BROOD STOCK NUTRITION: ENHANCEMENT OF EGG QUALITY

FOR THE PRODUCTION OF HYBRID CATFISH

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BROOD STOCK NUTRITION: ENHANCEMENT OF EGG QUALITY FOR THE PRODUCTION OF HYBRID CATFISH

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BROOD STOCK NUTRITION: ENHANCEMENT OF EGG QUALITY

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VITA

Herbert E. Quintero-Fonseca, eldest son of José Matias Quintero and Maria Villaldina Fonseca, was born on September 25th, 1967 in Bogotá, Colombia. He obtained a Bachelor of Science in Marine Biology from Universidad Jorge Tadeo Lozano in Bogota, Colombia. Following completion of his degree he began to work in a shrimp farm for eight years. During this time he obtained an Specialization degree in Business from Universidad de Cartagena. In August 1999, he moved to Puerto Rico and pursued a Master of Science in Marine Sciences from University of Puerto Rico. In January 2004 he entered Auburn University to pursue a Ph.D. in Fisheries and Allied Aquacultures. He married is to Mercy L. Peña and they are blessed with one daughter: Maria Paula.

DISSERTATION ABSTRACT

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FOR THE PRODUCTION OF HYBRID CATFISH

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The catfish industry is one of the largest and best developed aquaculture segments in the United States. The development of technological solutions to maintain a competitive level in the market is key to this success. The hybridization technique (channel catfish, *Ictalurus punctatus,* ♀ x blue catfish, *I. furcatus,* ♂) has been identified as one way to further improve production; however, reproductive limitations have affected its commercial application. One of the first steps to overcome this challenge is to conduct basic research on brood stock nutrition to optimize egg quality, hatch and fry production. To facilitate our understanding of the interaction of nutrition and reproductive performance from female channel catfish, two experiments were performed in earthen ponds. The first experiment

evaluated the interaction of feed quality (42 and 32% protein) and feed frequency (feed offered 3 or 6 times per week) in two strains (high and low spawning strains). The second experiment assessed the influence of different lipid sources and n3:n6 ratios using a commercial catfish feed containing 32% protein and 5% lipid as the basal diet.

Reproductive performance in terms of spawning and egg production were not influenced by changing protein level of the diet from 32 to 42%. Increasing the feeding frequency from 3 to 6 times per week negatively effected spawning in one of the strains, did not affect egg production. Age and period of spawning affected reproductive performance. In addition to having bigger eggs than their younger counterpart, older fish performed better than younger fish in terms of spawning success, and egg production. Biochemical composition of the eggs was affected significantly by dietary treatment in terms of lipid, fatty acids and free amino acid content.

Lipid supplementation on a 32% protein, 5% lipid, commercial catfish diet using soybean and /or, linseed oil, or menhaden fish oil enriched with docosahexaenoic acid (DHA) and arachidonic acid (ARA) had no significant effects ($p<0.05$) on spawning success neither egg production. The quantity of fry produced per female body weight and fry survival from fish fed top-coated feed with menhaden fish oil enriched ARA and DHA were two to five fold greater than those obtained from fish fed with feed supplemented with vegetable oils. This difference was not significant ($p = 0.08$) and their impact on a commercial basis could be very important. Based on the results of these studies, it is recommended that the minimum dietary requirements for ARA, eicosapentaenoic acid (EPA) and DHA be evaluated for enhancement of egg quality in the channel catfish.

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CHAPTER I

INTRODUCTION

Aquaculture development is feasible due to a favorable interaction of biological, ecological, and economical factors. The catfish industry in the US needs to be efficient, sustainable and profitable to survive in the local and global market. This may be accomplished using a variety of strategies. One potential strategy is to improve the biological characteristics of the catfish to improve the economic efficiencies of rearing the fish. One mechanism to greatly improve the overall culture characteristic (biology) of a fish is to cross it with another species to produce a hybrid. Hybridization programs in fish have as a goal to produce a fish that combined some of the more desirable characteristics of two parent species (Kerby & Harrell 1990). A number of hybrid crosses using catfish have been evaluated since the mid 1960s (Giudice 1966). The most favorable cross has been between the channel catfish female (*Ictalurus punctatus*) with blue catfish male (*I. furcatus*) (Dupree, Green & Sneed 1966) . Characteristics include better growth which are reportedly 20-100% faster than channel catfish (Yant, Smitherman & Green 1976; Chappell 1979; Dunham, Smitherman & Goodman 1987; Dunham, Brummet, Ella & Smitherman 1990; Jeppsen 1995; Dunham & Brummett 1999); improved feed conversion (10-20%); disease resistant to enteric septicemia of catfish (ESC) and columnaris (Ella 1984; Jeppsen 1995; Dunham & Brummett

1999), increase resistance to critically low oxygen levels (Dunham, Smitherman, & Webber 1983); easier to harvest by seining (Chappell 1979); susceptibility to angling (Tave, McGinty, Chappell & Smitherman 1981; Dunham, Smitherman, Goodman & Kemp 1986); dressout and nugget yield of the hybrid is higher than the channel catfish while shank fillet percentages for the two are equal (Argue, Liu & Dunham 2003).

Hybrid fry production through natural spawning is highly improbable due to reproductive isolating mechanisms between the parent species (Tave & Smitherman 1982). Thus, early research on hybrid catfish production focused on commercial traits evaluation, artificial fertilization and pen spawning using human chorionic gonadotropin (HCG) (Tave & Smitherman, 1982); additional efforts using other agents as carp pituitary extract (CPE) (Kim 1996; Dunham & Argue 2000); Kristanto (2004) evaluated the effect of different hormones (luteinizing hormone releasing hormone analogue (LHRHa), gonadotropin hormone releasing hormone (GnRH), OvaRH, and Ovaprim on hybrid production; and recently Hutson (2006) evaluated the use of LHRHa implants and injections. However, poor hatching and fry production rates remain as challenges to expand hybrids use in a large scale commercial setting.

One key factor in brood stock management is the nutritional status of those brood stock, since quality and quantity of the diet may influence gamete and fry quality (Bromage 1995). Food restriction itself can affect spawning success, and thus has been reported to cause an inhibition of gonadal maturation in several species such as goldfish *(Carassius auratus* L), European sea bass (*Dicentrarchus labrax* L), and male Atlantic salmon (*Salmo salar* L)(Izquierdo, Fernández-Palacios & Tacon 2001). Dietary components as diverse as

protein and fatty acids have all been shown to affect egg and embryo survival (Brooks, Tyler & Sumpter 1997). Proteins act as a source of amino acids and as a reservoir of materials used during the many biosynthetic activities that are essential for the early stages of embryogenesis (Metcoff 1986). Takeuchi, Watanabe, Ogino, Saito, Nishimura & Nose (1981) reported higher hatch rates for eggs from trout maintained at low (36%) protein diet, whereas Roley (1983) found that the eggs produced by trout brood fish fed on a 47% protein diet had higher survival than those produced by fish given either 27 or 37% protein diets. Lipid and fatty acid composition of brood stock diets have been identified as major dietary factors that determine successful reproduction and survival of offspring. Highly unsaturated fatty acids (HUFA) with 20 or more carbon atoms affect directly or through their metabolites, fish maturation and steroidogenesis (Fernandez-Palacios, Izquierdo, Robaina, Valencia, Salhi & Vergara 1995; Izquierdo *et al.* 2001). Altering the lipid composition of brood stock diet effected egg quality in the European sea bass. Eggs considered to be of better quality had a higher content of total n-3 fatty acids, which included enhanced levels of both docosahexaenoic acid and eicosapentaenoic acid (Brooks *et al.* 1997). In contrast to these studies, an analysis of the lipid and fatty acid composition of Atlantic halibut eggs showed that batches of eggs with widely differing viabilities had very similar lipid compositions (Bruce, Shields, Bell & Bromage 1993). In the case of Nile tilapia, performance was much higher in fish fed a basal diet supplemented with soybean oil (high in n-6 fatty acids) and relatively low in fish fed a diet supplemented with 5% cod liver oil (high in n-3 fatty acids) (Santiago & Reyes, 1993).

Currently, there is considerable interest in commercial production of hybrid catfish since it could alleviate some of the production problems facing catfish farmers (Kristanto 2004). However, fry production has been quite limited with highly variable results. Hence, the following research evaluated nutritional factors that may be influencing the production of hybrid fry.

CHAPTER II

LOGIT MODELS FOR EVALUATING SPAWNING PERFORMANCE OF CHANNEL CATIFSH *Ictalurus punctatus (Rafinesque)*

Abstract

Brood stock evaluations are often measured by variables such as the spawning success, fecundity, fertilization and hatching rates, usually expressed as percentage values. Outcomes are generally analyzed as continuous random variables assuming that they follow a normal distribution. Ordinary linear regression models (e.g. analysis of variance) as well as Chi-square analysis are typically applied. However, these models may not be the most appropriate as a number of test criteria may not be met. For example, spawning success outcomes are inherently discrete and non-negative data and hence their distribution is not likely to be normal. As these models may not be the most appropriate, a case study using logit analysis as an alternative method for the evaluation of this type of data is presented by considering the response as binary data (spawned vs did not spawn). An exact version of logit analysis was performed due to the sparseness of the data. Results demonstrate that appropriate statistical models provide better insight into the cause-effect relationships that exist between independent (i.e. treatments) variables and the dependent variable (likelihood of spawning in this case). As would be expected each strain of fish responded somewhat

differently to the test variables. Changing the protein level of the diet from 32 to 42% or increasing the feeding frequency from 3 to 6 times per week either did not influence spawning or negatively affected spawning respectively. Additionally, older fish performed better than younger and the early spawning period was better than the later spawning period, regardless of strain. These responses, however, were only detected by logit analysis, which is a more sensitive test and would thus be recommended for this type of data.

Introduction

Aquaculture development has been feasible due to a combination of factors, such as the application of controlled reproduction, which often rely on induced spawning or hypophysation, environmental manipulations and cryopreservation (Bromage 1995; Donaldson 1996). Multiple interrelated factors are involved in reproduction success including type and doses of hormones used in hypophysation, size and age of brood stock, condition factor, nutritional status of brood fish, and environmental conditions. Knowledge of these factors is useful in improving fry production. Spawning success (percentage of females ovulating) is one of the primary outcomes of this process and it has typically been evaluated using two approaches. The first approach is to consider it as a continuous variable and to further assume that it is normally distributed. In this case ordinary linear regression models are applied, such as analysis of variance (ANOVA), following some transformation such as an arcsine transformation (Lambert 1998; Celada, Antolin, Carral, Pérez & Sáez-Royuela 2006; Peck & Holste 2006; Rodríguez-González, García-Ulloa, Hernández-Llamas & Villareal 2006). The second approach is to use the Pearson Chi-square test for association in a cross tabulation where the independent variable is expressed as the number of successes in the spawning process (Bondari, Ware, Mullinix & Joyce 1985). However, the use of ordinary linear regression techniques for a dichotomous dependent variable could be considered inappropriate from a theoretical standpoint (Allison 1999) and interpretation of the results is not straightforward due to the transformation applied on the response variable. Pearson chi-square analysis tests for association between variables but is unable to give estimates of parameters (Stokes, Davis & Koch 2000).

Spawning success can be considered a *discrete random variable* instead of a *continuous random variable* since the possible measured or assigned values consist of a discrete set of categories: organisms that spawned successfully versus those that did not spawn. The analysis of continuous outcome data using linear regression models requires five assumptions: (1) the response is a linear function of the independent variables plus a random disturbance term or random error, (2) the independent variables and random error are independent, (3) variance of the random error is the same for all observations, (4) the random error for one observation is uncorrelated with the random error for any other observation, and (5) the errors are normally distributed with a mean of 0 (Allison 1999). Many statistical models make a distinction between response (or dependent) variables and explanatory (or independent) variables. In the classical linear models for regression and analysis of variance (ANOVA), one of the variables is treated as the response variable to be explained by the other explanatory variables (Friendly 2000). In this case the explanatory variables may be quantitative or categorical but the response variable must be quantitative. Since spawning success would be a response variable, and it is categorical in nature, the normality assumption imposed on the model residuals is violated. This invalidates the direct application of ordinary linear regression. Additionally, transformations (e.g. arcsine) often do not yield normally distributed data and may make the interpretation of regression coefficients difficult because they are not estimated on the original scale (Byers, Allore, Gill & Peduzzi 2003).

For a dichotomous response variable, such as spawning success coded as 0 for no spawn and 1 for spawn, it is convenient to construct a model relating a function of the

probability of successful spawning, say p, to a linear combination of the explanatory variables. A model such as this is commonly known as a generalized linear model (GLM). Our model is the logit model which is in the family of GLMs. Some of the advantages of this method are that the estimated probability of spawning is guaranteed to take values between 0 and 1, regardless of the values taken by the independent variables (Zelterman 1999, 2002), and that inference for the odds and odds ratios are derived directly from the estimated logit model. The odds of an event are the ratio of probability that it will occur to the probability that it will not occur. As the odds ratio is the ratio of two odds it provides a simple way of describing the dependence of the factors on each other (Allison 1999; Zelterman 2002).

The probabilistic approach for spawning success can also be reached from a biological stand point. Spawning is a result of a maturation process (Heino, Dieckmann & Godø 2002) and typically maturation has been evaluated as a deterministic process. This means that sexual maturity is fully determined by age and size of an individual. However, maturation is a complex process that is also influenced by other factors such as resource availability and body reserves (Bernardo 1993). As a result there will be some variability that cannot be explained by the size of maturing individuals at a given age. This highlights the probabilistic nature of the maturation process. Similarly spawning response would not occur with certainty once hormone injection is given. There are other factors, such as water quality and handling stress, which if maintained properly lead to ovulation and successful stripping. On the other hand, stress can cause egg retention in the body causing over-ripening which indicates failure in the process. In this paper, we investigate factors that significantly affect successful spawning within a probabilistic framework.

Logit analysis has previously been utilized in ecology, fisheries, and eco-toxicology studies, among others. Kellogg, Ligotino & Jinks (1984) developed a logistic regression model to predict thermal mortality in striped bass, *Morone saxatilis* (Walbaum). Munger, Wilde & Follis (1994) determined the relationship between size and age at sexual maturation in flathead catfish, *Pylodictis olivaris* (Rafinesque). DeMartini & Lau (1999) used this analysis to classify fish as either immature or mature, based on body length, ovary weight, and oocyte volume for two snappers *Etelis carbunculus* (Cuvier) and *Pristipomoides sieboldii* (Bleeker). Recently, Keating & Cherry (2004) have reviewed logistic regression use and interpretation for wildlife habitat selection studies. Finally, McHugh & Budy (2004) developed spawning habitat suitability models for Chinook salmon, *Oncorhyunchus tshawytscha* (Walbaum) in Idaho. As demonstrated with other species, logit analysis could provide a valuable tool for understanding catfish spawning success. Consequently, this case study was conducted to evaluate the applicability of logit analysis as compared to ANOVA, ANCOVA and Chi Square analyses for the interpretation of spawning data.

Methods

Data were obtained from a project performed to improve reproductive efficiency by producing hybrids from female channel catfish, *Ictalurus punctatus* (Rafinesque), with male blue catfish, *Ictalurus furcatus* (Lesueur), through nutrient manipulations. This study evaluated the influence/interaction of dietary protein level and feeding rate on egg production of two separate strains of catfish (each one with three ages). Two diets containing 42% and 32% protein level (ARKAT Feeds Inc, Dumas, AK), and two feed frequencies (six

times/week and three times/week), were used giving rise to four treatments 42%/6 , 42%/3, 32%/6, and 32%/3. Females were spawned during three periods (early, middle and late season). Evaluation of the dietary protein level and feeding rate treatments, were based on spawning success, total number of eggs, and egg production per unit of female weight.

Experimental fish

A total of 414 female channel catfish were maintained at the North Auburn Experimental Station, Auburn University. All brood stock were stocked in 16 ponds, using four ponds per treatment. The females which were given individual tags were divided into two strains on the basis of prior spawning behavior: high spawning strain (strain1) and low spawning strain (strain 2); based on that characteristic they were assigned proportionally in a randomized manner to each pond (Table 1). Female body weights ranged from 0.4 kg to 3.4 kg for strain 1 and 0.6 kg to 3.1 kg for strain 2, with a combined mean weight of 1.74 kg. The organisms were stocked in February 2004, in 0.04 ha ponds at a density of ~1,130 kg/ha, giving an acclimation period of approximately one month. The trial period was 70 to 90 days depending on the spawning period. Feeding was done during the warmest part of the day between 1500 and 1700 hours, at a rate of 1.7% of total biomass of brood fish stocked per pond. Water quality parameters were taken daily for dissolved oxygen and temperature; twice weekly for pH, ammonia-N, and nitrite-N. Alkalinity and hardness were recorded at the time of stocking and just before harvesting.

Table 1 - Number of females channel catfish by strain per treatment

Treatment	Strain		Total
	High spawning	Low spawning	
1 - 42% / 6	44	55	99
2 - 42% / 3	55	57	112
3 - 32% / 6	44	56	100
4 - 32% / 3	44	59	103
	187	227	414

For the first spawning period (early), two ponds of each treatment were drained, and 16 females (out of 32) were selected based on external characteristics (abdominal fullness, softness and palpability of the ovaries, redness or swollen appearance of the genitals). Evaluation of the second spawning period (middle) was performed using one pond of each treatment, selecting 16 females (out of 32). The last spawning period (late) utilized all the remaining females in all ponds.

Hormone injections

Selected females identifiable by dietary treatments were transferred to holding tanks supplied with continuous flow-through water, and placed individually in soft mesh bags. Total length, body weight, and girth were recorded. The luteinizing hormone releasing hormone analogue (LHRHa) GMP grade from American Peptide (Vista, CA) was utilized. Hormone injections were administered in two doses, a priming injection of 20 μg/kg LHRHa, followed 12 hours later by resolving dose of 100 μg/kg.

Collection and fertilization of gametes

Twenty four hours after the second injection, females were monitored for ovulation. Females with released eggs were removed from the holding tank and anesthetized in 250 mg/l tricaine methane sulfonate (MS-222) (Argent Chemical Laboratories, Redmond, WA). Females were then stripped and eggs were collected in aluminum containers lubricated previously with vegetable shortening. Those females that did not express eggs were returned and rechecked later. Stripping of gametes ceased when all females had been stripped or

attempts to strip them had been made. Females that spawned were classified in three different ways: by age of brood stock females, by period of spawning, and strain either as a proportion (Table 2) or as a frequency for each dietary treatment (Table 3).

Statistical analysis

Analysis of the spawning was performed using (1) ordinary linear regression (ANOVA and ANCOVA) after the response is arc-sine transformed; (2) Chi-square analysis and Fisher's exact test after the response is converted to percentages; and (3) logit analysis and exact logit analysis using binary spawn-no spawn response. All the models were constructed to include the effect of treatments (protein and frequency) on spawning success for each strain and use the age of fish and period of spawning as covariates. The GLM, Frequency, and Logistic procedures of SAS® version 9.1.3 were utilized for the analysis.

Performing ANOVA requires data transformation since percentages can not be used directly in this type of analysis. The percentage response is arcsine transformed before proceeding with ANOVA. The statistical model for this analysis is given by

$$y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk}$$

where μ represents the average response, α_i , i= 1,2, represents the effect of the ith level of protein, β_j , j = 1, 2, represents the effect of the jth level of feeding frequency, $(\alpha\beta)_{ij}$, i, j =1,2, represents the interactive effect of the ith level of protein and the jth level of feeding frequency, and ε_{ijkl} represent the random error associated with the arcsine transformed

Table 2 - Proportion of females channel catfish spawned (%) by age and period of spawning (early, middle, and late season) after being maintained on the various dietary treatments.

Treatment	Percentage spawned (%)			Percentage spawned (%)		
	Age 3	Age 4	Age 5	Period 1	Period 2	Period 3
Strain 1						
1 - 42%/6	59	33	78	79	100	39
2 - 42%/3	35	47	72	93	67	28
3 - 32%/6	44	29	90	79	67	54
4 - 32%/3	41	75	100	92	67	52
Total	44	46	85	85	75	42
Strain 2						
1 - 42%/6	44	83	73	88	50	58
2 - 42%/3	40	50	85	93	67	65
3 - 32%/6	36	50	63	87	71	38
4 - 32%/3	53	100	79	100	100	54
Total	43	71	75	92	74	54

Table 3 - Total number of brood stock spawned on the various dietary treatments, by strain, age, and period (Per.) of spawning (1 = Early, 2 = Middle, 3 = Late season). The total number of females that were evaluated on the various dietary treatments is in parentheses.

Treatment	Age 3			Age 4			Age 5		
	Per. 1	Per. 2	Per. 3	Per. 1	Per. 2	Per. 3	Per. 1	Per. 2	Per. 3
Strain 1									
1 - 42%/6	3 (4)	3 (3)	4 (10)	0 (1)	3 (3)	0 (5)	8 (9)	1 (1)	5 (8)
2 - 42%/3	6 (6)	0 (1)	1 (13)	4 (5)	2 (3)	2 (9)	3 (3)	4 (5)	6 (10)
3 - 32%/6	3 (6)	1 (2)	3 (8)	2 (2)	0 (1)	0 (4)	6 (6)	3 (3)	10(12)
4 - 32%/3	5 (5)	1 (2)	3 (15)	1 (2)	1 (2)	4 (4)	6 (6)	2 (2)	6 (6)
Total	17(21)	5 (8)	11(46)	7 (10)	6 (9)	6 (22)	23(24)	10(11)	27(36)
Strain 2									
1 - 42%/6	5 (5)	0 (2)	2 (9)	2 (2)	0	3 (4)	7 (9)	3 (4)	14(20)
2 - 42%/3	2 (3)	0 (1)	4 (11)	1 (1)	0	0 (1)	10(10)	4 (5)	20(25)
3 - 32%/6	2 (3)	0	3 (11)	1 (1)	0	1 (3)	10(11)	5(7)	9 (20)
4 - 32%/3	6 (6)	2 (2)	0 (7)	0	1 (1)	1 (1)	10(10)	5 (5)	18(27)
Total	15(17)	2 (5)	9 (38)	4 (4)	1 (1)	5 (9)	37(40)	17(21)	61(92)

response (y_{ijkl}) of the *k*th fish that was treated with the *i*th level of protein and the *j*th level of feeding frequency.

An extension of the ANOVA approach is the ANCOVA model given by

$$y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma_k + \eta x_{ijkl} + \varepsilon_{ijkl}$$

where γ_k , $k = 1,2,3,$ is the effect of period *k* and η is an unknown regression coefficient to be estimated from the data. Furthermore y_{ijkl}, x_{ijkl}, and ε_{ijkl} represent the response, age, and random error, respectively, of the *l*th fish that was treated with the *i*th level of protein and the *j*th level of feeding frequency in period *k*.

The Chi-square analysis tests whether the differences between the observed count and the expected count could have occurred by chance alone. Data are reported in raw frequencies (not percentages) and are assumed to be independent. Observed frequencies cannot be too small, otherwise the assumption of normal distribution of sample frequencies will not be met. When this happens the remedial measure involves performing Fisher's exact test, which is a non-parametric test that does not depend on any large-sample distribution assumption. The p-value of Fisher's exact test is just the proportion of tables that are as extreme as or more extreme than the table given by the observed sample in all possible permutations of the data (Hollander & Wolfe 1999). In our case we are testing for association in a cross classified table for each strain, where female age, and period of spawning are independent variables, between treatments and success (or failures) in spawning process (Table 3).

Logit analysis was performed by keeping the response in its simplest form as binary data where a response of 0 is recorded for a fish that did not successfully spawn and a response of 1 is recorded for a fish that successfully spawned. This enables one to consider the binary response corresponding to the fish in the cell *(i,j,k,l)* as a random variable that follows a Bernoulli distribution with an unknown probability of success p_{ijkl}. Logit analysis proceeds by linking this probability of success to the explanatory variables through a logit link function in the spirit of generalized linear models (McCullagh & Nelder 1989). This is given by

$$\log\left(\frac{p_{ijkl}}{1-p_{ijkl}}\right) = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma_k + \eta x_{ijkl}$$

where all the model parameters are as defined above. The left hand side of this equation is the natural logarithm of the odds of successful spawning (the ratio of the expected number of times of spawning success to the expected number of times of spawning failure), while the right hand side of this equation is a linear function composed of treatment effects and covariates. Solving for the probability of successful spawning as a function of the treatment effects and covariates we obtain the equivalent form given by

$$p_{ijkl} = \frac{e^{\mu+\alpha_i+\beta_j+(\alpha\beta)_{ij}+\gamma_k+\eta x_{ijkl}}}{1+e^{\mu+\alpha_i+\beta_j+(\alpha\beta)_{ij}+\gamma_k+\eta x_{ijkl}}}$$

where *e* is the base of the natural log function.

Traditional inference for logit analysis uses asymptotic distribution results based on parameters estimated using the unconditional maximum likelihood method. This method is asymptotic and hence requires a large sample size to provide a reliable inference. Our data,

however, was sparse in that it had a number of cells with small observed frequency counts. This was handled by implementing the exact logistic regression procedure given by Cox (1970), Hirji, Mehta & Patel (1987), and Mehta & Patel (1995). Given *n* observations, the exact logistic regression proceeds by determining how likely the observed response is among the 2^n possible response permutations in a manner that mimics Fisher's exact test. Inference is based on the exact distribution of parameters of interest conditional on the remaining parameters. A direct enumeration of all the possible permutations becomes infeasible quite quickly. For instance, for our data set of 414 fish there are $2^{414} = 4.23 \times 10^{124}$ possible permutations. To make this type of exact conditional inference computationally practical, we used the multivariate shift algorithm of Hirji *et al.* (1987).

Results

ANOVA and ANCOVA

For the ANOVA model, protein level, feed frequency and the interaction between the two were found to be non-significant (Table 4), with the exception of strain 2 (low spawning) where feed frequency was suggestive ($p = 0.0562$). ANCOVA showed that both covariates (age of fish and period of spawning) have a highly significant effect on the proportion of females that successfully spawned for both strains. Regarding the effects of protein level, feed frequency, and their interactions in the ANCOVA model, no significant effect (Table 5) on the proportion of females that successfully spawned was found with the exception of strain 2 (low spawning) fish where the feed frequency was found to have a significant effect ($p = 0.0408$).

