ENHANCING BLUEGILL PRODUCTION THROUGH LEAST-COST DIET DEVELOPMENT AND NOVEL REARING STRATEGIES

A Dissertation

presented to

the Faculty of the Graduate School

at the University of Missouri-Columbia

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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by

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JULY 2010

The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

ENHANCING BLUEGILL PRODUCTION THROUGH LEAST-COST DIET DEVELOPMENT AND NOVEL REARING STRATEGIES

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DEDICATION

I dedicate my dissertation to my parents, Masagounder Palanigounder and Karuppayammal Masagounder, my sisters, Devi and Rani and those whose livelihoods depend on aquaculture and fishing.

ACKNOWLEDGEMENTS

Looking back at my journey through my doctoral studies, I am both surprised and delighted with how MU has developed me as a responsible scholar and has led me to this position. It has certainly been a wonderful experience and I am deeply grateful for all that MU has provided me.

I am profoundly grateful to my dissertation supervisor, Dr. Robert Hayward for his guidance, encouragements and support from the beginning to the final stage of my Ph.D. He has always been generous with his time and knowledge and has helped in every element of my Ph.D. I am particularly thankful to him for editing my writings, and making me a better writer of manuscripts. I am also grateful to him for improving my understanding of fisheries management and fish bioenergetics, which ultimately helped me to secure a job at the USGS.

I am sincerely thankful to my committee members. Everyone contributed differently, but collectively helped me to carry out my experiments systematically and to do good science in the future. I am very indebted to Dr. Jeff Firman for his invaluable contribution in planning for my nutrition experiments and improving my knowledge of animal nutrition. Dr. Douglas Noltie has been a very helpful to me in planning for my studies, editing manuscripts and dissertation, and enriching my knowledge in fish behavior and conservation. My special thanks go to him. I am very thankful to Dr. Diana Papoulias for her invaluable direction in improving my study plan and my background in fish endocrinology, as well as for her constant support throughout my doctoral study. I

deeply owe Dr. Duane Keisler for his perspectives in planning and carrying out my studies. He has been more than willing whenever I needed his help.

I would like to express my gratitude to the North Central Regional Aquaculture Center and the Egg & Poultry Association for providing research funds. I deeply thank the J. N. Tata Endowment, Mumbai, India, for financial support of my travel to the U.S.

I am also sincerely thankful to Dr. Paul B. Brown (Purdue University), Dr. Santosh P. Lall (NRC, Canada) and Dr. David Ledoux (MU) for their assistance in my nutrition studies. I am very thankful to Dr. William R. Lamberson, Dr. Gene Pesti, and Ms. Lada Micheas for their help running my broken-line models – they were very generous with their time and knowledge.

I thank the American Fisheries Society, Missouri Department of Conservation, and Graduate Student Organization, Department of Fisheries and Wildlife Sciences for travel grants to attend various conferences.

I also gratefully acknowledge the staff of The School of Natural Resources, particularly Ms. Karen Decker, for giving me all necessary assistance during my studies. Karen was very helpful while establishing a contract with Texas A&M University for extruding my diets. I am also thankful to the library staff for giving me unlimited access to library resources. I also sincerely thank the staff of the Graduate School for all their assistance with my academic progress and the staff of the International Student Center at MU for their help with my visa issues.

I thank the staff of Food Protein R&D Center, Texas A&M University, particularly Mr. Chris Mack and Dr. Mian Riaz, for their support in extruding our

experimental diets. I also thank Nelson & Sons, Inc., Omega Protein, Inc., Archer Daniels Midland Company and Uniscope, Inc. for their continuous support in sending free feed ingredients for the study.

I am very grateful to Mr. Curtis Harrison for providing me all the facilities and manpower to conduct my pond study. I also thank Mr. Curtis Harrison (Harrison Fisheries, Inc.), Mr. Steve Kahrs (Osage Catfisheires Inc.), Dr. James Wetzel (Lincoln University) and Mr. Edward McMillan for their timely help in getting me bluegill for my lab studies. My heartfelt thanks go to Brandon Hanquist for his tremendous assistance with most of my experiments. I also thank Kelly Motaz for her assistance with the pond study. I am very thankful to Shane Simmons and Greg Brostoski for their help in carrying out my diet experiments. Shane was always available whenever I needed him. I also thank Peyton Bennett, Preston Feltrop, Sarah Ernst, Bailey Yotter and Amber for their assistance with my lab experiments.

I am very indebted to Dr. Chris Hardin for his generosity in allowing me to use his lab facilities for determining liver glycogen. Many thanks go to Ms. Tina Roberts and her lab students for their invaluable assistance in glycogen estimation. I always owe Dr. Sivanandan Chudalayandi for his help in lab analysis. I also thank Elisangela Guaiume, Elizabeth Jones, Dr. Sangsoo Sun and Dr. Mohammad Ali for their assistance in my lab experiments. My heartfelt thanks go to Mr. James Potter for his suggestions and immense help with my biochemical estimations, particularly protein.

I specially thank Dr. Shannon Brewer for her invaluable suggestions during my preparation for my comprehensive exams. I definitely owe my officemates, Aniruddha Belsare, Ryan Monello, Morgan Wehtje and Natalie Olifiers for their suggestions and

friendship throughout my Ph.D. My thanks also go to Abi-Tamin Vanak and Mihai Sun for their moral support. I thank Mr. Richard Spence for his friendship and support while I was working late night in the office.

My heartfelt thanks go to Vinoth, Kalai, Selvi, Nandhu, Kishore, Krishna, Siva and Anju for providing me moral support, transportation, fun and food during my Ph.D. I also thank my apartment mates Tom, Erick and Katy for their moral support during my dissertation writing.

I am deeply indebted to my family: without their love, support and patience, I would not have travelled this far. My Dad, Masagounder Palanigounder, Mom, Karuppayammal Masagounder, and my sisters, Devi & Rani stood by my ups & downs. I also thank my brother-in-laws, Nachimuthu and Veerappan, nephews, Rajesh, Gowtham and Karthi for their support during these years.

I thank the governments of both the India and the U.S. for helping me to gain a degree from the MU.

Above all, I wholeheartedly thank my mighty God for giving me the vision, strength, spirit and endurance to complete this interesting study.

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ENHANCING BLUEGILL PRODUCTION THROUGH LEAST-COST DIET DEVELOPMENT AND NOVEL REARING STRATEGIES

DISSERTATION ABSTRACT

My dissertation focuses on two broad issues: 1) developing a least-cost diet for juvenile bluegill *Lepomis macrochirus*, and 2) developing effective rearing strategies to enhance production of food-size bluegill. The diet development work involved a series of experiments conducted on juvenile bluegill to determine (i) digestibility of nutrients (amino acids, protein and energy) from common feedstuffs, (ii) dietary requirements for essential amino acids (EAAs), (iii) dietary protein and energy requirements for optimal fish growth performance, and finally (iv) a best, economically feasible diet, involving the formulation and evaluation of various experimental diets ranging from those with much fish meal (most expensive) to those containing no fish meal (least expensive).

The digestibility of nutrients (amino acids, protein and energy) from protein (blood meal, corn gluten meal, fish meal, meat and bone meal (MBM), poultry byproduct meal (PBM), soybean meal (SBM)) and energy (corn and wheat) sources was determined using single-test ingredients and the feces siphoning method. Available EAA levels were high from fish meal and PBM, whereas for other protein sources, one or a few amino acids were wanting. Available EAAs were low for corn and wheat. Digestible levels of protein and energy were generally high for all feedstuffs except wheat and corn, the available energy levels for which were low. Validation of the method used in the study (single ingredient test diet, siphoning method of feces collection) was also provided. The nutrient availabilities for each ingredient were then used in subsequent experiments to

determine digestible nutrient requirement levels, and a least-cost diet formulation for juvenile bluegill.

Dietary requirements for EAAs were determined for juvenile bluegills through two 60-d experiments, one in which bluegills were group-housed, and another with individually-housed bluegills. Both experiments were run to determine whether the two rearing approaches gave different indications of juvenile bluegill lysine requirements, due to the presence and absence, respectively, of social hierarchy formation. Optimal dietary lysine level (digestible basis) was estimated as 15 g Kg^{-1} based on broken-line regression analyses of relative growth rate and feed conversion ratio. No differences between the two rearing methods were observed, despite the higher growth rate shown by the individually-housed versus the group-housed bluegills. Requirements for other EAAs were determined from whole-body composition analyses and measured lysine levels, with values ranging from 2.4 g Kg^{-1} (tryptophan) to 15.3 g Kg^{-1} (leucine). After determining EAA requirements, a series of two additional 60-d experiments were run to determine the optimal levels of dietary protein and energy for juvenile bluegill. A dietary protein level of ~410 g Kg^{-1} and a dietary energy level of ~14.6 MJ Kg^{-1} were found to be optimal for juvenile bluegill. The study emphasized the importance of determining the appropriate dietary lipid to carbohydrate ratio for juvenile bluegill in order to reduce the expensive dietary protein requirement level.

In the final experiment, data concerning the digestibility of various feedstuffs and dietary nutrient requirements were used to formulate a fish-meal based diet (550 g Kg^{-1} fish meal). Subsequently, a series of diets was formulated by gradually replacing the costly fish meal component (0-550 g Kg^{-1} fish meal) with alternative protein sources to

reduce feed cost. A 60-day feeding trial showed no significant differences in fish feed consumption and growth performances among the different dietary groups, whereas the ingredient cost of the diet was reduced from \$816.23 tonne^{-1} (550 g Kg^{-1} fish meal) to \$559.41 tonne^{-1} (0 g Kg^{-1} fish meal). The trial included commercial catfish and trout diets as the industry-standard control diets. Results showed that the catfish diet produced poor bluegill growth, whereas the trout diets caused high body-fat deposition. Study results showed a diet formulation comprised predominantly of SBM (~37%) and MBM (~38%) to be the best, least-cost diet for juvenile bluegill.

Concerning rearing strategies for improving food-size bluegill production, two extensive studies were conducted, one involving "topping off", and the other size grading. The topping-off strategy was evaluated by rearing bluegill in 1000-L indoor recirculating tanks for 574 days. Evidence (significant positive relationships between bluegill relative weights (W_r) and fish length, fat content, and fish weight, as well as temporal increases in fish size variation) suggested that social hierarchies developed in bluegill by day 31 and continued to persist thereafter until "topping off" was initiated. The first-topping off removals were performed on day 376 by harvesting the upper 10th percentile (by weight) of bluegill and immediately replacing them with an equal number of juvenile bluegill. This approach was repeated twice before the final harvesting on day 574. In the no-topping off group, all the bluegill were harvested in a single batch on the final day of experimentation. The topping-off strategy apparently disrupted the bluegill social hierarchy, leading to a significant increase in growth and production of large bluegill (> 100 g) and improved yield, relative to the no topping-off group. However, this

strategy ultimately failed to produce food-size bluegill. Modifications to the topping-off strategy were suggested for enhancing bluegill growth and production.

The size-grading strategy (i.e., selectively removing the quartile of bluegill by length (≥ 85 mm)) produced a bluegill stock with predominantly fast-growing males. Size graded bluegills were evaluated versus mixed-size (ungraded) bluegill for producing large bluegill and increasing fish production in ponds. Graded and ungraded bluegill groups were reared in production ponds at an estimated density of 16,667 fish ha^{-1} for 584 days (April 2005 to Nov 2006). Results showed that size grading produced consistently larger bluegill over the study period, but no differences were observed between the groups in terms of growth rate and fish production. Surprisingly, evidence of social hierarchy establishment was detected in all production ponds by day 181, and persisted until the final sampling date. This study provides the first evidence of social hierarchy development in fish reared in production ponds. Apparent effects of social hierarchies on key production parameters including bluegill growth rates and feed efficiencies were also provided. The study further indicated that the development of social hierarchies among bluegills in ponds may have minimized the anticipated benefits of rearing predominantly male bluegills. Measures that would delay or prevent the formation of social hierarchies are discussed.

PREFACE

When I started my doctoral work in 2005, bluegill were emerging as an aquaculture species in the U.S.; unlike for trout and catfish, few aspects of bluegill culture technology had been standardized. Lack of a nutritionally complete, affordable diet was listed as a major constraint by bluegills producers. Consequently, the major objective of my doctoral study was to develop a least-cost diet for juvenile bluegill. Also, I evaluated two new rearing strategies, topping-off harvesting and size grading, to determine their efficiency in increasing bluegill growth and production. Experiments for these objectives were conducted from 2006 to 2009, and are detailed in 5 chapters in this dissertation.

The chapters of this dissertation are either published or in preparation for submission to peer-reviewed journals. Below, I detail the anticipated citation for and likely destination of each chapter.

Chapter 1: Masagounder, K., Firman, J., Hayward, R.S., Sun, S. & Brown, P. (2009) Apparent digestibilities of common feedstuffs for bluegill, *Lepomis macrochirus* and largemouth bass, *Micropterus salmoides* using individual test ingredients, *Aquacult. Nutr.*, **15** (1), 29-37.

Chapter 2: Masagounder, K., Hayward, R.S., & Firman, J. Comparison of dietary essential amino acid requirements determined from group- versus individually-housed juvenile bluegill, *Lepomis macrochirus*. *Aquacult. Nutr. (accepted).*

Chapter 3: Masagounder, K., Hayward, R.S., & Firman, J. Effects of dietary protein and energy levels on growth and body composition of juvenile bluegill *Lepomis macrochirus*. *Aquacult. Nutr. (in review).*

Chapter 4: Masagounder, K., Hayward, R.S., & Firman, J. Development of least-cost diet formulation for juvenile bluegill, *Lepomis macrochirus*. *Aquacult. Nutr. (to be submitted).*

Chapter 5: Masagounder, K., Hayward, R.S., Noltie, D. & Wang, H.P. Bluegill (*Lepomis macrochirus*) growth in indoor tanks and production ponds: evidence that social hierarchy development is a factor**.** *J. World Aquacult. Soc., (to be submitted).*

Masagounder, K., Hayward, R.S., & Noltie, D. Evaluation of novel rearing strategies for enhancing production of food-size bluegill, *Lepomis macrochirus Aquaculture (to be submitted).*

INTRODUCTION

Bluegill (*Lepomis macrochirus*) were historically grown in ponds throughout the U.S. as a forage fish for largemouth bass (*Micropterus salmoides*) and as a sport fish (Swingle 1946; Dupree & Huner 1984; McLarney 1987). Over the past decade, bluegill have also received substantial attention in the aquaculture sector as a food fish (225–340 g). Currently, about half (45%) of the ~250 fish growers in the North Central Region of the U.S. are involved in rearing bluegill (Morris & Mischke 2003).

In addition to the high market demand for bluegill, owing in part to a widespread familiarity with the species, bluegill also exhibit a number of favorable, production-related characteristics. These include, for examples, the ability to readily wean juvenile bluegill onto prepared feeds (Ehlinger 1989), high tolerance to handling stress and poor water quality (Heidinger 1975; Brunson & Robinette 1983), and year-round availability of spawn, owing to successful out-of-season spawning capacity (Mischke & Morris 1998).

With demand for food-size bluegill continuing to increase, rearing technology for bluegill and their hybrid crosses (e.g., ♂ bluegill × ♀ green sunfish *L. cyanellus*) has improved over the past two decades by (1) directing research efforts towards optimizing rearing densities (Hayward & Wang 2002; Loveshin & Matthews 2003), (2) indentifying practical feeds (Twibell *et al.* 2003), (3) optimizing feeding regimes (Wang *et al.* 1998), and (4) increasing fish growth and feed efficiency through compensatory growth feeding schedules (Hayward *et al.* 1997, 2000).

Despite improvements in bluegill culture techniques, running profitable businesses involving the production of food-size bluegill within two growing seasons has remained challenging to bluegill growers (Brunson & Morris 2000; Hayward & Wang 2002). A major impediment to bluegill aquaculture has been the lack of nutritionally-balanced, affordable diets for bluegill (Morris & Mischke 2003). Feed plays a critical role in the success of any intensive, fish farming operation, because feed costs alone often account for > 50 % of fish producer's total annual variable costs. The use of suboptimal diets not only reduces fish production and feed efficiency, but also increases production costs and nutrient pollution. Catfish diets and trout diets are two practical (readily available) diets that are often used by the fish producers raising bluegill. However, available data indicate that feeding catfish diets to bluegill leads to poor growth, whereas feeding trout diets causes high fat deposition in bluegill (37% by dry fish weight) (Twibell *et al.* 2003) and may lead to moribund condition. Hence, both of these diets are substantially suboptimal for bluegills, yet, they remain commonly used in bluegill culture. Information concerning the dietary nutrient requirements of bluegill is very limited. Hoagland *et al.* (2003) reported that bluegill require 44% dietary protein, but only 8% dietary lipid for high growth rates and feed efficiencies. Although these values were not on based on digestibility, the findings demonstrate that bluegill require lower percentages of dietary lipid than those (≥ 10%) that are available in practical trout diets. Excess fat deposition not only reduces fillet yield, but also feed consumption and ultimately fish production (de Pedro & Bjornsson 2001). Moreover, the expense of trout diets threaten the economic sustainability of bluegill farming (Curtis Harrison, Harrison Fisheries, Inc., MO, pers. comm.). The protein component in commercial trout diets is

largely represented by fish meal, the dietary inclusion levels of which range from 20% to 30% in the U.S., and up to 55% in other areas of the world (Tacon & Metian 2008). Fish meal has been used as the main protein source in aquafeeds because of its high nutrient digestibility, high protein level, and balanced essential amino acid profile. Consequently, the demand for fish meal in fish- and other animal-feed industries continues to increase, even though the commercial harvest of fishes used for fish meal production have continued to decline due to overharvest (Kureshy *et al.* 2000; Gatlin *et al.* 2007). As a result, fish meal has become a highly expensive protein source, with costs increasing from ~$400 tonne^{-1} (1999) to ~$1100 tonne^{-1} (2008) in the U.S. (Tacon & Metian 2008). Hence, although the development of a nutritionally-complete diet for bluegill is much needed for bluegill production, of equal importance is the determination of protein source alternatives to fish meal in order to reduce feed cost and conserve declining wild fish stocks that are harvested as fish meal sources. Fish meal can be effectively replaced by other protein sources only when information concerning a species' capacity to digest the alternative protein source is known, in addition to its dietary requirements for digestible EAAs and protein, and its acceptance of alternative protein ingredients. Information concerning optimal dietary energy requirement for bluegill also plays a key role in limiting the excess fat accumulation that typically results when fish are fed trout diets. Finally, formulating a diet on a digestibility basis promotes a more accurate meeting of the fishes' nutrient requirements, and tends to reduce the nutrient pollution that results from excess non-digestible dietary nutrients (Hertrampf & Piedad-Pascual 2000).

While a least-cost, complete diet formulation is much needed for profitable bluegill production, available data indicate that bluegill growth can be further enhanced

by reducing their social-interaction costs (Hayward & Wang 2002) as well as by rearing groups of predominantly male bluegills (Hayward & Wang 2006; Doerhoff 2007). Bluegill are highly aggressive and their well-known, agonistic interactions have been extensively reported (Poulsen & Chiszar 1974; Beitinger & Magnuson 1975; Henderson & Chiszar 1977; Colgan *et al.* 1979). Dominance hierarchy formation substantially reduces bluegill growth rates and feed efficiencies, while markedly increasing size variation, as has been documented for bluegill and their hybrids reared in indoor tanks (McComish 1971; Wang *et al.* 2000; Hayward & Wang 2002; Doerhoff 2007). Topping-off harvesting (also termed 'sequential harvesting' or 'cull harvesting') involves the removal of larger, market-size fish from a tank or pond, followed by stocking of additional fingerlings. This rearing strategy has been applied in semi-intensive and intensive aquaculture systems to enhance fish production by controlling size variation, dominance hierarchy formation, competition, and cannibalism. This rearing method has been applied to channel catfish *Ictalurus punctatus* (Hargreaves 2002), milkfish *Chanos chanos* (Avault 1996), sunshine bass (♂ *Morone saxatilis* × ♀ *M. chrysops)* (D'Abramo *et al.* 2002) as well as tilapia *Oreochromis shiranus* (Brummett 2002). The topping-off method appears to reduce dominance hierarchy formation among bluegill, thus allowing subordinates to grow at rates close to their maximum capacity. This rearing approach may allow fish producers to raise year-round supplies of desired, food-size bluegill.

Bluegill exhibit sexually dimorphic growth wherein males show substantially higher growth rates than females (Hayward & Wang 2006; Doerhoff 2007). Recent findings by Hayward & Wang (2006) indicate that male bluegill are capable of reaching market sizes within two growing season; males attained ~66 % of market size whereas

females reached only ~31 % of market size when housed individually in an indoor tank system for 234 d. In a follow-up study, Doerhoff (2007) demonstrated that when bluegills of the same intra-annual spawning cohort attain a size of ≥ 90 mm, size grading can be used effectively to select males from mixed-sex groups. Size grading for large fish is commonly done in fish farming to increase fish production (Avault 1996). Such rearing techniques may produce greater numbers of larger bluegill, thereby increasing fish production.

Overall, my dissertation research sought to enhance production of bluegill by:

1. developing a least-cost, complete diet for juvenile bluegills by determining (i) the digestibility of commonly-used feedstuffs, (ii) their dietary requirements (digestible basis) for essential amino acids, protein, and energy, and (iii) their capacity to use alternative protein sources to replace fish meal when balanced for dietary nutrient levels, and

2. evaluating the effectiveness of applying "topping-off" and "size-grading" techniques to increase bluegill growth and, in turn, the number of larger-size bluegill produced together with fish yield.

Chapters 1 through 4 document the multiple experiments that were carried out to develop a least-cost, complete diet for juvenile bluegill (Objective 1), whereas Chapter 5 describes the study that was conducted to determine the benefits of using the topping-off approach for enhancing bluegill production in an indoor-tank setting and in an outdoor pond setting.

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CHAPTER 1

APPARENT DIGESTIBILITIES OF COMMON FEEDSTUFFS FOR BLUEGILL *LEPOMIS MACROCHIRUS* USING INDIVIDUAL TEST INGREDIENTS

ABSTRACT

Apparent digestibility of dry matter and energy, and availability of amino acids from blood meal (BM), fish meal (FM), meat and bone meal (MBM), poultry byproduct meal (PBM), soybean meal (SBM), corn and wheat were determined for bluegill *Lepomis macrochirus* (mean weight, 57 g). To avoid nutrient interaction from a reference diet, diets containing 98.5% (985 g Kg^{-1}) of test ingredients were used. Feces were collected by a siphoning method. Apparent dry matter digestibility values ranged from 50% (corn) to 87% (BM) whereas apparent energy digestibility values ranged from 53% (corn) to 92% (BM) for bluegill. Apparent digestibility of most amino acids exceeded 90% for evaluated protein sources, except for MBM which showed slightly lower values (80-90%). Isoleucine digestibility from BM was relatively low (82%) for bluegill. High digestibility of SBM, PBM and BM, indicate their good potential for replacing FM in the diets of bluegill. Validation of the method (single ingredient test diet, siphoning method of feces collection) used in the study was also provided by comparing the digestibility values determined for largemouth bass *Micropterus salmoides* from this study versus the digestibility values reported for this species using the more commonly used method (compound test diet, sedimentation method of feces collection).

INTRODUCTION

A key aspect of developing diets for fishes is to determine their capacity to digest common feedstuffs (De Silva & Anderson 1995). Knowing availabilities of nutrients to the species aids selection of appropriate ingredients and formulation of cost-effective diets (Hajen *et al*. 1993). Inclusion of highly digestible feedstuffs will also reduce nutrient waste entering waterways and the water bodies. Young bluegill *Lepomis macrochirus* are planktivores. As bluegill grow, they increasingly consume small benthic invertebrates as well (Mischke & Morris 1998). From available reports (e.g., Yamamoto *et al*. 1998; De Silva *et al*. 2000; Lee 2002; Portz & Cyrino 2004), it is clear that energy and amino acid digestibility values differ among species and across feedstuffs, due to differences in fishes' digestive capacities. Dietary experiments conducted to date for bluegill (e.g., Hoagland *et al.* 2003; Twibell *et al.* 2003) have not been based on known digestibility values. Consequently, the findings are limited to the particular feed formulations used in these studies. Therefore, apparent digestibility coefficients (ADCs) of nutrients from various feed ingredients need to be determined for bluegills.

Test diets for evaluating digestibility values for fishes typically involve mixing a test ingredient (15-30 %) with a reference diet (70-85 %) that includes an indigestible indicator (often, 0.5 % chromic oxide) for indirect measurement of digestibility. Reference diet is included in the test diet to maintain adequate palatability and to satisfy the test species' requirements of essential nutrients. Digestibility of the test and reference diets are estimated independently and compared via a "difference" method to estimate the ADCs of nutrients in the test feedstuff (Cho *et al.* 1982). Although this approach is considered standard, inclusion of the reference diet can interfere with nutrient availability

from the test ingredient (Lupatsch *et al*. 1997) and cause erroneous digestibility values. For example, antagonistic effects of carbohydrate on digestibility of protein have been observed (Windell *et al*. 1978; Stone 2003; Krogdahl *et al.* 2005). Moreover, compound diets have had additive effects on digestibility of amino acids (Lupatsch *et al*. 1997). Therefore, variation in the composition of reference diets can cause differences in the apparent digestibility of a nutrient. Consequently, test ingredients would, preferably, be fed exclusively when evaluating their digestibility (Glencross *et al*. 2007). Problems associated with individual ingredient test diet, including poor water stability, nutrient deficiency, and poor acceptability, could be resolved by modifying test procedures. For example, poor water stability can be ameliorated by adding appropriate binders, while nutrient deficiencies and poor acceptance of test ingredients by fishes could be remedied by reducing feed trial durations and adding attractants, respectively. There can be exceptions for test ingredients such as oils, for which use of the "difference method" may be unavoidable due to difficulties with pelletizing.

The objective of this study was to determine digestibility of a range of feedstuffs for maturing bluegill using individual feedstuffs as test diets. In part, to evaluate the method of the present study that involved using individual feedstuff as test diet and collection of fish feces by siphoning, the study also determined digestibility values for juvenile largemouth bass *Micropterus salmoides* from few feedstuffs and compared those values versus the values of Portz & Cyrino (2004) who used more common procedures including compound feed as a test diet and feces collection by sedimentation for largemouth bass.

MATERIAL AND METHODS

Bluegill experiment

Test diets

Feedstuffs for evaluation were selected based on their local availabilities and relative importance in feed formulation. Seven feedstuffs, four being animal products, were evaluated for bluegill: fish meal (FM), pet-food grade poultry by-product meal (PBM), meat and bone meal (MBM), and blood meal (BM), as were four plant products: soybean meal (SBM), corn gluten meal (CGM), corn and wheat. Cereals such as corn and wheat are carbohydrate rich whereas other products are rich in protein. Ingredients were obtained from commercial sources.

Eight test feeds were prepared using single feedstuffs as the independent diet component representing 985 g Kg^{-1} (98.5 % test diets). An indigestible marker (chromic oxide), a feed attractant (betaine), and a commercial binder (Aqua-Tech, Uniscope Inc.), each at 5 mg g^{-1} (0.5 % of test diets), were added to the dry mix of each test feed. This approach, which differed from the more standard method (using 30 % test ingredient combined with 70 % reference diet) was used to minimize error from interactions of nutrients from the reference diet. Dry ingredients were sequentially mixed in a V-mixer (Patterson-Kelly, East Stroudsburg, Pennsylvania, USA), then transferred to a Hobart mixer (Hobart Corp., Troy, Ohio, USA), where water was added. All diets were pelleted, air dried and stored under air-tight conditions at 4 ˚C until used. Energy, ash and amino acid (AA) contents of the seven test feedstuffs are given in Table 1.

Experimental conditions

Eight, 945-L, elongated tanks (236 × 73 × 58 cm) equipped with biofiltration, water recirculation, aeration and temperature control capacities were used. Four hundred bluegill, 56.6 ± 20 g (mean weight ± SD), were randomly allotted to these tanks, 50 fish per tank. Tanks were half-filled to achieve approximate water volumes of 500 L per tank. Two feeds were simultaneously tested, using four tanks per test feed, with feces being collected from 200 bluegills per test feed. Water temperatures of 21.0 ± 1˚C and dissolved oxygen levels of 8.0 ± 0.5 ppm were maintained. Levels of NH_3-N and NO_2-N levels were monitored and maintained at < 1.0 ppm and < 0.1 ppm, respectively. A summer-like photoperiod (14 L: 10 D) was maintained throughout the experiment.

Feeding and feces collection

Bluegills were feed deprived for two days for gastric emptying prior to receiving the test feed. Fish were then fed the test feed twice daily at 09:00 and 17:00 hours, for three consecutive days. Finally, bluegills were provided a commercial feed (Aquamax-Grower-400, Purina 45 % crude protein, 16 % crude fat) for two consecutive days. This same 7-d procedure was repeated for each of the test feeds. Satiation feeding was followed throughout the experiment. Feeding bluegills the commercial diet between test diets was done to avoid nutrient deficiencies that might arise due to the use of a single-ingredient test feed. Two test feeds were evaluated in each week, with SBM and BM being exceptions for which feeding was continued to second weeks, in order to secure

adequate amounts of feces for analyses (6 g dry weight). All seven feeds were ultimately evaluated within a 5-week period.

A siphoning method (Windell *et al*. 1978) was used to collect feces associated with each test feed. Uneaten feed pellets and feces were siphoned from the tanks within 15 to 30 minutes after each feeding. Feces for each feedstuff were collected from the four tanks by slow siphoning three-times daily between 5 and 6 h after the first and second feedings, and again between 7 and 8 h after the second feces collection which occurred just prior to the first feeding of the next day. Care was taken to collect only unbroken feces to minimize nutrient leaching. During the 3-d test feeding period, feces collection was started on day 2. Feces voided on day 1 were not considered to avoid possible feces contamination from commercial feed fed previously. Each day, feces associated with a given feedstuff were collected from 200 bluegills, combined, and preserved at -20 °C until analyses were carried out. Just prior to analyses, feces were oven dried, finely ground and sieved (300 μm).

Largemouth bass experiment

An experiment that largely paralleled that for bluegill was conducted for juvenile largemouth bass. Four hundred largemouth bass (29.8 ± 15 g, mean weight ± SD) were allocated at random to the eight, previously described, 945-L elongated tanks, with 50 fish per tank. Three feedstuffs including FM, PBM and SBM were considered for evaluation**.** On week one, FM and PBM were evaluated with satiation feeding, from the feces produced by 200 largemouth bass in four tanks per feedstuff. Largemouth bass

would not readily consume SBM. Consequently the test feed prepared for SBM was force fed to 150 largemouth bass held in three tanks (50 fish per tank). Fish numbers for evaluating SBM were reduced because of practical difficulties in feeding > 150 fish. Force feeding was done once daily using a modified syringe connected to soft plastic tube (1.5 mm diameter, 7 cm length). The feed was macerated with water at a 1:3 ratio (feed:water) and mixed thoroughly. The wet feed (~3 % of body weight provided daily) was injected directly into the esophagus of the largemouth bass via the plastic tube. This feeding approach was continued for two weeks to obtain a sufficient quantity of feces. All other procedures including feces collection and preservation were as in the bluegill experiment.

Chemical and Statistical Analyses

All laboratory analyses for both feed and feces of bluegill and of largemouth bass followed procedures recommended by Association of Official Analytical Chemists (AOAC 2000). Gross energy content was analyzed using an adiabatic bomb calorimeter (Parr Instrument Co., Moline, IL, USA). Amino acids were analyzed with an automatic analyzer (Model 835-50, Hitachi Ltd., Tokyo, Japan) that included an ion exchange column. Ash content was determined by incinerating the feed samples at 550 ˚C for 12 h in a muffle furnace. Chromic oxide concentration was determined by a wet-acid-digestion method (Furukawa & Tsukahara 1966).

Apparent digestibility coefficients (ADCs) for dry matter, amino acids, and energy contents of the test diets were determined using the formula (Cho *et al*. 1982):

ADC of nutrients and energy (%)

$$= 100 - 100 \times \left(\frac{\text{\% chromium in feed}}{\text{\% chromium in feces}} \right) \times \left(\frac{\text{\% nutrient in feces}}{\text{\% nutrient in feed}} \right)$$

ADC of dry matter (%) = 100 – 100 (% chromium in feed / % chromium in feces)

Differences among mean ADCs for both dry matter and energy for the various test diets were evaluated with one-way ANOVA ($P < 0.05$) and, where appropriate, means were separated by Tukey's test for multiple comparisons (Statistical Analysis System, Version 9.1; SAS Institute Inc., Cary, NC, USA). Statistical tests were not applied to evaluate differences among amino acid digestibility means for the various feedstuffs due to lack of replication.

RESULTS AND DISCUSSION

Test diet acceptability

Test diets for FM, MBM and corn were immediately accepted by bluegill. The remaining four test feeds (PBM, SBM, BM and wheat) were consumed only modestly at the first offering, but were readily consumed upon the second offering. Largemouth bass immediately consumed FM and PBM, but would not accept SBM. SBM was ultimately force fed. Consistent with the observation concerning SBM, Kubitza *et al*. (1997) found that adding betaine at 0.6 % failed to stimulate largemouth bass to consume a diet in which 60 % of the fish meal had been replaced by soybean meal.

Nutrient deficiency has been a concern when using single-ingredient test diets to determine ADCs for feedstuffs (Bureau *et al*. 2002). However, no bluegill mortalities occurred and only four of the 400 largemouth bass perished (1 % mortality) during the experiments. Moreover, all surviving bluegill and largemouth bass appeared to remain healthy. The high survival rates and apparent good health of both bluegill and largemouth bass throughout the study could owe to having periodically provided the commercial diet. Alternatively, the duration of experiments (~1 month each) may simply not have been long enough to cause substantial nutrient deficiencies. Vitamins and minerals can be incorporated into deficient test diets in cases where experiments will run longer, as was done by Lupatsch *et al*. (1997).

Dry matter digestibility

For bluegill, mean ADC values for dry matter differed ($P < 0.05$) among all seven feedstuffs except for BM and for SBM and FM (Table 2). Results indicated the greatest and least utilization of nutrients from BM (87 %) and corn (50 %), respectively. Higher digestibility coefficients (> 77 %) for BM, FM, PBM and SBM than for wheat or corn, further indicate that bluegill are more efficient at utilizing protein and lipid sources than carbohydrate sources. Higher dry matter digestibility values for animal versus plant feedstuffs have been observed particularly among carnivorous fishes (Cho *et al*. 1982; Sullivan & Reigh 1995; Lee 2002). However, the high content of protein and the preheating process may increase the availability of nutrients from plant products. For example, high dry matter digestibility values (80 %) from protein–rich, extruded SBM

were reported for carnivorous Murray cod (*Maccullochella peelii peelii*) and Australian shortfin eel (*Anguilla australis*) (De Silva *et al*. 2000). Among the protein sources, the relatively low digestibility (~60 %) of MBM that was observed for bluegill may be attributable to the high ash content (198.7 g Kg^{-1}) of this feedstuff. Low digestibility (60-70 %) of MBM owing to high ash content (200-300 g Kg^{-1}) was also reported by Bureau *et al*. (1999) for rainbow trout *Oncorhynchus mykiss*. Similar to bluegills, high dry matter digestibility values (> 70 %) were also observed for largemouth bass from FM, PBM and SBM (Table 3). In accordance with the results of the present study, Portz & Cyrino (2004) obtained high dry matter digestibility values from FM (70 %), PBM (83 %), and SBM (70 %) for largemouth bass when studied with compound diet.

Energy Digestibility

Analyses of energy digestibility results indicated significant differences ($P < 0.05$) among the feedstuffs provided to bluegill (Table 2) and largemouth bass (Table 3). Higher apparent digestible energy values were observed for protein sources than for carbohydrate sources for bluegill. Higher energy digestibility values for protein-rich animal feedstuffs versus for carbohydrate-rich-plant products are commonly observed in fishes (Cho *et al*. 1982; Sullivan & Reigh 1995). Typical level of nitrogen free extract (NFE or carbohydrate) from animal protein sources (BM, FM, MBM and PBM) is < 4 % whereas, such value for SBM is ~30 % and for wheat and corn is ~80 % (Hertrampf & Piedad-Pascual 2000). Fish in general are poor in digesting carbohydrate, although omnivorous and herbivorous fishes are relatively better in utilizing carbohydrate

than piscivorous fishes (Stone 2003). Nitrogen free extract levels from the evaluated feedstuffs appear to have determined the degree of energy digestibility for bluegill and largemouth bass as have been commonly observed in other fishes (Stone 2003). MBM was poorly digested by bluegill, perhaps because of it high content of low digestible ash, as was also observed for rainbow trout (Bureau *et al*.1999) and gilthead seabream *Sparus aurata* (Robaina *et al.* 1999).

Digestibility of amino acids

Apparent digestibility coefficients of amino acids (AAs) for bluegill and for largemouth bass are given in Tables 2 and 3, respectively. Soybean meal showed the highest digestibility (~95 %) among the protein sources tested for bluegill and largemouth bass. High apparent digestibility of essential amino acids (EAAs) from SBM has been reported for many fishes including rainbow trout *Oncorhynchus mykiss* (95%) (Yamamoto *et al*. 1998), rock fish (> 85 %) (Lee 2002), Murray cod, and shortfin eel (> 85 %) (De Silva *et al*. 2000), while low digestibilities were found for yellowtail, *Seriola quinqueradiata* (53-85 %) (Masumoto *et al*. 1996) and channel catfish (81-93 %) (Wilson *et al*. 1981). Although largemouth bass were force fed SBM, no adverse effects were evident. Digestibility values for SBM for largemouth bass were similar to those of bluegill, suggesting that minimal stress on the digestive capacity of largemouth bass resulted from the force feeding. Wilson *et al*. (1981) also found no effect from force feeding on digestibility of EAAs from SBM for channel catfish.

As for SBM, FM and PBM showed high values (> 90 %) of AA digestibility for bluegill and largemouth bass. Apparent digestibilities of EAAs from FM have been found to be high (> 90 %) in fishes, e.g., Atlantic salmon *Salmo salar,* (Anderson *et al*. 1995), rainbow trout, red sea bream (*Pagrus majar*), common carp (*Cyprinus carpio*) (Yamamoto *et al*. 1998), and striped bass (Small *et al*. 1999). In contrast to our findings of high EAA digestibilities from PBM for bluegill and largemouth bass, Gaylord *et al*. (2004) found poor utilization of EAAs from this feedstuff by hybrid striped bass (mean digestibility of amino acids of 61 %). Poor utilization of amino acids from PBM by hybrid striped bass could partly be attributed to the composition of the reference diet that the study used – presence of ~8 % fiber (celufil) perhaps have diminished the absorption of other nutrients. Similar to the findings of the present study, Lupatsch *et al*. (1997) also observed high digestibility (80-91 %) of EAAs from PBM for seabream when using a single feed ingredient as the test diet. Further, Tidwell *et al*. (2005) completely replaced fish meal with PBM, in a diet fed to juvenile largemouth bass, and found no negative effects on growth rate or body composition, indicating the similarities in the available EAAs between fish meal and PBM for largemouth bass.

Blood meal showed the highest ADCs for majority of the amino acids (Table 4) among the protein sources that were evaluated for bluegills. Isoleucine was found to be least available to bluegills among all EAAs in BM. Similarly, least availability of isoleucine from BM were recorded also for hybrid striped bass *Morone chrysops* × *M. saxatilis,* (38 %) (Gaylord *et al*. 2004), and rockfish *Sebastes schlegeli* (65 %) (Lee 2002). The indicated low digestibility of isoleucine may owe to its low concentration in BM relative to other branched-chain amino acids such as leucine and valine that likely

compete with isoleucine for access to the blood stream (Gaylord *et al*. 2006)**.** This suggests an imbalance in the amino acid profile from BM which, despite its high percentage of total amino acid and energy availability, may ultimately suppress fish growth if the requirement exceeds the availability**.** Much as for dry matter and energy, the low digestibilities of amino acids from MBM that were determined for bluegill may owe to its high ash content (198.67 g Kg^{-1}) (Masumoto *et al*. 1996). Low digestibility of cystine from MBM to bluegill (54 %) is in agreement with findings for yellowtail (43 %) (Masumoto *et al*. 1996) and rock fish (64 %) (Lee 2002).

Despite the high percentage digestibilities of EAAs that were observed from the various feedstuffs for bluegills, quantitative availability of amino acids for bluegills (Table 4) varied substantially among the feedstuffs. For bluegill, all EAAs with the exceptions of isoleucine, and methionine, were available in large amounts from BM (Table 4). Relatively high amounts of arginine and isoleucine were determined from PBM, and likewise for methionine from FM. Meat and bone meal contained relatively low levels of lysine, methionine, and tryptophan, whereas SBM contained low amounts of methionine. Although cereals such as wheat and corn showed high percentage digestibilities for EAAs (80-90 %, Table 2), their quantitative availability values were very low (< 1 %) in bluegill (Table 4). A similar pattern of availability was observed for largemouth bass (Table 5) for which PBM showed the highest levels of quantitative availability for most EAAs, with exceptions being isoluecine, lysine, methionine, and tryptophan. Tryptophan was found to be highly available in SBM whereas the other three amino acids were high in FM.

Comparison of methods

Findings of the present study concerning ADCs of dry matter, energy, and EAAs for largemouth bass using the single-ingredient approach, compared well to those of Portz & Cyrino (2004) for FM, PBM, and SBM, based on the use of compound diets. For the most part, EAA digestibilities determined from the present study and that of Portz & Cyrino (2004) differed by no more than 10 % (Table 6). However, greater differences occurred between the studies for tryptophan from PBM (43 %) and FM (12 %) and for methionine from PBM (18 %) and SBM (14 %). The substantial difference for tryptophan may have resulted from error of analysis, owing to it's very low levels (0.46 %). Apparent digestibility coefficients for tryptophan have not been estimated in many studies (e.g., Yamamoto *et al*. 1998) largely because of problems posed from its low content and high-cost of analysis for accurate estimation. Portz & Cyrino (2004) likewise found a lower digestibility value for methionine relative to values reported in other studies, and also, that of other EAAs (except tryptophan) within their study. Methionine digestibility from fish meal or soybean meal has been reported to be > 90 % in other fish species including rainbow trout, common carp, red seabream (Yamamoto *et al.* 1998), and striped bass (Small *et al.* 1999). Variables including differences in quality of feedstuffs, ingredient processing methods, feces collection method, and fish age may also account for the differences observed in the present study and that of Portz & Cyrino's (2004). Thus, comparisons of methods under identical experimental conditions will give a more meaningful information as to the interaction of nutrients and their digestibility. Overall, comparisons of results of this study to those of Portz & Cyrino (2004) and the previously mentioned related studies indicate that similar outcomes occur from the two

methods for the majority of nutrients. An advantage associated with the use of single feed ingredients may be the elimination of inter-nutrient interactions (nutrient interaction across ingredients).

Conclusions

This experiment documents apparent dry matter, energy, and amino acid digestibility values for bluegill based on the use of a single feed ingredients as the test diets, and a slow-siphoning method for feces collection. High digestibility values determined for PBM, SBM, and BM, indicate opportunities for using these protein sources to replace expensive fish meal in the diets of bluegills. Potential levels for replacing FM can be predicted from nutrient requirements of bluegill. Results of the present study tend to support reasonable accuracy from the use of a slow-siphoning method for feces collection, coupled with using single feedstuffs almost exclusively in test diets. Such an approach should avoid problems that can arise from nutrient interactions when compound diets are used. Values provided from the present study can be used to more accurately formulate much needed feeds for bluegill.

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Table 1. Chemical composition (dry matter basis) of the test feedstuffs
(*n* = 2 samples for gross energy and ash).

