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Investigations Concerning Maximization of Cobia (Rachycentron candadum) Hatchery Production Including Incorporation of Microbial Management

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UNIVERSITY OF MIAMI

INVESTIGATIONS CONCERNING MAXIMIZATION OF COBIA (*Rachycentron candadum*) HATCHERY PRODUCTON INCLUDING INCORPORATION OF MICROBIAL MANAGEMENT

By

Ian C. Zink

A THESIS

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Master of Science

Coral Gables, Florida

May 2010

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INVESTIGATIONS CONCERNING MAXIMIZATION OF COBIA (*Rachycentron candadum*) HATCHERY PRODUCTON INCLUDING INCORPORATION OF MICROBIAL MANAGEMENT

Ian C. Zink

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As concerns regarding the growing human population, rising seafood demand, and up to present limited success of fisheries management intensify, aquaculture is increasingly posited as a means to more efficiently produce seafood commodities. However, aquaculture expansion raises contentious issues itself. The current study addresses a number of these issues in attempts to improve hatchery production and related activities. Investigation of less-harmful chemotherapeutants for disease reduction at the egg stage could lead to improved hatchery microbial management, increased survival during early larval stages, increased efficacy of bacterial probiotic incorporation, and reductions in disease transfer between hatcheries and locations. Attempts to surface sterilize and disinfect cobia *Rachycentron canadum* eggs with 3 and 2% hydrogen peroxide solutions significantly reduced survival through the yolk-sac larvae stage. Furthermore, timing of treatment application at differing stages of egg development was found to significantly impact survival, highlighting the importance of this compounding factor. Bacterial probiotics can remediate water quality, reduce target host stress, and improve survival and population growth rates of live feed organisms. Two of the following studies investigated the benefits of incorporation of a Bacillus spp. probiotic blend in aquaculture activities. During closed container mock shipment of yellowfin tuna

Thunnus albacares yolk-sac larvae, significant reductions in total ammonia nitrogen resultant from probiotic incorporation were observed. Furthermore, significantly reduced dissolved oxygen utilization might have resulted from stress reduction, as suggested by a non-significant lower degree in the breakdown of osmoregulation of the larvae. Incorporation of probiotics within rotifer *Brachionus plicatilis* cultures resulted in significantly higher daily mean populations, significantly lower population variability among replicates, and non-coincident logistic population growth regressions which yielded higher population growth rates and system carrying capacities. Broodstock management should not only maximize quantity of reproductive output, but quality as well. Significant increases in cobia egg diameter with increasing broodstock female age were detected, as well as significant increases in egg diameter with decreasing salinity. Confirmation of potentially increased larval growth rates resultant from increased energy stores of larger eggs when produced by older females, as demonstrated in other species, is warranted. Attempts to find production parameters which maximize cobia larval growth and survival would lead to increased sustainability via reducing demand for wild-collected *Artemia* and economic efficiency. Gaps in knowledge for achieving this goal continue to exist, but conclusions drawn from analysis of multiple production scale trials indicate temperatures of 29-31 °C maximize growth and survival. Improvement in sampling design and data analysis would increase statistical rigor and ease comparability of larvicultures outcomes across ranging influential factors. Further investigation of all of these matters is certainly warranted, although conclusions drawn could be effectively utilized to improve success of hatchery operations.

DEDICATION

To Humanity – May we realize that resources are finite, that we must share those finite resources, and not to do so flies against the basic rights of humanity. That human impact on the sustainability of the planet is an ever present problem, and the finite resources at our disposal should be used as minimally and as sustainably as possible or in order to maximize efficiency if we are to truly continue our society.

We could have saved the Earth but we were too damned cheap.

Kurt Vonnegut, Jr.

To My Colleagues – May the pursuit of science by synonymous with the pursuit of truth, whether it be in experimental procedures, statistical analysis, or presentation of results. To work in the sciences is to recognize patterns in the world and then to disseminate those observations for the whole public to utilize, whether for better or for worse. Inherent to this work is the necessity for obtaining and maintaining the trust of the public.

Ultimately, doing ethical work is a personal rather than an institutional responsibility. Thus, it cannot effectively be legislated. The only practical countermeasure is to alert people to the unethical procedures that can be used…and by so doing, hope to ferment a social backlash against those who engage in such malfeasance.

J.L. Crompton 2006

To My Parents – Who firstly told me to choose a profession I would enjoy, for I would be stuck doing it the rest of my life. For teaching me to be humble, modest, compassionate, honest, and sincere. For teaching me to follow my dreams, and to let *no* thing or person stand in the path of truth, joy, and belief. For always being there with a hug, smile, and the reassurance that this too shall pass.

There is a time in every man's education when he arrives at the conviction that envy is ignorance; that imitation is suicide; that he must take himself for better, for worse, as his portion; that though the wide universe is full of good, no kernel of nourishing corn can come to him but through his toil bestowed on that ground which is given to him to till.

R.W. Emerson

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Special thanks is also due to Dr. Nelson Ehrhardt and Mark Fitchett (I still owe you a copy of Applied Linear Statistical Models…), whose many discussions of data and statistical analysis have provided me a mirror in which to reflect ideas and deepen my understanding. Without their assistance, the work herein would be more lacking in sophistication.

Special thanks are also deserved by the RSMAS community, and to my home at the University of Miami. This journey began many years ago, and many individual's lives have threaded together, apart, and at times together again with my path in life and in Miami. Through work or play, hard times and good times, I have always felt I held a

special place as a member of these communities. I will *always* bleed orange and green (GO CANES!). To my friends, colleagues, and professors, both old and new, who have helped me on my journey thus far, and will continue doing so until the end of our days in this life. To my enemies, for what you have taught me often is more important than the guarded comments of my acquaintances. All of you have contributed to my life, and my home, in Miami.

I would also like to acknowledge you, the potential reader of this white elephant. Due to insistence upon the production of publications, in which everyone gains, able time was not allocated for a more thorough editing of this document. I ask for forgiveness and ask that judgement emphasize intellectual content rather than proper composition.

But most of all, I must thank my family, whose unwavering support has always insisted I pursue my dreams, do what is right, and never give up hope. Without you, I would not be here, and would not have gained my original perspective of how things ought to be….

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LIST OF ABBREVIATIONS

AA	Amino Acid
ANOVA	Analysis of Variance
ANCOVA	Analysis of Covariance
ARA	Arachidonic Acid, 20:4n-6
BG1	Broodstock Group 1
$BG2_{SP1}$	Broodstock Group 2, Spawn 1
$BG2_{SP2}$	Broodstock Group 2, Spawn 2
$BG2_{SP3}$	Broodstock Group 2, Spawn 3
$BG2_{SP4}$	Broodstock Group 2, Spawn 4
$BG3_{SP1}$	Broodstock Group 3, Spawn 1
$BG3_{SP2}$	Broodstock Group 3, Spawn 2
BMP(s)	Best Management Practice(s)
CFU	Colony Forming Units
CO_2	Carbon Dioxide
CV	Coefficient of Variation
df	degrees of freedom
DHA	Docosahexaenoic Acid, 22:6n-3
DO	Dissolved Oxygen
DPH	Days Post Hatch
EPA	Eicosapentaenoic Acid, 20:5n-3
ES1	Egg Sterilization 1
ES2	Egg Sterilization 2
ES3	Egg Sterilization 3
FAA	Free Amino Acids
FAO	Food and Agriculture Organization, United Nations
GAO	United States Government Accountability Office
GESAMP	Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection
GIT	Gastrointestinal Tract
GLM	Generalized Linear Model