Table 4 - Results of ANOVA evaluating differences in spawning success from channel catfish females, among two dietary protein levels, and two feed frequencies, for each strain.

a. Strain 1 (High spawning)

Source	DF	F	Pr > F
Protein	1	1.64	0.2024
Feed frequency	1	0.38	0.5719
Protein x feed frequency	1	0.78	0.3796

b. Strain 2 (Low spawning)

Source	DF	F	Pr > F
Protein	1	0.53	0.4820
Feed frequency	1	3.68	0.0562
Protein x feed frequency	1	0.78	0.3779

Table 5 - Results of ANCOVA evaluating differences in spawning success from channel catfish females, among two dietary protein levels, and two feed frequencies, for two strains (high and low spawning) using age of fish and period of spawning as covariates.

a. Strain 1 (high spawning)

Source	DF	F	Pr > F
Protein	1	2.14	0.1454
Feed frequency	1	0.02	0.8880
Protein x feed frequency	1	1.59	0.2094
Age of fish	1	28.01	<0.0001
Period of spawning	2	17.61	<0.0001

b. Strain 2 (low spawning)

Source	DF	F	Pr > F
Protein	1	1.18	0.2786
Feed frequency	1	4.23	0.0408
Protein x feed frequency	1	0.88	0.3499
Age of fish	1	23.11	<0.0001
Period of spawning	2	17.62	<0.0001

Pearson Chi-square and Fisher's exact test

We were unable to use the Pearson Chi-square analysis due to low frequency counts in the cells (5 or less). Fisher's exact test was used as a remedial measure. Analysis revealed that treatments had a significant effect ($p = 0.0041$) on spawning success only for strain 1, 4-year-old fish that were spawned in period 3 (late) (Table 6).

Logit analysis and exact logit analysis

Once again the low frequency counts make the performance of the traditional logistic regression analysis unsatisfactory. Thus, we performed the exact logistic analysis and the results obtained with this analysis are presented in Table 7. For strain 1, we found no significant effect of protein level, feed frequency, or their interaction on the probability of successful spawning. For strain 2, only feed frequency had a significant effect ($p = 0.0427$) on the probability of successful spawning. Considering the covariates, age of fish had a highly significant effect ($p < 0.0001$ in both strains), period 1 (early) had a significant effect ($p = 0.0022$ for strain 1, and $p < 0.0001$ for strain 2), and period 2 had no significant effect ($p = 0.9533$ for strain 1 and $p = 0.7776$ for strain 2) on the probability of successful spawning.

For strain 2, the odds of successful spawning of fish that are fed three times per week were estimated to be 1.9 times that of fish fed six times per week. Furthermore, for strain 1, the odds of successful spawning of female 5-year-old fish were estimated to be 8.4 times higher than female 3-year-old fish and 2.9 times higher than female 4-year-old fish. For

Table 6 - Results of Fisher's exact test evaluating differences in spawning success from channel catfish females, among two dietary protein levels, and two feed frequencies, for two strains (high and low spawning) using age of fish and period of spawning as variables.

	Period	Strain 1		Strain 2	
		Table probability	Pr ≤ p	Table probability	Pr ≤ p
Age 3	1	0.0134	0.0809	0.0662	0.1103
	2	0.0714	0.3571	0.1000	0.2000
	3	0.0052	0.2415	0.0120	0.3876
Age 4	1	0.0833	0.5000	N/A	N/A
	2	0.0714	0.4643	N/A	N/A
	3	0.0005	0.0041	0.0952	0.5714
Age 5	1	0.3750	1.0000	0.0401	0.1624
	2	0.4545	1.0000	0.0702	0.7953
	3	0.0082	0.2516	0.0005	0.1050

Table 7 - Results of Exact logistic regression analysis evaluating differences in spawning success from channel catfish females, among two dietary protein levels, and two feed frequencies, for two strains (high and low spawning) using age of fish and period of spawning as covariates

a. Exact logistic regression analysis - strain 1

Variable	DF	Parameter estimate	Pr > χ^2	Odds ratio
Protein	1	0.2595	0.1215	
Feed frequency	1	0.0399	0.8619	
Protein x feed frequency	1	0.2679	0.1184	
Age	1	1.0268	<0.0001	2.8
Period 1	1	1.0449	0.0022	8.6
Period 2	1	0.1936	0.9533	

*Period 1 and 2 correspond to the logistic regression coefficients where the levels of period are coded as period 1 (1,0) - period 2 (0,1) - period 3 (-1,1)

b. Exact logistic regression analysis - strain 2

Variable	DF	Parameter estimate	Pr > χ^2	Odds ratio
Protein	1	-0.1751	0.2707	
Feed frequency	1	0.315	0.0427	1.9
Protein x feed frequency	1	0.1213	0.4312	
Age	1	0.7988	<0.0001	2.2
Period 1	1	1.3963	<0.0001	16.3
Period 2	1	-0.2734	0.7776	

*Period 1 and 2 correspond to the logistic regression coefficients where the levels of period are coded as period 1 (1,0) - period 2 (0,1) - period 3 (-1,1)

strain 2, the odds of successful spawning of female 5-year-old fish were estimated to be 4.9 times higher than female 3-year-old fish and 2.2 times higher than female 4-year-old fish. Finally, the estimated odds of successful spawning during period 1 (early) were 8.6 and 16.3 times higher than the odds in period 3 (late) for strains 1 and 2, respectively, while no significant difference in the probabilities of spawning was detected between period 2 (middle) and period 3 (late) (Table 7).

Discussion

Logit analysis was developed as an alternative approach to ANOVA and ANCOVA. Conceptually this analysis makes more sense and has better statistical properties. It provides an optimal method for regression analysis of dichotomous dependent variables (Allison 1999). In addition to clear-cut violations of assumptions, ANOVA and ANCOVA employ an arcsine transformation which makes the interpretation of regression coefficients difficult since they are not estimated on the original scale (Byers *et al*. 2003). Logit analysis has previously been effectively utilized in fisheries to analyze fish maturation among other things. These studies used asymptotic distributional results that do not hold in our case due to small cell frequencies. Exact logit analysis was used to provide correct inference as recommended by Stokes *et al*. (2000).

In our exact logit analysis the effect of protein on spawning success, high protein levels (42%) versus low protein levels (32%), was not statistically significant for both strains (Table 7). Similar results were found for rainbow trout, *Salmo gairdneri* (Richardson) where fish fed at 27, 37, 47, and 56% protein level did not show a significantly different effect on

the success of spawning (Roley 1983). Feed frequency had a significant effect on spawning success only in strain 2 (low spawning), where fish fed every other day (three times per week) had 1.9 times the odds of spawning than those fed daily (six times per week). Commercial catfish brood stock operations have different feeding practices, thus Steeby & Wagner (2005) reported that about 23.5% of brood stock breeding operations feed their animals daily during Spring/Early Summer, while 68.8% feed every other day or every third day (35.7% every other day, and 33.1% every third day). Hence, results of the exact logit analysis could be used to reinforce commercial practices in feed management.

Age of fish is often considered to be a determinant factor in spawning performance. Santiago (1979) reported a very low spawning success in channel catfish 3-year-old females (12.7%), and our study shows that using 5-year-old female fish rather than younger females is likely to produce a significantly higher rate of successful spawning. Although older fish performed better, producers must carefully consider other factors, such as economic and genetic, along with the results presented in this paper in making their decision.

Results of this case study demonstrate that appropriate statistical models provide better insight into the cause-effect relationships that exist between control variables and the dependent variable (likelihood of spawning in this case). For spawning success or even other dichotomous variables (such as survival-mortality and infected-not infected), logistic and other binary regression models are generally considered to be better statistical tools since they offer detailed descriptions of the relationships among variables in addition to their theoretical optimality. Using exact logit analysis, channel catfish brood stock females from Auburn University, classified as strain 1 (high spawning) and strain 2 (low spawning), were

found to exhibit significantly different spawning success probabilities for different levels of feed frequency but not for different levels of protein. Strain 2 fish fed six times per week had a significantly lower success in spawning as compared to strain 2 fish fed three times per week. Whereas, feeding frequency did not have a significant effect on the spawning success of strain 1 fish. Age of fish and period of spawning also had a significant effect on the probability of spawning success.

In summary, as would be expected each strain of fish responded somewhat differently to the test variables. Changing the protein level of the diet from 32 to 42% or increasing the feeding frequency from 3 to 6 times per week either did not influence spawning or negatively affected spawning respectively. Additionally, older fish performed better than younger and the early spawning period was better than the later spawning period, irregardless of strain. These responses were only detected by logit analysis, which was a more sensitive test and would thus be recommended for this type of data.

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CHAPTER III

ZERO INFLATED DISCRETE STATISTICAL MODELS FOR FECUNDITY DATA ANALYSIS IN CHANNEL CATIFSH *Ictalurus punctatus (Rafinesque)*

Abstract

Traditionally fecundity is expressed in Aquaculture, either as number of eggs per body weight of individual female or number of eggs per spawn, and typically does not include females that do not spawn. Such spawning data contain a high proportion of zeroes either due to the absence of eggs or the inability to recover the eggs. This data is often excluded from the analysis. Zero-inflated discrete generalized linear models are an alternative method that can be developed to taking into account females that do not spawn. In this case study, we propose discrete generalized linear models that use specially constructed mixture models to handle the excess zeroes such as the zero-inflated Poisson (ZIP) and zero-inflated negative binomial (ZINB) models. These models have the advantage of modeling egg production simultaneously with the probability of spawning. The results show that age was the most significant factor influencing the number of eggs per gram of egg mass while period of spawning was the most significant factor influencing the number of eggs per female body weight. These were also the most important variables that significantly affected the probability of successful spawning. Model residual diagnostics show that zero-

inflated models exhibit superior performance compared to the traditional models like analysis of covariance, Poisson regression and negative binomial regression models.

Introduction

Reproduction in captivity has been the key which has opened the door to successful early rearing, metamorphosis and grow out to market size of economically important fin fish (Donaldson 1996). However, unpredictable and variable reproductive performance is an important limiting factor for the successful mass production of juveniles (Kjørsvik, Mangor-Jensen & Holmefjord.1990; Fernández-Palacios, Izquierdo, Robaina, Valencia, Salhi & Vergara1995; Izquierdo, Fernández-Palacios & Tacon 2001). Poor egg quality is one of the major constraints in the expansion of aquaculture of both marine and many freshwater fish species (Brooks, Tyler & Sumpter1997). Several methods have been developed to assess the egg quality of fish. One of these is fecundity, which is the total number of eggs produced by each fish expressed either in terms of eggs/spawn or eggs/body weight (Izquierdo *et al.* 2001). Fecundity for channel catfish, *Ictalurus punctatus* (Rafinesque), ranges from 677 to 14,360 eggs/kg of female (Walser & Phelps 1993). Maternal effects have been considered to be the source of the variation seen in egg enumeration estimates (Walser & Phelps 1993). On the other hand, stripping of females may underestimate realized fecundity because manipulation during stripping and/or the timing of stripping in relation to ovulation time may result in the incomplete recovery of ovulated eggs (Lambert & Thorsen 2003). This effect may be more pronounced in channel catfish considering that they are sequential spawners (Smitherman, Hussein & Reagan 1978).

Egg production either as number of eggs per body weight of female or number of eggs per spawn, has typically considered positive integer numbers with little attention to those females that did not spawn. Usually the only account for this is a summary figure in

terms of spawning success, and/or total number of eggs or fry produced per total body weight per unit of area. The spawning process is probabilistic in nature (Bernardo 1993). Age and size are not the only factors that affect sexual maturity, as there is a complex process that involves body reserves, water quality, handling stress, etc. These are variables that lead to ovulation and successful stripping or, on the other hand, to egg retention in the body causing over-ripening. The latter indicates failure in the spawning process. A similar probabilistic condition could be inferred for fecundity data since they are directly related. Thus, careful attention must be given to those females that did not spawn due to stress caused by an excess of manipulation, or environmental conditions that prevent recovery of ovulated eggs, either of which produces a high proportion of zeros in the total egg production of a given population of fish.

Analysis of fecundity is traditionally performed using analysis of variance (ANOVA) (Walser & Phelps 1993; Lambert 1998; Kristanto 2004), where it is assumed that the errors are independent and identically distributed according to the normal distribution with a mean of 0 and constant variance. Covariate analysis, simple linear and multiple regression are also performed to establish relationships between fecundity and total length or body weight. However, discrete data are usually not in line with these considerations and as a result other analytic methods should be considered (Byers, Allore, Gill & Peduzzi 2003). Response variables that result from a process of counting, such as number of eggs, are better modeled using discrete probability distributions such as the Poisson distribution whenever possible. However, one problem with using the Poisson distribution is that it assumes that the mean and variance are equal. This restriction does not hold in many studies where the variance of

the data is greater than the mean, a condition referred to as overdispersion. In this case a disturbance or error term must be included in the model giving rise to the negative binomial regression model (Zelterman 1999, 2002; Byers *et al.* 2003). Accounting for overdispersion in count data is necessary to draw correct inferences about the mean (or probability) parameters (Lindsey 1999). One way to measure overdispersion is to take the square root of the ratio of deviance to its degrees of freedom, where a value greater than one indicates the existence of overdispersion (Zelterman 2002).

Zero-inflated modeling has recently been suggested as an approach which gives a better fit to count data with excessive zero counts (Lambert 1992). These type of data have been reported in medical and public health studies, ecological studies, and fisheries, among others (Welsh, Cunningham & Chambers 2000; Lewsey & Thomson 2004; Mwalili, Lesaffre & Declerck 2004; Fletcher, Mackenzie & Villouta 2005; Martin, Wintle, Rhodes, Kuhnert, Field, Low-choy, Tyre & Possingham 2005). One of the objectives of this case study is to develop zero-inflated models that give better description of the egg production that can be used for inferences and predictions of future events.

Materials and Methods

Data were obtained from a project performed to improve reproductive efficiency to produce hybrids from female channel catfish, *Ictalurus punctatus*, with male blue catfish, *I. furcatus*, through nutrient manipulations. The study evaluated the influence and interaction of dietary protein level and feeding rate on egg production of two separate strains of catfish (each one with three ages). Two diets containing 42% and 32% protein level, and two feed

frequencies (six times/week and three times/week) were considered, defining the following four treatment combinations (TC): TC 1 - 42% protein level, 6 feedings per week; TC 2 - 42% protein level, 3 feedings per week; TC 3 - 32% protein level, 6 feedings per week; TC 4 - 32% protein level, 3 feedings per week. Females were spawned during three periods (early, middle and late season). Evaluation of the dietary protein level and feeding rate treatments, were based on spawning success, and egg production.

Experimental fish

A total of 414 female channel catfish were maintained at the North Auburn Experimental Station, Auburn University. All brood stock were stocked in 16 ponds, using four ponds per treatment. The females were divided into two strains on the basis of prior spawning behavior (high spawning strain (HSS) - strain 1 and low spawning strain (LSS) - strain 2) and based on that characteristic they were assigned proportionally in a randomized manner to each pond (Table 1). Female body weights ranged from 0.4 kg to 3.4 kg for HSS and 0.6 kg to 3.1 kg for LSS with a combined mean weight of 1.74 kg. The fish were stocked in February 2004, in 0.04 ha ponds at a density of ~1,130 kg/ha, giving an acclimation period of approximately one month before receiving the test diets. The trial period was 70 to 90 days depending on the spawning period. Feeding was done during the warmest part of the day between 1500 and 1700 hours, at a rate of 1.7% of total biomass of brood fish stocked per pond. Water quality parameters were taken daily for dissolved oxygen and temperature; twice weekly for pH, ammonia-N, and nitrite-N. Alkalinity and hardness were recorded at stocking and just before harvesting.

Table 1 - Number of females channel catfish by strain per treatment

Treatment combination	Protein level	Feed frequency	Strain		Total
			High	Low	
1	42%	6	44	55	99
2	42%	3	55	57	112
3	32%	6	44	56	100
4	32%	3	44	59	103
Total			187	227	414

For the first spawning period (early), two ponds of each treatment were drained, and 16 females (out of 32) were selected based on external characteristics (abdominal fullness, softness and palpability of the ovaries, redness or swollen appearance of the genitals). Second spawning period (middle) was performed using one pond of each treatment, selecting 16 females (out of 32). The last spawning period (late) selected all the remaining females in all ponds.

Hormone injections

Individually identifiable females were transferred to holding tanks (per treatment) supplied with continuous flow-through water, placed individually in soft mesh bags. Total length, body weight, and girth were recorded. Hormone injections were administered in two doses, a priming injection of 20 μg/kg luteinizing hormone-releasing hormone analogues (LHRHa), followed 12 hours later by resolving dose of 100 μg/kg.

Collection and fertilization of gametes

Twenty four hours after the second injection, females were monitored for ovulation. Females with released eggs were removed from holding tank and anesthetized in 250 mg/l tricaine methane sulfonate (MS-222). Then, females were stripped, and eggs were collected in aluminum containers lubricated previously with vegetable shortening. Those females that did not express eggs were returned to the holding tank and examined later. Stripping of eggs ceased when all females had been stripped or attempts to strip them were unsuccessful.

Egg enumeration

Egg samples were placed in vials previously weighted, and containing formalin 5%. Then, these vials were re-weighted and number of eggs was recorded for each sample. Total weight of the egg mass was determined gravimetrically and the number of eggs per gram of egg mass was recorded based on 186 samples. Egg number per gram of female body weight was estimated from total number of eggs in the egg mass divided by total female weight.

Statistical analysis

Egg production, either as eggs/g of egg mass or eggs/g of female body weight, was analyzed using five different approaches: analysis of covariance (ANCOVA), Poisson regression, negative binomial regression (NB), zero-inflated Poisson regression (ZIP), and zero-inflated negative binomial regression (ZINB). The explanatory variables examined were protein level, feed frequency, and their interactions, using strain, age of fish, and period of spawning as covariates. The Genmod and NLMixed procedures from SAS® (SAS Institute Inc., Cary, NC) were used.

The statistical model for the analysis of covariance (ANCOVA) was given by

$$y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma_k + \tau_l + \eta x_{ijklm} + \varepsilon_{ijklm}$$

where μ represents the average response, α_i , i= 1,2, represents the effect of protein i; β_j , j = 1, 2, represents the effect of feeding frequency j; $(\alpha\beta)_{ij}$, i, j =1,2, represents the interactive effect of protein i and feeding frequency j; γ_k , k = 1, 2, 3, is the effect of period k; τ_l , l = 1, 2, represents the effect of strain l, x_{ijklm} represents the age of fish m in the cell *(i, j, k, l)*, and

ε_{ijkl} represents the random error associated with fish *m* in the cell *(i, j, k, l)*. It is commonly assumed that ε_{ijkl} are independent and identically distributed (IID) random variables following the normal distribution with mean zero and constant variance σ^2, i.e. $\varepsilon_{ijkl} \sim IIDN(0, \sigma^2)$. In the notation of generalized linear models (McCullag & Nelder 1989), this ANCOVA model can be written as $y_{ijklm} \sim N(\mu_{ijklm}, \sigma^2)$, where μ_{ijklm} is linked to the explanatory variables as

$$\mu_{ijklm} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma_k + \tau + \eta x_{ijklm}$$

and σ^2 is left unrestricted.

Although this model is widely applied in the literature, assuming normality of the response variable under consideration is not very realistic it is a discrete-count type random variable. More appropriate models involve discrete probability distributions like the Poisson and negative binomial distributions.

The models we consider are members of the family of models known as generalized linear models (McCullagh and Nelder 1989). In the Poisson regression model we assume that $y_{ijklm} \sim Poisson(\lambda_{ijklm})$. Equivalently, the probability mass function (pmf) of the response variable is given by

$$P(y_{ijklm} = t) = \frac{\exp(-\lambda_{ijklm})\lambda_{ijklm}^{t}}{t!}, t = 0,1,2,\ldots,$$

where $\lambda_{ijklm} > 0$ is a parameter that is unknown. Note that the expected value and variance of y_{ijklm} are both equal to λ_{ijklm}, an restriction that may be questionable for the response variables that are considered in this paper. The next task is to link the parameter λ_{ijklm} to the

explanatory variables similar to the manner in which the normal distribution parameter μ_{ijklm} was linked to the explanatory variables in the ANCOVA model. However, in this case the link function must guarantee that $\lambda_{ijklm} > 0$. Although there are several link functions that satisfy this requirement, we shall use the simplest of them all given by

$$\log (\lambda_{ijklm}) = \mu_{ijklm} ,$$

where μ_{ijklm} is as defined in the ANCOVA model above.

One possible approach to alleviate the rather stringent restriction imposed by the equality of the mean and variance of y_{ijklm} is to use the negative binomial (NB) regression model assumes that the pmf of y_{ijklm} is given by

$$P(y_{ijklm} = t) = \frac{\Gamma(t + 1/r)(r\lambda_{ijklm})^t}{\Gamma(t+1)\Gamma(1/r)(1+r\lambda_{ijklm})^{t+1/r}}, t = 0,1,2,\ldots,$$

where $\lambda_{ijklm} > 0$ and $r > 0$ are unknown parameters and $\Gamma(.)$ is the gamma function defined by the integral $\Gamma(z) = \int_0^\infty w^{z-1} \exp(-w)dw$. The expected value and variance of y_{ijklm} are λ_{ijklm} and λ_{ijklm} $(1 + r\ \lambda_{ijklm})$, respectively. Note that the variance is always greater than the expected value and thus this distribution is appropriate to model response variables that may have overdispersion, a situation that cannot be adequately modeled using the Poisson distribution. Once a gain we shall employ the link function $\log (\lambda_{ijklm}) = \mu_{ijklm}$ and estimate r from the data in an unrestricted manner.

In some cases, the number of zero responses is in excess of that consistent with the Poisson and negative binomial distributions. A relatively easy way to build a model for this

zero-inflated data to attach a positive probability, say 1 - p for $0 < p < 1$, to the event that a zero value is not consistent with the Poisson or negative binomial distribution (Lambert 1992). This gives rise to the zero-inflated Poisson (ZIP) and zero-inflated negative binomial (ZINB) distributions that sample the respective distribution a proportion p times and zero a proportion 1 - p times.

Under the ZIP regression model pmf of y_{ijklm} is given by

$$P(y_{ijklm} = t) = \begin{cases} (1 - p) + \dfrac{p}{(1 + r\lambda_{ijklm})^{1/r}} & \text{if } t = 0 \\ p\dfrac{\Gamma(t + 1/r)}{\Gamma(t+1)\Gamma(1/r)}\dfrac{(r\lambda_{ijklm})^t}{(1 + \lambda_{ijklm})^{t + 1/r}} & \text{if } t > 0 \end{cases}$$

The mean and variance of y_{ijklm} are now given by $E(y_{ijklm}) = p\lambda_{ijklm}$ and $V(y_{ijklm}) = \lambda_{ijklm}(1 + \lambda_{ijklm}(1 - p))$, respectively (Simonoff 2003). The function $\log(\lambda_{ijklm}) = \mu_{ijklm}$ is used to link the parameter λ_{ijklm} to the explanatory variables.

Similarly, the zero-inflated negative binomial (ZINB) regression model uses a mixture distribution that assigns a mass 1 - p to the "extra" zeroes and a mass p to a negative binomial distribution, where $0 < p < 1$. The pmf of y_{ijklm} in the ZINB model is given by

$$P(y_{ijklm} = t) = \begin{cases} (1 - p) + p\exp(-\lambda_{ijklm}) & \text{if } t = 0 \\ \dfrac{p\exp(-\lambda_{ijklm})\lambda_{ijklm}^t}{t!} & \text{if } t > 0 \end{cases}$$

The mean and variance of $E(y_{ijklm}) = p\lambda_{ijklm}$ and $V(y_{ijklm}) = p\lambda_{ijklm}(1 + \lambda_{ijklm}(r+1 - p))$, respectively. It can be observed that this distribution approaches the ZIP distribution as $r \to 0$ and the negative binomial distribution as $p \to 1$ (Mwalili *et al.* 2004).

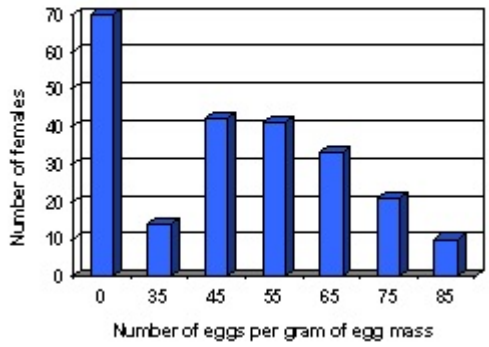
Assessment of fit was based on Akaike's Information Criterion (AIC) (Akaike 1987), which is a criterion for selecting the best model among a number of candidate models. The estimated AIC value is given by two times the number of estimated parameters minus two times the achieved log-likelihood. One is rewarded for larger values of the log-likelihood but penalized for larger number of parameters; thus lower AIC values correspond to better models. Since the models we are considering are not all properly nested, to make the AIC values comparable, we utilize the full log-likelihood in the computation of the AIC. Plots of residuals versus predicted values are used to perform graphical diagnosis of the fitted models. A visible pattern in a residual plot is indicative of misspecification problems with the corresponding model.

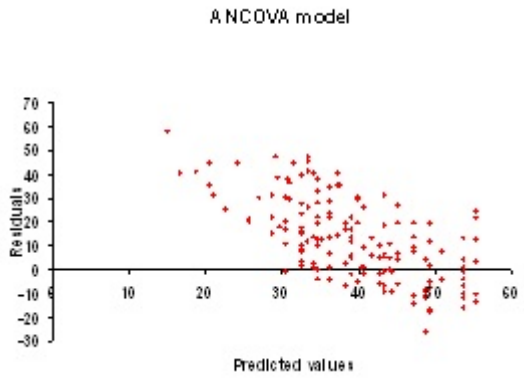
The Genmod, and NLMixed Procedures from SAS® version 9.1 (SAS Institute Inc., Cary, NC) were used. SAS codes for these analyses are presented in Appendix 1.

