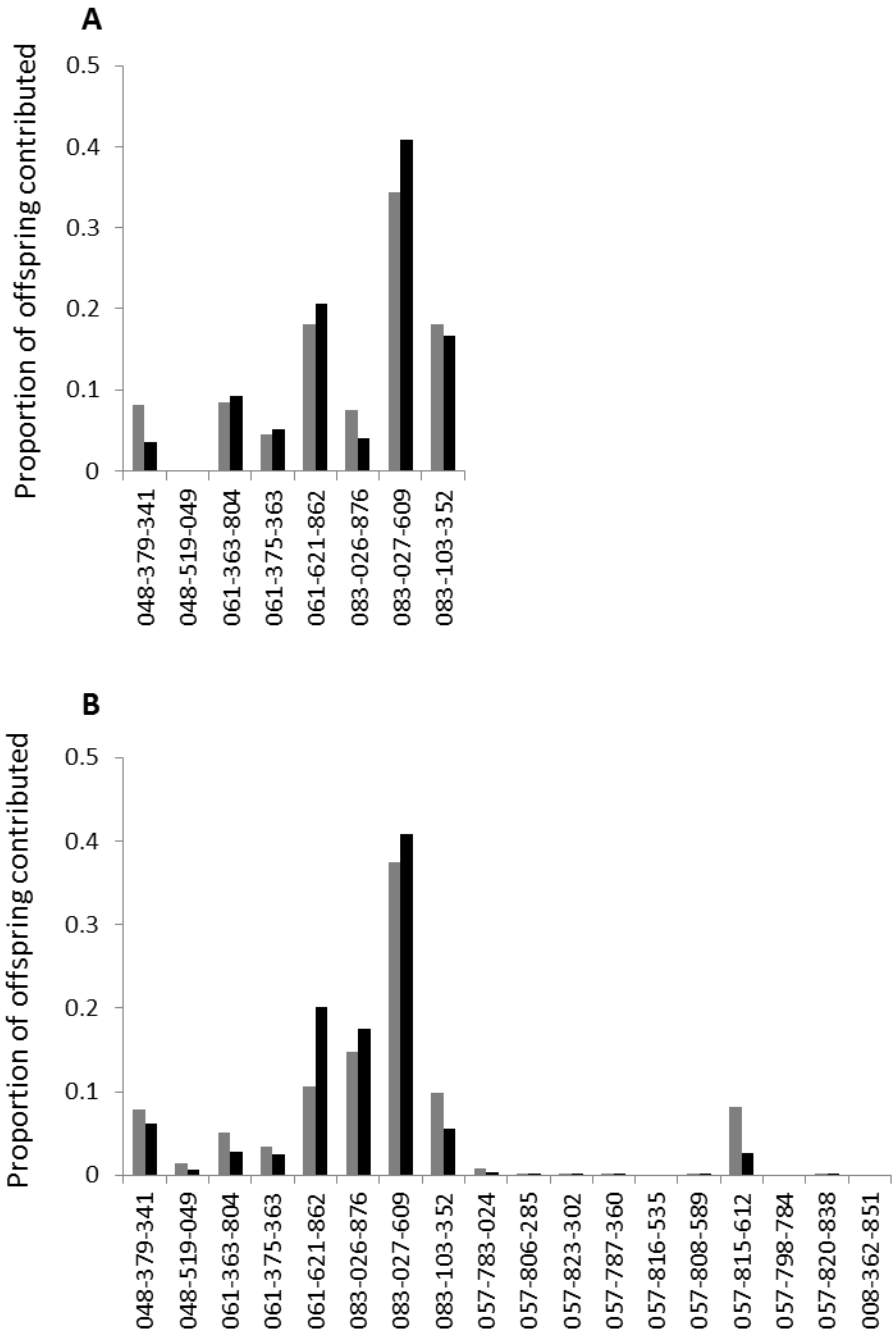

A
Proportion of offspring contributed
0.5
0.4
0.3
0.2
0.1
0
048-379-341
048-519-049
061-363-804
061-375-363
061-621-862
083-026-876
083-027-609
083-103-352
B
Proportion of offspring contributed
0.5
0.4
0.3
0.2
0.1
0
048-379-341
048-519-049
061-363-804
061-375-363
061-621-862
083-026-876
083-027-609
083-103-352
057-783-024
057-806-285
057-823-302
057-787-360
057-816-535
057-808-589
057-815-612
057-798-784
057-820-838
008-362-851

Figure 4: Frequency of female spawning intervals during the entire 2013 (black) and 2014 (gray) season, as explained by the intervals in individual female spawning. For both seasons, female intervals are binned by 5 days until day 20, then by 10 days.

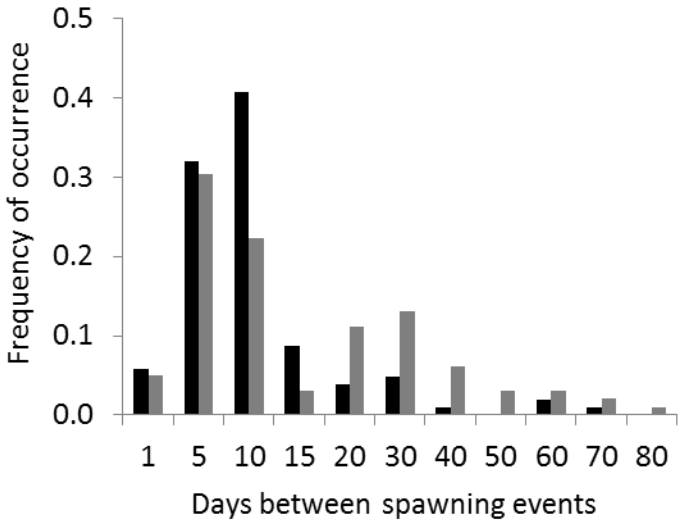

0.5
0.4
0.3
0.2
0.1
0.0
Frequency of occurrence
1
5
10
15
20
30
40
50
60
70
80
Days between spawning events

Figure 5: Proportional contribution of total eggs from each female from the 2013 (A) and 2014 (B) spawning seasons, as a function of average mass (kg). Original female brood fish (from both 2013 and 2014) are represented in black, with the added females from the 2014 spawning season in gray.

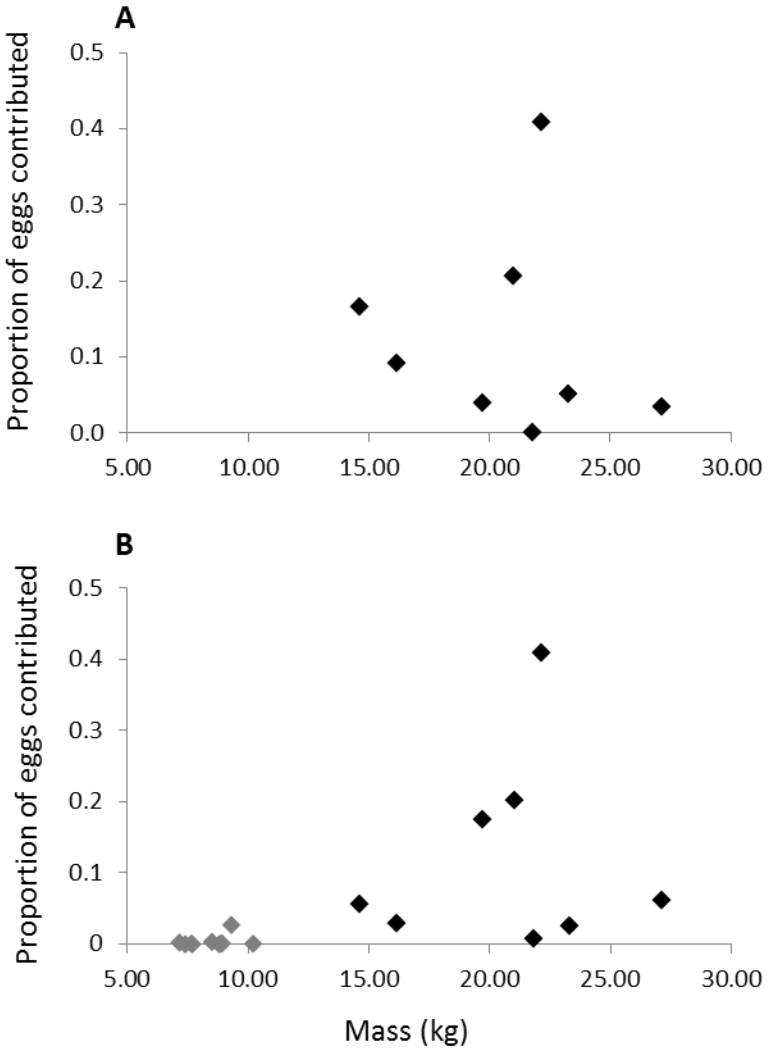
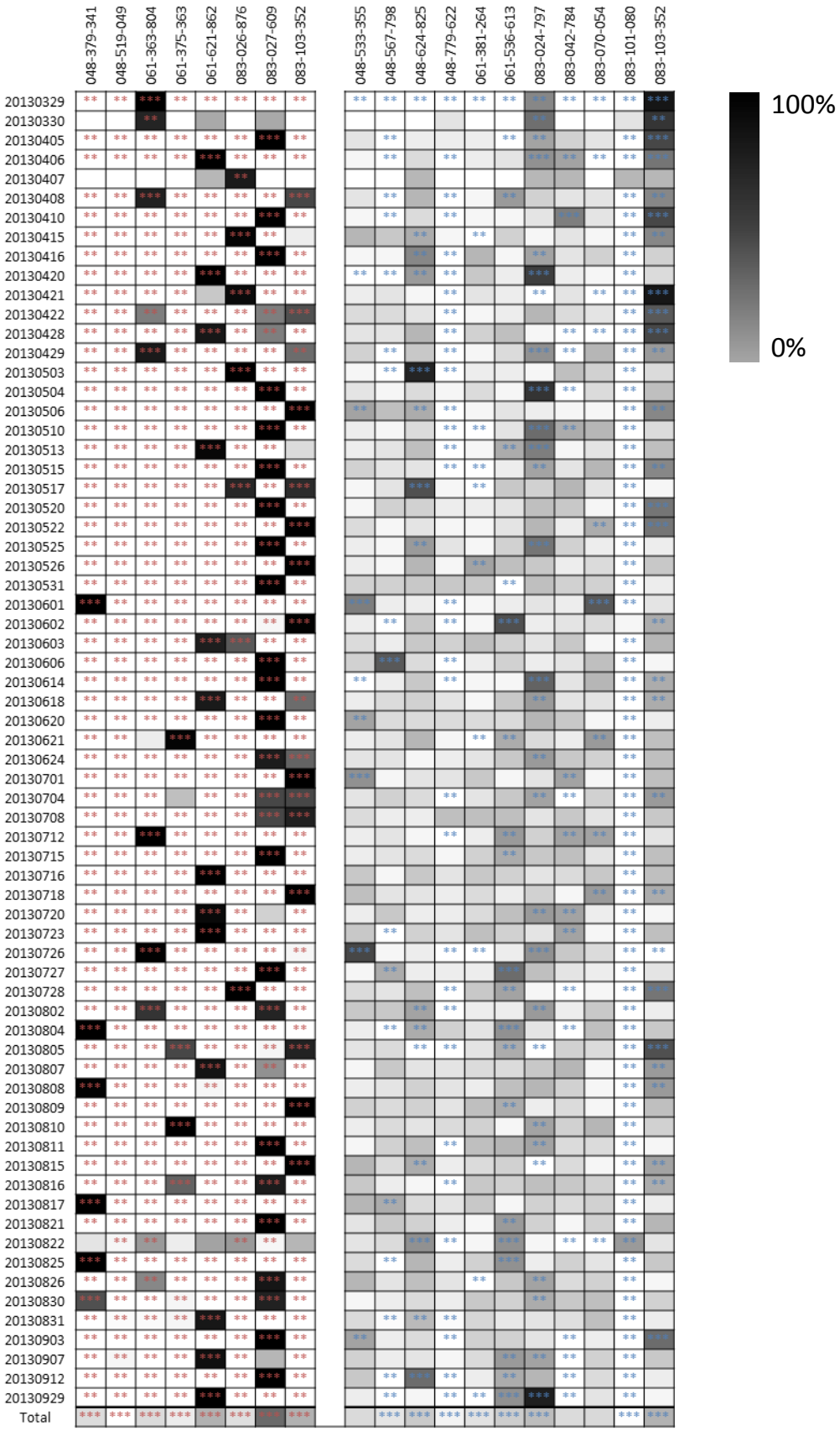

A
Proportion of eggs contributed
0.5
0.4
0.3
0.2
0.1
0.0
5.00
10.00
15.00
20.00
25.00
30.00
B
Proportion of eggs contributed
0.5
0.4
0.3
0.2
0.1
0
5.00
10.00
15.00
20.00
25.00
30.00
Mass (kg)

Figure 6: Proportion contribution to individual spawn for each fish from the 2013 spawning season, where black indicates 100% spawn contribution, and white indicating 0% contribution. Dates of spawn are given as yyyymmdd (first column), and all fish are represented by pit-tag number. Symbols within each cell indicate significance from binomial tests for females (red) and males (blue), evaluating whether observed parental contribution differs significantly from equal contribution, where blank cells are not significance, ** = $0.05 > P > 0.0001$, and *** = $P < 0.0001$.