HCG	Human Chorionic Gonadotropin
HPS	Hours Post Spawning
HSD	Honestly Significant Differences
HUFA	Highly Unsaturated Fatty Acid
IAA	Indispensible Amino Acid
O_2	Oxygen
NH_3	Ammonia
NH_4^+	Ammonium Ion
NMDA	N-methyl d-aspartate (specific type of glutamate receptor)
PUFA	Poly-Unsaturated Fatty Acid
RAS(s)	Recirculating Aquaculture System(s)
PGR	Population Growth Rate
SD	Standard Deviation
SL	Standard Length
T1E1	Trial 1 Experiment 1
T1E2	Trial 1 Experiment 2
T2E3	Trial 2 Experiment 3
T3E4	Trial 3 Experiment 4
T3E5	Trial 3 Experiment 5
T3E6	Trial 3 Experiment 6
T3E7	Trial 4 Experiment 7
T4E8	Trial 4 Experiment 8
T4E9	Trial 4 Experiment 9
T4E10	Trial 4 Experiment 10
T4E11	Trial 4 Experiment 11
T5E12	Trial 5 Experiment 12
T5E13	Trial 5 Experiment 13
TAA	Total Amino Acids
TAN	Total Nitrogen Ammonia
TL	Total Length
TSB	Tryptic Soy Broth

$UINH_3$	Un-Ionized Ammonia
UMEH	University of Miami Experimental Hatchery
USFDA	United States Food and Drug Administration
USGS	United States Geological Survey
UV	Ultraviolet

Chapter 1: Introduction – The Future of Global Seafood Production, Cobia as an Emerging Culture Species, and Microbial Management Incorporating Probiotics

1.1) Caught Between Disappearing Wild Stocks and Fish Culture – a Brief Statement of World Seafood Supply

Demand for consumable seafood continues to increase due to both the growing human population as well as increased consumer demand driven by reports and studies demonstrating the health benefits associated with seafood consumption (Rosenberg 2002, FAO 2008). Concern of pending crashes within the current wild capture fisheries, such as of "fishing down the food chain," permanent changes in size-at-spawning dynamics, and other fears associated with the over-exploitation of marine seafood resources (Schiermeier 2002, Worm et al. 2006, Baum and Worm 2009, Worm et al. 2009), leave little hope for sustaining current rates of exploitation without substantial changes to management and enforcement (Worm et al. 2009). Worldwide, wild-capture fisheries seem to have reached a production plateau (Watson and Pauly 2001, FAO 2005).

Only aquaculture production continues to increase while wild capture fisheries production stagnates and/or declines (Watson and Pauly 2001, FAO 2005, FAO 2006, FAO 2008). Aquaculture is anticipated to alleviate increasing seafood demand and concerns regarding wild-capture fisheries failures by supplementing or even replacing wild-capture fisheries with either the same or similar seafood products (Williams et al. 2000, Marra 2005). Not everyone shares the same idealistic hopes for aquaculture; some believe humanity's efforts would be better spent rebuilding wild fish stocks (Baum et al. 2005, Dalton 2004, Worm et al. 2009). Others contest the sustainability of culture based production which continues to heavily rely on the harvest of wild fisheries which may be more efficiently utilized when directly consumed by humans (Naylor et al. 2000,

Goldburg and Naylor 2005, Hall 2007, Worm et al. 2009). However, as long as aquaculture practices remain economically feasible and financially profitable, it is difficult to imagine holding back entrepreneurs from what they do best; where there is a market and a way, the will to meet that demand and make a profit follows.

Aquaculture has proven to provide a consistent supply of fresh, high grade product on demand. These qualities are not only beneficial, but crucial, for modern food service industries and maintaining consumer satisfaction. The economic feasibility of some aquaculture production system types has already been proven. Product distribution infrastructure, routes, methods, and markets are either already developed or can readily adapt those in place which were historically associated with wild capture fisheries. Investors and entrepreneurs continue to recognize this still emerging industry as a means to accumulate wealth. Meanwhile nations envision expansion of their Gross Domestic Product (GDP) and export products, creation of domestic employment, and a means to produce more wealth and foodstuffs for their domestic populations. Collectively, these factors facilitate the will for aquaculture expansion.

Wild capture fisheries are the last major source of food consumed by humans that is not cultured under some degree of controlled conditions. This means that production is limited by carrying capacities of the environment and it does not maximize efficiency of the transfer of energy from one trophic level to the next. Aquaculture, on the other hand, grows seafood and products in a controlled manner, analogous to terrestrial agriculture; thus, with continued understanding of growth processes, feeds assimilation, disease management, and other related factors, the production of these products can be achieved more efficiently (Marra 2005, Benetti et al. 2006). Independence or at least reduced

reliance on wild capture fisheries for fish meal and oil (Powell 2003) could allow the continued expansion of a perhaps more sustainable industry limited only by space and other biological factors, such as disease and production capabilities.

For all the positive aspects aquaculture may bestow upon modern seafood production, its practice can also result in a number of negative environmental impacts. These include increased organic loads and biological oxygen demand within bodies of water, increased disease prevalence and spread to wild species, increased demand for fishmeal and fish oil products, genetic manipulation of wild stocks due to escaped cultured individuals, intended and unintended introduction of exotic species and related ecosystem impacts, as well as a host of other concerns (Rosenthal 1994, Landesman 1994, Naylor et al. 2000, Pérez et al. 2002, Dalton 2004, Bunkely-Willams and Williams 2006, Benetti et al. 2006, Rapp et al. 2007). At the urging of the international community, continued development of aquaculture should include addressing these, and other sustainability, resource utilization, and social consequences, in order to continue to reap the benefits of this still emerging industry in balance with ecosystem considerations while minimizing negative consequences of its growth (FAO 1995).

1.2) Cobia as an Emerging Cultured Species

Cobia (*Rachycentron canadum*) has been selected as the model fish egg and larvae to be used in a majority of the subsequent experiments. Many aspects of this species warrant its consideration as a prime aquaculture candidate**.** The distribution of cobia provides for nearly pan-tropical and sub-tropical culture without resorting to introduction of an exotic species. However, multiple seemingly misidentifications have