Components	BM[a]	FM[b]	MBM[c]	PBM[d]	SBM[e]	Corn[f]	Wheat[g]
Gross Energy (MJ Kg^{-1})	23.6	17.7	16.9	20.8	18.4	16.7	16.4
Ash (g Kg^{-1})	46.8	146.5	198.7	84.9	57.3	14.4	20.9
AA (g Kg^{-1})							
EAA							
Arginine	36.3	34.9	34.1	45.0	35.4	4.3	6.3
Histidine	61.2	13.1	9.1	14.9	12.1	2.2	2.6
Isoleucine	7.6	24.9	12.8	25.9	21.5	3.0	4.0
Leucine	114.7	43.8	29.9	47.1	36.9	9.7	8.1
Lysine	73.2	45.7	25.2	44.3	29.4	2.7	3.4
Methionine	7.0	15.9	6.7	13.7	6.7	1.9	2.4
Phenylalanine	56.4	24.1	16.5	25.7	23.9	4.0	5.4
Threonine	27.0	24.4	15.3	25.4	18.4	3.0	3.6
Tryptophan	9.2	4.8	1.9	4.6	5.4		1.1
Valine	79.1	29.8	20.8	32.2	22.8	4.0	5.0
∑EAA	471.7	261.4	172.3	278.8	212.5	35.2	41.9
NEAA							
Aspartic Acid	98.5	53.6	35.7	54.1	54.3	5.6	6.7
Glutamic Acid	71.2	79.1	59.6	89.4	89.3	15.0	35.8
Alanine	68.9	38.1	34.7	41.6	20.9	6.1	4.7
Cysteine	6.9	4.8	5.1	6.8	6.7	1.7	2.8
Glycine	42.7	39.8	61.2	55.2	20.6	3.5	5.3
Serine	36.0	21.6	19.0	25.5	22.9	3.8	5.9
Proline	30.0	25.6	38.8	38.1	23.3	6.6	11.1
Tyrosine	11.5	15.4	8.5	18.7	14.5	2.1	2.4
∑NEAA	365.7	278.0	262.6	329.4	252.5	44.4	74.7
∑AA (Protein)	839.3	554.1	466.5	633.8	466.8	80.4	118.0

[a] Blood meal (BM), International feed number (IFN): 5-00-381
[b] Fish meal (FM), IFN: 5-02-009
[c] Meat and bone meal (MBM), IFN: 5-00-388
[d] Poultry byproduct meal (PBM), IFN: 5-03-798
[e] Soybean meal (SBM), IFN: 5-04-597
[f] Wheat, IFN: 4-05-205
[g] Corn, IFN: 4-02-935
Abbreviations: AA, amino acid; EAA, essential amino acid; NEAA, nonessential amino acid.

Table 2. Apparent dry matter, energy, and amino acid digestibility coefficients (%) of test ingredients for bluegills.

ADC (%)	BM	FM	MBM	PBM	SBM	Corn	Wheat
Dry matter	86.7[a]	77.6[c]	58.2[d]	83.4[b]	79.0[c]	50.0[f]	55.5[e]
Energy	91.8[a]	87.4[a]	72.3[c]	87.0b[ab]	79.8[b]	53.0[d]	55.3[d]
EAA							
Arg	91.5	93.9	83.6	94.7	97.0	91.8	92.7
His	94.0	91.9	86.0	93.5	96.4	90.5	91.9
Ile	81.9	92.6	83.4	90.6	94.2	81.9	85.4
Leu	92.4	93.1	84.3	91.0	94.1	91.9	90.8
Lys	95.1	94.8	85.8	95.4	95.7	80.1	82.7
Met	94.4	91.8	85.3	92.7	94.3	85.1	90.4
Phe	94.3	91.7	83.5	90.1	94.5	89.0	91.5
Thr	92.7	93.5	82.6	92.6	93.6	78.4	85.2
Trp	97.5	92.5	89.0	92.8	96.9		83.8
Val	92.4	91.7	82.1	89.0	93.7	90.6	89.3
∑EAA	93.2	93.1	83.0	92.4	95.0	94.8	89.2
NEAA							
Asp	93.5	91.0	82.3	91.9	96.0	86.0	87.8
Glu	94.4	93.2	83.3	92.9	96.8	94.2	97.6
Ala	94.4	91.7	83.3	90.7	92.6	87.9	83.6
Cys	85.8	82.9	53.6	82.3	94.7	80.1	90.4
Gly	93.0	88.2	80.6	91.0	93.0	81.2	85.7
Ser	93.8	92.1	81.1	91.7	95.5	88.5	92.9
Pro	92.6	90.8	80.3	91.6	95.3	90.2	95.6
Tyr	94.4	91.7	79.5	92.5	95.7	80.6	86.3
∑NEAA	93.6	91.3	80.0	91.7	94.7	95.4	89.6
∑AA	84.1	92.0	81.8	91.9	95.1	87.5	91.6

[a-f] Mean dry matter (n = 2 samples) within a row sharing different superscripts indicate significant differences ($P < 0.05$) between feedstuffs.

[a-d] Mean energy (n = 2 samples) within a row sharing different superscripts indicate significant differences ($P < 0.05$) between feedstuffs.

Abbreviations: AA, amino acid; EAA, essential amino acid; NEAA, nonessential amino acid.

Table 3. Apparent dry matter, energy and amino acid digestibility coefficients (%) of test ingredients for largemouth bass.

ADC (%)	FM	PBM	SBM
Dry matter	72.7[a]	75.9[a]	75.0[a]
Energy	87.2[a]	84.2[ab]	79.9[b]
EAA			
Arg	93.2	91.1	97.3
His	90.6	89.4	95.5
Ile	91.1	86.4	93.2
Leu	92.5	87.8	93.8
Lys	94.5	92.5	96.0
Met	91.6	89.7	94.4
Phe	90.7	87.6	93.7
Thr	92.3	89.1	91.9
Trp	93.8	94.3	96.3
Val	91.0	85.2	91.7
∑EAA	92.3	89.0	94.4
NEAA			
Asp	89.5	86.3	96.0
Glu	92.8	89.7	96.5
Ala	91.1	88.0	91.3
Cys	80.7	72.2	94.0
Gly	87.4	87.1	92.7
Ser	91.4	88.3	95.9
Pro	90.0	88.1	94.9
Tyr	92.0	90.3	96.0
∑NEAA	91.4	87.9	94.9
∑AA	91.3	88.4	94.7

[a] Mean dry matter (n = 2 samples) within a row sharing same superscript indicate no significant differences ($P < 0.05$) between feedstuffs.

[a-c] Mean energy (n = 2samples) within a row sharing different superscripts indicate significant differences ($P < 0.05$) between feedstuffs.

Abbreviations: AA, amino acid; EAA, essential amino acid; NEAA, nonessential amino acid.

Table 4. Availability of apparent digestible energy, and amino acids (dry matter basis) from various feedstuffs for bluegills.

	BM	FM	MBM	PBM	SBM	Corn	Wheat
Energy (MJ Kg^{-1})	21.7[a]	15.5[c]	12.2[d]	18.1[b]	14.7[c]	9.5[e]	9.1[e]
AA (g Kg^{-1})							
EAA							
Arg	33.2	32.8	28.5	42.6	34.3	3.9	5.8
His	57.5	12.0	7.8	13.9	11.7	2.0	2.4
Ile	6.2	23.1	10.7	23.5	20.2	2.5	3.4
Leu	106.0	40.8	25.2	42.9	34.7	8.9	7.4
Lys	69.6	43.3	21.6	42.3	28.1	2.2	2.8
Met	6.6	14.6	5.7	12.7	6.3	1.6	2.2
Phe	53.2	22.1	13.8	23.2	22.6	3.6	4.9
Thr	25.0	22.8	12.6	23.5	17.2	2.4	3.1
Trp	9.0	4.4	1.7	4.3	5.2		0.9
Val	73.1	27.3	17.1	28.6	21.4	3.6	4.5
∑EAA	439.5	243.3	143.0	257.5	201.8	33.0	37.4
NEAA							
Asp	92.1	48.8	29.4	49.7	52.1	4.8	5.9
Glu	67.2	73.7	49.6	83.1	86.4	14.1	35.0
Ala	65.1	34.9	28.9	37.7	19.4	5.4	3.9
Cys	5.9	4.0	2.7	5.6	6.3	1.4	2.5
Gly	39.7	35.1	49.3	50.2	19.1	2.8	4.5
Ser	33.8	19.9	15.4	23.4	21.9	3.4	5.5
Pro	27.8	23.2	31.2	34.9	22.2	6.0	10.6
Tyr	10.9	14.1	6.8	17.3	13.9	1.7	2.1
∑NEAA	342.3	253.7	210.0	301.9	239.1	42.3	66.9
∑AA	781.8	497.0	353.0	559.4	440.9	75.3	104.3

[a-e] Mean energy (n = 2 samples) within a row sharing different superscripts indicate significant differences ($P < 0.05$) between feedstuffs.

Abbreviations: AA, amino acid; EAA, essential amino acid; NEAA, nonessential amino acid.

Table 5. Availability of apparent digestible energy, and amino acids (dry matter basis) from various feedstuffs for largemouth bass.

	FM	PBM	SBM
Energy (MJ Kg^{-1})	15.5^{b}	17.5^{a}	14.7^{b}
AA (g Kg^{-1}) EAA			
Arg	32.5	41.0	34.4
His	11.9	13.3	11.5
Ile	22.7	22.4	20.0
Leu	40.5	41.3	34.6
Lys	43.2	41.0	28.2
Met	14.6	12.3	6.3
Phe	21.9	22.5	22.4
Thr	22.5	22.6	16.9
Trp	4.5	4.3	5.2
Val	27.1	27.4	20.9
∑EAA	241.4	248.2	200.6
NEAA			
Asp	48.0	46.7	52.1
Glu	73.4	80.2	86.1
Ala	34.7	36.6	19.1
Cys	3.9	4.9	6.3
Gly	34.8	48.1	19.1
Ser	19.7	22.5	22.0
Pro	23.0	33.6	22.1
Tyr	14.2	16.9	13.9
∑NEAA	254.1	289.5	239.6
∑AA	506.0	560.3	442.1

[a-b] Mean energy (n = 2 samples) within a row sharing different superscripts indicate significant differences ($P < 0.05$)

Abbreviations: AA, amino acid; EAA, essential amino acid; NEAA, nonessential amino acid.

Table 6. Difference between our results based on single-ingredient test diet versus those of Portz and Cyrino's study based on compound test diet on the digestibility of FM, PBM and SBM for largemouth bass.

ADC (%)	FM	PBM	SBM
Dry matter	2.7	-6.7	4.6
Energy	8.9	-1.0	4.5
EAA (essential amino acid)			
Arg	0.7	-0.1	-0.6
His	4.8	-3.7	4.5
Ile	2.2	0.6	-3.4
Leu	6.8	-0.9	-3.8
Lys	-1.3	1.7	-0.1
Met	8.9	18.4	14.1
Phe	-0.4	0.1	-1.0
Thr	4.3	3.0	-4.4
Trp	11.6	42.8	9.7
Val	-1.0	2.2	-6.9
∑EAA	2.5	2.1	-1.5

CHAPTER 2

DIETARY REQUIREMENTS FOR DIGESTIBLE ESSENTIAL AMINO ACIDS FOR GROUP- VERSUS INDIVIDUALLY- HOUSED JUVENILE BLUEGILL, *LEPOMIS MACROCHIRUS*

ABSTRACT

Two 60-d experiments were conducted sequentially to determine (i) lysine requirement of juvenile bluegill *Lepomis macrochirus* based on the dose-response method, (ii) requirements for other essential amino acids (EAAs) using whole-body amino acid profile, and (iii) whether differences in growth rates of group- versus individually-housed bluegills lead to different lysine requirement levels due to the presence and absence, respectively, of social hierarchies. Seven, semi-purified, experimental diets (isonitrogenous, isocaloric) were prepared to contain graded levels of digestible lysine (10-31 g Kg^{-1}). Experiment-1 involved group-housed bluegills (~27 g, n = 10 fish/chamber, 4 chambers/diet) whereas experiment-2 involved individually-housed bluegills (~30 g, n= 1 fish/chamber, 14 chambers/diet). Fish were fed twice daily to apparent satiation. Bluegill growth responses in both experiments generally improved ($P < 0.05$, ANOVA) with increasing dietary lysine levels from 10 to 16 g Kg^{-1}, and then leveled off with further increase in lysine level ($P > 0.05$). Optimal dietary lysine level (digestible basis) was estimated to be 15 g Kg^{-1} based on broken-line regression analyses of relative growth rate and feed conversion ratio with no differences being observed between the two rearing methods. Determined dietary requirement levels for other EAAs ranged from 2.4 g Kg^{-1} (tryptophan) to 15.3 g Kg^{-1} (leucine).

INTRODUCTION

Bluegill *Lepomis macrochirus*, once considered an emerging aquaculture species in the U.S. (Morris & Mischke 2003), now appears to be moving towards larger-scale production due to increased interest in this species as a food fish, coupled with evidence that it, and related hybrids, can be efficiently grown to food size through selective breeding (Hayward & Wang 2006; Hicks *et al.* 2009). However, no diets specifically developed for bluegill exist, due to a paucity of information concerning this species' nutritional requirements. Bluegill require high percentages of dietary protein. Hoagland *et al.* (2003), for example, determined the protein requirement of juvenile bluegills to be ≥ 44 %. Twibell *et al.* (2003) demonstrated that juvenile bluegill performed better with high-protein (≥ 44 %) trout diets than with lower-protein (≤ 36 %) catfish diets. However, protein-rich trout diets are expensive, adding substantially (> 60 %) to annual variable costs of commercial bluegill production (Curtis Harrison, Harrison Fisheries, Inc., MO, pers. comm.). One way to reduce feed costs for bluegill would be to minimize excessive dietary nutrients that are expensive, and to meet nutrient requirements to a greater extent through lower-cost dietary ingredients.

Fish do not require protein *per se*, rather, they require amino acids (AAs) that comprise protein. Ten essential AAs (EAAs) must be provided via dietary sources as they cannot be synthesized by fish (Wilson & Halver 1986). Dietary deficiency in any of the EAAs will impair protein synthesis and suppress fish growth in general (Wilson 2002). Diets based on fish meal protein are more likely to meet amino acid requirements of fishes (Gatlin *et al.* 2007). However, given the high and increasing costs of fish meal protein over recent years, much interest has emerged in identifying less costly alternative

protein sources for fish diets (Tacon & Metian 2008). When the fish meal component in a fish diet is replaced by alternative proteins, meeting adequate levels of dietary protein alone does not guarantee adequate levels of EAAs. This is because the amino acid profiles of alternative proteins tend not to match those of balanced diets to the extent that fish meal does (Hardy 2008). Hence, while diets based on alternative protein sources may provide sufficient protein, unless EAAs are adequately supplemented, such diets will suppress fish growth. The "ideal protein" refers to dietary protein that supplies exact requirements of amino acids with no deficiency and no excess, and supports optimal growth performance (Firman & Boling 1998). Determining the dietary requirement of EAAs is an essential aspect of developing a complete diet for a given fish species and life stage. To date, however, no data concerning amino acid requirements are available for bluegill or their hybrids (e.g., *Lepomis cyanellus* × *L. macrochirus*).

Requirements for EAAs have been determined conventionally by dose-response experiments for each amino acid. However, this approach is both time consuming and expensive. The pattern of EAA requirements for growth as determined by growth-response trials, is correlated with that of EAAs deposited in the whole-body tissue in fishes e.g., coho salmon *Oncorhynchus kisutch* (Arai 1981) and channel catfish *Ictalurus punctatus* (Wilson & Poe 1985). Such correlation tends to explain why fish meal has been considered an ideal protein source when the amino acid requirement of a fish species is not known. Accordingly, if the requirement of a single limiting amino acid is known, requirements for the remaining EAAs can be accurately estimated from the ratio of the whole-body amino acid pattern (A/E ratio) of a species, in much less time for far less cost (Akiyama *et al.* 1997). Lysine has been used as a reference amino acid in fish

(Small & Soares 1998; Montes-Girao & Fracalossi 2006), and higher animals (Emmert & Baker 1997), mainly because of its key role in protein deposition. Requirements of EAAs based on this approach have been determined in many fishes including striped bass *Morone saxatilis* (Brown 1995), jundia *Rhamdia quelen* (Montes-Girao & Fracalossi 2006), and largemouth bass *Micropterus salmoides* (Dairiki 2007). Moreover, amino acid requirements determined through this approach have been found not to differ significantly from those determined via the more arduous dose-response method in fishes including channel catfish (Wilson 2002). Consequently, the present study sought to develop the "ideal protein" for juvenile bluegills by determining (i) their dietary lysine requirement, and (ii) their requirement for other EAAs, based on the whole-body amino acid profile and determined dietary lysine level.

Social hierarchy effects in bluegill studies

Bluegills are aggressive fish (Henderson & Chiszar 1977) and are known to form social hierarchies when reared in groups, which typically lead to a relatively few dominant individuals acquiring high percentages of the feed provided, while the remaining fish, to varying degrees receive less feed. Consequences of social hierarchy formation among group-reared bluegills may include reduced mean consumption, poor growth, decreased feed efficiency and increased size variation (Hayward & Wang 2002). Rearing bluegills individually in test chambers offers an alternative approach for eliminating social hierarchies and their undesirable effects on diet performance studies. However, individual rearing has been viewed as exceeding the bounds of standard rearing methods for diet studies by some fish nutritionists, who maintain that such evaluations

must involve fish that are reared in confined groups. On the other hand, the reduced overall growth rates of bluegills that typically occur under group rearing from dominance hierarchy formation have also been criticized, for possible inadequate growth separation among dietary groups.

Despite the criticisms that have been leveled by fish nutritionists at both rearing approaches, it has not been shown whether differences in bluegill's growth performance under the two rearing methods, in fact lead to different outcomes concerning lysine requirement. Consequently, the study compared the lysine requirement determined from group-reared bluegills to that determined from multiple, individually-housed bluegills, in order to elucidate whether the two rearing approaches in fact lead to different indications of lysine requirement for juvenile bluegills.

MATERIAL AND METHODS

Determination of dietary lysine requirement level

Digestibility of Feedstuffs and Experimental Diets

Corn gluten meal, fish meal, soybean meal and wheat were used as intact protein sources. Digestibility values of amino acids and energy from fish meal, soybean meal, and wheat for juvenile bluegill were taken from Masagounder *et al.* (2009), whereas such values for corn gluten meal were determined from procedures similar to those described by Masagounder *et al.* (2009). The test diet for corn gluten meal was prepared by mixing 985 g Kg^{-1} corn gluten meal, 5 g Kg^{-1} chromic oxide, 5 g Kg^{-1} betaine, and 5 g Kg^{-1} of a

commercial binder (Ultra-Bond™, Uniscope, Incorporated, Johnstown, CO, USA). Ingredients were mixed in a Hobart mixer (Hobart Corporation, Troy, OH, USA), and extruded with a twin-screw extruder. Duplicate bluegill groups (35.5 ± 15 g, mean weight ± SD) of 50 fish each were fed the test diets until sufficient feces were collected. Procedures for fish feeding, feces collection (siphoning method), and analyses, again, followed those used in an earlier study (Masagounder *et al.* 2009). Digestibility values obtained for corn gluten meal (Table 1) and for other ingredients were used to determine digestible lysine values in the test diets. Basal diets were formulated to provide 10 g Kg^{-1} of digestible lysine. Glutamic acid in the basal diet was gradually replaced (on a weight basis) by lysine-HCl, giving seven experimental diets in total with digestible lysine levels (dry weight basis) of 10 g Kg^{-1}, 13 g Kg^{-1}, 16 g Kg^{-1}, 19 g Kg^{-1}, 22 g Kg^{-1}, 25 g Kg^{-1} and 31 g Kg^{-1} (designated as Lys10, Lys13, Lys16, Lys19, Lys22, Lys25 and Lys31, respectively). A commercial binder (Ultra-Bond™, Uniscope, Inc., Johnstown, CO, USA) was added in the experimental diets to minimize leaching of nutrients. Formulations of experimental diets are summarized in Table 2. Gross protein levels from fish meal, soybean meal and wheat were estimated to be 589.1 g Kg^{-1}, 445.8 g Kg^{-1} and 111.4 g Kg^{-1}, respectively. Digestible protein levels for these feedstuffs were determined from their digestibility value of total amino acids (Masagounder *et al.* 2009) and estimated gross protein levels. Digestible protein levels from the synthetic amino acids were assumed to be 100 %. Digestibility of energy from fish oil and lecithin was assumed to be 90 %, and that of synthetic amino acids was assumed to be 100 % (NRC 1994). Diets were formulated to be isonitrogenous and isocaloric with digestible protein and energy levels being ~390 g Kg^{-1} and ~16.6 MJ Kg^{-1}, respectively.

Coarse ingredients were ground and sieved with a 500 µm screen in a Fitzmill (W. J. Fitzpatrick Company, Chicago, Illinois, USA). Dry ingredients were then sequentially mixed in a Hobart mixer (Hobart Corporation, Troy, OH, USA). All diets were extruded with a twin-screw extruder at the Food Protein R&D Center, Texas A&M University, College Station, TX, USA. Diets were then air dried, packaged in air-tight bags, transported to the University of Missouri, Columbia, MO, USA, and stored under air-tight conditions at 4 °C until used. Nutrient compositions of the diets are given in Table 3. Gross protein and energy levels of the experimental diets were ~500 g Kg^{-1} and ~21 MJ Kg^{-1}, respectively. Total lysine levels in the semi-purified experimental diets ranged from 12 g Kg^{-1} to 33 g Kg^{-1}. Levels of all other amino acids were kept above those recommended by NRC (1993) for common freshwater fishes.

Feeding Trial and Data Collection

Group rearing

Juvenile bluegill were purchased from a commercial fish producer (Harrison Fisheries, Inc., Hurdland, MO, USA) and transported to the Fish Growth and Nutrition Laboratory at the University of Missouri, Columbia, MO, USA. Upon arrival, the fish were acclimated to laboratory conditions for 2 weeks. Four rectangular tanks (236 × 73 × 58 cm; water holding capacity = 945 L) each equipped with biofiltration, water-recirculation/re-aeration and temperature-control capacities were used in the study. Seven perforated, plastic test chambers (43 × 30 × 43 cm) whose screen-covered tops protruded above tank water levels, were placed in each of the four tanks giving 28 test chambers.

Tanks were filled to 75 % of their heights such that water volumes of 40 L resulted in each chamber. Water from a head tank and biofilter was trickled into each chamber via a perforated PVC pipe that ran above each fish tank. Acclimated mixed-sex, juvenile bluegills (~27 g) were randomly allotted to the test chambers at 10 fish per chamber, and further acclimated for seven days. In each of the four tanks, the seven test diets were randomly allotted to the seven test chambers, giving one replicate per tank for each test diet (total N=4 replicates per test diet). The experiment followed a randomized complete block design.

Fish were hand-fed twice daily to apparent satiation at 0800 and 1600 h. Feces were siphoned out prior to each feeding. Feeding was continued for ~1 h at each feeding time. Any feed pellets that remained in a chamber as of 30 min post feeding were removed by siphoning under no-flow conditions and stored at -20 °C until the end of the study. After completion of the 60-d feeding trial, preserved, uneaten pellets from each chamber were dried at 70 °C for 48 h and weighed. Leaching of test diets was accounted when determining weights of unconsumed pellets. Upon completion of the study, and after removing fish from test chambers, test diets of known dry weights were immersed for 1 h in water-filled chambers and then siphoned out and dried. The percentage weight loss from leaching was then added to the weights of uneaten pellets. The dry weight of the unconsumed feed was then subtracted from the total feed weight provided to determine total feed consumption by bluegills in each chamber. Means ± 1SD of daily recorded tank water temperatures and dissolved oxygen levels were 22.3 ± 1.2 °C and 7.2 ± 0.3 mg L^{-1}, respectively. Weekly determined NH_3-N and NO_2-N levels remained

< 0.1 mg L^{-1}, while a summer-like photoperiod (14 L: 10 D) was continued throughout the 60-d experiment.

Live weights of fish from each chamber were determined on days 0 and 60. At the end of the experiment, bluegill were euthanatized with an overdose of MS222 (Aquatic Eco-systems, Apopka, FL, USA). Six randomly selected fish from each chamber were used to determine whole-body proximate composition. Values of the following indices were determined from all fish in each of the replicate test chambers over the 60-d experiment period, and averaged across the four replicates for each of the seven experimental diets:

Total feed consumption (g/fish) = (total feed provided (g) – total unconsumed feed (g)) / N_f, where N_f is the average number of fish fed per day in a chamber.

$N_f = (n_1+n_2+n_3+\ldots+n_{60}) / 60$, where n_1, n_2, n_3, n_{60} are the total number of fish fed in a chamber on days 1, 2, 3, 60, respectively,

Relative growth rate, RGR (g $100g^{-1}$ d^{-1}) = (wet weight gain (g) × 100 / average fish weight (g) / t), where average fish weight = (final weight + initial weight) / 2, and t is the duration of the experiment (60 d). Because bluegills of 30 g had surpassed the early logarithmic growth phase, RGR was used rather than specific growth rate (SGR) to report fish growth (Hopkins 1992).

Feed conversion ratio (FCR) = total dry feed fed (g) / wet weight gain (g),

Protein efficiency ratio (PER) = wet weight gain (g)/ total protein fed (g),

Apparent protein utilization (APU) (%) = protein gain (g) ×100/ total protein fed (g).

Individual rearing

Juvenile bluegills were purchased from a local fish producer (Harrison Fisheries, Inc., Hurdland, MO, USA) and acclimated to laboratory conditions for 2 weeks. Seven rectangular tanks (236 × 73 × 58 cm; water holding capacity = 945 L), each equipped with water recirculation, biofiltration, aeration and temperature-control capacities, were used for the feeding trial. Fourteen perforated, plastic test chambers (30 × 24 × 40 cm) were placed in each of the seven tanks, giving 98 test chambers. Tanks were filled to one-half of their heights such that water volumes of 15 L resulted in each chamber. Acclimated bluegills weighing 30.48 ± 7.43 g (mean weight ± SD) were then randomly allotted to the test chambers, one fish per chamber. In each of the seven tanks, the seven test diets were randomly allotted to the 14 chambers, giving two replicates for each test diet per tank (total N = 14 per test diet). The experiment followed a randomized complete block design. The feeding protocol followed the previous trial with the exception that uneaten pellets were not collected. Collecting uneaten pellets from the 98 chambers on twice daily basis was not feasible. However, strong efforts were made not to provide excess amounts of feed by observing feeding activity and feeding multiple times during each feeding. Mean ± SD daily recorded tank water temperatures and dissolved oxygen levels were 22.0 ± 1.3 °C and 7.7 ± 0.6 mg/L, respectively. Weekly determined NH_3-N and NO_2-N levels remained < 0.1 mg L^{-1}, while a summer-like photoperiod (14 L: 10 D) was continued throughout the 60-d experiment. Live weights of individual fish were measured on days 0 and 60. Growth performance of bluegills was assessed via RGR, FCR, PER and APU. At the end of the experiment, fish were euthanized and their whole-body proximate composition was determined for all fish in each dietary group.

Change in the coefficient of size variation (CV) was determined among the individually- as well as group-housed bluegills, to evaluate potential social hierarchy establishment among the group-housed fish. Change in CV = (final CV – initial CV) ×100/ initial CV, where CV = standard deviation of weight × 100 / mean weight.

Determination of dietary requirements for other EAAs

Ten wild-caught juvenile bluegills (31.2 ± 16.4 g, mean weight ± SD) were euthanized, placed in crushed ice and transported to the laboratory. After removing intestinal contents, the fish were dried at 70 °C for 3 d, ground, mixed, and sieved with 1-mm mesh. Four randomly selected fish samples were used to determine amino acid compositions of whole-body tissue. Ratios of essential amino acids (A/E ratios) were calculated as:

A/E ratio = individual essential amino acid content in whole body × 1000 / (total essential amino acid content including cystine and tyrosine).

The ratio of EAAs (Table 6) was then used to calculate the dietary requirements of amino acids based on the determined lysine requirement levels.

Chemical and Statistical Analyses

All laboratory analyses (moisture, crude protein, amino acids, crude lipid, ash, and chromium) followed procedures recommended by the Association of Official

Analytical Chemists (AOAC 2000). Gross energy content was analyzed using an adiabatic bomb calorimeter (Parr Instrument Company, Moline, IL, USA). Crude protein contents of feed and fish samples were determined by the combustion method using a *LECO* FP-528 (Leco Corporation, St. Joseph, MI, USA). Amino acids were analyzed using an automatic analyzer (Model 835-50, Hitachi Ltd., Tokyo, Japan) with an ion exchange column at Experiment Station Chemical Laboratories, University of Missouri, Columbia, MO. Whole body-lipid content was estimated using the ether extraction method. Ash content was determined by incinerating the feed samples at 600 ˚C for 12 h in a muffle furnace. Chromium content in the corn gluten test feed as well as in fecal samples were determined spectrophotometrically after digestion with nitric acid and perchloric acid (Furukawa & Tsukahara 1966).

One-way analysis of variance (ANOVA) was used to determine whether mean responses for each metric relating to feed consumption, feed efficiency, growth, body composition and survival, differed across the seven diets containing increasing levels of lysine ($P < 0.05$). All data were tested for homogeneity of variances and normality. Survival data were arcsine square-root transformed prior to ANOVA. Tanks were used as a blocking factor for dietary treatments in both experiments. Where appropriate, means across diet types were separated by Tukey's test for multiple comparisons.

Lysine requirement was determined by fitting the response variables, RGR and FCR, with broken-line regression models (Robbins *et al.* 2006) as well as second-order polynomial regression models (Zhang *et al.* 2008). Goodness of fit (R^2) and corrected Akaike information criterion (*AICc*) (Robbins *et al.* 2006; Anderson 2008) were used for selecting the best model.

Differences between group- and individually- housed bluegills in feed consumption, RGR, FCR and change in CV (%) were determined by the Student's t-test. Mean values from each of the seven dietary treatments were used while determining differences between the two types of rearing for each of the response variable.

All statistical analyses were performed via the Statistical Analysis System (SAS, Version 9.1; SAS Institute Inc., Cary, NC, USA). The GLM procedure of SAS was used to analyze growth responses for the individually-housed bluegills, in that one fish perished among the fish fed diets Lys16 and Lys25, giving a slightly unbalanced design. The ANOVA procedure was used for the growth analyses of the group-housed bluegills.

RESULTS

Determination of dietary lysine requirement level

Group rearing

Bluegill survival ranged from 85 % (Lys21) to 92.5 % (Lys12), with no significant differences observed among dietary treatments ($P > 0.05$). No overt symptoms of lysine deficiency were observed among fish fed diets containing low lysine levels. However, dietary lysine concentration significantly affected ($P < 0.01$) growth performance of bluegills (Table 4a).

Final mean weights of bluegills differed significantly ($P < 0.05$) among dietary groups, with fish fed the lowest (Lys10) and highest (Lys31) lysine levels showing the

lowest (34 g) and highest (41 g) final mean weights, respectively, while fish fed intermediate levels of lysine showed no significant differences in their final mean weights. Relative growth rate (RGR) ranged from 0.35 (Lys10) to 0.67 g $100g^{-1}$ d^{-1} (Lys31) with fish fed higher levels of dietary lysine (≥ 13 g Kg^{-1}) showing significantly higher RGR values ($P < 0.01$) than those fed 10 g Kg^{-1} dietary lysine. Feed consumption (g $fish^{-1}$) did not differ among dietary groups ($P > 0.05$). Nevertheless, FCR differed significantly ($P < 0.01$), with fish fed Lys10 showing significantly higher (poorer) values (2.05) than those fed diets containing ≥ 16 g Kg^{-1} lysine. Fish fed Lys13 did not differ significantly ($P \geq 0.05$) from any other groups in terms of FCR.

Protein efficiency ratio (PER) differed significantly ($P < 0.01$) among dietary groups. Fish fed lowest level of lysine (Lys10) showed significantly lower PER than those fed higher levels of dietary lysine (≥ 16 g Kg^{-1}). Similarly, fish fed Lys13 showed poorer PER than those fed Lys16, but did not differ significantly from those fed the other diets. Results indicated that PER reached a maximum at the dietary lysine level of 16 g Kg^{-1}, and then declined slightly with further increases in dietary lysine level (≥ 19 g Kg^{-1}). Apparent protein utilization (APU) significantly increased when the dietary lysine level was increased from 10 g Kg^{-1} to 16 g Kg^{-1}, and then leveled off despite further increase in dietary lysine level.

Proximate composition (moisture, crude protein, crude lipid, and ash) of whole-body estimates of bluegills fed graded levels of dietary lysine did not differ significantly among groups ($P > 0.05$) (Table 4b). Moisture content of bluegills ranged from 71.78 % (Lys13) to 73.01 % (Lys16), while crude lipid content ranged from 7.34 % (Lys16,

Lys19) to 9.23 % (Lys13). Crude protein level ranged from 13.52 % (Lys13) to 14.62 % (Lys25), and, ash content ranged from 4.09 % (Lys13) to 4.72 % (Lys25).

Better values of R^2 and *AICc* were obtained for the broken-line model than for the second-order polynomial model for both RGR and FCR data (Table 5). Consequently, the broken-line model was selected for reporting lysine requirement values. Break points based on broken-line regression analyses were estimated to be 14.0 g Kg^{-1} digestible lysine level for RGR (Fig 1a.) and 15.0 g Kg^{-1} digestible lysine level for FCR (Fig 1b.).

Individual rearing

No deficiency symptoms were observed in the fish fed the experimental diets during the 60-d study period. One fish each perished in the fish groups fed the diets Lys16 and Lys25, with no significant differences observed among the dietary groups in percentage survival ($P > 0.05$). Similar to the group-reared bluegills, dietary lysine level significantly affected growth performance of individually-reared bluegills ($P < 0.05$) (Table 4a.). Values of RGR ranged from 0.50 g $100g^{-1}$ d^{-1} (Lys10) to 0.94 g $100g^{-1}$ d^{-1} (Lys16, Lys25), FCR ranged from 1.28 (Lys25) to 2.08 (Lys10), PER ranged from 1.12 (Lys10) to 1.66 (Lys25) and APU ranged from 8.10 % (Lys10) to 22.31 % (Lys31). Fish fed the lowest dietary lysine levels exhibited the lowest growth rate, FCR, PER and APU while increasing dietary lysine levels from 10 to 16 g Kg^{-1} significantly increased RGR, FCR, PER and APU ($P < 0.05$); further increases in dietary lysine levels did not improve growth performance ($P > 0.05$).

Whole-body moisture, crude protein and crude lipid levels were significantly affected by dietary lysine levels ($P < 0.05$). Crude protein level generally increased whereas crude lipid level generally declined as the dietary lysine level increased up to 16 g Kg^{-1} and then leveled off when the dietary lysine level was further increased (Table 4b). Whole-body ash content did not differ across dietary groups ($P > 0.05$).

Again, better values of R^2 and *AICc* were obtained for the broken-line model than for the second-order polynomial model for both RGR and FCR data (Table 5). Broken-line analyses for RGR (Fig. 2a) and FCR (Fig. 2b) showed break points at 15.3 g Kg^{-1} and 15.4 g Kg^{-1} digestible, dietary lysine levels, respectively.

Determination of dietary requirements for other EAAs

Amino acid profiles for whole-body tissue of juvenile bluegill are given in Table 6. A dietary lysine level of 15 g Kg^{-1} (digestible basis) was considered to be the requirement level for bluegills as this lysine level produced maximum RGR and minimum FCR values in both of the experiments. Dietary requirements for other EAAs, determined from the whole-body amino acid profile, as well as lysine level (15 g Kg^{-1}), are given in Table 6.

Growth performances of group- versus individually- housed bluegills

Feed consumption and RGR were significantly lower ($P < 0.05$), whereas change in CV (%) was significantly higher ($P < 0.05$), for group-housed bluegills relative to individually-housed bluegills. Mean values (mean ± S.D.) of feed consumption (g $fish^{-1}$), FCR, RGR (g $100g^{-1}$ day^{-1}) and change in CV (%) were determined to be 14.49 ± 1.44, 1.36 ± 0.33, 0.57 ± 0.11 and 70 ± 25.33, respectively, for group-housed bluegills, and 24.77 ± 3.72, 1.48 ± 0.29, 0.77 ± 0.14 and 4.38 ± 12.12, respectively, for individually-housed bluegills.

DISCUSSION

The study, based on broken-line regression analyses of RGR and FCR, indicates that bluegill require 15 g of digestible lysine per kilogram of diet for adequate growth. Determined dietary lysine value (15 g Kg^{-1}) corresponds to a digestible lysine level of 38.1 g per kilogram of digestible protein which is within the range of values (32-62 g Kg^{-1} of dietary protein) reported for other fishes (Wilson 2002). The present study reinforces earlier findings (Hayward & Wang 2002) that group-housed bluegills develop social hierarchies, as indicated by increased size variation, and that individually-housed bluegills consume more feed and grow larger than their group-housed counterparts. However, despite the differences we observed in mean growth rates of bluegills reared in groups versus individually, their dietary requirement for lysine did not differ substantially (0.10 % difference for RGR and 0.04 % difference for FCR). Hence, the study results indicate that nutrient requirements of fish, such as that for lysine, can be

accurately determined for aggressive fishes, whether they are reared individually or in confined groups. This finding is of particular importance in studies of nutrient requirements in fishes that tend to form strong social hierarchies, and typically exhibit reduced consumption, growth and feed efficiency when reared in groups.

Growth responses to dietary lysine level

Depending on the level of dietary lysine deficiency, responses observed in fishes have ranged from reduced growth rate to poor survival rates. Fin erosion, mortality, and poor growth were observed in rainbow trout fed lysine deficient diets (Ketola 1983). High mortality rates were observed also in Japanese flounder (36 % mortality) when the dietary lysine level was inadequate (Forster & Ogata 1998). However, in the present study neither deficiency symptoms nor significant mortality was observed in response to low dietary lysine levels. The observed mortalities in the group-housed bluegills are believed to have been caused by social hierarchy effects, much more so than from any dietary or water quality effects. Growth rates (RGRs) remained positive for fish fed at the lowest lysine level (10 g Kg^{-1}), indicating that as little as 10 g of dietary lysine was adequate, not only for meeting maintenance requirements, but to elicit some growth. Other studies have likewise observed no deficiency symptoms or mortality due to insufficient dietary lysine level, e.g., Nile tilapia (Santiago & Lovell, 1988); mrigal, *Cirrhinus mrigala* (Ahmed & Khan 2004); largemouth bass (Dairiki 2007); turbot, *Scophthalmus maximus* (Peres & Oliva-Teles 2008).

Insufficient dietary lysine leading to reduced feed intake has been observed in many fishes, e.g., catfish *Mystus nemurus* (Tantikitti & Chimsung 2001); Japanese flounder *Paralichthys olivaceus* and red sea bream *Pagrus major* (Forster & Ogata 1998); striped bass (Small & Soares 1998). Similarly, feed consumption of bluegills generally differed among dietary groups with the fish group fed the lowest dietary lysine level showing the least feed consumption.

Growth rates of bluegill observed in the present study compare well with those observed in other studies. Absolute growth rates (g d^{-1}) in the present study ranged from 0.10 (Lys10) to 0.22 (Lys16) for group-housed bluegills and from 0.17 (Lys10) to 0.40 (Lys22) for individually-housed bluegills. Similar values were observed by Hayward & Wang (2002) for group-housed bluegills (~0.2 g d^{-1}) as well as for individually-housed bluegills (0.3 g d^{-1}). Determinations of AGR for group-reared bluegills from other nutrition studies have shown similar values: 0.1 g d^{-1}AGR for 6 g bluegill reared for 75 days (Hoagland *et al.* 2003), and 0.14-0.23 g $d^{-1}$of AGR for 8-14 g bluegill reared for 56 d (Twibell *et al.* 2003).

According to a review of lysine requirements of fish (Hauler & Carter 2001), live weight gain of ~54 mg is generally achieved in fish for every 1 mg of lysine consumed. A similar calculation from this study for fish that were fed the diet Lys16 showed a live weight gain of ~58 mg for group-housed bluegills and 50 mg for individually-housed bluegills for 1 mg of lysine consumption. Furthermore, fish generally exhibit a lysine utilization efficiency of 18.5 g lysine per kg of live weight gain at marginal lysine intake (Hauler & Carter 2001). In the present study, diets considered to provide marginal to sufficient dietary lysine levels (≤ 16 g Kg^{-1} digestible lysine), produced similar lysine

utilization efficiencies ranging from 17.2 g (Lys16) to 19.8 g (Lys13) of lysine per kg live weight gain for group-housed bluegills. The individually-housed bluegills showed lysine utilization efficiencies ranging from 20.4 g (Lys10) to 21.5 g (Lys13) of lysine per kg live weight gain. The similarity of estimates of bluegill growth and lysine efficiency to those of Hauler & Carter (2001) indicate that the growth responses observed for bluegill from the present study are similar to those observed for other fishes fed various levels of dietary lysine.

Despite having used fixed levels of dietary protein and energy, the low PER and APU values determined for bluegills fed low dietary lysine levels ($\leq$ 13 g Kg^{-1}) suggest that the lower lysine levels may have contributed to imbalances in the dietary amino acid ratio, which may have impaired protein deposition and weight gain. Yet, increasing the dietary lysine level to above 16 g Kg^{-1} did not produce further increases in weight gain, suggesting that the excess lysine may have been used for energy rather than for further protein deposition.

When the lysine level is deficient, a portion of the dietary protein is diverted to energy use, wherein the excess available energy may be deposited as fat. Also, lysine and methionine serve as precursors for the synthesis of carnitine which is involved in fatty acid metabolism (Walton *et al.* 1984). Consequently, deficiency of lysine likely impedes normal fat metabolism and increases body fat deposition. Lysine deficiency leading to high fat deposition has been observed in fishes including rainbow trout (Cheng *et al.* 2003) and yellow croaker (Zhang *et al.* 2008). Similarly in the portion of the present study involving individually-housed bluegills, high body fat deposition was observed for the fish fed a low dietary lysine level (13 g Kg^{-1}). However, fish fed the lowest level of

lysine (10 g Kg^{-1}) did not exhibit significantly higher fat deposition. It should be noted, however, that the fish group fed the lowest dietary level consumed the least amount of feed. Therefore, the absolute amounts of available energy for bluegills fed the lowest lysine level and those fed higher levels of dietary lysine (> 16 g Kg^{-1}) may not have differed sufficiently to cause differences in body fat deposition. In contrast to the individually-housed bluegills, no significant differences in body fat content were observed in the group-housed bluegills due to lysine deficiency. Conceivably, this occurred because the group-housed bluegills had used substantial amounts of energy to cope with the social stress associated with dominance hierarchies. Also, dietary lysine effects were likely less pronounced among subordinate individuals from their suppressed feeding. This may have obscured the dietary treatment effect on body fat deposition.

Model estimation of lysine requirements

Although both the quadratic (second-order polynomial) and broken-line regression models assume that deficiency of a test nutrient impedes fish growth, the quadratic model assumes a decline in fish growth performance whereas the broken-line model assumes no change in growth performance, under excessive levels of the test nutrient (Forster 2000). Over the range of dietary lysine used in the present study (10-31 g Kg^{-1}), increase in dietary lysine from 16 g Kg^{-1} to 31 g Kg^{-1} did not reduce growth performance of bluegill. This was shown by a better fit to the response variables (RGR and FCR) by the broken-line model than by the quadratic model. Similarly, better fit to growth responses by a broken-line model than by a quadratic model were also

observed by Dairiki (2007) and Zhang et al. (2008). The broken-line, regression model approach has frequently been used to estimate nutrient requirements of fishes (Hauler & Carter 2001), despite its reputation for underestimating nutrient requirements in some cases (Shearer 2000).

Excessive levels of dietary lysine have been observed to reduce utilization efficiency of arginine and growth performance in poultry (Balnave & Barke 2002) and in canine (Czarnecki *et al.* 1985), but not in swine (Edmonds & Baker 1987) or feline (Fascetti *et al.* 2004). In fishes, lysine-arginine antagonism was not observed in channel catfish (Robinson *et al.* 1981) or hybrid striped bass (Griffin *et al.* 1994). Similarly, that excess lysine did not affect growth performance of bluegills, suggests that lysine-arginine antagonism is absent in this fish species as well.

EAA requirements for bluegills and other fishes

In recent years, fishes' dietary requirements for all EAAs have often been determined from whole-body amino acid profiles. Examples include studies of striped bass (Brown 1995; Small & Soares 1998), European seabass *Dicentrarchus labrax*, gilthead seabream *Sparus aurata* and turbot *Psetta maxima* (Kaushik 1998), jundia (Montes-Girao & Fracalossi 2006), and largemouth bass (Dairiki 2007). Determining EAA requirements via this method is considered an expedient and effective approach for building an ideal dietary protein for an emerging aquaculture species. Nevertheless, the accuracy of this approach has been questioned in that, arguably, it ignores individual differences in maintenance requirement among EAAs (the method assumes that

maintenance requirements of lysine and those of others EAAs are similar) (Green & Hardy 2002). The deletion method, originally developed for pigs (Wang & Fuller, 1989), likely considers maintenance requirements for EAAs. In this approach, change in nitrogen retention when removing a fixed proportion of each EAA, is used to determine the ideal dietary essential amino acid pattern in which all amino acids are equally limiting. The deletion method assumes nitrogen retention to be linearly correlated to dietary EAA content, when a particular amino acid is limiting. This approach could produce erroneous results if there is substantial deviation in the linear relationship between any EAA levels and nitrogen gain (Green & Hardy 2002). Relatively few studies have determined dietary requirements of EAAs for fishes via the deletion method (e.g., Green & Hardy 2002; Rollin *et al.* 2003; Peres & Oliva-Teles 2009). However, Peres & Oliva-Teles (2009) found strong positive correlation (0.99) between EAA requirement values determined by whole-body amino acid composition, and those determined by the deletion method for gilthead seabream. Similarly, Green & Hardy (2002) observed no differences in growth responses of rainbow trout groups fed diets containing dietary EAA patterns based on (i) whole-body amino acid ratio, (ii) requirements determined by the dose-response method, or (iii) requirements determined by deletion method. These studies indicate that maintenance requirements of EAAs may be minimal for fishes or proportionately equivalent to that of lysine. Additional research may be warranted to determine whether advantages exist in using the “deletion method” versus the “whole-body amino acid ratio method”, particularly for slow growing or adult fish that may require a higher proportion of amino acids for maintenance than do faster growing ones.