048-379-341
048-519-049
061-363-804
061-375-363
061-621-862
083-026-876
083-027-609
083-103-352
048-533-355
048-567-798
048-624-825
048-779-622
061-381-264
061-536-613
083-024-797
083-042-784
083-070-054
083-101-080
083-103-352
20130329
20130330
20130405
20130406
20130407
20130408
20130410
20130415
20130416
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20130422
20130428
20130429
20130503
20130504
20130506
20130510
20130513
20130515
20130517
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20130522
20130525
20130526
20130531
20130601
20130602
20130603
20130606
20130614
20130618
20130620
20130621
20130624
20130701
20130704
20130708
20130712
20130715
20130716
20130718
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20130723
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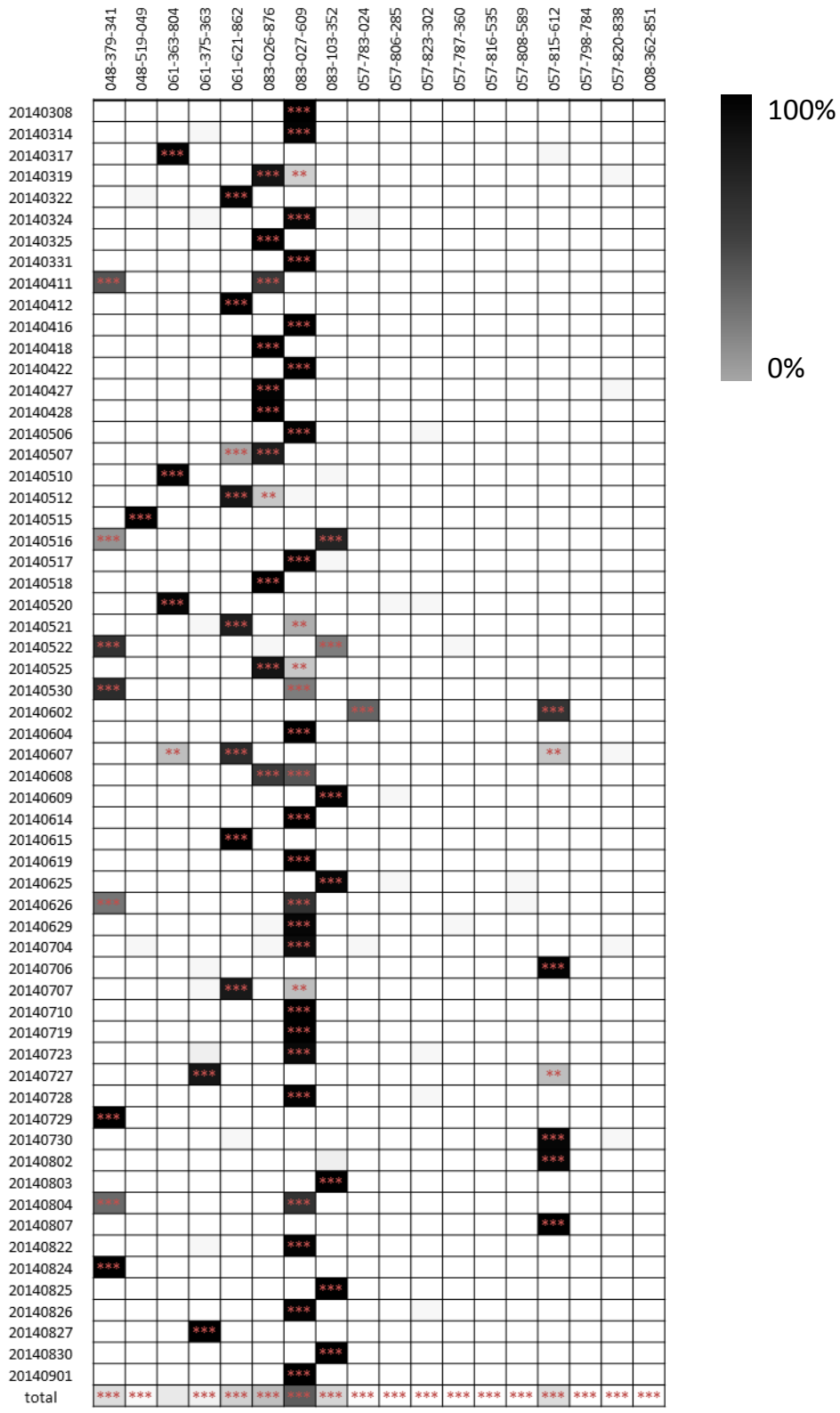
20130728
20130802
20130804
20130805
20130807
20130808
20130809
20130810
20130811
20130815
20130816
20130817
20130821
20130822
20130825
20130826
20130830
20130831
20130903
20130907
20130912
20130929
Total
100%
0%

Figure 7: Proportion contribution to individual spawn for female fish from the 2014 spawning season, where black indicates 100% spawn contribution, and white indicating 0% contribution. Dates of spawn are given as yyyymmdd (first column), and all fish are represented by pit-tag number. Symbols within each cell indicate significance from binomial tests, evaluating whether observed parental contribution differs significantly from equal contribution, where blank cells are not significance, ** = $0.05 > P > 0.0001$, and *** = $P < 0.0001$.


048-379-341
048-519-049
061-363-804
061-375-363
061-621-862
083-026-876
083-027-609
083-103-352
057-783-024
057-806-285
057-823-302
057-787-360
057-816-535
057-808-589
057-815-612
057-798-784
057-820-838
008-362-851
20140308
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20140322
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20140723
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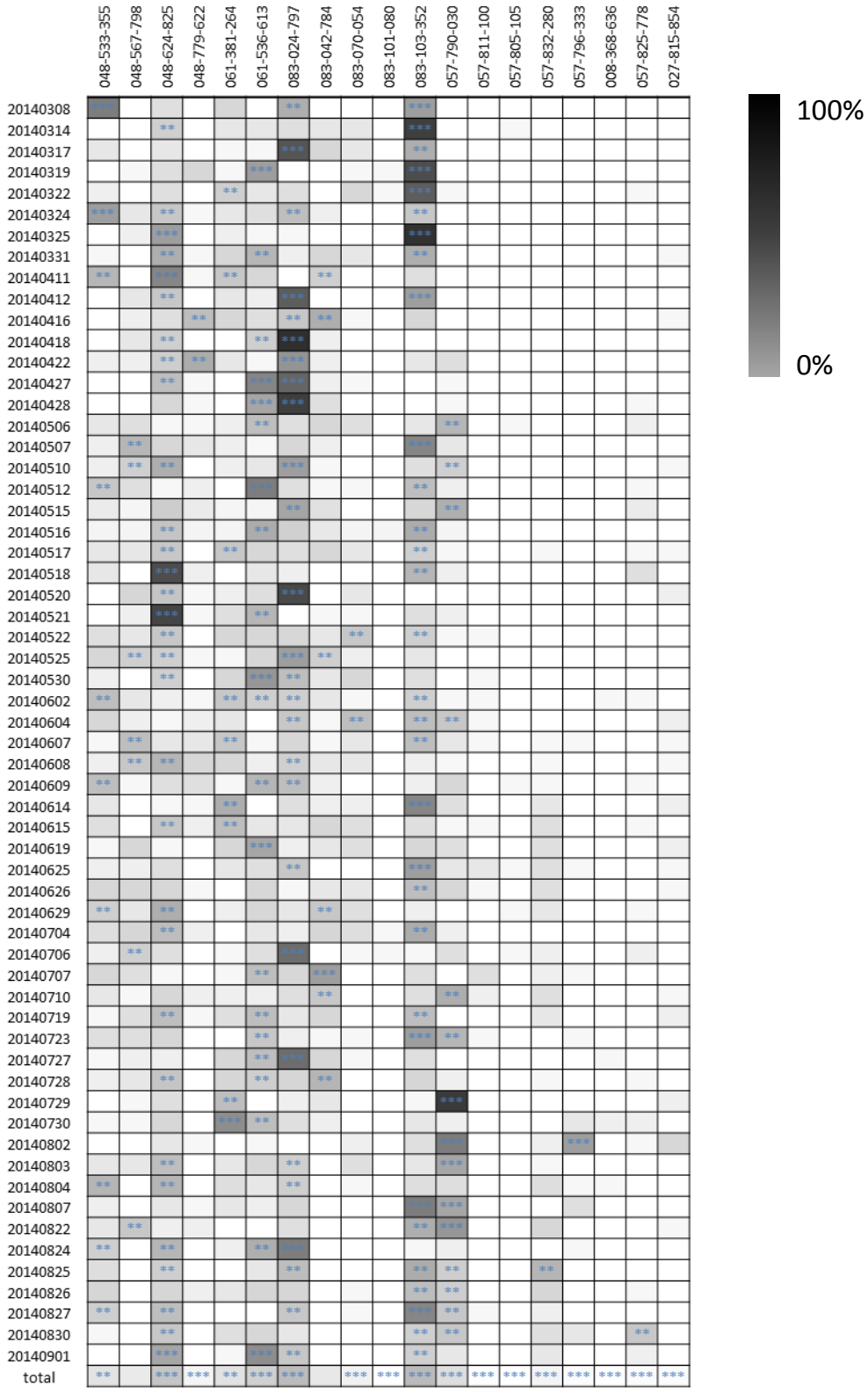
20140729
20140730
20140802
20140803
20140804
20140807
20140822
20140824
20140825
20140826
20140827
20140830
20140901
total
100%
0%

Figure 8: Proportion contribution to individual spawn for male fish from the 2014 spawning season, where black indicates 100% spawn contribution, and white indicating 0% contribution. Dates of spawn are given as yyyymmdd (first column), and all fish are represented by pit-tag number. Symbols within each cell indicate significance from binomial tests, evaluating whether observed parental contribution differs significantly from equal contribution, where blank cells are not significance, ** = $0.05 > P > 0.0001$, and *** = $P < 0.0001$.


048-533-355
048-567-798
048-624-825
048-779-622
061-381-264
061-536-613
083-024-797
083-042-784
083-070-054
083-101-080
083-103-352
057-790-030
057-811-100
057-805-105
057-832-280
057-796-333
008-368-636
057-825-778
027-815-854
20140308
20140314
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20140324
20140325
20140331
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20140704
20140706
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20140728
20140729
20140730
20140802
20140803
20140804
20140807
20140822
20140824
20140825
20140826
20140827
20140830
20140901
total
100%
0%

Table 2: Summary statistics for microsatellite loci including repeat motif, allele number allele number (k), total number of fish genotyped (N), observed (HObs) and expected (HExp) heterozygosity, and conformance to Hardy-Weinberg equilibrium (HWE).