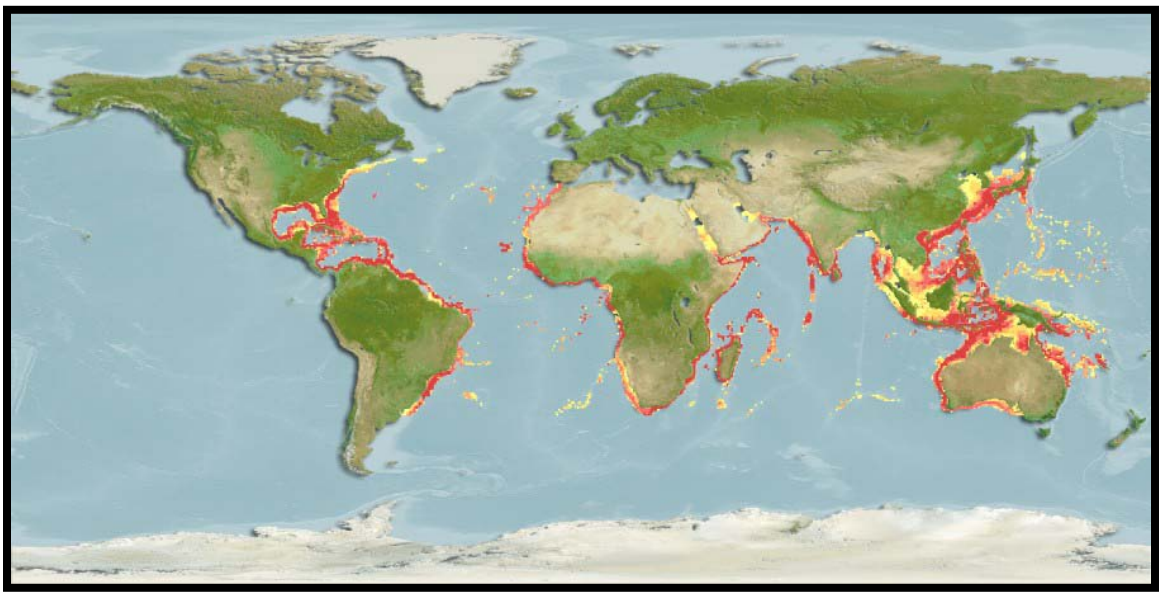
resulted in confusion as whether to list the species as present in the Eastern Equatorial Pacific (Springer 1982). These early reports, as well as repeated citation of them by others, such as by Briggs (1960), has led others to include the Eastern Tropical Pacific region as part of the cobia's native range (Benetti et al. 2008b, Benetti et al. 2010). These issues warrant clarification in order to properly determine whether cobia is a proper species to be cultured in certain regions or whether doing so would constitute introduction of an exotic species.

Older reports state having caught cobia off the coast of western South America or state that locals report having caught the species (Fowler 1944, reviewed in Springer 1982). It has been suggested that confusion of species identification and local nomenclature for fishes was the cause of these reports (Springer 1982). More recent reviews do not include the Eastern Equatorial Pacific as part of the native range of cobia. Shaffer and Nakamura (1989) cite the native range of the species in the equatorial waters of the Western Pacific, Indian, and Atlantic Oceans while Collette (1999) reports that cobia are found in all tropical waters but is "absent from the eastern Pacific and the Pacific Plate, except marginally." Further discussion with Dr. Collette confirms its absence from the eastern Equatorial Pacific (B. Collette Pers. Com.). Easily accessible information, such as distribution maps published as part of FishBase, could eliminate confusion by human-verification of the computer-generated distribution maps. Computer-generated maps are initially constructed based the environmental tolerances a species (Fig 1.1) and only after human-verified versions are published can they truly be relied upon (Reyes 2007, Fishbase 2009).

Besides a broad native range, cobia exhibit other characteristics which lends the species to mass aquaculture production. Aquaculture research programs of the United States have recognized the value of these fish for their high growth rates, high resistance to disease, and high quality flesh (Benetti et al. 2007, Schwarz et al. 2007a, Holt et al. 2007a, Weirich et al. 2007, Benetti et al. 2010). These observations are not new; cobia culture and production were first developed in the 1990's in Taiwan, where aquaculture production continues to increase (Liao et al. 2004, Liao et al. 2007). Rapid larval growth makes this species relatively cheap and easy to produce in the hatchery setting: larvae pass rapidly through live feed stages where the majority of mortalities occur while prolonged larval stage of other species requires greater labor and live feeds production inputs (Feeley et al. 2007). The lack of competition with a wild fishery for the species in the United States allows for an open, unimpeded market for the sale of cobia. However, this can also be a disadvantage as consumer unfamiliarity with the species could hinder its acceptance.

In 2007, world product was just short of 30,000 mt. (FAO 2009); other recent reports label it a species whose production is "about to take off" (FAO 2008). Currently, this species is cultured in Taiwan, China, the Philippines, Japan, Vietnam, Thailand, French Territories (Reunion Is.- introduced for aquaculture purposes [Letourneau et al. 2004]), Indonesia, Iran, Australia, United States, Dominican Republic, Belize, Mexico, Panama, the Bahamas, Brazil, and Ecuador. Expansion of production is expected to continue, requiring study and development of protocols that maximize production and minimize costs at all stages of production from egg to market. Despite the intense

Fig. 1.1: Reviewed distribution map of cobia *Rachycentron canadum* reproduced from www.fishbase.org (Reyes 2007).



interest in cobia aquaculture and its expansion, a number of gaps in the literature remain regarding the understanding of various parameters affecting cobia production (Schwarz et al. 2007b). A more detailed review of the current understanding of factors influencing cobia larval growth and survival is included in Chapter 5. By better understanding factors which most influence production outcomes, such as growth, survival, and numbers of juveniles production, improved production protocols could be established which maximize these production characteristics.

As cobia aquaculture continues to expand, care and prudence must be taken to ensure this species does not develop the same negative repertoire as has developed for Atlantic salmon. Cobia aquaculture has already been blamed for introduction of exotic species into a region where it was not reported to previously occur (Letourneur et al. 2004), releases of measureable wastes from cage culture (Rapp et al. 2007), and introductions of diseases to regions where they were previously not reported to occur (Bunkley-Williams and Williams 2006). Although the potential negative ecological impacts of any of these has yet to be thoroughly studied, the initial evidence already points to similar continuous issues which have surrounded Atlantic salmon cage culture. Improvement of best management practices (BMPs), protocols, and regulations, both at national and international levels, is warranted.

1.3) Microbial Management

Efforts to control microbes is certainly not a recent concept to humanity; this desire has pervaded in subject areas such as medicine and food processing since realization of microbes' relation to infection, disease, and food spoilage (Lawrence and

Block 1968). In more recent decades, efforts to standardize microbial management along with other safety factors in industrial production has led to creation of systems such as and similar to Hazards Analyses Critical Control Points (HACCAP). In aquaculture settings, the industrial production of marine finfish fingerlings in intensive, high density systems creates conditions that allow proliferation of opportunistic and potentially pathogenic microbes (Vereschuere et al. 2000). This is analogous to terrestrial industrial farming systems which create the conditions for proliferation of microbes and disease in intensive operations (Samah et al. 2006).

Aquaculture culture conditions often result in nutrient-rich, high concentrations of dissolved and/or particulate organic matter in 'artificial,' non-steady state environments. These conditions allow opportunist microbes employing an 'r' selection strategy to rapidly overload culture systems and dominate microbial communities (Andrews and Harris 1986, Vadstein et al. 1993). Attempts to control the climax microbial community composition which dominates a system constitute microbial management in the aquaculture setting (Douillet and Holt 1997, Douillet 2000a, Blackshaw 2001, Lee 2003). These actions include reactive, such as antibiotic and chemotherapeutant utilization, and preventative actions, such as care and cleanliness of systems and prophylactic antibiotic and chemotherapeutant utilization.

1.4) Negative Consequences of Antibiotic and Chemotherapeutant Utilization in Aquaculture

Antibiotic and other chemotherapeutant utilization has been adapted as a standard practice for the culture of animal protein destined for human consumption. Recent figures state approximately 50% (of tonnage) of antimicrobials produced in North

America and Europe is destined for consumption by food-producing animals (including uses as prophylactics, growth supplements, and treatment of diseases) (WHO 2002). Although analysis was hindered by lack of consistent national monitoring and tracking programs, a recent review revealed that, for many of the nations analyzed, the majority of antibiotics usage related to agriculture were administered at sub-therapeutic concentrations as growth promoters (Samah et al. 2006). Similar to terrestrial agriculture, utilization of antimicrobials in aquaculture is a proven method to increase the survival of cultured organisms (Shelbourne 1963, Blaxter and Hunter 1982, Gatesoupe 1989, Benetti 1997, Benetti et al. 1998, Rotman et al. 2005).