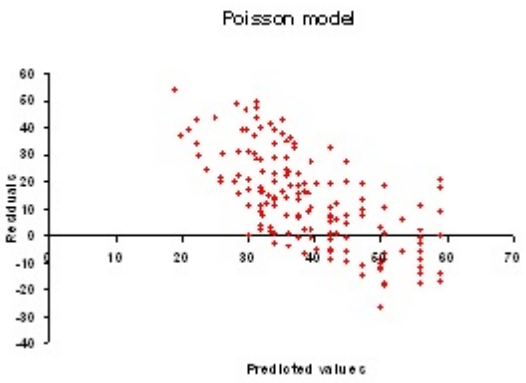
Results

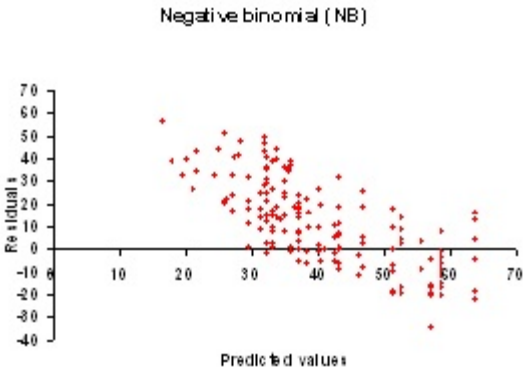
The frequency histogram of number of eggs/g of egg mass indicates the presence of a relatively high number of zeroes (Fig. 1). A similar observation is made regarding the distribution of number of eggs/g of female body weight (Fig. 2). Evaluation of egg production as number of eggs/g of egg mass detected overdispersion using the ANCOVA, with an overdispersion value of 24.8, whereas the overdispersion values were reduced to 5.2

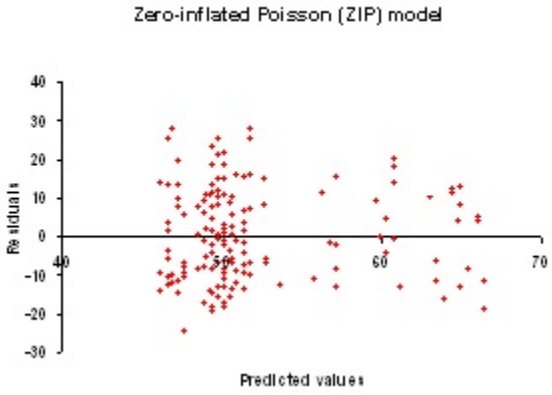
Figure 1 - Number of eggs per gram of egg mass from channel catfish females (*Ictalurus punctatus*), and residual plots for statistical models











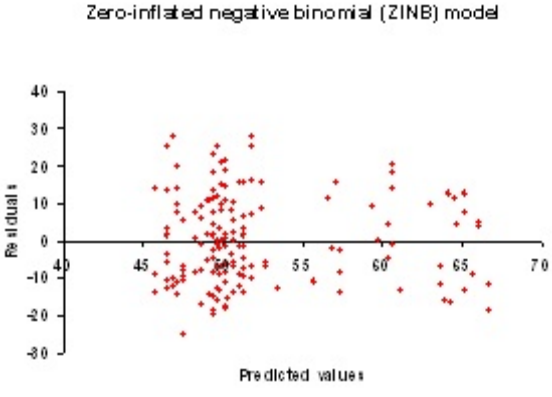


Figure 2 - Number of eggs per gram of channel catfish female (*Ictalurus punctatus*) body weight, and residual plots for statistical models

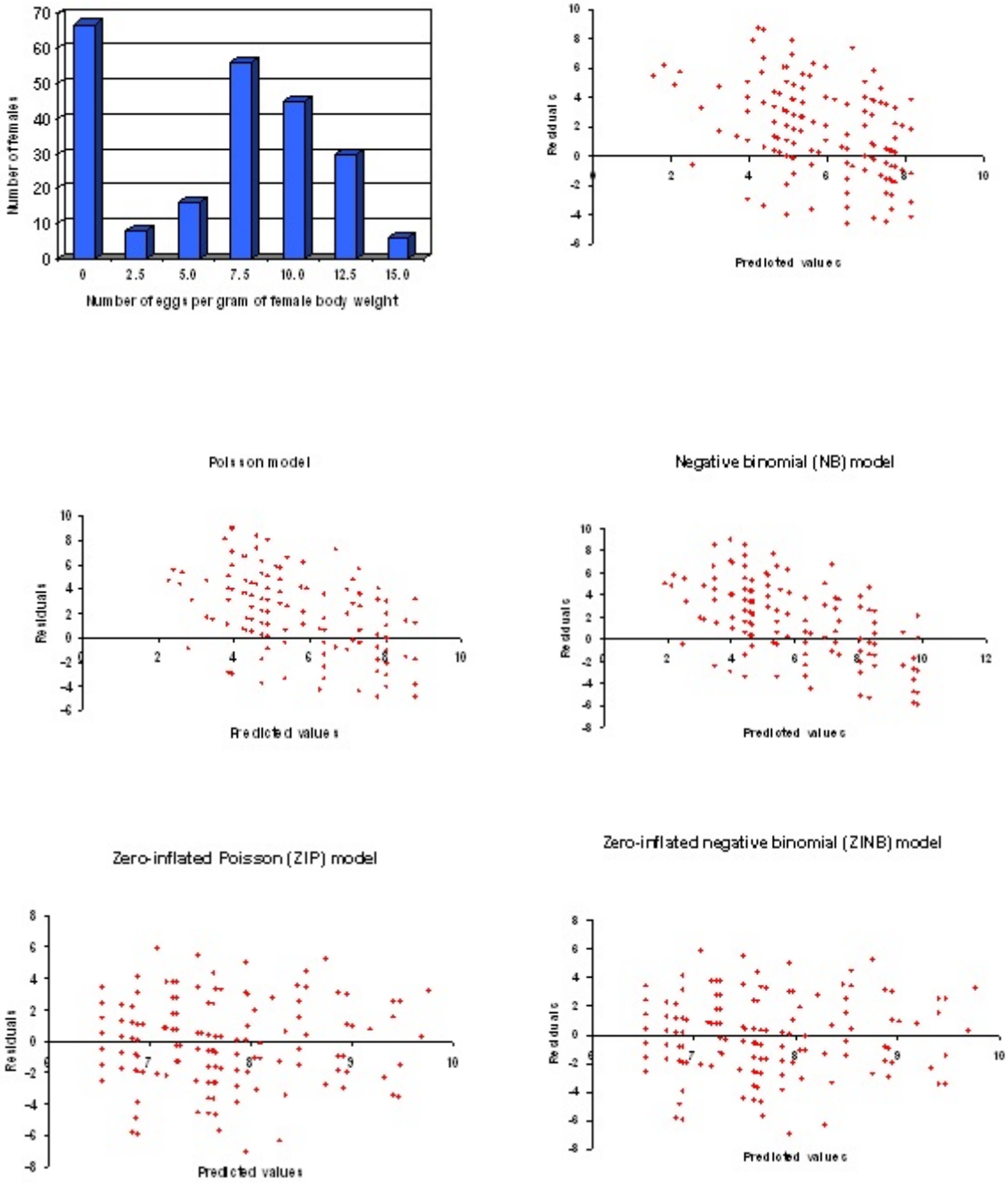


Table 2 - Summary of the dispersion and Akaike's Information Criterion (AIC) of the statistical models for number of eggs/g of egg mass and eggs/g of female body weight (0.001 kg)

Estimate	ANCOVA	Poisson	NB[a]	ZIP[b]	ZINB[c]
Eggs per gram of egg mass					
Overdispersion	24.8	5.2	2.4	---	---
AIC	2148.6	6898.6	2042.9	1619.7	1510.5
Eggs per gram of female body weight (0.001 kg)					
Overdispersion	3.9	2	0.96	---	---
AIC	1289.4	1549.4	1260.8	1016.2	1018.2

a. NB: Negative binomial model
b. ZIP: Zero Inflated Poisson distribution
c. ZINB: Zero Inflated Negative Binomial distribution

and 2.4 for the Poisson and negative binomial models, respectively (Table 2). The AIC value on the other hand was extremely high for the Poisson model with a value of 6898.6, followed by the ANCOVA model with 2148.6, the NB model with 2042.9, the ZIP model with 1619.7, and the ZINB model with 1510.5 (Table 2). Analysis of fecundity as number of eggs per gram of female body weight exhibited a similar pattern though to a lower degree. The overdispersion values were closer to each other and they drop from 3.9 to 0.96 as we move from the ANCOVA model to the NB model (Table 2). The AIC values were 1549.4 for the Poisson model, 1289.4 for ANCOVA, 1260.8 for NB, 1016.2 for ZIP, and 1018.2 for ZINB (Table 2). These AIC values indicate that zero-inflated models provide fits that are superior to the traditional models for these data sets. These results are not unexpected and they are consistent with our observations from the frequency histograms.

All the Poisson regression model parameters were found to be significant at the 5% level of significance, while the ANCOVA model found feed frequency and age of fish as significant, and early period of spawning as highly significant ($P < 0.0001$) in determining the number of eggs per gram of egg mass (Table 3). The NB regression model detected only age and early period of spawning as highly significant. The ZIP and ZINB models found age to be the only variable that is highly significant ($P < 0.0001$) for the same response variable (Table 3). The variables that significantly affect zero-inflation, and hence the probability of successful spawning, are found to be feed frequency, age, and early period of spawning in both the ZIP and ZINB models for number of eggs per gram of egg mass. Residual plots for number of eggs per gram of egg mass, with zeroes dropped for better visual inspection, are presented in Fig.1. ANCOVA, Poisson and NB models exhibited a pattern that is generally

Table 3 - Summary of estimates of model parameters for number of eggs/g of egg mass from channel catfish females, *Ictalurus punctatus*

Parameters		ANCOVA	Poisson	NB[a]	ZIP[b]	ZINB[c]
Protein level	○	-4.3697	-0.1030	-0.1354	-0.0245	-0.0231
	◊	(-13.29, 4.55)	(-0.16, -0.04)	(-0.71, 0.44)	(-0.08, 0.03)	(-0.12, 0.07)
	★	-0.3355	-0.0006	-0.6429	-0.4098	(0.6384)
Feed frequency	○	-10.2593	-0.2766	-0.3113	-0.0323	-0.0328
	◊	(-19.74, -0.78)	(-0.34, -0.21)	(-0.92, 0.30)	(-0.10, 0.03)	(-0.14, 0.07)
	★	-0.0340	(<0.0001)	-0.3159	-0.3274	-0.5453
Protein level x	○	8.1034	0.2123	0.3364	-0.0260	-0.0298
feed frequency	◊	(-4.63, 20.83)	(0.12, 0.30)	(-0.49, 1.17)	(-0.11, 0.06)	(-0.17, 0.12)
	★	-0.2110	(<0.0001)	-0.4249	-0.5608	-0.6858
Strain	○	-1.6655	-0.0519	-0.0813	-0.0064	-0.0088
	◊	(-8.20, 4.86)	(-0.10,- 0.01)	(-0.50, 0.34)	(-0.05, 0.04)	(-0.08, 0.06)
	★	-0.6158	-0.0224	-0.7021	-0.7781	-0.8125
Age	○	6.8203	0.2061	0.2520	-0.1255	-0.1263
	◊	(3.06, 10.58)	(0.18, 0.23)	(0.01, 0.49)	(-0.15, -0.10)	(-0.17, -0.08)
	★	-0.0004	(<0.0001)	-0.0420	(<0.0001)	(<0.0001)
Early season	○	14.7158	0.3983	0.4623	0.0156	0.0140
	◊	(7.65, 21.78)	(0.35, 0.45)	(0.01, 0.92)	(-0.03, 0.06)	(-0.06, 0.09)
	★	(<0.0001)	(<0.0001)	-0.0464	-0.5226	-0.7272
Middle season	○	3.7879	0.1234	0.1407	-0.0096	-0.00153
	◊	(-5.12, 12.70)	(0.06, 0.19)	(-0.44, 0.72)	(-0.07, 0.05)	(-0.12, 0.09)
	★	-0.4031	-0.0002	-0.6307	-0.7681	-0.7723
Inflation						
Protein level	○				0.5764	0.4363
	◊				(-0.39, 1.54)	(-0.52, 1.39)
	★				-0.2409	-0.3697

Feed frequency	○	1.1902	1.0105
	♢	(0.18, 2.20)	(0.01, 2.01)
	★	-0.0211	-0.0478
Protein level x	○	-1.1846	-0.9455
feed frequency	♢	(-2.50, 0.14)	(-2.26, 0.37)
	★	-0.0789	-0.1582
Strain	○	0.1695	0.1350
	♢	(-0.50, 0.84)	(-0.53, 0.80)
	★	-0.6184	-0.6914
Age	○	-1.0327	-1.0448
	♢	(-1.41, -0.66)	(-1.42, -0.67)
	★	(<0.0001)	(<0.0001)
Early season	○	-1.5307	-1.5849
	♢	(-2.33, -0.73)	(-2.39, -0.78)
	★	-0.0002	-0.0001
Middle season	○	-0.2699	-0.3329
	♢	(-1.11, 0.57)	(-1.18, 0.51)
	★	-0.5282	-0.4382

a. NB: Negative binomial model
b. ZIP: Zero Inflated Poisson distribution
c. ZINB: Zero Inflated Negative Binomial distribution
○. Parameter estimate
♢ 95% confidence interval for the parameter
★ p-value of the parameter estimate

Table 4 - Summary of estimates of model parameters for number of eggs/g of female body weight from channel catfish females, *Ictalurus punctatus*

Parameters		ANCOVA	Poisson	NB[a]	ZIP[b]	ZINB[c]
Protein level	○	-0.2174	-0.0286	-0.0499	0.0535	0.0535
	◊	(-1.61, 1.17)	(-0.18, 0.13)	(-0.45, 0.35)	(-0.10, 0.21)	(-0.15, 0.26)
	★	-0.7581	-0.7146	-0.8039	-0.4946	-0.6095
Feed frequency	○	-1.2111	-0.2312	-0.2931	0.0445	0.0445
	◊	(-2.69, 0.26)	(-0.40, -0.06)	(-0.72, 0.13)	(-0.13, 0.22)	(-0.16, 0.25)
	★	-0.1072	-0.0081	-0.1758	-0.6103	-0.6686
Protein level x	○	0.9036	0.1694	0.3281	-0.0674	-0.0674
feed frequency	◊	(-1.08, 2.89)	(-0.06, 0.40)	(-0.25, 0.91)	(-0.30, 0.16)	(-0.39, 0.25)
	★	-0.3700	-0.1462	-0.2653	-0.5679	-0.6802
Strain	○	-0.4302	-0.0935	-0.1529	-0.0495	-0.0495
	◊	(-1.45, 0.59)	(-0.21, 0.02)	(-0.45, 0.14)	(-0.17, 0.07)	(-0.20, 0.10)
	★	-0.4054	-0.1150	-0.3060	-0.4063	-0.5125
Age	○	1.4213	0.3106	0.3744	-0.0300	-0.0300
	◊	(0.84, 2.01)	(0.23, 0.39)	(0.20, 0.55)	(-0.11, 0.05)	(-0.14, 0.08)
	★	(<0.0001)	(<0.0001)	(<0.0001)	-0.4528	-0.5856
Early season	○	2.5629	0.4975	0.5998	0.1062	0.1062
	◊	(1.46, 3.66)	(0.37, 0.63)	(0.28, 0.92)	(-0.02, 0.24)	(-0.02, 0.24)
	★	(<0.0001)	(<0.0001)	-0.0002	-0.1080	-0.1107
Middle season	○	1.9993	0.4109	0.4467	0.2626	0.2626
	◊	(0.61, 3.39)	(0.25, 0.57)	(0.05, 0.84)	(0.10, 0.42)	(0.10, 0.42)
	★	-0.0049	(<0.0001)	-0.0268	-0.0013	-0.0016
Inflation						
Protein level	○				0.4345	0.4350
	◊				(-0.53, 1.40)	(-0.53, 1.40)
	★				-0.3767	-0.3762

Feed frequency	○	1.1424	1.1421
	♢	(0.13, 2.15)	(0.13, 2.15)
	★	-0.0268	-0.0269
Protein level x	○	-0.9762	-0.9762
feed frequency	♢	(-2.30, 0.35)	(-2.30, 0.35)
	★	-0.1477	-0.1478
Strain	○	0.1131	0.1129
	♢	(-0.56, 0.79)	(-0.56, 0.79)
	★	-0.7406	-0.7409
Age	○	-1.0715	-1.0715
	♢	(-1.45, -0.69)	(-1.45, -0.69)
	★	(<0.0001)	(<0.0001)
Early season	○	-1.7153	-1.7156
	♢	(-2.53, -0.90)	(-2.52, -0.90)
	★	(<0.0001)	(<0.0001)
Middle season	○	-0.4285	-0.4285
	♢	(-1.28, 0.42)	(-1.28, 0.42)
	★	-0.3215	-0.3215

a. NB: Negative binomial model
b. ZIP: Zero Inflated Poisson distribution
c. ZINB: Zero Inflated Negative Binomial distribution
○. Parameter estimate
♢ 95% confidence interval for the parameter
★ p-value of the parameter estimate

decreasing while residuals of the zero-inflated models were evenly distributed above and below the zero line with no visible pattern. This suggests that the ZIP and ZINB models are more appropriate for this type of data.

When analyzing the number of eggs per gram of female body weight, the Poisson model found feed frequency, age of fish, and spawning period to be variables that have a significant effect on this response (Table 4). On the other hand, ANCOVA and NB models found only age and spawning period as significant (Table 4). Finally ZIP and ZINB models found a significant effect of the middle spawning period. In both the ZIP and ZINB models the variables that significantly affected zero-inflation were found to be feed frequency, age of fish and early period of spawning. Residual plots show once again that ZIP and ZINB models provide superior fits when compared to all the other models (Fig. 2).

Discussion

Egg production data analysis enables hatchery producers to estimate their broodstock requirement. As a result a model for egg production is considered to be a critical tool to optimize fry production. We approach egg production analysis using two indices: number of eggs/g of egg mass, and number of eggs/g of female body weight. Total egg production is a trait that is a function of the size or age of fish. According to some authors the ability to reproduce does not depend on longevity but rather on the attainment of an adequate body size (Sokołowska & Skóra 2001). Thus, most of the egg production analysis has mainly considered female body weight (Froese & Luna 2004). On the other hand, the effect of age in egg production of freshwater fishes remained under-explored in contrast to marine species

(Shatunovskii 2006). In a catfish aquaculture setting, female age has been found responsible for spawning performance rather than its size (Santiago 1979). In fact fecundity has been found to be poorly correlated with female body weight (Bice 1981; Walser & Phelps 1993; Argue 1996; Lambert 1998). It is also not uncommon, especially in commercial hatcheries, to have several females that do not spawn. We provide statistical support to the fact that fish age has significant effect in egg production even when there were several females that did not spawn, and which likely were younger females, since the odds of spawning of 5 years-old females were higher than the odds of spawning of 3 years-old females (Quintero, Abebe & Davis 2007). In this particular analysis female size was highly variable. For instance, the range for 3 year-old females was 1.16 -3.46 kg, 4 year-old females was 0.74-2.8 kg, and 5 year-old females 0.94-4.04 kg, which explains the poor correlation between egg production and female body weight.

Egg production as number of eggs/g of egg mass showed a significant effect of age on this trait. We believe that this is due to the fact that the reference point is the egg mass which is a result of female condition that includes age, size, length, nutrition, etc. Thus, older females had significantly lower number of eggs than younger females, in other words they produce bigger eggs. According to Shatunovskii (2006) this phenomenon can be attributed to an increased reproductive function in ontogeny, which is realized as a more active synthesis of ovovitellin in the liver and its storage in oocytes as well as to an elongated period of trophoplasmatic growth of oocytes. This situation is also seen in walleye, where female age accounted for a greater amount of variation in the quantity of eggs mass than brood stock length or size (Johnston 1997).

Egg production as number of eggs/g of female body weight did not show significant effect of age on this trait. This is very likely a result of confounding, because the reference point is size, and as a result effect from age is eliminated. In fact, period of spawning came up as a variable with a significant effect on this trait. This is not a surprise because fecundity increased from the beginning of the season and then declined as the spawning season is ending (Lambert 1998). This also could be related to the fact, that brood stock females were selected based on external characteristics for the first two spawning periods, while the last period accounted for the remaining females.

For this data set zero-inflated models performed better than ANCOVA, Poisson, and negative binomial (NB) models. This was evident from the investigation of Akaike's Information Criterion (AIC) values and residual plots. Residual plots for number of eggs/g of egg mass display a clear systematic pattern for the ANCOVA, Poisson and negative binomial models (Fig. 1). This is points out the violation of the constant variance assumption for these models. Residual plots for the zero-inflated models appear to be evenly distributed around the zero line and do not have any visually discernible pattern (Fig. 1). This indicates that there is no evidence of violation of the constant variance assumption for the ZIP and ZINB models. Similar patterns are observed for the residual plots corresponding to the number of eggs/g of female body weight (Fig. 2).

The ZINB regression model predicted the mean values of the number of eggs/g per egg mass for age 3, 4 and 5 females to be 63, 55, and 49, respectively. These mean values were 53, 52, and 51 for early, middle and late spawning periods, respectively. When analyzing number of eggs/g of female body weight using the ZINB regression model, the

predicted means for females of age 3, 4, and 5, were 8.2, 8.3, and 7.5, respectively. For the same response variable the mean values were 7.8 for early season, 8.2 for middle season, and 6.9 for late season periods of spawning.

The significance of period of spawning and age in determining zero-inflation coincided with the results of a previous study of spawning success using exact logistic regression analysis, where these same variables were found to significantly affect the probability of successful catfish spawning (Quintero *et al.* 2007).

Conclusions

Reproductive performance is critical in hatchery management, and quantitative models that describe variation in fecundity can be used to optimize resources in this field. Predictions related to number of eggs that can be spawned for a group of fish can help us choose the best spawners. In this regard zero-inflated models gave better performance than the traditional ANCOVA, Poisson, and negative binomial (NB) models. This was evident from investigations of the Akaike's Information Criterion (AIC) values and residual plots. Given that results from the zero-inflated models provided a statistical resolution that corresponded with expected biological responses, it appears that this analysis is an optimal choice for these types of data.

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Appendix 1 - SAS Codes

```
data spawning;
input pro fre str age grp Y3 Y4;
/*Y3 is number of eggs per gram of egg mass, and Y4 is number of eggs per gram of
        female body weight*/
data spawning3;
set spawning;
if (fre eq 3) then fre = 0; if (fre eq 6) then fre = 1;
if (pro eq 32) then pro = 0; if (pro eq 42) then pro = 1;
if (str eq 1) then str = 0; if (str eq 2) then str = 1;
if (age eq 3) then age3 = 1; else age3 = 0; if (age eq 4) then age4 = 1; else age4 = 0;
if (grp eq 1) then grp1 = 1; else grp1 = 0;if (grp eq 2) then grp2 = 1; else grp2 = 0;
run;

proc nlmixed data=spawning3;
title 'eggs per gram - Ancova';
parms b0=0 b1=0 b2=0 b12=0 b31=0 b32=0 b4=0 b5=0;
mean= b0 + b1*fre + b2*pro + b12*pro*fre + b31*grp1 + b32*grp2 +  b4*str + b5*age;
model y3 ~ normal (mean, v);
predict mean out=est;
run;

proc nlmixed data=spawning3;
title 'Eggs per gram - Poisson using nlmixed';
parms b0_nb=0 b1_nb=0 b2_nb=0 b12_nb=0 b31_nb=0 b32_nb=0 b4_nb=0 b5_nb=0
k=20;
eta_nb = b0_nb + b1_nb*fre + b2_nb*pro + b12_nb*pro*fre + b31_nb*grp1 +
b32_nb*grp2 +  b4_nb*str + b5_nb*age;
mean = exp(eta_nb);
model y3 ~ poisson(mean);
predict mean out=est;
run;

proc nlmixed data=spawning3;
title 'Eggs per gram - Negative Binomial using nlmixed';
parms b0_nb=0 b1_nb=0 b2_nb=0 b12_nb=0 b31_nb=0 b32_nb=0 b4_nb=0 b5_nb=0;
eta_nb = b0_nb + b1_nb*fre + b2_nb*pro + b12_nb*pro*fre + b31_nb*grp1 +
b32_nb*grp2 +  b4_nb*str + b5_nb*age;
mean = exp(eta_nb);
p_else = exp(lgamma(y3+(1/k)) - lgamma(y3+1) - lgamma(1/k) +          y3*log(k*mean)
- (y3+(1/k))*log(1+k*mean));
loglike = log(p_else);
```

```
model y3 ~ general(loglike);
predict mean out=est;
run;

proc nlmixed data=spawning3;
title 'Eggs per gram - Zero-Inflated Poisson';
parms b0_zip=0 b1_zip=0 b2_zip=0 b12_zip=0 b31_zip=0 b32_zip=0 b4_zip=0
b5_zip=0
b0_nb=0 b1_nb=0 b2_nb=0 b12_nb=0 b31_nb=0 b32_nb=0 b4_nb=0 b5_nb=0;
eta_zip = b0_zip + b1_zip*fre + b2_zip*pro + b12_zip*pro*fre + b31_zip*grp1 +
b32_zip*grp2 + b4_zip*str + b5_zip*age;
p0_zip = 1 / (1 + exp(-1*eta_zip));
eta_nb = b0_nb + b1_nb*fre + b2_nb*pro + b12_nb*pro*fre + b31_nb*grp1 +
b32_nb*grp2 +  b4_nb*str + b5_nb*age;
mean = exp(eta_nb);
p0 = p0_zip + (1-p0_zip)*exp(y3*log(mean) - mean - lgamma(y3+1));
p_else = (1-p0_zip)* exp(y3*log(mean) - mean - lgamma(y3+1));
if y3=0 then loglike = log(p0);
else         loglike = log(p_else);
model y3 ~ general(loglike);
predict mean out=est;
run;

proc nlmixed data=spawning3;
title 'Eggs per gram - Zero-Inflated Negative Binomial';
parms b0_zip=0 b1_zip=0 b2_zip=0 b12_zip=0 b31_zip=0 b32_zip=0 b4_zip=0
b5_zip=0
b0_nb=0 b1_nb=0 b2_nb=0 b12_nb=0 b31_nb=0 b32_nb=0 b4_nb=0 b5_nb=0;
eta_zip = b0_zip + b1_zip*fre + b2_zip*pro + b12_zip*pro*fre + b31_zip*grp1 +
b32_zip*grp2 + b4_zip*str + b5_zip*age;
p0_zip = 1 / (1 + exp(-1*eta_zip));
eta_nb = b0_nb + b1_nb*fre + b2_nb*pro + b12_nb*pro*fre + b31_nb*grp1 +
b32_nb*grp2 +  b4_nb*str + b5_nb*age;
mean = exp(eta_nb);
p0 = p0_zip + (1-p0_zip)* exp(lgamma(y3+(1/k)) - lgamma(y3+1) - lgamma(1/k) +
y3*log(k*mean) - (y3+(1/k))*log(1+k*mean));
p_else = (1-p0_zip)* exp(lgamma(y3+(1/k)) - lgamma(y3+1) - lgamma(1/k) +
y3*log(k*mean) - (y3+(1/k))*log(1+k*mean));
if y3=0 then loglike = log(p0);
else         loglike = log(p_else);
model y3 ~ general(loglike);
predict mean out=est;
run;          /*Similar codes for Y4*/_____
```

CHAPTER IV

EFFECTS OF VARYING DIETARY PROTEIN LEVELS AND FEEDING FREQUENCIES ON CONDITION AND REPRODUCTIVE PERFORMANCE OF CHANNEL CATFISH TO PRODUCE HYBRID CATFISH

Abstract

The catfish industry is one of the largest and best developed aquaculture segments in the United States. The development of technological solutions to maintain a competitive level in the market is key to this success. The interspecific hybridization technique channel catfish, *Ictalurus punctatus* female x blue catfish, *I. furcatus* male has been identified as one way to further improve production; however, lack of spawning success has affected its commercial application. One of the first steps to overcome this challenge is to conduct basic research on brood stock nutrition to optimize egg production, hatch, and fry production. To facilitate our understanding of the interaction of nutrition and fry production we evaluated the interaction of feed quality (42 and 32% protein level) and feed frequency (feed offered 3 or 6 times per week). The treatments were offered during the spring season 70-90 days prior to the start of the spawning season. Induced reproduction was performed using luteinizing hormone releasing hormone analogue (LHRHa), and the eggs were fertilized using blue catfish sperm to produce catfish hybrids. Condition of the fish at the beginning

and at the end of the diet trial was evaluated, as well as reproductive performance using spawning success, egg production, egg size and egg viability after 48 hours. Changing protein level of the diet from 32 to 42% did not influence spawning, fecundity or fertilization, but affected egg size. Increasing the feeding frequency from 3 to 6 times per week negatively affected spawning in one of the two strains, did not affected egg production and egg fertilization, but had a significant effect on egg size. Older fish performed better than younger fish in terms of spawning success, and egg production, in addition to having bigger eggs than their younger counterpart. Biochemical composition of the eggs was affected significantly by dietary treatment in terms of lipid, fatty acids and free amino acid content, but not in the protein content.