Table 2: Genetic statistics from each spawning season (2013 / 2014)

Locus	Repeat	k	N	HObs	HExp	HWE
Sequ 38	GT	20 / 21	3190 / 2725	0.858 / 0.868	0.892 / 0.892	NS / NS
Sequ 77	CA	17 / 23	3181 / 2793	0.814 / 0.821	0.892 / 0.887	NS / NS
Sdu gA3D	GT	12 / 19	3183 / 2818	0.891 / 0.888	0.859 / 0.871	NS / NS
Sdu 46	GA	13 / 14	3101 / 2795	0.646 / 0.668	0.636 / 0.659	NS / NS
Sdu 4	^	7 / 10	3119 / 2821	0.568 / 0.544	0.566 / 0.534	NS / NS
Sequ 320	CA	19 / 21	3182 / 2816	0.918 / 0.908	0.889 / 0.890	NS / NS
Sequ 230	CA	8 / 8	3192 / 2828	0.709 / 0.689	0.664 / 0.648	NS / NS
Sdu 10	GAA	13 / 15	3153 / 2820	0.738 / 0.747	0.758 / 0.747	NS / NS
Sdn 06	GATA	17 / 22	3085 / 2819	0.850 / 0.853	0.830 / 0.853	NS / NS
All loci		**14 / 17**	**3196 / 2902**	**0.7767 / 0.7757**	**0.7761 / 0.7526**	

^ Repeat motif for Sdu 4: GACA,GGCA,GACA

Table 3: Spawn date, census and effective broodstock populations for females (n_f / Ne_f), males (n_m / Ne_m) and both sexes (n_e / Ne) for the 2013 spawning season.

Table 3: 2013 census (*n*) and effective (*Ne*) breeding size per spawn.

yyyymmdd	n_f	Ne_f	n_m	Ne_m	n_e	N_e
20130329	1.00	1.00	2.00	1.67	3.00	2.50
20130330	3.00	2.74	4.00	2.96	7.00	5.70
20130405	1.00	1.00	8.00	3.98	9.00	3.20
20130406	1.00	1.00	7.00	4.43	8.00	3.26
20130407	2.00	2.12	5.00	7.20	7.00	6.55
20130408	2.00	1.96	8.00	5.43	10.00	5.75
20130410	1.00	1.00	8.00	3.49	9.00	3.11
20130415	2.00	1.09	9.00	5.71	11.00	3.66
20130416	1.00	1.00	9.00	5.59	10.00	3.39
20130420	1.00	1.00	7.00	3.55	8.00	3.12
20130421	2.00	1.29	7.00	1.96	9.00	3.11
20130422	3.00	2.93	9.00	4.81	12.00	7.29
20130428	2.00	1.72	7.00	3.97	9.00	4.80
20130429	2.00	1.82	7.00	5.43	9.00	5.44
20130503	1.00	1.00	8.00	3.05	9.00	3.01
20130504	1.00	1.00	9.00	3.73	10.00	3.15
20130506	1.00	1.00	9.00	5.32	10.00	3.37
20130510	1.00	1.00	8.00	4.94	9.00	3.33
20130513	2.00	1.18	9.00	5.54	11.00	3.90
20130515	1.00	1.00	8.00	5.68	9.00	3.40
20130517	2.00	2.00	9.00	4.36	11.00	5.48
20130520	1.00	1.00	10.00	5.83	11.00	3.41
20130522	1.00	1.00	10.00	5.37	11.00	3.37
20130525	1.00	1.00	10.00	5.27	11.00	3.36
20130526	1.00	1.00	10.00	7.10	11.00	3.51
20130531	1.00	1.00	9.00	8.46	10.00	3.58
20130601	1.00	1.00	9.00	3.98	10.00	3.20
20130602	2.00	1.04	9.00	4.31	11.00	3.36
20130603	2.00	1.90	10.00	8.34	12.00	6.18
20130606	1.00	1.00	9.00	4.83	10.00	3.31
20130614	1.00	1.00	8.00	4.55	9.00	3.28
20130618	2.00	1.82	10.00	7.10	12.00	5.78
20130620	1.00	1.00	10.00	7.75	11.00	3.54
20130621	2.00	1.09	9.00	6.33	11.00	3.72
20130624	2.00	1.86	10.00	7.39	12.00	5.94
20130701	1.00	1.00	10.00	6.15	11.00	3.44
20130704	3.00	2.60	8.00	6.48	11.00	7.43
20130708	2.00	1.96	10.00	8.46	12.00	6.36
20130712	1.00	1.00	9.00	5.92	10.00	3.42
20130715	1.00	1.00	10.00	7.92	11.00	3.55
20130716	1.00	1.00	10.00	8.73	11.00	3.59
20130718	1.00	1.00	10.00	6.92	11.00	3.50
20130720	2.00	1.23	10.00	6.52	12.00	4.15
20130723	1.00	1.00	9.00	7.29	10.00	3.52
20130726	2.00	1.04	7.00	3.65	9.00	3.25
20130727	1.00	1.00	10.00	5.25	11.00	3.36
20130728	1.00	1.00	8.00	5.05	9.00	3.34

Table 3: Continued

yyyymmdd	n_f	Ne_f	n_m	Ne_m	n_e	N_e
20130802	2.00	1.99	9.00	6.40	11.00	6.08
20130804	1.00	1.00	8.00	6.19	9.00	3.44
20130805	3.00	2.05	7.00	4.27	10.00	5.54
20130807	2.00	1.61	10.00	7.15	12.00	5.27
20130808	2.00	1.04	10.00	7.10	12.00	3.64
20130809	1.00	1.00	10.00	8.03	11.00	3.56
20130810	1.00	1.00	10.00	7.75	11.00	3.54
20130811	1.00	1.00	9.00	6.88	10.00	3.49
20130815	1.00	1.00	9.00	6.84	10.00	3.49
20130816	2.00	1.86	9.00	7.54	11.00	5.96
20130817	1.00	1.00	10.00	7.97	11.00	3.55
20130821	1.00	1.00	10.00	7.10	11.00	3.51
20130822	8.00	5.25	8.00	4.96	16.00	10.20
20130825	1.00	1.00	9.00	6.76	10.00	3.48
20130826	2.00	1.67	9.00	7.15	11.00	5.41
20130830	3.00	2.02	10.00	7.97	13.00	6.46
20130831	4.00	2.03	10.00	12.73	14.00	6.99
20130903	1.00	1.00	8.00	5.03	9.00	3.34
20130907	3.00	1.46	9.00	6.55	12.00	4.79
20130912	1.00	1.00	7.00	4.79	8.00	3.31
20130929	1.00	1.00	6.00	2.34	7.00	2.80
Total	8.00	4.87	11.00	8.63	19.00	12.46
Average	1.63	1.37	8.65	5.87	10.28	4.25
Std. dev.	1.06	0.69	1.53	1.88	1.79	1.45
Min	1.00	1.00	2.00	1.67	3.00	2.50
Max	8.00	5.25	10.00	12.73	16.00	10.20
Median	1.00	1.00	9.00	5.77	11.00	3.51

Table 4: Spawn date, census and effective broodstock populations for females (n_f / Ne_f), males (n_m / Ne_m) and both sexes (n_e / N_e) for the 2014 spawning season.

Table 4: 2014 census (n) and effective (Ne) breeding size per spawn.

yyyymmdd	n_f	Ne_f	n_m	Ne_m	n_e	N_e
20140308	1.00	1.00	5.00	4.08	6.00	3.21
20140314	2.00	1.04	8.00	3.32	10.00	3.18
20140317	2.00	1.04	8.00	3.71	10.00	3.26
20140319	3.00	1.35	8.00	3.28	11.00	3.83
20140322	2.00	1.04	10.00	4.34	12.00	3.36
20140324	3.00	1.09	9.00	6.40	12.00	3.73
20140325	1.00	1.00	6.00	2.22	7.00	2.76
20140331	1.00	1.00	10.00	6.37	11.00	3.46
20140411	2.00	1.98	7.00	5.10	9.00	5.70
20140412	1.00	1.00	6.00	3.59	7.00	3.13
20140416	1.00	1.00	10.00	7.34	11.00	3.52
20140418	1.00	1.00	5.00	2.24	6.00	2.77
20140422	1.00	1.00	10.00	6.01	11.00	3.43
20140427	2.00	1.04	7.00	3.36	9.00	3.19
20140428	1.00	1.00	7.00	2.98	8.00	3.00
20140506	2.00	1.04	13.00	8.94	15.00	3.74
20140507	2.00	1.56	10.00	5.71	12.00	4.90
20140510	2.00	1.04	10.00	6.29	12.00	3.58
20140512	3.00	1.41	11.00	5.35	14.00	4.45
20140515	1.00	1.00	11.00	6.88	12.00	3.49
20140516	2.00	1.68	12.00	6.83	14.00	5.39
20140517	2.00	1.04	13.00	9.56	15.00	3.76
20140518	1.00	1.00	8.00	3.62	9.00	3.13
20140520	3.00	1.09	8.00	3.57	11.00	3.35
20140521	3.00	1.58	9.00	3.38	12.00	4.31
20140522	4.00	2.01	11.00	9.02	15.00	6.57
20140525	2.00	1.34	10.00	6.97	12.00	4.49
20140530	2.00	1.82	9.00	6.33	11.00	5.64
20140602	2.00	1.93	14.00	8.87	16.00	6.34
20140604	1.00	1.00	12.00	8.03	13.00	3.56
20140607	4.00	2.05	15.00	9.02	19.00	6.69
20140608	2.00	1.98	13.00	8.87	15.00	6.47
20140609	2.00	1.04	13.00	7.92	15.00	3.69
20140614	1.00	1.00	11.00	5.68	12.00	3.40
20140615	1.00	1.00	13.00	9.73	14.00	3.63
20140619	1.00	1.00	12.00	8.40	13.00	3.57
20140625	3.00	1.09	13.00	7.92	16.00	3.83
20140626	3.00	1.95	13.00	9.73	16.00	6.51
20140629	3.00	1.09	12.00	8.40	15.00	3.86

Table 4: Continued

yyyymmdd	n_f	Ne_f	n_m	Ne_m	n_e	N_e
20140704	5.00	1.20	11.00	7.90	16.00	4.16
20140706	2.00	1.04	13.00	5.20	15.00	3.48
20140707	3.00	1.46	11.00	7.06	14.00	4.85
20140710	1.00	1.00	13.00	8.87	14.00	3.59
20140719	1.00	1.00	11.00	8.09	12.00	3.56
20140723	3.00	1.14	11.00	6.52	14.00	3.88
20140727	2.00	1.39	10.00	4.75	12.00	4.31
20140728	2.00	1.04	11.00	8.14	13.00	3.70
20140729	1.00	1.00	8.00	2.85	9.00	2.96
20140730	3.00	1.09	12.00	6.55	15.00	3.74
20140802	2.00	1.09	10.00	4.79	12.00	3.55
20140803	1.00	1.00	10.00	7.39	11.00	3.52
20140804	2.00	1.91	11.00	7.72	13.00	6.12
20140807	1.00	1.00	9.00	4.96	10.00	3.33
20140822	2.00	1.04	9.00	5.86	11.00	3.54
20140824	1.00	1.00	8.00	4.57	9.00	3.28
20140825	1.00	1.00	7.00	6.19	8.00	3.44
20140826	2.00	1.04	11.00	8.82	13.00	3.74
20140827	1.00	1.00	8.00	5.15	9.00	3.35
20140830	1.00	1.00	10.00	8.53	11.00	3.58
20140901	1.00	1.00	8.00	5.20	9.00	3.35
Total	15.00	5.00	19.00	9.79	34.00	13.24
Average	1.90	1.21	10.07	6.24	11.97	3.98
Std. dev.	0.93	0.33	2.30	2.12	2.77	1.03
Min	1.00	1.00	5.00	2.22	6.00	2.76
Max	5.00	2.05	15.00	9.73	19.00	6.69
Median	2.00	1.04	10.00	6.35	12.00	3.58

Table 5: Summary statistics of effective population sizes for females (Ne_f), males (Ne_m) and both sexes (N_e), as well as estimates of fecundity, and spawning intervals for females across both the 2013 and 2014 spawning seasons.