Widespread antibiotic utilization has yielded a number of negative results that only more recently have been fully recognized. Alexander Fleming (1946), who is credited with the discovery and properties of penicillin, foretold of the abusive reliance on antimicrobial compounds in a speech given just a few years after the benefits of penicillin were realized. Increasingly, alarm is being raised regarding reliance on antibiotics and some chemotherapeutants as a means to bolster animal production.

Studies and reports of antibiotic resistant bacteria warn of the risks, both to humans and to future farming production, associated with unnecessary and prophylactic utilization of antibiotics (Shah et al. 1993, Gold and Moellering 1996, Witte 1998, Walsh 2000, Falkow and Kennedy 2001). Bacterial resistance to antibiotics has been associated with aquaculture practices and this problem is feared to perpetuate under the current practices of some operations (Björklund et al. 1991, Herwig et al. 1997, Alderman and Hastings 1998, Chelossi et al. 2003, Sarmah et al. 2006). Demonstration of transference of antibiotic resistance acquired by microbes from aquaculture environments to microbes

commensal to humans (Rhodes et al. 2000) highlights concerns relating to human health. Analogous to terrestrial agriculture, continued aquaculture development relying on antibiotics will only compound these problems (Austin 1985, Jank and Rath 2002, Holmström et al. 2003, Sarmah et al. 2006).

Governments and international organizations are taking steps to reduce nonessential antibiotic utilization and develop BMPs which either reduce or eliminate their necessity (FAO 1995, FAO 1997, GAO 1999, WHO 2002, GAO 2004). Denmark, Finland, and Sweden have prohibited antibiotic utilization as growth promoters in attempts to limit future negative impacts (Jank and Rath 2002, Sarmah 2006). Evidence suggests these changes in usage patterns have already resulted in positive consequences with recognition of a reversal in resistance trends (Lipstich 2001, Sarmah 2006). More recently, the European Union has followed suite and banned the prophylactic utilization of antibiotics, and the United States national government is currently considering their prohibition of prophylactic utilization as well (Oyeleye 2009).

Many chemotherapeutants are currently utilized by the world wide aquaculture industry. As new production species and techniques are investigated, an even wider array is utilized in aquaculture-related research. Chemotherapeutants and chemicals of concern in the aquaculture setting include chemicals associated with structural materials, sanitation of equipment, soil and water treatments, disinfectants, pesticides, herbicides/algaecides, feed additives, hormones, and anesthetics (GESAMP 1997). Concerns related to chemotherapeutant utilization involve health and safety of the treated organisms, health and safety of workers administering treatments, effects on the ambient environment and non-target organisms, potential development of resistance to the

treatment from diseases or parasites, and consumer safety from bioaccumulation or residues found within the tissues of treated organisms (GESAMP 1997).

Similar to antibiotic utilization, many ill effects or undesired repercussions stemming from utilization of certain chemotherapeutants have been recognized. In response, governments and international organizations have acted to promote BMPs that do not necessitate chemotherapeutant utilization, or in some instances, have banned utilization of particular chemicals (FAO 2006). Despite these actions, some chemicals known to be toxic to workers and/or consumers are still utilized, either illegally or legally within countries with less stringent regulations (FAO 2006). Environmental, worker, and consumer safety are not the only issues at risk. The consumer threat has prompted more stringent testing of imported seafood for chemical and drug residues, resulting in difficult entry and/or banning of products from repetitively offending nations thus resulting in economic impacts as well.

Aquaculture operations represent an investment and people depend upon return on their efforts in order to maintain livelihoods, their position in a company, and the functioning of economies – this notion relates to any aquaculturist from small-scale, individual or family operations which contribute to the livelihood of a household to multi-national corporations that employ thousands of individuals and handle the investments of many more. Disease management is essential for efficient production that minimizes wastes and maximizes profits. If production stock is compromised by disease, then "aquaculturists often do not have or do not see any choice other than applying disease control chemicals to treat or prevent such disease outbreaks" (GESAMP 1997). Ultimately the development of protocols and BMPs utilizing chemotherapeutants with the

least toxic effects which maintain high production efficiency, maximize worker safety, result in safe products for consumers, and yet minimize potential negative environmental impacts should be the expected goal of continued aquaculture research.

1.5) Microbial Management and the Incorporation of Probiotics

The concerns surrounding antibiotic resistance and limitation of utilization of both antibiotics and certain chemotherapeutants have promoted renewed interest into a relatively new and peripheral field within microbial research. This field investigates how selected microbial organisms can promote host growth and health via various direct and indirect routes. Certain microorganisms have been recognized for health benefits they bestow upon their host or benefits realized when utilized as modifiers of bacterial communities. When intentionally administered to inoculate host organisms or their ambient environments, these microbes are called probiotics. Attempts to limit pathogenic microbial presence, proliferation, virulence, and transference via incorporation of probiotics into production protocols are transforming traditional views of microbial management. Compounding research results suggest probiotic incorporation in hatchery production protocols should be included as part of the larger strategy of microbial management (Douillet and Holt 1997, Douillet 2000a, Verschuere et al. 2000, Blackshaw 2001, Lee 2003).

The term probiotic was first coined by Lilly and Stillwell (1965); they defined probiotic as "…growth-promoting factors produced by microorganisms." After recognition that these growth promoting abilities can extend to host organisms as well, the definition was modified by Fuller (1989) to "a live microbial feed supplement which

beneficially affects the host animal by improving its intestinal microbial balance." Some authors continue to consider probiotics to constitute the narrow definition of live microbial adjuncts, administered orally, which colonize the gastrointestinal tract (GIT) (Gatesoupe 1999, Vine et al. 2006). However, others broaden the definition of the term. Verschuere et al. (2000) defined probiotic in such a way that reflects all potential microbial benefits to aquatic host organisms: "A live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment." Hereafter, utilization of the term probiotic will reflect this latter, broader definition.

Probiotics have the potential to be applied to nearly every organism humanity deems necessary to improve the health and growth thereof, including ourselves. Probiotics are capable of inhibiting potentially pathogenic bacteria in a number of ways: competition for available adhesion substrate, competition for available nutrients and energy, production of bacteriostatic and bactericidal compounds, modification of ambient physical conditions, and stimulation the host organism's innate and adaptive immune response (Fuller 1989, Gatesoupe 1999, Verschuere et al. 2000, Vine et al. 2006). Besides these mechanisms, probiotics can be utilized as promoters of feed conversion which leads to improved growth and weight gain (Fuller 1989). Probiotics may also act in more indirect means via improvement of ambient water quality conditions; examples include removal of toxins or environmental pollutants, such as ammonia, which may have negative effect on fish physiology and growth (Verschuere et al. 2000).

1.6) Complete Integration of Probiotics in Hatchery Protocols

Responsible, proactive hatchery management should attempt to prevent disease before it occurs. Antibiotics and chemotherapeutants have been traditionally utilized as prophylactics to prevent these circumstances and, more often, are deferred to once disease causes mortalities or reduced growth. Probiotics, as a part of a broader microbial management plan, can eliminate or at least minimize the need for antibiotic and chemotherapeutant utilization by limiting disease before it occurs. Microbial management can incorporate probiotic utilization into proactive disease control protocols associated with all aspects of hatchery production: broodstock maintenance, egg care and incubation, microalgae and live feeds production, larval finfish cultures, and transport of eggs, yolk-sac larvae, or fingerlings.