Bluegills are often fed high-protein trout diets, and were reported to perform better on such diets than on catfish diets (Twibell *et al.* 2003). Dietary requirements of EAAs for bluegills versus those for rainbow trout and channel catfish are provided in Fig. 3. Dietary requirements for most EAAs appear to be lower for channel catfish than for bluegill, suggesting that channel catfish diets may be deficient in certain EAAs for bluegills. Moreover, requirements for the most common limiting amino acids such as lysine and methionine are likely lower for bluegill than for rainbow trout. This suggests that higher levels of dietary fish meal can be replaced by alternative protein sources for bluegill than for rainbow trout, while no amino acid supplementation is needed.

Group- versus individually- housed fishes in nutrition studies

Dominance hierarchies have frequently developed among fishes reared indoors, such as in tanks (Sloman & Armstrong 2002). As social hierarchies form, a relatively few, dominant individuals monopolize the feed provided, and grow at their inherent capacity. Fish occupying lower hierarchical positions eat and grow at progressively lesser rates, avoid agonistic interactions with more dominant individuals (Sloman & Armstrong 2002). Consequently, fish growth responses that are due exclusively to test nutrients are likely distorted by the intense and persistent social interaction. For fishes that tend to establish social hierarchies, the effect of a given dietary nutrient on growth physiology may be best evaluated under individual rearing conditions versus group rearing. However, determination of fishes' nutrient requirement under group rearing may hold advantages including the fact that fish are typically group-reared in commercial

production systems. Hence, nutrient requirements of fish determined under such growing conditions may better reflect their true requirements. For example, dietary requirements for energy, and for some nutrients that are involved in the production of stress hormones, can be higher under group- versus individual rearing, particularly for fishes that tend to develop dominance hierarchies. Also, if a given study seeks to observe changes in the concentration of certain nutrients in serum samples (e.g., Griffin *et al.* 1994), blood samples may be required from multiple individuals to secure adequate amounts for which group housing can hold advantages over individual housing. Moreover, some fishes (e.g., African catfish *Clarias gariepinus*, Martins *et al.* 2006) are intolerant of social isolation and may grow far below their inherent capacity when reared under such conditions. Therefore, while the present study indicated no differences between the two rearing conditions in terms of dietary lysine requirement for juvenile bluegill, careful consideration may be warranted when selecting a rearing method for other diet-related studies.

In recent years, efforts have been directed to enhance bluegill production through genetic selection (Hicks *et al.* 2009), as well as through rearing mono-sex male populations (Hayward & Wang 2006; Wang *et al.* 2009). Requirements of dietary lysine have been shown to vary according to strain, sex, and age of agricultural animals, e.g., swine (NRC 1998) and poultry (NRC 1994). Consequently, the EAA requirements reported in the present study for “standard”, mixed-sex juvenile bluegills, may warrant redetermination for male-only, or genetically altered bluegills.

The present study has, for the first time, determined dietary EAA requirements that can be used to select appropriate practical protein sources for juvenile bluegills.

When formulating diets for juvenile bluegills, a margin of safety (10% higher than the reported level) should be added for each EAA to compensate for variations in ingredient composition and environmental effects.

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Table 1. Gross nutrient levels, percentage digestibility, and availability of digestible nutrients from corn gluten meal.

Components	Gross nutrients (MJ Kg^{-1} for energy, and g Kg^{-1} for protein and amino acids (AAs))	Digestibility (%)	Available nutrients (MJ Kg^{-1} for energy, and g Kg^{-1} for protein and amino acids)
Energy	22.34	81.74	18.26
Protein	625.0	83.68	523.0
EAA (essential AA)			
Arginine	20.3	91.03	18.5
Histidine	14.4	88.47	12.7
Isoleucine	28.7	88.29	25.3
Leucine	109.6	82.06	89.9
Lysine	13.7	90.66	12.4
Methionine	14.9	94.36	14.1
Phenylalanine	41.6	88.37	36.8
Threonine	21.1	90.14	19.0
Tryptophan	3.8	95.26	3.6
Valine	30.9	82.72	25.6
∑EAA	299.0	86.27	257.9
NEAA (nonessential amino acid)			
Aspartic Acid	39.1	83.38	32.6
Glutamic Acid	125.9	83.34	104.9
Alanine	56.1	84.74	47.5
Cysteine	10.4	93.46	9.7
Glycine	17.9	91.40	16.4
Serine	28.3	84.52	23.9
Proline	60.3	89.02	53.7
Tyrosine	33.9	87.43	29.6
∑NEAA	371.9	85.61	318.4
∑AA	670.9	85.90	576.3

Table 2. Formulations of seven experiment diets used in the study.

Ingredients ($g\ Kg^{-1}$)	Diets						
	Lys10	Lys13	Lys16	Lys19	Lys22	Lys25	Lys31
Menhaden fish meal	40.0	40.0	40.0	40.0	40.0	40.0	40.0
Corn gluten meal	611.6	611.6	611.6	611.6	611.6	611.6	611.6
Soybean meal	15.0	15.0	15.0	15.0	15.0	15.0	15.0
Wheat	171.0	171.0	171.0	171.0	171.0	171.0	171.0
Fish oil	77.2	77.2	77.2	77.2	77.2	77.2	77.2
Lecithin	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Vitamin premix[1]	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Vitamin C-PP	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Choline-Cl	0.9	0.9	0.9	0.9	0.9	0.9	0.9
Mineral premix[2]	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Dicalcium phosphate	22.5	22.5	22.5	22.5	22.5	22.5	22.5
Lime stone	8.7	8.7	8.7	8.7	8.7	8.7	8.7
Sodium chloride	3.8	3.8	3.8	3.8	3.8	3.8	3.8
Lysine.HCl	0.0	3.9	7.7	11.5	15.3	19.1	26.7
Glutamic acid	26.7	22.8	19.0	15.2	11.4	7.6	0.0
L-Tryptophan	0.4	0.4	0.4	0.4	0.4	0.4	0.4
L-Arginine	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Betaine	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Binder[3]	9.0	9.0	9.0	9.0	9.0	9.0	9.0
Total	1000	1000	1000	1000	1000	1000	1000

[1]Vitamin premix contains (amount per kg of dry feed): vitamin A, 44092 IU; vitamin D3, 19290 IU; vitamin E, 69 IU; niacin, 276 mg; D-pantothenic acid, 83 mg; riboflavin, 33 mg; menadione, 11 mg; folic acid, 8 mg; thiamin, 7 mg; biotin, 6 mg; vitamin B12, 1 mg.

[2]Mineral premix contains (amount per kg of dry feed): Ca (as calcium carbonate), 25 mg; Mn (as manganese sulfate), 110 mg; Zn (as zinc sulfate), 110 mg; Fe (as ferrous sulfate), 60 mg; Mg (as magnesium oxide) 27 mg.

[3]Ultra-Bond™, Uniscope, Inc., Johnstown, CO, USA.

Table 3. Proximate composition (*n* = 2 for gross estimation) of the experimental diets used in the study (values in the parentheses indicate nutrient levels on a digestible basis).

Composition	Lys10	Lys13	Lys16	Lys19	Lys22	Lys25	Lys31
Gross Energy (MJ Kg^{-1})	21.28 (16.54)	21.25 (16.56)	20. 72 (16.59)	20.80 (16.62)	21.60 (16.64)	21.84 (16.67)	21.20 (16.72)
Crude Protein (g Kg^{-1})	488.2 (393.4)	491.4 (393.4)	493.8 (393.4)	494.1 (393.4)	496.9 (393.4)	498.2 (393.4)	495.8 (393.4)
Crude Lipid (g Kg^{-1})	112.2	108.5	105.3	110.6	112.2	115.1	104.7
Crude Ash (g Kg^{-1})	51.8	50.7	46.7	49.0	45.3	52.4	52.4
EAA (g Kg^{-1})[a]							
Arginine	16.0 (15.1)						
Histidine	9.5 (8.8)						
Isoleucine	19.8 (17.3)						
Leucine	72.4 (58.4)						
Lysine	11.9 (10.2)	14.9 (13.2)	17.9 (16.2)	20.9 (19.2)	23.9 (22.2)	26.9 (25.2)	32.9 (31.2)
Methionine	10.5 (9.7)						
Phenylalanine	28.4 (24.6)						
Threonine	14.7 (13.3)						
Tryptophan	3.7 (3.0)						
Valine	21.9 (17.8)						
∑ AA	496.8	496.8	496.8	496.8	496.8	496.8	496.8

[a]Essential amino acid (EAA) levels were estimated only for the diet Lys10, and for the other diets, dietary lysine level was calculated from Table 2 based on synthetic lysine levels added.

Table 4a. Growth responses of juvenile bluegills fed the experimental diets for 60 days. Values are presented as means ± SD*.

Variable	Lys10	Lys13	Lys16	Lys19	Lys22	Lys25	Lys31	*P*-value (ANOVA)
Group reared (n =4)								
Initial weight (g)	27.46±1.04	26.47±1.09	26.25±1.55	27.23±1.98	26.62±1.30	26.93±1.23	27.42±1.87	0.86
Final weight (g)	33.97±3.56^{a}	37.40±2.32^{ab}	39.43±4.08^{ab}	38.54±1.23^{ab}	37.78±1.27^{ab}	38.60±2.75^{ab}	41.05±1.20^{b}	0.04
RGR (g^{-1} $100g^{-1}$ d^{-1})	0.35±0.17^{a}	0.57±0.07^{b}	0.66±0.09^{b}	0.58±0.07^{b}	0.58±0.08^{b}	0.59±0.05^{b}	0.67±0.08^{b}	<0.01
Feed consumption (g $fish^{-1}$)	12.39±1.76	16.31±1.79	13.91±2.26	13.79±0.97	13.96±1.05	14.73±1.50	16.37±2.85	0.09
FCR	2.05±0.70^{a}	1.51±0.23^{ab}	1.07±0.13^{b}	1.21±0.18^{b}	1.25±0.08^{b}	1.23±0.14^{b}	1.20±0.13^{b}	<0.01
Change in CV (%)	33.79±41.96	80.48±22.66	63.78±21.88	111.12±48.88	62.77±22.53	97.77±35.31	80.22±16.45	
PER	1.09±0.34^{a}	1.37±0.21^{ab}	1.92±0.24^{c}	1.65±0.16^{bc}	1.61±0.11^{bc}	1.59±0.17^{bc}	1.70±0.18^{bc}	<0.01
APU (%)	3.34±5.41^{a}	6.98±1.90^{ab}	17.20±5.80^{c}	15.28±4.22^{bc}	14.29±2.72^{bc}	16.06±7.48^{bc}	17.30±2.40^{c}	<0.01
Survival (%)	92.50±9.57	90.00±0.00	87.50±5.00	85.00±5.77	90.00±14.14	87.50±9.57	90.00±8.16	0.74
Individually reared (n = 13 or 14)								
Initial weight (g)	27.79±5.66	31.90±8.81	30.35±9.20	27.99±9.10	31.37±8.57	29.87±7.07	32.40±6.35	0.69
Final weight (g)	37.77±8.10^{a}	49.18±15.74^{ab}	50.57±13.44^{ab}	44.88±15.58^{ab}	51.96±14.84^{ab}	54.14±15.23^{b}	52.92±9.35^{ab}	0.03
RGR (g^{-1} $100g^{-1}$ d^{-1})	0.50±0.16^{a}	0.69±0.192^{ab}	0.87±0.22^{b}	0.80±0.18^{b}	0.81±0.19^{b}	0.94±0.29^{b}	0.80±0.25^{b}	<0.01
Feed consumption (g $fish^{-1}$)	18.59±5.6^{a}	26.61±10.3^{ab}	25.45±6.26^{ab}	20.60±6.28^{ab}	26.68±6.69^{ab}	28.81±9.42^{b}	26.67±8.94^{ab}	0.01
FCR	2.08±0.67^{a}	1.65±0.39^{ab}	1.30±0.25^{b}	1.33±0.37^{b}	1.39±0.41^{b}	1.28±0.34^{b}	1.35±0.31^{b}	<0.01
Change in CV (%)	4.48	17.88	-12.28	6.98	4.55	18.94	-9.88	
PER	1.12±0.39^{a}	1.29±0.28^{ab}	1.62±0.32^{b}	1.62±0.38^{b}	1.54±0.33^{b}	1.66±0.40^{b}	1.57±0.35^{b}	<0.01
APU (%)	8.10±4.94^{a}	11.46±4.26^{a}	21.15±5.57^{b}	21.09±5.33^{b}	21.11±6.21^{b}	21.83±7.76^{b}	22.31±7.18^{b}	<0.01
Survival (%)	100	100	93	100	100	93	100	0.55

*Values within a row sharing different superscript alphabets are significantly different, $P < 0.05$).

Table 4b. Whole-body composition of juvenile bluegills fed the experimental diets for 60 days. Values are presented as means ± SD*.

Variable	Lys10	Lys13	Lys16	Lys19	Lys22	Lys25	Lys31	*P*-value (ANOVA)
Group reared								
Moisture (%)	72.43±0.94	71.78±0.82	73.01±0.53	72.81±0.86	72.40±0.47	71.84±1.12	71.83±2.08	0.58
Crude Protein (%)	14.27±0.40	13.52±0.13	14.14±0.71	14.13±0.60	14.44±0.41	14.62±0.49	14.56±0.2	0.10
Crude lipid (%)	7.54±0.8	9.23±0.96	7.34±1.16	7.34±0.87	7.99±0.37	8.55±2.06	8.64±2.08	0.33
Ash (%)	4.44±0.62	4.09±0.81	4.46±0.23	4.54±0.11	4.33±0.16	4.72±0.45	4.27±0.26	0.57
Individually reared								
Moisture (%)	71.71±1.47[a]	70.51±0.49[b]	70.87±0.98[ab]	70.61±0.79[b]	70.10±0.42[b]	71.08±1.28[ab]	70.09±0.32[b]	<0.01
Crude Protein (%)	14.40±0.29[a]	14.12±0.17[a]	15.24±0.49[b]	15.50±0.20[b]	15.62±0.53[b]	15.22±0.53[b]	15.57±0.29[b]	<0.01
Crude lipid (%)	8.84±0.84[ab]	9.49±0.59[b]	8.27±1.25[a]	8.34±0.81[a]	7.99±0.20[a]	7.91±0.78[a]	8.33±0.31[a]	<0.01
Ash (%)	3.97±0.11	3.87±0.18	3.94±0.17	3.82±0.14	3.75±0.16	3.85±0.57	3.75±0.38	0.38

*Values within a row sharing different superscript alphabets are significantly different, $P < 0.05$).

Table 5. Model selection statistics* for the RGR and FCR data of bluegill.

Response Variable	Model	R^2	*AICc*	Requirement
Group housing				
RGR	Broken line	0.89	-48.8	14.0
	Polynomial	0.61	-31.0	26.7
FCR	Broken line	0.97	-3.8	15.0
	Polynomial	0.76	2.5	24.2
Individual housing				
RGR	Broken line	0.85	-46.1	15.3
	Polynomial	0.80	-31.2	24.1
FCR	Broken line	0.98	-5.4	15.4
	Polynomial	0.84	0.4	24.5

* Higher R^2, lower *AICc* values indicate better fit.

Table 6. Essential amino acid (EAA) profile of whole-body tissue of juvenile bluegill, and dietary requirements for EAAs.

EAA	g Kg^{-1} dry weight	A/E ratio	Digestible requirements (g Kg^{-1} diet)
Arginine	25.3	114.82	11.9
Cysteine	4.1	18.67	1.9
Histidine	8.8	39.87	4.1
Isoleucine	20.8	94.43	9.8
Leucine	32.5	147.35	15.3
Lysine	31.9	144.72	15.0
Methionine	13.0	58.81	6.1
Phenylalanine	21.0	95.33	9.9
Threonine	18.5	84.01	8.7
Tryptophan	5.2	23.56	2.4
Tyrosine	14.2	64.43	6.7
Valine	25.1	113.91	11.8

Figure 1a. Broken-line regression model fitted to RGRs of group-housed bluegills (mean ± 1SD) fed the experimental diets containing graded levels of dietary lysine: RGR = 0.62 – 0.07 (14.0 – Lysine), where (14.0 – Lysine) = 0 when Lysine > 14.0.

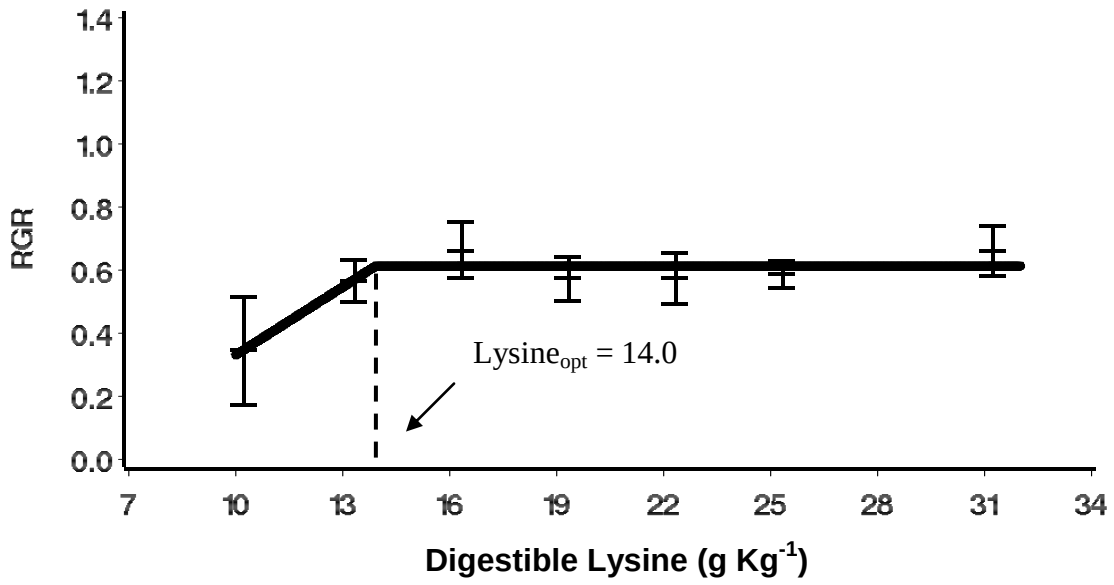


Figure 1b. Broken-line regression model fitted to FCR of group-housed bluegills (mean ± 1SD) fed the experimental diets containing graded levels of dietary lysine: FCR = 1.19 + 0.18 (15.0- Lysine), where (15.0 – Lysine) = 0 when Lysine > 15.0.

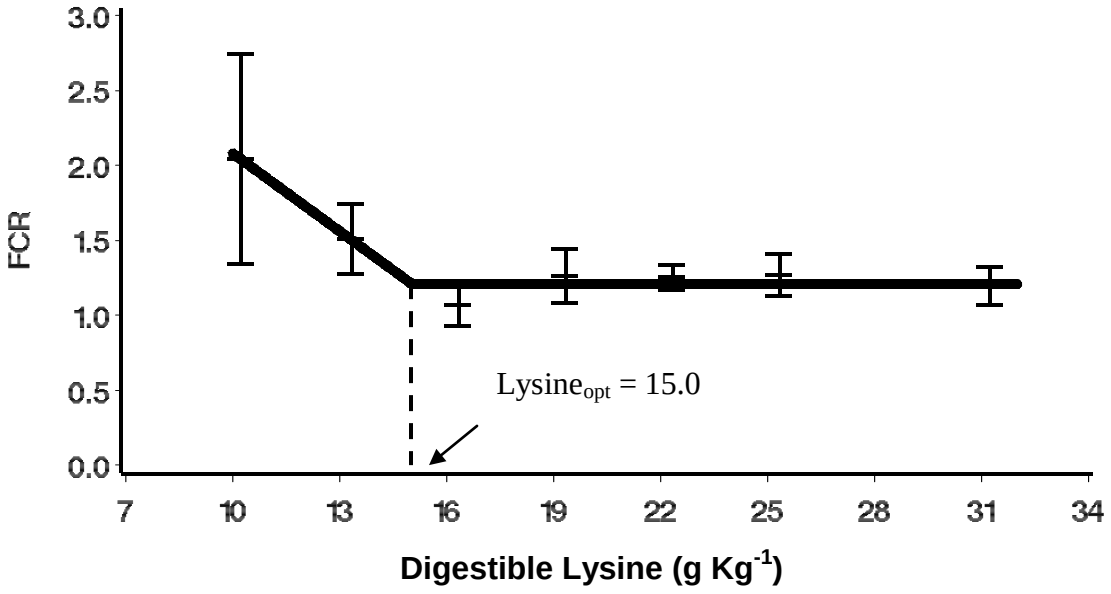


Figure 2a. Broken-line regression model fitted to RGRs of individually-housed bluegills (mean ± 1SD) fed the experimental diets containing graded levels of dietary lysine: RGR = 0.84 – 0.06 (15.3 – Lysine), where (15.3 – Lysine) = 0 when Lysine > 15.3.

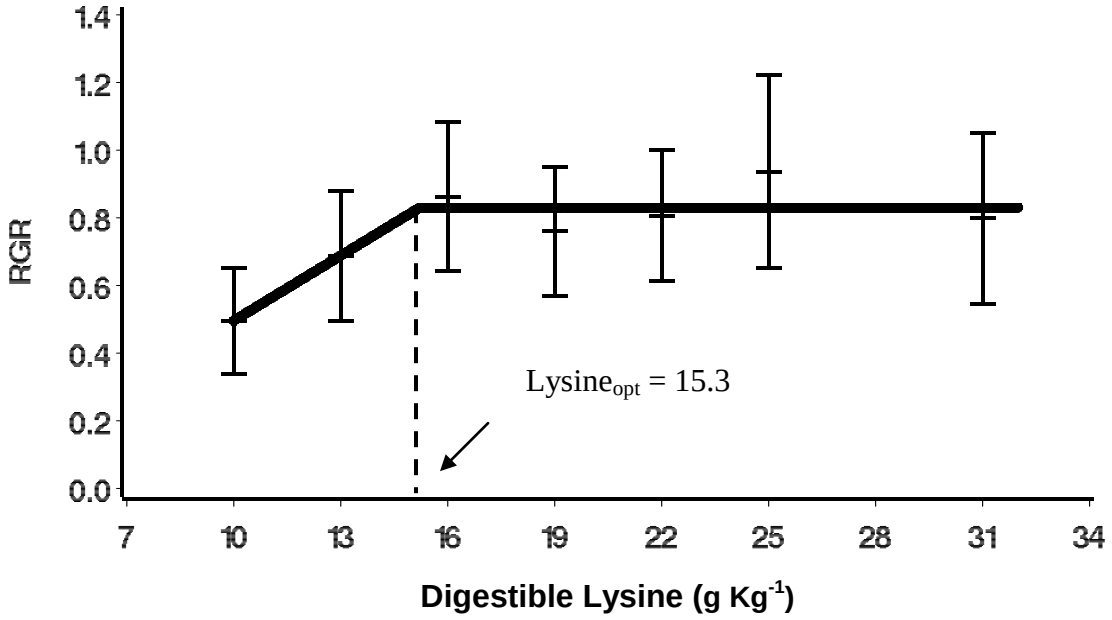


Figure 2b. Broken-line regression model fitted to FCR of individually-housed bluegills (mean ± 1SD) fed the experimental diets containing graded levels of dietary lysine: FCR = 1.34 +0.14 (15.39- Lysine), where (15.39 – Lysine) = 0 when Lysine > 15.39.

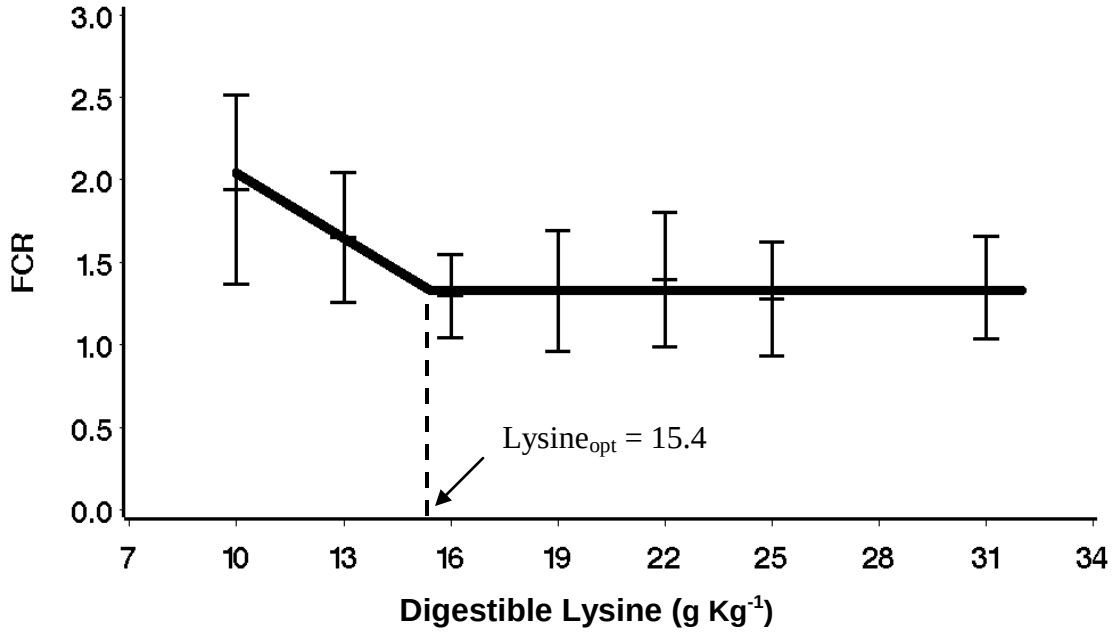
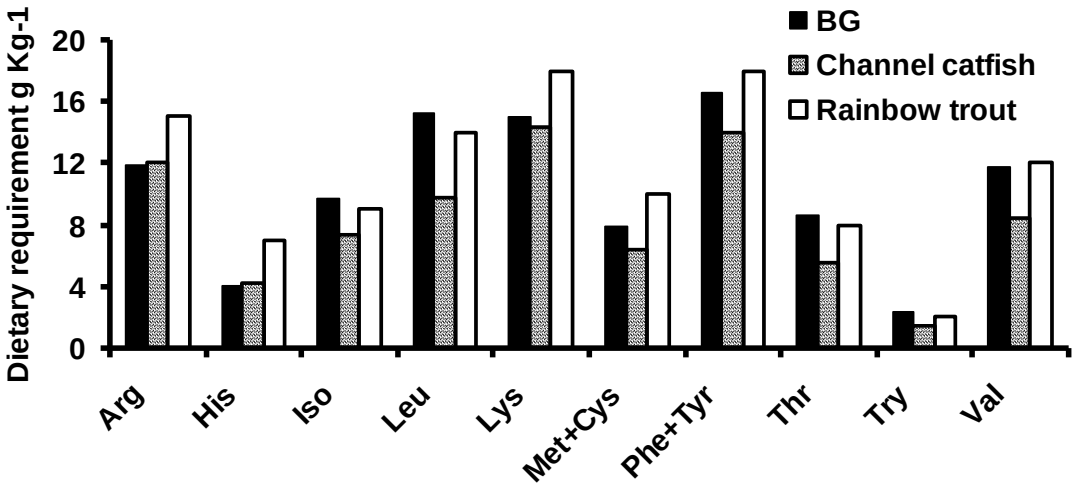


Figure 3. Comparison of essential amino acid requirements (digestible basis) of bluegills versus those of rainbow trout and channel catfish. Values (digestible basis) for channel catfish and rainbow trout were taken from NRC (1993).



CHAPTER 3

DIETARY REQUIREMENTS OF DIGESTIBLE PROTEIN AND ENERGY LEVELS FOR JUVENILE BLUEGILL, *LEPOMIS MACROCHIRUS*

ABSTRACT

Information on optimal dietary levels of digestible protein and energy for developing a cost-efficient diet is not available for juvenile bluegill, *Lepomis macrochirus.* A series of two, 60-d experiments was conducted to determine optimal levels of dietary protein and energy for juvenile bluegill. In experiment-1, eight experimental diets were formulated to contain digestible protein levels ranging from 355 g Kg^{-1} to 495 g Kg^{-1} at 20 g Kg^{-1} increments, at a fixed digestible energy level of 15.91 MJ Kg^{-1}. In experiment 2, seven experimental diets were formulated to contain digestible energy levels ranging from 12.55 MJ Kg^{-1} to 17.57 MJ Kg^{-1} at ~0.84 MJ Kg^{-1} increments, with digestible protein levels fixed at 412 g Kg^{-1} across diets. In both experiments, quadruplicate bluegill groups (~20 g, n = 10 fish/group) were fed the experimental diets twice daily to apparent satiation for 60 d. Fish fed the lowest protein level generally showed poorer feed consumption and relative growth rate (RGR) but a better protein efficiency ratio than those fed ≥ 470 g Kg^{-1} protein. Fish fed at a 17.57 MJ Kg^{-1} dietary energy level generally produced higher hepato-, viscero-somatic indices and whole body fat contents than did those fed ≤ 15 MJ Kg^{-1} dietary energy levels. Optimal dietary digestible protein level was estimated to be ~410 g Kg^{-1} based on a broken-line fit to RGR. Optimal dietary digestible energy level was estimated to be ~14.6 MJ Kg^{-1} based on a quadratic fit to RGR and protein gain. Results indicate that bluegill require a

relatively high protein/energy ratio of ~28 g MJ^{-1}. The study results emphasized the importance of determining lipid-to-carbohydrate ratio in order to maximize bluegill's efficiency in utilizing non-protein energy source or minimize the inclusion levels of expensive dietary protein level.

INTRODUCTION

Fish producers in the U.S. often use commercial catfish or trout diets to grow bluegill *Lepomis macrochirus*. However, a recent study by Twibell *et al*. (2003) showed that juvenile bluegill (~4 g) fed a catfish diet (320-360 g Kg^{-1} crude protein and 40-60 g Kg^{-1} crude fat) exhibited poor growth, whereas those fed a protein-rich trout diet (440-470 g Kg^{-1} crude protein and 110-150 g Kg^{-1} crude fat) grew substantially better but with higher body fat deposition. Although superior in some respects, trout diets are expensive, often accounting for > 60% of total annual variable costs in bluegill farming operations (Curtis Harrison, Harrison Fisheries, Inc., Hurdland, MO, pers. comm.). The absence of a diet formulated specifically for bluegill is arguably impeding the economic sustainability of bluegill aquaculture. Consequently, development of a nutritionally balanced diet for bluegill has been considered to be highly important for effective and profitable commercial bluegill culture (Hoagland *et al.* 2003; Masagounder *et al.* 2009; Masagounder *et al.* accepted).

Although fish can derive energy from protein, fat, or carbohydrate, only protein containing balanced levels of amino acids can directly support fish growth by protein deposition (Wilson & Halver 1986). As a result of its key role in diets, protein has

become the most costly nutrient in fish and other animal diets. It is desirable that fish energy requirements be met using non-protein sources in order that expensive dietary protein is reserved primarily for somatic growth (Wilson 2002). On the other hand, excess dietary energy levels can be costly in that fish may deposit more body fat, limiting feed consumption, and thereby limiting fish production. Consequently, balancing the protein and energy levels in fish diets has long been emphasized, not only to maintain rapid growth rates with minimal body fat deposition, but to also minimize undesirable nitrogenous output and nutrient effluents (Wilson & Halver 1986). Formulating diets in this fashion has been a primary goal in fish nutrition. However, given that fish differ in their capacities for using protein and non-protein energy sources (Wilson & Halver 1986), determining protein and energy requirements for individual fish species has been emphasized.

Hoagland *et al.* (2003) showed that bluegill (1.76 g) require 440 g Kg^{-1} protein but only 80 g Kg^{-1} fat using experimental diets containing 320-440 g Kg^{-1} protein and 60-120 g Kg^{-1} lipid. However, the study left open the possibility that yet higher levels of dietary protein may be beneficial, given that the fish performed significantly better at the upper end (440 g Kg^{-1}) of the study's protein range. Furthermore, the study did not provide information concerning nutrient digestibility of the feedstuffs used, nor the availability of dietary amino acid levels, limiting the results to the formulation that the study adopted. The digestibilities of common feedstuffs (Masagounder *et al.* 2009) and the dietary requirements of digestible essential amino acids (EAAs) (Masagounder *et al.*; accepted) have been determined for juvenile bluegill. Using this information to determine their dietary requirements for protein and energy levels constitutes the next step in these

studies. Furthermore, knowing their nutrient requirement values on a digestible basis may allow feed formulators to assure adequate levels of dietary nutrients for a formulation other than the one that was used during the determination of dietary nutrient requirement levels. Moreover, fish producers typically stock larger bluegills (10-20 g) into ponds than those used in the previous studies (Hoagland *et al.* 2003; Twibell *et al.* 2003). Consequently, the present study was conducted to determine dietary requirements of digestible levels of protein and energy for stock-size juvenile bluegill.

MATERIAL AND METHODS

A series of two, 60-d experiments was conducted to determine dietary requirements for digestible protein (Experiment 1) and digestible energy (Experiment 2) for juvenile bluegill.

Experiment 1: Protein diets

Experimental diets

Fish meal, blood meal, soybean meal, corn gluten meal, and wheat were used as intact protein sources. The gross levels of energy, protein, and amino acids measured for these feedstuffs are given in Table 1. Digestible nutrient levels (Table 1) were calculated from the digestibility values determined for the respective feedstuffs (Masagounder *et al.* 2009; Masagounder *et al.* accepted). Digestibility of energy from dextrin, fish oil, and

lecithin were assumed to be 90%, with their digestible energy levels being 15.1, 34 and 28.6 MJ Kg^{-1}, respectively (Table 1). Eight semi-purified diets were formulated to contain digestible protein levels ranging from 355 g Kg^{-1} to 495 g Kg^{-1} at 20 g Kg^{-1} increments by increasing the dietary fish meal from 500 g Kg^{-1} (diet 1) to 804 g Kg^{-1} (diet 8). Digestible energy level was maintained at 15.9 MJ Kg^{-1} across these diets. Desired levels of protein and energy were obtained across diets by varying the levels of soybean meal, wheat, fish oil, and dextrin. A minimum of 40 g Kg^{-1} of fish oil was provided in all the diets to ensure the availability of adequate levels of essential fatty acids, as recommended for rainbow trout *Oncorhynchus mykiss* by Hardy (2002). Formulations and proximate compositions of the Experiment-1 diets are given in Table 2. All these diets were formulated to provide EAAs at levels that were determined to be adequate for optimal growth performance of juvenile bluegill (Masagonder *et al.* 2009).

All coarse dietary ingredients were ground and sieved with a 500 µm screen in a Fitzmill (W. J. Fitzpatrick Company, Chicago, IL, USA). Dry ingredients for each experimental diet were then mixed in a Hobart mixer (Hobart Corporation, Troy, OH, USA). All diets were extruded with a twin-screw extruder at the Food Protein R&D Center, Texas A&M University, College Station, TX, USA. Diets were then air dried, packaged in air-tight bags, transported to the University of Missouri, Columbia, MO, USA, and stored under air-tight conditions at 4 °C until used. Gross protein levels varied from 418 g Kg^{-1} to 570 g Kg^{-1}, gross energy levels varied from 19.1 MJ Kg^{-1} to 21.0 MJ Kg^{-1}, and ash content varied from 112 g Kg^{-1} to 168 g Kg^{-1} across diets (Table 2).

Experimental design

Juvenile bluegill were purchased from a local fish producer (Osage Catfisheries, Inc., Osage Beach, MO, USA) and transported to the University of Missouri, Columbia, MO, USA. Fish were then acclimated to laboratory conditions for 10 d. Five rectangular tanks (236 × 73 × 58 cm; water holding capacity = 945 L), each equipped with biofiltration, water-recirculation/re-aeration and temperature-control capacities, were used in the study. Seven perforated plastic test chambers (43 × 30 × 43 cm) whose screen-covered tops protruded above tank water surfaces were placed in each of the five tanks, yielding 35 test chambers. Thirty-two chambers were chosen to allow four replicates for each of the eight diets. Three additional chambers in tank 5 were used such that the fish density was equivalent to that of the other tanks. Tanks were filled to 75% of their heights such that water volumes of ~40 L resulted in each chamber. Water from a head tank and biofilter were trickled into each chamber via a spray bar mounted above each fish tank. Acclimated juvenile bluegills (~20 g) were then randomly allotted to the test chambers at 10 fish per chamber and acclimated for another 5 days. Eight test diets were randomly assigned to the test chambers, giving four replicates for each test diet. The experiment followed a completely randomized design.

Experiment 2: Energy diets

Experimental diets

Seven experimental diets were formulated to contain energy levels ranging from 12.55 MJ Kg^{-1} (3000 Kcal Kg^{-1}) to 17.57 MJ Kg^{-1} (4200 Kcal Kg^{-1}) at ~0.84 MJ Kg^{-1} (200 Kcal Kg^{-1}) increments. Fish meal and corn gluten meal were used as the main protein sources, whereas fish oil was used as the primary energy source. Digestible levels of nutrients for the ingredients used in this experiment for bluegill were determined as in Experiment 1. Energy levels were increased across the experimental diets by gradually replacing indigestible α-cellulose with fish oil. All other ingredients were added at a fixed amount across all the diets (Table 1). All diets were kept isonitrogenous by fixing the digestible protein level at 412 g Kg^{-1}, which was found to be optimal for juvenile bluegills in Experiment 1. In all the Experiment-2 diets, digestible EAAs were maintained above the levels determined to be ideal for juvenile bluegill (Masagounder *et al.* accepted). Formulations and proximate compositions of the Experiment 2 diets are given in Table 4.

Dietary ingredients were finely ground, mixed, and extruded as in Experiment 1. Gross protein levels varied from 452 g Kg^{-1} to 560 g Kg^{-1}, gross energy levels varied from 18.6 MJ Kg^{-1} to 22.8 MJ Kg^{-1}, and dietary ash content varied from 112 g Kg^{-1} to 168 g Kg^{-1} across these diets (Table 2).

Experimental design

Juvenile bluegill were transported to the University of Missouri, Columbia, MO, USA from the Cooperative Research & Extension Unit, Lincoln University, Jefferson City, MO, USA. Fish were then acclimated to laboratory conditions for seven days. The experimental design was identical to that used in Experiment 1, except that only four tanks (water holding capacity = 945 L per tank) were used to accommodate 28 chambers (seven chambers per tank). A water volume of 40 L was maintained in all chambers throughout the study as in Experiment 1. Acclimated juvenile bluegills (~21 g) were then randomly allocated to the test chambers at 10 fish per chamber, and allowed five additional days to complete acclimation. Seven test diets were randomly allocated to the test chambers, giving four replicates per test diet.

Feeding procedure and measurements

In both experiments, bluegills were hand-fed twice daily to apparent satiation at 0800 and 1600 h. Feces were siphoned out before each feeding. Each feeding was handled by two persons and continued for ~1 h. Any feed pellets that remained in a chamber 30 min post-feeding were removed by siphoning under no-flow conditions and stored at -20 °C until the end of the study. After completion of the 60-d feeding trial, preserved, uneaten pellets from each chamber were dried at 70 °C for 48 h and weighed. Leaching of test diets was accounted for as follows when determining the weights of

unconsumed pellets. Upon completion of the study, after removing fish from the test chambers, test diets of known dry weights were immersed for 1 h in water-filled chambers, siphoned out and dried. The percentage weight loss from leaching was then added to the weights of uneaten pellets for each test diet. The dry weight of the unconsumed feed was then subtracted from the total feed weight provided to determine total feed consumption by the bluegill in each chamber. Water temperature and dissolved oxygen were recorded daily from a randomly chosen chamber in each tank in both experiments. A constant water temperature and dissolved oxygen levels were maintained at ~22.0 °C and ~7 mg L^{-1}, respectively. Weekly determined NH_3-N and NO_2-N levels remained < 0.1 mg L^{-1}, while a summer-like photoperiod (14 L: 10 D) was continued throughout the 60-d periods of both the experiments.

Live weights of fish from each chamber were determined on days 0 and 60. At the end of the experiment, bluegill were euthanized by an overdose of MS222 (Aquatic Eco-systems, Apopka, FL, USA). Six randomly-selected fish from each chamber were used to determine whole-body proximate composition. In Experiment 2 (energy study), before beginning the feeding trial, 10 additional fish were acclimated and euthanized to determine initial fish protein content.

Values of the following indices were determined from fish in each of the replicate test chambers over the 60-d experiment period, and averaged across the four replicates for each of the experimental diets in both feeding trials.

Total feed consumption (g/fish) = (total feed provided (g) – total unconsumed feed (g)) / N_f, where N_f is the average number of fish fed per day in a chamber.

$N_f = (n_1+n_2+n_3+\ldots+n_{60}) / 60$, where n_1, n_2, n_3, n_{60} are the total number of fish fed in a chamber on days 1, 2, 3, 60, respectively.

Relative growth rate, RGR (g 100 g^{-1} d^{-1}) = (wet weight gain ×100 / average fish weight)/ t, where wet weight gain in a chamber = final weight (g) – initial weight (g), average fish weight (g) = (initial weight + final weight) / 2, and t is duration of the experiment (60 d).

Feed conversion ratio (FCR) = total dry feed fed (g) / wet weight gain (g).

Protein efficiency ratio (PER) = wet weight gain (g)/ total protein fed (g).

Because fish fed high energy diets deposited more body fat, protein gain was determined in the experiment to indicate true somatic weight gain.

Protein gain (g $fish^{-1}$) = mean final fish protein (g) - mean initial fish protein (g).

Hepatosomatic index (HSI) and viscerosomatic index (VSI) were measured on day 60 for individual bluegills within each dietary group in both the experiments to determine the influences of dietary nutrients on fat deposition.

HSI = liver weight × 100/ whole body weight

VSI = visceral weight × 100/ whole body weight

Chemical and Statistical Analyses

All laboratory analyses (moisture, crude protein, amino acids, crude lipid, and ash) followed procedures recommended by the Association of Official Analytical Chemists (AOAC, 2000). Gross energy content was analyzed using an adiabatic bomb calorimeter (Parr Instrument Company, Moline, IL, USA). Crude protein contents of feed

and fish samples were determined by the combustion method using a LECO FP-528 (Leco Corporation, St. Joseph, MI, USA). Amino acids were analyzed using an automatic analyzer (Hitachi Model 835-50, Tokyo, Japan) with an ion exchange column at the Experiment Station Chemical Laboratories, University of Missouri, Columbia, MO, USA. Whole-body lipid content was estimated using the ether extraction method. Ash content was determined by incinerating the feed samples at 600 ˚C for 12 h in a muffle furnace.

One-way analysis of variance (ANOVA) was used to determine whether mean responses of each metric (feed consumption, feed efficiency, growth, body composition, survival), differed across the diets that contained increasing levels of protein or energy ($P < 0.05$). All data were evaluated for homogeneity of variances and normality. Survival data were arcsine square-root transformed prior to ANOVA. Where appropriate, means across diet types were separated by Tukey's test for multiple comparisons.

Protein requirement was determined by fitting a broken-line regression model (Robbins *et al.* 2006) to growth data, whereas energy requirement was determined by fitting growth responses with second-order polynomial models (Shearer 2000). Broken-line models were used to determine optimal dietary protein levels in order to best utilize the expensive protein for supporting fish growth; this is because protein levels that support maximum growth rate may not be cost effective. The polynomial model, on the other hand, was chosen for determining optimal energy requirement in order to exploit the greatest efficiency of low-cost, non-protein energy for maximizing protein accretion. All statistical analyses were performed using the Statistical Analysis System (SAS, Version 9.1; SAS Institute Inc., Cary, NC, USA).

RESULTS

Protein study

The overall fish survival rate was 93.7 ± 2.8% (mean ± 2.8); no differences were detected among the dietary groups ($P = 0.74$). Dietary protein levels significantly affected final mean weight, total feed consumption, RGR, and PER (ANOVA; $P < 0.05$), but not FCR, his, or VSI (Table 3).

Bluegill fed 355 g Kg^{-1} dietary protein showed the lowest final weights which differed significantly from those of fish fed 495 g Kg^{-1} dietary protein ($P < 0.05$).

Relative growth rate (RGR) of bluegill ranged from 0.90 (355 g Kg^{-1} dietary protein) to 1.36 g $100g^{-1}$ d^{-1} (495 g Kg^{-1} dietary protein) with fish fed 355 g Kg^{-1} dietary protein showing significantly lower RGRs ($P < 0.01$) than those fed ≥ 415 g Kg^{-1} dietary protein. Similarly, bluegill fed 375 g Kg^{-1} dietary protein exhibited a poorer RGR (1.04 g $100g^{-1}$ d^{-1}) than did those fed 495 g Kg^{-1} dietary protein, but showed no differences from other fish groups.