Table 5: General genetic statistics from 2013 and 2014 spawning seasons

2013	Ne_f	Ne_m	N_e	Annual fecundity (total eggs)	Batch fecundity (eggs/spawn)	Female interval (days)
Minimum	1.00	1.66	2.50	55,000	18,000	1.00
Mean	1.38	5.89	4.26	8,200,000	460,000	11.16
Median	1.00	5.77	3.53	4,700,000	460,000	7.50
Maximum	5.25	12.73	10.20	27,000,000	790,000	64.00
Std. dev.	0.69	1.89	1.46	8,800,000	250,000	4.77
2014						
Minimum	1.00	2.22	2.75	0	0	1.00
Mean	1.21	6.23	3.98	3,100,000	250,000	19.57
Median	1.04	6.31	3.58	290,000	98,000	18.64
Maximum	2.05	9.78	6.69	23,000,000	1,200,000	77.00
Std. dev.	0.33	2.11	1.03	6,000,000	350,000	10.01

Discussion

All microsatellites loci were in HWE, and 100% of offspring were assigned to parental pairs using Cervus software. No clear spawning patterns from this system were reasonably attributed to environmental factors, as correlations were not significant. Effective population was lower than census population of brood fish, which was also reported in a similar study where cultured Asian seabass (*Lates calcarifer*) contributed unequally to offspring (Liu *et al.*, 2012). When looking at annual totals, however, this effective population number more closely represented the number of brood fish in the tank (Table 2-3). While each male contributed nearly equally, usually one female contributed to each spawn, however, the primary spawning female changed daily (Figure 6-8). This primary female lottery polygyny system was also observed in a similar study on white seabass (Gruenthal and Drawbridge, 2012).

Wild CYT, along with the majority of pelagic finfish, reproduce with eager males following ripe females, synchronizing sperm release at the time of her egg release (Gonçalves and Oliveira, 2010). This appears to be the same breeding system in these captive CYT, but has now been linked to one female clearly out-performing others by contributing ~40% of the offspring during both spawning seasons. It is important to note that only fertilized eggs were studied for this parentage analysis, and that many of the unfertilized eggs could have easily been from a different female. If our primary female had a genetic compatibility with the other males, or otherwise produced eggs more likely to be fertilized than the other females, then our results are biased towards her. However, we could not

assess unfertilized eggs for parentage analysis, for obvious reasons. This female was not the largest female in the tank, but was in the larger and older subset of brood fish, at 22.6 kg. This disproportionate offspring contribution linked to female size class has also been observed in many other fish parentage studies (Beldade *et al.*, 2012). Interestingly, the largest female, number 048-379-341, was 5 kg heavier than this primary female, but contributed only 6.2% to the annual production of offspring (Figure 3). This could be due to the largest female perhaps having added metabolic costs which reduced energy for egg production, a factor that plagues very old fish (Hixon *et al.*, 2013). For 2013, there was a slight negative correlation between fish mass and annual female fecundity; however, this correlation had a strong positive trend in 2014. The lack of wider variety in fish masses from 2013 is thought to contribute to this observation, as this correlation was not found to be significant.

Rearing at 26.5 °C has been found to lead to optimal growth rates in *Seriola lalandi* (Abbink *et al.*, 2011), and it is possible future studies could assess whether there may be similar optimal temperatures regimes for reproductive output. Aside from temperature, it is possible that these fish have some kind of hormonal or behavioral hierarchy, which was not tested for in this work. As courtship has been previously observed in CYT, it is possible that females respond to visual cues, as seen Nile tilapia (*Oreochromis niloticus*) (Castro *et al.*, 2009). In coral and pelagic fish, elevated levels of testosterone and cortisol were linked to aggressive and bold behaviors in confrontation (Chang *et al.*, 2012). These two behavioral attributes had been previously linked to successful increases

in reproduction at the cost of survival (Smith and Blumstein, 2008). In a closed system, like those used in aquaculture, these aggressive fish would be able to reproduce at increased rates with no negative effects from confrontation, unless survival was based on intraspecific interactions. However, as no fish were removed from this system without dependence on humans, this is likely not the case for our breeding system. Female 083-027-609, who provided 40% of all offspring, could have elevated hormones, or another behavioral or genetic factor involved making her the primary female in our system, which would provide interesting analysis in future studies. However, other cues for female mating choice or social hierarchy, such as male-male competition or other chemical/visual cues (Gonçalves-de-Freitas *et al.,* 2009), must not be ruled out, as these were not directly assessed in this study.

The average spawning time for 2013 and 2014 was every 5 – 19 days per female (Figure 4). The primary female of both years, female 083-027-609, spawned consistently every 5 – 6 days throughout both spawning seasons. This time was surely spent reforming eggs internally, as up to 3% loss in weight has been reported from spawning carangid species (Clarke and Privitera, 1995). However, averages for female spawning intervals presented here are much higher than other studies reported. Yellowfin (*Thunnus albacares*) and bluefin tuna (*T. thynnus*) share a similar life history of CYT in terms of being large pelagic migratory fish that broadcast spawn. These tuna spawn every 1.5 – 4.5 days, with larger females spawning more frequently (McPherson, 1991; Chen *et al.*, 2006), as was seen in our study. Other carangid fish, such as bigeye scad (*Selar*

crumenophthalmus) and round scad (*Decapturus macarellus*), spawn every 3 days (Clarke and Privitera, 1995). It is important to note, that these values are averages based upon oocyte development from histology, rather than with genetic markers for parentage as our study was done. Therefore results from previous studies based on histology may complicate comparisons of results.

Comparisons of fecundity estimate also become complicated. In carangid females, eggs are produced in high quantity >100,000 per female per spawning event) but with little per-egg energetic investment. Batch fecundity for two species of scad was reported at 92,000 and 136,000 eggs (Clarke and Privitera, 1995), 344,700 eggs for horse mackerel (*Trachurus trachurs*) (Macer, 1974), and an estimated 95,000 eggs from CYT (Stuart and Drawbridge, 2013). These values represent calculations based on oocyte development, ovarian weight and/or number of females present. Other studies of the local *Seriola dorsalis* estimate batch fecundity around 450,000 – 940,000 eggs (Baxter, 1960). These measurements of fecundity do not track individual reproduction, or fecundity of individual females. Our results were within the same orders of magnitude, with larger females producing ~500,000 eggs per spawn, and smaller females producing 35,000 eggs per spawn. Also like the mackerel and scad species above, batch fecundity increased with mass of the female in CYT. Annual fecundity and female mass (Figure 5) were positively correlated in CYT ($\rho = 0.753$), as seen in many other fish species, including estimates from wild *Seriola dorsalis* (Baxter, 1960; Beldade *et al.*, 2012; Hixon *et al.*, 2013).

This study provides the first detailed evaluation on mating system, measurements of fecundity, and spawning intervals for CYT, based on genetic assignment of parentage. These data are on the same order of magnitude as those previously reported for wild carangid species, using histologic evaluation of ovarian development only. By understanding this lottery polygyny spawning system behavior, brood fish management could be implemented to potentially enhance egg production. While we did not directly test mating strategies, a similar study in minnows (*Hybognathus amarus*) had more successful offspring when allowed to mate in naturalized settings (e.g. environmental or hormonal cues) compared to forced monogamy (Osborne *et al.*, 2013). In terms of our study, for example, lesser contributing females may produce more in the absence of the primary female in this tank (female 083-027-609). By removing these females to a new brood tank without the presence of female 083-027-609, they might begin to produce more offspring. Sex ratios could also be skewed in favor of females, as was done for white seabass after the conclusions from Gruenthal and Drawbridge (2012).

In conclusion, CYT spawning was dynamic, but predictable in our system, with males spawning equally during each spawning event, and females spawning at fairly consistent intervals. Female 083-027-609 is clearly the primary female of both spawning seasons, as she spawned more frequently and produced more eggs than any other female for 2013 and 2014. Females that were ~20 kg produced more than double the amount of eggs as females in lower weight classes, with some smaller females not producing at all. Measurements of batch and annual

fecundity were consistent with wild estimates, despite these data coming from cultured fish. These data can be used to better understand how wild fish interact during the spawning season, and will also benefit the direct management of this breeding program in San Diego.

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CONCLUSION TO THESIS

Spawning behavior in fish species is varied and dynamic. Having a thorough understanding of reproduction will aide in management of wild fisheries, or in aquaculture breeding programs. Similar to other pelagic teleosts, CYT broadcast spawn in aggregations. This makes understanding intricacies of spawning nearly impossible without genetic markers, which was done for the first time in the present study on this species. Using microsatellite loci genotyped to a group of brood fish CYT from San Diego, CA, parentage was assigned and analyzed over two spawning seasons.

To gain a better understanding of spawning dynamics, subsampling needed to be adequate for relative brood fish contributions to be meaningfully assigned. By assigning parental contribution to a range of sample sizes of offspring, both in actual practice and via computer simulation, a minimum sampling size was determined to be $n \geq 30$ offspring per spawning event. In contrast, when a power analysis was applied under theoretical conditions, assuming infinite sample sizes and equal allelic contribution from each potential parent, subsampling was determined to be $n = 385$ offspring per spawn. Our results show that an $n \geq 30$ offspring, statistically significant means and 95% confidence intervals are achieved in comparison to "true" proportional parental contribution. This is similar to other studies which use microsatellite markers for population genetic assessments, but is the first study to directly evaluate microsatellites for parentage studies.

Using this method justification, n = 47 offspring were assigned to parental pairs from every spawn occurring during the 2013 and 2014 spawning season of CYT. Using n = 47 offspring per spawn, allowed us to use a statistically meaningful sample size that also fit into a typical 96-well plate, universally used for genetic lab work. There was no obvious novel correlation between spawning time and environmental factors. For each spawn, there was one primary female in egg production, with males contributing sperm nearly equally. This lottery polygyny system occurred in both 2013 and 2014. However, the additional smaller fish added in 2014 contributed disproportionately fewer offspring than the larger fish available both years tested. Female mass was positively correlated with offspring production, as seen in many other species of fish. Larger females usually spawned weekly or bimonthly, while larger males contributed to offspring production nearly every day.

These patterns could represent hormonal dominance or another form of social hierarchy seen in these fish. Characterization of the individual spawning events showed one female contributing nearly 40% of all offspring over each year. This could lead to interesting follow up studies to test her levels of testosterone or cortisol, or direct observation of the lesser female behavior if removed from this primary female. If this primary female was removed, and the social hierarchy of females allowed to reestablish, it is possible that the lesser contributing females may increase their egg production. This would directly affect the egg production at the HSWRI facility, at least relative to diversity. This

information could also help with management of other aquaculture facilities with similar fish.

This study will lead to the advancement of these brood fish in captivity, but can also be used to better understand fish on the global scale. As these fish were wild-caught, and results regarding fecundity and female spawning intervals found from this study are consistent with wild data, it is possible to apply results here to support fisheries management. These data are the first of their kind reported in CYT, and this sample collection justification one of the first for genetic assignment of parentage. The data from this study not only characterized individual spawning events from up to 19 fish, over two years, but also successfully found a statistically significant –and reasonable- sub-sample size to analyze parental pair patterns.

Appendix 1 –In-house made reagents.