Ultimately, it is hoped that probiotic utilization would circumvent the necessity to apply potentially more detrimental and less environmentally acceptable treatments to stop bacterial diseases. Hatchery activities form an integrated process with the end goal of maximizing efficiency in fingerling production. As such, the continuity between different hatchery activities demand attention to each production step for the most complete and efficient application of probiotics.

This concept of continuity between different hatchery production steps leads to the concept of HACCAP, as developed for other industries (King and Nardi 2006). Microbial contamination sources can include the culture water itself (Douillet and Pickering 1999), settlement from ambient air, injected air utilized to oxygenate and circulate culture water, culture vessels and equipment utilized during production, and workers themselves (Skjermo and Vadstein 1999). Attempts to control all potential

sources of contamination must be made for the successful implementation of microbial management.

Larvicultures incorporating microbial management protocols and probiotics utilization should begin with protocols which relate to treatment of eggs immediately after spawning. Bacteria have been observed colonizing fish eggs removed from ovaries under sterile conditions in as little as two hours post spawning; by the time hatching occurs, eggs can be heavily overgrown with bacteria (Hansen and Olafsen 1989, Bergh et al. 1992, Douillet and Holt 1994). Sterilization of newly spawned eggs would ensure a "tabula rasa" onto which probiotic species could adhere and gain a foot hold before other, potentially pathogenic bacteria, colonize while also breaking the potential link of vertical transmission of microbes from broodstock and their tank system.

"Matured water" production, or the act of filling tanks with seawater and allowing bacterial communities to mature to a stable, climax community composition, is reported to be a traditional practice of Chinese fish farmers (Lee 2003). Utilization of matured water is believed to increase larval fish survivorship and growth due to stability of water quality and/or the microbial community (Skjermo et al. 1997, Skjermo and Vadstein 1999, Lee 2003). The theory behind microbially matured water lies in r/K selection concept, in which K-strategists eventually come to dominate tank microbial ecosystems. These matured ecosystems should include high diversity of microorganisms that utilize narrow niche specialization and exhibit high resilience against perturbation (Skjermo et al. 1997).

Microbial maturation via direct additions of probiotics to egg incubators after disinfection/sterilization could ensure the intended probiotic bacterial floras become

established. The larval fish GIT is initially colonized by bacteria before the introduction of live feed organisms from either there external surfaces or from ambient culture water (Bergh et al. 1992, Douillet and Holt 1994, Reitan et al. 1998, Hansen and Olafsen 1999, Lee 2003). It is known that larval fish develop rudimentary stomodeum and proctodeum sphincters and begin drinking water before exogenous feeding begins in order to osmoregulate (Tytler and Blaxter 1988a, 1988b). Furthermore, yolksac larval fish are known concentrate bacteria at this early stage (Reitan et al. 1998) which may relate to pre-exogenous feeding energy uptake or early ontogeny of immune systems and bacteria recognition (Olafsen and Hansen 1992).

Transfer of yolk-sac larvae from incubators to larval rearing tanks presents another opportunity to incorporate probiotics. Probiotic microbial maturation of larviculture tank systems could also be employed. Continued application of probiotics to larval rearing systems would depend upon system design: recirculating systems present an opportunity for probiotic retention within the system while flow-through production units would reduce retention potential.

"Greenwater systems," or direct additions of microalgae culture water to larvicultures, are often utilized in cobia larviculture (Kaiser and Holt 2005, Benetti et al. 2007, Schwarz et al. 2007a). Microalgae are known to produce antimicrobial compounds and limit the growth of opportunistic pathogenic bacteria (i.e. *Vibrio spp.*) (Austin et al. 1992, Lee 2003, Lio-Po et al. 2005). Furthermore, greenwater utilization has been recognized as a repetitive source of bacterial inoculation (Salvesen et al. 2000). Under these pretenses, the incorporation of probiotics within gnotobiotic microalgae cultures could continue to inoculate and maintain probiotic flora within culture tanks.

Once exogenous feeding commences, microbial management application also necessitates consideration of live feeds production systems. Live feed organisms are traditionally required as feeds during finfish larvicultures of most species (Watanebe et al. 1983, Lubzens et al. 1997, Lee and Ostrowski 2001). Despite attempts to develop microdiets to reduce the reliance on live feed, success has been mixed, depending upon the species of finfish; thus live feeds remain in prevalent use (Lee 2003).

Addition of live food organisms to larviculture tanks has long been recognized as a source of bacterial contamination (Skjermo and Vadstein 1993, Verdonck et al. 1997, Lee 2003, Vine et al. 2006). After exogenous feeding begins, larval finfish intestinal microbial communities reflect those species present in live feed organisms (Hansen and Olafsen 1999, Vine et al. 2006). Live feeds have been recognized for the capabilities as bioecapsulators (Hansen and Olafsen 1999, Lee 2003, Vine et al. 2006). Probiotics could potentially colonize not only external surfaces of live feed organisms, but also colonize their respective digestive tracts and be actively accumulated by as prey items for live feeds. Thus, larvae feeding upon live feeds which were previously inoculated with probiotics acts as a direct method for repetitive inoculation of the larval fish's gas GIT.

Rotifer (*Brachionus* spp.) cultures typically harbor bacterial loads on the order of 10^3 colony forming units (CFU) ind.$^{-1}$ but can reach as high as 10^8 CFU ind.$^{-1}$ (Skjermo and Vadstein 1993, Eddy and Jones 2002). Typical production cycles of rotifers span many days and semi-continuous cultures can be continued for months on end. Often, bacterial species present tend to be dominated by K-strategists, or those bacterial species which exhibit optimal community dominance when faced with "crowded" environments and limitations in terms of food supply, toxic metabolites, or predation (Andrews and

Harris 1986). Although, this balance can be upset and include higher numbers of opportunistic species during system perturbations, such as increased nutrient loading resultant from rotifer enrichment feeds (Skjermo and Vadstein 1993, Eddy and Jones 2002, Tinh et al. 2006).

Artemia spp. are routinely utilized within marine finfish hatcheries as live feeds once larvae have outgrown and developed beyond the efficient utilization of rotifers as prey items (Sorgeloos et al. 2001, Bengston 2003). It is well documented that *Artemia* cultures harbor high loads of opportunistic pathogenic bacteria (Vereschuere et al. 1997, Sorgeloos et al. 2001, Eddy and Jones 2002, Villamil et al. 2003). *Artemia* cultures are generally short in duration, with initial hatching to enrichment cycles generally being completed over a 48 hr, or shorter, cycle. Furthermore, these cultures are very dense and nutrient rich due to organic loading from hatching, metabolic waste production, and/or the addition of enrichment diets. These nutrient rich, short duration systems represent conditions for the proliferation of opportunistic, r-selected bacterial species (Andrews and Harris 1986, Skjermo and Vadstein 1993, Eddy and Jones 2002).

Many studies have already investigated the incorporation of differing types of probiotics into rotifer and *Artemia* cultures, either to improve their population growth and/or survival and stability of culture or as bioencapuslators intended for inoculation of larval fish (Gatesoupe 1991; Rombaut et al. 1999; Douillet 2000b, 2000c; Makridis et al. 2000; Planas et al. 2004; Rotman et al. 2005). A majority of these studies indicate that probiotic utilization can improve different aspects of larval rearing production, such as consistency of production, as well as affect growth via increased efficiency of digestion

and stimulation of non-specific immune defenses (Skjermo and Vadstien 1999, Vereschuere et al. 2000, Rotman et al. 2005, Vine et al. 2006).