Introduction

The catfish industry in the US started in the late 1960's, and has grown rapidly to become the largest segment of the aquaculture industry in the United States (Quagrainie & Engle 2002; Engle 2003). Commercial catfish production generated over 42% of the total value of aquaculture production in the US during 2005 with nearly 462 million dollars (USDA 2006). Since 2000, the U.S. farm-raised catfish industry has suffered because of low prices in the market-place caused by the influx of imported fish, depressed prices for competing meats, and a weak economy (Hanson, Dean & Spurlock 2004). These economic challenges will require developing new production technologies, market infrastructure, and market development (Engle 2003; Hanson *et al.* 2004) that lead to enhanced productivity.

Hybridization techniques have identified the interspecific hybrid channel catfish female (*Ictalurus punctatus*) x blue male (*I. furcatus*) as the most suitable for culture conditions, due to its better growth, higher resistance to low dissolved oxygen levels, resistance to diseases, ease of harvesting, and higher carcass yield (Giudice 1966; Yant, Smitherman & Green 1976; Brooks 1977; Chappell 1979; Tave, McGinty, Chappell & Smitherman 1981; Dunham, Smitherman & Webber, 1983b; Smitherman, Dunham & Tave 1983; Stephens & Dorman 1984; Ella 1984; Dunham, Smitherman & Goodman 1987; Dunham, Brummett, Ella & Smitherman 1990; Jeppsen 1995; Argue, Liu & Dunham 2003). However, reproductive problems have limited its application in commercial scale (Tave & Smitherman 1982; Dunham & Smitherman 1987). Increasing interest in developing techniques to manipulate and control hybrid production has shown a significant increase during the past twenty five years, in areas as physiological responses, genetic aspects,

artificial and husbandry methods (Tave & Smitherman 1982; Bart 1994; Kim 1996; Bart & Dunham 1996; Dunham, Liu & Argue 1998; Lambert 1998; Dunham, Bart & Kucuktas 1999; Dunham & Argue 2000; Kristanto 2004; Hutson 2006). However, a lack of information in nutritional status of brood stock is common, not only in channel catfish but in other aquaculture species. This lack of information is due in part to the need for appropriate indoor and/or outdoor culture facilities for maintaining large groups of adult fish and the associated costs with those requirements (Izquierdo, Hernández-Palacios & Tacon 2001). Nutritional aspects of brood stock channel catfish were considered by Santiago (1979), who studied the effects of feeding, and age on reproductive performance. The objective of this research was to evaluate the effect of dietary protein levels, and frequency of feeding on the egg performance of channel catfish females to produce hybrid catfish, using a holistic approach that lead us to better understanding of the effects that interact in the reproduction process. Strain as genetic component, age as physiological component, and period of spawning as an environmental component were evaluated in conjunction with the dietary treatments.

Methods

The influence/interaction of dietary protein level and feeding rate on reproductive performance from female channel catfish, *Ictalurus punctatus* (Rafinesque), crossed with male blue catfish, *Ictalurus furcatus* (Lesueur), to produce hybrid catfish was evaluated. Two diets containing 42% and 32% protein level were manufactured by ARKAT Feeds Inc (Dumas, AK) using a commercial formulation for catfish. Two feeding frequencies (six

times/week and three times/week), were used giving rise to four treatment combinations: 42 / 6 - 42% protein level, 6 feedings per week; 42 / 3- 42% protein level, 3 feedings per week; 32 / 6 - 32% protein level, 6 feedings per week; 32 / 3 - 32% protein level, 3 feedings per week. Two channel catfish strains (high and low spawning), each one with three ages (3, 4 and 5-year-old), were spawned during three periods (early, middle and late season) (Table 1). Evaluation of the dietary protein level and feeding rate treatments, were performed through spawning success, egg production (number of eggs/g of egg mass, and number of eggs/g of female body weight), and egg viability 48 hours after fertilization.

Experimental fish

A total of 414 female channel catfish were maintained at the E. W. Shell Experimental Station, Auburn University. All brood stock were stocked in 16 ponds, using four ponds per treatment. The females were divided into two strains on the basis of prior spawning behavior: high spawning strain (strain1) and low spawning strain (strain 2); based on that characteristic they were assigned proportionally in a randomized manner to each pond (Table 1). Females were tagged with PIT tags, in order to keep track of body weight, length, and girth on individual basis. The fish were stocked in February 2004, in 0.04 ha ponds at a density of ~1,130 kg/ha. The acclimation period was approximately one month, and the trial period was 70 days to 90 days depending on the spawning period. Feeding was done during the warmest part of the day between 1500 and 1700 hours, at a rate of 1.7% of total biomass of brood fish stocked per pond. Water quality parameters were taken twice daily for dissolved oxygen and temperature; twice weekly for pH, ammonia-N, and nitrite-N.

Table 1. Classification of channel catfish females (*Ictalurus punctatus*) by strain, age class, and period of spawning per dietary treatment

Treatment	1	2	3	4	
Protein level	42%	42%	32%	32%	Total
Feed frequency	6	3	6	3	
Females by strain					
High spawning	44	55	44	44	187
Low spawning	55	57	56	59	227
Females by age class					
3 years-old	33	35	30	37	135
4 years-old	15	19	11	10	55
5 years-old	51	58	59	56	224
Females by spawning period					
Early	30	28	29	29	116
Middle	13	15	13	14	55
Late	56	69	58	60	243

For the first spawning period (early), two ponds of each treatment were drained, and 16 females (out of 32) were selected based on external characteristics (abdominal fullness, softness and palpability of the ovaries, redness or swollen appearance of the genitals). The second spawning period (middle) was performed using one pond of each treatment, selecting 16 females (out of 32). The last spawning period (late) selected all the remaining females in all ponds.

Selected females per treatment were transferred to holding tanks (3.0 x 0.47 x 0.61 m with a water volume from 670 – 837 liters) supplied with continuous flow-through water, and placed individually in soft mesh bags. Total length, body weight, and girth were recorded. The luteinizing hormone-releasing hormone analogues (LHRHa) GMP grade from American Peptide (Vista, CA) was utilized. Hormone injections were administered in two doses, a priming injection of 20 μg/kg LHRHa, followed 12 hours later by resolving dose of 100 μg/kg.

Collection and fertilization of gametes

Twenty four hours after the second injection, females were monitored for ovulation. Females with released eggs were removed from the holding tank and anesthetized in 250 mg/l tricaine methane sulfonate (MS-222) (Argent Chemical Laboratories, Redmond, WA) buffered with sodium bicarbonate. Females were stripped and eggs were collected in metal pie pans lubricated previously with vegetable shortening. Those females that did not express eggs were returned and rechecked later. Stripping of gametes ceased when all females had been stripped or attempts to strip them had been made. Collected egg masses were weighted

and then number of eggs/g of egg mass, number of eggs/g of female body weight , and total number of eggs, were estimated and recorded individually. The eggs and blue catfish sperm were gently swirled together, allowing to sit for two to ten minutes until they formed a mass, then they were placed in a water hardening trough for 15 minutes. Finally, they were transferred to an egg basket in a hatching trough and held until hatch. The troughs had an air supply and a paddle wheel which was turned on when the youngest egg mass in the trough was at least 3 hours old. Eggs were treated with formalin (100 ppm) and copper sulfate (32 ppm) to prevent fungus growth.

Egg viability

The egg masses were evaluated 48 hours after addition of sperm to determine the total number of developing eggs in the egg mass/total number of eggs in egg mass. A sample of eggs was placed in a clear petri dish and a light shown through the bottom to view the eggs and estimate the percent that was developing (Dunham *et al.* 1998).

Egg measurements

Two samples of eggs were taken from the first set of eggs during the stripping of each female. One of the samples was placed in the freezer (-80 ^{o}C) for later biochemical analysis, and the other sample was preserved using formalin (5%). The latter was used to determine the egg weight, as well as the egg diameter. Egg weight was determined from total number of eggs in known weights of samples. Egg diameter was measured for 78 individual females randomly chosen from treatments 42/3 and 32/3, between ages 3 and 5 years-old. A sub-

sample randomly chosen, consisting of 15 eggs from each of the 78 individuals was photographed. The software Image Pro-Express v. 4.5.1.3 (Media cybernetics, Bethesda, MD) was used to determine egg diameter measurements from preserved eggs.

Analytical procedures

Analysis of diets was conducted to determine crude protein, crude lipids, energy content, and moisture content. Crude protein content of the diets was analyzed by micro Kjeldahl method and the crude lipid by the ether extraction method (Soxtec Avanti 2055 Manual Extraction Unit, Foss Tecator, Höganäs, Sweden).

Characterization of egg diameter, and determination of protein, lipid, and fatty acids profile on eggs, were performed on 78 samples from treatments 42/3 and 32/3, for 3 and 5 years-old females. Protein content, crude lipid determinations, and fatty acid profiles from eggs were performed in two sub-samples of each individual. The crude lipid of the egg samples were extracted by using the method of Folch, Lee & Sloane-Stanley (1957). Approximately 400 mg of eggs were placed in 20 x 125mm borosilicate glass screw-cap tubes. Samples were homogenized in chloroform/methanol solution, followed by addition of water. Two separate phases were obtained, the upper phase was discarded, and the lower phase was placed in a new tube (previously weighted). The sample was dried by flushing with nitrogen. Lipid fraction was obtained as a percentage on a wet weight basis. Following lipid extraction, began methylation of the fatty acids. Methanolic potassium hydroxide (1 mL, 0.5 N KOH) was added to each tube, capped tightly, vortexed, and heated in a water bath at 70 ºC for 20 min. After cooling, esterification agent (1 mL 14% boron trifluoride-

methanol, Sigma-Aldrich, Dallas, Texas, USA) was added to each tube, flushed with nitrogen, capped tightly, reheated in water bath at 70 ºC for 45 min to finalize esterification then cooled. Extraction of fatty acid methyl esters (FAMEs) was facilitated by separate additions and mixing of exactly 2 mL of hexanes (Fisher Scientific) followed by 1mL saturated NaCl solution, and vortexed for 1 min. Upper (hexane) phase containing FAMEs was transferred via Pasteur pipette to 13 x 100 mm borosilicate glass. These samples were flushed with nitrogen and stored in a freezer, for chromatographic analysis. FAMEs were analyzed using a hydrogen flame ionization gas chromatograph (GC-17A Ver. 3, Columbia, MD) equipped with capillary column (Omegawax 530, 30 m x 0.53 mm x 0.5 μm film thickness, Supelco 2-4019, Sigma-Aldrich, Oslo, Norway), using helium as the carrier gas. Injector port and detector temperatures were maintained at 260 and 270 ºC, respectively. Samples containing FAMEs were injected on column in 1 μL of dichloromethane (DCM-methylene chloride HPLC - GC/MS, Fisher Scientific, Fair Lawn, NJ) using an autosampler (AOC-20i, Shimadzu, Columbia, MD). Column oven temperature was initially 140 ºC, and then was increased to 260 ºC at a rate fo 3.0 ºC/min. Total run time was 42.0 min per sample, Sample FAMEs were identified and quantified by comparing peak retention times and area counts to those of serially diluted mixtures of reference standards PUFA-3, Supelco 37 Component FAME Mix, and GLC 90 (Supelco, Bellefonte, PA). Nonadecanoic acid methyl ester (C19:0) (Sigma-Aldrich Inc, St. Louis, MO) served as the internal standard. The results of the individual fatty acids were expressed as relative percentage of total identified FAMEs and as mg per individual egg.

Free amino acids were determined as total ninhydrin positive substances (TNPS) using a colorimetric determination (Lee & Takahashi 1966). Approximately 380 mg of egg sample were macerated and placed in 5 mL of 80% ethanol at 4 ^{o}C for 48 hours to extract intracellular NPS. Then a volume of 0.1 mL was added to 1.9 ml of a ninhydrin-citrate-glycerol mixture (0.5 mL of 1% ninhydrin solution in 0.5 M citrate buffer (pH=5.5), 1.2 mL of glycerol, and 0.2 mL of 0.5 M citrate buffer (pH=5.5)). This solution was heated in a boiling water bath for 12 min, and then cooled in a tap-water bath at room temperature. The tube was shaken and read at 570 μm within 1 hr from the procedure. A reagent blank and a standard amino acid -norleucine- solution were run at the same time to verify and standardize the determination. TNPS were reported as μmol g^{-1} of eggs and μmol per 100 eggs.

Statistical analysis

Relative weight (W_r) was used as condition index to determine whether or not feeding conditions and / or the quality of the food had an effect on the organisms. The relative weight (Wr) is determined by the equation:

$$W_r = (W / W_s) \times 100$$ (Anderson & Neumann 1996),

Where W is the weight of an individual and W_s is a length-specific standard weight predicted by a weight-length regression constructed to represent the species. The form of the W_s equation for catfish is:

$$\log_{10}(W_s) = -5.800 + 3.294 (\log_{10} TL)$$ (Brown, Jaramillo, Gatlin & Murphy 1995)

where TL is total length for channel catfish that are 70 mm or longer.

Relative weight (W_r) values were determined at the beginning and at the end of the trial, as well as the difference between these two values for each strain, and classified by age class. Then, an ANOVA procedure was performed to detect differences due to protein levels, feed frequencies, or the interactions of these two.

Analysis of the spawning success was performed using exact logit analysis. The model was constructed to include the effect of treatments (protein and frequency) on spawning for each strain, using the age of fish and period of spawning as covariates (Quintero, Abebe & Davis 2007a). Number of eggs/gram of egg mass and number of eggs/gram of female body weight was evaluated using the zero-inflated negative binomial regression (ZINB). This model used protein level, feed frequency, and their interactions as the explanatory variables, and strain, age of fish, and period of spawning as covariates (Quintero, Abebe & Davis 2007b). Analysis of variance (ANOVA) was performed for egg viability following an arcsin transformation, and for egg diameter measurements to detect treatment differences due to protein levels, feed frequency and/or their interaction for each age class by strain (high and low spawning). Analysis of protein and lipid content on the egg were performed using the Beta regression model proposed by Ferrari & Cribari-Neto (2004). Fatty acid composition for treatments 42/3 and 32/3 was analyzed using t-test in terms of relative percentage (original values were transformed by taking the arcsin of their square root). If the arcsin variances were not normally distributed, the Kruskal-Wallis non-parametric test was applied to the non-transformed data. Ratios of essential fatty acids (DHA:EPA, ARA:DHA, ARA:EPA) were also analyzed using t-test. Statistical procedures from SAS® version 9.1 (SAS Institute Inc., Cary, NC) were used.

Results

Water quality parameters are given in Table 2. No significant difference among the treatments was detected for any of these parameters, except nitrite in ponds held under 42% protein level, feed offered 6 times per week, which had significantly higher amounts of nitrite. The observed levels were suitable for culture of this species. Aerators were used sporadically to maintain adequate dissolved oxygen levels. Mean dissolved oxygen values were kept over 7.0 mg/L in the morning, and over 8.0 mg/L in the afternoon.

Condition of the fish was characterized as poor, moderate and good according to relative weight. Three year-old fish, either strain 1 (high spawning) or 2 (low spawning), were stocked in good condition (range 120-130), and were harvested in the same range (110-130) (Table 3 and 4). Four year-old strain 1 fish had the poorest condition (range 65-78), while fish of the same age in strain 2 were in moderate condition (range 106-112), but at the end of the trial, all fish were in good condition (mean relative weight values were 117 and 143 for strain 1 and 2, respectively). Five year-old fish from both strains were initially in moderate condition (relative weight between 80 and 92), and similar to four year-old fish, they reached a good condition by the end of the experiment (relative weight values from 115 to 125). In general, fish under high feed frequencies (42/6 and 32/6) had better final condition (higher relative weight values) than fish under low feed frequencies (42/3 and 32/3), except for four year-old fish which did not exhibit significant differences in relative weights for any of the treatments (Tables 3 and 4).

Table 2 - Water quality parameters from female channel catfish, *Ictalurus punctatus*, held in earthen ponds for the entire acclimation and trial period (mean ± SD)

Treatment	1	2	3	4
Protein level	42%	42%	32%	32%
Feed frequency	6	3	6	3
Parameters				
D.O. (mg/L) AM	7.0 ± 0.4	7.3 ± 0.6	7.0 ± 0.6	7.1 ± 0.4
D.O. (mg/L) PM	10.5 ± 1.9	10.5 ± 0.3	10.3 ± 0.3	11.4 ± 0.6
Temp. (^{o}C) AM	20.1 ± 0.2	20.3 ± 0.2	20.2 ± 0.2	20.0 ± 0.3
Temp. (^{o}C) PM	23.2 ± 0.2	23.4 ± 0.2	23.5 ± 0.1	23.3 ± 0.1
pH	8.0 ± 0.3	8.2 ± 0.2	8.0 ± 0.3	8.1 ± 0.3
TAN (mg/L)	0.16 ± 0.13	0.07 ± 0.03	0.06 ± 0.01	0.07 ± 0.04
NO_2 (mg/L)	0.009 ± 0.002*	0.005 ± 0.0005	0.004 ± 0.0004	0.005 ± 0.003

* Significantly different p- value = 0.0056

Table 3. Initial relative weight, final relative weight and difference between relative weights of channel catfish females (*Ictalurus punctatus*) after being maintained in dietary treatments by age class, for strain 1

Treatment	Initial Wr	Final Wr	Difference
Age 3			
1 - 42 / 6	124.6 ± 12.8	126.7 ± 10.6 [a]	2.0 ± 14.1
2 - 42 / 3	121.0 ± 11.1	113.2 ± 10.4 [b]	-7.8 ± 8.8
3 - 32 / 6	124.2 ± 8.8	125.5 ± 11.8 [a]	1.3 ± 13.0
4 - 32 / 3	126.2 ± 13.0	118.8 ± 12.2 [ab]	-7.3 ± 13.2
P-value	0.5419	0.0015	0.0213
Age 4			
1 - 42 / 6	65.3 ± 11.0	117.5 ± 9.4	52.2 ± 8.3
2 - 42 / 3	75.3 ± 19.8	118.5 ± 21.7	43.2 ± 16.9
3 - 32 / 6	68.4 ± 12.4	117.5 ± 10.2	49.2 ± 10.4
4 - 32 / 3	77.7 ± 17.2	112.8 ± 11.1	35.1 ± 12.4
P-value	0.3542	0.8743	0.0687
Age 5			
1 - 42 / 6	86.9 ± 7.8	120.2 ± 12.9 [ab]	33.3 ± 15.4 [ab]
2 - 42 / 3	90.4 ± 11.9	120.4 ± 11.5 [ab]	30.0 ± 14.4 [ab]
3 - 32 / 6	85.8 ± 11.2	125.8 ± 9.9 [a]	40.0 ± 12.9 [a]
4 - 32 / 3	91.5 ± 14.4	114.9 ± 9.0 [b]	23.4 ± 12.1 [b]
P-value	0.3960	0.0458	0.0076

a, b means followed by the same letter are not different (P>0.05, Tukey-Kramer method) within each column

Table 4. Initial relative weight, final relative weight and difference between relative weights of channel catfish females (*Ictalurus punctatus*) after being maintained in dietary treatments by age class, for strain 2

Treatment	Initial Wr	Final Wr	Difference
Age 3			
1 - 42 / 6	130.1 ± 15.3	128.7 ± 10.3 [a]	-1.4 ± 13.2 [ab]
2 - 42 / 3	129.8 ± 16.5	118.8 ± 9.1 [b]	–10.9 ± 11.4 [b]
3 - 32 / 6	125.9 ± 11.8	129.3 ± 9.2 [a]	3.5 ± 13.4 [a]
4 - 32 / 3	129.5 ± 19.0	123.8 ± 10.6 [ab]	-5.6 ± 10.6 [ab]
P-value	0.8797	0.0175	0.0172
Age 4			
1 - 42 / 6	108.8 ± 38.0	140.3 ± 61.8	31.5 ± 24.7
2 - 42 / 3	111.6 ± 26.6	150.0 ± 41.3	38.3 ± 14.7
3 - 32 / 6	106.0 ± 19.2	130.8 ± 29.8	24.8 ± 11.4
4 - 32 / 3	117.0 ± 59.5	168.1 ± 67.7	51.2 ± 8.2
P-value	0.9862	0.8681	0.4766
Age 5			
1 - 42 / 6	85.9 ± 10.9	123.7 ± 12.8 [a]	37.8 ± 13.8 [ab]
2 - 42 / 3	84.4 ± 13.5	115.9 ± 10.6 [b]	31.5 ± 13.8 [b]
3 - 32 / 6	80.8 ± 11.6	122.6 ± 12.5 [a]	41.8 ± 15.6 [a]
4 - 32 / 3	83.4 ± 11.3	115.4 ± 10.8 [b]	32.1 ± 10.1 [b]
P-value	0.3154	0.0019	0.0019

a, b means followed by the same letter are not different (P>0.05, Tukey-Kramer test) within each column

moderate condition (relative weight between 80 and 92), and similar to four year-old fish, they reached a good condition by the end of the experiment (relative weight values from 115 to 125). In general, fish under high feed frequencies (42/6 and 32/6) had better final condition (higher relative weight values) than fish under low feed frequencies (42/3 and 32/3), except for four year-old fish which did not exhibit significant differences in relative weights for any of the treatments (Tables 3 and 4).

Spawning success was significantly affected by age of fish and period of spawning for both strains (Table 5). The odds of spawning for strain 1 were not affected either by dietary protein level or feed frequency, but age of fish had a highly significant effect ($p < 0.0001$). Thus, five-year-old females had 8.4 times higher odds of spawning than three year-old females and 2.9 times higher than four year-old female. Spawning success of females from strain 2, was affected significantly by the change in frequency of feeding, but not by the change in the dietary protein level. For this reason, the odds of spawning from fish fed either 42/3 or 32/3 were 1.9 times that of fish fed 42/6 or 32/6. There was also, a significant effect of age, with older fish having higher odds of spawning. Five year-old females had 4.9 times and 2.2 times higher odds of spawning than fish three and four year-old, respectively. Finally, as mentioned above, period of spawning had a significant effect on spawning success, with higher odds of spawning at the beginning of the spawning season than in mid or late season (8.6 and 16.3 times higher, respectively).