Similar to the pattern of RGR, feed consumption generally increased as the dietary protein level increased. Fish fed 355 g Kg^{-1} dietary protein level consumed significantly less feed than those fed ≥ 455 g Kg^{-1} dietary protein ($P < 0.05$). Likewise, fish fed the 375 and 395 g Kg^{-1} dietary protein levels consumed significantly less than those fed 495 g Kg^{-1} ($P < 0.05$), whereas fish fed intermediate levels of dietary protein (415-435 g Kg^{-1}) did not differ from their dietary counterparts. No differences were observed, however, in FCR among dietary groups ($P > 0.05$).

Protein efficiency ratio (PER) varied inversely withRGR, with fish fed low levels (≤ 395 g Kg^{-1}) of dietary protein generally exhibiting better utilization of dietary protein than those fed ≥ 475 g Kg^{-1} dietary protein ($P < 0.05$).

No significant differences ($P > 0.05$) were detected among the dietary groups in either HSI (1.36 ± 0.29 %, overall mean ± S.D.) or VSI (12.48 ± 2.06 %, overall mean ± S.D.).

Similar to what was observed for body condition indices (HSI or VSI), dietary protein levels did not affect fish fat levels or other proximate components ($P > 0.05$) (Table 3). Overall values (mean ± S.D.) of moisture, crude protein, crude lipid, and ash contents were 69.66 ± 0.84%, 16.01 ± 0.39%, 8.0 ± 0.89% and 4.5 ± 0.11%, respectively.

Broken-line regression analysis of RGR versus digestible protein yielded a break point at 412 g Kg^{-1} digestible protein level (Fig. 1).

Energy study

Survival rate of fish fed different levels of energy varied from 86.7 % to 100.0 %, but no significant differences were detected among the dietary groups ($P > 0.05$). Surprisingly, dietary energy levels affected neither feed consumption, RGR, FCR, nor protein gain ($P > 0.05$) (Table 5). However, increases in dietary energy levels significantly increased HSI and VSI values ($P < 0.05$) (Table 5). Fish fed the highest level of energy (17.57 MJ Kg^{-1}) showed significantly higher values of HSI than those fed ≤ 15.06 MJ Kg^{-1} dietary energy, whereas those fed 15.90-16.74 MJ Kg^{-1} dietary energy

exhibited intermediate HSI values ($P > 0.05$). Similarly, fish fed 17.57 MJ Kg^{-1} showed higher VSI values than those fed ≤ 13.39 MJ Kg.

As indicated by body condition indices (HSI and VSI), increased dietary energy levels also affected final fish whole-body moisture and crude lipid contents. Fish fed 17.57 MJ Kg^{-1} digestible energy produced significantly lower levels of moisture and higher levels of body fat content versus those fed ≤ 15.06 MJ Kg^{-1}, whereas no differences were observed in either of the response variables in the fish groups fed intermediate levels of energy (Table 5). Unlike for body fat or moisture content, dietary energy levels did not influence body protein or ash contents ($P > 0.05$) (Table 5). Estimated levels (overall mean ± S.D.) of crude protein and ash contents were 15.6 ± 0.33 % and 4.3 ± 0.22 %, respectively.

Based on the polynomial model, optimal levels of dietary digestible energy were determined to be 14.61 MJ Kg^{-1} (~3500 Kcal Kg^{-1}) and 14.65 MJ Kg^{-1} (~3500 Kcal Kg^{-1}) for the maximum values of RGR (Fig. 2a) and protein gain (Fig. 2b), respectively.

DISCUSSION

This study demonstrates that juvenile bluegill require ~410 g Kg^{-1} of dietary digestible protein and ~14.6 MJ Kg^{-1} of dietary digestible energy for optimal growth performance. Our study results further show that the growth rates of bluegill did not decline even at 490 g Kg^{-1} dietary protein level, indicating that this level of protein neither caused any metabolic disorder to the bluegill, nor did it reduce their energy

budget. The present study also demonstrates that high dietary energy inclusion (17.57 MJ Kg^{-1}) does not reduce either fish appetite or growth rate, but does result in elevated levels of fat deposition in bluegill.

Protein study

Dietary protein requirement varies among fishes. Planktivorous and omnivorous fishes typically require only 300-400 g Kg^{-1} of dietary protein (e.g., channel catfish *Ictalurus punctatus*, common carp *Cyprinus carpio* and Nile tilapia *Tilapia nilotica*), whereas piscivorous fishes require higher levels of dietary protein, 450-550 g Kg^{-1} (e.g., Atlantic salmon *Salmo salar*, plaice *Pleuronectes platessa*, red seabream *Chrysophrys major* and yellowtail *Seriola quinqueradiata*) (NRC 1993; De Silva & Anderson 1995). The present study shows that bluegill, an omnivore, require slightly higher levels of dietary protein (~410 g Kg^{-1}) relative to other common omnivorous fishes such as channel catfish and Nile tilapia. However, the protein requirement of bluegill is similar to those reported for its hybrids *L. cyanellus* ♀ × *L. macrochirus* ♂ (≥ 370 g Kg^{-1}, Tidwell *et al.* 1992; Webster *et al.* 1997; 440 g Kg^{-1} dietary protein, Stinefelt *et al.* 2004), and for other centrarchid fishes (e.g., ~400 g Kg^{-1} for largemouth bass *Micropterus salmoides*; Anderson *et al.* 1981; Portz *et al.* 2001 and ~450 g Kg^{-1} for smallmouth bass *M. dolomieui*; Anderson *et al.* 1981). Furthermore, the bluegill protein requirement value (~410 g Kg^{-1} digestible protein or ~450 g Kg^{-1} gross protein) determined in the present study did not differ substantially from that (440 g Kg^{-1}gross protein) reported by Hoagland *et al.* (2003) for juvenile bluegill. Although the diet containing 350 g Kg^{-1}

protein did provide adequate levels of EAAs for bluegill, the observed poor growth rate at this protein level indicates that bluegill consume protein in excess of the level required to meet EAA requirements for optimal growth performance. This is likely because that bluegill needed more amounts of non EAAs. On the other hand, the lack of a significant increase in bluegill growth for > 410 g Kg^{-1} digestible protein, as well as an observed decline in PER, suggest that excess dietary protein levels were directed towards energy utilization instead of into further protein accretion. Similar to this observation, decline in protein efficiency as the dietary protein level exceeds the requirement for somatic growth, has often been observed in other fish studies (e.g., Hafedh 1999; Ng *et al.* 2001; Schulz *et al.* 2007). Therefore, supplementing excess levels of expensive protein will also not be beneficial given that it does not increase fish growth, but rises feed cost.

Despite having fixed levels of energy across the diets, our bluegill HSI and VSI values showed marginal differences across the diets, with fish fed lower protein levels (≤ 375 g Kg^{-1}) exhibiting higher values. This likely resulted from poor utilization of the dextrin that was added at high levels in the low protein diets. Enlarged livers have often been observed in fishes fed elevated levels of dietary carbohydrate (Wilson 1994). Finding no differences in whole-body protein or fat contents in the present study parallels what was observed for bluegill by Hoagland *et al.* (2003) or for Mexican silverside *Menidia estor* by Martinez-palacios *et al.* (2007). However, whole-body lipid levels often decline with increasing dietary protein level in fishes, e.g., in Nile tilapia (Hafedh 1999), bagrid catfish *Mystis nemurus* (Ng *et al.* 2001) and pike perch *Sander lucioperca* (Schulz *et al.* 2007). Studies (e.g., Hafedh 1999; Ng *et al.* 2001; Schulz *et al.* 2007) that observed significant differences in whole-body lipid contents typically involved fish fed an amount

of feed proportional to fish weight in each dietary group. Doing so likely increased the availability of non-protein energy to fish that were fed low-protein diets, and produced in them increased body fat. However, in the present study or in other similar studies (e.g., Hoagland *et al.* 2003; Martinez-palacios *et al.* 2007) that did not observe significant differences in fat deposition, fish were fed to apparent satiation and were observed to have increased their feed consumption for the increasing levels of dietary protein. Increased feed consumption in these studies likely caused no substantial differences in the absolute amount of non-protein energy across dietary groups and therefore, produced no significant differences in body fat deposition.

The absolute growth rates (AGR) of bluegill in the present study varied from 0.27 g d^{-1} to 0.45 g d^{-1} which is, indeed, higher than those observed for bluegills in other related studies: 0.10 g d^{-1} for 6 g bluegill reared for 75 days (Hoagland *et al.* 2003), and 0.14-0.23 g d^{-1} for 8-14 g bluegill reared for 56 d (Twibell *et al.* 2003). This difference could be attributed to differences in the initial size of bluegills and the diet composition among studies: fish have been observed to generally exhibit higher AGRs at intermediate size than at small or large size in their life stage (Hopkins 1992). Also, high levels of dietary fish meal (> 500 g Kg^{-1}) used in the present study may have supported high bluegill growth.

Shearer (2000) showed that a polynomial model provides better fits than broken-line regression models in many studies of fish nutrient requirements, and that the former model produces a higher nutrient requirement level than the latter. Feeding bluegill a high percentage of dietary protein to achieve modest increases in growth rate may not be economically beneficial to fish producers. This is because increasing the protein level

results in the poor protein utilization we observed in the present study. Also, the present study showed no significant differences in the growth rate of bluegill fed ~410 g Kg^{-1} dietary protein versus ~500 g Kg^{-1} dietary protein. Because of the such reasons, the broken-line regression model has been preferred by many researchers when determining optimal dietary protein requirements for fishes (e.g., Kim *et al.* 2001; Luo *et al.* 2004; Meyer & Fracalossi 2004; Mohanta *et al.* 2008).

Energy study

Energy requirements (digestible basis) for freshwater fishes generally range from ~12 MJ Kg^{-1} (channel catfish, Nile tilapia, hybrid striped bass *Morone chrysops* ♀ ×*M. saxatilis* ♂) to ~17 MJ Kg^{-1} (rainbow trout) (NRC 1993). In the present study, where as little as 12.55 MJ Kg^{-1} dietary energy appears sufficient for bluegill, increasing the dietary energy level through non-protein energy source to 14.65 MJ Kg^{-1} was beneficial in terms of sparing more protein for slightly higher somatic growth, as was indicated by the polynomial model results. Directing expensive dietary protein for protein accretion rather than for energy utilization will eventually reduce feed cost and thereby increase the economic gain of bluegill aquaculture.

Increasing the lipid energy level to the dietary optimum has increased feed efficiency in fishes (e.g., Williams & Robinson 1988; Thoman *et al.* 1999). On the other hand, excess dietary energy and the resultant fat accumulation has often been shown to suppress fish feed consumption ("lipostatic regulation of feed intake") (Jobling & Miglavs 1993; Shearer *et al.* 1997; Johansen *et al.* 2002 & 2003). Interestingly,

increasing the dietary energy level in the present study affected neither feed consumption nor feed efficiencies. The absence of fish appetite regulation due to increased dietary energy levels in the present and other like studies (e.g., Lee *et al.* 2000, De Silva *et al.* 2002) may relate to the restricted experimental periods (~8 weeks) that these studies used; studies that did show suppressed feed intake from high fat deposition levels had been run for longer periods (> 13 weeks).

Despite increased dietary energy levels, bluegill did not exhibit improved protein efficiency. However, the excess dietary energy levels elicited significant increases in HSI, VSI, and whole-body fat content. This result indicates the limited ability of bluegill to use lipid as an energy source and to spare protein for somatic growth. Hence, bluegill require high percentages of dietary protein not only for somatic growth but also for energy utilization. Hoagland *et al.* (2003) also observed for juvenile bluegill (1.76 g) that increasing dietary lipid from 8% to 12% did not result in a protein sparing effect. Weight gain by fat deposition may not be reflective of true somatic growth in fishes (Lovell 1998). Although the present study did not show evidence of reduced feed consumption, high fat deposition in the long run will likely reduce fish appetite, growth rate, and production. As motioned above, high fat deposition resulting in reduced feed intake has been demonstrated in fishes particularly salmonids (Shearer *et al.* 1997; Johansen *et al.* 2002 & 2003) when the studies were run for > 13 weeks; these studies suggest that fish do possess lipostatic regulatory mechanism of food intake (adipose tissue, due to excess fat deposition, signaling brain via hormones such as leptin and limiting feed intake).

Capacity of non-protein energy sparing dietary protein for body-protein accretion differs across species depending on the source of the non-protein energy. For example,

lipid acts as the primary protein-sparing source in salmonids (NRC 1993), whereas carbohydrate acts as the primary-protein sparing source for tilapia, catfish, and carps (NRC 1993; Stone 2003). Differences in energy utilization capacity among species may be associated with energy requirement. For example, rainbow trout generally require 15-17 MJ Kg^{-1} dietary energy, whereas catfish, Nile tilapia, and common carp require about 12-13 MJ Kg^{-1} (NRC 1993; Medale & Guillaume 2001). Collectively, these findings suggest that fish with lower energy requirements will exhibit a greater protein sparing effect from carbohydrate versus lipid, and vice versa. Accordingly, the limited capacity of bluegill to use lipid may be associated with their relatively low levels of dietary energy requirements (~14.6 MJ Kg^{-1}). Further research may be warranted to determine the energy sources that best maximize protein sparing in bluegill.

The absolute growth rates (AGR) of bluegill in the energy study ranged from 0.18 g d^{-1} to 0.23 g d^{-1}. While such growth rates are comparable to those (0.10 - 0.23 g d^{-1}) observed in related studies (Hoagland *et al.* 2003; Twibell *et al.* 2003), bluegill from the energy experiment exhibited poorer feed consumption and growth rates than did those in the protein experiment. Conceivably, this lower consumption and growth could be due to our having added an indigestible fiber material (α-cellulose) to the diets to obtain desired levels of protein and energy. Reduced growth rates resulting from dietary inclusion of α-cellulose have been noted in other related studies (e.g., Hilton *et al.* 1983; Lee *et al.* 2003). However, studies that determine requirements for dietary energy level often include indigestible materials such as α-cellulose to maintain constant protein levels across diets while also increasing energy levels and therefore, reduced growth rates due to α-cellulose in this type of nutritional studies appears inevitable.

Protein to energy ratio (P/E ratio)

Protein:energy ratio is not a nutritional requirement, but is often determined for fishes as a measure of protein sparing capacity from non-protein energy sources. P/E ratios range from about 20 to 26 g MJ^{-1} for common freshwater fishes (NRC 1993; Medale & Guillaume 2001). Low values indicate that fish can better utilize non-protein energy, and that protein is mainly used for growth and maintenance requirements. Higher ratios indicate that fish are relatively poor users of non-protein energy and that the protein is allocated to meeting energy as well as growth and maintenance requirements.

A high P/E ratio of 28.1 g MJ^{-1} (118.3 mg $Kcal^{-1}$) was observed for bluegill, as has been observed in many other fishes: e.g., 25.4-32.8 g MJ^{-1} (106-137 mg $Kcal^{-1}$) for largemouth bass (Bright *et al.* 2005), 27.7 g MJ^{-1} for amberjack *Seriola dumerili* (Takakuwa *et al.* 2006), and 26.7 g MJ^{-1} for pikeperch (Schulz *et al.* 2007). However, the value of 28.1 g MJ^{-1} for bluegill is substantially greater than that determined for channel catfish (23.2 g MJ^{-1} by Robinson & Li 2002) or rainbow trout (22-25 g MJ^{-1} by NRC 1993). Hence, the present study indicates that commonly used, "practical" industry diets that are optimized mainly for rainbow trout or catfish, are likely suboptimal for bluegill. Protein sparing can be effectively achieved by feeding diets that include particular carbohydrate and lipid level combinations from certain sources. Indeed, carbohydrate-to-lipid ratios have been optimized in many fishes (e.g., Asian seabass *Lates calcarifer* Catacutan & Coloso 1997, walking catfish *Clarias batrachus* Erfanullah & Jafri 1998 and piracanjuba *Brycon orbignyanus* Borba *et al.* 2006) to increase protein efficiency for somatic growth. Such a study may be warranted for bluegills, to further increase protein sparing efficiency via non-protein energy sources.

In summary, the present study demonstrates that juvenile bluegill require high levels of dietary protein (~410 g Kg^{-1} digestible protein) and low levels of dietary energy (~14.6 MJ Kg^{-1}) with a P/E ratio of ~28 g MJ^{-1}. The study also provides evidence that lipid is not an appropriate protein-sparing source for juvenile bluegill, emphasizing that appropriate dietary lipid-to-carbohydrate ratios should be determined for juvenile bluegill to reduce expensive dietary protein levels.

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Table 1. Nutrient profile of ingredients used in the study.

Nutrients*	Fish Meal	Blood Meal	SBM	CGM	Wheat	Dextrin	Fish Oil	Lecithin
Protein ($g\ Kg^{-1}$)	592.0 (544.6)	915.8 (770.2)	447.5 (425.5)	625.0 (523.0)	112.5 (103.1)			
Energy ($MJ\ Kg^{-1}$)	17.7 (15.5)	23.6 (21.7)	18.4 (14.7)	15.9 (14.4)	16.4 (9.1)	16.7 (15.1)	37.7 (34.0)	31.8 (28.6)
Amino acids ($g\ Kg^{-1}$)								
Arginine	34.6 (32.5)	36.3 (33.2)	35.5 (34.4)	20.0 (18.2)	06.3 (05.8)			
Histidine	12.9 (11.9)	42.2 (39.7)	11.9 (11.5)	18.9 (16.7)	02.6 (02.4)			
Isoleucine	24.5 (22.7)	07.6 (06.2)	21.2 (20.0)	27.7 (24.5)	04.0 (03.4)			
Leucine	43.5 (40.5)	114.7 (106.0)	36.8 (34.6)	123.6 (101.4)	08.1 (07.4)			
Lysine	45.6 (43.2)	73.2 (69.6)	29.5 (28.2)	11.4 (10.3)	03.4 (02.8)			
Methionine	15.9 (14.6)	07.0 (06.6)	06.7 (06.3)	15.8 (14.9)	02.4 (02.2)			
Cysteine	04.7 (03.9)	06.9 (05.9)	06.7 (06.3)	11.8 (11.0)	02.8 (02.5)			
Phenylalanine	23.9 (21.9)	56.4 (53.2)	23.7 (22.4)	40.3 (35.6)	05.4 (04.9)			
Tyrosine	15.4 (14.1)	11.5 (10.9)	14.5 (13.9)	35.1 (30.7)	02.4 (02.1)			
Threonine	24.4 (22.8)	27.0 (25.0)	18.1 (16.9)	22.2 (20.0)	03.6 (03.1)			
Tryptophan	04.8 (04.4)	09.2 (09.0)	05.4 (05.2)	03.8 (03.6)	01.1 (00.9)			
Valine	29.8 (27.3)	79.1 (73.1)	22.8 (21.4)	33.6 (27.8)	05.0 (04.5)			

*Values in the parenthesis represent digestible amount calculated from their respective percentage digestibility values (Masagounder *et al.* 2009; Masagounder *et al.* accepted). Energy digestibility values of dextrin, fish oil and lecithin were assumed to be 90%.

Table 2. Formulation of the experiment diets used in the study.

	Diets							
	D1	D2	D3	D4	D5	D6	D7	D8
Ingredients (g Kg^{-1})								
Menhaden fish meal	500.3	535.9	571.4	611.7	654.6	697.4	748.4	804.4
Blood meal	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Soybean meal	70.0	70.0	70.0	70.0	70.0	70.0	59.9	43.0
Corn gluten meal	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0
Wheat	161.6	164.2	166.8	147.2	115.4	83.5	53.5	26.9
Dextrin	99.8	61.7	23.6	10.0	10.0	10.0	10.0	7.5
Fish oil	90.0	90.0	90.0	82.9	71.9	60.8	50.0	40.0
Lecithin	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8
Vitamin premix	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Vitamin C	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Choline chloride	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
Mineral premix	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Binder	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Total	1000	1000	1000	1000	1000	1000	1000	1000
Proximate composition (digestible values)								
Protein (g Kg^{-1})	355	375	395	415	435	455	475	495
Energy (MJ Kg^{-1})	15.9	15.9	15.9	15.9	15.9	15.9	15.9	15.9
Amino acids (g Kg^{-1})								
Arg	21.0	22.2	23.3	24.5	25.7	27.0	28.1	29.2
His	8.8	9.2	9.6	10.1	10.5	10.9	11.4	11.8
Iso	14.4	15.2	16.0	16.9	17.8	18.6	19.5	20.3
Leu	30.1	31.5	33.0	34.5	36.0	37.5	39.0	40.4
Lys	25.8	27.4	28.9	30.6	32.4	34.1	36.0	37.8
Met	8.8	9.4	9.9	10.4	11.0	11.5	12.1	12.8
Cys	3.4	3.5	3.6	3.8	3.8	3.9	4.0	4.0
Phen	15.8	16.6	17.4	18.2	19.0	19.7	20.5	21.2
Tyr	9.8	10.3	10.8	11.4	11.9	12.4	12.9	13.4
Thr	14.4	15.2	16.0	16.9	17.8	18.6	19.5	20.5
Trp	3.0	3.2	3.4	3.5	3.7	3.8	4.0	4.1
Val	18.5	19.4	20.4	21.4	22.5	23.5	24.5	25.6
Gross Protein (g Kg^{-1})	418	439	444	447	474	513	543	570
Gross Energy (MJ Kg^{-1})	20.8	20.9	20.3	19.7	22.0	20.2	19.1	19.9
Crude Ash (g Kg^{-1})	112	117	114	118	131	149	159	168

*Vitamin and mineral premixes were similar to the one used by Masagounder *et al.* (accepted).

Table 3. Growth responses and proximate composition (means ± SD) of juvenile bluegills fed the experimental diets for 60 days (Values within a row sharing different superscript alphabets are significantly different, $P < 0.05$).

Variable	Experiment Diets (Digestible protein level g Kg^{-1})								*P*-value (ANOVA)
	D1 (355)	D2 (375)	D3 (395)	D4 (415)	D5 (435)	D6 (455)	D7 (475)	D8 (495)	
Growth Responses									
Initial weight (g)	21.7±1.4	18.3±1.1	19.5±1.7	20.9±0.8	19.6±1.1	21.5±1.7	20.6±0.9	20.0±2.2	0.86
Final weight (g)	37.8±2.7^{a}	38.0±7.2^{ab}	40.1±3.7^{ab}	46.3±3.4^{ab}	41.7±0.4^{ab}	48.0±5.2^{ab}	46.7±7.2^{ab}	47.7±5.6^{b}	0.04
Feed consumption (g $fish^{-1}$)	22.1±2.1^{a}	25.3±4.3^{ab}	24.5±2.7^{ab}	29.1±2.5^{abc}	27.8±1.3^{abc}	32.8±3.7^{bc}	32.8±6.0^{bc}	34.7±1.3^{c}	<0.01
RGR (g $100g^{-1}$ d^{-1})	0.90±0.15^{a}	1.04±0.13^{ab}	1.15±0.04^{abc}	1.26±0.08^{bc}	1.20±0.08^{bc}	1.26±0.19^{bc}	1.21±0.14^{bc}	1.36±0.05^{c}	<0.01
FCR	1.36±0.17	1.39±0.12	1.17±0.11	1.15±0.03	1.26±0.13	1.26±0.13	1.28±0.13	1.27±0.14	0.11
PER	2.18±0.40^{a}	1.95±0.16^{ab}	2.15±0.34^{a}	2.10±0.06^{ab}	1.83±0.18^{ab}	1.76±0.07^{ab}	1.65±0.17^{b}	1.61±0.17^{b}	<0.01
Survival (%)	96.7±5.8	90.0±10.0	95.0±10.0	93.0±5.8	90.0±17.3	92.5±9.6	97.5±5.0	95.0±10.0	0.74
HSI	1.51±0.29	1.61±0.17	1.27±0.23	1.47±0.45	1.25±0.35	1.17±0.14	1.23±0.24	1.22±0.19	0.09
VSI	13.87±2.68	12.08±1.15	14.11±2.40	12.56±3.31	10.81±0.70	11.54±2.01	11.83±1.11	11.86±1.24	0.10
Proximate composition									
Moisture	68.66±1.55	70.02±1.52	68.56±0.78	68.91±1.92	70.07±0.53	70.55±0.51	70.68±0.59	69.85±0.28	0.14
Protein	16.50±1.14	15.29±0.57	16.08±0.23	15.73±0.59	15.84±0.56	16.02±0.63	16.20±0.50	16.42±0.24	0.11
Crude lipid	8.20±0.87	8.35±0.28	8.72±0.79	9.38±0.72	7.79±0.45	6.99±0.74	6.65±0.52	7.67±0.44	0.28
Ash	4.35±0.42	4.54±0.20	4.49±0.08	4.66±0.29	4.67±0.13	4.58±0.25	4.45±0.35	4.43±0.19	0.73

Table 4. Formulations of the diets containing graded levels of energy.

	Diets						
	D1	D2	D3	D4	D5	D6	D7
Ingredients (g Kg^{-1})							
Menhaden fish meal	613.2	613.2	613.2	613.2	613.2	613.2	613.2
Corn gluten meal	150.0	150.0	150.0	150.0	150.0	150.0	150.0
Fish oil	24.1	48.7	73.4	98.0	122.6	147.3	171.9
α-Cellulose	194.5	169.8	145.2	120.6	95.9	71.3	46.7
Lecithin	2.8	2.8	2.8	2.8	2.8	2.8	2.8
Vitamin premix*	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Vitamin C	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Choline chloride	1.2	1.2	1.2	1.2	1.2	1.2	1.2
Mineral premix*	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Binder	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Total	1000	1000	1000	1000	1000	1000	1000
Proximate composition (digestible values)							
Protein (g Kg^{-1})	412	412	412	412	412	412	412
Energy (MJ Kg^{-1})	12.55	13.39	14.23	15.06	15.90	16.74	17.57
(Kcal Kg^{-1})	3000	3200	3400	3600	3800	4000	4200
Amino acids (g Kg^{-1})							
Arg	22.7	22.7	22.7	22.7	22.7	22.7	22.7
His	9.1	9.1	9.1	9.1	9.1	9.1	9.1
Iso	17.6	17.6	17.6	17.6	17.6	17.6	17.6
Leu	40.0	40.0	40.0	40.0	40.0	40.0	40.0
Lys	28.0	28.0	28.0	28.0	28.0	28.0	28.0
Met	11.2	11.2	11.2	11.2	11.2	11.2	11.2
Cys	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Phen	18.8	18.8	18.8	18.8	18.8	18.8	18.8
Tyr	13.3	13.3	13.3	13.3	13.3	13.3	13.3
Thr	17.0	17.0	17.0	17.0	17.0	17.0	17.0
Trp	3.2	3.2	3.2	3.2	3.2	3.2	3.2
Val	20.9	20.9	20.9	20.9	20.9	20.9	20.9
Gross Protein	459	460	456	457	452	458	452
Gross Energy	18.61	19.09	19.62	20.67	21.48	21.66	22.81
Crude Ash	135	132	131	130	133	138	135

*Vitamin and mineral premixes were similar to the one used by Masagounder *et al.* (accepted).

Table 5. Growth responses and proximate composition (means ± SD) of juvenile bluegills fed the energy diets for 60 days (Values within a row sharing different superscript alphabets are significantly different, $P < 0.05$).

Variable	Experimental Diets (Digestible energy level MJ Kg^{-1})							*P*-value (ANOVA)
	D1 (12.55)	D2 (13.39)	D3 (14.23)	D4 (15.06)	D5 (15.90)	D6 (16.74)	D7 (17.57)	
Growth Responses								
Initial weight (g)	20.8±0.4	21.7±1.4	22.8±0.3	21.5±1.4	21.4±0.5	20.5±1.6	19.6±1.7	0.17
Final weight (g)	31.7±1.2	33.8±2.6	36.7±1.2	34.0±3.3	33.1±1.9	31.6±1.6	30.6±2.4	0.07
Feed consumption (g $fish^{-1}$)	16.4±8.8	16.4±1.2	18.4±0.8	15.0±6.6	17.3±3.8	18.5±4.9	17.4±1.6	0.95
RGR (g $100g^{-1}$ d^{-1})	0.70±0.03	0.74±0.10	0.79±0.04	0.76±0.08	0.73±0.05	0.72±0.07	0.71±0.08	0.75
PER	1.59±0.81	1.8±0.01	1.83±0.16	2.2±0.64	1.67±0.17	1.51±0.31	1.52±0.05	0.55
Protein Gain (g $fish^{-1}$)	1.90±0.22	2.08±0.10	2.18±0.28	2.02±0.22	2.06±0.21	1.92±0.07	1.91±0.31	0.73
FCR	1.33±0.30	1.35±0.25	1.34±0.12	1.22±0.25	1.46±0.15	1.51±0.29	1.59±0.23	0.42
Survival (%)	90.0±14.1	87.50±5.0	86.7±11.5	87.5±12.6	93.3±11.5	90.0±8.2	100.0±0.0	0.28
HSI	$1.00±0.06^{a}$	$0.99±0.03^{a}$	$0.97±0.12^{a}$	$0.97±0.07^{a}$	$1.03±0.12^{ab}$	$1.02±0.03^{ab}$	$1.22±0.08^{b}$	<0.01
VSI	$8.83±0.49^{a}$	$8.46±0.67^{a}$	$8.93±0.65^{ab}$	$8.99±0.64^{ab}$	$8.94±0.60^{ab}$	$9.19±0.33^{ab}$	$9.92±0.36^{b}$	<0.01
Proximate Composition								
Moisture	$71.0±0.6^{a}$	$71.0±1.8^{a}$	$71.0±1.1^{a}$	$70.6±0.7^{a}$	$69.5±0.4^{ab}$	$69.4±1.0^{ab}$	$68.2±0.7^{b}$	<0.01
Protein	15.6±0.4	15.4±1.0	15.3±0.6	15.2±0.4	16.2±0.4	15.7±0.3	15.5±0.4	0.58
Crude lipid	$9.6±0.5^{a}$	$9.7±0.3^{a}$	$10.0±0.6^{a}$	$10.2±0.4^{a}$	$10.9±0.6^{ab}$	$11.0±0.8^{ab}$	$12.1±0.8^{b}$	<0.01
Ash	4.1±0.1	4.3±0.2	4.1±0.2	4.4±0.4	4.5±0.2	4.6±0.6	4.6±0.5	0.30

Figure 1. Broken-line regression model fitted to RGRs of bluegills (mean ± 1SD) fed the experimental diets containing graded levels of dietary digestible protein: RGR = 1.261 – 0.006 (411.9 – Protein), where (411.9 – Protein) = 0 when Protein > 411.9.

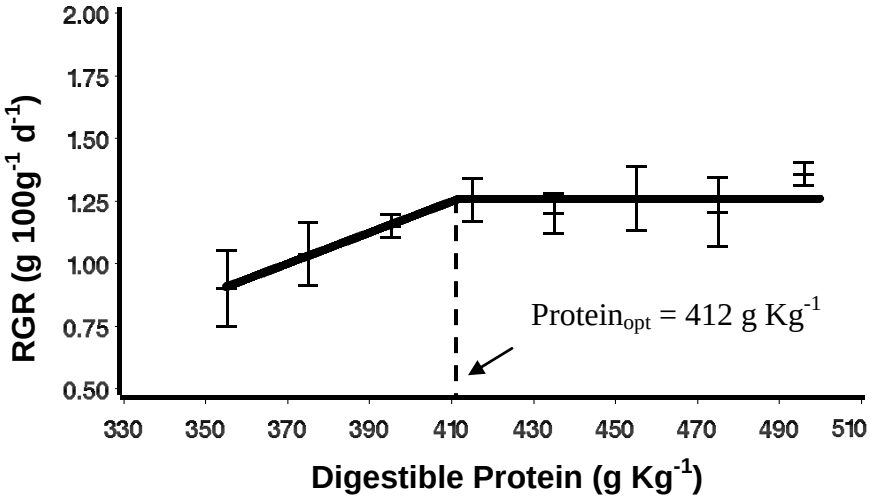


Figure 2a. Second-order polynomial model fitted to RGR of bluegills (mean ± 1SD) fed the experimental diets containing graded levels of dietary digestible energy: RGR = -0.009 (Digestible Energy)^2 + 0.263 (Digestible Energy) – 1.204.

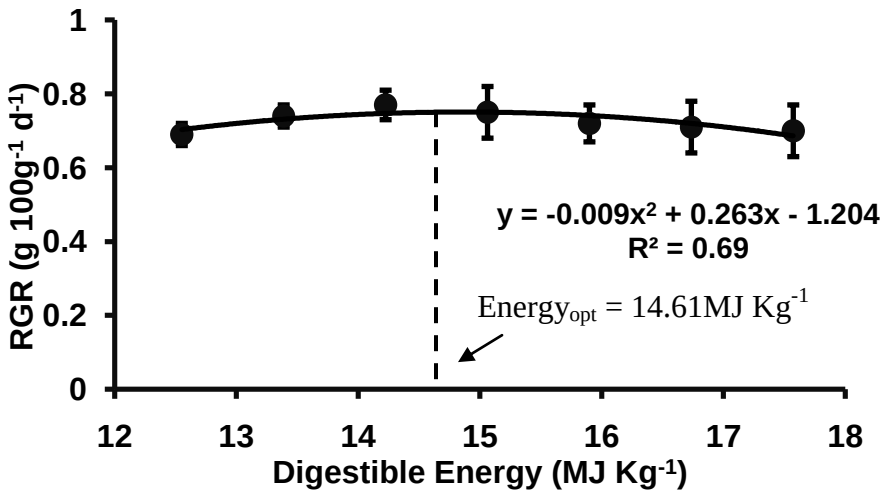
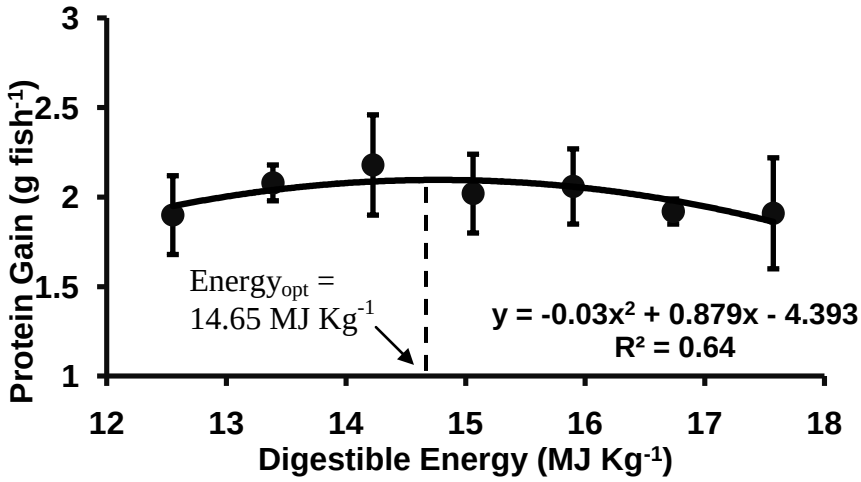


Figure 2b. Second-order polynomial model fitted to protein gain of bluegills (mean ± 1SD) fed the experimental diets containing graded levels of dietary digestible energy: Protein gain = -0.03 (Digestible Energy)^2 + 0.879 (Digestible Energy) – 4.393.



CHAPTER 4

DEVELOPMENT OF A FISH- MEAL-FREE, LEAST-COST DIET FORMULATION FOR JUVENILE BLUEGILL, *LEPOMIS MACROCHIRUS*

ABSTRACT

A 60-d study was conducted to determine the least-cost diet formulation for juvenile bluegill *Lepomis macrochirus*. Seven experimental diets were computer formulated for evaluation by gradually replacing fish meal with a blend of alternative protein sources. Fish meal inclusion levels in experimental diets 1 through 6 ranged from 550 g Kg^{-1} (diet 1) to 0 g Kg^{-1} (diet 6). Optimal digestible energy (DE) (14.64 MJ Kg^{-1}), digestible protein (410 g Kg^{-1}) and essential amino acid levels determined in previous studies were maintained across the six diets. Ingredient costs for the six diets ranged from \$ 899.69 $tonne^{-1}$ (diet 1) to \$ 616.62 $tonne^{-1}$ (diet 6). Additional effort was put forth to further reduce feed cost; diet 7 was prepared by slightly reducing digestible protein (400 g Kg^{-1}) and energy (13.95 MJ Kg^{-1}) levels, which lowered the ingredient cost to \$ 587.41 $tonne^{-1}$ (0 g fish meal). Sources of protein feedstuffs considered in the software program were menhaden fish meal, poultry byproduct meal, porcine meat and bone meal, blood meal, soybean meal and corn gluten meal. Three commercial diets were included in the study as practical control diets: a high-energy trout diet (450 g Kg^{-1} protein, 160 g Kg^{-1} fat), a low-energy trout diet (400 g Kg^{-1}protein, 100 g Kg^{-1} fat) and a catfish diet (350 g Kg^{-1} protein, 70 g Kg^{-1} fat). Quintuplicate bluegill groups (~22 g, n = 10 fish per group) were fed the experimental diets twice daily to apparent satiation for 60 d. No significant differences in feed consumption, feed efficiency nor growth rate were detected among

bluegill groups fed experimental diets 1 through 7. Fish fed the catfish diet exhibited a poorer growth rate than did those fed diets with high levels of fish meal (≥ 300 g Kg^{-1}) or the high-energy trout diet. The trout diets produced higher whole-body-lipid deposition than did diets 3 through 7. Fish fed diets 6 and 7 produced significantly higher gain:cost ratios (weight gain (g) / feed consumed (g) × ingredient cost per gram feed) than did fish fed diets 1 through 5. Relative to diet 1, ingredient costs of diets 6 and 7 were lower by 32% and 35%, respectively. Nevertheless, diet 6 produced slightly better overall fish growth performance than did diet 7. Relative to fish fed the high-energy and low-energy trout diets, fat content of fish fed diet 6 was lower by 34% and 27%, respectively. Study results indicate diet 6, comprising predominantly SBM (~37%) and MBM (~38%), to be the best, least-cost diet for juvenile bluegill.

INTRODUCTION

The demand for large, food-size bluegill *Lepomis macrochirus* has increased in recent years and rearing techniques for this species have improved concurrently (NCRAC 2005; Hayward & Wang 2006; Wang *et al.* 2009; Hicks *et al.* 2009). A survey (NCRAC 2005) by the Industry Advisory Council of the North Central Regional Aquaculture Center (NCRAC) involving 71 fish growers throughout the North Central Region of the U.S. showed bluegill to be among the top two fish species reared in this region. Hicks *et al.* (2009) recently demonstrated that selective breeding for growth is a promising rearing technique for producing food-size bluegill within two growing seasons. Rearing male-only bluegill appears to be another approach for producing food-size bluegill within two

growing seasons (Hayward & Wang 2006; Wang *et al.* 2009). However, despite the increasing market demand for bluegill and associated advancements in bluegill rearing technology, a much-needed, cost-effective bluegill diet remains to be identified. It is notable that available practical diets for bluegill are not only expensive, but also produce poor growth rates (e.g., catfish diets) and often lead to high levels of body fat deposition (e.g., when feeding trout diet) (Twibell *et al.* 2003). A survey by NCRAC (2005) indicated that fish producers considered the lack of a nutritionally balanced, affordable diet for bluegill to be a major current constraint to sunfish aquaculture.

Development of a least-cost, economic feed formulation for a farm animal requires information concerning their dietary nutrient requirements, nutrient availability from commonly used feedstuffs, cost and availability of individual feedstuffs, and their dietary inclusion limits (Cheeke 2005). However, such data are readily available for only a limited number of aquaculture species including rainbow trout *Oncorhynchus mykiss* and channel catfish *Ictalurus punctatus*. Recently, Allan *et al.* (2000) developed a least-cost diet formulation for Australian silver perch *Bidyanus bidyanus* after conducting sequential studies on digestibility, nutrient requirements, and feedstuff inclusion limit. A similar approach was used to develop a cost effective complete diet formulation for juvenile bluegill based on the digestibilities of common feedstuffs (Masagounder *et al*. 2009), dietary requirements for essential amino acids (EAAs) (Masagounder *et al*. *accepted*) and for protein and energy (Chapter 3). Information concerning the palatability of feedstuffs was not readily available, yet the recent study on bluegill's digestibility (Masagounder *et al*. 2009) that used individual ingredients as the test diets provided evidence that the palatability of common feedstuffs (including soybean meal) was not an

impediment. Given that fish prefer diets containing fish meal (De Silva & Anderson 1995; Tacon & Metian 2008), which tend to produce desirable growth rates (Rumsey 1993; Hardy 2008), alternative less expensive protein feedstuffs are typically evaluated for palatability and fish growth performance by feeding fish test diets containing increasing levels of the substitute. Consequently, the objective of the present study was to develop an economically favorable diet formulation for juvenile bluegill by gradually replacing fish meal with a blend of alternative protein feedstuffs, using a least-cost feed formulation program.

MATERIAL AND METHODS

Experimental diets

All experimental diets were formulated using least-cost formulation software, WUFFDA (Windows-based User Friendly Feed Formulation, Research Bulletin 438, 2003, University of Georgia, USA). The software is programmed in an Excel® using separate work sheets for the ingredient profile (cost, nutrient profile), nutrient requirements, and formulation, respectively.

In the 'ingredient profile' work sheet, all ingredients considered (Table 2) in the experimental diet formulations are provided. The protein sources we considered included menhaden fish meal (FM), poultry byproduct meal (pet-food grade) (PBM), porcine meat and bone meal (MBM), blood meal (BM), soybean meal (SBM), and corn gluten meal (CGM). Ingredient costs for the protein sources, as well as for corn and wheat, were

determined from the weekly newspaper 'feedstuffs'. Prices for these feedstuffs, published during the first week of each month for the years 2008 and 2009 for nearby major cities (Kansas City, MO; Memphis, TN), were averaged and used. Prices for other ingredients were obtained from the respective commercial suppliers (Table 2). Ingredient cost, however, did not include freight charges. Digestible nutrient levels (amino acids, protein, and energy) from each of the ingredients were calculated using the estimated gross nutrient levels and the nutrient digestibility values determined by Masagounder *et al.* (2009) and Masagounder *et al.* (*accepted*) for bluegill (Table 1). For amino acids, protein, and energy, these digestible levels were provided in the ingredient profile work sheet, whereas for vitamins and minerals, values were taken from NRC (1993) for the respective feedstuffs.

In the 'nutrient requirement' work sheet, for protein and energy, the levels determined to be ideal (410 g Kg^{-1} digestible protein and ~14.65 MJ Kg^{-1} or 3500 Kcal Kg^{-1} digestible energy, Chapter 3) for optimal growth performance of juvenile bluegill were provided as fixed nutrient constraints. For digestible EAAs, the nutrient requirement values (Masagounder *et al. accepted*) were increased by 10% and provided as minima, considering that the EAAs are the critical determinants of fish growth (Masagounder *et al. accepted*).

Similarly, for lipid, a range of 80 to 100 g Kg^{-1} was provided in the 'nutrient requirement' work sheet, as the level to be met in the diet formulations, following the value (80 g Kg^{-1}) determined to be ideal for a 6-g bluegill (Hoagland *et al.* 2003).

In the 'formulate' worksheet, constraints were given for the FM to be selected at levels, 550 g Kg^{-1}, 400 g Kg^{-1}, 300 g Kg^{-1}, 200 g Kg^{-1}, 100 g Kg^{-1} and 0 g Kg^{-1} for diets 1 through 6, respectively. The graded replacement of fish meal was tested in the experimental diets, given that information on an inclusion limit for each feedstuff was not available for bluegill. Constraints on fish oil, lecithin, vitamin, mineral, and binder were provided to fix the amount for each (Table 2), and to thereby balance the diets for other essential nutrients including fatty acids, vitamins, and minerals, following the recommendations of NRC (1993) for general freshwater fishes. The computer solved the constraints for the least-cost formulation for all the six diets (Table 2), and the feed formulation software selected increasing levels of MBM and SBM as the dietary fish meal level was gradually reduced (Table 2). Ingredient costs of the six diets varied from \$ 899.69 $tonne^{-1}$ or \$ 816.23 ton^{-1} (diet 1) to \$ 616.62 $tonne^{-1}$ or \$ 559.41 ton^{-1} (diet 6). Diets 1-6 were made isocaloric and isonitrogenous on a digestible basis (Table 2).

Additional effort was made to further reduce feed cost; diet 7 was prepared by reducing the protein level from 410 g Kg^{-1} to 400 g Kg^{-1} and the energy level from 14.64 MJ Kg^{-1} (3500 Kcal Kg^{-1}) to 13.95 MJ Kg^{-1} (3333 Kcal Kg^{-1}), thereby, reducing the ingredient cost from \$ 899.69 $tonne^{-1}$ or \$ 559.41 ton^{-1} (0 g fish meal Kg^{-1}) to \$ 587.41 $tonne^{-1}$ or \$532.94 ton^{-1} (0 g fish meal Kg^{-1}) (Table 2). In the ingredient constraint list for diet # 7, no changes were made beyond those from diet 6, except for (i) dicalcium phosphate which was removed, as MBM, itself, is a good source of calcium and phosphorus, and (ii) fish oil, the level of which was reduced from 40 g Kg^{-1} (diet 1-6) to 30 g Kg^{-1}. Although a level of 40 g Kg^{-1} fish oil was considered sufficient to supply adequate levels of highly unsaturated fatty acids for fishes such as rainbow trout (Hardy

2002), the level was reduced to 30 g Kg^{-1} given that freshwater fish generally grow well on lower levels of EFAs (Sargent *et al.* 2002).