1.7) *Bacillus* spp. as Probiotics

EcoAqua (EcoMicrobials, LLC, Miami, FL, www.ecomicrobials.com), the probiotic blend utilized in the following investigations, is a commercial product consisting of a blend of four *Bacillus* species: *B. licheniformis, B. subtilis, B. laterosporous,* and *B. megaterium* (P. Douillet, Pers. Comm.). The genus *Bacillus* is characterized as rod-shaped, Gram-positive staining, endospore forming aerobic bacteria. However, *Bacillus* spp. can also act as facultative anaerobes, allowing for the potential to survive conditions necessary for establishment and proliferation in GITs. The genus has been grouped with other genera of both spore and non-spore forming bacteria within the family *Bacillales* (Fritze 2004).

One essential requirement for this *Bacillus* spp. blend to succeed as marine finfish probiotics is the capability of growth in saline aqueous environments. The strains utilized in EcoAqua were originally isolated from marine environments (P. Douillet, Pers. Comm.) and their ability to grow in saline environments was later demonstrated (Didoha 2004). Other studies have also demonstrated the ability of *Bacillus* spp. to grow in saline aqueous environments (Rengpipat et al. 1998; Douillet 2000b, 2000c; Decamp and Moriarty 2007) and in salinities of up to 40 ppt (Decamp et al. 2007). Although *Bacillus* spp. are traditionally considered common inhabitants of terrestrial and marine sediments, recent studies are providing evidence that transience through animal digestive systems may be a part of these bacteria's life cycle (Hong and Duc 2004).

For *Bacillus* spp. to act as probiotics, at least survival, if not reproduction as well, within anaerobic GIT systems is essential. This capability has been demonstrated by mammalian model studies. Mice orally administered *B. subtilis* spores subsequently excreted spores in faeces for up to seven days after initial inoculation and final cumulative counts were up to seven-fold greater than the original inoculate dose (Hoa et al. 2001). Another study demonstrated the ability of a majority of *Bacillus* spores to survive simulated physio-chemical environments similar to those encountered in the stomach and small intestine (Duc et al. 2003). Although, a majority of vegetative cells, including those of other genera utilized as references, did not survive (Duc et al. 2003). These results may not be directly applicable to marine fishes as the physio-chemical regimes of the respective GIT tracts would be slightly different. Even so, these studies prove that spores can survive some of the harshest conditions which would be similar: low pH, an enzymatic environment, and anaerobic conditions (Duc et al. 2003).

Bacillus spp. have been isolated from fish GITs (Kennedy et al. 1998, Sugita et al. 1998, Eddy and Jones 2002). It therefore seems plausible to conclude that *Bacillus* spores and potentially vegetative cells could be bioencapsulated by live feeds, received within larval fish GITs, and survive to colonize their new host. It is recognized that Gram-negative spp. and Gram-positive spp. other than *Bacillus* spp. are more commonly and consistently associated with fish GITs (Olafsen 2001, Eddy and Jones 2002, Vine et al. 2006). Thus, repetitive inoculations would most likely be necessary in order to maintain *Bacillus* spp. as substantial members of the larval fish GIT (Vine et al. 2006).

The goal of probiotics utilization is to improve host-related or ambient microbial communities with the intent of eliciting a beneficial response. Thus, a potential probiotic

ought to demonstrate such a response else it is ostensibly not a good candidate probiotic. The blended strains of *Bacillus* spp. utilized in EcoAqua were selected for their demonstrated ability to inhibit the growth of opportunistic pathogenic *Vibrio* spp. (Didoha 2004). Indeed, researchers have described a number of lytic enzymes, bacteriocins and bacteriocin-like inhibitory substances (BLIS), and natural antibiotics produced by these and other *Bacillus* spp. (Urdaci and Pinchuk 2004). Sugita et al. (1998) isolated a *Bacillus* sp. bacterium from coastal fishes of Japan; a siderophore isolated from this bacterium consistently inhibited the growth of 227 of 363 other bacterial strains isolated within the same study.

Researchers have demonstrated that *Bacillus* spp. probiotic utilization results in induction of innate immune responses such as increased phagocytic activity (Rengpipat et al. 2000), increased phenoloxidase production (Rengpipat et al. 2000, Gullian et al. 2004), increased antibacterial activity (Rengpipat et al. 2000), and increased hyaline cell counts (Gullian et al. 2004). *Bacillus* spp. can also act as ambient water quality conditioners. Dissolved organic matter can act as growth substrates and Bacillus spp. possess biochemical machinery to directly uptake ammonia (NH_3) for utilization as a nitrogen source (Donohue and Bernlohr 1981). Indeed, several studies report *Bacillus* spp. have reduced ammonia concentrations when utilized as probiotic incorporations within shipping protocols (Gomes et al. 2008, Gomes et al. 2009) and recirculating aquaculture systems (Chen and Chen 2001, Lallo et al. 2007). Furthermore, in recirculating aquaculture systems, their utilization improved survival rate and quality of fish produced in one study (Chen and Chen 2001) while reductions in background pathogen reduction was realized in another (Lallo et al. 2007).

Thus far, the application of *Bacillus* spp. probiotics has focused on invertebrate live feeds production systems and shrimp production ponds (Moriarty 1998; Rengpipat et al. 1998; Rengpipat et al. 2000; Douillet 2000b, 2000c; Rengpipat et al. 2003; Vaseeharan and Rasmasamy 2003; Guillan et al. 2004; Wilson and Priya 2004; Zaiei-Nejad et al. 2006; Decamp and Moriarty 2007; Decamp 2007). These studies have found *Bacillus* spp. probiotics helpful in reducing *Vibrio* loads (Moriarty 1998, Vaseeharan and Rasmasamy 2003, Wilson and Priya 2004, Guillan et al. 2004) and mortality associated when hosts were challenged with pathogens (Rengpipat et al. 1998, Rengpipat et al. 2003, Vaseeharan and Rasmasamy 2003). Positive outcomes from their utilization include improved immune response (Rengpipat et al. 2000, Guillan et al. 2004, Zaiei-Nejad et al. 2006), survival (Moriarty 1998, Rengpipat et al. 1998, Rengpipat et al. 2000, Rengpipat et al. 2003, Zaiei-Nejad et al. 2006), weight gain (Guillan et al. 2004, Zaiei-Nejad et al. 2006), individual growth (Rengpipat et al. 1998, Rengpipat et al. 2000, Rengpipat et al. 2003, Ziaei-Nejad et al. 2006), feed conversion ratio (Zaiei-Nejad et al. 2006), and population growth (Douillet 2000b, 2000c).

Fewer studies have been conducted investigating the use of *Bacillus* spp. as probiotics with the production of marine finfish larvae. Kennedy et al. (1998) concluded that *Bacillus* spp. and other gram-positive bacteria were associated with improved survivorship of larval snook *Centropomus undecimalis*. In the same study, *Bacillus* isolates from well-performing snook larvicultures were later utilized as probiotics during the larviculture of spotted seatrout *Cynoscion nebulosus* and striped mullet *Mugil cephalus* resulting in improvements in both growth rate and uniformity in growth (Kennedy et al. 1998). Flounder (species not specified), when administered *Bacillus*

probiotics via feeds and by direct additions to ambient tank water, exhibited a marked improvement in weight gain over control fish (Decamp and Moriarty 2007). Application of *Bacillus* spp. to turbot larviculture has yielded increased larval survival as well as reduced mortality when challenged with *Vibrio* spp. (Gatesoupe 1993). Benetti et al. (2008b) described trials in which EcoAqua was utilized as an additive during live feeds production which did not cause differences in growth nor survival. However, these results were most likely heavily influenced by lack of replication and a heavy mortality event (I. Zink, Pers. Obs.).