10% (w/v) Chelex resin:

10g Chelex resin

100 ml milli-Q water

10X PCR Buffer; pH 8.8:

670 mM Tris

166 mM $(NH_4)_2SO_4$

20 mM $MgCl_2$

100 mM ß-mercaptoethanol

Bovine Serum Albumin (BSA): 20 mg / mL

0.22 µm filtered and UV sterilized.

dNTP Mix 2 mM each dNTP:

4 µl dATP (100 mM)

4 µl dCTP (100 mM)

4 µl dGTP (100 mM)

4 µl dTTP (100 mM)

184 µl milli-Q water

Appendix 2 -- All PCR mixes

Reagent	Per reaction (µl)	Master mix (µl x100)	Fluorescent label
PCR 1			
Milli-Q water	7.20	720	
10X PCR buffer	1.00	100	
dNTPs (2 mM)	1.00	100	
BSA	0.25	25	
Sequ 38 F (10 mM)	0.25	25	5HEX
Sequ 38 R (10 mM)	0.25	25	
Taq Polymerase	0.05	5	
DNA	1.00	--	
PCR 2			
Milli-Q water	5.79	579	
10X PCR buffer	1.00	100	
dNTPs (2 mM)	1.00	100	
BSA	0.25	25	
Sequ 77 F (10 mM)	0.25	25	5HEX
Sequ 77 R (10 mM)	0.25	25	
Sdu gA3D F (10 mM)	0.15	15	56-FAM
Sdu gA3D R (10 mM)	0.15	15	
Sdu 46 F (10 mM)	0.25	25	56-TAMN
Sdu 46 R (10 mM)	0.25	25	
Sdu 4 F (10 mM)	0.30	30	56-FAM
Sdu 4 R (10 mM)	0.30	30	
Taq Polymerase	0.06	6	
DNA	1.00	--	
PCR 3			
Milli-Q water	5.89	589	
10X PCR buffer	1.00	100	
dNTPs (2 mM)	1.00	100	
BSA	0.25	25	
Sequ 320 F (10 mM)	0.20	20	5HEX
Sequ 320 R (10 mM)	0.20	20	
Sequ 230 F (10 mM)	0.15	15	56-FAM
Sequ 230 R (10 mM)	0.15	15	
Sdu 10 F (10 mM)	0.25	25	56-TAMN
Sdu 10 R (10 mM)	0.25	25	
Sdn 06 F (10 mM)	0.30	30	56-FAM
Sdn 06 R (10 mM)	0.30	30	
Taq Polymerase	0.06	6	
DNA	1.00	--	

Appendix 3: Mean values and 95% confidence intervals (CI) of all CYT brood fish from the 2013. Pit tag numbers are listed above each set of values. Date of spawn and simulated sample size (S n-value) is listed in first column. Note as simulated sample sizes increase, CI become closer to the mean for each brood fish at each spawn.

Appendix 3: Mean values and 95% confidence intervals for CYT brood fish bootstap simulation									
mmddyy -	048-379-341			048-519-049			061-363-804		
S n-value	Mean	CI 2.5%	CI 97.5%	Mean	CI 2.5%	CI 97.5%	Mean	CI 2.5%	CI 97.5%
04/29/13 - 10	0.0000	0.0000	0.0000	0.6030	0.3000	0.9000	0.0000	0.0000	0.0000
04/29/13 - 20	0.0000	0.0000	0.0000	0.6023	0.4000	0.8000	0.0000	0.0000	0.0000
04/29/13 - 30	0.0000	0.0000	0.0000	0.6028	0.4333	0.7667	0.0000	0.0000	0.0000
04/29/13 - 40	0.0000	0.0000	0.0000	0.6032	0.4500	0.7500	0.0000	0.0000	0.0000
04/29/13 - 47	0.0000	0.0000	0.0000	0.6041	0.4681	0.7234	0.0000	0.0000	0.0000
04/29/13 - 60	0.0000	0.0000	0.0000	0.6024	0.4833	0.7167	0.0000	0.0000	0.0000
04/29/13 - 100	0.0000	0.0000	0.0000	0.6035	0.5200	0.6800	0.0000	0.0000	0.0000
04/29/13 - 150	0.0000	0.0000	0.0000	0.6030	0.5467	0.6600	0.0000	0.0000	0.0000
04/29/13 - 200	0.0000	0.0000	0.0000	0.6029	0.5600	0.6450	0.0000	0.0000	0.0000
04/29/13 - 250	0.0000	0.0000	0.0000	0.6025	0.5680	0.6360	0.0000	0.0000	0.0000
04/29/13 - 300	0.0000	0.0000	0.0000	0.6025	0.5800	0.6233	0.0000	0.0000	0.0000
05/17/13 - 10	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
05/17/13 - 20	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
05/17/13 - 30	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
05/17/13 - 40	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
05/17/13 - 47	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
05/17/13 - 60	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
05/17/13 - 100	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
05/17/13 - 150	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
05/17/13 - 200	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
05/17/13 - 250	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
05/17/13 - 300	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
06/21/13 - 10	0.0000	0.0000	0.0000	0.0527	0.0000	0.2000	0.9473	0.8000	1.0000
06/21/13 - 20	0.0000	0.0000	0.0000	0.0523	0.0000	0.1500	0.9477	0.8500	1.0000
06/21/13 - 30	0.0000	0.0000	0.0000	0.0519	0.0000	0.1333	0.9481	0.8667	1.0000
06/21/13 - 40	0.0000	0.0000	0.0000	0.0518	0.0000	0.1250	0.9482	0.8750	1.0000
06/21/13 - 47	0.0000	0.0000	0.0000	0.0523	0.0000	0.1277	0.9477	0.8723	1.0000
06/21/13 - 60	0.0000	0.0000	0.0000	0.0516	0.0000	0.1000	0.9484	0.9000	1.0000
06/21/13 - 100	0.0000	0.0000	0.0000	0.0519	0.0200	0.0900	0.9481	0.9100	0.9800
06/21/13 - 150	0.0000	0.0000	0.0000	0.0521	0.0267	0.0800	0.9479	0.9200	0.9733
06/21/13 - 200	0.0000	0.0000	0.0000	0.0519	0.0300	0.0700	0.9481	0.9300	0.9700
06/21/13 - 250	0.0000	0.0000	0.0000	0.0518	0.0360	0.0680	0.9482	0.9320	0.9640
06/21/13 - 300	0.0000	0.0000	0.0000	0.0519	0.0400	0.0600	0.9481	0.9400	0.9600
07/26/13 - 10	0.0028	0.0000	0.1000	0.9944	0.9000	1.0000	0.0000	0.0000	0.0000
07/26/13 - 20	0.0027	0.0000	0.0500	0.9946	0.9500	1.0000	0.0000	0.0000	0.0000
07/26/13 - 30	0.0026	0.0000	0.0333	0.9948	0.9667	1.0000	0.0000	0.0000	0.0000
07/26/13 - 40	0.0026	0.0000	0.0250	0.9948	0.9750	1.0000	0.0000	0.0000	0.0000
07/26/13 - 47	0.0027	0.0000	0.0213	0.9947	0.9787	1.0000	0.0000	0.0000	0.0000
07/26/13 - 60	0.0026	0.0000	0.0167	0.9948	0.9833	1.0000	0.0000	0.0000	0.0000
07/26/13 - 100	0.0026	0.0000	0.0100	0.9948	0.9800	1.0000	0.0000	0.0000	0.0000
07/26/13 - 150	0.0026	0.0000	0.0067	0.9947	0.9867	1.0000	0.0000	0.0000	0.0000
07/26/13 - 200	0.0027	0.0000	0.0050	0.9947	0.9900	1.0000	0.0000	0.0000	0.0000
07/26/13 - 250	0.0027	0.0000	0.0040	0.9947	0.9920	1.0000	0.0000	0.0000	0.0000
07/26/13 - 300	0.0027	0.0000	0.0033	0.9947	0.9933	1.0000	0.0000	0.0000	0.0000
08/26/13 - 10	0.0000	0.0000	0.0000	0.2336	0.0000	0.5000	0.0000	0.0000	0.0000
08/26/13 - 20	0.0000	0.0000	0.0000	0.2345	0.0500	0.4000	0.0000	0.0000	0.0000
08/26/13 - 30	0.0000	0.0000	0.0000	0.2343	0.1000	0.4000	0.0000	0.0000	0.0000
08/26/13 - 40	0.0000	0.0000	0.0000	0.2331	0.1250	0.3500	0.0000	0.0000	0.0000
08/26/13 - 47	0.0000	0.0000	0.0000	0.2345	0.1277	0.3410	0.0000	0.0000	0.0000
08/26/13 - 60	0.0000	0.0000	0.0000	0.2341	0.1333	0.3333	0.0000	0.0000	0.0000
08/26/13 - 100	0.0000	0.0000	0.0000	0.2341	0.1700	0.3000	0.0000	0.0000	0.0000
08/26/13 - 150	0.0000	0.0000	0.0000	0.2334	0.1867	0.2800	0.0000	0.0000	0.0000
08/26/13 - 200	0.0000	0.0000	0.0000	0.2337	0.1950	0.2700	0.0000	0.0000	0.0000
08/26/13 - 250	0.0000	0.0000	0.0000	0.2342	0.2080	0.2600	0.0000	0.0000	0.0000
08/26/13 - 300	0.0000	0.0000	0.0000	0.2338	0.2200	0.2467	0.0000	0.0000	0.0000

Appendix 3: Mean values and 95% confidence intervals for CYT brood fish bootstap simulation									
mmddyy -	061-375-363			061-621-862			083-026-876		
S n-value	Mean	CI 2.5%	CI 97.5%	Mean	CI 2.5%	CI 97.5%	Mean	CI 2.5%	CI 97.5%
04/29/13 - 10	0.0029	0.0000	0.1000	0.0000	0.0000	0.0000	0.0032	0.0000	0.1000
04/29/13 - 20	0.0027	0.0000	0.0500	0.0000	0.0000	0.0000	0.0028	0.0000	0.0500
04/29/13 - 30	0.0029	0.0000	0.0333	0.0000	0.0000	0.0000	0.0030	0.0000	0.0333
04/29/13 - 40	0.0028	0.0000	0.0250	0.0000	0.0000	0.0000	0.0029	0.0000	0.0250
04/29/13 - 47	0.0027	0.0000	0.0213	0.0000	0.0000	0.0000	0.0028	0.0000	0.0213
04/29/13 - 60	0.0028	0.0000	0.0167	0.0000	0.0000	0.0000	0.0028	0.0000	0.0167
04/29/13 - 100	0.0028	0.0000	0.0100	0.0000	0.0000	0.0000	0.0028	0.0000	0.0100
04/29/13 - 150	0.0028	0.0000	0.0067	0.0000	0.0000	0.0000	0.0028	0.0000	0.0067
04/29/13 - 200	0.0028	0.0000	0.0050	0.0000	0.0000	0.0000	0.0028	0.0000	0.0050
04/29/13 - 250	0.0028	0.0000	0.0040	0.0000	0.0000	0.0000	0.0028	0.0000	0.0040
04/29/13 - 300	0.0028	0.0000	0.0033	0.0000	0.0000	0.0000	0.0028	0.0000	0.0033
05/17/13 - 10	0.0000	0.0000	0.0000	0.5018	0.2000	0.8000	0.0029	0.0000	0.1000
05/17/13 - 20	0.0000	0.0000	0.0000	0.4987	0.3000	0.7000	0.0027	0.0000	0.0500
05/17/13 - 30	0.0000	0.0000	0.0000	0.4982	0.3333	0.6667	0.0028	0.0000	0.0333
05/17/13 - 40	0.0000	0.0000	0.0000	0.5000	0.3500	0.6500	0.0028	0.0000	0.0250
05/17/13 - 47	0.0000	0.0000	0.0000	0.4977	0.3617	0.6383	0.0028	0.0000	0.0213
05/17/13 - 60	0.0000	0.0000	0.0000	0.4987	0.3833	0.6167	0.0029	0.0000	0.0167
05/17/13 - 100	0.0000	0.0000	0.0000	0.4978	0.4200	0.5800	0.0028	0.0000	0.0100
05/17/13 - 150	0.0000	0.0000	0.0000	0.4984	0.4333	0.5600	0.0028	0.0000	0.0067
05/17/13 - 200	0.0000	0.0000	0.0000	0.4984	0.4550	0.5450	0.0028	0.0000	0.0050
05/17/13 - 250	0.0000	0.0000	0.0000	0.4986	0.4640	0.5320	0.0028	0.0000	0.0040
05/17/13 - 300	0.0000	0.0000	0.0000	0.4986	0.4767	0.5200	0.0028	0.0000	0.0033
06/21/13 - 10	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
06/21/13 - 20	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
06/21/13 - 30	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
06/21/13 - 40	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
06/21/13 - 47	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
06/21/13 - 60	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
06/21/13 - 100	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
06/21/13 - 150	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
06/21/13 - 200	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
06/21/13 - 250	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
06/21/13 - 300	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
07/26/13 - 10	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
07/26/13 - 20	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
07/26/13 - 30	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
07/26/13 - 40	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
07/26/13 - 47	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
07/26/13 - 60	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
07/26/13 - 100	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
07/26/13 - 150	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
07/26/13 - 200	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
07/26/13 - 250	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
07/26/13 - 300	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
08/26/13 - 10	0.0000	0.0000	0.0000	0.0031	0.0000	0.1000	0.7633	0.5000	1.0000
08/26/13 - 20	0.0000	0.0000	0.0000	0.0030	0.0000	0.0500	0.7624	0.5500	0.9500
08/26/13 - 30	0.0000	0.0000	0.0000	0.0031	0.0000	0.0333	0.7626	0.6000	0.9000
08/26/13 - 40	0.0000	0.0000	0.0000	0.0030	0.0000	0.0250	0.7639	0.6500	0.8750
08/26/13 - 47	0.0000	0.0000	0.0000	0.0031	0.0000	0.0213	0.7624	0.6383	0.8723
08/26/13 - 60	0.0000	0.0000	0.0000	0.0031	0.0000	0.0167	0.7628	0.6667	0.8500
08/26/13 - 100	0.0000	0.0000	0.0000	0.0031	0.0000	0.0100	0.7629	0.6900	0.8300
08/26/13 - 150	0.0000	0.0000	0.0000	0.0031	0.0000	0.0067	0.7635	0.7133	0.8133
08/26/13 - 200	0.0000	0.0000	0.0000	0.0030	0.0000	0.0050	0.7633	0.7250	0.8000
08/26/13 - 250	0.0000	0.0000	0.0000	0.0031	0.0000	0.0040	0.7627	0.7400	0.7880
08/26/13 - 300	0.0000	0.0000	0.0000	0.0031	0.0000	0.0033	0.7631	0.7500	0.7767