Three commercial diets (Nelson's Silver cup fish feed, Nelson & Sons, Inc., UT) that are commonly used by commercial fish producers were included in the study as the practical control diets: high-energy trout diet (diet 8: 450 g protein Kg^{-1}; 160 g fat Kg^{-1}), low-energy trout diet (diet 9: 400 g protein Kg^{-1}; 100 g fat Kg^{-1}) and catfish diet (diet 10: 350 g protein Kg^{-1}; 70 g fat Kg^{-1}). In total, ten diets, seven being the experimental diets and three being the practical control diets, were evaluated for bluegill.

Experimental design

Juvenile bluegill were purchased from a commercial fish grower (Osage Catfisheries, Incorporated, Osage Beach, MO, USA) and professionally transported to the University of Missouri, Columbia, MO, USA. Upon arrival, fish were acclimated to laboratory conditions for two weeks. Eight rectangular tanks (236 × 73 × 58 cm; water holding capacity = 945 L) equipped with biofiltration, water- recirculation/re-aeration, and temperature-control capacities were used in the study. Seven perforated, plastic test chambers (43 × 30 × 43 cm) whose screen-covered tops protruded above tank water surfaces were placed in each of the eight tanks, giving 56 test chambers. Fifty chambers were chosen to allocate five replicates for each of the ten diets. Six additional chambers in tank 8 were used such that the fish density was equivalent to that of the other tanks. Tanks were filled to three-fourths of their heights such that water volumes of 40 L resulted in each chamber. Acclimated bluegills were then randomly allocated to the test

chambers at 10 fish per chamber, and further acclimated for seven days. Just before the feeding trial, 10 additional fish were acclimated in a separate chamber and euthanatized to determine the initial whole-body protein content of bluegill. Bluegills were weighed (~22 g) on day 0, prior to commencement of the feeding trials. Diets 1 to 10 were randomly allocated to the 50 chambers, giving five replicates for each test diet. The experiment followed a completely randomized design.

Fish were hand-fed twice daily to apparent satiation at 0800 and 1600 h. Feeding was handled by two persons and continued for ~1 h at each feeding time. Any feed pellets that remained in a chamber at 30 min postfeeding were removed by siphoning under no-flow conditions and stored at -20 °C until the end of the study. After completion of the 60-d feeding trial, the preserved, uneaten pellets from each chamber were dried at 70 °C for 72 h and weighed. Leaching of all the diets was accounted for by following the method of Masagounder *et al.* (*accepted*) when determining weights of unconsumed pellets. Here, known dry weights of test diets were immersed for 1 h in water-filled chambers and then siphoned out and dried. The percentage weight loss from leaching was then added to the weights of uneaten pellets. The dry weight of the unconsumed feed was then subtracted from the total feed weight provided to determine total feed consumption by the bluegill in each chamber. Means ± 1SD of daily recorded tank water temperatures and dissolved oxygen levels were 23.4 ± 0.8 °C and 7.2 ± 0.2 mg L^{-1}, respectively. Weekly NH_3-N and NO_2-N levels remained < 0.1 mg L^{-1} and < 0.2 mg L^{-1} while a summer-like photoperiod (14 L: 10 D) was continued throughout the 60-d experiment.

Live weights of fish from each chamber were determined on days 0 and 60. At the end of the experiment, bluegill were euthanatized with an overdose of MS222 (Aquatic

Eco-systems, Apopka, FL, USA). Six randomly selected fish from each chamber were used to determine whole-body proximate composition (moisture, crude lipid, crude protein, and crude ash).

Values of the following indices were determined from all fish in each of the replicate test chambers over the 60-d experiment period, and averaged across the five replicates for each of the ten test diets;

Total feed consumption (g/fish) = (total feed provided (g) – total unconsumed feed (g)) / N_f, where N_f is the average number of fish fed per day in a chamber.

$N_f = (n_1+n_2+n_3+\ldots+n_{60}) / 60$, where n_1, n_2, n_3, n_{60} are the total number of fish fed in a chamber on days 1, 2, 3, 60, respectively.

Relative growth rate, RGR (g $100g^{-1}$ d^{-1}) = (wet weight gain × 100 / average fish weight)/ t, where wet weight gain in a chamber = final weight (g) – initial weight (g), average fish weight (g) = (initial weight + final weight) / 2, and t is duration of the experiment (60 d).

Feed conversion ratio (FCR) = total dry feed consumed (g) / wet weight gain (g).

Apparent protein utilization (APU) = protein gain (g) / total protein fed (g), where total protein fed = gross protein content of a diet (%) × feed consumed (g). Gross protein content was used in that digestible protein levels from the practical control diets are unknown for bluegill.

Gain-to-cost ratio = wet weight gain (g) ×100 / feed cost, where feed cost = ingredient cost ($/g) × dry feed consumed (g).

Hepatosomatic index (HSI) and Viscerosomatic indexes (VSI) were determined for bluegills at the end of the feeding trial in each of the dietary treatments:

HSI = liver weight × 100 / fish weight (g),

VSI = visceral weight × 100 / fish weight (g).

Chemical and Statistical Analyses

Laboratory analyses of moisture, crude protein, amino acids, crude lipid, crude ash and gross energy for feed and fish samples followed procedures recommended by the Association of Official Analytical Chemists (AOAC, 2000). Gross energy content was analyzed using an adiabatic bomb calorimeter (Parr Instrument Company, Moline, IL, USA). Crude protein contents were determined by the combustion method using a LECO FP-528 (Leco Corporation, St. Joseph, MI, USA). Amino acids of feed samples were analyzed using an automatic analyzer (Hitachi Model 835-50, Tokyo, Japan) with an ion exchange column at the Experiment Station Chemical Laboratories, University of Missouri, Columbia, MO. Whole-body lipid content was estimated using the ether extraction method. Ash content was determined by incinerating the feed samples at 600˚C for 12 h in a muffle furnace.

One-way analysis of variance (ANOVA) was used to determine whether the mean responses for each metric (feed consumption, feed efficiency, growth, body composition, survival) differed across the 10 diets ($P < 0.05$). All data were tested for variance homogeneity and normality. Survival data were arcsine, square-root transformed prior to ANOVA. Where appropriate, means across diet types were separated by Tukey's test for multiple comparisons.

RESULTS

Bluegill survival rates ranged from 95.4% (catfish diet) to 98% (diet 2) with no significant differences being detected across the 10 diets ($P > 0.05$). Among fish fed experimental diets (#1-7) no significant differences were observed in RGR, feed consumption, FCR, APU, HSI, or VSI ($P > 0.05$) (Table 4). Values (overall mean ± S.D.) of RGR, feed consumption, and FCR across dietary groups were 1.11 g $100g^{-1}$ d^{-1}, 27.66 g $fish^{-1}$ and 1.27, respectively, whereas those for HSI and VSI (overall mean ± S.D.) were 1.40 % and 9.93 %, respectively. Interestingly, diet 1, which contained high levels of fish meal (550 g Kg^{-1}) produced higher body fat levels and lower tissue moisture contents than did diets 3 through 7 which contained relatively low levels of fish meal (0-440 g Kg^{-1}) ($P < 0.05$). No significant differences were observed among the fish fed experimental diets (#1-7) in terms of whole-body protein and ash content. With regard to cost, fish fed diets 6 and 7 showed significantly higher gain-to-cost ratios than those fed diets 1 through 5, whereas no differences in this ratio were observed for diets 6 and 7. Diet 1, containing 550 g Kg^{-1} fish meal, produced the lowest gain-to-cost ratio.

Significant differences ($P < 0.05$) were detected in growth responses between fish fed the experimental diets (#1-7) and those fed the practical control diets (#8-10) (Table 4). Fish fed the catfish diet (#10) exhibited poorer RGRs (0.9 g $100g^{-1}$ d^{-1}) than did those fed the experimental diets (#1-3) containing higher levels of dietary fish meal (≥300 g Kg^{-1} fish meal) (1.13-1.23 g $100g^{-1}$ d^{-1}) or those fed the high-energy trout diet, diet 8 (1.16 g $100g^{-1}$ d^{-1}). Similarly, fish consumed significantly lesser amounts of catfish diet (16.4 g $fish^{-1}$) relative to any of the experimental diet (25.7-29.6 g $fish^{-1}$ for diets 1-7). However, the low-protein catfish diet elicited higher APU values than did any other diets

(diet #1-9) ($P < 0.05$). Also, fish fed the catfish diet exhibited the highest liver-somatic index values, which significantly differed from experimental diets 2 through 7 (Table 4). However, neither VSI nor body composition of fish fed the catfish diet differed from those fed the experimental diets (#1-7).

Unlike for the catfish diet, the two trout diets (#9 &10) did not differ from the experimental diets in terms of fish feed consumption or fish growth rate (RGR). However, the high-energy trout diet (#8), but not the low-energy trout diet (#9), produced a significantly lower FCR ($P < 0.05$) relative to the experimental diets 3 through 7, the fish meal contents of which were ≤ 300 g Kg^{-1}: ~1 (FCR) for diet #8, versus ~1.3 (FCR) for diets # 3-7). Similarly, the two trout diets produced better protein utilization values than did experimental diets #3-7, but 6 ($P < 0.05$). Both trout diets produced significantly higher ($P < 0.05$) fat indices, HSI and VSI, relative to the diets containing low levels of fish meal (≤ 200 g Kg^{-1}), particularly diets 4, 5, and 6 (Table 4). Similarly, both trout diets (#8 and 9) elicited lower whole-body-moisture content, but higher body lipid deposition than the fish fed diets #2-7 ($P < 0.05$). No significant differences were detected in the whole-body protein content nor the ash content among the dietary groups ($P > 0.05$, ANOVA).

A comparison of ingredient costs for diets 6 and 7 to those of diet 1 showed the costs of diets 6 and 7 to be ~32% and 35% lower, respectively. However, fish fed diet 7 (75 g fat Kg^{-1}) exhibited slightly lower feed consumption and slightly higher whole-body lipid deposition than those fed diet 6 (67 g fat Kg^{-1}). Although the feed cost of diet 6 is ~32% below that of diet 1, diet 6 also reduced whole-body lipid deposition by 34% and 27%, respectively, versus diets 8 (high-energy trout diet) and 9 (low-energy trout diet).

Cost comparisons against the commercial diets could not be made as the dietary formulations of trout and catfish diets are not reported.

DISCUSSION

As the fish meal level was reduced in the experimental diets, a protein feedstuff blend containing various levels of MBM, SBM and CGM, was proportionately increased. Reducing the dietary fish meal level from 550 g Kg^{-1} (55 %) to 0 g Kg^{-1} (0 %) did not lead to significant changes in bluegill feed consumption, growth rates, FCR, or protein utilization. Indeed, fish fed diets containing ≤ 300 g Kg^{-1}(30 %) fish meal showed significantly less body fat than those fed the fish-meal-based diet (diet # 1). Furthermore, diets #6 & 7, containing no fish meal, became the most economical diets whereas the 550 g Kg^{-1} (55 %) fish meal diet was the least-economical diet. Between diets #6 and 7, given that the latter produced a slightly higher body fat level as well as a modest decrease in feed consumption, diet 6 was considered to be the best diet for bluegill. Diet 6, containing zero fish meal, has potential to reduce the total ingredient feed cost from ~4 % (100 g Kg^{-1} fish meal diet) to ~35 % (550 g Kg^{-1} fish meal diet), depending on the level of fish meal in the control diet. The present study also demonstrates that trout diets lead to significantly higher body fat deposition in bluegill, whereas catfish diets tend to elicit significantly poorer bluegill growth, consistent with previous work by Twibell *et al.* (2003). Additional findings from the present study were that the trout diet, particularly diet 8, contained higher levels of energy (20.1 MJ Kg^{-1}) than that which bluegill required (14.6 MJ Kg^{-1}), whereas the catfish diet contained lower levels of methionine (5.5 g Kg^{-1},

gross estimate) and protein (350 g Kg^{-1}, gross estimate) than the optimal levels (6.1 g Kg^{-1} digestible methionine, 410 g Kg^{-1} digestible protein) required by juvenile bluegill. Overall, the present study demonstrates that fish meal can be completely replaced in the diet of juvenile bluegill by a mixture of plant and animal protein sources containing predominantly MBM, SBM, and CGM.

Fish meal replacement: single ingredient approach

Numerous studies have investigated the potential of individual, rendered animal (PBM, MBM, BM, feather meal) and plant (SBM, CGM) protein products to effectively replace fish meal in fish diets. One commonly evaluated alternative animal protein source is MBM (e.g., El-Sayed 1998; Bharadwaj *et al.* 2002; Bureau *et al.* 2000; Ai. *et al.* 2006; Li *et al.* 2009), because its protein level closely matches that of fish meal, and because its price is often less than half the cost of fish meal. Success rates in replacing dietary fish meal with MBM have ranged from 10 % (e.g., Cuneate drum *Nibea miichthioides*, Wang *et al*. 2006) to 83 % (e.g., hybrid striped bass *Morone saxatilis* × *M. chrysops*, Bharadwaj *et al.* 2002) and, in some cases, up to 100 % (e.g., Nile tilapia *Oreochromis niloticus*, Wu *et al.* 1999; El-Sayed 1998), with no adverse effects observed in fish feed consumption or growth rate. However, the high ash content (up to 37 %, Li *et al.* 2008) in MBM has been thought to reduce nutrient availability and feed efficiency in fishes, including Nile tilapia (El-Sayed 1998), rainbow trout *Oncorhynchus mykiss* (Bureau *et al.* 2000), Malabar grouper *Epinephelus malabaricus* (Li *et al.* 2009) and gibel carp *Carassius auratus gibelio* (Zhang *et al.* 2006). In the present study, all the experimental diets were balanced

for digestible nutrients, favoring adequate feed efficiencies and growth rate. It should be noted, however, that when the fish meal level was reduced to ≤ 400 g Kg^{-1} with increasing levels of MBM and SBM, slight reductions in feed efficiency were detected. Yet, better cost-to-gain ratio for the diets (# 6 and 7) containing high levels of MBM than for the diets (#1 and 2) containing high levels of fish meal indicates that because of much lower ingredient cost, MBM based diets would yield substantial profits to bluegill producers despite its producing somewhat slightly lower feed efficiency.

Soybean meal is another alternative protein source that has been widely evaluated, mainly because of its more favorable nutrient profile and high digestibility values versus other plant protein sources. However, lysine, methionine, threonine and phosphorous can all be limiting in soybean meal-based diets (Gatlin *et al.* 2007). The success in replacing fish meal with SBM has varied widely in fishes depending on species feeding behavior, digestive capacity, and dietary nutrient requirements. Omnivorous fishes such as catfish and tilapia tend to show high levels of tolerance for SBM: Nile tilapia fed diets (zero fish meal diet) containing 550 g Kg^{-1} SBM and supplemental amino acids (0.5 % lysine and 1 % methionine) exhibited better growth rates and feed efficiencies than those fed a control diet containing 200 g Kg^{-1} fish meal and 300 g Kg^{-1} SBM (El-Saidy & Gaber 2002). Similarly, fish species such as blue catfish *Ictalurus furcatus* (Webster *et al.* 1992) and channel catfish *I. punctatus* (Peres *et al.* 2003) were reported to perform well with diets containing predominantly SBM protein (heat treated) and no fish meal. High tolerance levels (> 500 g Kg^{-1}) for SBM were also observed for red drum *Sciaenops ocellatus* (McGoogan & Gatlin 1997) and for sunshine bass (Keembiyehetty & Gatlin 1997). Similarly, in the present study, diets 4 & 5 containing 500 g Kg^{-1} SBM resulted in

no differences in growth rate, feed consumption, or FCR relative to when fish-meal-based diets were fed, indicating that juvenile bluegill can tolerate high SBM levels in their diets. Diets containing 500 g Kg^{-1} SBM included 100 or 200 g Kg^{-1} fish meal which likely permitted adequate palatability. Unlike for omnivorous fishes, the inclusion level of SBM in the diets of salmonids is often limited to ≤ 20% (Olli *et al.* 1995; Hardy 2002; Sealey *et al.* 2009). Antigenic and antinutritional factors in the SBM were found to limit its dietary inclusion level for salmonids (Baeverfjord *et al.* 1996; Sealey *et al.* 2009). A high SBM inclusion level not only altered gut morphology, but also affected immune responses for Atlantic salmon *Salmo salar* (Baeverfjord *et al.* 1996) and rainbow trout (Rumsey *et al.* 1994). Nevertheless, heating and extrusion treatments for SBM inactivated its antinutritional factors and thereby increased nutrient digestibility for fishes including rainbow trout (Barrows *et al.* 2007) and channel catfish (Peres *et al.* 2003). The extrusion process that we applied in the present study likely enhanced bluegill capacity for high inclusion levels. Although wild bluegills often consume benthic invertebrates and zooplankton, they also frequently ingest plant material in their diets (Mischke & Morris 1998; Michaletz 2006) indicating that bluegill likely possess some inherent capacity to utilizing plant products. The study results suggest that bluegill do have ability to exhibit satisfactory growth performances from plant based diets.

Among all animal protein sources, PBM most closely resembles fish meal's nutrient profile (Yu 2008). Like MBM and SBM, PBM has also been used widely, with replacement success levels ranging from partial (e.g., Fowler 1991; Nengas *et al.* 1999; Abdel-Warith *et al.* 2001) to complete (e.g., Yang *et al.* 2006; Hernandez *et al.* 2009). Recent studies that have attained 100% replacement success with PBM have used pet-

food-grade versus feed-grade PBM. However, the higher price of pet-food-grade PBM relative to that of MBM, SBM, or CGM (Table 2), diminishes its value as a high-level, fish-meal replacement animal protein source. Accordingly, the least-cost software used in the present study omitted PBM in the formulation not only because PBM is expensive, but also because the nutrient requirements of juvenile bluegill can be met from other, relatively inexpensive protein sources.

Blood meal, although rich in protein, due to its poor amino acid profile, is usually mixed with MBM to obtain a better amino acid balance in the diet (Li *et al.* 2008). Yet, the maximum inclusion level of BM is often limited to ≤ 100 g Kg^{-1} (≤ 10%). For example, dietary inclusion of blood meal at 150 g Kg^{-1} along with 200 g Kg^{-1} MBM to completely replace fish meal resulted in reduced growth rates and feed efficiencies in Nile tilapia (El-Sayed 1998). Similar results were obtained for juvenile grouper *Epinephelus coioides* (Millamena 2002) and channel catfish (Li *et al.* 2003). However, neither fish growth rate nor feed efficiency was affected when BM was included at 50 g Kg^{-1} for channel catfish (Li *et al.* 2002) and at 120 g Kg^{-1} for rainbow trout (El-Haroun *et al.* 2009). In the present study, the software included ~50 g Kg^{-1} BM in the control diet formulation, but not in the other diet formulations, again because of this feedstuff's very high cost relative to other alternative feedstuffs, as well as it's lower content (6.2 g Kg^{-1}) of isoleucine relative to bluegill's requirement (9.8 g Kg^{-1}).

Corn gluten meal was included up to 190 g Kg^{-1} in the zero fish meal diets in the present study. Although CGM is rich in protein and contains low levels of indigestible fiber, it is deficient in certain EAAs including lysine and arginine (Pereira & Oliva-Teles 2003). Consequently, its upper inclusion level is often limited to 200-250 g Kg^{-1} for

salmonids (Gatlin *et al.* 2007). A few other studies that evaluated CGM have shown partial success at replacing dietary fish meal protein -- CGM added to the diets at ~410 g Kg^{-1}, ~230 g Kg^{-1} and 330 g Kg^{-1} could replace dietary fish meal only at levels of 60%, 40% and 33% for gilthead seabream *Sparus aurata* (Pereira & Oliva-Teles 2003), rainbow trout (Morales *et al.* 1994) and European seabass (Ballestrazzi *et al.* 1994), respectively. Supplementing the limiting AAs in the CGM based diets appears to increase the fish meal replacing success, yet the success rate can be species specific -- for example, high inclusion of CGM in the diets of rainbow trout resulted in reduced palatability and fish growth (Morales *et al.* 1994).

Numerous studies have attempted to replace fish meal with single alternative protein sources. However, such approaches have tended to achieve limited success (< 50%), largely because individual alternative protein sources tend to cause the diet to be deficient in certain nutrients when one attempts to replace fish meal beyond a moderate level. Also, this type of formulation is unlike most industry-standard diet formulations ("practical diet formulations"), which usually involve mixtures of multiple protein sources versus only a few and therefore can provide a better nutrient profile. Yet, the single protein approach to replacing fish meal has merits in that it provides accurate information concerning an individual ingredient's capacity to replace fish meal in terms of nutrient profile and palatability.

Fish meal replacement: multiple ingredient approach

In contrast to the single ingredient approach, formulating diet with a mixture of ingredients (practical diet formulation) can provide a better nutrient profile that more closely matches the nutrient profile of a fish-meal-based diet. Also, when fish nutrient requirements are known, nutrient deficiency become a rare issue in practical diet formulations, given that deficiency of a nutrient from one ingredient can easily be supplemented from other ingredients. However, high inclusion level of certain ingredients in practical diet formulation can negatively affect fish palatability and therefore, for such ingredients, information obtained from single ingredient approach will help determine their inclusion levels in practical diet formulations. When such information is not available for individual ingredients, practical diet formulations may need to be evaluated by gradually replacing fish meal, as was done in the present study, in order to ensure adequate diet palatability. Recent studies (e.g., Bureau *et al.* 2000, Millamena 2002 and El-Haroun *et al.* 2009) that have used combinations of animal protein sources have shown evidence that diets with alternative protein sources provide better nutrient profiles, adequate palatability, and, ultimately, more successful nutrient replacement. For example, Hu *et al.* (2008) showed that fish meal could be replaced completely by a mixture of rendered animal protein sources (PBM, MBM) for gibel carp. Alternatively, studies have used a mixture of plant protein sources to substitute for dietary fish meal: Kaushik *et al.* (2004) demonstrated that the fish meal inclusion level in the diets of European seabass *Dicentrarchus labrax* could be reduced from 52 % to only 5 % using a mixture of SBM, CGM, rapeseed meal, and wheat gluten meal, supplemented with lysine and dicalcium phosphate. Moreover, studies have shown that

channel catfish have exhibited growth performance on a plant-protein-based diet that is as favorable as when fed a fish meal based diet (e.g., Robinson & Li 1998 & 1999; Li *et al.* 2003). Similarly, Nguyen *et al.* (2009) demonstrated that combinations of plant and animal protein sources have the capacity to completely replace animal protein sources in the diets of juvenile tilapia *Oreochromis spp.* without limiting fish growth performance. Similarly, the present study used a blend of plant and animal proteins to balance dietary nutrient levels and to produce a least-cost diet formulation. However, unlike for bluegill, most replacement successes for fish meal were recorded for fishes such as catfish and tilapia that require less dietary protein (< 40%), and therefore lower levels of dietary fish meal. The ability to completely replace fish meal with a blend of animal and plant protein sources, as was achieved in the present study, suggest that adequate palatability was maintained even in the non-fish meal diets, and that all the experimental diets were balanced for adequate levels of essential nutrients for fish growth. Growth rates (0.37g d^{-1}) observed in the present study for the experimental diet containing no fish meal (#6) are comparable to those reported for juvenile bluegill in previous studies: 0.2-0.3 g d^{-1} (Hayward & Wang 2002), 0.1 g d^{-1} (Hoagland *et al.* 2003), 0.14-0.23 g d^{-1} (Twibell *et al.* 2003). Similar growth rates of bluegill fed the least-cost diet from this study versus those fed commercial or experimental diets in other studies provide additional evidences that our least-cost diet do maintain desirable bluegill growth (assuming that all other growth determining factors are similar in these studies).

Experimental versus practical diets: Growth and feed efficiency

Although all experimental diets and practical control diets produced similar fish growth rates, the high-energy trout diet and, to an extent, the catfish diet as well, elicited better FCR values than did the experimental diets containing low levels of fish meal (≤ 300 g Kg^{-1}). Although the experimental diets were balanced for essential nutrients, the underlying cause for the observed differences in feed efficiency between the low-fish-meal diets and the high-energy trout diet is difficult to explain, but likely stems from differences in the extrusion processes used to manufacture the diets. Both the high-energy diet and the catfish diets were provided as floating pellets, whereas the experimental diets and the low-energy trout diet were provided as sinking pellets. Moreover, many studies that have included high levels of MBM in low fish-meal diets, have reported poor feed efficiency, potentially due to the high levels of ash that occur in MBM-based diets, or the associated poor nutrient availability (e.g., El-Sayed 1998, Bureau *et al.* 2000, Zhang *et al.* 2006 and Li *et al.* 2009). In the present study, the possibility that a high ash content in the MBM component caused feed efficiency to be unfavorable cannot be dismissed, given that the ash content of all experimental diets (~15 %), as well as that of the industry standard trout (~9 %) and catfish diets (~7 %) differed substantially (Table 9). Despite these concerns, the observed FCR of ~1.3 for the low-fish-meal diet was within the acceptable range (Tacon & Metian 2008). Also, as mentioned before in this section, MBM based diets produced better cost-to-gain ratios than fish meal based diets suggesting that the much reduced cost of MBM (current MBM's price is ~57 % lower than that of fish meal, Table 2) negated its slightly reduced feed efficiency. Therefore, the

MBM based diet should ultimately produce greater economic benefits for bluegill farming.

Protein utilization did not differ across fish groups fed the experimental diets. However, these values were lower than those observed for fish fed the catfish diet. This difference reflects differences in the protein levels of the experimental and catfish diets; the catfish diet contained only 350 g Kg^{-1} (35 %) dietary protein whereas the experimental diets contained ~440 g Kg^{-1} (~44 %) of dietary protein (gross level). Declines in the levels of protein utilization with increasing levels of dietary protein occur in fishes, e.g., Hafedh (1999), Ng *et al.* (2001), and Schulz *et al.* (2007). Similarly, better protein utilization from the low-energy trout diet relative to the experimental diets containing low levels of fish meal (≤ 300 g Kg^{-1}) may reflect the low dietary protein level of the latter (400 g Kg^{-1} gross protein). Although it is evident that differences in the protein level across the diets largely reflected their protein efficiency, it is unclear whether the differences in the extrusion process (floating pellets for catfish, and high-energy trout diet versus sinking pellets for other diets) played any significant role in the protein utilization values given that differences in the extrusion temperatures can affect protein bioavailability (Riaz 2008). Although catfish diet produced better protein efficiency, it reduced bluegill growth relative to fish meal based diet and also, produced enlarged liver relative to the least-cost diet (discussed later in this section), which negate its protein utilization value. Similarly, the benefit of better protein utilization from trout diets relative to the least-cost diet is negated by its producing enlarged liver and higher body fat in bluegill.

Experimental versus practical diets: fat deposition

Results of the whole-body crude lipid and moisture content and liver-somatic index values indicate that the high-energy trout diet produced significantly higher body fat in bluegills than did the experimental diets containing high levels of MBM and SBM. Our findings from the trout diet which led to high body fat deposition, and the catfish diet which produced poor growth rates, are consistent with what was reported for juvenile bluegill by Twibell et al. (2003). High dietary energy leading to increased body fat deposition and reduced fish appetite (via "lipostatic regulation of feed intake, i.e., adipose tissue, due to excess fat deposition, signaling brain via hormones such as leptin and limiting feed intake") as well as growth has frequently been observed in fishes (Jobling & Miglavs 1993; Johansen *et al.* 2003). In the present study, the trout diet containing high levels of energy did not reduce fish feed consumption even though it produced significantly higher body fat deposition. The absence of fish appetite regulation from increased dietary energy levels in the present study and other similar studies (e.g., Lee *et al.* 2000, De Silva *et al.* 2002) could owe to the restricted experimental periods (~8 weeks) associated with these studies. Other studies (e.g., Jobling & Miglavs 1993; Johansen *et al.* 2003) that showed suppressed feed intake due to high fat deposition were run for longer periods (> 13 weeks). Also, the absence of a lipostatic mechanism for the control of feed intake is consistent with what was observed for juvenile bluegill fed high-energy diets (Chapter 3.). Because the trout diet elicited high fat deposition while also being high in cost, this negates better feed utilization versus the experimental diets that contained low levels of fish meal ($\leq$ 300 g Kg^{-1}). The catfish diet and experimental diets (#1-7) contained similar levels of energy (Table 3). Given that the catfish diet was similar

to the experimental diets in terms of fat content, but contained lower levels of dietary protein, the former apparently contained higher levels of carbohydrate than the later. Omnivorous fishes such as tilapia and catfish tolerate high levels of carbohydrate (up to 40 %) (Stone 2003), whereas carnivorous fishes such as salmonids exhibit poor tolerance of carbohydrate, with increased carbohydrate (> 10 %) often leading to enlarged livers (Stone 2003). Similarly, the high HSI values observed for the juvenile bluegill we fed the catfish diet suggesting that bluegill poorly tolerate elevated levels of dietary carbohydrate.

The significantly higher level of body fat that resulted from the fish-meal-based control diet (#1) versus the low-fish meal diet (≤ 300 g Kg^{-1}) was unexpected, given that all the experimental diets were balanced for digestible energy. It should be noted, however, that the juvenile bluegill consumed fish-meal-based feed (#1) at a slightly higher level than the other experimental diets, which likely increased the absolute amount of energy available to them, thereby producing higher body fat content. Therefore, this increase in fat deposition from fish meal based diet (#1) is likely because of bluegill's strong desire for fish meal based diet. However, the same cannot be said for the trout diets given that high-energy trout diet (# 8) produced slightly higher fat deposition (10.1 % versus 9.0 % body fat) even for less amount of feeding (24.2 g versus 29.4 g) than did the fish meal based diet (# 1) (Table 4).

Overall, high body fat deposition from the trout diets and poor bluegill growth as well as enlarged liver from the catfish diet suggest that under a commercial rearing system that typically involves longer rearing period, trout and catfish diets will likely lead

to reduced bluegill production relative to the least-cost diet developed in the present study.

Conclusions

Study results demonstrate that the fish-meal component of the diets of juvenile bluegill can be completely replaced by a protein feedstuff blend consisting primarily of MBM and SBM with no loss in fish growth performance. The present study also showed the potential to reduce the feed cost for juvenile bluegill by 4-32 %, depending on the level of fish meal (100-550 g Kg^{-1}) in the control diet. This study further demonstrates that while both the trout and catfish diets promote enlarged livers in bluegill, the trout diet elicits high body fat deposition whereas the catfish diet yields reduced fish growth rates, indicating that both diets are suboptimal for juvenile bluegill. Also, in comparison to trout diets, the bluegill diet developed in this study resulted in a ~30 % reduction in body fat in juveniles. The present study also demonstrates that diets formulated from a mixture of alternative animal and plant protein sources, balanced for digestible nutrients, can reduce dietary fish meal levels to low levels. Long-term studies of the effects of least-cost diets on growth, feed efficiencies, fish health, and fillet quality in bluegill should provide additional information regarding the utility of the least-cost diet formulation we derived.

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Table 1. Proximate composition of the ingredients used for least-cost experimental diets. Values in the parenthesis indicate digestible nutrient level.

Composition	FM	MBM	BM	SBM	CGM	Corn	Wheat
Gross Energy (MJ Kg^{-1})	17.7 (15.5)	16.9 (12.2)	23.6 (21.7)	18.4 (14.7)	22.3 (18.3)	17.9 (9.5)	16.4 (9.1)
Crude Protein (g Kg^{-1})	592.4 (545.0)	546.7 (447.2)	915.8 (764.2)	448 (426.0)	625.0 (523.0)	78.8 (69.0)	112.5 (103.1)
Crude Lipid (g Kg^{-1})	13.3	9.6	0.4	4.6	3.3	4.3	2.2
Crude Ash (g Kg^{-1})	146.5	198.7	46.8	57.3	25.0	14.4	20.9
EAA* (g Kg^{-1})							
Arginine	36.9 (34.6)	34.1 (28.5)	36.3 (33.2)	34 (33.0)	21.5 (19.6)	4.3 (3.9)	5.6 (5.2)
Histidine	12.5 (11.5)	9.1 (7.8)	61.2 (57.5)	12.5 (12.1)	13.4 (11.9)	2.3 (2.1)	2.6 (2.4)
Isoleucine	23.6 (21.9)	12.8 (10.7)	7.6 (6.2)	21.9 (20.6)	27.6 (24.4)	2.9 (2.4)	3.9 (3.3)
Leucine	41 (38.2)	29.9 (25.2)	114.7 (106.0)	36.7 (34.5)	113.4 (93.1)	9.3 (8.5)	7.6 (6.9)
Lysine	41.9 (39.7)	25.2 (21.6)	73.2 (69.6)	30.7 (29.4)	11.7 (10.6)	3 (2.4)	3.6 (3.0)
Methionine	15.9 (14.6)	6.7 (5.7)	7.0 (6.6)	6.7 (6.3)	14.9 (14.1)	1.9 (1.6)	2.4 (2.2)
Phenylalanine	22.2 (20.3)	16.5 (13.8)	56.4 (53.2)	23.8 (22.5)	42.8 (37.8)	3.9 (3.5)	5.1 (4.7)
Threonine	23.5 (22.0)	15.3 (12.6)	27.0 (25.0)	18.8 (17.6)	22 (19.8)	2.9 (2.3)	3.2 (2.7)
Tryptophan	4.8 (4.4)	1.9 (1.7)	9.2 (9.0)	5.4 (5.2)	3.8 (3.6)		1.1 (0.9)
Valine	28.6 (26.2)	20.8 (17.1)	79.1 (73.1)	23.1 (21.7)	30.6 (25.3)	4.0 (3.6)	5.0 (4.5)
∑ AA**	497.0	353.0	781.8	440.9	576.3	75.3	104.3

* EAA, essential amino acid
** AA, amino acid

Table 2. Computer formulated least-cost experimental diets used in the study.

		Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
Total ingredient cost ($ tonne^{-1})	➡	899.7	791.8	739.7	687.7	642.9	616.6	587.4
Ingredients (g Kg^{-1})	Cost ($ tonne^{-1})							
Fish meal[1]	975.9	550.0	400.0	300.0	200.0	100.0	0.0	0.0
PBM	731.9	0	0	0	0	0	0	0
Porcine meal & bone meal[2]	421.6	0.0	132.1	164.9	197.7	255.6	380.1	520.7
Blood meal[2]	955.1	52.5	0.0	0.0	0.0	0.0	0.0	0.0
Soybean meal[3]	376.8	85.2	293.7	392.3	490.9	521.1	369.9	142.5
Corn gluten meal[4]	622.5	0.0	0.0	0.0	0.0	32.8	152.9	190.7
Corn[5]	171.5	0.0	113.1	81.7	50.3	29.3	36.0	97.0
Wheat[5]	259.3	251.3	0.0	0.0	0.0	0.0	0.0	0.0
Fish oil[6]	1477.1	40.0	40.0	40.0	40.0	40.0	40.0	30.0
Lecithin[7]	4188.7	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Dicalcium phosphate[8]	4133.6	2.0	2.0	2.0	2.0	2.0	2.0	0.0
Vitamin premix[9]	11022.9	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Vitamin C[10]	1543.2	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Choline chloride[10]	1543.2	1.4	1.4	1.4	1.4	1.4	1.4	1.4
Mineral mix[11]	1543.2	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Binder[12]	2314.8	3.0	3.0	3.0	3.0	3.0	3.0	3.0

[1]Eldon C. Stutsman, Inc., Hills, IA, USA.
[2]American Midwest Distributors, LLC, Kansas City, MO, USA.
[3]ADM Soybean Meal Plant, Mexico, MO, USA.
[4]Grain Processing Corporation, Muscatine, IA, USA.
[5]Bourn Feed, Columbia, MO, USA.
[6]Refined Menhaden Oil (Virginia Prime Gold), Omega Protein, Inc., Houston, TX, USA.
[7]Archer Daniels Midland Company, Decatur, IL, USA.
[8]American livestock and pet supply, Inc., Madison, WI, USA.
[9, 11] Nelson's Silvercup Fish Feed, Nelson & Sons, Inc., Murray, UT, USA.
[10]MP Biomedicals, Solon, OH, USA.
[12]Ultra-Bond™, Uniscope, Inc., Johnstown, CO, USA.

[9]Vitamin premix contains (amount per kg of dry feed): vitamin A, 9650 IU; vitamin D3, 6598 IU; vitamin E, 130 IU; niacin, 21.6 mg; D-pantothenic acid, 46.3 mg; riboflavin, 9.6 mg; menadione, 1.1 mg; folic acid, 2.49 mg; thiamin, 8.82 mg; biotin, 0.33 mg; vitamin B6, 13.23; vitamin B12, 0.03 mg; inositol, 599.66.

[11]Mineral premix (US Fish and Wildlife Service trace mineral premix # 3) contains (amount per kg of dry feed): Mn (as manganese sulfate), 20 mg; Zn (as zinc sulfate), 75 mg; Cu (as copper sulphate) 1.54; Iodine (as potassium iodide) 10

Table 3. Proximate composition of the diets fed to juvenile bluegill. Values in the parenthesis indicate digestible nutrient level.

Composition	Req*	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Trout1	Trout2	Catfish
Gross Energy (MJ Kg^{-1}	(14.6)	18.6 (14.6)	18.5 (14.6)	18.3 (14.6)	18.4 (14.6)	18.3 (14.6)	18.4 (14.6)	18.5 (14.0)	20.1	18.6	18.0
Crude Protein (g Kg^{-1})	(410)	424.7 (410)	445.2 (410)	439.8 (410)	443.9 (410)	433.9 (410)	443.3 (410)	438.4 (400)	450.0	400.0	350.0
Crude Lipid (g Kg^{-1})	8.0	83.0	63.5	64.6	72.6	63.7	81.8	81.5	160.0	100.0	70.0
Crude Ash (g Kg^{-1})		140.5	155.4	151.3	140.3	143.1	151.3	165.0	85.9	85.2	74.8
EAA (g Kg^{-1})**											
Arginine	(11.9)	26.9(24.9)	29.8 (27.7)	30.9 (28.3)	32.7 (28.9)	31.7 (28.7)	30.3 (26.2)	29.4 (23.7)	27.5	24	22.8
Histidine	(4.1)	12.5 (11.0)	10.9 (9.4)	10.8 (9.6)	11.4 (9.9)	11.0 (9.9)	10.8 (9.3)	10.4 (8.2)	10.7	12.1	10.1
Isoleucine	(9.8)	16.6 (14.9)	18.6 (16.5)	18.6 (16.6)	19.2 (16.7)	18.0 (16.5)	17.9 (15.5)	16.0 (13.4)	19	15.2	14
Leucine	(15.3)	34.5 (31.2)	33.1 (29.7)	33.1 (29.9)	34.4 (30.0)	34.8 (31.6)	41.4 (36.9)	43.1 (36.6)	35.3	36.2	29.5
Lysine	(15.0)	30.9 (28.8)	30.5 (27.6)	30.1 (27.2)	30.7 (26.8)	28.2 (25.2)	25.1 (20.8)	23.1 (17.7)	27	24.4	21
Methionine	(6.1)	9.1 (9.5)	9.2 (8.6)	8.4 (7.9)	8.3 (7.2)	7.9 (6.7)	7.1 (6.7)	7.7 (6.7)	9.3	7.9	5.5
Phenylalanine	(9.9)	19.2 (17.1)	19.2 (17.0)	19.5 (17.5)	20.7 (18.0)	20.2 (18.6)	21.6 (19.5)	21 (17.9)	19.7	19.6	17.4
Threonine	(8.7)	16.3	17.0 (15.9)	17.1 (15.8)	17.8 (15.6)	17.1 (15.3)	16.4 (14.4)	16.2 (13.1)	18	15.9	13.9
Tryptophan	(2.4)	(3.6)	(3.5)	(3.6)	(3.8)	(3.7)	(3.1)	(2.3)			
Valine	(11.8)	24 (21.2)	22.7 (19.5)	22.5 (19.5)	22.9 (19.4)	21.5 (19.2)	22.2 (18.5)	21.2 (17.2)	23.6	21.9	19.9

*Req: digestible requirement values determined to be optimal for juvenile bluegills
** EAA, essential amino acid

Table 4. Growth responses and body composition of juvenile bluegills fed the experimental diets for 60 days. Values are presented as means ± SD (Values within a row sharing different superscript alphabets are significantly different, $P < 0.05$).

Variables	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Trout1	Trout2	Catfish	ANOVA
Growth Responses											
Initial weight (g)	21.2±1.6	23.0±1.2	20.4±0.7	21.6±2.0	21.3±1.6	22.5±1.4	21.7±1.1	22.7±2.0	21.9±0.7	21.6±1.3	0.64
Final weight (g)	46.2±4.8^{a}	48.2±4.6^{a}	41.3±3.4^{ab}	41.5±2.5^{ab}	40.4±3.1^{ab}	44.4±2.7^{ab}	42.2±2.3^{ab}	46.9±4.1^{a}	42.2±2.8^{ab}	37.7±3.8^{b}	0.03
RGR (g^{-1} $100g^{-1}$ d^{-1})	1.23±0.09^{a}	1.17±0.11^{a}	1.13±0.09^{ab}	1.05±0.05^{ab}	1.02±0.04^{ab}	1.09±0.07^{ab}	1.05±0.06^{ab}	1.16±0.1^{a}	1.05±0.07^{ab}	0.90±0.14^{b}	<0.01
Feed consumption (g $fish^{-1}$)	29.4±4.1^{a}	29.6±2.2^{a}	28.5±8.1^{a}	26.3±2.2^{a}	25.7±2.3^{a}	27.9±2.7^{a}	25.8±3.1^{ab}	24.2±2.0^{ab}	23.0±5.2^{ab}	16.4±2.6^{b}	<0.01
FCR	1.18±0.04^{abc}	1.19±0.13^{abc}	1.34±0.19^{b}	1.33±0.09^{b}	1.34±0.06^{b}	1.27±0.01^{bc}	1.27±0.10^{b}	1.01±0.07^{a}	1.12±0.16^{abc}	1.04±0.09^{ac}	<0.01
APU (%)	38.55±1.7^{bc}	36.0±3.0^{bc}	32.5±3.4^{c}	33.5±3.4^{c}	34.5±3.2^{c}	34.7±2.2^{bc}	34.4±1.3^{c}	42.2±3.5^{b}	44.0±6.6^{b}	50.9±4.0^{a}	<0.01
Gain-to-cost ratio	104.1±3.9^{c}	117.8±13.0^{bc}	112.1±14.3^{bc}	120.6±8.4^{b}	127.8±5.4^{b}	142.6±7.1^{a}	146.1±11.1^{a}				<0.01
HSI	1.8±0.3^{abc}	1.4±0.3^{bc}	1.4±0.1^{bc}	1.2±0.1^{b}	1.3±0.1^{b}	1.2±0.2^{b}	1.4±0.2^{bc}	1.9±0.5^{ac}	1.9±0.3^{ac}	2.1±0.2^{a}	<0.01
VSI	11.6±1.5^{abc}	10.6±1.3^{abc}	9.2±1.8^{c}	9.5±1.2^{bc}	9.0±2.4^{c}	9.7±1.3^{bc}	9.8±1.0^{abc}	12.6±1.7^{a}	12.4±0.9^{ab}	11.0±1.1^{abc}	<0.01
Survival (%)	96.6±7.6	98.0±4.5	97.4±5.8	97.9±3.6	97.1±5.5	96.7±4.7	97.1±3.8	97.8±3.6	96.3±3.7	95.4±5.3	0.99
Proximate composition											
Moisture (%)	69.3±0.8^{bc}	70.0±0.8^{ab}	71.6±0.7^{a}	71.4±0.7^{a}	70.9±0.6^{ab}	71.3±0.8^{a}	71.1±1.0^{a}	68.4±0.7^{c}	68.4±0.8^{c}	70.3±1.0^{ab}	<0.01
Crude protein (%)	15.9±0.3	15.8±0.6	15.6±0.2	15.8±0.5	15.9±0.7	16.0±0.6	15.5±0.5	15.8±0.3	15.8±0.7	15.1±0.4	0.23
Crude lipid (%)	9.0±0.6^{ab}	8.1±0.5^{bc}	7.1±0.7^{cd}	7.1±0.3^{cd}	7.3±0.6^{cd}	6.7±0.5^{d}	7.5±0.6^{cd}	10.1±0.8^{a}	9.2±0.4^{ab}	8.2±0.6^{bc}	<0.01
Crude ash (%)	4.2±0.2	4.1±.4	4.2±0.3	4.1±0.1	4.3±0.2	4.3±0.3	4.2±0.1	4.2±0.2	4.4±0.2	4.2±0.1	0.29

CHAPTER 5

EVALUATION OF NOVEL REARING STRATEGIES FOR ENHANCING PRODUCTION OF FOOD-SIZE BLUEGILL

ABSTRACT

This study evaluated two distinct rearing strategies, "topping off" and "size grading", to determine their relative effectiveness in increasing bluegill *Lepmomis macrochirus* growth rates, in order to produce large, food-size bluegill. Furthermore, the study examined the extent to which social hierarchies developed among bluegill reared in indoor tanks and production ponds, and their effects on bluegill growth and feed efficiency. To evaluate "topping off", 300 juvenile bluegills (~12 g) were stocked into each of two, 1000-L indoor recirculating aquaculture tank system (RAS). Seventy-five bluegill were individually marked in each tank with visible implant elastomer tags. Fish were fed three times daily throughout the 574-d study and were sampled for length and weight approximately every 30-60 d in both tanks. In one of the tanks (the "topping-off group"), the upper 10 % of bluegill (by weight) were removed and immediately replaced by an equal number of juvenile bluegill (~15 g) on day 376. This was repeated twice (2nd and 3rd topping-off harvests) at 60-d intervals before final harvesting on day 574. All bluegill in the control tank (no topping-off group) were also harvested on day 574. Analysis of the individually-marked fish showed that the topping-off group grew significantly faster and produced significantly more large bluegill (> 100 g) versus the control group that experienced no "topping off".