Probiotic species selection requires cautious testing of potential species benefits both *in vitro* and *in vivo*. Slight nuances between similar strains and species of bacteria necessitate careful screening and selection before incorporation as a probiotic. Investigation of *Bacillus toyoi* as a potential probiotic illustrates this point. When utilized via spraying on pellet feed for European eel *Anguilla anguilla*, this bacterium was concluded to be unable to colonize the GIT tract due to lack of detection in incubated samples collected from 24 hr starved specimens (Chang and Liu 2002). Furthermore, when challenged with *Edwardsiella tarda*, final survival of the *A. aguilla* was not significantly different from controls despite demonstration of *in vitro* test cultures of *B. toyoi* as capable of inhibition of *E. tarda* growth (Chang and Liu 2003).

Other than beneficial effects on the intended host, endospore forming *Bacillus* spp. exhibit many production and logistical benefits. The endospores of these species are resistant to heat, radiation, desiccation, chemical and enzymatic degradation, and can withstand dry or aqueous storage for many years and yet remain viable (Nicholson 2004, Wang et al. 2008). These qualities allow for ease of storage (long shelf life with no

refrigeration required) as well as resilience to the shocks of transportation (physical stresses such as higher or lower temperatures than optimal range for vegetative cells) as well as resistance to UV radiation (no need to store in opaque containers). UV resistance could be beneficial in recirculating aquaculture systems employing UV water sterilization. Although, it should be anticipated that a majority of the bacterial cells would be in a vegetative rather than a sporulated state, thus this advantage may not be realized. Heat resistance may also ease their incorporation into the production of pelletized feeds often used for on-growing of aquacultured fishes.

1.8) Summary and Purpose of Investigations

Efforts to improve and optimize aquaculture practices and protocols, from both economical as well and environmental standpoints, is ongoing. The following studies were conducted to facilitate the efficient expansion and consistent hatchery production of cobia *Rachycentron canadum* juveniles by investigation of methods to maximize their growth, survival, and production via application of microbial management and better understanding of their ecology/life history. Through development of protocols which incorporate chemotherapeutants which are more environmentally acceptable than others currently in extensive use and incorporation of probiotics into hatchery production activities, such as shipment of live larvae and juveniles, improved control over the potential proliferation and transference of diseases can be established while concurrently improving larval fish growth and survival. A thorough background investigation of knowledge regarding the life history and natural ecology aspects of a species, along with investigation of biological, physical, and circumstantial factors affecting the growth of

larval fish in general, can reveal those combinations of factors which maximize growth and production. Ultimately, efficient production and high survival make not only good sense in terms of economic efficiency of production, but also environmental sustainability as well.

Chapter 2: Microbial Surface Sterilization of Cobia Eggs

2.1) Background

Intensive incubation of marine finfish eggs can allow microbial communities to proliferate in conditions vastly different than would be experienced in natural settings. Traditional strategies for the prevention and control of diseases in aquaculture settings call for non-selective reduction of bacteria (Vadstein et al. 1993). This can be achieved in egg and early life stages via mechanical rinsing, disinfection, and chemical treatment of embryos and larvae (Brock and Bullis 2001).

Surface disinfection and/or sterilization of fish eggs would allow for improved application of probiotics at this stage via the elimination of bacteria already colonizing the egg. The necessity for microbial and disease control extends beyond the operations and functionality of one laboratory or production hatchery when eggs and other living materials are transferred between facilities, regions, or even nations. Thus, not only would disease control improve survival at the hatchery were the eggs were spawned, but can also limit the transfer of pathogens to other locations.

Much research has already linked the potentially deleterious effects of bacterial colonization on external surfaces of finfish eggs, which can lead to decreased hatchability and survival of marine fish larvae (Oppenheimer 1955, Shelbourne 1963, Hansen and Olafsen 1989, Hansen et al. 1992, Vadstein et al. 1993, Hansen and Olafsen 1999, Lee 2003). Studies have detected 'substantial' bacterial adhesion to temperate fish eggs as early as two hours post spawning and observed complete overgrowth at hatching 18 days later (Hansen and Olafsen 1989). Similarly, a study of a tropical species detected rapid growth and high community numbers ($> 10^4$ CFU egg^{-1}) which were dominated by

opportunistic pathogenic species (Verner-Jeffreys et al. 2006). Furthermore, more ubiquitous marine microbes remained at lower colonization loads (~10^2 CFU egg^{-1}) when opportunistic pathogens were not present (Verner-Jeffreys et al. 2006).

Pomacentrid eggs transferred from wild reefs to laboratory conditions have been observed to become quickly diseased; bacterial isolates from the diseased batches were later utilized as inoculates to demonstrate their pathogenicity (Nelson and Ghiorse 1999). Opportunistic pathogenic species isolated from intensively incubated marine finfish eggs were also shown to be deleterious to hatching and early yolk-sac larvae when challenged by bath emersions, resulting in significantly reduced survival by 3 days post hatch (DPH) (Verner-Jeffreys et al. 2006). Concern of excessive bacterial growth is not limited to disease as overgrowth itself may be detrimental to respiration of developing embryos (Hansen and Olafsen 1989).

Other studies have further demonstrated that the larval fish GIT is initially colonized by bacteria before the onset of exogenous feeding and the introduction of live feed organisms from either their external surfaces or from ambient culture water (Bergh et al. 1992, Douillet and Holt 1994, Reitan et al. 1998, Hansen and Olafsen 1999, Lee 2003). Utilizing yolksac larvae have been observed to uptake and retain bacteria in their rudimentary guts (Olafsen 1984, Reitan et al. 1998). Yolksac larvae have been observed to ‘drink’ fluorescent dyed water before complete formation of an open mouth and rectum with the onset of exogenous feeding, demonstrating that rudimentary stomodeum and proctodeum sphincters are functional before they are readily detected by gross analysis (Tytler and Blaxter 1988a, 1988b). Yolksac larvae have also been observed to

uptake antigens from bacteria, which may be related to early ontogeny of immune systems and bacterial recognition (Olafsen and Hansen 1992).

In order to maximize early larval survival via reduction of opportunistic pathogens, a number of investigations have been conducted in order to determine the efficacy of surface disinfection or sterilization of marine finfish eggs. Egg sterilization/disinfection has become standard in protocols aimed at obtaining microbial management control within incubation systems (Vadstein et al. 1993, Douillet and Holt 1994, Skjermo and Vadstein 1999, Hansen and Olafsen 1999, Brock and Bullis 2001, Benetti et al. 2008a, Benetti et al. 2008b). Furthermore, egg disinfection/surface sterilization has been considered necessary for maximizing successful inoculation with probiotic bacteria via reduction, or complete elimination, of bacteria competing to colonize egg surfaces (Douillet and Holt 1994). At any rate, the incorporation of quick and efficient bath disinfection in hatchery protocols would impose a "hygiene barrier" between broodstock and incubation and/or larval rearing hatchery areas (Verner-Jeffreys et al. 2007), reducing the probability of vertical transmission of a number of pathogens.