Appendix 3: Mean values and 95% confidence intervals for CYT brood fish bootstap simulation									
mmddyy -	083-027-609			083-103-352			048-533-355		
S n-value	Mean	CI 2.5%	CI 97.5%	Mean	CI 2.5%	CI 97.5%	Mean	CI 2.5%	CI 97.5%
04/29/13 - 10	0.3910	0.1000	0.7000	0.2006	0.0000	0.5000	0.0338	0.0000	0.2000
04/29/13 - 20	0.3922	0.2000	0.6000	0.2000	0.0500	0.4000	0.0331	0.0000	0.1000
04/29/13 - 30	0.3914	0.2333	0.5667	0.1991	0.0667	0.3333	0.0344	0.0000	0.1000
04/29/13 - 40	0.3911	0.2500	0.5250	0.1999	0.1000	0.3250	0.0339	0.0000	0.1000
04/29/13 - 47	0.3905	0.2553	0.5319	0.1994	0.1064	0.3191	0.0332	0.0000	0.0851
04/29/13 - 60	0.3920	0.2833	0.5000	0.2011	0.1167	0.3000	0.0337	0.0000	0.0833
04/29/13 - 100	0.3908	0.3100	0.4700	0.1997	0.1300	0.2700	0.0337	0.0100	0.0700
04/29/13 - 150	0.3913	0.3333	0.4533	0.2002	0.1533	0.2467	0.0339	0.0133	0.0533
04/29/13 - 200	0.3915	0.3450	0.4350	0.1998	0.1650	0.2350	0.0338	0.0150	0.0500
04/29/13 - 250	0.3918	0.3600	0.4240	0.1999	0.1720	0.2280	0.0338	0.0200	0.0440
04/29/13 - 300	0.3918	0.3700	0.4133	0.1999	0.1800	0.2167	0.0338	0.0267	0.0400
05/17/13 - 10	0.4953	0.2000	0.8000	0.0454	0.0000	0.2000	0.1009	0.0000	0.3000
05/17/13 - 20	0.4987	0.3000	0.7000	0.0451	0.0000	0.1500	0.1005	0.0000	0.2500
05/17/13 - 30	0.4989	0.3333	0.6667	0.0448	0.0000	0.1333	0.1003	0.0000	0.2000
05/17/13 - 40	0.4972	0.3500	0.6500	0.0449	0.0000	0.1250	0.1014	0.0250	0.2000
05/17/13 - 47	0.4995	0.3617	0.6383	0.0453	0.0000	0.1064	0.1017	0.0213	0.1915
05/17/13 - 60	0.4984	0.3833	0.6167	0.0454	0.0000	0.1000	0.1010	0.0333	0.1667
05/17/13 - 100	0.4995	0.4200	0.5800	0.0449	0.0100	0.0800	0.1012	0.0500	0.1500
05/17/13 - 150	0.4988	0.4400	0.5600	0.0451	0.0200	0.0733	0.1015	0.0667	0.1400
05/17/13 - 200	0.4988	0.4550	0.5450	0.0452	0.0250	0.0650	0.1016	0.0750	0.1300
05/17/13 - 250	0.4986	0.4640	0.5320	0.0450	0.0320	0.0600	0.1015	0.0800	0.1200
05/17/13 - 300	0.4986	0.4767	0.5200	0.0451	0.0367	0.0533	0.1015	0.0867	0.1133
06/21/13 - 10	0.0000	0.0000	0.0000	0.0602	0.0000	0.2000	0.0928	0.0000	0.3000
06/21/13 - 20	0.0000	0.0000	0.0000	0.0597	0.0000	0.2000	0.0923	0.0000	0.2500
06/21/13 - 30	0.0000	0.0000	0.0000	0.0598	0.0000	0.1667	0.0930	0.0000	0.2000
06/21/13 - 40	0.0000	0.0000	0.0000	0.0606	0.0000	0.1250	0.0932	0.0250	0.1750
06/21/13 - 47	0.0000	0.0000	0.0000	0.0602	0.0000	0.1277	0.0932	0.0213	0.1702
06/21/13 - 60	0.0000	0.0000	0.0000	0.0603	0.0167	0.1167	0.0923	0.0333	0.1667
06/21/13 - 100	0.0000	0.0000	0.0000	0.0600	0.0200	0.1000	0.0927	0.0500	0.1400
06/21/13 - 150	0.0000	0.0000	0.0000	0.0601	0.0333	0.0867	0.0928	0.0600	0.1267
06/21/13 - 200	0.0000	0.0000	0.0000	0.0600	0.0400	0.0800	0.0929	0.0650	0.1200
06/21/13 - 250	0.0000	0.0000	0.0000	0.0602	0.0440	0.0760	0.0929	0.0720	0.1120
06/21/13 - 300	0.0000	0.0000	0.0000	0.0600	0.0467	0.0700	0.0930	0.0767	0.1067
07/26/13 - 10	0.0028	0.0000	0.1000	0.4455	0.2000	0.7000	0.0081	0.0000	0.1000
07/26/13 - 20	0.0027	0.0000	0.0500	0.4463	0.2500	0.6500	0.0079	0.0000	0.0500
07/26/13 - 30	0.0026	0.0000	0.0333	0.4454	0.2667	0.6000	0.0079	0.0000	0.0333
07/26/13 - 40	0.0026	0.0000	0.0250	0.4460	0.3000	0.6000	0.0078	0.0000	0.0500
07/26/13 - 47	0.0026	0.0000	0.0213	0.4458	0.3191	0.5745	0.0078	0.0000	0.0426
07/26/13 - 60	0.0026	0.0000	0.0167	0.4455	0.3333	0.5667	0.0081	0.0000	0.0333
07/26/13 - 100	0.0026	0.0000	0.0100	0.4451	0.3600	0.5300	0.0079	0.0000	0.0200
07/26/13 - 150	0.0027	0.0000	0.0067	0.4452	0.3867	0.5067	0.0079	0.0000	0.0200
07/26/13 - 200	0.0027	0.0000	0.0050	0.4453	0.3950	0.4950	0.0080	0.0000	0.0150
07/26/13 - 250	0.0027	0.0000	0.0040	0.4459	0.4120	0.4840	0.0080	0.0000	0.0120
07/26/13 - 300	0.0027	0.0000	0.0033	0.4458	0.4200	0.4700	0.0080	0.0033	0.0100
08/26/13 - 10	0.0000	0.0000	0.0000	0.1728	0.0000	0.4000	0.0831	0.0000	0.3000
08/26/13 - 20	0.0000	0.0000	0.0000	0.1720	0.0500	0.3500	0.0831	0.0000	0.2000
08/26/13 - 30	0.0000	0.0000	0.0000	0.1728	0.0667	0.3000	0.0834	0.0000	0.2000
08/26/13 - 40	0.0000	0.0000	0.0000	0.1722	0.0750	0.3000	0.0832	0.0250	0.1750
08/26/13 - 47	0.0000	0.0000	0.0000	0.1722	0.0851	0.2766	0.0831	0.0213	0.1702
08/26/13 - 60	0.0000	0.0000	0.0000	0.1727	0.0833	0.2504	0.0833	0.0167	0.1500
08/26/13 - 100	0.0000	0.0000	0.0000	0.1718	0.1100	0.2300	0.0832	0.0400	0.1300
08/26/13 - 150	0.0000	0.0000	0.0000	0.1724	0.1267	0.2133	0.0831	0.0533	0.1133
08/26/13 - 200	0.0000	0.0000	0.0000	0.1723	0.1400	0.2050	0.0831	0.0600	0.1050
08/26/13 - 250	0.0000	0.0000	0.0000	0.1722	0.1480	0.1921	0.0831	0.0640	0.1000
08/26/13 - 300	0.0000	0.0000	0.0000	0.1724	0.1600	0.1833	0.0831	0.0733	0.0900