Results showed significant positive relationships between bluegill relative weight (W_r) and length, body fat and fish weight, relative weight and body fat. The significant increase in fish weight variation throughout the study period provided evidence of social hierarchy development. Social hierarchy development was apparently occurred by day 31 in the indoor tanks, and continued to persist thereafter until "topping off" was initiated. The study demonstrated that the topping-off approach can be used to disrupt social hierarchies and thereby increase bluegill growth and production.

To evaluate the size-grading strategy, each of three, 0.12-ha, outdoor production ponds were stocked with 2000 bluegill (~12 g mean weight) that had not been size graded, whereas three additional ponds were stocked with 2000, size-graded bluegill (upper 25 percentile by length) the mean sizes of which were ~21 g. Stocking density was ~16,667 bluegill ha^{-1}. The pond-stocked fish were fed a commercial feed to apparent satiation once daily, five days a week, excluding weekends. The study ran from April 2005 to November 2006, covering 584 days. As for the tanks, bluegill in ponds were sampled every ~60 days excluding winter period (Nov, 2006 – Mar, 2007) to determine lengths and weights. Size-graded bluegill showed higher fish weight throughout the experiment and larger bluegill (> 100 g, live weight) production in the final harvest. However, no differences were observed between the two groups in terms of growth rate or large-fish yield. It is noted that our observations of apparent social hierarchy development in production ponds are the first to be recorded in pond systems. Evidence of the presence of social hierarchies among bluegill in ponds was first detected on day 181; the apparent hierarchies were observed to persist for the remainder of the study in both the fish groups. The study provides evidence of the likely effects of social hierarchy

development on key production parameters such as fish growth rate and feed efficiency for both the fish groups.

Overall, the topping-off and size-grading evaluations in both indoor tanks and production ponds demonstrated their potential benefits for increasing fish yield. Nonetheless, both rearing strategies failed to produce food-size bluegill. Discussion concerning additional measures that may effectively diminish effects of social hierarchies on bluegill production in pond and indoor tank systems is included.

INTRODUCTION

Bluegill (*Lepomis macrochirus*) have historically been reared in the U.S. by commercial fish producers and government agencies for stocking recreational ponds (Lewis & Heidinger 1971; Brunson & Robinette 1986; Heidinger 1999; Brunson & Morris 2000). Over the past ~15 years, however, interest has developed among U.S. fish producers in rearing bluegill to substantially large sizes (227-340 g; 0.5-0.75 lbs) in response to a demand for this species as a food fish (Chopak 1992; NCRAC 1999; Brunson & Morris 2000). Producing food-size bluegill within two growing seasons is considered necessary by fish producers to make this business profitable (Hayward & Wang 2006). Bluegill producers are thought to be hesitant to assume the higher risk of product loss associated with this longer grow-out time (Hayward & Wang 2006; Lovshin & Matthews 2003), and may instead continue rearing smaller bluegill for which there is a continuing demand that can be met with less risk.

One factor that impedes bluegills from achieving their inherent growth capacities is their tendency to form social hierarchies. Indeed, hybrids of bluegill (♂ *L. macrochirus* × ♀ *L. cyanellus*) have long been thought to possess greater growth capacity than bluegill, until Hayward & Wang (2002) demonstrated that the later tend to form social hierarchies that impede them from achieving their true growth capacity. Dominance hierarchy formation substantially reduces growth rate as well as feed efficiency. It also significantly increases size variation in bluegill and their hybrids reared indoors (McComish 1971; Wang *et al.* 2000; Hayward & Wang 2002; Doerhoff 2007). Although, rearing bluegill individually, (e.g., in chambers) negates social hierarchy formation (Hayward & Wang 2002), such a rearing approach is impractical in commercial production settings. Appropriate modifications of rearing strategies for bluegill are much needed to reduce or eliminate the adverse effects of the social hierarchy formation. Topping-off harvesting (also termed 'sequential harvesting' or 'cull harvesting') involves the removal of larger, market-size fish from a tank or pond, followed by the stocking of a new batch of fingerlings to maintain the original fish density. This rearing strategy has been applied in semi-intensive and intensive aquaculture systems for fishes including channel catfish *Ictalurus punctatus* (Hargreaves 2002), milkfish *Chanos chanos* (Avault 1996), sunshine bass (♂ *Morone saxatilis* × ♀ *M. chrysops*) (D'Abramo *et al.* 2002), and tilapia *Oreochromis shiranus* (Brummett 2002), to enhance fish production by controlling size variation, competition, and cannibalism. The topping-off method warrants further evaluation for its potential to effectively impede dominance hierarchy formation among bluegills, thus allowing subordinates to grow at rates close to their inherent capacity. This rearing approach may allow fish producers to supply food-size bluegill year-round.

Bluegill exhibit sexually dimorphic growth wherein males show substantially higher growth rates than females (Hayward & Wang 2006; Doerhoff 2007). Recent findings by Hayward & Wang (2006) indicate that male bluegill are capable of reaching market sizes within two growing season; males attained ~66 % of market size, whereas females reached only ~31 % of market size when housed individually in an indoor tank system for 234 d. In a follow-up study, Doerhoff (2007) demonstrated that when bluegills of the same intra-annual cohort attain a size of ≥ 90 mm, the upper 25 % of these fish is largely represented (80-100 %) by males. Accordingly, size grading can be effectively applied to select males from mixed-sex groups. However, Doerhoff (2007) showed that rearing predominantly male bluegills in indoor recirculating aquaculture systems (RASs) results in higher weight gain relative to mixed-sex groups, although social hierarchy development was again an impediment. Alternatively, large rearing volumes of ponds and the differences therein (e.g., presence of natural feed, turbidity, aquatic plants) may lesser social hierarchy development. Accordingly, the greater growth capacity of male bluegill may be better exploited in pond systems than in tank systems when seeking to produce large bluegill and increase fish production.

With this as background, the present study was conducted to determine:

(1) whether a "topping off" strategy could disrupt social hierarchy formation, and thereby increase fish growth rates, numbers of large fish, and fish production in commercial scale indoor RASs, and
(2) whether size-grading bluegill in production ponds could increase fish growth rates, numbers of large fish, and fish production.

Given that social hierarchy development has not been documented in large rearing systems such as production ponds, this study also examines (i) whether or not bluegill stocked at typical sizes (~100 mm) into commercial-scale indoor RASs form social hierarchies, (ii) if so, the time periods required for social hierarchies to develop, (iii) whether social hierarchy development influences key production parameters, and finally, (iv) time periods over which social hierarchies persist.

Understanding which factors impede bluegill growth in large rearing systems should help fish producers to improve rearing approaches so that food-market weights can be achieved within acceptable grow-out periods.

MATERIAL AND METHODS

Experiment 1 – Evaluation of a topping-off strategy for bluegills reared indoors

Juvenile bluegills (~12 g) were obtained from Harrison Fisheries, Inc., Hurdland, MO, in September, 2006. Following transport to the University of Missouri-Columbia, the fish were acclimated to laboratory conditions for two weeks. An indoor RAS comprised four, 1000-L tanks, each of which was stocked with 300 juveniles (11.47 ± 1.07 g; average of four separate tank means ± 1 SD) selected at random. In each of the four tanks, 75 fish were randomly selected and individually marked with a visible implant elastomer tag (Northwest Marine Technology Inc., Shaw Island, WA, USA) on day 0, using a combination of any two of four marking locations (right caudal, left caudal, right dorsal and left dorsal), and any one or two of four tag colors (blue, pink, green, and red).

During tagging, each fish was anesthetized with ~40 ppm of MS222 (Tricaine Methanesulfonate) to minimize tagging stress, measured for length and weight, and injected with ~0.1 ml of elastomer solution beneath the skin.

The fish were fed to apparent satiation thrice daily (0800, 1300 and 1800 h) with a high-protein, floating pellet diet (Aquamax Grower-400® diet; St. Louis, MO; 45 % crude protein and 16 % lipid). The presence of a few uneaten pellets after ~20 min of feeding was considered to indicate that apparent-satiation-feeding had been achieved. Feeding level was adjusted periodically, based on the number of uneaten pellets observed.

After ~6 months of rearing, all bluegill from two tanks perished due to an accidental overnight loss of tank water. The study was continued with the remaining two tanks that shared the same water via the RAS.

Tank water temperatures were maintained at 22 ± 0.75 ^{o}C (mean ± SD) under a summer-like photoperiod (14 h light: 10 h dark). Throughout the study, daily dissolved oxygen readings remained above 6.5 ppm, whereas ammonia and nitrite concentrations did not exceed 0.20 ppm and 0.50 ppm, respectively. The RAS biofilter was back-washed every three days during the first three months, and every two days throughout the remainder of the study period.

To track changes in fish lengths and weights over time, 100 bluegill were randomly selected from each tank every ~30 d for the first three months, and every ~ 60 d for the remainder of the 574-d study period.

After rearing the bluegill for 376 days, the topping-off strategy was implemented in one of the two remaining fish tanks (called the "topping-off group"). Fish in the top 10th percentile of weight were removed with an equivalent number of juvenile bluegill being added to maintain the same fish density. This procedure was repeated three times throughout the study, at ~60-d intervals. Juveniles that were added at each topping-off occasion were batch marked by clipping the right pelvic fin (first topping off) or left pelvic fin (second topping off) or both the pelvic fins (third topping off). Growth rates of each batch of newly added juvenile bluegill were tracked over time by periodic length and weight measurements. On day 574, all fish from the two tanks were measured and weighed. During each "topping-off" harvest, and also on the final day of experimentation (day 574), individually-marked fish from each tank were measured for length and weight.

Fish growth in the recirculating tanks was tracked over time from the length and weight data collected on each sampling date. The metrics evaluated were: relative growth rate (RGR), feed efficiency (FE), relative weight (W_r), and coefficient of weight variation (CV_w).

Relative growth rate, RGR (g $100g^{-1}$ d^{-1}) = (wet weight gain (g) × 100 / average fish weight (g) / t), where average fish weight = (final weight + initial weight) / 2, and t is the rearing period in days (Hopkins 1992; Peres & Oliva-Teles 2008).

Feed efficiency was calculated as FE = (wet weight gain) / (dry feed fed). The amount of feed provided was adjusted based on consumption as described above. However, this does not represent true feed consumption because the amount of uneaten

feed was not determined. Mean fish weights for each sampling period were used to determine RGR and FE.

At each sampling, the relative weight (Wege & Anderson, 1978) of each fish was determined to indicate fish energetic condition, where W_r (%) = (W / W_s) × 100 %, W being a bluegill's observed weight (g), and W_s being the standard weight (g) expected according to its length (mm) as per Hillman's (1982) standard weight equation: $\text{Log}_{10} W_s = -5.374 + (3.316 \text{ x } \text{Log}_{10} L)$ where L = total length (mm). This standard weight equation was developed from bluegills collected from the wild in impoundments located throughout Missouri.

The coefficient of weight variation was also calculated to determine the extent to which bluegill weight ranges increased over time among the individuals within each replicate, with CV_w (%) = (sample standard deviation × 100 %) / sample mean weight. Fish mortality was also tracked throughout the study to determine survival rates.

The development of social hierarchies among bluegill was assessed by examining the extent that CV_w increased over time, and by examining the presence of significant positive relationships between W_r and fish length on every sampling outing. Twenty, randomly selected fish from the final harvest of each of the two fish groups, and six randomly selected fish from the group harvested during each topping off, were used to determine whole-body fat content. Relationships between parameters including body fat content versus fish weight, and W_r versus body fat, were used as additional indicators of social hierarchy development. Energy reserves such as hepatic glycogen content and plasma glucose level (review by Sloman & Armstrong 2002) or body lipid content (Li &

Brocksen 1977) were used to indicate social dominance in fish. In the present study that was for > 1 year, body lipid content was used as a measure of energy reserve to indicate the influence of social stress. Whole body-lipid content was estimated using the ether extraction method as described by the Association of Official Analytical Chemists (AOAC 2000).

Statistical analysis

Statistical analyses were performed using Student's *t*-test, regression analyses (linear and brokenline) as well as ANOVA. Appropriate procedures (detailed below) were used to determine (i) when social hierarchies developed, (ii) temporal changes in fish growth performances and finally, (iii) the effectiveness of "topping off" for disrupting established social hierarchies, and improving fish growth performance.

Growth

The growth pattern of bluegills reared in tanks was assessed by fitting the von Bertalanffy growth model to bluegill weight data (Hopkins 1992; Isely & Grabowski 2007) that were collected periodically until the beginning of "topping-off" harvesting on day 376. Mean fish weights derived from individually recorded fish weights in each tank for each sampling period were used to fit the model to the weight data.

Differences in weights between the two groups, "topping-off" (TO) and "no topping–off" (NTO), were determined from a *t*-test based on individual weight data

collected on day 0, day 376 (day of first "topping–off") and day 574 (final harvest day). Newly added juveniles were excluded from the comparison. Weights of bluegills removed during the three "topping-off" episodes were included in the TO group while comparing the two groups on the final day (day 574). The extent that the two groups differed in weight on the final day was used to determine the ability of the "topping–off" strategy to produce large-size bluegill and total fish yield.

Differences in bluegill growth rates between the TO and the NTO groups were again determined by applying a *t*-test. Overall growth rates between groups of individually-marked fish were compared over the period encompassing the three TO efforts. As of day 574, only ~25% of fish had retained their elastomer tags. Tag losses were observed to increase over time, mainly due to gradual deposition of tissue over them.

Social hierarchy development

Simple linear regression analyses were run to determine whether significant positive relationships developed between bluegill's W_r values and length for each sampling date over the duration of the study. Significant positive relationships between W_r and bluegill length would tend to indicate that larger bluegill were maintaining higher condition levels relative to smaller bluegills, which would be consistent with, and indicative of the development of a social hierarchy. For the TO group, regression analysis was carried out with the newly-added juveniles being excluded. Similarly, CV_w was regressed against days, from day 0 to the day when "topping-off" was initiated, and

again, from the day when "topping-off" was initiated, to the final harvest day for both the groups, in order to evaluate whether temporal increases in size variation occurred and whether the "topping-off" strategy disrupted social hierarchy establishment.

The presence of a relationship between fish fat content and fish weight was evaluated separately for the TO and the NTO groups, based on fish harvested on the final day. Moreover, one-way ANOVA was applied to determine whether differences in mean fish fat content existed among five groups (three groups being associated with the three episodes of "topping off", and two groups representing the final harvest, TO and NTO groups.

Influence of social hierarchy development on fish growth performance

Progressive changes in bluegill growth rate and feed efficiency as well as time-related changes in the relationship of W_r versus fish length were tracked to evaluate a possible linkage between social hierarchy development and change in bluegill growth performance. Plots of growth rate and feed efficiency versus day interval for the "pre-topping-off" period (day 0-376) indicated a marked initial decline towards zero followed by a leveling out of both growth rate and feed efficiency. Day interval (i.e., a midpoint for each sampling period) was calculated by averaging two successive days of sampling (e.g., day interval for RGR determined for the period spanning days 0-30 was 15.5). Also, the *P*-value of W_r -versus-length relationships determined for each sampling day showed a similar pattern as that of growth rate and feed efficiency. Accordingly, broken-line regressions were performed separately for RGR and feed efficiency versus day interval,

as well as for *P*-value versus days. When assessing fish social hierarchy development and its influences on bluegill growth performance, newly-added juvenile bluegill were excluded. All analyses were performed using SAS (Statistical Analysis System, Version 9.1; SAS Institute Inc., Cary, North Carolina, USA).

Experiment 2 – Evaluation of size-grading strategy for bluegills reared in ponds

In April, 2006, juvenile bluegill were seined from a 0.8-ha nursery pond at the Harrison Fish Farm, a commercial fish-rearing facility in northern Missouri (near Hurdland, MO) and stocked into raceways. One hundred bluegill were randomly selected from the raceways and measured for total length (nearest 1 mm TL). Bluegill measuring ≥ 85 mm represented the upper 25th percentile (upper quartile) of the group. Prior to grading, 6000 fish were randomly selected from the mixed, size-group and stocked into three 0.12-ha ponds at 2,000 fish per pond (~ 16,667 fish ha^{-1}); these fish represented the "ungraded group". From the remaining mixed-size fish, a floating, in-pond fish grader was used to select the required 6,000 fish ≥ 85 mm TL. The graded fish were stocked into three additional 0.12-ha ponds at 2000 fish per pond, representing the "graded group". Initial weights (mean ± S.D.) of bluegill in the graded and the ungraded groups were 21.35 ± 0.62 g and 11.54 ± 3.12 g, respectively.

The stocked bluegill were fed to apparent satiation once daily, five days per week excluding weekends. They were provided floating feed pellets (Aquamax Grower-400® diet; St. Louis, MO; 45% crude protein and 16% fat) which were hand broadcasted over at least 50 % of each pond's surface. Cessation of feeding activity and presence of a

small number of uneaten pellets after ~30 min of each feeding was considered to indicate satiation feeding. Feeding was adjusted periodically based on the amount of pellets that remained uneaten. The amount of feed provided was also reduced on cloudy or rainy days (~20 days) given that dissolved oxygen levels may drop due to reduced photosynthesis during those days. The study continued until the end of the subsequent growing season (November, 2007; 584 d; ~ 20 months). The fish were not fed during the 138-d over-winter period (November, 2006 – March, 2007) in the middle of the experiment, nor during 16 days in August, 2007 when excessive heat led to unfavorably warm water temperatures and markedly reduced feeding by the fish.

To track changes in bluegill's lengths and weights in each pond, 30 fish were sampled by seining half of each pond's surface area every ~2 months during the first growing season (April through October, 2006), whereas 100 fish were sampled by seining the whole pond surface area every ~2 months during the second growing season (April through November, 2007). Fish were selected at random during each sampling effort. Individual fish length (nearest 1 mm TL) and weight (nearest 0.1 g) were determined using a measuring board and a portable electronic balance (Denver Instrument, Bohemia, NY, USA), respectively. All fish were returned to their respective ponds after each sampling. Sampling of fish was not conducted during winter (November, 2006 – March, 2007). Aquatic macrophyte growth was observed in two of the ponds, one being graded and the other being ungraded. This was manually removed from both ponds in July 2006 and also in April 2007. Because of the weed infestation, the ungraded pond was not sampled in April 2007.

Bluegill spawning activity was observed in all the ponds during mid-June 2006 in the first growing season, and large numbers of young-of-the-year bluegill were present throughout the second year. To minimize their influence, all the ponds were seined early in the second growing year to remove as many young bluegill as possible.

The overall survival rate of bluegill was determined by counting the number of bluegill remaining at the final harvest of each pond relative to the number of bluegill initially stocked. The development of social hierarchies among bluegills in the production ponds was evaluated as in Experiment 1, by examining temporal changes in social-hierarchy indicators including CV_w, and particularly, whether significant positive linear relationships developed between bluegills relative weight (W_r) and length, based on periodic collections.

At the end of each experiment, all fish were seined from each production pond, with the total biomass of all harvested fish representing each pond's total production (gross yield). Final counts of bluegills from each pond were used to determine final survival rate. Mean survival rate for each group was determined as the average of the three pond means within each group. From the harvested bluegills, 50 fish were randomly selected from each pond and used to determine sex by dissection.

Statistical analysis

Effects of size grading

Differences between size-graded and ungraded groups as regards RGR, fish production, FE, W_r and CV_w were determined by applying *t*-tests at regular time intervals. The growth trajectories of the pond reared bluegills were also assessed by fitting the von Bertalanffy growth model to the weight data for each separate group. This was done to gain a better understanding of possible differences in the general growth pattern of bluegills in the different groups (predominantly male versus mixed sex) and to compare them to the growth pattern of bluegills obtained from indoor tanks (Experiment 1). Bluegill weighing > 100 g were considered large fish. The percentage bluegill weighing > 100 g from the final harvest were compared between the two groups by *t*-test with the percentage data determined separately for each pond being arcsine square-root transformed.

Effects of social hierarchy development

Social hierarchy development was separately assessed for graded and ungraded bluegill groups based on temporal changes in the positive linear relationships between W_r and fish length, as well as trends in CV_w over time. The influence of social hierarchy development on bluegill growth rate and feed efficiency was examined separately for the size-graded and ungraded groups as those determined for bluegill in the Experiment 1 – broken-line regression was run separately for RGR and feed efficiency versus day

interval and also for *P*-values of W_r-versus-length relationships versus days to assess the possible linkage between social hierarchy development and change in bluegill growth performance in the two groups. However, ponds infested with weeds were excluded from this analysis, given the potential for confounding effects on growth parameters. All analyses were performed using SAS.

RESULTS

Tank Study

Weight

For both the "no topping-off" (NTO) and "topping-off" (TO) groups, mean weights of bluegills increased with time (Table 1; Fig. 1, upper panel), following the asymptotic relationship: Weight = 101.60 $(1\text{-}e^{-0.004(\text{Days}+25.03)})$; $r^2 = 0.97$, von Bertalanffy growth model), up to the time when TO was initiated. The greatest proportional increase in bluegill weight occurred immediately following stocking (i.e., Day 0 to Day 65; ~70 % increase) (Table 1). Thereafter, proportional weight increases between weighing dates generally declined over time. The fish achieved a mean weight of 84.15 ± 6.83 g (average of two tank means ± S.D.), representing a 6.87-fold increase relative to the starting value of 12.25 g during the pre-harvest period (0-376 days). Over the period of cull harvesting (days 376-574), there was little increase in fish weight for the NTO group. Percent weight gain declined drastically from 2.9 % (days 376-436) to 0.1 % (days 510-574), whereas there was a 10-15 % increase in weight gain for the TO group, which matched the percent

weight gain (~11.5 %) that occurred over the period, days 161-376. Mean weights of the fish removed during TO harvests 1, 2 and 3, were 182.24 g (n = 24), 135.63 g (n = 23) and 123.75 g (n = 16), respectively, whereas the mean weight of harvested fish on the final day was 72.57 g for the TO group (excluding juvenile bluegill). For the TO group, the true final mean weight determined by including all of the originally stocked bluegill that were harvested at different times was 96.46 g, whereas the mean weight for the NTO group that was harvested on the final day (day 576) was only 82.4 g, with the mean weights of the two groups being statistically different ($P < 0.05$, t-test). However, no significant differences were observed between the two groups on day 0 nor on day 376 when the initial TO was applied ($P > 0.05$, t-test). The percentage of fish weighing more than 100 g increased from 37.7 % to 46.2 % between days 376 and 574 for the TO group, whereas for the NTO group, there was a negligible increase, with the percentage of fish > 100 g increasing only from 30.7 % (day 378) to 30.8 % (day 574). Bluegill survival rates did not differ substantially between the two groups, the values being 75.00 % for the NTO group versus 72.86 % for the TO group. Approximately 50 % of the total mortality in each group occurred within first 60 d.

Final mean weights of the juvenile bluegills added during TO episodes 1, 2 and 3 were 36.31 g, 22.92 g and 28.11 g, respectively (Table 1). Overall, weight gain (%) for the juvenile bluegills added during the three subsequent TO harvests averaged 149.0 %, 74.56 % and 63.72 %, respectively.

Weights of individually-marked bluegill

Of the 75 bluegill that were initially marked, only 30 were recovered in each of the fish groups (TO and NTO) on day 376 (just prior to "topping off"), and only 19 and 17 bluegill were found to have marks on the final day of harvesting for the NTO and TO groups, respectively. Ultimately, the individuals identified on the final day were used to compare the two groups over the TO period. Initial weights (mean ± S.D.) (day 0) for the NTO group and the TO group were 12.48 ± 2.79 g and 10.69 ± 2.31 g, respectively. Weights of individually-marked fish were lower than those observed for randomly selected fish in the later samplings carried out on days 376 and 574. For the NTO group, mean weights (mean ± S.D.) were 63.12 ± 37.12 g and 73.14 ± 44.55 g for the days 376 and 574, respectively, whereas for the TO group, the values were 51.59 ± 19.39 g and 65.70 ± 25.58 g for days 376 and 574, respectively (Table 4).

RGR

Pre-harvest period (day 0-376): Relative growth rate (RGR) was highest immediately after stocking (1.63 g $100g^{-1}$ d^{-1} for the NTO group and 1.73 g $100g^{-1}$ d^{-1} for the TO group; Days 0 – 31) for both groups (Table 3). Subsequently, RGR declined significantly (RGR = 1.37 -0.005 Days; r^2 = 0.80 for the NTO group and RGR = 1.40 - 0.005 Day; r^2 = 0.79 for the TO group; $P < 0.05$, regression) with the values (mean of two groups) declining from 1.68 g $100g^{-1}$ d^{-1} (Days 0-31) to 0.14 g $100g^{-1}$ d^{-1} (Days 285 - 358) (Table 3) over the pre-harvest period. A plot of the RGR values for both groups across days yielded a broken-line regression (Fig. 3, middle panel; SAS non-linear

regression procedure, r^2 = 0.97) having a distinct breakpoint for the sampling period 161-223 days, the decline being significant ($P < 0.01$) beforehand, and non-significant ($P > 0.05$) thereafter. The overall RGR averaged 0.40 ± 0.0 g $100g^{-1}$ d^{-1} (mean ± S.D.) for the pre-harvest period for both groups.

Topping off period (day 376-574): Regression analysis for the three mean RGR values corresponding to the three TO periods versus days, showed a nonsignificant decline (RGR = 0.18 - 0.0003 Days; r^2 = 0.90 and P = 0.21) and a nonsignificant increase (RGR = 0.10 + 0.0001 Days; r^2 = 0.08 and P = 0.83) for the NTO and TO groups, respectively. However, the analysis of RGR for the individually-marked fish showed a significant difference between the two groups over the TO period ($P < 0.05$, *t*-test). For the pre-harvest period, RGR (mean ± S.D.) of individually-marked bluegill from the NTO and TO groups were 0.33 ± 0.08 g $100g^{-1}$ d^{-1} and 0.34 ± 0.04 g $100g^{-1}$ d^{-1}, respectively, there being no significant differences between the two groups, whereas for the TO period, the TO group exhibited significantly higher ($P < 0.05$, *t*-test) RGRs than the NTO group, with the respective values (mean ± S.D.) being 0.14 ± 0.09 g $100g^{-1}$ d^{-1} (TO group) and 0.07 ± 0.07 g $100g^{-1}$ d^{-1} (NTO group).

Growth rates of newly added juveniles generally declined over the subsequent samplings (Table 3). Overall, the RGRs (g $100g^{-1}$ d^{-1}) of the juvenile bluegill added during three TO events were 0.43, 0.39 and 0.76, respectively.

FE

Mean feed efficiency (Table 3) was high in the month following stocking, reached a peak in the subsequent month, and then steadily declined, with the overall decline being significant for the pre-harvest period for both the NTO and TO groups (FE = 1.00 - 0.003 Days; r^2 = 0.71 and $P < 0.05$ for NTO group and FE = 1.00 - 0.003 Days; r^2 = 0.78 and $P < 0.01$ for TO group). A plot of FE values for both groups across days for the pre-harvest period, yielded a broken-line regression (r^2 = 0.92; SAS non-linear regression procedure) with a distinct breakpoint over the sampling period 161-223 days (Fig. 3, lower panel). The decline was significant ($P < 0.01$) beforehand, and non-significant ($P > 0.05$) thereafter. The overall FE (g wet weight gain / g dry feed fed) was 0.47 ± 0.01% (mean ± S.D.) for the pre-harvest period. Mean FE (mean ± S.D.) values over the TO period (days 376-574) were 0.04 ± 0.09 and 0.27 ± 0.06 for the NTO and TO groups, respectively.

W_r

For both groups, initial fish relative weight (mean value) was only ~85%; however, it surpassed 100 % on day 61, and remained at ~100 % until day 161. Subsequently, the fish's W_r levels dropped over each consecutive sampling episode, with the mean value being ~88 % just prior to TO (Fig. 1, middle panel). During the TO period, the overall mean W_r (mean ± S.D.) of the three final sampling episodes (days 436, 510 and 574) were 85.04 ± 3.27% and 88.03 ± 1.85% for the NTO and TO groups,

respectively, with no significant differences being detected between them ($P > 0.05$, *t*-test).

The "cloud" of fish relative weight (W_r) versus length values for each tank (Fig. 2) moved progressively to the right as time progressed, consistent with the growth that was occurring. Simultaneously, the shapes of the data clouds changed from roughly spherical to increasingly elongate ovals, evidence that the individuals in each tank were of an expanding range in length but of a narrowing breadth in length-specific relative weights. Interestingly, the lengths of the smallest individuals failed to increase between day 161 and day 376 (pre-harvest period), stalling at ca. 110 mm for both fish groups. However, between day 376 and the final harvest day, 574 ("topping off" period), length of the smallest individuals remained at ~110 mm for the NTO group, whereas for the TO group, the data cloud indicated that the smallest individuals moved from ~110 mm (day 376) to ~130 mm (day 574).

At stocking (Day 0), there was no correlation between the relative weight (W_r) and length of individual fish (Fig. 2) in either group. However, a significant positive relationship between these two variables developed in both tanks by Day 31; this persisted throughout the pre-harvest period (day 376) (Fig. 2). Correspondingly, for the pre-harvest period a plot of the statistical significance of the $\mathbf{W_r}$ versus length relationships (i.e., *P*-values) through time yielded a broken-line regression (SAS, non-linear regression procedure; r^2=0.78) showing a distinct breakpoint at Day 32 (Fig. 3, upper panel), with the decline being significant ($P < 0.01$) beforehand and non-significant ($P > 0.05$) thereafter. Fish that perished within first 60 days post-stocking were observed to be poorer in body condition compared to other fish in the group (Fig. 2).

For the NTO group, the significant positive relationships between W_r and length ($P < 0.05$, regression analysis) persisted throughout the study period, whereas for the TO group, a non-significant relationship ($P > 0.05$, regression analysis) was observed between W_r and length after the second topping off (Table 2 and Fig. 2).

CV_w

The coefficient of variation in weight (Table 2 and Fig. 1, lower panel) was lowest at stocking (Day 0; 28.14 %) and increased significantly ($P < 0.05$, regression) for both groups by day 376 (CV_w = 40.13 + 0.06 Days, r^2 = 0.52 for NTO group and CV_w = 38.06 + 0.07 Days, r^2 = 0.74 for TO group). However, over the period of topping off (days 376-574), no change in CV_w (CV_w = 49.20 + 0.01 Days, P = 0.34 & r^2 = 0.44) was observed for the NTO group whereas a significant decline in CV_w (CV_w = 87.47 - 0.09 Days, P = 0.04 & r^2 = 0.93) was observed for the TO group.

Fat Content

There were no differences in mean fat content between the NTO and TO groups harvested on the final day. However, fish that were removed during each TO episode (~30 % body fat, dry weight basis) exhibited significantly higher body fat contents than did the groups that were removed on the final day of harvest (~22 % body fat, dry weight basis) ($P < 0.01$, ANOVA), whereas no differences were observed in fat content among the fish harvested during the three TO episodes (Table 5). Also, the fat contents of fish

that were harvested on the final day exhibited a significant positive relationship with fish weight (Fat = 13.81 + 0.10 Weight; r^2 = 0.71 & P < 0.01 for NTO group, and Fat = 14.58 + 0.11 Weight; r^2 = 0.84 & P < 0.01 for TO group) indicating that larger fish stored in greater energy reserves with energy reserve declining with declining fish weight. Also, fat content of fish showed a significant positive relationship with relative weight (W_r, %) (Fat = -4.67 + 0.29 W_r; r^2 = 0.23 & P < 0.01 for NTO group, and Fat = -8.73 + 0.35 W_r; r^2 = 0.52 & P = 0.04 for TO group) suggesting that relative weight can be used as a reliable indicator of body fat content or fish condition for bluegill.

Pond study

Weight

The percentage of males in the graded and the ungraded groups were 70.20 and 48.42, respectively. For the pond fish, as was observed for tank bluegill, mean weight increased with time (Fig. 4), the relationship being asymptotic for both the ungraded (Weight = 84.56 $(1-e^{-0.004(Days+35.9)})$; r^2 = 0.89) and graded (Weight = 99.89 $(1-e^{-0.004(Days+50.90)})$; r^2 = 0.85) groups (Fig. 4, upper panel). However, the mean weights of the graded group were significantly (P < 0.05) or marginally (P = 0.05-0.1) higher than for the ungraded group throughout the study period (Table 6).

The greatest proportional increase in weight occurred immediately following stocking (i.e., between Day 0 and Day 65; 1.81 % d^{-1} for the ungraded group and 1.09% d^{-1} for the graded group), as was observed for bluegills in the laboratory. The

proportional increases between weighing dates generally declined until Day 352, after which they leveled off, varying between 0 % d^{-1} and ~0.3 % d^{-1} for both groups (Table 6). The fish achieved mean final weights (mean ± S.D.) of 82.14 ± 4.24 g and 103.97 ± 14.59 g, in the ungraded and the graded groups respectively, with group means differing only marginally (P = 0.07, *t*-test). The ungraded groups showed an 8.2-fold increase in weight, whereas the graded groups showed only a 5.4-fold weight increase relative to their respective initial weights (~10 g for ungraded fish and ~19 g for graded fish).

Fish survival did not differ between the groups ($P > 0.05$, *t*-test), the values being 41.41 ± 8.43% and 34.95 ± 5.16% for the ungraded and the graded groups, respectively. However, survival values were only about half of those experienced in the laboratory setting. Despite the differences in the mean weights, non-significant differences ($P > 0.05$, *t*-test) were observed in final production for the two groups. Final total production was 558.00 ± 113.18 Kg ha^{-1} for the ungraded group, and 594.51 ± 166.87 Kg ha^{-1} for the graded group.

The percentage of bluegills weighing > 100 g at final harvest was higher for the graded group (48.16 ± 12.45 %) than for the ungraded group (22.33 ± 9.11 %) ($P < 0.05$, *t*-test).

RGR

No significant differences were observed in RGR between the ungraded and graded groups (Table 8; Fig. 6.). Although the RGR for the ungraded group (0.78-0.89 g $100g^{-1}$ d^{-1}) was slightly higher than for the graded group (0.66-0.78 g $100g^{-1}$ d^{-1}) throughout the first year, no significant differences were detected on any sampling date ($P > 0.05$, *t*-test). Non-significant differences ($P > 0.05$, *t*-test) were likewise observed between the two groups in year two. However, both groups exhibited a significantly higher growth rate (g $100g^{-1}$ d^{-1}) in year one (0.84 for the ungraded group and 0.71 for graded group) than for year two (0.15 for ungraded group and 0.19 for graded group) ($P < 0.01$, *t*-test). Growth rates generally declined over the sampling periods for both groups. A plot of RGR values over days yielded a broken-line regression with a distinct breakpoint associated with the winter period (sampling interval 181-352 days or Oct 2006-Apr 2007) (Fig. 7, middle panel) for both the graded and the ungraded groups. The overall RGR (mean ± S.D.) was 0.26 ± 0.02 g $100g^{-1}$ d^{-1} for the ungraded groups and 0.23 ± 0.01 g $100g^{-1}$ d^{-1} for the graded groups.

FE

Feed efficiency largely followed the temporal pattern of RGR. No significant differences in FE were detected between the graded and ungraded groups throughout the study ($P > 0.05$, *t*-test) (Table 8; Fig. 6.). Feed efficiency, however, declined over time for both groups; the ungraded group exhibited significantly greater FE (g wet weight gain / g feed fed) in year one (0.42) than in year two (0.18) ($P < 0.05$, *t*-test), whereas the

graded group exhibited marginally higher FE in year one (0.51) than in year two (0.23) (P = 0.08, t-test). A plot of the FE values over days yielded a broken-line regression with a distinct breakpoint for the winter period (sampling interval: 181-352 days; Oct 2006-Apr 2007) (Fig. 7, lower panel) for both the groups. The overall FE (mean ± S.D.) was slightly higher for the graded group (0.30 ± 0.05) than for the ungraded group (0.25 ± 0.05) with differences between the groups being non-significant (P > 0.05, t-test).

W_r

Despite differences in mean weight between the ungraded and graded groups, no significant differences were detected in relative weight (W_r) (Fig. 4, middle panel; P value > 0.05, t-test). At stocking (Day 0), the majority of bluegill in all ponds exhibited W_r values > 80 %, with the mean W_r values being ~83 % for each of the two groups (Fig. 4, middle panel, and Fig. 5). In subsequent sampling, days 65, 129, and 181 (June through October, first summer), the condition of most bluegills improved substantially in each group: most individuals exhibited W_r values > 90% with the mean W_r values being ≥ 100 % (Fig. 5). Interestingly, individuals with W_r values of < 90 % were again observed in substantial numbers on Day 352 (April) after over-wintering (Fig. 4, middle panel and Fig. 5) and such a body condition continued to persist thereafter for both the groups until the final day.

For the pond fish, the "cloud" of fish relative weight (W_r) versus length values for the graded and ungraded groups (Fig. 5), moved progressively to the right with time, indicating that growth was occurring, similar to that which occurred in the laboratory

tank setting (Fig. 2). For the fish in ponds, changes in the data "cloud" shape through time (Fig. 5) were less pronounced than in the laboratory fish (Fig. 2). Ovoid data clouds having significant positive slopes were detected for both the ungraded and graded groups in the outdoor ponds only from day 181 onward (Fig. 5), whereas this was the case in the laboratory from day 31 onward (Fig. 2).

Correspondingly for the pond fish, a plot of the statistical significance of the W_r versus length relationships (i.e., *P*-values, r^2) through time (Table 7), yielded a broken-line regression that had a distinct breakpoint for the sampling interval 181-352 days (Oct, 2006-Apr, 2007) for both graded and ungraded groups (Fig. 7, upper panel), with the decline being significant ($P < 0.01$) beforehand and non-significant ($P > 0.05$) thereafter.

CV_w

The coefficient of variation (CV_w) in weight was significantly higher ($P < 0.05$, *t*-test) for the ungraded groups (~50%) than for the graded bluegill groups (~30%), throughout the entire growing year one (day 0-181), whereas, CV_w did not differ between the groups ($P > 0.05$, *t*-test) (~30% for both the groups) for the majority of growing year two (day 352-584) (Table 7 and Fig. 4, lower panel). Corresponding to this observation, CV_w of the ungraded group declined significantly over growing year one (CV_w = 58.68 - 0.07 Days; $P < 0.01$ and $r^2 = 0.99$; regression), whereas that of the graded group showed a non-significant decline (CV_w = 55.17 - 0.04 Days; $P = 0.17$ and $r^2 = 0.54$, regression). However, non-significant declines ($P > 0.05$, regression) were observed for both groups (CV_w = 55.17 - 0.04 Days; $P = 0.17$ and $r^2 = 0.54$ for ungraded, and CV_w =

32.02 - 0.005 Days; P = 0.17 and r^2 = 0.54 for graded) over growing year two (day 352-584).

DISCUSSION

Of the two novel rearing strategies that I evaluated, the topping-off approach demonstrated the greater capacity to increase fish growth, produce more large bluegill (> 100 g), and increase fish production. Moreover, the present study tends to support previous findings (e.g., Doerhoff 2007) that size grading can be effectively applied to form bluegill groups that are predominantly composed of male bluegill (70 %). The presence of additional male bluegills (20 % of total) within the reared groups, while not markedly increasing total bluegill production, did significantly increase mean bluegill weight, as well as the percentage of large bluegills (> 100 g) present. This study has, for the first time, provided evidence that bluegill do establish social hierarchies in large rearing systems, e.g., production ponds, and that the establishment of these hierarchies negatively affects key fish production parameters.

Indicators of fish production

Weight

The topping-off strategy produced larger bluegill as well as higher fish yields relative to single-batch harvesting. This is likely due to the growth spurt that was

exhibited by subordinates once they were released from the dominating force of the social hierarchy (discussed later in this section). Partial or sequential harvesting has been used to increase fish yields by reducing competition among coexisting individuals, in catfish (Tucker & Robinson 1990), tilapia (Brummett 2002), and rainbow trout (Westers & Weeks 2003). Similarly, size grading has been applied to increase final fish weights and fish production in Atlantic salmon, *Salmo salar* (Gunnes 1976) and yellow perch (Wallat *et al.* 2005). In the present study, size grading did result in an increased mean fish weight, as well as a greater number of larger-size fish. Large bluegill (> 200 g) are of substantial economic value and command substantially higher prices than do small-to-intermediate-size bluegill (Curtis Harrison, Harrison Fisheries, Inc., MO, 2010, pers. comm.). However, both the size-grading and topping-off rearing strategies yielded bluegill with final weights of only ~100 g, well below the desired food-market weight of 227 g. In comparison to the control group that achieved a final weight of ~82 g the modified rearing strategies in the present studies increased bluegill weights only by 15-20 g.

Topping off

Bluegill removed during the initial topping-off episode had reached 80.72 % of food-market weight (227 g), whereas those removed during the second and third topping-off episodes had reached only 59.75 % and 54.52 % of market size, respectively. This indicates that the bluegills that were released from the dominating forces of social hierarchy, although exhibiting a modest growth spurt, did not exhibit sufficient weight

gain to achieve food-market size, or even the sizes of bluegill that were culled during first topping-off episode. Moreover, the weights of the remaining stock continued to decline over the successive topping-off removals, with mean weights of the unremoved fish declining from 88.98 g on day 376, to 72.57 g on day 574. Hence, although topping-off did increase the number of large bluegill, this rearing strategy clearly requires further refreshment to determine, for example, when to initiate topping-off, the numbers of larger fish to be removed in each removal episode, as well as the optimal time intervals between successive topping-off harvests. Westers & Weeks (2003) demonstrated that including two cohorts per rearing cycle, versus using a single-cohort rearing strategy, increased the production of rainbow trout by as much as 60%. Similarly, Yu & Leung (2006) demonstrated that the extent to which a partial rearing strategy is successful versus a single harvesting strategy, depends, in part, on how well the sequential harvesting strategy is designed to maximize fish production. Therefore, further refinements in the "topping off" strategy will likely increase bluegill growth and production.

Size grading

Size grading of fishes has led to a wide range of outcomes in aquaculture. Size grading resulting in no improvements in weight gain or yield was reported for Arctic charr *Salvelinus alpinus* (Baardvik & Jobling 1990) and Atlantic cod *Gadus morhua* (Lambert & Dutil 2001), whereas improvement in weight gain has reported for juvenile Atlantic salmon (Gunnes 1976) as well as gilthead sea bream *Sparus auratus* (Popper *et al.* 1992). In the present study, size grading showed potential to produce higher

numbers of large, food-size bluegill, but showed no improvement in fish yield. The lack of significant differences between the ungraded and graded groups as regards final fish production suggests such factors as the development of social hierarchy and a slightly higher mortality recorded for the graded versus the ungraded group may have obscured benefits from size grading. For example, although male bluegill possess higher growth capacity than female bluegill, if the growth of a majority individuals is suppressed by a few dominant individuals, advantages from size grading or from rearing male-only bluegills will not be substantial. Wang *et al.* (2009) demonstrated the advantage of size grading in producing food-size bluegill when reared them for a short duration (11 month) involving two different rearing phases (~4 month tank rearing and ~7 month pond rearing) -- social hierarchy may not have played a major role in their study, given that the study was run for only 11 months and the fish were moved to a different culture setting which may have disrupted the hierarchy development. Therefore, using measures that would disrupt social hierarchy development will likely show benefits from size grading.