Egg production, either as number of eggs/g of egg mass or number of eggs/g of female body weight, included a relatively high number of zeroes (Fig. 1, 2). The presence of those zeros was consequence of the significant effect of fish age, period of spawning and

Table 5 - Results of Exact logistic regression analysis evaluating differences in spawning success from channel catfish females, among two dietary protein levels, and two feed frequencies, for two strains (high and low spawning) using age of fish and period of spawning as covariates

a. Logistic regression analysis - strain 1

Variable	DF	Parameter estimate	Pr > χ^2	Odds Ratio
Protein	1	0.1215	0.1215	
Feed frequency	1	0.8619	0.8619	
Protein x feed frequency	1	0.1184	0.1184	
Age	1	<0.0001	<0.0001	2.8
Period 1*	1	0.0022	0.0022	8.6
Period 2*	1	0.9533	0.9533	

b. Logistic regression analysis - strain 2

Variable	DF	Parameter estimate	Pr > χ^2	Odds Ratio
Protein	1	-0.1751	0.2707	
Feed frequency	1	0.3150	0.0427	1.9
Protein x feed frequency	1	0.1213	0.4312	
Age	1	0.7988	<0.0001	2.2
Period 1*	1	1.3963	<0.0001	16.3
Period 2*	1	-0.2734	0.7776	

* Period1 and period 2 correspond to the logistic regression coefficients where the levels of period are coded as period 1 (1, 0) - period 2 (0, 1) - period 3 (-1, 1)

Figure 1 - Distribution of number of channel catfish females, *Ictalurus punctatus*, versus number of eggs per gram of egg mass

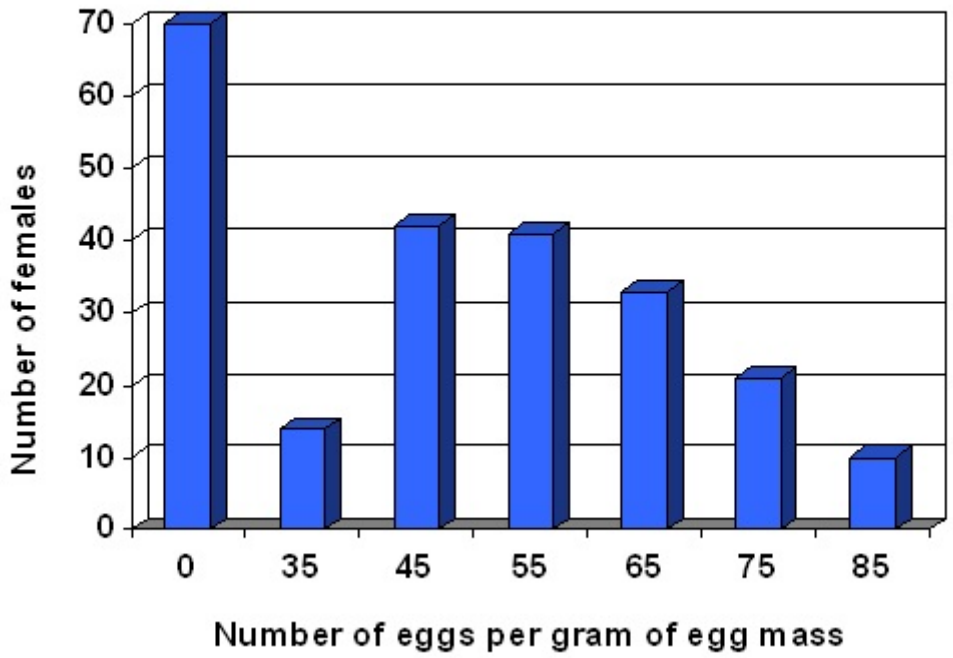
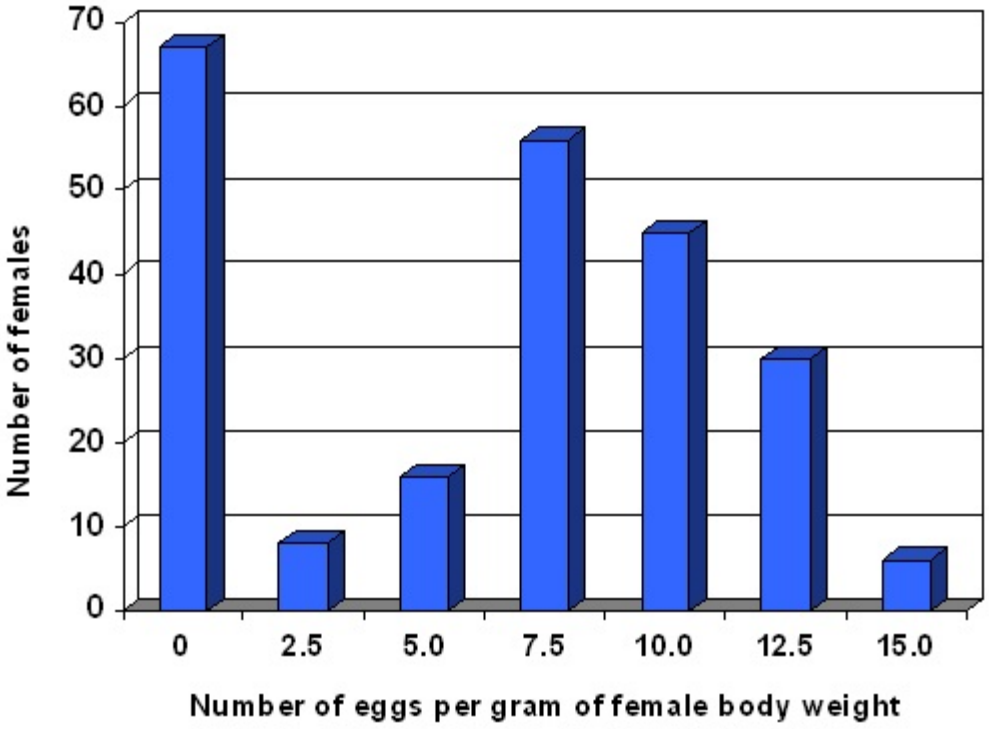


Figure 2 - Distribution of number of channel catfish females, *Ictalurus punctatus*, versus number of eggs per gram of female body weight



feed frequency on spawning success. Also, fish age was found to be the only variable that significantly affects the number of eggs/g of egg mass ($P < .0001$), while spawning period (middle period) significantly affected the number of eggs/g of female body weight (Quintero *et al.* 2007b).

The ZINB regression model predicted the mean values of the number of eggs per gram of egg mass for age 3, 4 and 5 females to be 63, 55, and 49, respectively, and 53, 52, and 51 for early, middle and late spawning periods, respectively. When analyzing number of eggs per gram of female body weight, the predicted means for females of age 3, 4, and 5, were 8.2, 8.3, and 7.5, respectively. For the same response variable the mean values were 7.8 for early season, 8.2 for middle season, and 6.9 for late season periods of spawning.

The relationship between total number of eggs versus total weight, and total length are described for each strain (Figures 3, 4). Variation in total female body weight explained 55% and 40% of the variability in the total number of eggs for strain 1 and strain 2, respectively. The percentage variability in total number of eggs that was explained by length was 48% for strain 1 and 26% for strain 2. Variation in biomass gained (lost) explained 24% and 3% of the variability in the total number of eggs for strain 1 and strain 2, respectively.

Fertilization rates from eggs produced by females of the high spawning strain (strain 1) were in the range of 42% to 90%, with an average of 67.5% (coefficient of variance CV = 24.9%). Fertilization rates from females of the low spawning strain (strain 2) were in the range of 36% to 95%, with a mean value equal to 64.4% (CV = 29.6%). There was not significant difference among fertilization rates from treatment combinations in each one of the strains (Table 6).

Figure 3a - Total number of eggs produced by channel catfish females, *Ictalurus punctatus*, versus total female body weight -(Strain 1, high spawning)

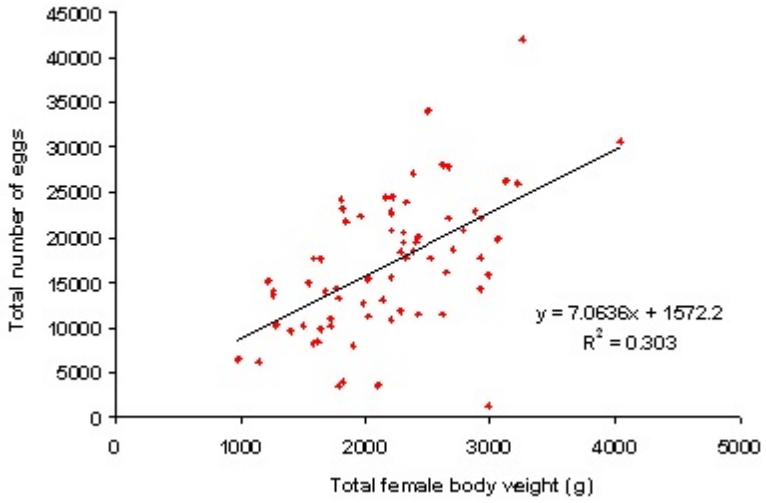


Figure 3b - Total number of eggs produced by channel catfish females, *Ictalurus punctatus*, versus total female body length -(Strain 1, high spawning)

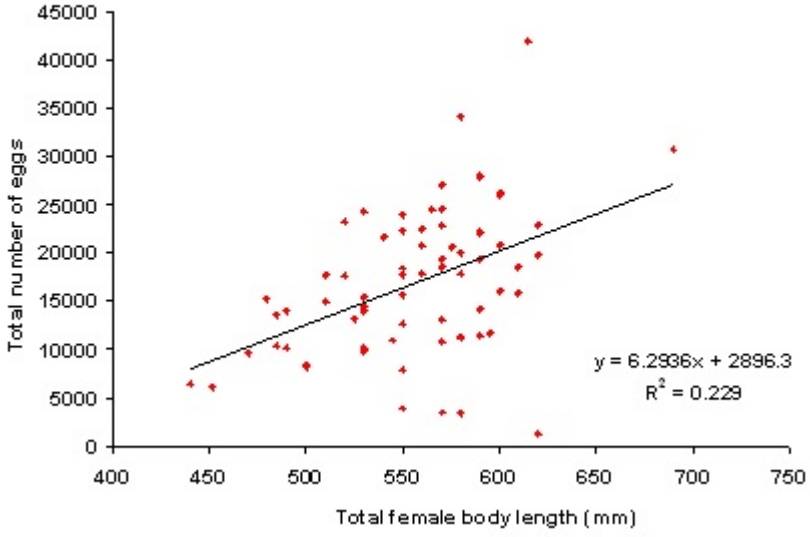


Figure 4a - Total number of eggs produced by channel catfish females, *Ictalurus punctatus*, versus total female body weight -(Strain 2, low spawning)

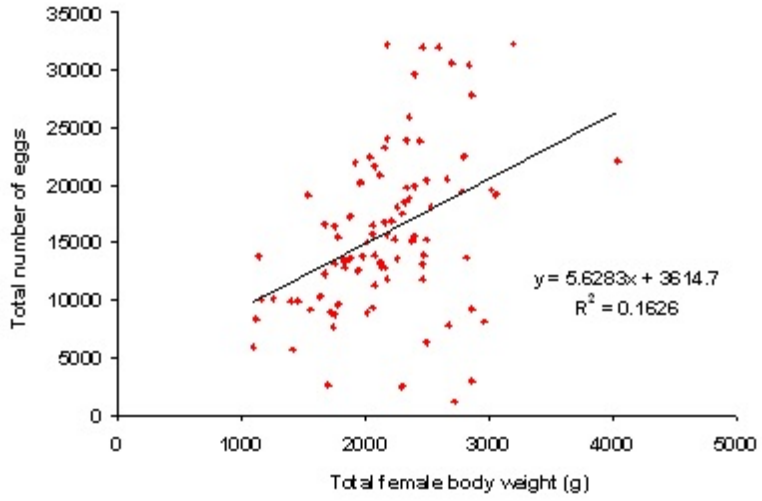


Figure 4b - Total number of eggs produced by channel catfish females, *Ictalurus punctatus*, versus total female body length -(Strain 1, high spawning)

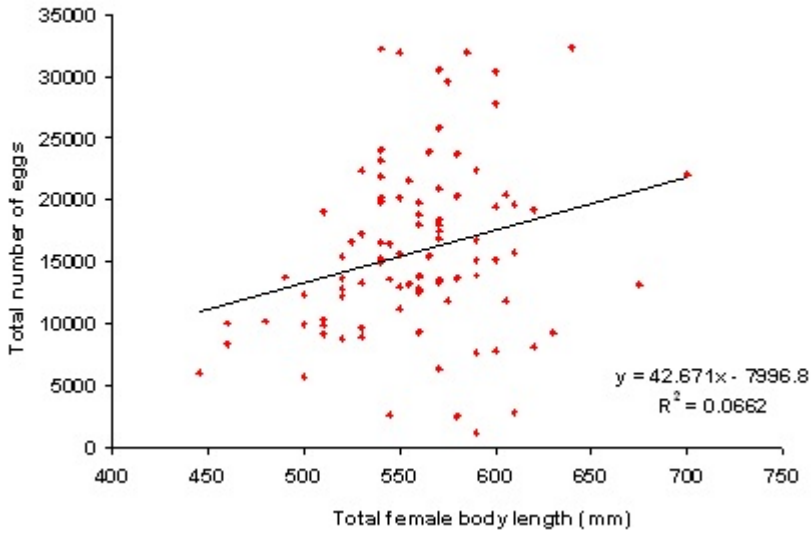


Table 6 - Percent of egg viability from eggs of channel catfish females, *Ictalurus punctatus*, after 48 hours of fertilization with sperm from blue catfish males, *Ictalurus furcatus*, related to female brood stock dietary treatments by strain (high and low spawning strain) (P-values from Tukey-Kramer test)

Treatment combination	Egg viability (%)	P-value
High spawning (strain 1)		
1 - 42 / 6	73.5 ± 2.8	0.0606
2 - 42 / 3	61.9 ± 3.7	
3 - 32 / 6	74.2 ± 3.3	
4 - 32 / 3	65.2 ± 3.4	
Low spawning (strain 2)		
1 - 42 / 6	72.9 ± 3.2	0.1480
2 - 42 / 3	61.2 ± 5.8	
3 - 32 / 6	61.0 ± 4.5	
4 - 32 / 3	66.9 ± 4.1	

Egg diameters from high spawning strain (strain 1) females averaged 3.35 mm (CV = 9.11%), and low spawning strain females had average egg diameters of 3.36 mm (CV = 7.64%). Egg diameters from three year-old females were significantly different from five year-old females (p-value<0.0001) with mean values of 3.15 and 3.45 mm, respectively. Considering egg diameters for three year-old females, there was a significant difference between strains (strain 1 - 3.11 mm, strain 2 - 3.20 mm), as well as for five year-old females (strain 1 - 3.46 mm, strain 2 - 3.42 mm), with p-values equal to 0.0001, and 0.0153, respectively. Differences among treatments for each strain were detected when data was compartmentalized as a function of female age (three and five years-old) (Table 7).

Biochemical analysis from feed and channel catfish eggs produced by dietary treatments are displayed in table 8. Moisture, protein, lipid and energy content from the feed were determined for dietary treatments. Brood stock reared on the 42% protein diet had significantly higher values for each of the variables analyzed than those reared on the 32% protein diet (Table 8). Egg composition, protein and lipids relative percentages were not significantly different among treatments, but free amino acids were significantly higher for 42% protein diet. Egg fatty acid composition is displayed in Table 8 and 9. The most abundant fatty acids were 16:0, 18:0, 18:1n9, 18:2n6, 20:3n3, and 22:6n3. Except for 14:0, 16:0, 18:2n6, and 20:1n9 there was a significant effect of protein level on the fatty acid composition. Proportions and absolute values of linolenic acid and highly unsaturated fatty acids (ARA, EPA, and DHA), as well as the n3:n6 ratio were significantly higher in eggs from fish fed 42% protein diet (Table 8, 9). The ratios DHA:EPA, ARA:EPA, and ARA:DHA were significant higher in eggs from fish fed 32% protein diet (Table 8).

Table 7 - Egg diameter (mm) measurements (mean ± SD) from channel catfish females, *Ictalurus punctatus*, related to dietary treatments, by strain (high and low spawning strain, strain 1 and 2, respectively) and age class

Treatment combination	Age 3	Age 5
High spawning (strain 1)		
1 - 42 / 6	3.21 ± 0.14 [a]	3.57 ± 0.31 [a]
2 - 42 / 3	3.15 ± 0.21 [a]	3.53 ± 0.24 [ab]
3 - 32 / 6	3.15 ± 0.09 [a]	3.44 ± 0.20 [b]
4 - 32 / 3	2.94 ± 0.21 [b]	3.28 ± 0.34 [c]
P-value	<0.0001	<0.0001
Low spawning (strain 2)		
1 - 42 / 6	3.22 ± 0.17	3.36 ± 0.26 [b]
2 - 42 / 3	3.15 ± 0.20	3.47 ± 0.19 [a]
3 - 32 / 6	3.23 ± 0.09	3.52 ± 0.29 [a]
4 - 32 / 3	3.19 ± 0.23	3.31 ± 0.22 [b]
P-value	0.3438	<0.0001

a, b, c means followed by the same letter are not different (P>0.05 Tukey-Kramer test) within each column

Table 8 - Proximate analysis from commercial channel catfish feed, and biochemical composition from eggs of channel catfish females, *Ictalurus punctatus*, including proteins, lipids, free amino acids as total ninhydrin positive substances (TNPS), essential fatty acids, and ratios between essential fatty acids from dietary treatments 42% and 32% protein level offered three times per week

Parameter	42% - 3	32% - 3	p-values
Feed			
Moisture (%)	9.41 ± 0.09	7.12 ± 0.19	0.0048
Protein (%)	40.74 ± 0.83	33.99 ± 1.03	0.0187
Lipids (%)	6.22 ± 0.05	5.48 ± 0.11	0.0137
Energy (cal)	4,437 ± 6	4,231 ± 17	0.0036
Eggs			
Protein (%)	17.80 ± 1.93	17.21 ± 1.71	0.1590*
Protein (mg/individual egg)	3.20 ± 0.98	2.95 ± 0.94	0.1531
Lipids (%)	6.82 ± 1.05	6.49 ± 1.05	0.6159*
Lipids (mg/individual egg)	1.12 ± 0.42	1.02 ± 0.39	0.1415
TNPS[a] (μmol/gr egg mass)	3.84 ± 1.89	2.43 ± 2.05	<0.0001
TNPS[a] (μmol/individual egg)	6.91 ± 3.60	3.74 ± 3.09	<0.0001
Fatty acids - absolute values (mg / 100 eggs)			
Linoleic acid (18:2n6)	9.18 ± 3.53	8.08 ± 3.01	0.0933
Linolenic acid (18:3n3)	0.53 ± 0.22	0.31 ± 0.13	<0.0001
ARA[b] (20:4n6)	0.11 ± 0.05	0.07 ± 0.03	<0.0001
EPA[c] (20:5n3)	1.60 ± 0.76	0.36 ± 0.23	<0.0001
DHA[d] (22:6n3)	9.41 ± 3.70	5.13 ± 2.27	<0.0001
Ratios			
DHA:EPA	6.46 ± 1.98	13.27 ± 3.09	0.0001
ARA:DHA	0.012 ± 0.005	0.015 ± 0.003	0.0004

ARA:EPA	0.08 ± 0.07	0.19 ± 0.07	<0.0001

*The smallest p-value ≥0.05 from Beta regression coefficients
a - TNPS: Total ninhydrin positive substances
b - ARA: Arachidonic acid
c - EPA: Eicosapentaenoic acid
d - DHA: Docosahexaenoic acid

Table 9 - Fatty acid analysis from eggs of channel catfish females, *Ictalurus punctatus*, by dietary treatments, commercial catfish diet 42% and 32% protein level offered three times per week

Fatty acid	42% / 3	32% / 3	p - value
14:0	0.99 ± 0.01	0.90 ± 0.04	0.0961
16:0	18.78 ± 0.02	18.37 ± 0.03	0.0702
16:1n7	3.08 ± 0.02[a]	2.37 ± 0.04[b]	<0.0001
18:0	12.17 ± 0.02	13.10 ± 0.02	<0.0001
18:1n9	28.67 ± 0.11	30.70 ± 0.05	0.0001
18:2n6	7.25 ± 0.07	6.97 ± 0.04	0.2471
18:3n6	ND	ND	ND
19:0	5.92 ± 0.04	5.89 ± 0.04	0.8472
18:3n3	0.41 ± 0.01	0.26 ± 0.002	<0.0001
20:1n9	1.08 ± 0.01	1.13 ± 0.01	0.1607
20:2n6	1.16 ± 0.004	1.42 ± 0.01	<0.0001
20:3n6	2.65 ± 0.02	3.25 ± 0.01	<0.0001
20:3n3	3.88 ± 0.04	5.72 ± 0.01	<0.0001
20:4n6	0.08 ± 0.002	0.06 ± 0.001	<0.0001
20:5n3	1.20 ± 0.03	0.35 ± 0.01	<0.0001
22:4n6	0.24 ± 0.003	0.37 ± 0.002	<0.0001
22:5n6	1.11 ± 0.02	2.56 ± 0.02	<0.0001
22:5n3	0.74 ± 0.004	0.55 ± 0.01	<0.0001
22:6n3	7.34 ± 0.03	4.38 ± 0.08	<0.0001
∑ n - 6	11.37 ± 0.08	13.10 ± 0.05	<0.0001
∑ n - 3	13.66 ± 0.02	11.31 ± 0.07	<0.0001
n - 3 / n - 6	1.22 ± 0.18	0.84 ± 0.12	<0.0001

Discussion

Channel catfish brood stock females were evaluated in their reproduction performance as a function of dietary treatments. However, reproduction is a very complex response. For instance, environmental cues and physiological status of the organisms must come together in order to achieve a successful mating process. Hence, this research considered the evaluation of a nutritional component, interacting with other components (genetics, physiological and environmental) that lead us to a better understanding of the factors involved in the reproductive function of channel catfish. A genetic component was defined by classification of females for their previous performance. The physiological aspect was related to fish age, whereas the environmental component was linked to the spawning period. A general overview considered factors as condition, size, and age of the fish, and their influence on spawning success and number of eggs produced per female. Then, evaluation of biochemical aspects on the eggs is related to the dietary treatment.

Condition of the fish, as Relative weight (W_r) has been considered as a robust predictor of fecundity rather than of growth (Neumann & Murphy 1992), in fact, some studies have found influence of that condition on the quantity and quality of the eggs (Chambers, Leggett & Brown 1989; Brown and Taylor 1992). Although, condition of the fish was affected by dietary treatments, the relative weight values did not exhibit a clear relationship with the egg mass, or the number of eggs per gram of female body weight obtained from spawned females.

Age of the fish was found to significantly effect the probability of spawning for both strains, with older fish having higher probability of successful spawning than younger fish.

Several authors have pointed out this effect. Santiago (1979) reported a very low spawning success in channel catfish 3-year-old females (12.7%). Dunham, Smitherman, Horn & Bice (1983a) found that four-year-old fish of the Kansas strain had a greatly improved spawning rate, suggesting they had just reached sexual maturity. Moore (1986) showed that for older fish as fish weight increased, percent success and hatch generally increased, and a similar trend was observed in younger fish but data was not conclusive. Davis, Simco & Silverstein (2005), also suggested that age rather than size is a more important component of maturation. These authors proposed to expose fish to shortened seasonal changes that mimic increased age combined with intensive feeding of fish, as a solution to overcome the age as limiting factor in brood stock performance (Davis *et al.* 2005).

The spawning period had a significant effect in the estimated odds of successful spawning in both strains, with higher probabilities of spawning during the early season. Broussard & Stickney (1981) evaluating four channel catfish strains (Minessota, Uvalde, Auburn, and Rio Grande strains) found distinctive patterns in spawning periods for each strain. Similar results were found by Dunham *et al.* (1983a) who evaluated crossbreed and pure-strain mating from Kansas, Marion and Rio Grande populations. Also, strain of the fish was found to exhibit significantly different spawning success probabilities for different levels of feed frequency but not for different levels of protein, which states the genetic component variability in the spawning response (Table 6). Regarding the effect of protein level on spawning success, Roley (1983) did not find an effect of protein levels on the spawning for rainbow trout, *Salmo gairdneri* (Richardson) which where fish fed at 27, 37, 47, and 56% protein levels.

Fecundity is a trait that is a function of the size or age of the fish. According to some authors the ability to reproduce does not depend on age but rather on the attainment of an adequate body size (Sokołowska & Skóra 2001). Thus, most of the fecundity analysis has primarily considered female body weight (Froese & Luna 2004). However, fecundity in channel catfish studies have been found to be poorly correlated with female body weight (Bice 1981; Walser & Phelps 1993; Argue 1996; Lambert 1998). For instance, an earlier study from Brauhn & McCraren (1975) found that pre-spawning gonadosomatic indices (GSI = gonad weight / body weight x 100) were not uniform among size classes. In other words, ovary weights were not increasing proportionally with increasing fish size. This can be related to the fact that growth in wild channel catfish displays a high degree of overlap in lengths between age groups, and also this range in length within the different age groups increases with an increase in age (Appelget & Smith 1951). Thus, female body weight used in the current study ranged from 0.4 kg to 3.4 kg for strain 1 and 0.6 kg to 3.1 kg for strain 2, with a combined mean weight of 1.74 kg. The observed weight discriminated by age revealed overlapping of weights. For instance, the range for three year-old females was 1.16 -3.46 kg, four year-old females was 0.74-2.8 kg, and five year-old females 0.94-4.04 kg. These factors explained the poor correlation observed between total number of eggs and total weight, biomass gain, and/or total length of the female (Table 8, Figures 3, 4).

On the other hand, the effect of age on fecundity indices of freshwater fishes remains under-explored in contrast to marine species (Shatunovskii 2006). In channel catfish aquaculture, female age has been found responsible for spawning performance rather than its size (Santiago 1979; Dunham *et al.* 1983). Braunhn & McCraren (1975), observed that

the pre-spawning GSI by age class did not show any trend, however, their sample size was very low (three individuals for classes three to six years-old). In the present study, results from the ZINB analysis found that number of eggs per gram of egg mass had a significant effect of age on this trait. Thus, older females had significantly lower number of eggs than younger females, in other words they produce bigger eggs. According to Shatunovskii (2006) this phenomenon can be attributed to an increased reproductive function in ontogeny, which is realized as a more active synthesis of ovovitellin in the liver and its storage in oocytes as well as to an elongated period of trophoplasmatic growth of oocytes. This situation is also seen in walleye, where female age accounted for a greater amount of variation in egg mass than fork length or size (Johnston 1997).

Regarding egg diameter and female size, there are reports of significant relationship among these variables (Gall 1974; Mann & Mills 1985; Wright & Shoesmith 1988; Bromage 1995). Other authors, however, did not find any increase of egg size with parental size (Kamler 1992). Abdoly, Pont & Sagnes (2005) suggested that female body size *per se* does not directly affect egg size, but rather the apparent effect of body size may result from age differences. Markmann and Doroshov (1983) monitored ovarian maturation in channel catfish finding a highly significant correlation ($r=0.903$, $p<0.001$) between mean oocyte diameter and GSI, which is indicator of ovarian maturation. Similar results were described for the maturation cycle, where GSI was found to be low after spawning, increased from September to January, no change in January-March, and a profound increase in GSI to maximum values in April (MacKenzie, Thomas & Farrar 1989). However, channel catfish female weight has been negatively correlated with average egg weight (Broussard & Stickney

1981). In the present study, there was not significant relation between egg size and either fish weight or fish length, but there was a significant difference in egg diameters due to fish age (older females had bigger eggs). Also, females given higher protein diets, and fed more frequently tended to have larger eggs.