Appendix 3: Mean values and 95% confidence intervals for CYT brood fish bootstap simulation									
mmddyy -	048-567-798			048-624-825			048-779-622		
S n-value	Mean	CI 2.5%	CI 97.5%	Mean	CI 2.5%	CI 97.5%	Mean	CI 2.5%	CI 97.5%
04/29/13 - 10	0.2126	0.0000	0.5000	0.0000	0.0000	0.0000	0.0647	0.0000	0.2000
04/29/13 - 20	0.2126	0.0500	0.4000	0.0000	0.0000	0.0000	0.0647	0.0000	0.2000
04/29/13 - 30	0.2146	0.1000	0.3667	0.0000	0.0000	0.0000	0.0652	0.0000	0.1667
04/29/13 - 40	0.2140	0.1000	0.3250	0.0000	0.0000	0.0000	0.0646	0.0000	0.1500
04/29/13 - 47	0.2148	0.1064	0.3191	0.0000	0.0000	0.0000	0.0650	0.0000	0.1277
04/29/13 - 60	0.2134	0.1167	0.3167	0.0000	0.0000	0.0000	0.0647	0.0167	0.1333
04/29/13 - 100	0.2144	0.1500	0.2800	0.0000	0.0000	0.0000	0.0650	0.0300	0.1100
04/29/13 - 150	0.2140	0.1667	0.2602	0.0000	0.0000	0.0000	0.0647	0.0333	0.0933
04/29/13 - 200	0.2143	0.1750	0.2500	0.0000	0.0000	0.0000	0.0647	0.0400	0.0850
04/29/13 - 250	0.2143	0.1840	0.2400	0.0000	0.0000	0.0000	0.0648	0.0480	0.0800
04/29/13 - 300	0.2140	0.1967	0.2300	0.0000	0.0000	0.0000	0.0647	0.0533	0.0733
05/17/13 - 10	0.3698	0.1000	0.7000	0.0091	0.0000	0.1000	0.0085	0.0000	0.1000
05/17/13 - 20	0.3670	0.1500	0.6000	0.0085	0.0000	0.0500	0.0085	0.0000	0.0500
05/17/13 - 30	0.3696	0.2000	0.5333	0.0083	0.0000	0.0333	0.0084	0.0000	0.0333
05/17/13 - 40	0.3682	0.2250	0.5000	0.0083	0.0000	0.0500	0.0086	0.0000	0.0500
05/17/13 - 47	0.3690	0.2340	0.4894	0.0085	0.0000	0.0426	0.0084	0.0000	0.0426
05/17/13 - 60	0.3695	0.2667	0.4833	0.0085	0.0000	0.0333	0.0085	0.0000	0.0333
05/17/13 - 100	0.3688	0.2900	0.4500	0.0085	0.0000	0.0200	0.0085	0.0000	0.0200
05/17/13 - 150	0.3692	0.3133	0.4267	0.0084	0.0000	0.0200	0.0085	0.0000	0.0200
05/17/13 - 200	0.3691	0.3250	0.4100	0.0084	0.0000	0.0150	0.0085	0.0000	0.0150
05/17/13 - 250	0.3690	0.3360	0.4000	0.0085	0.0000	0.0120	0.0085	0.0040	0.0120
05/17/13 - 300	0.3692	0.3467	0.3900	0.0085	0.0033	0.0100	0.0084	0.0033	0.0100
06/21/13 - 10	0.1750	0.0000	0.4000	0.0115	0.0000	0.1000	0.0225	0.0000	0.1000
06/21/13 - 20	0.1754	0.0500	0.3500	0.0111	0.0000	0.0500	0.0221	0.0000	0.1000
06/21/13 - 30	0.1753	0.0667	0.3000	0.0112	0.0000	0.0667	0.0214	0.0000	0.0667
06/21/13 - 40	0.1751	0.0750	0.3000	0.0111	0.0000	0.0500	0.0212	0.0000	0.0750
06/21/13 - 47	0.1742	0.0851	0.2766	0.0110	0.0000	0.0426	0.0219	0.0000	0.0638
06/21/13 - 60	0.1743	0.0833	0.2667	0.0109	0.0000	0.0333	0.0221	0.0000	0.0667
06/21/13 - 100	0.1748	0.1100	0.2400	0.0111	0.0000	0.0300	0.0219	0.0000	0.0500
06/21/13 - 150	0.1743	0.1267	0.2200	0.0108	0.0000	0.0202	0.0220	0.0067	0.0400
06/21/13 - 200	0.1749	0.1400	0.2100	0.0110	0.0000	0.0200	0.0219	0.0100	0.0350
06/21/13 - 250	0.1748	0.1480	0.2000	0.0109	0.0040	0.0160	0.0218	0.0120	0.0320
06/21/13 - 300	0.1747	0.1567	0.1933	0.0109	0.0067	0.0133	0.0218	0.0133	0.0267
07/26/13 - 10	0.0522	0.0000	0.2000	0.0162	0.0000	0.1000	0.0101	0.0000	0.1000
07/26/13 - 20	0.0530	0.0000	0.1500	0.0159	0.0000	0.1000	0.0111	0.0000	0.0500
07/26/13 - 30	0.0524	0.0000	0.1333	0.0157	0.0000	0.0667	0.0106	0.0000	0.0667
07/26/13 - 40	0.0533	0.0000	0.1250	0.0162	0.0000	0.0500	0.0105	0.0000	0.0500
07/26/13 - 47	0.0536	0.0000	0.1277	0.0160	0.0000	0.0638	0.0108	0.0000	0.0426
07/26/13 - 60	0.0532	0.0000	0.1167	0.0159	0.0000	0.0500	0.0107	0.0000	0.0333
07/26/13 - 100	0.0527	0.0200	0.0900	0.0162	0.0000	0.0400	0.0105	0.0000	0.0300
07/26/13 - 150	0.0531	0.0267	0.0800	0.0159	0.0000	0.0333	0.0107	0.0000	0.0267
07/26/13 - 200	0.0532	0.0300	0.0750	0.0157	0.0050	0.0250	0.0106	0.0000	0.0200
07/26/13 - 250	0.0531	0.0360	0.0680	0.0159	0.0080	0.0240	0.0106	0.0040	0.0160
07/26/13 - 300	0.0530	0.0400	0.0633	0.0159	0.0100	0.0200	0.0106	0.0033	0.0133
08/26/13 - 10	0.1264	0.0000	0.3000	0.0490	0.0000	0.2000	0.0519	0.0000	0.2000
08/26/13 - 20	0.1268	0.0000	0.3000	0.0498	0.0000	0.1500	0.0530	0.0000	0.1500
08/26/13 - 30	0.1256	0.0333	0.2333	0.0494	0.0000	0.1333	0.0523	0.0000	0.1333
08/26/13 - 40	0.1270	0.0500	0.2250	0.0489	0.0000	0.1250	0.0519	0.0000	0.1250
08/26/13 - 47	0.1264	0.0426	0.2128	0.0491	0.0000	0.1064	0.0518	0.0000	0.1064
08/26/13 - 60	0.1253	0.0500	0.2000	0.0490	0.0000	0.1000	0.0522	0.0000	0.1000
08/26/13 - 100	0.1257	0.0700	0.1800	0.0490	0.0200	0.0900	0.0525	0.0200	0.0900
08/26/13 - 150	0.1260	0.0867	0.1667	0.0493	0.0267	0.0733	0.0523	0.0267	0.0800
08/26/13 - 200	0.1262	0.0950	0.1550	0.0492	0.0300	0.0650	0.0525	0.0350	0.0700
08/26/13 - 250	0.1262	0.1040	0.1440	0.0492	0.0360	0.0600	0.0524	0.0400	0.0640
08/26/13 - 300	0.1261	0.1133	0.1367	0.0492	0.0400	0.0533	0.0523	0.0433	0.0567

Appendix 3: Mean values and 95% confidence intervals for CYT brood fish bootstap simulation									
mmddyy -	061-381-264			061-536-613			083-024-797		
S n-value	Mean	CI 2.5%	CI 97.5%	Mean	CI 2.5%	CI 97.5%	Mean	CI 2.5%	CI 97.5%
04/29/13 - 10	0.0641	0.0000	0.2000	0.1728	0.0000	0.4000	0.0114	0.0000	0.1000
04/29/13 - 20	0.0661	0.0000	0.2000	0.1723	0.0500	0.3500	0.0112	0.0000	0.0500
04/29/13 - 30	0.0650	0.0000	0.1667	0.1717	0.0667	0.3000	0.0111	0.0000	0.0667
04/29/13 - 40	0.0652	0.0000	0.1500	0.1716	0.0750	0.2750	0.0114	0.0000	0.0500
04/29/13 - 47	0.0649	0.0000	0.1277	0.1718	0.0851	0.2766	0.0114	0.0000	0.0426
04/29/13 - 60	0.0653	0.0167	0.1333	0.1715	0.0833	0.2667	0.0113	0.0000	0.0333
04/29/13 - 100	0.0645	0.0300	0.1100	0.1718	0.1100	0.2400	0.0112	0.0000	0.0300
04/29/13 - 150	0.0647	0.0333	0.0933	0.1720	0.1267	0.2200	0.0113	0.0000	0.0267
04/29/13 - 200	0.0650	0.0400	0.0900	0.1716	0.1350	0.2050	0.0112	0.0000	0.0200
04/29/13 - 250	0.0647	0.0480	0.0800	0.1717	0.1440	0.1960	0.0113	0.0040	0.0160
04/29/13 - 300	0.0648	0.0533	0.0733	0.1719	0.1533	0.1867	0.0112	0.0067	0.0133
05/17/13 - 10	0.1487	0.0000	0.4000	0.1096	0.0000	0.3000	0.1094	0.0000	0.3000
05/17/13 - 20	0.1502	0.0000	0.3000	0.1115	0.0000	0.2500	0.1104	0.0000	0.2500
05/17/13 - 30	0.1505	0.0333	0.2667	0.1099	0.0000	0.2333	0.1097	0.0000	0.2333
05/17/13 - 40	0.1497	0.0500	0.2500	0.1099	0.0250	0.2000	0.1095	0.0250	0.2000
05/17/13 - 47	0.1488	0.0638	0.2553	0.1100	0.0426	0.1915	0.1096	0.0426	0.1915
05/17/13 - 60	0.1482	0.0667	0.2333	0.1102	0.0500	0.1833	0.1099	0.0500	0.1833
05/17/13 - 100	0.1496	0.0900	0.2100	0.1099	0.0600	0.1600	0.1096	0.0600	0.1600
05/17/13 - 150	0.1488	0.1067	0.1933	0.1099	0.0733	0.1467	0.1096	0.0733	0.1467
05/17/13 - 200	0.1492	0.1150	0.1800	0.1098	0.0800	0.1400	0.1096	0.0800	0.1400
05/17/13 - 250	0.1492	0.1240	0.1720	0.1098	0.0880	0.1320	0.1099	0.0880	0.1280
05/17/13 - 300	0.1492	0.1333	0.1633	0.1098	0.0933	0.1233	0.1097	0.0933	0.1233
06/21/13 - 10	0.2407	0.0000	0.5000	0.0559	0.0000	0.2000	0.0936	0.0000	0.3000
06/21/13 - 20	0.2459	0.1000	0.4500	0.0577	0.0000	0.1500	0.0927	0.0000	0.2500
06/21/13 - 30	0.2439	0.1000	0.4000	0.0570	0.0000	0.1333	0.0927	0.0000	0.2000
06/21/13 - 40	0.2436	0.1250	0.3750	0.0576	0.0000	0.1250	0.0929	0.0250	0.1750
06/21/13 - 47	0.2438	0.1277	0.3617	0.0571	0.0000	0.1277	0.0926	0.0213	0.1702
06/21/13 - 60	0.2435	0.1500	0.3500	0.0574	0.0167	0.1167	0.0925	0.0333	0.1667
06/21/13 - 100	0.2434	0.1700	0.3200	0.0575	0.0200	0.1000	0.0929	0.0500	0.1400
06/21/13 - 150	0.2435	0.1933	0.2933	0.0574	0.0267	0.0867	0.0932	0.0600	0.1267
06/21/13 - 200	0.2428	0.2050	0.2800	0.0575	0.0350	0.0800	0.0930	0.0650	0.1200
06/21/13 - 250	0.2432	0.2120	0.2720	0.0573	0.0400	0.0720	0.0929	0.0720	0.1120
06/21/13 - 300	0.2433	0.2233	0.2633	0.0574	0.0467	0.0667	0.0929	0.0800	0.1067
07/26/13 - 10	0.0571	0.0000	0.2000	0.1521	0.0000	0.4000	0.1975	0.0000	0.5000
07/26/13 - 20	0.0566	0.0000	0.1500	0.1527	0.0000	0.3000	0.1954	0.0500	0.3500
07/26/13 - 30	0.0563	0.0000	0.1333	0.1534	0.0333	0.2675	0.1971	0.0667	0.3333
07/26/13 - 40	0.0555	0.0000	0.1250	0.1535	0.0500	0.2750	0.1961	0.0750	0.3250
07/26/13 - 47	0.0555	0.0000	0.1277	0.1535	0.0638	0.2553	0.1961	0.0851	0.2979
07/26/13 - 60	0.0557	0.0167	0.1167	0.1537	0.0667	0.2500	0.1966	0.1167	0.3000
07/26/13 - 100	0.0554	0.0200	0.1000	0.1545	0.1000	0.2200	0.1968	0.1300	0.2602
07/26/13 - 150	0.0559	0.0267	0.0867	0.1540	0.1067	0.2000	0.1963	0.1467	0.2467
07/26/13 - 200	0.0557	0.0350	0.0750	0.1540	0.1200	0.1850	0.1965	0.1600	0.2350
07/26/13 - 250	0.0556	0.0400	0.0720	0.1536	0.1280	0.1800	0.1963	0.1680	0.2240
07/26/13 - 300	0.0557	0.0433	0.0667	0.1537	0.1333	0.1700	0.1963	0.1767	0.2167
08/26/13 - 10	0.1166	0.0000	0.3000	0.1780	0.0000	0.4000	0.0739	0.0000	0.3000
08/26/13 - 20	0.1167	0.0000	0.2500	0.1777	0.0500	0.3500	0.0734	0.0000	0.2000
08/26/13 - 30	0.1171	0.0333	0.2333	0.1777	0.0667	0.3333	0.0745	0.0000	0.1667
08/26/13 - 40	0.1161	0.0250	0.2250	0.1786	0.0750	0.3000	0.0739	0.0000	0.1500
08/26/13 - 47	0.1162	0.0426	0.2128	0.1797	0.0851	0.2766	0.0739	0.0213	0.1489
08/26/13 - 60	0.1165	0.0500	0.2000	0.1787	0.1000	0.2667	0.0742	0.0167	0.1333
08/26/13 - 100	0.1171	0.0700	0.1700	0.1790	0.1200	0.2400	0.0738	0.0300	0.1200
08/26/13 - 150	0.1171	0.0800	0.1533	0.1783	0.1333	0.2267	0.0736	0.0467	0.1067
08/26/13 - 200	0.1167	0.0900	0.1450	0.1788	0.1450	0.2100	0.0737	0.0500	0.0950
08/26/13 - 250	0.1167	0.0960	0.1360	0.1787	0.1560	0.2000	0.0738	0.0560	0.0880
08/26/13 - 300	0.1170	0.1067	0.1267	0.1783	0.1667	0.1900	0.0739	0.0667	0.0800