Mean production of bluegill achieved in the present study (558 Kg ha^{-1} for ungraded and 595 Kg ha^{-1} for graded) was slightly greater than that reported by Lane (2001) for bluegill reared in middle-latitude ponds. Bluegill reared in production ponds in Iowa for 384 d at an initial density of 12,000 ha^{-1} yielded 250 kg ha^{-1} of fish production, with a final mean weight of 33 ± 13.8 g and a survival rate of 62 % (Lane 2001). However, this production level was somewhat lower than that (757 kg ha^{-1}) reported by Schmittou (1965), and much lower (2080 Kg ha^{-1} to 2973 Kg ha^{-1}) than in the studies reported by Lovshin & Matthews (2003) for bluegill. Much as for production, higher survival rates were also recorded in these other studies: 85% (Schmittou 1965), 38 %

(high stocking density), 81% (low stocking density) (Lovshin & Matthews 2003) versus 38% (mean survival among six ponds) in the present study. The studies of Schmittou (1965) and Lovshin & Matthews (2003) were conducted at southern latitudes (Auburn University, mid-Alabama), whereas the present study was conducted at a middle latitude (northern Missouri). Latitude effects, including differences in temperature, winter severity, and length of growing season, have likely accounted for differences among studies in terms of survival rate as well as fish production. It is, in fact, noteworthy that in the present study (in Northern Missouri), that the pond fish gained no weight over winter (171 days; October, 2006 - April, 2007; Table 2). Thus, although bluegill spent 584 d in the ponds, the no-growth winter period restricted their growth to the 413 remaining days of the trial. Severe winter effects, as well as low survival likely reduced bluegill production in the present study, as was previously recorded for bluegill reared at a middle latitude by Lane (2001). Therefore, while it is important to add measures that would disrupt social hierarchy development in ponds, it will be further advantageous if bluegills are moved to indoor rearing systems during winter season in order to reduce the adverse seasonal effects.

Growth rate

Topping off

Despite the favorable rearing conditions provided for bluegills reared indoors, their growth rates declined continually. The growth of individually-marked bluegills in the present study showed that the topping-off harvesting strategy did elicit a significant

increase in bluegill growth rate. Similarly, partial harvesting has been reported to increase fish growth rate as well as production in some fishes including tilapia (Paessun & Allison 1984), trout (Watten 1992), as well as in shrimp (Moss *et al.* 2005). Yet, the growth rate increase due to topping-off in the present study was inadequate to allow bluegill to reach food-market size. In order to produce food-size bluegill of 227 g from an initial weight of 12 g in 574 days, a projected growth rate (RGR) of 0.313 g $100g^{-1}$ d^{-1} would be required. A higher initial growth through day 161 decreased the required growth rate to 0.29 g $100g^{-1}$ d^{-1} for the NTO group and to 0.27 g $100g^{-1}$ d^{-1} for the TO group for the remaining growth period (day 161-574). However, the NTO group exhibited almost zero growth over the remainder of the study period, whereas the TO group exhibited a modest growth spurt of ~0.15 g $100g^{-1}$ d^{-1} (0.13-0.19 g $100g^{-1}$ d^{-1}), which was less than the growth rate required to produce food-size bluegill. This resulted in a continuous decline in bluegill weights over the successive TO harvesting episodes. Therefore, while the TO harvesting increased bluegill growth rates and appeared to improve fish production, the resulting growth rate was insufficient to produce food-size bluegill within the rearing period.

Size grading

Similar to what was observed for bluegill reared in tanks, bluegill from the graded and ungraded groups that were reared in ponds exhibited continuous growth rate declines throughout the rearing period. Some seasonal growth fluctuations were observed, with growth cessation occurring over winter. Despite the male bluegill's higher capacity for

growth relative to females (Hayward & Wang 2006), and the fact that size grading favored sex ratios skewed towards males, the similarity in growth rates between the two groups suggests from the outset, that size grading may not be greatly beneficial for bluegills reared communally in ponds. However, the possibility that social hierarchies largely negated the benefits from rearing predominantly male bluegill is discussed later in the section.

For the ungraded and graded groups, a projected growth rate of 0.31 g $100g^{-1}$ d^{-1} and 0.29 g $100g^{-1}$ d^{-1}, respectively, would be required throughout the study (584 days) in order to produce food-size bluegill. During the initial rearing period (until day 181), both groups exhibited better growth (~0.80 g $100g^{-1}$ d^{-1} for the ungraded group and ~0.70 g $100g^{-1}$ d^{-1} for the graded group) than their projected required growth rates – this high initial growth reduced the required growth rate to 0.30 g $100g^{-1}$ d^{-1} for the ungraded group and to only 0.24 g $100g^{-1}$ d^{-1} for the graded group for the reminder of the study period. However, both the groups exhibited only 0.15 g $100g^{-1}$ d^{-1} (mean growth rate covering Oct 2006-Nov 2007) during the remaining period, ultimately resulting in smaller-size (80-100 g) bluegill. Wang *et al.* (2009) observed a significant difference in the absolute growth rate (AGR) of size-graded group versus ungraded bluegill. Calculation of RGR for the mean weights they reported showed that the ungraded group grew at 0.27 g $100g^{-1}$ d^{-1}, a value similar to ours (0.26 g $100g^{-1}$ d^{-1}), whereas the graded group grew at only 0.15 g $100g^{-1}$ d^{-1} (in the top 25%) or 0.16 g $100g^{-1}$ d^{-1} (in the top 50%), lower than what the present study recorded for the graded group (0.23 g $100g^{-1}$ d^{-1}). Likely, the lower RGR owes to the much higher initial weight that

Wang *et al.* (2009) used for the graded (90.5 g for top 25%, 67.3 g for top 50%) versus for the ungraded group (30.1 g).

Numerous studies have examined the benefits of size grading in fishes. Size grading improved growth rates of Atlantic salmon *Salmo salar* (Gunnes 1976), gilthead sea bream, *Sparus auratus* (Popper *et al.* 1992) and Nile tilapia, *Oreochromis niloticus* (Brzeski & Doyle 1995), but not for Arctic charr (Wallace & Kolbeinshavn 1988; Baardvik & Jobling 1990), turbot (Sunde *et al.* 1998), or yellow perch (Wallat *et al.* 2005). It has been suggested that high levels of intraspecific competition and agonistic interaction among individuals of similar size may limit the advantage of size grading (Baardvik & Jobling 1990; Sunde *et al.* 1998). Similarly, in the present study, the establishment of social hierarchies likely confounded the benefits of size grading, as discussed later in this section.

Feed efficiencies

Topping off

For the tank-reared fish, mean feed efficiency was highest following fall stocking (~1.10), and then declined continually. In the NTO group, FE declined to near zero, whereas in the TO group, FE remained above 0.20. Feed efficiency determined for the TO group was based on multiple cohorts, whereas that for the NTO group, was based solely on the original stock. Although FE from the TO group was partly influenced by the newly added juveniles, increased growth rate of the originally stocked bluegill indicate

that the improved FE were also stemmed from the latter. Sequential harvesting has been viewed as a strategy for improving growth rate and fish production more so than to improve feed efficiency, e.g., Paessun & Allison (1984), Watten (1992) and Moss *et al.* (2005). Yet, the present study showed the evidence of enhancement of bluegill's feed efficiency under cull harvesting, indicating the additional benefits from this strategy for bluegill aquaculture

In both tanks, the fish's initial vigorous feeding levels were observed to wane within about six months of stocking, even among the larger, dominant individuals. Increases in the numbers of feed pellets that remained uneaten after feeding prompted downward adjustment of the feed amounts provided. This reduction in feed consumption is thought to have been in response to the accumulation of body fat in dominant bluegill and the consequent suppression of feed intake. The commercial diet used in this study contained 16 % lipid, whereas Hoagland *et al*. (2003) showed that juvenile bluegill diets need only contain 8 % lipid. Body fat deposition and reduced appetites have often been observed in fishes provided such high-energy diets, e.g., Arctic charr *Salvelinus alpines* (Jobling & Miglavs 1993), chinook salmon *Oncorhynchus tshawytscha* (Shearer *et al.* 1997) and Atlantic salmon *Salmo salar* (Johansen *et al.* 2002).

Size grading

As observed for growth rate, size grading did not promote FE, but produce a modest increase in FE throughout the study period. No changes in feed conversion from size grading have often been reported, e.g., Wallace & Kolbeinshavn (1988) for Arctic

charr, Carmichael (1994) for channel catfish, Sunde *et al.* (1998) for turbot and Wallat *et al.* (2005) for yellow perch. Overall FE values for fishes reared in ponds (0.25 for ungraded and 0.30 for graded) are comparable to values obtained in studies described by Loveshin & Matthews (2003) (0.28-0.37 for FE or 2.7-3.6 for FCR) for bluegills reared in ponds. The pond fish FEs exhibited apparent seasonality, whereas this association was less prominent in indoor tanks. For pond fish, low overwinter FEs suggest that winter conditions markedly influenced feeding and growth. Overall, differences in FE magnitude and their fluctuations over time further support the notion that pond and tank rearing environments are dissimilar, in terms of factors that influenced feeding and growth therein (see below).

Indicators of social hierarchy

Relative weight and body fat

Topping off

Increased levels of stress hormone, observed particularly in subordinate fish, and associated declines in energy reserves, body condition, and growth, have been commonly observed in fishes that tend to establish social hierarchies (review by Sloman & Armstrong 2002). Bluegill agonistic interactions have been extensively recorded (Poulsen & Chiszar 1974; Beitinger & Magnuson 1975; Henderson & Chiszar 1977; Colgan *et al.* 1979). The present study recorded neither behavioral responses nor changes in stress hormone levels due to practical difficulties associated with the large rearing systems,

particularly for recording behavioral responses. However, physiological responses based on body fat content (an indicator of energy reserve), relative weight (W_r) (also an indicator of body condition) as well as change in size variation (CV_w) were examined as indicators of social hierarchy establishment. With dominance hierarchy formation, larger/dominant individuals would be expected to exhibit levels of condition in excess of what smaller/subordinate individuals would show. Moreover, this inequity would be expected to increase with time. Consistent with this prediction, the tank fish exhibited distinct positive relationships between relative weight and length from Day 31. In other words, as time passed, larger fish exhibited increased levels of condition as their lengths increased, whereas smaller fish did not. Broken-line regression analysis of the statistical significance of the $\mathbf{W_r}$ versus length relationship ($P < 0.05$) suggested that the dominance effect was evident from Day 31 onward: one would expect rapid dominance hierarchy formation under more confined conditions. After the second topping off, no regression relationships were observed between W_r and fish length, indicating that the removal of bluegill in the topped-off group markedly reduced the previously existing dominance hierarchies. However, for the NTO group, the presence of a significant positive relationship between W_r and fish length across all sampling dates indicated that social hierarchies persisted through the end of the study. The study results overall suggest that "topping-off" can be used as an effective strategy to disrupt bluegill social hierarchies.

In addition to the W_r versus fish-length relationship, a significant positive relationship was observed in the present study for body fat content versus fish weight, as well as for body fat content versus relative weight. These relationships provide evidence that social hierarchies did become established in the tanks, and that relative weight can,

indeed, be used as a metric to identify the establishment of social hierarchies in groups of bluegill. Larger bluegill exhibited higher amounts of body fat than smaller bluegill within groups, indicating that larger bluegill had monopolized the feed, whereas small bluegill (subordinates) likely expended more energy in avoiding agonistic social interactions and also consumed considerably less feed--a phenomenon that is often observed in fishes forming social hierarchies (Sloman & Armstrong 2002).

Plots of bluegill relative weight versus length relationships (Fig. 2) suggest that fish perished likely when their body condition dropped below 50 %. Also, the observation that subordinates approached W_r levels close to 50 % with time, particularly for the NTO group, indicates the dire effects of social hierarchies on fish's energetic states. Mean W_r of fish harvested on the final day, although not significantly different between the two groups (TO and NTO), the topped off group was largely represented by fish that likely were subordinates in the past. This suggests that body condition of subordinates apparently have improved to a level that could match the W_r of the NTO group. Overall, the study results overall indicate that the "topping off" approach can be applied to efficiently disrupt the strong social relationships that developed among bluegill, and thereby improve the body condition of subordinates.

Size grading

Despite the fact that substantial differences were observed in the weights of graded versus ungraded bluegill, no differences were observed in relative weight for the groups. This indicates that predominantly male bluegill group increased not only in

weight, but concomitantly in length as well (Fig. 5). The results of the pond trials yielded complimentary findings in that distinct positive relationships between relative weight and length of bluegills developed over time for the graded as well as the ungraded group. However, it was not until day 181 that statistical significance between W_r and fish length was first observed. In the less confined pond environment versus tanks, it is not surprising that the development of social hierarchies required more time to develop. Interestingly, the smallest individuals in ponds surpassed 100 mm TL by day 181 (Fig. 5); the same could not be said for the tank-reared fish under the NTO harvest regime, even by day 285 (Fig. 2). Thus, the less confined pond environment apparently provided less growth suppression of smaller bluegills, than occurred in the more confined, tank-rearing environment. Given that bluegill exhibited a significant relationship between W_r and fish length during both the pre- and post-winter period, it appears that social hierarchies, once established, persist through winter (Fig. 5 and Fig. 7). These data provide the first evidence that social hierarchy development occurs in commercial-scale pond rearing environments, and that social hierarchies can influence the condition of such fish. Consequently, the strength of the W_r versus length regression relationships may be the best diagnostic of social hierarchy formation especially in larger rearing systems, signaling the need for remedial action. Also, despite initial differences in size ranges of bluegills in the ungraded and graded groups, it was fish in the upper end of the length distributions that, over time, began to exhibit enhanced condition, suggesting that size grading did not minimize the development of a social hierarchy (Fig. 5).

Coefficient of size variation

Topping off

The coefficient of variation (either in terms of fish weight (CV_w) or length (CV_l)) is commonly used to detect social hierarchy presence. It was expected that dominance hierarchy formation would result in increasing coefficients of weight variation through time, due to smaller, more subordinate fish growing progressively lower rates than few larger, more dominant fish. Increased size variation through time from the development of social hierarchies have been observed in a variety of species (e.g., eleotrid goby *Odontobutis obscurus*, Yamagishi *et al.* 1974; Arctic charr *Salvelinus alpinus*, Jobling 1995; sunfish hybrids *L. cyanellus* × *L. macrochirus*, Wang *et al.* 2000). Fish reared in tanks exhibited significant increases in CV_w until ~day 376 when "topping off" was initiated, indicating that fish in both tanks exhibited significant increases in size variation. However, from day 376 to the end of the rearing period (day 574), the NTO group exhibited no further increase in size variation. From this observation, one might conclude that the bluegill social hierarchy had reached a stable state where all members exhibited some growth according to their social rank, with the most dominant individuals exhibiting the most growth, and with growth rate declining with declining social rank. However, during this period (days 376-574), both mean growth rate and feed efficiency declined to almost zero for the NTO group. The lack of change in size variation and absence of growth from days 376-574 indicated that growth, even among the more dominant bluegills, ceased during this period for the NTO group. This cessation of growth likely resulted from dominant fish having attained their fat requirement which tends to markedly reduce fishes' appetites (Jobling & Miglavs 1993; Shearer *et al.* 1997;

Johansen *et al.* 2002). Yet, the reduction of appetite in dominant individuals did not allow poor-condition subordinate bluegills increase their feed consumption and exhibit better body condition as well as growth rates. In the TO group, once the dominant bluegills were removed, subordinates had increased their feed consumption and exhibited growth spurt (as indicated by individually-marked bluegills) which led to a decrease in their CV_w. Overall, results indicate the benefit of the topping-off strategy which disrupted the bluegill social hierarchy and increased fish growth performance.

Size grading

Unexpectedly, while the tank fish exhibited the anticipated increase in CV_w with time (Table 1), the pond fish did not (Table 2). Similar differences have been observed for hybrid bluegill reared in tanks (Wang *et al.* 2000) versus ponds (Sager & Winkelman 2006). Indeed, the ungraded pond fish exhibited a significant decline in size variation, whereas the graded fish showed no change in size variation. Similarly, a reduction in CV_w was reported by Wang *et al.* (2009) for bluegills reared for 44 weeks that included ~5 months of rearing in indoor tanks (400 L) and ~6 months of rearing in outdoor cages (1 m^3). For tank-reared fish, the confined space and potential for repeated inter-individual interaction likely have reinforced any hierarchy-based access to feed, yielding positive feed-back and thereby increasing fish size variation. Low survival of the pond fish (38 %, versus 74 % in the tanks) might also have influenced their CV_w values, with fish in poor condition being more susceptible to the extremes of both winter (Murphy *et al.* 1991) and summer (Schneider 1998). Furthermore, for pond-reared fish, large individuals may exert

their dominance when feeding occurs, but smaller subordinate individuals may be able to escape ongoing agonistic interactions via spatial avoidance (e.g., by moving elsewhere within the larger habitat that a pond provides). In addition, the presence of natural feed may have provided the stocked fish alternative feed choices at least early in the study. Such factors likely have influenced the observed CV_w and delayed social hierarchy establishment in the ponds. By examining just the CV_w, one might arrive at a different conclusion concerning the development of social hierarchies in ponds. However, because the W_r versus length relationship indicated that social hierarchy indeed developed in the ponds by day 181, the W_r versus length regression relationship appears to be a more accurate diagnostic tool than the size variation for identifying the development of social hierarchies in large rearing systems.

Social hierarchy development and effects on production parameters

The establishment of a social hierarchy was indicated in the present study by significant, positive relationships between bluegills' W_rs and fish length (in both ponds and tanks), and by the relationship between body fat content and fish weight, and by changes in fish size variation (in tanks). Most studies of social hierarchy development in fishes have been restricted to laboratory settings (Sloman & Armstrong 2002), partly because of the lack of a practical indicator of social hierarchy development. The present study demonstrated that the positive linear relationships between fish W_r values and their associated lengths is an easily-implemented, reliable metric for determining the social hierarchy establishment.

Based on the relationship between W_r and fish length, two stages of social hierarchy formation may be classified: 1) the developing phase, 2) the post-development or social-intensification phase. The developing phase is the period from the day of stocking to the day when a significant positive relationship is first recorded between W_r and fish length. Social hierarchy is considered fully developed when a significant relationship between W_r and fish length is first observed. The post-development phase covers the period from the day when the significant relationship first occurs to the day of final harvest. Based on this classification, for the tank fish, the developing phase lasted for only ~30 days, whereas for the pond fish, the developing phase was ~180 days. On the other hand, the post-development or social-intensification phase was much longer for the tank fish than that for the pond fish. Social intensification during this period can be seen via significant increases in size variation (CV_w). Differences in the duration of each phase of development for the pond versus the tank bluegill stem from differences in the two rearing environment. However, regardless of whether bluegills experienced the developing phase for a relatively brief period (tank fish) or a more protracted period (pond fish), significant declines in their growth and feed efficiency (FE) were recorded in both rearing systems. Similarly, for the tank system, although bluegill moved quickly into the post-development phase, their growth performance continued to decline until it reached almost zero, due likely to social intensification. Interestingly, in both the culture systems, the break point (the time from when the least growth occurred) was observed for fish growth (Fig. 3 and Fig. 7, middle panels) after about 3-4 months (~134 days for tanks and ~86 days for ponds) from the day when the significant relationship was first recorded.

While declines in RGR may stem from reduced feeding opportunities due to social hierarchies, declines in FE were likely due to more energy having been spent by bluegills through social interaction. Greater FE variation was observed for bluegill in the ponds versus the tanks, likely because of the seasonal variation that the pond bluegills experienced. Also, the conversion of feed to pond fish biomass that was lost via fish mortality was not quantifiable; consequently, differences in mortality rates among ponds may also have played a role in the observed FE variations.

Previously, Hayward & Wang (2002) showed that bluegill reared in groups exhibited a reduction in absolute growth rate (AGR) by 32%, mean daily consumption by 23 %, and gross growth efficiency by about 5% relative to those reared individually. Furthermore, Hayward & Wang (2006) achieved a growth rate of 0.58 g d^{-1} for males and 0.24 g d^{-1} for females reared indoors in individual chambers for 200 days (30-35 g initial weight) in these same re-circulating aquaculture tanks at similar temperatures and photoperiods. Similar growth (AGR) calculations for the bluegill in the NTO group of the present study showed a rate of only 0.12 g d^{-1} increments. Thus, for the current experiment, it appears that group-holding and its consequences (social hierarchy development) may be a primary underlying cause for the lower-than-anticipated growth that was observed.

No differences were observed between the graded and ungraded pond groups in the W_r versus length relationships suggesting that the presence of 20 % more males in the former did not affect social hierarchy development. Hence, reducing the initial size variation of bluegills by excluding smaller fish did not delay or impede social hierarchy formation. Results from the pond trials, therefore, clearly refute the study's original

presumptions that social hierarchies do not develop in ponds because of differences (e.g., large water volume, presence of natural feed and underwater cover) in the rearing environment relative to tanks, and that the advantage of greater growth capacity of male bluegills can be better exploited in ponds.

Aquaculture implications

The present study showed that "topping off" can be an effective method for "disrupting" social hierarchies and increasing fish production. However, in indoor systems, although hierarchies became fully established as early as day 31, "topping off" was not initiated until day 376. This timing may have allowed subordinate bluegill to sustain the chronic stress imposed by dominance hierarchies for prolonged periods, and this may have impeded their ability to respond with compensatory growth when periodically "freed" from the social dominance. Given indications that social hierarchies were established well in advance, initiating cull harvesting much earlier may allow the subordinates to respond with better growth rates. While this may seem advantageous, frequent cull harvesting may not be profitable either, given that additional costs of harvesting may be more than the profit gained from such removals. The present study showed that bluegill growth declined drastically after day 161; therefore, the first "topping off" should likely be initiated by this time to minimize the social hierarchy effects and to increase growth and production. On the other hand, measures that would delay or prevent the development of social hierarchy would be more beneficial. This might involve (alone or in combination) the use of physical structures (e.g., arrays of

anchored or suspended artificial foliage or PVC tubing) to increase visual isolation (Arndt *et al.* 2002), or perhaps duoculture (rearing bluegill with another fish species of value) (Nortvedt & Holm 1991; Jobling *et al.* 1998; Karakatsouli *et al.* 2006).

For bluegills reared in ponds, the present study has demonstrated a social hierarchy development by day 181. Size grading, although resulting in 20 % additional males, did not lead to marked differences in fish growth rate or production. The study results suggest that social hierarchy development may have confounded any benefits from size grading. Therefore, the measures suggested above (physical structures, duoculture) may be beneficial for minimizing the effects of social hierarchy in ponds, and for increasing the benefits of size grading. Fish reared in ponds containing appropriate physical structure may also benefit from being moved to indoor rearing systems during winter to reduce their winter growth decline (Hayward & Wang 2006). The use of diets that are specifically developed for different bluegill life stages may lead to less body fat deposition, thus allowing bluegill to avoid appetite suppression. Over the past two years, development of a nutritionally complete diet for juvenile bluegill has been an area of substantial focus, with such a diet for juvenile bluegill having been fully formulated recently (Masagounder *et al.* 2009; Masagounder *et al.* accepted). The use of selective breeding to promote more rapid growth in bluegill may be also be highly beneficial for producing food-size bluegill in less than two growing seasons: Hicks *et al.* (2009) showed that pond rearing coupled with indoor winter rearing of bluegill that had been selected for rapid growth may be effective in rearing food-size bluegill within very reasonable grow-out periods.

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Table 1. Progressive changes in the mean weights of bluegills reared in indoor re-circulating aquaculture system tanks.

(i) No topping off (NTO) and topping off (TO) bluegill groups reared for 574 days.

Month	Day Number	Mean weight (g)		Percent change in mean weight over sampling interval*	
		NTO	TO	NTO	TO
Oct 2006	0	11.60	12.90		
Nov 2006	31	19.47	22.38	67.84 (2.19% d^{-1})	73.49 (2.37% d^{-1})
Dec 2006	61	28.50	32.56	46.38 (1.55% d^{-1})	45.49 (1.52% d^{-1})
Jan 2007	92	36.46	41.46	27.93 (0.90% d^{-1})	27.33 (0.88% d^{-1})
Mar 2007	161	57.78	64.23	58.48 (0.85% d^{-1})	54.92 (0.80% d^{-1})
May 2007	223	62.53	70.90	8.22 (0.13% d^{-1})	10.38 (0.17% d^{-1})
July 2007	285	68.27	80.46	9.18 (0.15% d^{-1})	13.48 (0.22% d^{-1})
Oct 2007	376	79.32	88.98	16.19 (0.18% d^{-1})	10.59 (0.12% d^{-1})
Oct 2007 (after first removal)	376		71.48		
Dec 2007	436	81.62	77.49	2.90 (0.05% d^{-1})	8.41 (0.14% d^{-1})
Dec 2007 (after second removal)	436		66.51		
Mar 2008	510	82.32	76.74	0.86 (0.01% d^{-1})	15.38 (0.21% d^{-1})
Mar 2008 (after third removal)	510		65.85		
May 2008	574	82.40	72.57	0.10 (0.002% d^{-1})	10.21 (0.16% d^{-1})
May 2008 (Actual mean weight)	574	82.40	96.46	3.88	8.41

* percentage change in weight between successive sampling days was calculated as: $(W_{t+1} - W_t)\times 100 / W_t$, where W_t = weight recorded at time 't' and W_{t+1} = weight recorded at time 't+1'.

(ii) Harvested bluegills and newly added juvenile bluegills in the topping off (TO) group

Month	Mean weight (g)			
	Top 10 percentile removed during TO	Juveniles added during first TO	Juveniles added during second TO	Juveniles added during third TO
Oct 2007	183.24 (n=24)	14.58		
Dec 2007	135.63 (n=23)	21.81	13.13	
Mar 2008	123.75 (n=15)	30.55	18.85	17.17
May 2008		36.31	22.92	28.11

Table 2. Progressive changes in *P*-values of W_r versus length regression and CV_w (%) of bluegills reared in indoor re-circulating aquaculture system tanks for 574 days.

Month	Day Number	P-value of W_r versus length regression		CV_w (%)	
		No Topping off	Topping off	No Topping off	Topping off
Oct 2006	0	0.22	0.66	26.20	29.90
Nov 2006	31	0.017	3.9×10^{-4}	40.93	39.41
Dec 2006	61	6.28×10^{-9}	1.3×10^{-5}	50.43	42.52
Jan 2007	92	4.76×10^{-11}	1.79×10^{-10}	51.55	50.33
Mar 2007	161	4.83×10^{-5}	3.44×10^{-9}	55.95	50.88
May 2007	223	6.0×10^{-4}	1.68×10^{-5}	53.37	56.39
July 2007	285	2.73×10^{-9}	1.06×10^{-18}	57.21	59.78
Oct 2007 (first removal)	376	3.17×10^{-5}	1.31×10^{-13}	55.15	55.46
Dec 2007 (second removal)	436	5.59×10^{-8}	1.62×10^{-5}	53.02	44.09
Mar 2008 (third removal)	510	8.16×10^{-6}	0.20	55.73	40.83
May 2008	574	7.21×10^{-11}	0.53	56.97	37.52

Table 3. Progressive changes in RGR and FE of bluegills reared in indoor re-circulating aquaculture system tanks.

(i) No topping off (NTO) and topping off (TO) bluegill groups reared for 574 days.

Time Interval	Day Span	RGR		FE	
		NTO	TO	NTO	TO
Oct-Nov, 2006	0-31	1.634	1.734	0.88	1.01
Nov-Dec, 2006	31-61	1.255	1.235	1.12	1.09
Dec, 2006-Jan, 2007	61-92	0.791	0.776	0.83	0.70
Jan-Mar, 2007	92-161	0.656	0.624	0.66	0.60
Mar-May, 2007	161-223	0.127	0.159	0.18	0.25
May-July, 2007	223-285	0.142	0.204	0.22	0.33
July-Oct, 2007	285-376	0.165	0.111	0.31	0.23
Oct-Dec, 2007	376-436	0.048	0.134	0.09	0.24
Dec, 2007-Mar , 2008	436-510	0.012	0.193	0.02	0.34
Mar-May, 2008	510-574	0.002	0.152	0.002	0.23
Oct, 2007-May, 2008	376-574	0.019	0.041		

(ii) Newly added juvenile bluegills in the topping off (TO) group.

Month	RGR		
	Juveniles added during first TO	Juveniles added during second TO	Juveniles added during third TO
Oct-Dec, 2007	0.66		
Dec, 2007-Mar, 2008	0.45	0.48	
Mar-May, 2008	0.27	0.30	0.76
Overall period	0.43	0.39	0.76

Table 4. Progressive changes in mean weight and RGR (mean ± S.D.) of individually-marked bluegill concerning no topping off (NTO) and topping off (TO) groups reared in indoor re-circulating aquaculture system tanks for 574 days.

Month	Day	Sample size (n)		Weight		*P*-value (*t*-test)	Time Interval	Percent change in weight over sampling interval	
		NTO	TO	NTO	TO			NTO	TO
Oct, 2006	0	19	17	12.48±2.79	10.69±2.31	0.06	Oct, 2006-Oct, 2007	405.77	382.60
Oct, 2007	376	19	17	63.12±37.12	51.59±19.39	0.27	Oct-Dec, 2007	8.03	7.04
Dec, 2007	436	19	17	68.19±41.04	55.00±22.09	0.26	Dec, 2007-Mar, 2008	2.98	10.99
Mar, 2008	510	19	17	70.22±42.00	61.59±23.28	0.49	Mar-May, 2008	4.16	5.51
May, 2008	574	19	17	73.14±44.55	65.70±25.58	0.56	Oct, 2007-May, 2008	15.87	25.35

Time Interval	Day Span	RGR		*P*-value (*t*-test)
		NTO	TO	
Oct, 2006-Oct, 2007	0-376	0.33±0.08	0.34±0.04	0.53
Oct-Dec, 2007	376-436	0.12±0.10	0.08±0.15	0.44
Dec, 2007-Mar, 2008	436-510	0.04±0.09	0.16±0.16	<0.01
Mar-May, 2008	510-574	0.06±0.18	0.07±0.14	0.65
Oct, 2007- May, 2008	376-574	0.07±0.06	0.12±0.06	0.03

Table 5. Fat content* (mean ± S.D.) of bluegills harvested during each topping off and on the final experimental day (day 574).

First topping off	Second topping off	Third topping off	Topping off (final day)	No topping off (final day)	*P*-value (ANOVA)
31.27±1.79[a]	29.36±2.65[a]	28.97±2.64[a]	22.15±3.63[b]	22.46±4.46[b]	<0.01

*values with different superscripts indicate significant differences

Table 6. Progressive changes in the mean weights of graded and ungraded bluegills reared in production ponds for 584 days.

Month	Day	Mean Weight (g) ± S.D.			Percent change in mean weight over sampling interval		Percent change in mean weight over growing seasons		
		Ungraded	Graded	*P*-value (*t* test)	Ungraded	Graded	Season	Ungraded	Graded
Apr, 2006	0	10.00±1.79	19.27±2.02	<0.01					
Jun, 2006	65	21.77±7.65	32.87±3.88	0.09	117.71 (1.81% d^{-1})	70.55 (1.09% d^{-1})	Apr, 2006-Oct, 2006	476.34 (2.63%/d)	303.20 (1.68%/d)
Aug, 2006	129	38.33±5.56	55.07±8.34	0.04	76.08 (1.19% d^{-1})	67.57 (1.06% d^{-1})			
Oct, 2006	181	57.63±3.93	77.70±8.19	0.02	50.35 (0.97% d^{-1})	41.08 (0.79% d^{-1})			
Apr, 2007	352*	59.05±6.29	73.57±7.19		2.46 (0.01% d^{-1})	-5.32 (-0.03% d^{-1})			
May, 2007	402	58.50±7.98	74.03±7.87	0.07	-0.93 (-0.02% d^{-1})	0.63 (0.01% d^{-1})			
July, 2007	442	63.43±9.56	83.53±2.58	0.02	8.43 (0.21% d^{-1})	12.83 (0.32% d^{-1})	Apr, 2007-Nov, 2007	40.41 (0.17%/d)	40.44 (0.17%/d)
Oct, 2007	527	77.15±4.14	93.08±7.82	0.04	21.62 (0.25% d^{-1})	11.43 (0.13% d^{-1})			
Nov, 2007	584	82.14±4.24	103.97±14.59	0.07	6.47 (0.11% d^{-1})	11.70 (0.21% d^{-1})			

* n = 2 for ungraded ponds (one pond was not sampled due to weed infestation)

Table 7. Progressive changes in *P*-values of W_r versus length regression and CV_w (%) of graded and ungraded bluegills reared in production ponds for 584 days.

Month	Day	P-value of W_r versus length regression ± S.D. (positive slope from day 181)		CV_w (%) ±S.D.		
		Ungraded	Graded	Ungraded	Graded	*P*-value (*t* test)
Apr, 2006	0	0.85 ± 0.08	0.86 ± 0.09	58.57±10.20	33.37±3.15	0.02
Jun, 2006	65	0.84 ± 0.09	0.59 ± 0.06	54.51±19.44	26.24±6.60	0.08
Aug, 2006	129	0.39 ± 0.44	0.59 ± 0.40	48.93±6.50	27.50±2.59	<0.01
Oct, 2006	181	$1.76\times10^{-6} \pm 2.91\times10^{-6}$	$1.6\times10^{-3} \pm 2.8\times10^{-3}$	46.08±1.77	29.53±1.38	<0.01
Apr, 2007	352*	$7.16\times10^{-6} \pm 9.96\times10^{-6}$	0.05 ± 0.06	37.58±6.30	31.71±1.42	
May, 2007	402	$1.16\times10^{-5} \pm 1.16\times10^{-5}$	0.07 ± 0.09	43.97±4.47	28.62±2.37	<0.01
July, 2007	442	0.06 ± 0.06	0.12 ± 0.03	38.38±1.41	30.10±6.05	0.08
Oct, 2007	527	0.03 ± 0.04	0.04 ± 0.04	30.61±4.68	29.36±4.22	0.75
Nov, 2007	584	0.02 ± 0.02	0.02 ± 0.01	33.05±1.40	29.91±2.23	0.11

* n = 2 for ungraded ponds (one pond was not sampled due to weed infestation)

Table 8. Progressive changes in relative growth rate (RGR) and feed efficiency (FE) of graded and ungraded bluegills reared in production ponds for 584 days.

Month Time Interval	Day	Mean RGR ± S.D.			Mean FE ± S.D.		
		Ungraded	Graded	*P*-value (*t* test)	Ungraded	Graded	*P*-value (*t* test)
Apr-Jun, 2006	0-65	0.89±0.27	0.68±0.21	0.19	0.59±0.27	0.65±0.24	0.81
Jun-Aug, 2006	65-129	0.84±0.23	0.78±0.22	0.75	0.33±0.05	0.47±0.15	0.24
Aug-Oct, 2006	129-181	0.78±0.31	0.66±0.09	0.57	0.35±0.13	0.41±0.01	0.47
Oct, 2006-Apr, 2007*	181-352	0.03±0.01	-0.06±0.05		0.25±0.14	-0.40±0.10	
Apr-May, 2007*	352-402	0.13±0.31	0.10±0.23		0.19±0.44	0.02±0.19	
May-Jul, 2007	402-442	0.19±0.24	0.31±0.20	0.54	0.25±0.33	0.48±0.29	0.42
Jul-Oct, 2007	442-527	0.17±0.03	0.12±0.06	0.31	0.16±0.02	0.15±0.08	0.80
Oct-Nov, 2007	527-584	0.13±0.16	0.21±0.11	0.41	0.12±0.15	0.25±0.16	0.32
Apr, 2006-Nov, 2007	0-584	0.26±0.02	0.23±0.01	0.11	0.25±0.02	0.30±0.05	0.26

*n = 2 for ungraded ponds (one pond was not sampled due to weed infestation)

Figure 1. Progressive changes in weight, coefficient of weight variation (CV_w %) and relative weight (W_r %) of bluegill reared in indoor recirculating aquaculture system (RAS) tanks.

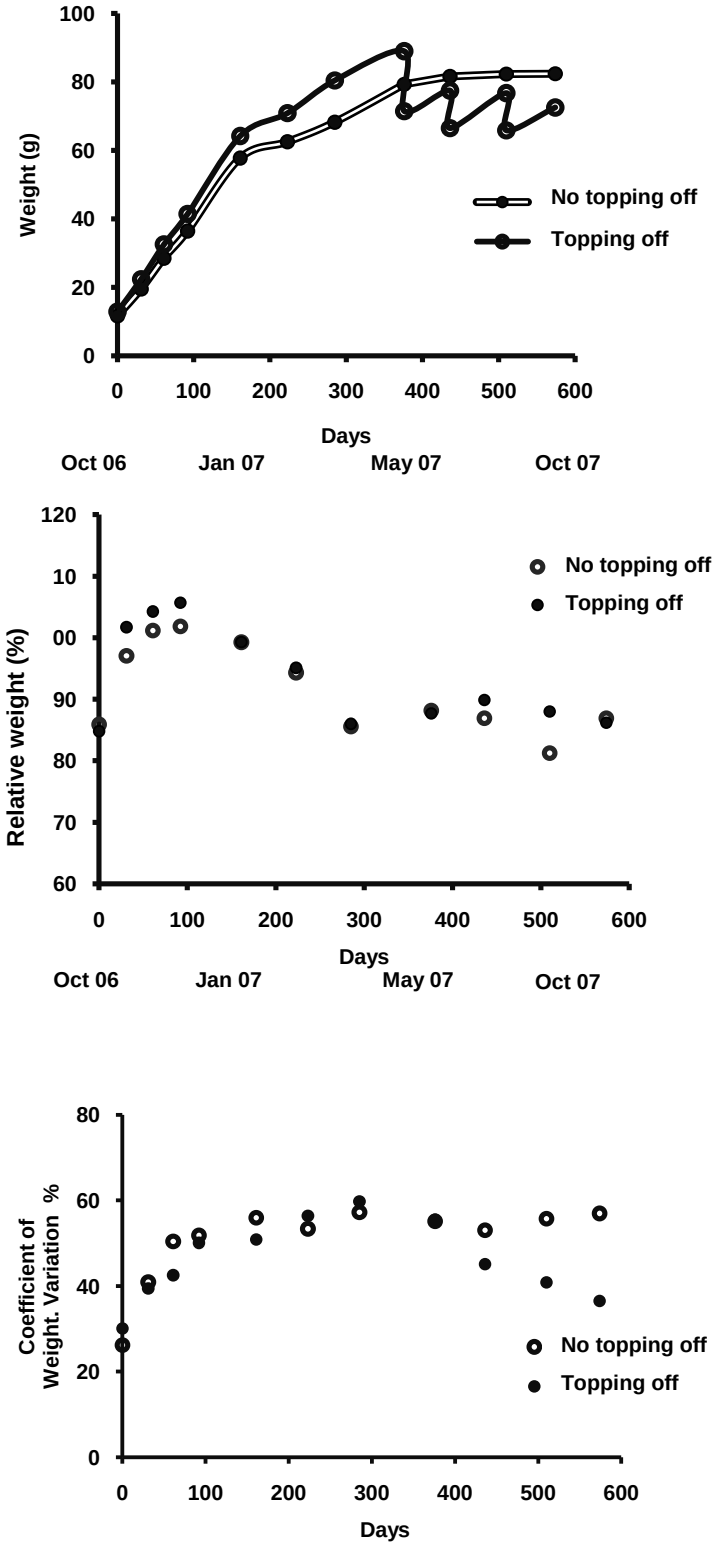
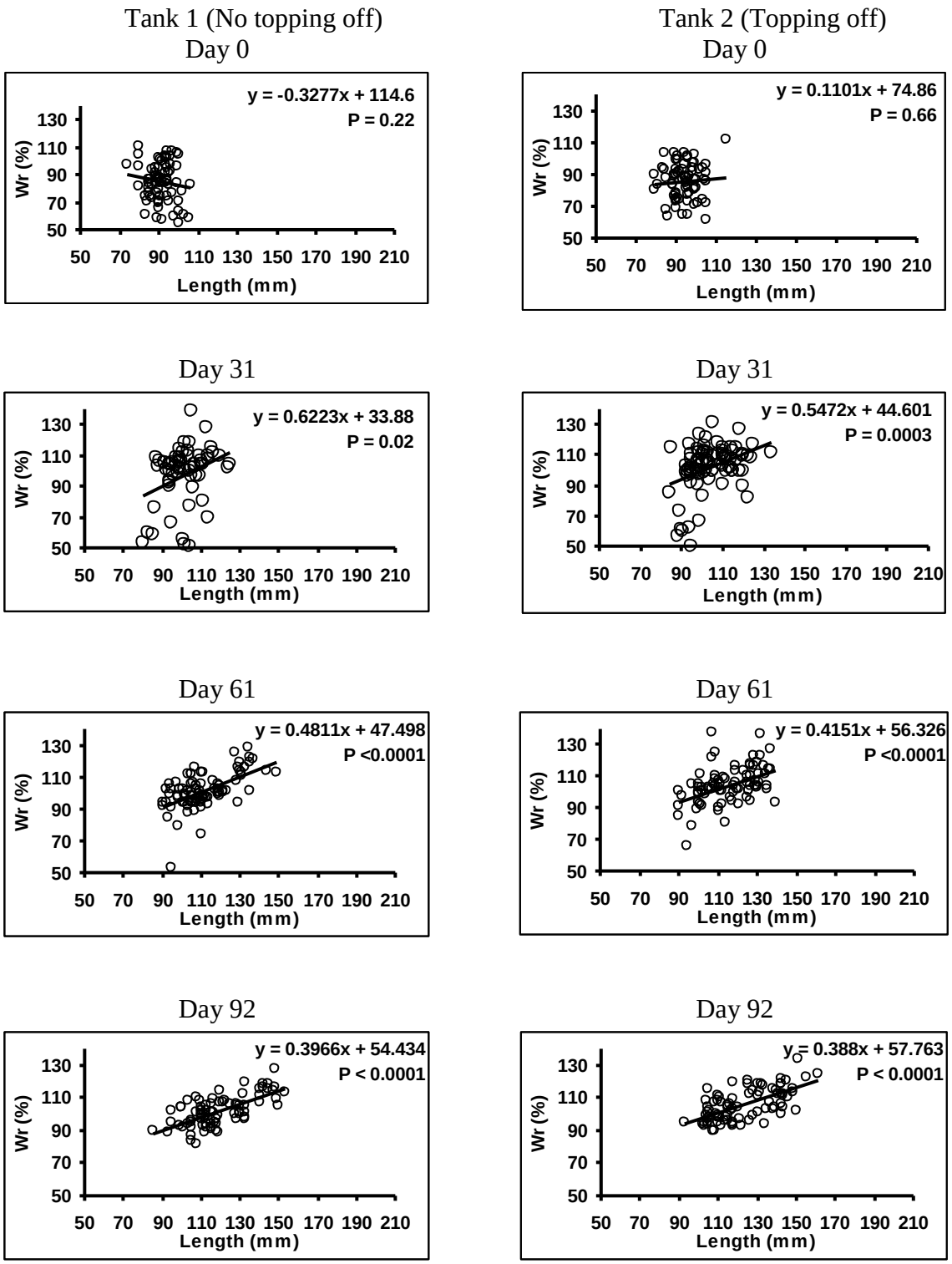
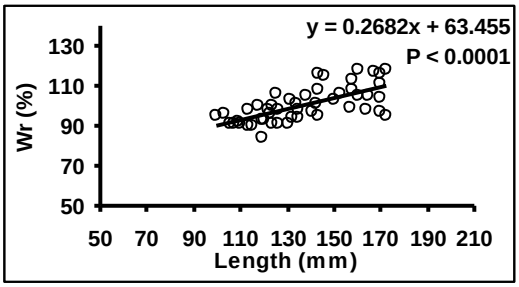


Figure 2. Progressive changes in the relationship of fish length and W_r for bluegills reared in indoor RAS tanks.