Egg viability at 48 hours was not significantly different among treatments for each strain (p-value = 0.0606 for high spawning strain, and p-value = 0.1480 for law spawning strain) (Table 6)., and may be considered in the normal range from previous studies, where 70-80% fertilization rates has been achieved (Dunham *et al.* 1998). Biochemical and fatty acid composition of the eggs were affected by dietary treatments, however, the influence of that composition on fry production was not evaluated.

Dietary treatment: Protein level and feed frequency

Daily and seasonal rates of feeding of brood stock diets have direct effects on fecundity and egg size (Bromage, Jones, Randall, Thrush, Springate, Duston & Barker 1992). Bromage *et al.* (1992) evaluating different protocols for feeding rainbow trout found that fish fed on high rations perform better than fish fed on low rations, however feeding high rations during the latter stages of the cycle did not appear to effect the numbers of eggs produced by each individual fish but did increase the weight of the brood fish (Bromage 1995). Santiago (1979) evaluated high protein and low protein diets for channel catfish brood fish, with and without inclusion of forage fish, as well as, one vitamin-deficient diet, one treatment gives only forage fish, and the last treatment with no feeding at all. Some of his findings were that lack of vitamin supplementation to the pelleted diet effected reproductive performance, and

spawning success was effected by age of the fish. Fecundity in terms of number of eggs per g body weight were not significantly different among those treatments, except for fish that were under fed. Egg diameters were not significantly different, except for fish under fed and fed the vitamin-deficient diet. Abidin, Hashim & Chong (2006) evaluating three different protein levels (30%, 35% and 40%) with the bagrid catfish (*Mystus nemurus*) found higher growth rates and higher relative fecundity in treatments with 35% and 40% protein level, concluding that 35% protein level is adequate to support growth and egg quality parameters.

Observations of feeding activity, performed by MacKenzie *et al.* (1989) indicated that it roughly paralleled water temperature, decreasing in November to lowest levels in December through February, and increasing from March to June, reaching highest levels in July and August. Our observations are limited to late winter (February) and the spring season (March to June), and they followed this same pattern, very low response during February, and then, it was increasing during the spring. Obviously, their effects were observed as a growth response, and increase in the condition index (relative weight), which was strongly marked in 4 and 5 year-old catfish, but very poor or even negatively effected in the 3 year-old fish.

One of the reported benefits from protein contribution is an earlier maturation of gonads and eggs in larger brood stock (El-Sayed, Mansour & Ezzat 2003, Chong, Ishak, Osman & Hashim 2004), this was not the case in channel catfish, since spawning success and fecundity parameters were related to fish age rather than fish size. Evaluation of dietary effects on rainbow trout brood fish with variation in protein and carbohydrate levels did not find any effect on the histomorphology of ovarian development, neither on egg proximate

composition, but there was a significant effect on survival to the eye-up stage, hatchability and relative fecundity, being higher in those under low and intermediate protein levels than higher protein levels (Washburn, Frye, Hung, Doroshov & Conte 1990). In the performance of channel catfish, there was not significant difference in terms of fecundity, neither egg viability (48 hours), but there was an effect on the egg size.

Conclusion

The approach from our study evaluated the effect of high versus low protein (42% and 32%) diets, offered every other day (three times per week) versus every day (six times per week) during spring/early summer. Female brood stock increase their weight by the end of the trial period (except for females three years-old, fed three times per week), and that response was a significantly different according to the dietary treatment. In terms of spawning response, changing the protein level of the diet from 32 to 42% or increasing the feeding frequency from three to six times per week either did not influence spawning rate, respectively. Additionally, older fish performed better than younger and the early spawning period was better than the later spawning period, irregardless of strain. Number of eggs/g of egg mass, and number of eggs/g of female body weight were significantly affected by period of spawning and female age. Egg viability at 48 hours was not significantly affected either from protein level or feed frequency in any of the strains, however, the dietary treatment effected significantly egg size. Period of spawning and fish age, were identified as significant factors in brood stock performance, using spawning and egg production data analysis.

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CHAPTER V

EFFECT OF LIPID SUPPLEMENTATION ON REPRODUCTIVE PERFORMANCE OF CHANNEL CATFISH TO PRODUCE HYBRID CATFISH

Abstract

The influence of different lipid sources and n3:n6 ratios on reproductive performance for female channel catfish, *Ictalurus punctatus*, crossed with male blue catfish, *I. furcatus*, to produce hybrid catfish was evaluated. Four dietary treatments were evaluated using a commercial catfish feed 32% protein level, 5% lipids as a basal diet. The basal diet was top coated with 2% oil with the following ratios diet 1, soybean oil 0.95% and linseed oil 1.05%; diet 2, soybean oil 1.75% and linseed oil 0.25%; diet 3, 2.00% linseed oil, and diet 4, 1.00% menhaden fish oil, supplemented with 0.5% arachidonic acid (ARA), and 0.5% docosahexaenoic acid (DHA). Fish were fed 70 to 85 days prior to the spawning season, and then eggs were stripped and fertilized. Spawning success, number of eggs per gram of egg mass, number of eggs per gram of female body weight, fry production, and fry survival were evaluated, in conjunction with biochemical parameters. Multivariate statistics was used to evaluate the differences and similarities in the results due to the effect of linoleic acid, linolenic acid, ARA, eicosapentaenoic acid (EPA), and DHA in the egg samples. Fatty acid composition of the eggs reflects the effect of dietary treatment, and thus, evidenced nutrient

deposition during spring season. Principal component analysis (PCA) described 83% of the variance within the data using two principal components axes, and demonstrated differences in fatty acid composition due to dietary treatments. Vegetables oils could have a negative impact on egg quality due to the presence of significantly higher proportions of C20:3n6, which effects immune cell functions. Supplementation of ARA, EPA and DHA in commercial catfish feed in the form of menhaden fish oil with purified liquid algae extracts of ARA and DHA had a beneficial effect on reproductive performance parameters. Hence, commercial producers may consider their use in catfish brood stock diets to improve their fry production.

Introduction

The interspecific hybrid between channel catfish, *Ictalurus punctatus* (Rafinesque) females and blue catfish, *I. furcatus* (Lesueur) males has been described as the most suitable for culture conditions compared to channel catfish due to better growth, increased resistance to low oxygen levels and diseases, ease of harvest, and higher carcass yield (Giudice 1966; Yant, Smitherman & Green 1976; Brooks 1977; Chappell 1979; Tave, McGinty, Chappell & Smitherman 1981; Dunham, Smitherman & Webber, 1983; Smitherman, Dunham & Tave 1983; Stephens & Dorman 1984; Ella 1984; Dunham, Brummett, Ella & Smitherman 1990). Inconsistent spawning and fry rearing are currently bottlenecks to the continued commercialization of hatchery technologies (Tave & Smitherman 1982; Dunham & Smitherman 1987). Hence, improvement and/or development of new techniques dealing with broodstock management, spawning, gamete quality, fertilization, and hatchery protocols are required to provide strategies that will advance this technology. Genetic, physiological, environmental, and nutritional background from broodstock can influence gamete production in terms of quality and quantity (Brooks, Tyler & Sumpter 1997; Schreck, Contreras-Sanchez & Fitzpatrick 2001). In particular, the nutritional status of the broodstock is critical due to the fact that all the contents of an egg, that will determine its quality, must be incorporated into an egg when it is an oocyte within the ovary in a process called vitellogenesis (Sargent 1995; Wiegand 1996; Brooks *et al.* 1997). Lipids can be incorporated into egg yolk from dietary sources, or from reserves that are stored prior to vitellogenesis and subsequently mobilized, or possibly synthesized *de novo* (Wiegand 1996). A considerable amount of research has addressed the effect of dietary essential fatty acid content on reproductive

performance, with varying results. In some cases, as in gilthead seabream (*Sparus aurata* L.), fecundity increased significantly with an increase in dietary n–3 highly unsaturated fatty acids (HUFAs), but an excess also caused detrimental effects (Fernández-Palacios, Izquierdo, Robaina, Valencia, Salhi & Vergara 1995; Izquierdo, Fernández-Palacios & Tacon 2001). Also, there are studies where no correlation between egg quality and levels of fatty acids could be demonstrated, such as in goldfish (Wiegand , Kitchen & Hataley 1991), and Atlantic halibut (Bruce, Shields, Bell & Bromage 1993; Wiegand 1996). Regarding the channel catfish, there are a few studies that evaluate the effect of dietary lipids on fatty acid composition of fry (Yingst & Stickney 1979), and also in young fish (Gatlin & Stickney 1982; Hedrick, Popma & Davis 2005). However, there is limited information on the effects on broodstock performance and egg quality.

The aim of the present study was to evaluate the effect of polyunsaturated fatty acids PUFAs (linolenic acid, 18:2n6 and linolenic acid, 18:3n3) in different ratios, as well as the effect of highly unsaturated fatty acids HUFAs (arachidonic acid, 20:4n6 (ARA); eicosapentaenoic acid, 20:5n3 (EPA); and docosahexaenoic acid, 22:6n3 (DHA)) on channel catfish, *Ictalurus punctatus* brood stock females to produce hybrid catfish fry.

Methods

Dietary treatments

Four experimental diets were formulated using a commercial formulation feed 32% protein level, 5% lipids (manufactured by ARKAT Feeds Inc, Dumas, AK). The feed was then top-coated with 2% lipids, from different sources. Diet 1 to 3 used soybean and linseed

oil as primary sources. Diet 1 was top coated with 0.95% of soybean oil and 1.05% of linseed oil (SBO-LSO); diet 2 was top coated with 1.75% of soybean oil and 0.25% linseed oil (SBO); diet 3 was top coated with 2% linseed oil (LSO). Diet 4 was top coated with 1% of menhaden fish oil, 0.5% of docosahexaenoic acid (DHA) rich oil, and 0.5% of arachidonic acid (ARA) rich oil, these latter as a liquid algae extracts with about 40% purity of the DHA or ARA (manufactured by Advanced Bionutrition ABN, Columbia, MD) (MFO)(Table 1). Lipid supplementation in those proportions targeted the following n3:n6 ratios 1:1, 1:4, 4:1, and 3:2 for diets 1 to 4, respectively. Lipid supplementation was added by spraying the feed as it was tumbled in a rotary mixer. The feed was stored in 4-pound plastic bags in a freezer at -20 ^{o}C.

Experimental fish

A total of 187 female channel catfish, Kansas strain, 4 year-old were maintained at the E. W. Shell - Auburn Experimental Station, Auburn University. The fish were randomly stocked in January 2005, in eight ponds (0.04 ha each one) at a density of ~600 kg/ha, using two ponds per treatment. Females were weighed and measured at the beginning and at the end of the experiment. The acclimation period was approximately two months, and the trial period was 70 days to 85 days depending on the spawning period. Feed was offered during the warmest part of the day between 1500 and 1700 hours, at an estimated rate of 2.75% of total biomass of brood fish stocked per pond. Water quality parameters were taken twice daily for dissolved oxygen and temperature; twice weekly for pH, ammonia-N, and nitrite-N.

Table 1. Dietary treatments based on lipid supplementation (2% top coated oil) from channel catfish commercial diet (32% protein level, 5% lipid), and final observed n3:n6 ratios in supplemented oil and experimental feed

Diet	Soybean oil (high n6)	Linseed oil (high n3)	MFO[a] +ARA[b] +DHA[c]	n3:n6 (Oil)	n3:n6 (Feed)
1 - SBO[d]-LSO[e]	0.95%	1.05%	---	0.98	0.42
2 - SBO[d]	1.75%	0.25%	---	0.32	0.22
3 - LSO[e]	---	2.00%	---	2.37	0.68
4 - MFO[a]	---	---	2.00%	1.62	0.40

a. MFO: Menhaden Fish Oil
b. ARA: Arachidonic Acid (algal extract - 40% ARA)
c. DHA: Docosahexaenoic acid (algal extract - 40% DHA)
d. SBO: Soybean oil
e. LSO: Linseed oil

Two spawning periods were evaluated using 95 and 92 fish, respectively in each period. Upon initiation of the spawning cycle, one pond from each treatment (randomly selected) was drained, and all the females were harvested and selected based on external characteristics (abdominal fullness, softness and palpability of the ovaries, redness or swollen appearance of the genitals). At this time the total length, body weight, and girth were recorded and the selected fish placed in labeled soft mesh bags. Selected females were transferred to holding tanks (3.0 x 0.47 x 0.61 m with a water volume from 670 – 837 liters) supplied with continuous flow-through water. The luteinizing hormone-releasing hormone analogues (LHRHa) GMP grade from American Peptide (Vista, CA) was utilized to stimulate egg release. Interperitoneal hormone injections were administered in two doses, a priming injection of 30 μg/kg LHRHa, followed 12 hours later by resolving dose of 150 μg/kg. Data pertaining to the number of fish stocked and those selected for strip spawning are presented in Table 4.

Collection and fertilization of gametes

Twenty four hours after the second injection, females were monitored for ovulation. Females with released eggs were removed from the holding tank and anesthetized in 250 mg/l tricaine methane sulfonate (MS-222) (Argent Chemical Laboratories, Redmond, WA) buffered with sodium bicarbonate. Females were then stripped and eggs were collected in metal pie pans lubricated previously with vegetable shortening. Those females that did not express eggs were rechecked later. Stripping of gametes ceased when all females had been stripped or attempts to strip them had been made. Collected eggs were weighed and total

number of eggs were estimated per spawn, as well as per female body weight. After weighing, eggs and sperm were gently swirled together and allowed to sit for two to ten minutes until they formed a mass, which was transferred to a water hardening trough for 15 minutes. Finally, they were transferred to an egg basket in a hatching trough. The troughs had an air supply and a paddle wheel which was turned on when the youngest egg mass in the trough was at least 3 hours old. Eggs were treated with formalin (100 ppm) and copper sulfate (32 ppm) to prevent fungus growth.

The egg masses were evaluated 24 hours before hatching to determine the proportion of eggs that were alive. Each mass was weighed and inspected visually to estimate the number of eggs that were developing. This allowed an estimation of number of fry per egg mass, which later was related to the total number of fry obtained in each trough to determine individual female performance.

Egg measurements

Two samples of eggs were taken from the first set of eggs during the stripping of each female. One of the samples was placed in the freezer (-70 ºC) for later biochemical analysis, and the other sample was preserved using a 5% solution of formalin. The latter was used to determine the egg weight, as well as the egg diameter. Egg weight was determined from total number of eggs in the sample related to the total weight of the sample. A sub-sample randomly chosen, consisting of 15 eggs was measured. The software Image Pro-Express v. 4.5.1.3 (Media cybernetics, Bethesda, MD) was used to determine egg diameter measurements.

Analytical procedures

Analysis of diets was conducted to determine crude protein, crude lipids, energy content, and humidity. Crude protein content of the diets was analyzed using the micro Kjeldahl method and the crude lipid by the ether extraction method (Soxtec Avanti 2055 Manual Extraction Unit, Foss Tecator, Höganäs, Sweden). Energy content was evaluated using a 1425 micro bomb calorimeter (Parr Instrument Company, Moline, IL, USA), a 15 mg sample was flame-combust and the heat released was measured. Moisture content was determined by drying a 1 g sample feed in 100 ºC oven to a constant weight.

Characterization of egg diameter, and determination of protein, lipid, free amino acids, and fatty acids profile on eggs, were performed on samples from females spawned on the first spawning period. A total of 43 individual samples were performed by triplicate for the parameters mentioned previously, except for free amino acids which was analyzed by duplicate. The crude lipid of the egg samples were extracted by using the method of Folch, Lee & Sloane-Stanley (1957). Approximately 400 mg of eggs were placed in 20 x 125mm borosilicate glass screw-cap tubes. Samples were homogenized in chloroform/methanol solution, followed by addition of water. Two separate phases were obtained, the upper phase was discarded, and the lower phase was placed in a new tube (previously weighted). The sample was dried by flushing with nitrogen. Lipid fraction was obtained as a percentage on a wet weight basis. Following lipid extraction, began methylation of the fatty acids. Methanolic potassium hydroxide (1 mL, 0.5 N KOH) was added to each tube, capped tightly, vortexed, and heated in a water bath at 70 ºC for 20 min. After cooling, esterification agent (1 mL 14% boron trifluoride-methanol, Sigma-Aldrich, Dallas, Texas, USA) was added to

each tube, flushed with nitrogen, capped tightly, reheated in water bath at 70 ºC for 45 min to finalize esterification then cooled. Extraction of fatty acid methyl esters (FAMEs) was facilitated by separate additions and mixing of exactly 2 mL of hexanes (Fisher Scientific) followed by 1mL saturated NaCl solution, and vortexed for 1 min. Upper (hexane) phase containing FAMEs was transferred via Pasteur pipette to 13 x 100 mm borosilicate glass. These samples were flushed with nitrogen and stored in the freezer, for chromatographic analysis. FAMEs were analyzed using a hydrogen flame ionization gas chromatograph (GC-17A Ver. 3, Columbia, MD) equipped with capillary column (Omegawax 530, 30 m x 0.53 mm x 0.5 μm film thickness, Supelco 2-4019, Sigma-Aldrich, Oslo, Norway), using helium as the carrier gas. Injector port and detector temperatures were maintained at 260 and 270 ºC, respectively. Samples containing FAMEs were injected on column in 1 μL of dichloromethane (DCM- methylene chloride HPLC - GC/MS, Fisher Scientific, Fair Lawn, NJ) using an autosampler (AOC-20i, Shimadzu, Columbia, MD). Column oven temperature was initially 140 ºC, and then was increased to 260 ºC at a rate fo 3.0 ºC/min. Total run time was 42.0 min per sample. Sample FAMEs were identified and quantified by comparing peak retention times and area counts to those of serially diluted mixtures of reference standards PUFA-3, Supelco 37 Component FAME Mix, and GLC 90 (Supelco, Bellefonte, PA). Nonadecanoic acid methyl ester (C19:0) (Sigma-Aldrich Inc, St. Louis, MO) served as the internal standard. The results of the individual fatty acids were expressed as relative percentage of total identified FAMEs and as mg per 100 eggs.

Free amino acids were determined as total ninhydrin positive substances (TNPS) using a colorimetric determination (Lee & Takahashi 1966). Approximately 380 mg of eggs

sample were macerated and placed in 5 mL of 80% ethanol at 4 °C for 48 hours to extract intracellular NPS. Then a volume of 0.1 mL was added to 1.9 ml of a ninhydrin-citrate-glycerol mixture (0.5 mL of 1% ninhydrin solution in 0.5 M citrate buffer (pH=5.5), 1.2 mL of glycerol, and 0.2 mL of 0.5 M citrate buffer (pH=5.5). This solution was heated in a boiling water bath for 12 min, and then cooled in a tap-water bath at room temperature. The tube was shaken and read at 570 μm within 1 hr from the procedure. A reagent blank and a standard amino acid -norleucine- solution were run at the same time to verify and standardize the determination. Free amino acids were reported as μmol g^{-1} of eggs and μmol per 100 eggs.

Statistical analysis

The experiment was conducted using a randomized complete block design. The ponds were grouped into two blocks according to period of spawning. Relative weight (W_r) (Anderson & Neumann 1996) was used as a condition index to determine whether or not feeding had an effect on the organisms during the spring season. Analysis of spawning success was performed using logit models, considering spawning as binary response. The model included the effect of treatments (lipid supplementation) on spawning using period of spawning as a covariate. Number of eggs per gram of egg mass, and number of eggs per gram of female body weight were determined using the zero-inflated negative binomial regression (ZINB). This model used treatment (lipid supplementation) and period of spawning as the explanatory variables. Analysis of variance (ANOVA) was performed for egg diameter measurements to detect treatment differences due to lipid treatments.

Percent protein and lipid egg content were analyzed using beta regression modelling (Ferrari & Cribari-Neto 2004). Fatty acid composition was analyzed using the ANOVA procedure following an arcsin transformation. Tukey's test was applied for multiple comparison of mean values. If the responses were not normally distributed, the Kruskal-Wallis non-parametric test was applied to the non-transformed data. Regression analyses were performed on ratios of essential fatty acids (DHA:EPA, ARA:DHA, ARA:EPA) and fry hatch. We also studied the correlation between arachidonic acid and linolenic acid. Multivariate statistics was used to evaluate the differences and similarities in the results. The relative amounts of linoleic acid, linolenic acid, ARA, EPA, and DHA in the samples were transformed using arcsin square root of the percentage values, and then they were subjected to principal component analysis. Two new coordinates, the principal components (PCs) were generated in the direction of the largest and second largest variation of the samples. The relation among the samples was then displayed by projecting them on the plane. This allowed a display of the major trends within the data set without significant loss of total original variation. The GLM, Logistic, Genmod, NLMixed, and Princomp Procedures from SAS® version 9.1 (SAS Institute Inc., Cary, NC) were used.

Results

The observed water quality parameters were suitable for culture of this species (Table 2). There was no significant difference among the treatments for any of these parameters, except nitrite in ponds held under diet 3 (LSO) which had significantly higher level of nitrite.

Table 2 - Water quality parameters from female channel catfish, *Ictalurus punctatus*, held in earthen ponds for the entire acclimation and trial period (mean ± SD)

Diet	1	2	3	4
	SBO-LSO	SBO	LSO	MFO
Parameters				
D.O. (mg/L) AM	8.6 ± 1.6	8.3 ± 1.5	8.3 ± 1.8	8.1 ± 1.6
D.O. (mg/L) PM	10.9 ± 1.2	10.9 ± 1.2	11.0 ± 1.9	10.7 ± 1.1
Temp. (^{o}C) AM	16.0 ± 5.3	15.8 ± 5.2	15.9 ± 5.2	15.9 ± 5.3
Temp. (^{o}C) PM	19.5 ± 6.4	19.5 ± 6.4	19.5 ± 6.3	19.4 ± 6.3
pH	8.1 ± 0.5	7.9 ± 0.3	7.9 ± 0.6	7.9 ± 0.3
TAN (mg/L)	0.19 ± 0.28	0.15 ± 0.21	0.50 ± 0.46	0.11 ± 0.12
NO_2 (mg/L)	0.03 ± 0.025	0.02 ± 0.014	0.04 ± 0.0018*	0.02 ± 0.013

* Significantly different p- value = 0.0001

The test diets were accepted and consumed by females brood stock, which was reflected in the condition index. For instance, final condition index indicated that fish improved their condition during the spring season, with final values over 120 (Table 4), which represented an increase over 60% from their initial condition. However, final relative weights were not significantly affected by dietary treatments.

The parameters evaluated to determine reproductive performance were restricted to the first spawning period due to fertilization problems across all the treatments during the second period, probably linked to sperm quality. Additionally fish evaluated in treatment four (MFO) during the second spawning period were stressed at their harvest causing around 38% mortality (due to a broken water line). Thus, reproductive performance in brood stock females from the first spawning period in terms of spawning success, number of eggs per gram of egg mass, number of eggs per female body weight, egg mass per female body weight, fry produced per body weight and hatch were not affected significantly by dietary treatments. Logistic regression analysis for spawning success evidenced that this variable was not significantly affected either by dietary treatments or period of spawning (Table 3). Number of females spawned per period of spawning is given in Table 4. Number of eggs per gram of egg mass was not significantly affected by dietary treatments, although there was a trend for bigger eggs in fish fed diets 4 (MFO) with a mean value of 53, followed by fish from diet 1 (SBO-LSO) with 55, then fish from diet 3 (LSO) with 57, and smaller eggs in fish fed diet 2 (SBO) with 63 eggs per gram of egg mass (Table 4). Number of eggs per body weight (kg) were higher in fish fed diet 2 (SBO) with a mean value of 9,410 eggs/kg, followed by diet 4 (MFO) with 8,805 eggs/kg, then diet 1 (SBO-LSO) with 8,592 eggs/kg,

Table 3 - Results of logistic regression analysis evaluating differences in spawning success from channel catfish females, *Ictalurus punctatus*, Kansas strain, 4 year-old after being maintained in dietary treatments using period of spawning as covariate

Variable	DF	Parameter estimate	Pr >χ^2
Diet 1 - SBO[a] - LSO[b]	1	-0.0120	0.9635
Diet 2 - SBO[a]	1	0.0615	0.8270
Diet 3 - LSO[b]	1	0.0250	0.9244
Period 1	1	-0.1482	0.3446

* Diet 1, Diet 2, and Diet 3 correspond to the logistic regression coefficients where the levels of diet are coded as diet 1 (1, 0, 0) - diet 2 (0, 1, 0) - diet 3 (0, 0, 1) - diet 4 (-1, -1, -1); and Period 1 is the logistic regression coefficient where period is coded as period 1 (1), period 2 (-1)

a. SBO: Soybean oil
b. LSO: Linseed oil

Table 4 - Relative weight condition (Wr) before and after dietary treatments; number of channel catfish females, *Ictalurus punctatus*, Kansas strain, 4 year-old, spawned from total number of fish per dietary treatment (in parentheses); number of eggs and egg diameter from channel catfish females per dietary treatment; fry per kilogram of female, and hatching percentage of eggs after being fertilized with sperm from blue catfish males, *Ictalurus furcatus*, related to female brood stock dietary treatments (P-values from Tukey-Kramer test)

Diet	1- SBO-LSO	2- SBO	3- LSO	4- MFO	p-values
Relative weight (Wr)					
Initial Wr	76.8 ± 8.5 [b]	83.3 ± 7.8 [a]	74.9 ± 11.7 [b]	77.6 ± 10.9 [b]	0.0008
Final Wr	125.7 ± 13.2	126.3 ± 11.9	121.8 ± 13.6	125.5 ± 12.4	0.3572
Difference	48.8 ± 15.4	42.5 ± 14.1	46.9 ± 19.8	47.6 ± 16.2	0.3766
Fish spawned					
1st period	12 (24)	10 (24)	8 (23)	13 (24)	
2nd period	12 (23)	12 (21)	16 (24)	6(15)*	
Number of eggs - 1st period of spawning					
Eggs / g [1]	55 ± 6	63 ± 10	57 ± 13	53 ± 9	0.1405
Eggs / kg [2]	8592 ± 1381	9410 ± 1514	7748 ± 2025	8805 ± 1352	0.1687
Egg mass / kg [3]	158.3 ± 34.7	154.7 ± 34.8	136.0 ± 26.9	170.9 ± 38.1	0.1817
Number of eggs - 2nd period of spawning					
Eggs / g [1]	45 ± 6	56 ± 13	51 ± 13	53 ± 9	0.1279
Eggs / kg [2]	8497 ± 1717	8356 ± 2343	8813 ± 2519	8378 ± 1267	0.9486
Egg mass / kg [3]	189.4 ± 34.5	150.0 ± 27.6	180.8 ± 56.3	175.5 ± 32.6	0.2161
Eggs - first period of spawning					
Diameter (mm)	3.69 ± 0.18[a]	3.45 ± 0.30[b]	3.66 ± 0.26 [a]	3.71 ± 0.23[a]	<0.0001
Fry - first period of spawning					
Fry/kg female	630 ± 341	796 ± 397	293 ± 412	1602 ± 341	0.0800
Hatch (%)	7.16 ± 5.34	8.05 ± 5.02	3.78 ± 4.52	20.85 ± 8.23	0.0686

* Females were reduced from 24 to 15 due to mortality at harvest
a, b means followed by the same letter are not different (P>0.005, Tukey-Krammer test)

1. Eggs / g: number of eggs per gram of egg mass
2. Eggs / kg: number of eggs per kilogram of spawning female body weight
3. Egg mass / kg: total weight of egg mass (g) per kilogram of female body weight

and the lowest number for fish fed diet 3, with a mean value of 7,748 eggs/kg. Egg masses per female body weight were not significantly affected by lipid supplementation, with bigger masses produced by females fed under diet 4 (MFO) with a mean value of 170.9 g/kg, and the smallest masses in diet 3 (LSO) with 136.0 g/kg. Linear function of the total weight of females, and total length explained 86.7% and 81.1%, respectively of the total variability in the number of egg (Fig 1 a, b). Egg diameters from females fed diet 2 (SBO) were significantly smaller than those from females fed diets 1, 3, and 4 (Table 4).