Appendix 3: Mean values and 95% confidence intervals for CYT brood fish bootstap simulation												
mmddyy -	083-042-784			083-070-054			083-101-080			083-103-352		
S n-value	Mean	CI 2.5%	CI 97.5%	Mean	CI 2.5%	CI 97.5%	Mean	CI 2.5%	CI 97.5%	Mean	CI 2.5%	CI 97.5%
04/29/13 - 10	0.0747	0.0000	0.3000	0.0026	0.0000	0.1000	0.1627	0.0000	0.4000	0.1627	0.1627	0.2373
04/29/13 - 20	0.0732	0.0000	0.2000	0.0028	0.0000	0.0500	0.1640	0.0500	0.3500	0.1640	0.1140	0.1861
04/29/13 - 30	0.0732	0.0000	0.1667	0.0028	0.0000	0.0333	0.1628	0.0333	0.3000	0.1628	0.1295	0.1372
04/29/13 - 40	0.0739	0.0000	0.1500	0.0028	0.0000	0.0250	0.1628	0.0500	0.2750	0.1628	0.1128	0.1122
04/29/13 - 47	0.0729	0.0213	0.1489	0.0029	0.0000	0.0213	0.1637	0.0638	0.2553	0.1637	0.0999	0.0916
04/29/13 - 60	0.0732	0.0167	0.1333	0.0027	0.0000	0.0167	0.1630	0.0833	0.2500	0.1630	0.0797	0.0870
04/29/13 - 100	0.0734	0.0300	0.1200	0.0028	0.0000	0.0100	0.1636	0.1000	0.2300	0.1636	0.0636	0.0664
04/29/13 - 150	0.0732	0.0400	0.1067	0.0028	0.0000	0.0067	0.1633	0.1200	0.2067	0.1633	0.0433	0.0434
04/29/13 - 200	0.0733	0.0500	0.0950	0.0028	0.0000	0.0050	0.1634	0.1300	0.1950	0.1634	0.0334	0.0316
04/29/13 - 250	0.0733	0.0560	0.0920	0.0028	0.0000	0.0040	0.1634	0.1400	0.1880	0.1634	0.0234	0.0246
04/29/13 - 300	0.0733	0.0600	0.0833	0.0028	0.0000	0.0033	0.1634	0.1467	0.1800	0.1634	0.0167	0.0166
05/17/13 - 10	0.0594	0.0000	0.2000	0.0000	0.0000	0.0000	0.0395	0.0000	0.2000	0.0395	0.0395	0.1606
05/17/13 - 20	0.0589	0.0000	0.1500	0.0000	0.0000	0.0000	0.0393	0.0000	0.1500	0.0393	0.0393	0.1107
05/17/13 - 30	0.0595	0.0000	0.1667	0.0000	0.0000	0.0000	0.0390	0.0000	0.1000	0.0390	0.0390	0.0610
05/17/13 - 40	0.0594	0.0000	0.1250	0.0000	0.0000	0.0000	0.0400	0.0000	0.1000	0.0400	0.0400	0.0600
05/17/13 - 47	0.0591	0.0000	0.1277	0.0000	0.0000	0.0000	0.0398	0.0000	0.1064	0.0398	0.0398	0.0666
05/17/13 - 60	0.0591	0.0167	0.1167	0.0000	0.0000	0.0000	0.0398	0.0000	0.0833	0.0398	0.0398	0.0436
05/17/13 - 100	0.0594	0.0200	0.1000	0.0000	0.0000	0.0000	0.0394	0.0100	0.0700	0.0394	0.0294	0.0306
05/17/13 - 150	0.0593	0.0333	0.0867	0.0000	0.0000	0.0000	0.0396	0.0133	0.0667	0.0396	0.0262	0.0271
05/17/13 - 200	0.0592	0.0400	0.0800	0.0000	0.0000	0.0000	0.0394	0.0200	0.0550	0.0394	0.0194	0.0156
05/17/13 - 250	0.0593	0.0440	0.0760	0.0000	0.0000	0.0000	0.0394	0.0240	0.0520	0.0394	0.0154	0.0126
05/17/13 - 300	0.0592	0.0467	0.0700	0.0000	0.0000	0.0000	0.0394	0.0300	0.0467	0.0394	0.0094	0.0073
06/21/13 - 10	0.1399	0.0000	0.4000	0.0000	0.0000	0.0000	0.1079	0.0000	0.3000	0.1079	0.1079	0.1921
06/21/13 - 20	0.1380	0.0000	0.3000	0.0000	0.0000	0.0000	0.1052	0.0000	0.2500	0.1052	0.1052	0.1448
06/21/13 - 30	0.1397	0.0333	0.2667	0.0000	0.0000	0.0000	0.1061	0.0000	0.2333	0.1061	0.1061	0.1273
06/21/13 - 40	0.1384	0.0500	0.2500	0.0000	0.0000	0.0000	0.1065	0.0250	0.2000	0.1065	0.0815	0.0935
06/21/13 - 47	0.1399	0.0426	0.2340	0.0000	0.0000	0.0000	0.1061	0.0213	0.1915	0.1061	0.0848	0.0854
06/21/13 - 60	0.1393	0.0667	0.2167	0.0000	0.0000	0.0000	0.1074	0.0333	0.1833	0.1074	0.0741	0.0759
06/21/13 - 100	0.1394	0.0800	0.2000	0.0000	0.0000	0.0000	0.1062	0.0600	0.1600	0.1062	0.0462	0.0538
06/21/13 - 150	0.1394	0.1000	0.1800	0.0000	0.0000	0.0000	0.1066	0.0667	0.1467	0.1066	0.0399	0.0401
06/21/13 - 200	0.1396	0.1050	0.1700	0.0000	0.0000	0.0000	0.1066	0.0800	0.1350	0.1066	0.0266	0.0284
06/21/13 - 250	0.1394	0.1160	0.1640	0.0000	0.0000	0.0000	0.1065	0.0840	0.1280	0.1065	0.0225	0.0215
06/21/13 - 300	0.1394	0.1233	0.1567	0.0000	0.0000	0.0000	0.1066	0.0900	0.1200	0.1066	0.0166	0.0134
07/26/13 - 10	0.0454	0.0000	0.2000	0.0000	0.0000	0.0000	0.0158	0.0000	0.1000	0.0158	0.0158	0.0842
07/26/13 - 20	0.0454	0.0000	0.1500	0.0000	0.0000	0.0000	0.0158	0.0000	0.1000	0.0158	0.0158	0.0842
07/26/13 - 30	0.0450	0.0000	0.1333	0.0000	0.0000	0.0000	0.0163	0.0000	0.0667	0.0163	0.0163	0.0504
07/26/13 - 40	0.0453	0.0000	0.1250	0.0000	0.0000	0.0000	0.0159	0.0000	0.0500	0.0159	0.0159	0.0341
07/26/13 - 47	0.0448	0.0000	0.1064	0.0000	0.0000	0.0000	0.0162	0.0000	0.0638	0.0162	0.0162	0.0477
07/26/13 - 60	0.0448	0.0000	0.1000	0.0000	0.0000	0.0000	0.0159	0.0000	0.0500	0.0159	0.0159	0.0341
07/26/13 - 100	0.0448	0.0100	0.0800	0.0000	0.0000	0.0000	0.0160	0.0000	0.0400	0.0160	0.0160	0.0240
07/26/13 - 150	0.0450	0.0200	0.0733	0.0000	0.0000	0.0000	0.0160	0.0000	0.0333	0.0160	0.0160	0.0174
07/26/13 - 200	0.0451	0.0250	0.0650	0.0000	0.0000	0.0000	0.0159	0.0050	0.0250	0.0159	0.0109	0.0091
07/26/13 - 250	0.0451	0.0280	0.0600	0.0000	0.0000	0.0000	0.0159	0.0080	0.0240	0.0159	0.0079	0.0081
07/26/13 - 300	0.0451	0.0333	0.0533	0.0000	0.0000	0.0000	0.0159	0.0100	0.0200	0.0159	0.0059	0.0041
08/26/13 - 10	0.0991	0.0000	0.3000	0.0000	0.0000	0.0000	0.0492	0.0000	0.2000	0.0492	0.0492	0.1508
08/26/13 - 20	0.0979	0.0000	0.2500	0.0000	0.0000	0.0000	0.0495	0.0000	0.1500	0.0495	0.0495	0.1005
08/26/13 - 30	0.0986	0.0000	0.2000	0.0000	0.0000	0.0000	0.0487	0.0000	0.1333	0.0487	0.0487	0.0847
08/26/13 - 40	0.0987	0.0250	0.2000	0.0000	0.0000	0.0000	0.0494	0.0000	0.1250	0.0494	0.0494	0.0756
08/26/13 - 47	0.0985	0.0213	0.1702	0.0000	0.0000	0.0000	0.0492	0.0000	0.1064	0.0492	0.0492	0.0572
08/26/13 - 60	0.0988	0.0333	0.1667	0.0000	0.0000	0.0000	0.0493	0.0000	0.1000	0.0493	0.0493	0.0507
08/26/13 - 100	0.0983	0.0500	0.1500	0.0000	0.0000	0.0000	0.0496	0.0200	0.0900	0.0496	0.0296	0.0404
08/26/13 - 150	0.0987	0.0667	0.1333	0.0000	0.0000	0.0000	0.0492	0.0267	0.0733	0.0492	0.0225	0.0241
08/26/13 - 200	0.0984	0.0750	0.1250	0.0000	0.0000	0.0000	0.0493	0.0300	0.0650	0.0493	0.0193	0.0157
08/26/13 - 250	0.0984	0.0800	0.1160	0.0000	0.0000	0.0000	0.0492	0.0360	0.0600	0.0492	0.0132	0.0108
08/26/13 - 300	0.0984	0.0867	0.1067	0.0000	0.0000	0.0000	0.0493	0.0400	0.0533	0.0493	0.0093	0.0041