Day 161



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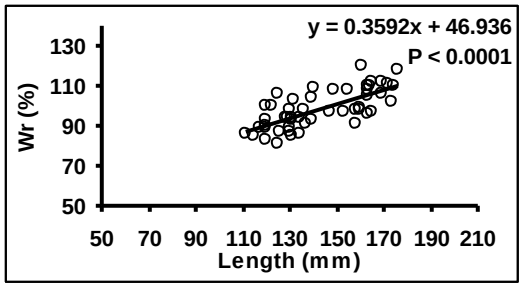
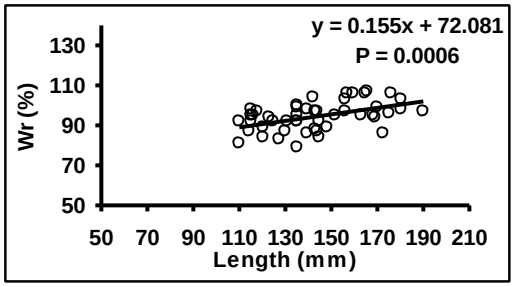


Day 223



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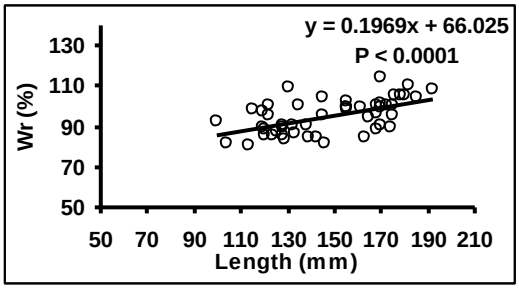
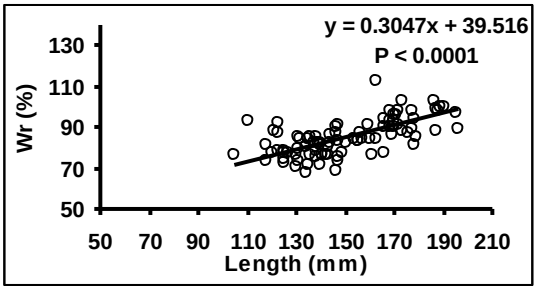


Day 285



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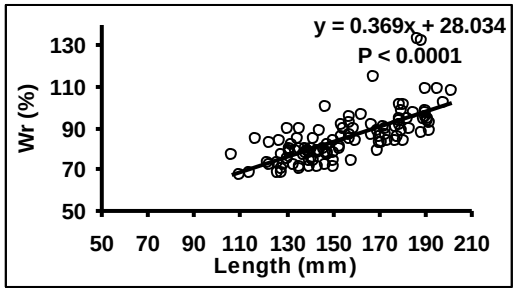
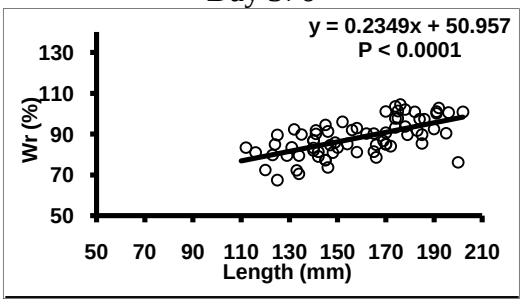
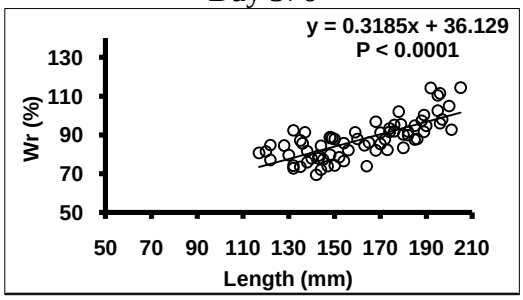


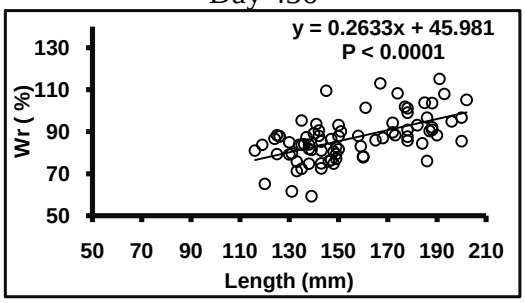
Day 376



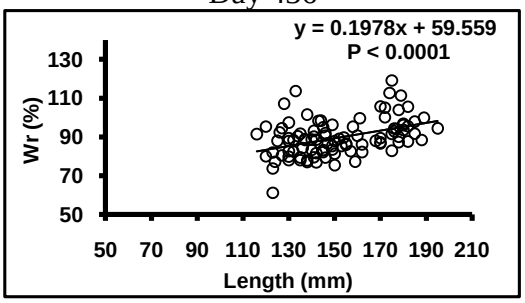
Day 376



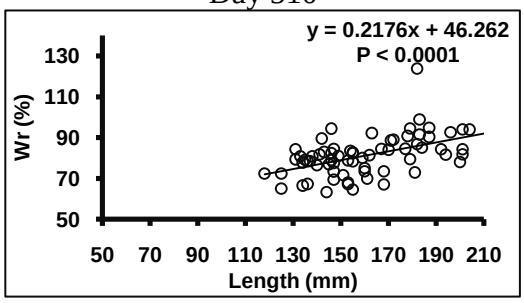
Day 436



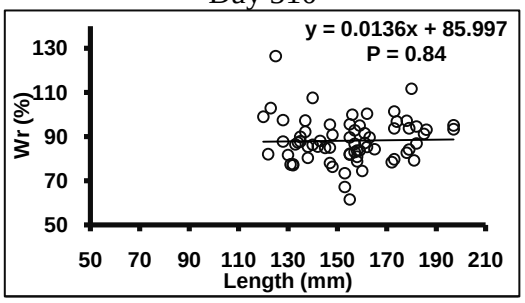
Day 436



Day 510



Day 510



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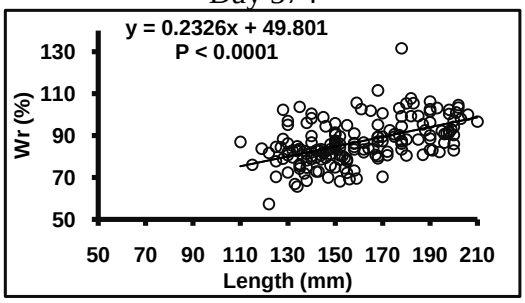


Day 574

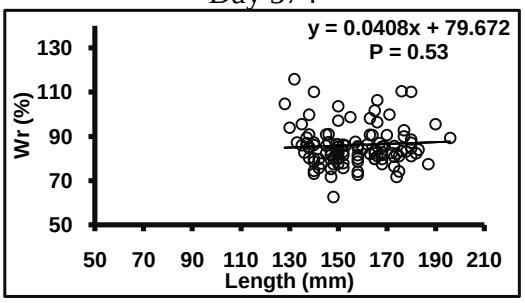


Figure 3. Broken-line regression model fitted to *P*-value of W_r versus length regression, RGR and FE of bluegills reared for 358 days in indoor RAS tanks.

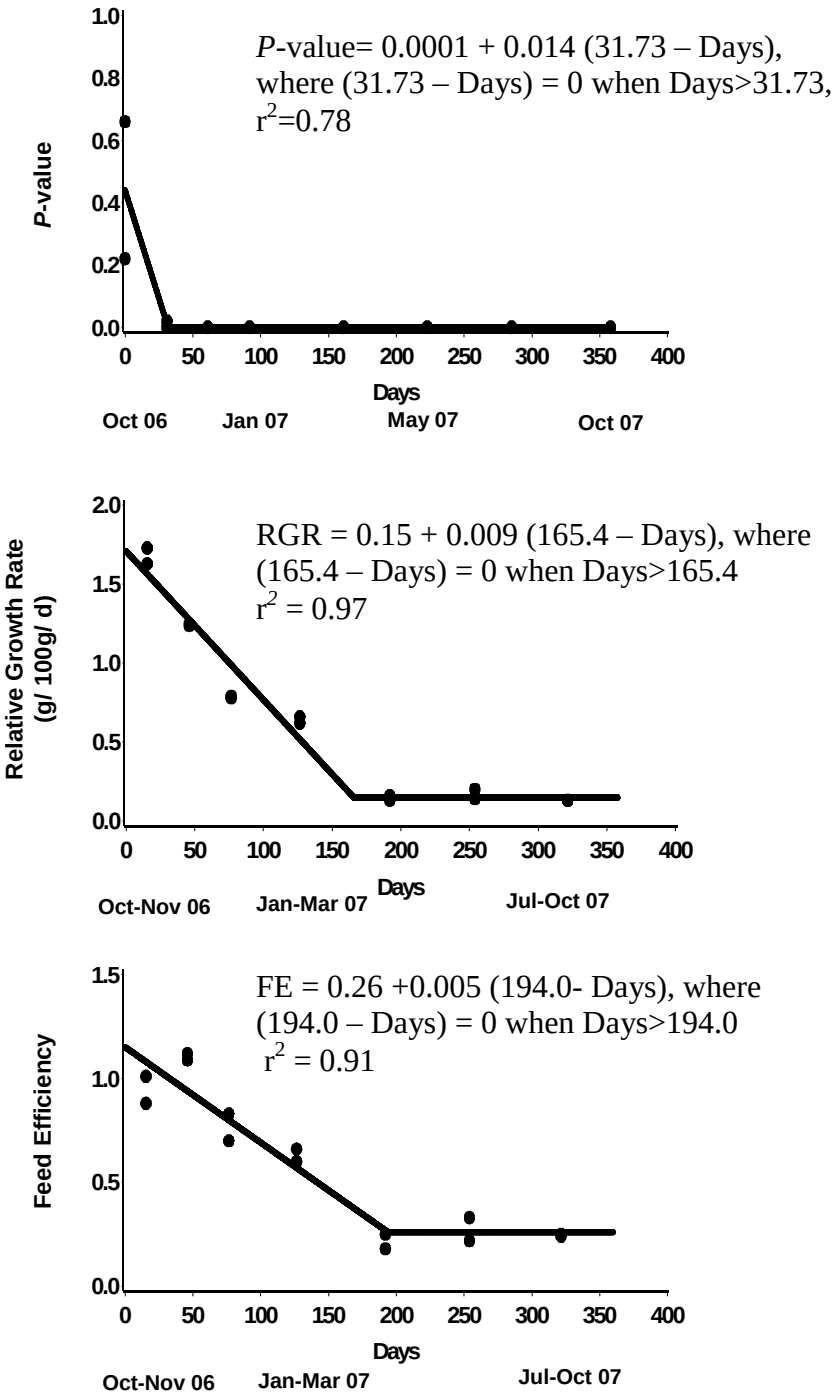


Figure 4. Progressive changes in weight, coefficient of weight variation (CV_w %) and relative weight (W_r %) of bluegill reared in ponds.

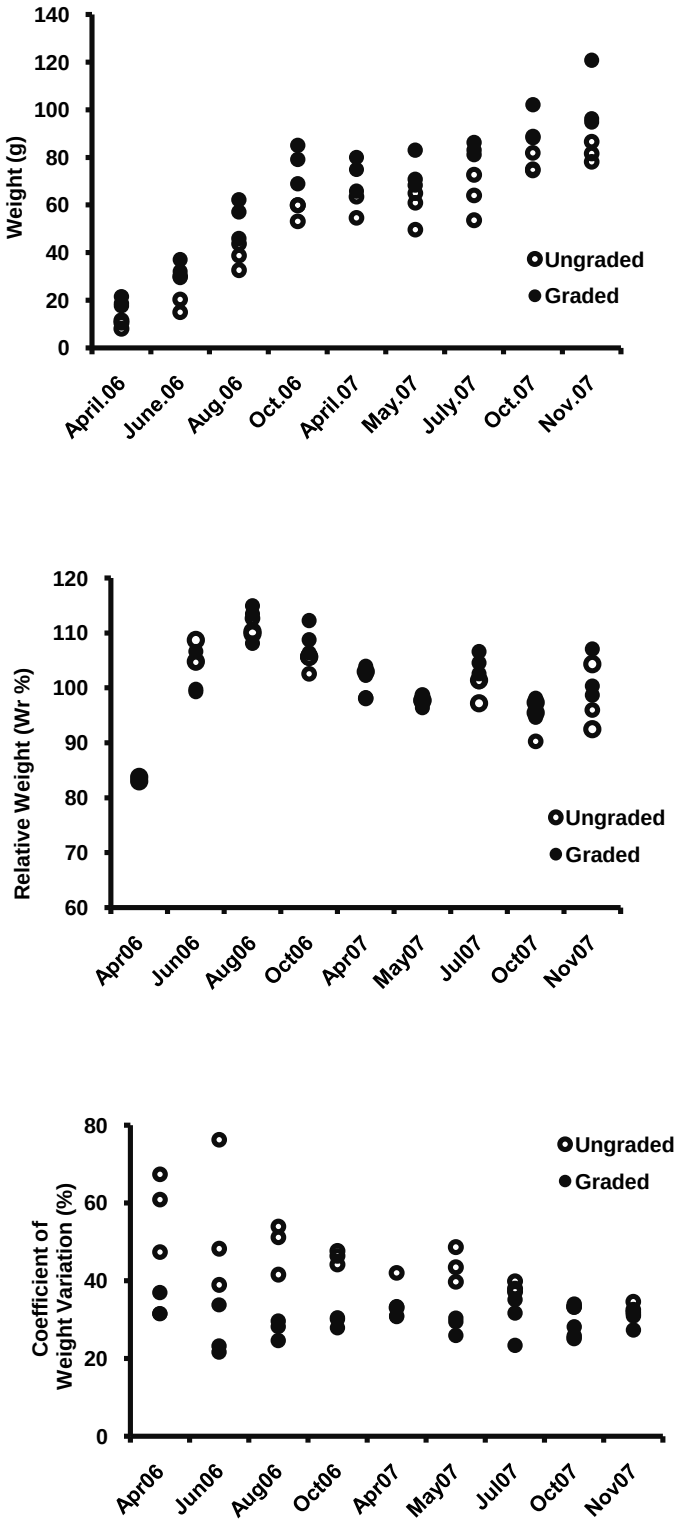
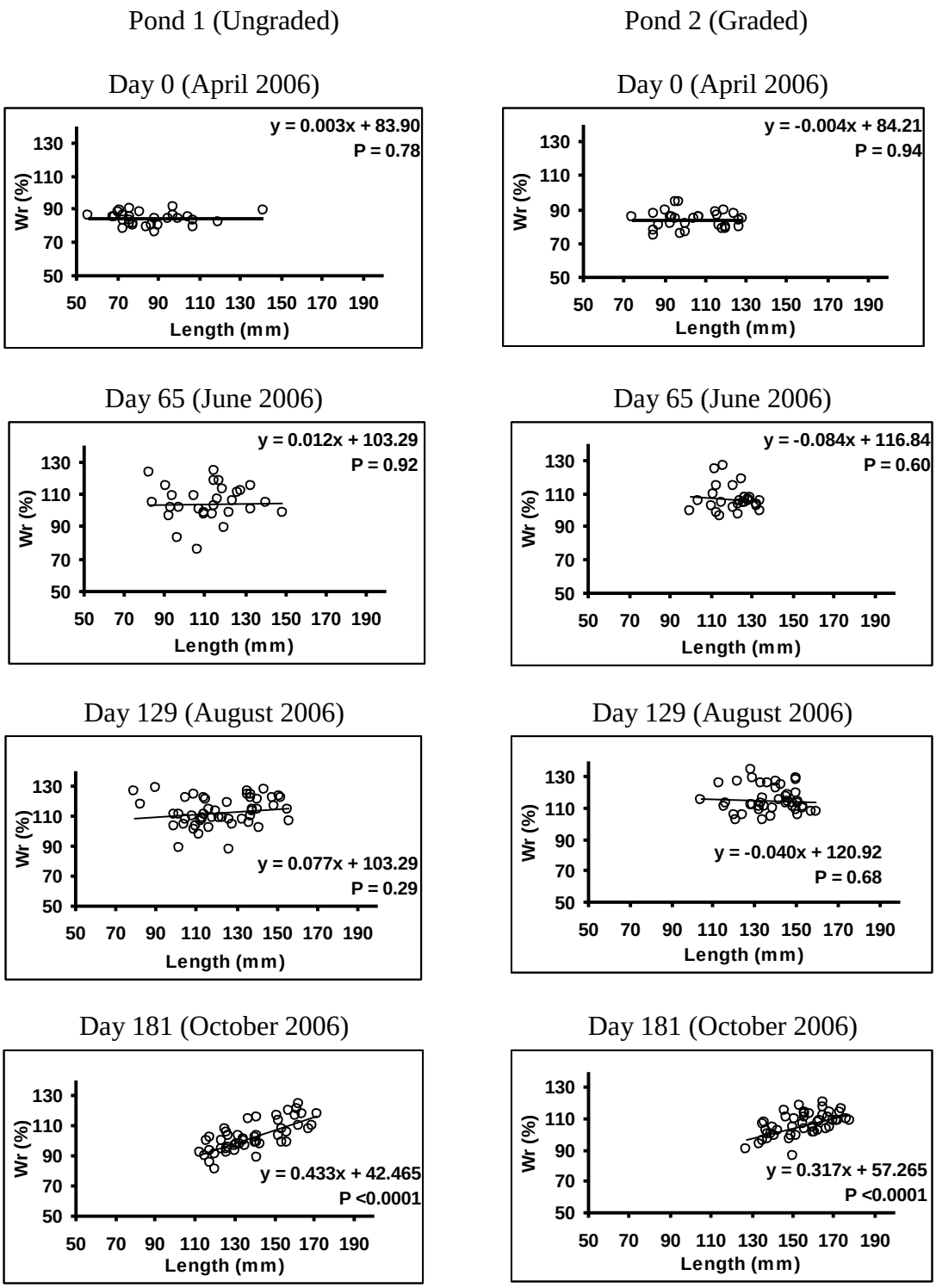
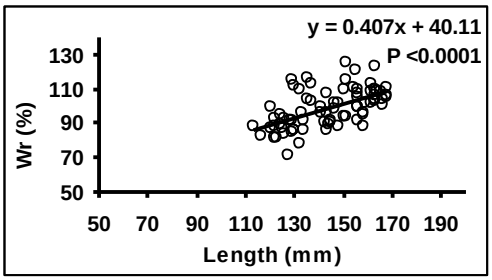


Figure 5. Progressive changes in the relationship of fish length and W_r for bluegills reared in production ponds.



Day 352 (April 2007)



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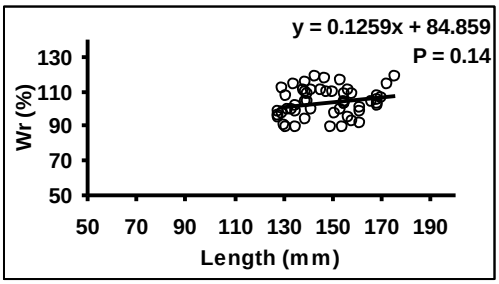
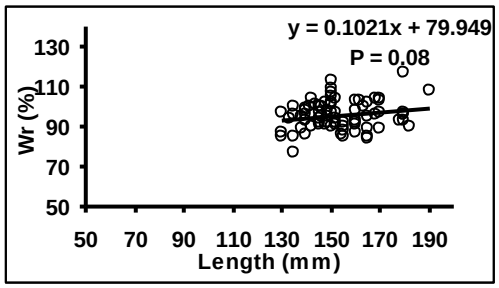


Day 584 (Nov 2007)



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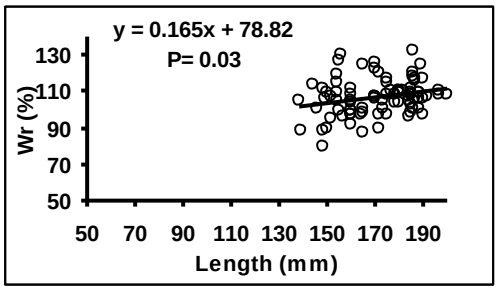

Pond 3 (Graded)

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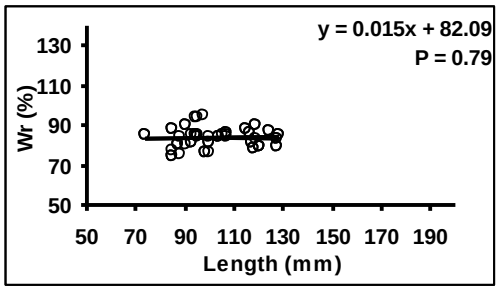
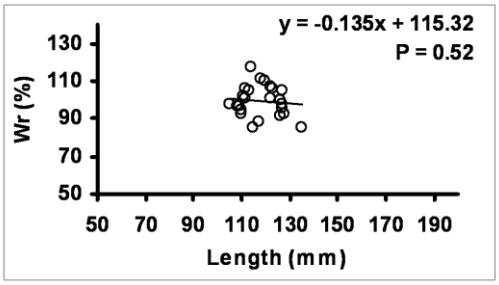
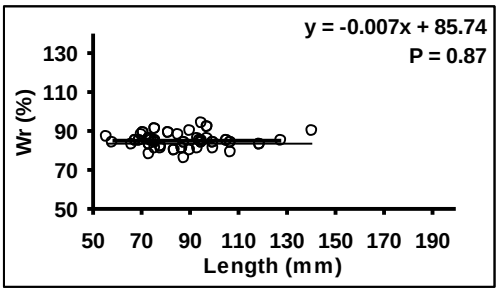


Day 65 (June 2006)



Pond 4 (Ungraded)

Day 0 (April 2006)



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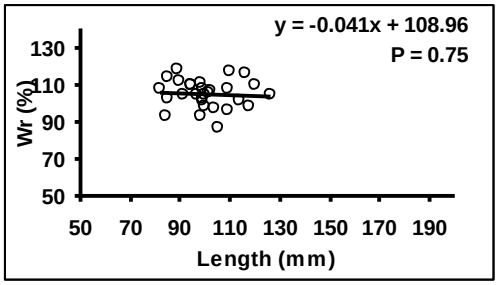
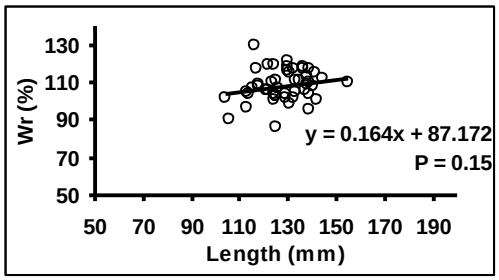


Day 129 (August 2006)



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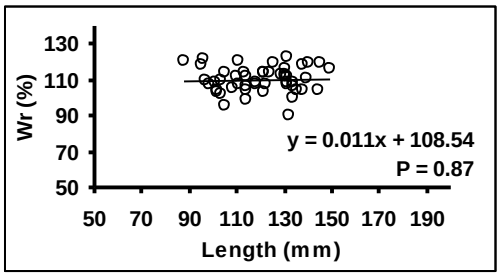

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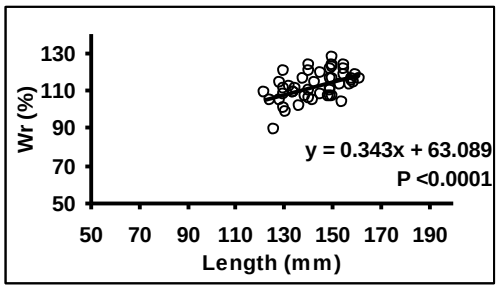

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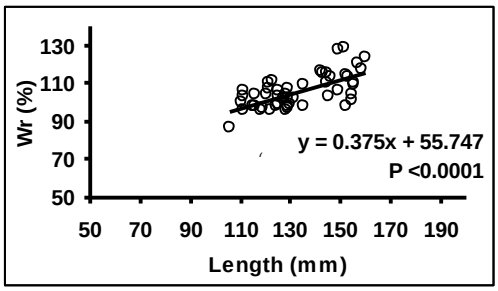

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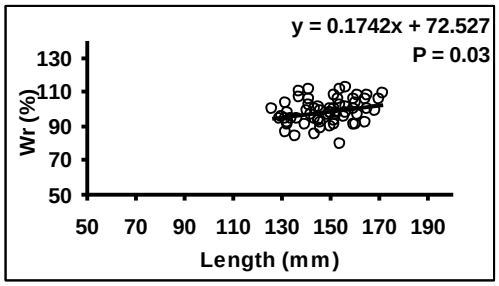

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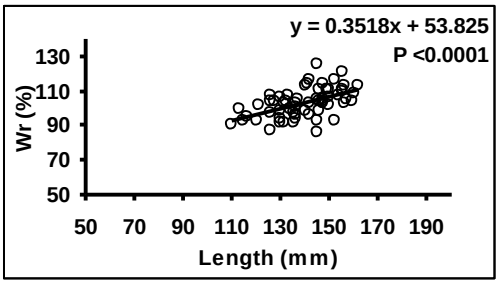
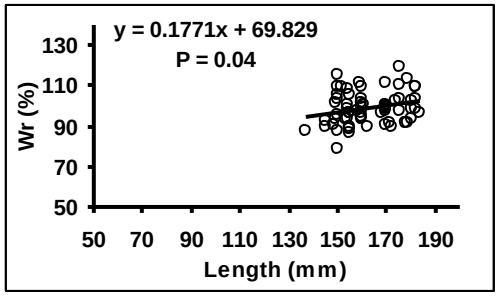


Day 584 (Nov 2007)



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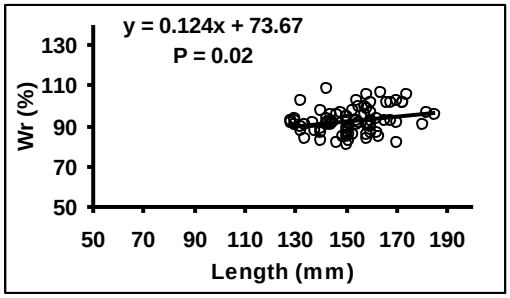


Figure 6. Pattern in changes of RGR and FE of ungraded and graded bluegills reared in production ponds for 584 d.

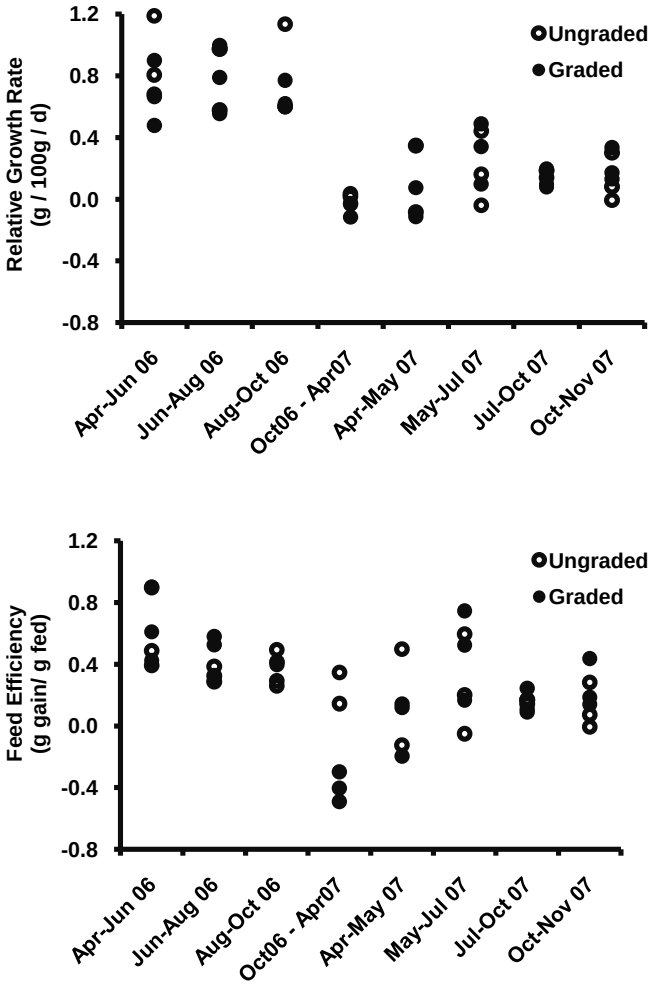
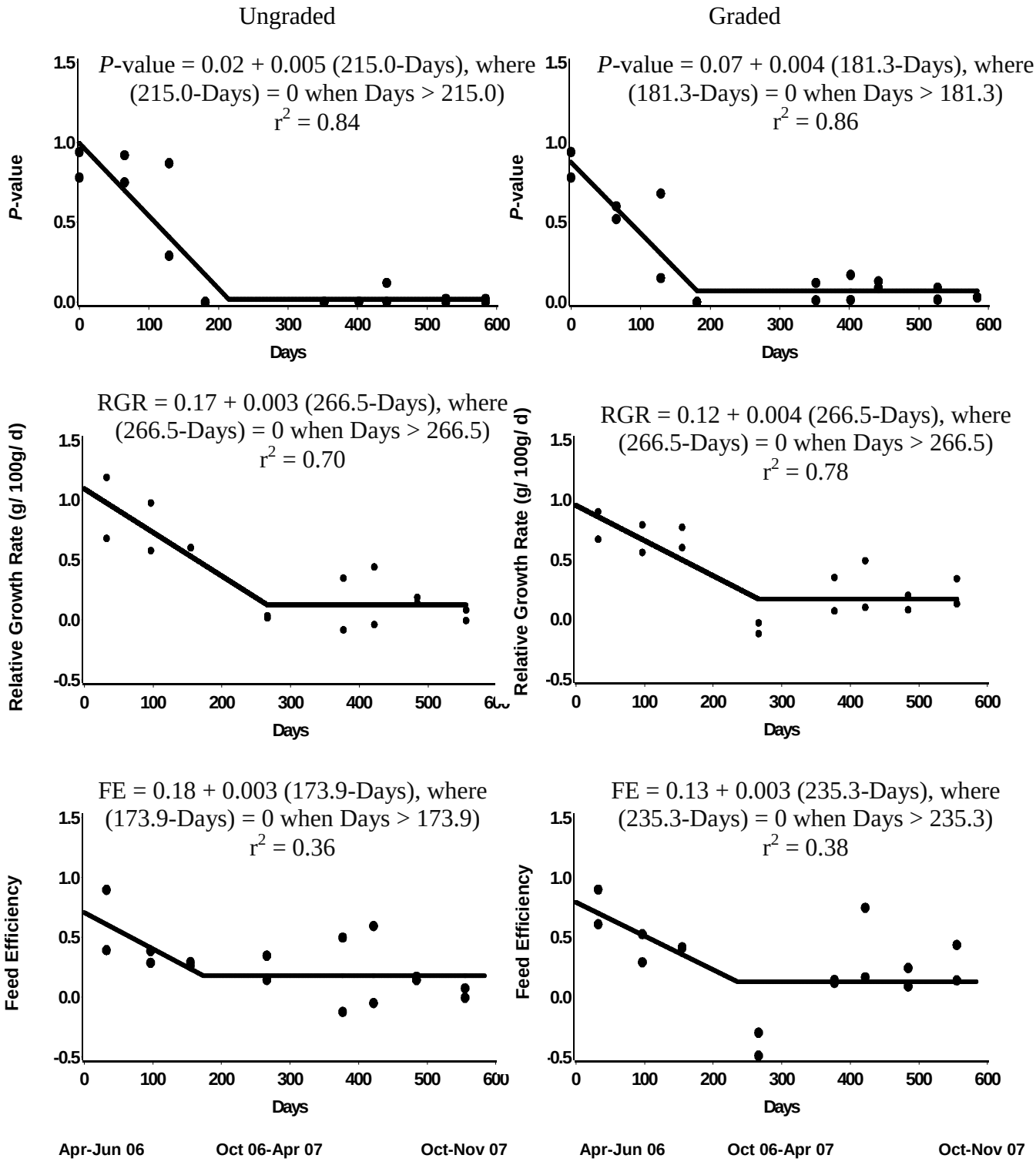


Figure 7. Broken-line regression model fitted to *P*-values of W_r versus length regression, RGR and FE of ungraded and graded bluegills reared in production ponds for 584 d.



SUMMARY AND CONCLUSION

Over the past decade, bluegill *Lepomis macrochirus* have received much attention in the aquaculture sector as a food fish (225–340 g). Considerable progress had been made in bluegill culture technology over the last 15 years. Yet, the lack of a nutritionally balanced, affordable diet for bluegill remains a major concern for running a profitable business with this species. The major part of my dissertation study focused on developing a specific diet for juvenile-stage bluegill. The study followed a systematic four-step process to develop a nutritionally balanced, least-cost diet for juvenile bluegill, the steps being (i) determining the digestibility of commonly available feedstuffs that could form a balanced diet and reduce feed cost, (ii) determining bluegill digestible nutrient requirements, including essential amino acids (EAAs), protein, and energy, (iii) formulating various experimental diets ranging from those with much fish meal (most expensive) to those containing no fish meal (least expensive), the constraint on each experimental diet being that the optimal nutrient levels be met, and (iv) evaluating the formulated diets versus the commercial trout and catfish diets for bluegill growth performance and identifying the best, most economically-feasible experimental diet.

Development of least-cost, complete diet

Digestibility

The study determined the apparent digestibility of energy, and amino acids from protein sources including blood meal (BM), fish meal (FM), meat and bone meal (MBM), poultry

byproduct meal (PBM), soybean meal (SBM), and corn gluten meal (CGM), and carbohydrate sources including corn and wheat for bluegill. Apparent digestibility of most amino acids exceeded 90 % for the evaluated protein sources, except for MBM which showed slightly lower values (80-90 %). Available amino acid profile of PBM closely matched that of fish meal, a feed ingredient that is well known for its balanced EAA profile and highly digestible protein. For most other sources, some EAAs were lower relative to fish meal, e.g., isoleucine for BM; lysine, methionine, and tryptophan for MBM; methionine and lysine for SBM; arginine and lysine for CGM. As expected, EAA profiles for carbohydrate-rich corn and wheat were poor. Relative to fish meal, digestible levels of protein (total content of amino acids) and energy were higher for BM, PBM, and CGM, and slightly lower for SBM and MBM. Corn and wheat showed much lower levels of digestible protein and energy. Although the present study showed that most feedstuffs lacked in a few nutrients relative to fish meal, the importance of such differences can be better judged only when the nutrient requirement values for bluegill are known. While the study's major interest was to determine the digestibility of common feedstuffs, it also, in part, validated the method adopted. Studies in the past determined the digestibility of nutrients by using compound diets, whereas the present study demonstrated that digestibility can also be determined using single test feedstuff, with the advantage of adopting the latter method being no interactions of nutrients across feedstuffs that can possibility produce erroneous results in digestibility. For evaluating the method, the study determined the digestibility of nutrients for largemouth bass *Micropterus salmoides* from selective feedstuffs for which digestibility values were reported adopting compound test diets. However, the digestibility values obtained from the present study did not differ greatly those reported for largemouth bass. The study suggested that

methods comparisons using parallel experiments may be more valuable for determining the advantage of using single-ingredient method.

Digestible nutrient requirements

After determining digestibility of nutrients from common feedstuffs, a series of four experiments was conducted to determine dietary requirements for digestible amino acids, protein, and energy. Initially, two 60-d experiments were conducted sequentially to determine (i) lysine requirement for juvenile bluegill based on the dose-response method, (ii) requirements for other essential amino acids (EAAs) using whole-body amino acid profiles, and (iii) whether differences in growth rates of group- versus individually-housed bluegills lead to different lysine requirement levels due to the presence and absence, respectively, of social hierarchies. The study demonstrated that group-reared bluegills did develop social hierarchies, and that individually-housed bluegills consumed more feed and grew larger than their group-housed counterparts. Interestingly, despite the growth differences between group- versus individually- housed bluegills, their dietary requirement for lysine did not differ substantially. The study, based on broken-line regression analyses of relative growth rate (RGR) and feed conversion ratio (FCR), indicated that bluegill require 15 g of digestible lysine per kilogram of diet for adequate growth. For all other EAAs, the dietary requirement level was determined using bluegill's whole-body EAA composition and the values ranged from 2.4 g Kg^{-1} (tryptophan) to 15.3 g Kg^{-1} (leucine). All the subsequent experiments were carried out with group-held bluegills because fish are typically group-reared in commercial production systems. Hence the nutrient requirements of fish determined under such growing conditions may better reflect their true requirements.

In the next two experiments, the optimal level of dietary protein (digestible basis) was determined to be 412 g Kg^{-1} based on broken-line fit of RGR, and the optimal dietary energy level (digestible basis) was estimated to be 14.62 MJ Kg^{-1} based on quadratic fit of RGR, whereas the estimated protein/energy ratio was found to be 28.1 g MJ^{-1} for these requisite levels of protein and energy. The study results showed that very high levels of dietary energy (17.57 MJ Kg^{-1}) did not reduce fish appetite or growth rate, but did result in elevated levels of fat deposition in bluegill. The study provided evidence that lipid is not an appropriate protein sparing source for juvenile bluegill and thereby emphasized that an appropriate dietary lipid to carbohydrate ratio needs to be determined for juvenile bluegill in order to reduce the expensive dietary protein level.

Least-cost diet formulation

The digestible nutrient levels from various feedstuffs and optimal dietary requirement levels of EAAs, protein, and energy determined in the previous experiments for juvenile bluegill were used to formulate a fish meal-based diet (550 g Kg^{-1} fish meal). Fatty acid, vitamin, and mineral levels were maintained at levels that are known to be the requirements for general freshwater fishes (NRC 1993). To reduce the feed cost of fish meal based diet, a series of experimental diets were formulated by gradually replacing fish meal with alternative protein sources using least-cost computer formulation software. All the formulated diets were extruded and evaluated for bluegill growth performance with a 60-d feeding trial. The study results showed no differences in fish growth performance across the experimental diets, while the feed

cost of the no fish meal diet ($ 616.6 tonne^{-1}), containing predominantly MBM and SBM was reduced by ~35 % relative to a fish meal based diet (550 g Kg^{-1} fish meal diet) ($ 899.7 tonne^{-1}).

Harvesting fish for feeding fish versus humans -- are we dumb?

Fish meal has long been the protein source of choice, for reasons including its high nutrient digestibility, well balanced amino and fatty acids profiles, protein, palatability, and absence of antinutritional factors. This fish meal is generally processed from shoaling marine fishes (e.g., anchovies, herring, mackerel and menhaden), but concerns are raised over the status of these fishes, with most of them are either fully exploited (e.g., Atlantic menhaden *Brevoortia tyrannus*, gulf menhaden *Brevoortia patronus*, Atlantic mackerel *Scomber scombrus*, Atlantic herring *Clupea harengus*) or over exploited (e.g., blue whiting *Micromesistius poutassou*, South American pilchard *Sardinops sagax*) (FAO 2005; Watson *et al.* 2006). Furthermore, using fish in the form of fish meal for producing fish is considered an inefficient way of producing fish protein. In the year 2006, the aquaculture sector consumed 23.8 mt of small pelagic forage fish in the form of feed inputs such as fish meal and fish oil to produce 51.7 mt of aquatic animals (fish, crustacean and molluscs) (Tacon & Metian 2009). This indicates that 46.00% (23.8 mt of 51.7 mt) of total aquaculture production is arguably fish caught from the ocean. Also, the marine pelagic fishes are in fact the food of cash-poor people within developing countries. Competition for these fish from the non-food use sector makes the small pelagic fish catch simply unavailable for human consumption as it is processed into fish meal on board. Using fish in the form of fish meal for raising another fish is an inefficient way of contributing to global food security (Tacon & Metian 2009). Using the least-cost diet containing zero fish meal developed in the present

study will help minimize depletion of marine fish resources, as well as people living in protein-hungry regions.

Environmental impacts

The environmental impacts of unregulated aquaculture development have received much attention over the years (review by Subasinghe *et al.* 2009). Phosphorous is a limiting nutrient in fresh water, and excess phosphorous levels in aquaculture effluent can lead to eutrophication. When the typical phosphorous levels of various ingredients (NRC 1993) used in the present study were taken into consideration, the least-cost diet formulation is calculated to contain a total phosphorous level of 2.59 %, whereas a similar calculation for a fish meal based diet (550 g Kg^{-1}) shows a phosphorous level of 1.79 %. The phosphorous level in the least-cost diet results largely from MBM that typically contains higher levels of phosphorus than fish meal (~4.5 % phosphorous in MBM versus ~3.0 % phosphorous in fish meal) (NRC 1993). Therefore, while the diet developed for bluegill is a lot cheaper than a fish meal diet, it simultaneously increases dietary phosphorus level. The availability of dietary phosphorous from different feedstuffs and the dietary phosphorous requirements for bluegill are unknown; consequently, further studies may need to determine the serious consequences of phosphorous excretion from the least-cost diet formulation that this study developed. Similarly, the amount of available phosphorous from commercial diets may need to be estimated to determine the current status of phosphorous pollution from bluegill farming.

While the study's major focus centered on developing a least-cost complete diet for juvenile bluegill, the study also evaluated two novel rearing techniques ("topping off" and size grading) to determine their efficiency in increasing bluegill growth, and fish production.

Novel rearing strategies

Topping off

Social hierarchy development in bluegill was shown in the past (Hayward & Wang 2002; Doerhoff 2007) to be a major impediment in the production of bluegill. The present study evaluated the ability of a "topping off" strategy to disrupt bluegill social hierarchies and thereby increase fish growth and production. The strategy was evaluated for bluegill reared in 1000-L indoor recirculating aquaculture systems for 574 days. The significant positive relationship between relative weight (W_r %) and fish length was used as a key indicator of social hierarchy development. Significant relationships between bluegill fat content and fish weight, as well as the increase in coefficient of weight variation (CV_w) over time were used as additional indicators of social hierarchy development. Social hierarchies developed in indoor tank bluegills by day 31 and persisted until the "topping off" harvesting was performed by periodically removing the upper 10th percentile (by weight) of bluegill and immediately replacing them with an equal number of juvenile bluegill. The study also demonstrated that W_r is an indicator of energetic condition given the significant positive relationship between W_r and fish fat content. Progressive declines in growth, feed efficiency, and body condition were recorded over time and were suggested to be the consequences of social hierarchy development. The study results showed that "topping off" can be used to disrupt social hierarchies and increase bluegill growth and

production. The topping off (TO) group exhibited a significant increase in fish growth (116 % increase or 0.041 g $100g^{-1}$ d^{-1} for TO group and 0.019 g $100g^{-1}$ d^{-1} for no topping off (NTO) group) as well as in the number of large bluegill > 100 g, (8.5 % increase vs. 0.1 % increase) relative to the NTO group. Yet, the discouraging fact is that bluegill released from dominant force, despite a modest growth spurt, did not exhibit the substantial weight gains that would take them to the desired food size (227 g) or to the size of fish that were culled during first topping off. The TO group attained a final mean weight of ~96 g and the NTO group attained ~82 g. Bluegill removed during the first topping off, after ~12 month of rearing, attained 80.72 % of food-market weight, whereas fish removed at ~2 month intervals during the subsequent TOs attained only 59.75 % and 54.52 % of market size. Based on the observed results, modifications to the "topping off" strategy may further enhance bluegill growth and fish production.

Size grading

Previous studies (Hayward & Wang 2006; Doerhoff 2007) showed that size grading help produce market size bluegill and increase fish production, given that males grow substantially larger than females and that size grading for larger bluegill formed a mostly male fish stock.

In evaluating the size grading strategy for producing large bluegill and increasing bluegill production, juvenile bluegills (~21 g) of upper quartile by length (≥ 85 mm) were selected from a mixed-size group and stocked in production ponds at a density of ~16,667 bluegill ha^{-1}. The growth performance of size graded bluegill as compared against a mixed-size bluegill stock (~12 g) reared at the same density. Bluegills of both the groups (n=3 replicates per group) were reared in the ponds for two growing seasons, Apr 2005 to Nov 2006, covering 584 days. As anticipated,

size grading resulted in a skewed sex ratio with the majority individuals being males (70.20 % males) and no size grading produced an even sex ratio (48.42 % males). Those in the size graded group were consistently larger than in the ungraded group throughout the study, with their final mean weights being 82.14 g for the ungraded group and 103.97 g for the graded group. Furthermore, the percentage of large bluegill (> 100 g) on the day of harvest was higher for the graded group (48.16 %) than for the ungraded group (22.33 %). However, neither the growth rate (0.26 g $100g^{-1}$ d^{-1} for ungraded group and 0.23 g $100g^{-1}$ d^{-1} for graded group) nor the final fish yield (558.00 Kg ha^{-1} for ungraded group and 594.51 Kg ha^{-1} for graded group) differed between the two groups. Surprisingly, social hierarchy establishment was detected in all production ponds by day 181 with these hierarchies persisting thereafter until the final harvest day (day 584). This study provides the first evidence of social hierarchy development in fish reared in production ponds. Parallel to what was observed for bluegills reared in tanks, progressive declines in growth and feed efficiency as well as in body condition (with some seasonal fluctuations) were recorded through time, and such declines were suggested to be the adverse consequences of social hierarchies. The study further suggested that social hierarchy development in bluegill may have confounded the benefits of rearing predominantly male bluegills. Consequently, the benefits of size grading for rearing predominantly male bluegill might be better exploited after finding a remedy for the social hierarchy effects. Measures such as the addition of appropriate physical structure that would increase visual isolation among bluegill, and duoculture, i.e., rearing bluegill with another species of value, may delay or prevent social hierarchy development.

Overall, the study provided a least-cost diet formulation for juvenile bluegill and enhanced knowledge of the nutritional requirements of juvenile bluegill. The study also increased understanding of the beneficial effects of “topping off” and size grading to enhance the

yield of large bluegill and production. The study also provided insights into the development of social hierarchies in bluegill reared in commercial-scale rearing systems and their adverse effects in bluegill production.

The study also suggested several follow-up studies that may advance the culture technology and production of bluegill, the important ones being (i) determine the optimal ratio of carbohydrate and lipid in bluegill diets, (ii) develop a specific diet for adult bluegill, (iii) determine whether the developed least-cost diet for juvenile bluegill leads to increased nutrient pollution, (iv) evaluate the ability of early "topping off" to further enhance bluegill growth and thereby produce food-size bluegill, when using the bluegill diet developed in the present study, (v) evaluate the ability of physical structure to curtail social hierarchy development and increase bluegill growth performance, and (vi) re-evaluate the benefits of size grading in producing food-size bluegill and increasing bluegill production after incorporating measures that would delay or prevent the social hierarchy development.

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