Although, fry produced per female body weight was not significantly affected by treatments ($p=0.08$), there was a big difference in number of fry obtained for fish fed diet 4 (MFO). Brood females given diet 4 (MFO) produced five times more fry than females given diet 3 (LSO), and over two times those produced given diet 1 (SBO-LSO) and diet 2 (SBO). Fry hatch displayed a similar pattern to that of fry per female body weight, with only a weak relationship to dietary treatment (p-value= 0.0686)(Table 4).

Biochemical composition of the test diets did not differ significantly for moisture, protein, lipids, and energy (Table 5). Protein content of the eggs was affected significantly by the diets. Eggs from fish fed diet 4 (MFO) had significantly lower level of proteins than eggs from other treatments. In terms of the amount of protein per unit egg, eggs from fish fed diet 2 (SBO) had significantly lower amounts of protein than eggs from other treatments (2.73 ± 0.39 mg/egg). This is related to the fact that those eggs were significantly smaller (Table 4 and 5). There was not significant effect from dietary treatments on lipid content, but in terms of amount of lipids per unit egg, there was an effect related to the size of the egg, with eggs from diet 2 (SBO) displaying the lowest amount of lipids (Table 5). The content

Figure 1a - Total number of eggs produced by channel catfish females, *Ictalurus punctatus*, versus total female body weight

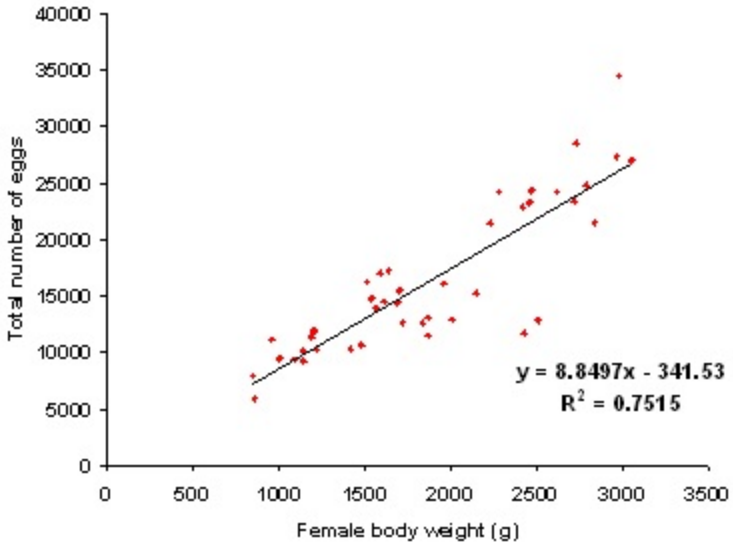


Figure 1b - Total number of eggs produced by channel catfish females, *Ictalurus punctatus*, versus total female body length

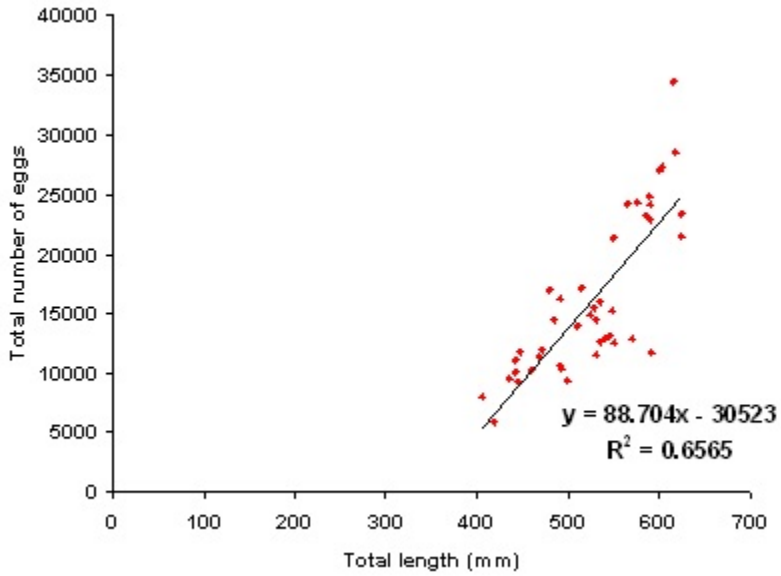


Table 5 - Proximate analysis from commercial channel catfish feed, and biochemical composition from eggs of channel catfish females, *Ictalurus punctatus*, including proteins, lipids, free amino acids as total ninhydrin positive substances (TNPS), and ratios between essential fatty acids from dietary treatments

Diet	1- SBO[a]-LSO[b]	2- SBO[a]	3- LSO[b]	4- MFO[c]	p-values
Feed					
Moisture (%)	7.36 ± 0.25	7.15 ± 0.20	7.55 ± 0.10	7.18 ± 0.12	0.5512
Protein (%)	33.43 ± 0.40	33.58 ± 0.17	33.53 ± 0.31	33.39 ± 0.36	0.9726
Lipids (%)	7.12 ± 0.04	6.94 ± 0.20	7.12 ± 0.48	7.21 ± 0.26	0.7402
Energy (cal)	4,128 ± 15	4,207 ± 47	4,230 ± 66	4,220 ± 72	0.5312
Eggs					
Proteins					
Percentage	16.81 ± 0.18[a]	16.65 ± 0.12[a]	16.74 ± 0.24[a]	16.33 ± 0.13[b]	0.0360*
mg / egg	3.07 ± 0.30[a]	2.73 ± 0.39[b]	3.02 ± 0.46[a]	3.16 ± 0.42[a]	0.0001
Lipids					
Percentage	7.27 ± 0.13	7.19 ± 0.09	7.40 ± 0.17	7.54 ± 0.09	0.2681**
mg / egg	1.12 ± 0.19[ab]	0.97 ± 0.12[b]	1.14 ± 0.28[ab]	1.27 ± 0.40[a]	0.0003
Free AminoAcids as total ninhydrin psoitve substances (TNPS)					
µmol/gr	3.29 ± 2.26[c]	5.85 ± 1.83[b]	3.72 ± 2.02[c]	7.37 ± 1.63[a]	<0.0001
µmol/eggs	5.92 ± 4.15[c]	9.62 ± 3.45[b]	7.06 ± 4.74[bc]	14.48 ± 4.45[a]	<0.0001
Ratios					
DHA[d]: EPA [e]	7.54 ± 2.09[b]	10.93 ± 3.62[a]	8.14 ± 1.96[b]	8.60 ± 1.97[b]	<0.0001
ARA[f]: DHA[d]	0.021±0.004[b]	0.016±0.003[c]	0.026±0.005[a]	0.013±0.002[d]	<0.0001
ARA[f]: EPA[e]	0.149±0.02[c]	0.173±0.054[b]	0.204±0.034[a]	0.108±0.028[d]	<0.0001

1. Total amount of free aminoacids (µmol) per 100 eggs
* The largest p-value <0.05 from Beta regression coefficients
** The smallest p-value ≥0.05 from Beta regression coefficients
a. SBO: Soybean oil
b. LSO: Linseed oil

c. MFO: Menhaden oil, supplemented with arachidonic acid (ARA~40%) and docosahexaenoic acid (DHA~40%)
d. DHA: docosahexaenoic acid
e. EPA: eicosapentaenoic acid
f. ARA: arachidonic acid

of free amino acids differ significantly among treatments, with the highest amount either as μmol per gram, or μmol per 100 eggs in eggs from diet 4 (MFO), followed by diet 2 (SBO), and lowest values for diets 1 (SBO-LSO) and 3 (LSO) (Table 5).

Fatty acid composition from experimental diets exhibited marked differences reflecting the composition of oils used to top coat the feed (Table 6, Figures 2a to 2e). In general terms C16:0, C18:1n9, C18:2n6 and C18:3n3 accounted for around 88% of total fatty acids in diets 1 to 3, while these same fatty acids represented only 70% from diet 4 (MFO). Similarly, C20:4n6 (ARA), C20:5n3 (EPA), and C22:6n3 (DHA) represented less than 1% in diets to 3, but almost 11% in diet 4 (MFO). Regarding linoleic (18:2n6) and linolenic (18:3n3) acids, diet 1 (SBO-LSO) had intermediate values of both fatty acids, diet 2 (SBO) had higher linoleic acid, and lower linolenic, while diet 3 (LSO) had higher linolenic and lower linoleic acid (Table 6). Differences in fatty acid compositions were reflected in the n-3 to n-6 ratio. Diet 2 had a significantly lower ratio (0.22) than all the other diets, diet 1 and 4 were intermediate (0.42 and 0.40, respectively), and diet 3 had the highest ratio (0.68). Although, diet 1 and 4 had similar ratios, the source of n-3 was prominently linolenic acid in diet 1 (SBO-LSO), while EPA and DHA were for diet 4 (MFO) (Table 6).

Relative proportions of fatty acid composition from egg samples for each treatment are displayed in Table 7, Figures 2a to 2e. The most abundant fatty acids were 16:0, 18:0, 18:1n9, 18:2n6, 20:3n3, and 22:6n3. Comparison of linoleic, linolenic, ARA, EPA, and DHA acids as relative proportions in feed and eggs, as well as calculated values in mg per 100 eggs are displayed in Table 8. Linoleic acid (18:2n6) was significantly higher in eggs from females given diets 1 (SBO-LSO), and 3 (LSO) (Figure 2 a). Linolenic acid (18:3n3)

Table 6 - Fatty acid analysis from dietary treatments (commercial catfish diets 32% protein, 5% lipids, top-coated with 2% oil)

Fatty acid	Diet 1 - SBO[1] - LSO[2]	Diet 2 - SBO[1]	Diet 3 - LSO[2]	Diet 4 -MFO[3]
14:0	0.83 ± 0.03	0.89 ± 0.07	0.78 ± 0.03	2.99 ± 0.13
16:0	16.19 ± 0.32	16.69 ± 0.26	14.27 ± 0.14	16.73 ± 0.21
16:1n7	1.75 ± 0.04	1.78 ± 0.08	1.71 ± 0.07	3.37 ± 0.14
18:0	4.65 ± 0.11	4.64 ± 0.01	3.91 ± 0.07	4.82 ± 0.10
18:1n9	30.15 ± 0.42	31.67 ± 0.23	28.94 ± 0.06	26.90 ± 0.27
18:2n6	30.31 ± 0.64	32.41 ± 0.57	27.77 ± 0.36	24.01 ± 0.68
18:3n6	ND*	ND*	ND*	0.42 ± 0.03
19:0	1.33 ± 0.19	2.45 ± 0.11	1.45 ± 0.16	2.25 ± 0.07
18:3n3	11.76 ± 0.46	6.11 ± 0.34	18.28 ± 0.21	2.83 ± 0.12
20:1n9	0.78 ± 0.04	0.89 ± 0.05	0.69 ± 0.15	1.03 ± 0.03
20:2n6	0.30 ± 0.01	0.33 ± 0.03	0.32 ± 0.04	0.35 ± 0.02
20:3n6	0.18 ± 0.02	0.20 ± 0.01	0.21 ± 0.01	0.50 ± 0.03
20:3n3	0.21 ± 0.004	0.23 ± 0.002	0.21 ± 0.01	ND*
20:4n6	ND*	ND*	ND*	3.54 ± 0.18
20:5n3	0.33 ± 0.02	0.38 ± 0.02	0.33 ± 0.02	2.13 ± 0.10
22:4n6	0.02 ± 0.02	0.04 ± 0.002	0.04 ± 0.002	0.07 ± 0.01
22:5n6	0.08 ±0.01	0.09 ± 0.01	0.11 ± 0.01	0.14 ± 0.01
22:5n3	0.09 ± 0.001	0.11 ± 0.01	0.10 ± 0.004	0.41 ± 0.02
22:6n3	0.37 ± 0.04	0.35 ± 0.01	0.31 ± 0.01	5.30 ± 0.24
∑ n - 6	30.60 ± 0.63	32.74 ± 0.57	28.12 ± 0.34	28.69 ± 0.45
∑ n - 3	12.76 ± 0.50	7.17 ± 0.38	19.24 ± 0.20	11.53 ± 0.41
n - 3 / n - 6	0.42 ± 0.03	0.22 ± 0.01	0.68 ± 0.01	0.40 ± 0.02

*ND: Not detected

1. SBO: Soybean oil
2. LSO: Linseed oil
3. MFO: Menhaden oil, supplemented with arachidonic acid (ARA~40%) and docosahexaenoic acid (DHA~40%)

Table 7 - Fatty acid analysis from eggs of channel catfish females, *Ictalurus punctatus*, held under dietary treatments, commercial catfish diet 32% protein , 5% lipids, top-coated with 2% oil, and offered three times per week

Fatty acid	1- SBO[1] -LSO[2]	2 - SBO[1]	3 - LSO[2]	4 -MFO[3]	p - values
14:0	0.63 ± 0.12[b]	0.67 ± 0.14[b]	0.71 ± 0.11[ab]	0.75 ± 0.10[a]	0.0003
16:0	17.49 ± 0.75	17.25 ± 0.70	17.27 ± 0.26	17.62 ± 0.57	0.0485
16:1n7	2.06 ± 0.45[b]	1.91 ± 0.29[b]	2.35 ± 0.25[a]	2.35 ± 0.38[a]	<0.0001
18:0	13.09 ± 1.44[b]	14.51 ±1.34[a]	13.04 ± 1.31[b]	13.47 ± 1.39[b]	0.0001
18:1n9	29.54 ± 1.15	29.48 ± 1.27	29.95 ± 1.22	30.21 ± 1.38	0.0537
18:2n6	7.46 ± 0.81[a]	6.79 ± 0.90[b]	7.08 ± 1.11[ab]	6.53 ± 0.79[b]	0.0001
18:3n6	0.45 ± 0.39[b]	0.59 ± 0.26[a]	0.58 ± 0.23[a]	0.40 ± 0.11[ab]	0.0075
19:0	6.61 ± 1.07[ab]	7.09 ± 1.32[a]	6.99 ± 0.78[a]	6.25 ± 0.93[b]	0.0040
18:3n3	0.85 ± 0.18[b]	0.45 ± 0.20[c]	1.11 ± 0.22[a]	0.50 ± 0.11[c]	<0.0001
20:1n9	0.97 ± 0.17[b]	0.98 ± 0.15[b]	0.92 ± 0.15[b]	1.14 ± 0.19[a]	<0.0001
20:2n6	1.18 ± 0.14[ab]	1.25 ± 0.13[a]	1.14 ± 0.13[b]	1.24 ± 0.20[ab]	0.0348
20:3n6	3.04 ± 0.26[a]	3.05 ± 0.25[a]	2.87 ± 0.31[a]	2.63 ± 0.25[b]	<0.0001
20:3n3	6.00 ± 0.42[b]	6.38 ± 0.79[a]	5.42 ± 0.49[c]	5.38 ± 0.33[c]	<0.0001
20:4n6	0.12 ± 0.03[b]	0.08 ± 0.03[c]	0.16 ± 0.03[a]	0.09 ± 0.02[c]	<0.0001
20:5n3	0.81 ± 0.23[a]	0.54 ± 0.37[b]	0.77 ± 0.14[a]	0.87 ± 0.27[a]	<0.0001
22:4n6	0.40 ± 0.04[a]	0.41 ± 0.04[a]	0.34 ± 0.05[b]	0.36 ± 0.03[b]	<0.0001
22:5n6	1.68 ± 0.35[b]	2.42 ± 0.60[a]	1.53 ± 0.22[b]	1.56 ± 0.37[b]	<0.0001
22:5n3	0.94 ± 0.16[a]	0.72 ± 0.22[b]	0.89 ± 0.17[a]	0.76 ± 0.13[b]	<0.0001
22:6n3	5.73 ± 0.69[b]	4.77 ± 0.94[c]	6.05 ± 0.58[b]	6.98 ± 0.60[a]	<0.0001
∑ n - 6	13.14 ± 1.07[ab]	13.33 ± 0.75[a]	12.56 ± 1.35[b]	11.56 ± 0.75[c]	<0.0001
∑ n - 3	14.33 ± 1.20[a]	12.86 ± 1.70[b]	14.25 ± 0.79[a]	14.49 ± 1.08[a]	<0.0001
n - 3 / n - 6	1.09 ± 0.07[b]	0.96 ± 0.10[c]	1.14 ± 0.09[b]	1.26 ± 0.10[a]	<0.0001

a, b, c means followed by the same letter are not different (P>0.005, Tukey-Krammer test)

1. SBO: Soybean oil

2. LSO: Linseed oil
3. MFO: Menhaden oil, supplemented with arachidonic acid (ARA~40%) and docosahexaenoic acid (DHA~40%)

Figure 2 - Relative proportions of linoleic, linolenic, ARA, EPA and DHA acids from feed and eggs, and calculated values in mg of fatty acid per 100 eggs

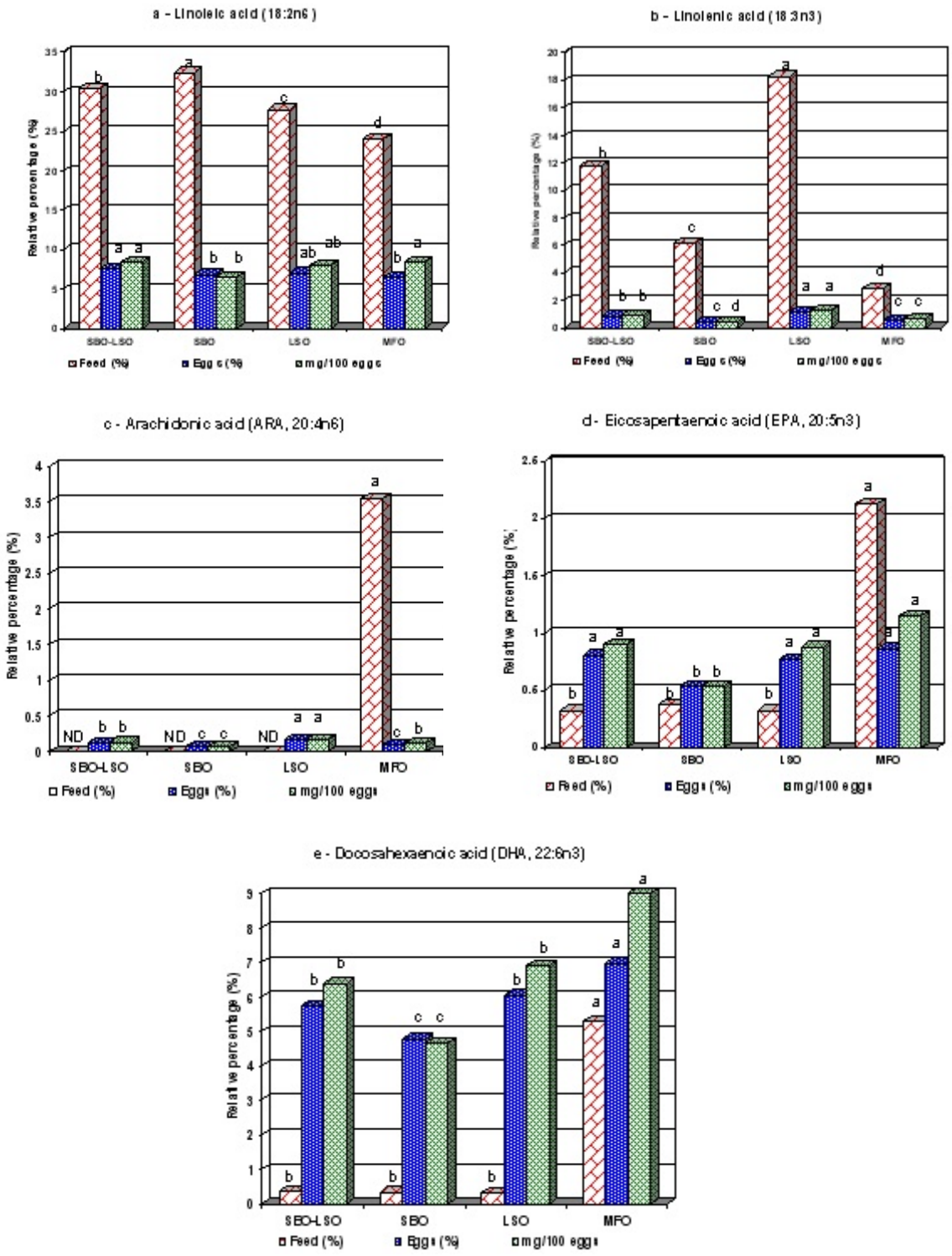


Table 8 - Percentage of essential fatty acids from feed corresponding to dietary treatments (commercial catfish diets top-coated with 2% oil); and percentage and absolute values (mg/100 eggs) of essential fatty acids from eggs of channel catfish females, *Ictalurus punctatus*, by dietary treatments

Diets	1 - SBO[1]-LSO[2]	2 - SBO[1]	3 - LSO[2]	4 - MFO[3]	P-values
Fatty acids - Feed					
Percentage (%)					
18:2n6	30.31 ± 0.64[b]	32.41 ± 0.57[a]	27.77 ± 0.36[c]	24.01 ± 0.68[d]	<0.0001
18:3n3	11.76 ± 0.46[b]	6.11 ± 0.34[c]	18.28 ± 0.21[a]	2.83 ± 0.12[d]	<0.0001
20:4n6	ND*	ND*	ND*	3.54 ± 0.18[a]	<0.0001
20:5n3	0.33 ± 0.02[b]	0.38 ± 0.02[b]	0.33 ± 0.02[b]	2.13 ± 0.10[a]	<0.0001
22:6n3	0.37 ± 0.04[b]	0.35 ± 0.01[b]	0.31 ± 0.01[b]	5.30 ± 0.24[a]	<0.0001
Fatty acids - Eggs					
Percentage (%)					
18:2n6	7.46 ± 0.81[a]	6.79 ± 0.90[b]	7.08 ± 1.11[ab]	6.53 ± 0.79[b]	0.0001
18:3n3	0.85 ± 0.18[b]	0.45 ± 0.20[c]	1.11 ± 0.22[a]	0.50 ± 0.11[c]	<0.0001
20:4n6	0.12 ± 0.03[b]	0.08 ± 0.03[c]	0.16 ± 0.03[a]	0.09 ± 0.02[c]	<0.0001
20:5n3	0.81 ± 0.23[a]	0.54 ± 0.37[b]	0.77 ± 0.14[a]	0.87 ± 0.27[a]	<0.0001
22:6n3	5.73 ± 0.69[b]	4.77 ± 0.94[c]	6.05 ± 0.58[b]	6.98 ± 0.60[a]	<0.0001
Absolute values (mg / 100 eggs)					
18:2n6	8.36 ± 1.82[a]	6.61 ± 1.42[b]	8.02 ± 1.88[ab]	8.42 ± 3.35[a]	0.0070
18:3n3	0.94 ± 0.25[b]	0.44 ± 0.23[d]	1.24 ± 0.24[a]	0.66 ± 0.32[c]	<0.0001
20:4n6	0.13 ± 0.04[b]	0.08 ± 0.03[c]	0.17 ± 0.03[a]	0.12 ± 0.05[b]	<0.0001
20:5n3	0.91± 0.31[a]	0.54 ± 0.42[b]	0.88 ± 0.24[a]	1.15 ± 0.70[a]	<0.0001
22:6n3	6.40 ± 1.38[b]	4.66 ± 1.23[c]	6.89 ± 1.64[b]	8.99 ± 3.24[a]	<0.0001

* ND: Not detected

a, b, c, d means followed by the same letter are not different (P>0.005, Tukey-Krammer test)

1. SBO: Soybean oil

2. LSO: Linseed oil
3. MFO: Menhaden oil, supplemented with arachidonic acid (ARA~40%) and docosahexaenoic acid (DHA~40%)

and arachidonic acid (ARA - 20:4n6) displayed similar patterns, being significantly higher in eggs from females given diet 3 (LSO), and significantly lower in diets 2 (SBO) and 4 (MFO)(Figures 2b and 2c). Eicosapentaenoic acid (EPA - 20:5n3) was significantly lower in eggs from diet 3 (LSO)(Figure 2d). Docosahexaenoic acid (DHA - 22:6n3) was significantly higher in diet 4 (MFO), and eggs from diet 2 (SBO) were significantly lower in terms of relative proportion and absolute values (mg/100 eggs)(Figure 2e). The n3:n6 ratio was significantly different among treatments, diet 4 (MFO) was significantly higher with 1.26, diets 1 (SBO - LSO) and 3 (LSO) were similar with 1.09 and 1.14, respectively, and diet 2 (SBO) was significantly lower with 0.96. Ratios among highly unsaturated fatty acids exhibited significant differences among treatments. DHA:EPA was significantly higher in diet 2 (SBO) than the others treatments. Ratios between ARA:DHA and ARA:EPA were significantly higher in diet 3 (LSO), and the lowest in diet 4 (MFO).

Linoleic acid, linolenic acid, ARA, EPA, and DHA composition, as individual fatty acids did not provide evidence of relationship with fry hatch. A strong positive correlation was evidenced for linolenic acid and arachidonic acid content from eggs (r^2=0.843, $p<0.0001$) (Figure 3). Weak negative correlations were found among ratios of ARA:DHA and ARA:EPA versus fry survival regardless of dietary treatment(r^2=0.1087, p=0.0308; and r^2=0.1036, p=0.0353, respectively) (Figure 4 and 5, respectively).

Principal component analysis (PCA) allowed the description of 83% of the variance in the data set and is therefore a good representation of the relationship among the diets and the fatty acids present in egg samples. Figure 6 shows the coefficients plotted for the first two principal components. The first PC accounted for 63% of the variance, with all five fatty

Figure 3 - Linear regression of relative proportion of Arachidonic acid vs linolenic acid in unfertilized eggs from females channel catfish, *Ictalurus punctatus*

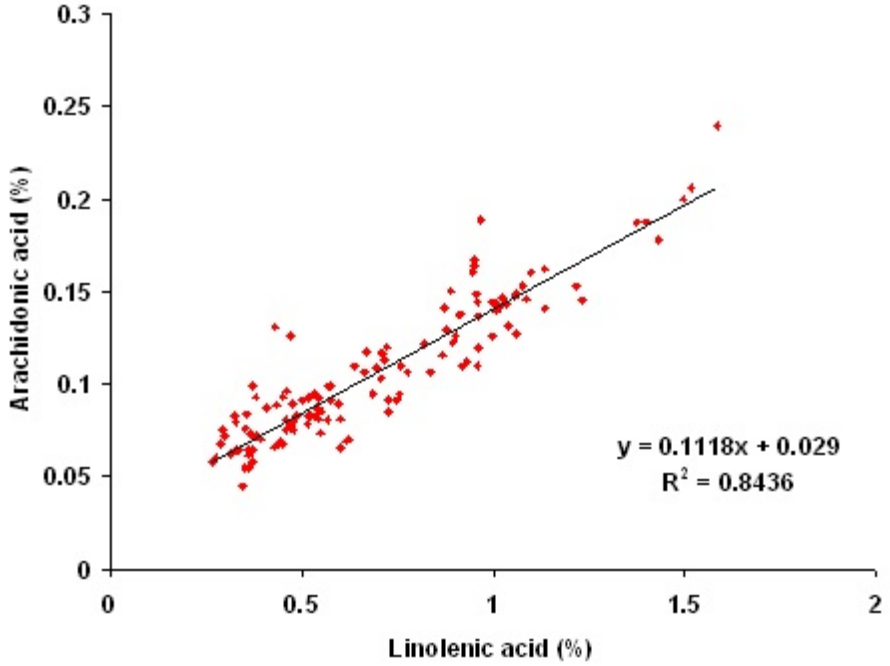


Figure 4 - Linear regression of ratio arachidonic acid (ARA): eicosapentaenoic acid (EPA) from unfertilized eggs vs fry hatch from females channel catfish, *Ictalurus punctatus*, when hybridized with blue catfish, *I. furcatus*, males

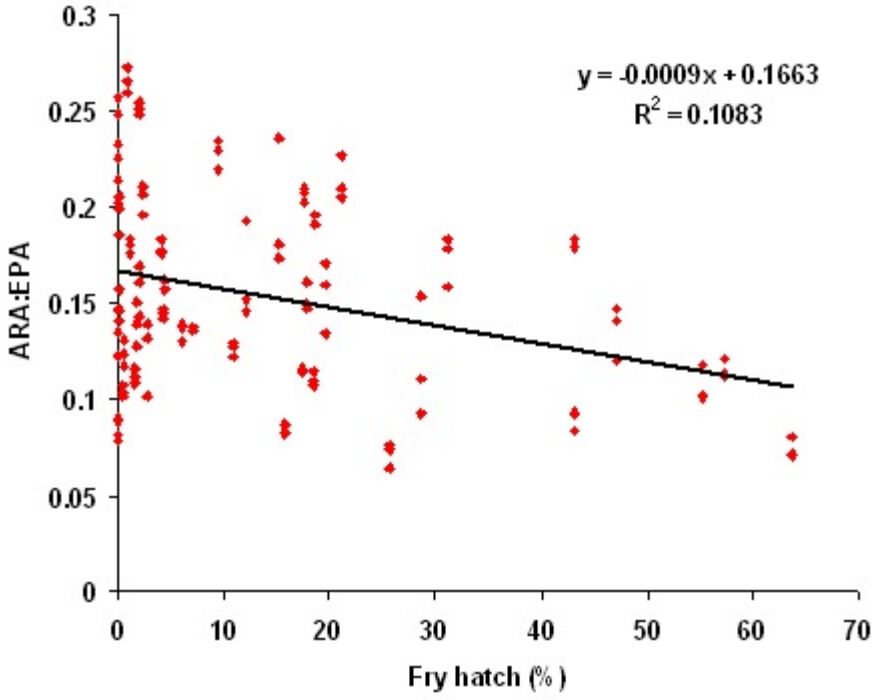


Figure 5 - Linear regression of ratio arachidonic acid (ARA): docosahexaenoic acid (DHA) from unfertilized eggs vs fry hatch from females channel catfish, *Ictalurus punctatus*, when hybridized with blue catfish, *I. furcatus*, males

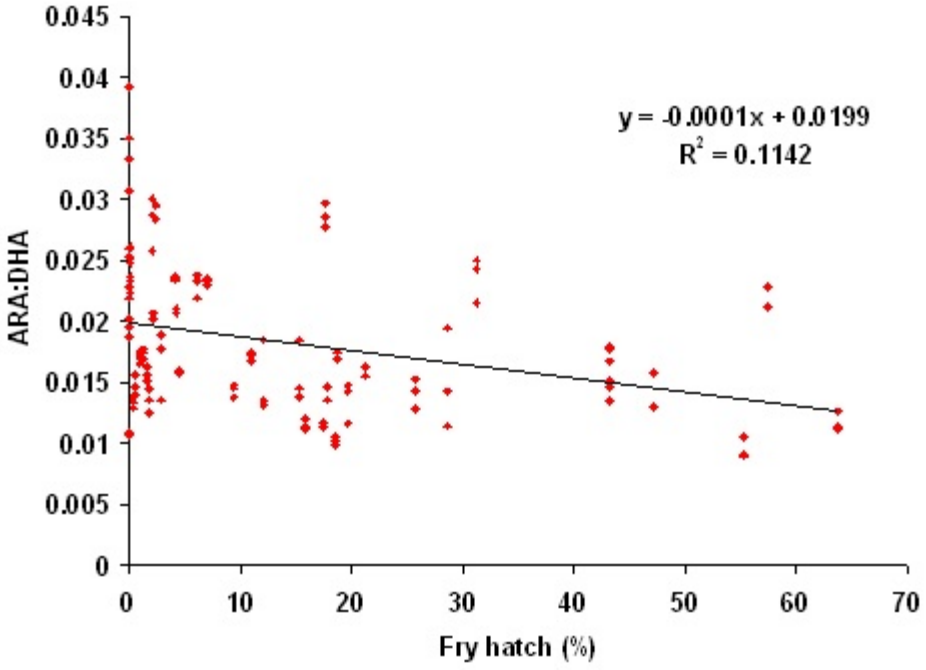
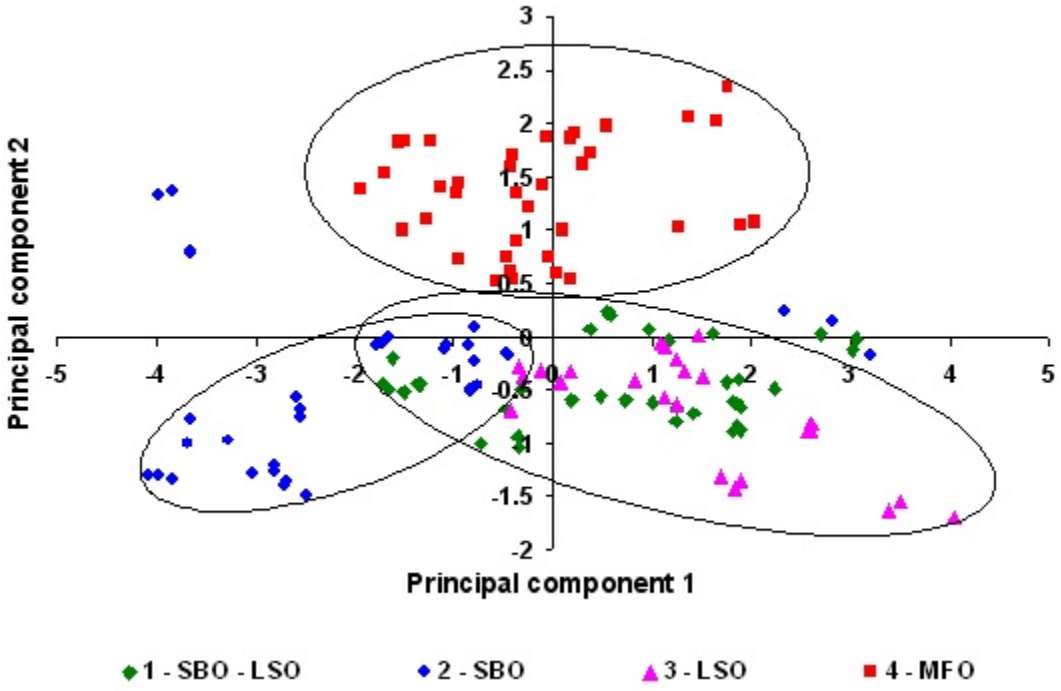


Figure 6 - Plot of factor scores from five fatty acids (linoleic, linolenic, ARA, EPA, and DHA) present in unfertilized eggs from females channel catfish, *Ictalurus punctatus*, after principal components analysis with respect to first (PC 1) and second (PC 2) principal components



acids loaded positively onto the axis. The PC2 axis represented 20% of the variance. EPA and DHA were positively loaded on this axis, whereas linoleic, linolenic and arachidonic acid were negatively loaded. Scores for diet 4 (MFO) grouped together on the positive side of PC2, and both sides of PC1. Scores for diet 2 (SBO) grouped mostly in the negative side of PC1 and PC2, whereas scores for diets 1 (SBO-LSO) and 3 (LSO) gathered in the positive side of PC1 and negative from PC2.

Discussion

The results of this study showed that fatty acid composition of the eggs was affected by broodstock dietary treatment provide prior to the spawning season. This has been observed in species that eat during sexual maturation and throughout the spawning season (Harel, Tandler, Kissil & Applebaum 1994). Results suggest that dietary essential fatty acids are readily incorporated into the eggs, and also mechanisms to elongate and desaturate fatty acids are very active in channel catfish. Preferential accumulation of certain fatty acids was also observed especially with regards to saturated fatty acids such as C16:0 and C18:0, and monoenes as C:16:1 and C18:1n9, which represented from 62.2% to 63.7% of total fatty acids in eggs. This characteristic has been noted in other freshwater fish and could be related to the fact that these fatty acids are heavily catabolized to generate metabolic energy in fish (Kaitaranta & Linko 1984; Tocher & Sargent 1984; Henderson & Tocher 1987; Anderson, Arthington & Anderson 1990; Wiegand 1996; Sargent, Tocher & Bell 2002).

Linoleic acid (C18:2n6) and linolenic acid (C18:3n3) are considered primary precursors of highly unsaturated fatty acids, especially in freshwater fishes (Sargent *et al.*

2002), were found in lower proportions than those found in the feeds. For instance, proportions of linoleic acid were around 21% to 27% of those found in diets 1 to 4, while linolenic acid were 6.1% to 7.4% in diets 1 to 3, and 17.7% in diet 4. These differences are more likely to be related to active processes in generation of HUFAs, such as ARA, EPA, and DHA, which tend to be deposited selectively into fish eggs (Henderson & Tocher 1987; Wiegand 1996; Sargent *et al.* 2002). Thus, a relatively high presence of ARA (C20:4n6), EPA (C20:5n3), and DHA (C22:6n3) in eggs from diets 1 to 3 indicate either a selective mobilization of this fatty acid from other tissues or elongation and desaturation of C18:2n6 and C18:3n3. The elongation and desaturation of linolenic acid (C18:3n3) occurs in the absence of long-chain fatty acids of the n3 configuration in order to prevent essential fatty acid deficiencies (Farkas, Csengeri, Majoros & Oláh 1977). This elongation and desaturation mechanism is more likely to explain the differences observed in eggs produced by fish fed diets 2 and 3, therefore, eggs from diet 3 (LSO) had significantly higher amount of DHA (C22:6n3) than eggs from diet 2 (SBO). A similar mechanism is observed for ARA (C20:4n6) which is the main end product of desaturation and elongation of linoleic acid (C18:2n6). Although, in this case, eggs from fish fed diet 2 (SBO) have significantly lower amounts of C20:4n6, but the presence of C22:5n6 is significantly higher, which could be related to the fact that C20:4n6 can be further desaturated and elongated to C22:5n6 (Sargent *et al.* 2002).

A remarkable finding is the positive correlation between linolenic acid and arachidonic acid content from eggs, and their relation with fry hatch. Diet 3 (LSO) had significant higher amounts of linolenic acid (C18:3n3) also had significantly higher amounts

of ARA (C20:4n6) but lower fry hatch, although not significant (p=0.0686) at 0.05 level it is highly suggestive of a lower fry hatch. Diet 2 (SBO) had the lowest amount of linolenic acid, and although it was richer in linoleic acid (C18:2n6), proportion of ARA (C20:4n6) was maintained when it was compared to other treatments, in fact, it seems like excess of ARA was turned into C22:5n6 (Table 7). Diet 1 (SBO-LSO) which had linoleic (C18:2n6) and linolenic (C18:3n3) acids in similar proportions, yielded into eggs with intermediate levels of linolenic acid (C18:3n3) and ARA (C20:4n6) relative to diet 1 and 3. Finally, diet 4 (MFO), which had significantly higher amounts of ARA available in the feed did not result in eggs with the highest ARA level but eggs with a very low proportion, but closely related to linolenic composition.

There was not a defined trend in proportions of n3 HUFAs in the eggs, specifically EPA and DHA, when they were related to spawning success, egg production, and/or fry survival, in fact none of those parameters were affected significantly by dietary treatments. This is exemplified with eggs from fish fed diet 4 (FMO), and those from diet 3 (LSO), both displayed the highest proportions of DHA (C22:6n3), but those from diet 4 had the highest hatch, while those from diet 3 had the lowest. Conversely, eggs from fish fed diet 2 (SBO), which had the lowest proportions of EPA (C20:5n3) and DHA (C22:6n3A), displayed an intermediate fry hatch. Thus, we can not infer that low or high concentrations of n3 HUFAs by themselves have a negative consequence on fry survival (Ashton, Farkvam & March 1993; Harel *et al.* 1994; Navas, Bruce, Thrush, Farndale, Bromage, Zanuy, Carrillo, Bell & Ramos 1997; Almansa, Pérez, Cejas, Badia, Villamandos & Lorenzo 1999; Fernández-Palacios *et al.* 1995; Bruce, Oyen, Bell, Asturiano, Farndale, Carrillo, Zanuy, Ramos &

Bromage 1999; Furuita, Tanaka, Yamamoto, Suzuki & Takeuchi 2002). Egg quality of channel catfish, it appears, is affected by more than the relative abundance of n3 HUFAs.

The ratio between ARA (20:4n6) and EPA (20:5n3), or DHA (22:6n3) could be more determinant in that outcome, as was suggested by Bell & Sargent (2003). They consider that both the concentrations and ratios of the essential HUFAs (DHA, EPA and ARA) are likely to have important influences on both fertilization rates and survival of fish eggs, and make the generalization that a high ARA:EPA in fish eggs may be mandatory for survival. Moreover, embryogenesis could be influenced by essential fatty acids C20:3n6, C20:4n6, C20:5n3, and C 20:6n3, since they are precursors for eicosanoid production, which in turn result in metabolites that include prostaglandins, leukotrienes and lipoxins (Leray, Nonnotte, Roubaud & Leger 1985; Mokoginta, Takeuchi, Moeljohardjo, Sumawidjaja & Fardiaz 1998; Bell & Sargent 2003). Prostaglandins, especially ARA-derived PGE2 and leukotriene B4 (LTB4) are associated with modulation of immune cell function. Additionally, low concentrations of these substances are required for normal immune function, while high concentrations are immunosuppresive (Kinsella & Lokesh 1990; Bell & Sargent 2003). This effect could explain the fact that high concentration of ARA evidenced in diet 3 (LSO) would be affecting normal development of hybrid catfish embryos, possibly resulting in lower survival.

Regarding the effect of linseed oil in this matter, it has been observed that Atlantic salmon fed diets containing this oil showed significant reductions in nonspecific immune parameters (Bell, Ashton, Secombes, Witzel, Dick & Sargent 1996, Bell & Sargent 2003). Although, the mechanism involved in this apparent immunosuppresion is not known, it has

been hypothesized that increased levels of membrane 20:3n6, in fish fed vegetable oils, may increase competition between ARA (C20:4n6) and 20:3n6 for prostaglandin production, which in turns alter immune cell composition and function (Kinsella & Lokesh 1990; Kelley & Daudu 1993; Bell & Sargent 2003). It is remarkable that proportion of 20:3n6 was significantly lower in diet 4 (MFO), while diets 1 to 3, top coated with vegetable oils, soybean and linseed oil, had significantly higher proportions of this fatty acid, which eventually could be affecting normal development of hybrid catfish embryos.

Concentration and ratio of DHA:EPA:ARA has been suggested as important in larval marine fish nutrition (Sargent, McEvoy, Estevez, Bell, Bell, Henderson & Tocher 1999). From this study, the ratio that displayed the highest fry production per body weight, and hence fry survival was found to be 77.5:9.7:1.0 from eggs produced under diet 4 (MFO), while the lowest fry survival from diet 3 (LSO) displayed a ratio 37.8:4.8:1.0, which again, highlighted the higher proportion of ARA.

Principal component analysis (PCA) performed on the fatty acids targeted in this research, lead us to differentiate diet 4 (MFO) from diets 1 to 3 (vegetable oil based). Also, scores from diet 1 (SBO-LSO) and 3 (LSO) tended to be mixed in the same region, whereas diet 2 (SBO) appeared to isolate from the others (Figure 6). Multivariate statistics have been previously conducted on fish egg composition and between different developmental stages (Copeman & Parrish 2002), as well as in nutrition studies (Johnsen, Grahl-Nielsen and Roem 2000; Cunha, Saborido-Rey & Planas 2003).

Another important component of the egg is the free amino acid pool. Free amino acids (FAA) are used in protein synthesis, as well as fuel in the energy metabolism of

developing of marine fish eggs and larvae, and some degree in osmoregulatory functions (Rønnestad & Fyhn 1993). However, in freshwater fish a higher FAA pool is not established during ovulation, possibly because the eggs will not be laid in a hyper-osmotic environment, and also, they may not be utilized as an energy source during early ontogeny (Gunasekera, de Silva & Ingram 1999). Free amino acids for channel catfish eggs have not been reported previously, but Wilson & Poe (1985) characterized amino acid composition from eggs (4-5 days old). They found higher levels of isoleucine, leucine, methionine, threonine, valine, phenylalanine and tyrosine, and lower levels of arginine and lysine when compared to amino acid composition of the whole body tissue. In our case we found significant differences among treatments but there was not clear relation with fry hatch or fry produced per female body weight.

Production parameters as fry produced per female body weight and fry hatch were affected by treatments, p=0.08 and 0.0686, respectively. From a commercial point of view, the effect of top coated commercial catfish feed 32% with highly unsaturated fatty acids (HUFAs) could have a positive impact on those parameters. This was evidenced with a production from two to five folds the number fry produced per female body weight when compared to fish fed commercial catfish feed 32% top coated with vegetable oils.

Conclusions

Reproductive parameters, such as spawning success, number of eggs either as per gram of egg mass or per female body weight did not exhibit significant effect from dietary treatments. Channel catfish females fed with feed top-coated with soybean oil (high n6)

produced eggs that were found to be significant smaller than the eggs produced by females under other treatments. Fatty acid composition of the eggs reflected the dietary lipid supplementation, making clear the incorporation of essential fatty acids during the pre-spawning season. Multivariate statistics using Principal Components Analysis (PCA) for the five fatty acids (linoleic acid, linolenic acid, ARA, EPA, and DHA) contained in the egg samples, lead us to differentiate groups of eggs based on scores assigned for each principal component which were reflection of the dietary treatments. Linseed oil, rich in linolenic acid C18:3n3 displayed the lowest hatch, which could be related to higher levels of ARA (C20:4n6), causing alteration in the immune cell composition. Similarly, higher proportions of C20:3n6 observed in diets 1 (SBO-LSO) and 2 (SBO) could be affecting embryo development due to immune response alterations. Supplying ARA, EPA, and DHA directly to brood stock females in diet 4 (MFO) increased fry production from two to five times when compared to females fed with diets top coated with soybean and/or linseed oil. Hence, commercial producers may consider to use highly unsaturated fatty acids as lipid supplement on their brood stock diets to improve their fry production. Finally, further investigation is required to elucidate the mechanism that regulates fatty acid composition in eggs, in particular that related to proportions of linolenic acid and arachidonic acid, and the physiological implications of such relation. reproductive performance parameters evaluated on channel catfish females, either spawning success, number of eggs per gram of egg mass, number of eggs per body weight, egg mass per body weight, fry production, or fry hatch.

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CHAPTER VI

SUMMARY & CONCLUSIONS

Channel catfish industry is one of the largest sectors of the aquaculture industry in the US. Currently the industry is facing economic challenges due to international competition and depressed prices. In order to ensure long term viability of the industry, production efficiencies and costs of production must be improved. One way to improve production efficiencies is to facilitate the commercialization of the of the hybrid catfish (channel x blue catfish). To facilitate the continued development and improvement of hybrid technologies two studies were carried out to investigate the effect of nutrition on the reproductive performance of channel catfish females and subsequently on hybrid fry production.

To enhance our understanding of what factors influence fry production we took the data from the first experiment and conducted a case study on proper statistical analyses. Traditionally, spawning success is evaluated using either ANOVA or Chi-square analysis. However, these models may not be the most appropriate as a number of test criteria may not be met. We found that logit analysis is a more suitable alternative method for the evaluation of this type of data. Results using this method lead us to identify age and period of spawning as determinant factors in spawning success. Additionally, five statistical models, ANCOVA, Poisson distribution, negative binomial distribution, zero inflated Poisson (ZIP), and zero

inflated negative binomial (ZINB) were used to model a set of data corresponding to number of eggs per gram of egg mass, and number of eggs per female body weight, including females that did not spawn, since they were part of the treatment response. Zero inflated discrete generalized linear models exhibited superior performance compared to other models used, and determined age and period of spawning as the most significant factors in determine eggs per gram of egg mass, and eggs per gram of female body weight, respectively. These findings coincided with those found using logit analysis for spawning success. Hence, they are recommended to analyze this type of data.

Effect of dietary treatments using protein level and feeding frequency as factors in determining reproductive performance are described in the fourth chapter. Protein level variation did not affect hatchery production parameters (spawning success, production of eggs, and fertilization rate). Increasing feeding frequency from three to six times per week negatively affected spawning success, but did not influence the number of eggs produced either by gram of egg mass or by female body weight. Fish age was found to be a significant factor in determining spawning success and production of eggs. Hence, five year-old channel catfish females had higher odds of spawning than younger females (three or four year-old), and produced significantly lower number of eggs per gram of egg mass, which is also related to having significantly bigger eggs. Spawning period significantly affected spawning success and production of eggs. Thus, higher odds of spawning were found during early season, and higher production of eggs per female body weight were related to middle season.

To further investigate the effect of different sources of lipids and different n3:n6 ratios on egg composition and their relation as indicator of egg quality a second spawning

experiment was conducted. Reproductive parameters, such as spawning success, number of eggs either as per gram of egg mass or per female body weight did not exhibit significant effect from dietary treatments ($p<0.05$). Channel catfish females fed with feed top-coated with soybean oil (high n6) produced eggs that were found to be significant smaller than the eggs produced by females under other treatments. Fatty acid profile from channel catfish eggs reflected the effect of dietary treatments. Thus, Principal Component Analysis (PCA) using linoleic, linolenic, ARA, EPA, and DHA acids from egg samples lead us to differentiate associated scores for each diet. Fish fed with feed top-coated with menhaden fish oil enriched with ARA and DHA produced two to five times more fry than those fed with feed top-coated with soybean and/or linseed oil. This difference, although, not statistically different ($p = 0.08$) is meaningful for commercial producers, which could increase their fry production.

The studies conducted in this research have increased the knowledge of nutritional effects on brood stock performance. Nutrient deposition and their impact on egg quality depends not only on dietary treatments but is influenced by internal and external factors, which act previous and during reproduction (physiology of the fish, genetic information, and environmental stressors). There are also, other factors related with incubation and hatching, which interact and determine the final outcome of fry production. Based on the results of these studies the supplementation of HUFA sources to catfish brood stock diets is warranted and further research should be conducted to elucidate minimal dietary requirements that result in improved fry production.

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