Antibiotics (Oppenheimer 1955, Shelbourne 1963, Alderman 1988), malachite green (Alderman 1988, Sudova et al. 2007), benzalkonium chloride (Douillet and Holt 1994), iodine (Douillet and Holt 1994, Salvesen and Vadstein 1995, Katharios et al. 2007), bronopol (Treasurer et al. 2005), glutaraldehyde (Harboe et al. 1994, Salvesen and Vadstein 1995, Escaffre et al. 2001, Morehead and Hart 2003, Katherios et al. 2007), chloramine-T (Salvesen and Vadstein 1995), sodium hypochlorite (Douillet and Holt 1994, Salvesen and Vadstein 1995), formalin (Douillet and Holt 1994, Tamaru et al. 1999, Benetti et al. 2008a, Benetti et al. 2008b), thimerosal (Douillet and Holt 1994), and

hydrogen peroxide (Douillet and Holt 1994, Peck et al. 2004, Verner-Jeffreys et al. 2007, Wagner et al. 2008) have all been utilized as disinfecting or sterilizing agents during egg incubation with varying degrees of success. Utilization of most of these treatments would most likely cause backlash regarding worker and environmental safety.

Malachite green is known to be highly mutagenic and teratogenic. Concerns of toxicity towards fish, workers administering treatments, the environment at large, and the bioaccumulation of malachite green and its direct metabolites within fish tissues has prompted many governments, including the United States, Canada, and the European Union, to prohibit its utilization in aquaculture (Anderson et al. 2004, Sudova et al. 2007). Although it is physiological intermediary metabolite commonly found in mammals, formalin (aqueous 40% formaldehyde solution) can be toxic at high concentrations (Pandey et al. 2000) and studies link chronic exposure to increased risk of cancer (Libeling et al. 1984, Hauptmann et al. 2003). Despite these risks, formalin is approved by the United States Food and Drug Administration (USFDA) for treatment of Spaprolegniacea fungal infection for all finfish species (USFDA 2009). Similarly, the carcinogenic properties of glutaraldehyde also raises concern for worker safety (Ballantyne and Jordan 2001, Zeiger et al. 2005) as well as waste disposal and environmental harm associated with its use (Leung 2001, Emmanuel et al. 2005). Though it is a common treatment in aquaculture experiments, it has not been approved as a commercial treatment (USFDA 2009).

Hydrogen peroxide has been approved for utilization by the aquaculture industry; although the labeled use does not apply to marine species, veterinarians may approve extra-label to these species (New Animal Drugs; Hydrogen Peroxide 2007). Besides the

obvious advantage of legally approved use, hydrogen peroxide is considered a relatively safe compound in terms of worker and environmental safety. However, it's high oxidative potential warrants caution from those handling it (USGS 2007). It is also readily accepted by the general public as a 'safe' chemical, most likely due to its familiarity as an over-the-counter surface disinfectant for minor injuries. Hydrogen peroxide is ubiquitous in the environment and upper layers of the ocean, is a by-product of oxidative metabolism, is produced by a wide variety of organisms as a natural bactericide (Douillet and Holt 1994). Furthermore, it is stable at normal operating temperatures, and does not leave unwanted residues in fish tissues or hatchery effluents (Douillet and Holt 1994).

Hydrogen peroxide has been successful used as a egg surface disinfectant in a number of studies. Douillet and Holt (1994) achieved successful surface sterilization of red drum *Sciaenops ocellatus* eggs when utilizing a 3% solution bath for 5 minutes of exposure which lead to axenic larvae at hatching. Timing of treatments was found to significantly influence later survival, with early tail-free and tail-free stages yielding the highest survival (Douillet and Holt 1994). Similar trials conducted on yellowtail snapper (*Ocyurus chrysurus*) and spotted seatrout (*Cynoscion nebulosus*) detected 1 and 2% concentrations, respectively, as the limits these species could withstand without reducing hatchability and 24 hr survival (Douillet and Holt 1994). Of the group of disinfectants tested, Douillet and Holt (1994) concluded hydrogen peroxide was the most desirable to work with and the only treatment that resulted in sterilized egg surfaces, which provided a best-case scenario for maximal success of inoculation with probiotic bacteria.

In a comparative study of the effects on the survival and disinfection of cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) eggs of differing disinfectants, 5 minute 3% hydrogen peroxide bath treatments yielded statistically similar hatching success as other treatments and controls, indicating little or no toxicity to the developing embryos (Peck et al. 2004). Although complete sterilization was not achieved, this treatment led to the second highest reduction of egg-surface colonized microbes and ranked second to the highly lethal 0.1% sodium hypochlorite treatments (Peck et al. 2004). Variable survival results for both species and at different egg development stages of each species further indicate successful application of hydrogen peroxide is both species-specific and developmental stage specific (Peck et al. 2004).

Similarly, hydrogen peroxide has been demonstrated to 'effectively,' although not completely, sterilize both amberjack *Seriola rivoliana* and Pacific threadfin *Polydactylus sexfilis* eggs when used as 1.1% (w/v) bath treatments for 5 minutes exposure time (Verner-Jeffreys et al. 2007). Bath concentrations of 3.4% compromised Pacific threadfin hatching success, but amberjack hatching success was not significantly different than controls if treated approximately three hours post fertilization at this concentration (Verner-Jeffreys et al. 2007).

Hydrogen peroxide is a good candidate as an inexpensive, reliable, and relatively safe treatment for the reduction and elimination of bacteria colonizing the surface of marine finfish eggs. Variability in the tolerance among species, as well as timing and concentration of treatment, are all important factors to consider for successful application. The purpose of the following study was to investigate the effects of hydrogen peroxide on survival and the ability to achieve sterilization of cobia eggs.

2.2) Materials and Methods

These trials predominately emulated procedures described in Douillet and Holt (1994). During the 2007 season, a total of five trials utilizing hydrogen peroxide as an egg surface sterilization agent were initiated; three of these yielded results that were deemed acceptable for further analysis and are included here. Eggs were collected within hours or early during the morning after a spawning event from broodstock held at the University of Miami Experimental Hatchery (UMEH). Collected eggs were assessed for fertilization and deemed acceptable for experimental use and stocked in 1,000 L incubators. Egg Sterilization 1 (ES1) was conducted approximately 18 hours post spawning (HPS); immediately preceding treatment the eggs were observed to have reached late stage embryonic development. Egg Sterilization 2 (ES2) and Egg Sterilization 3 (ES3) were both treated approximately 14 hours after being spawned; these eggs had progressed to early to mid tail-free stage embryo stages.

Eggs were treated within 250 mL separatory funnels; this procedure aided the experimental process by allowing rapid draining of treatment solution when once eggs were allowed to float to the solution surface. Separatory funnels were first sterilized by submergence in a 10% HCl bath for 5 min, followed by a rinse with sodium bicarbonate and tap water solution to neutralize the acid. Treatment seawater was prepared in bulk by disinfecting 10 μm filtered seawater for 30 min with sodium hypochlorite, which was then neutralized via sodium thiosulfate. Disinfected seawater of the appropriate volumes was then transferred via acid-sterilized, as previously described, graduated cylinders to experimental separatory funnels. Volumes of Perox-Aid (FDA approved 35% [w/w] hydrogen peroxide solution; Western Chemical Inc., Ferndale, WA, USA) hydrogen

peroxide necessary to yield final desired concentrations were added to immediately prior to egg sterilization in order to minimize reductions in treatment concentration. Hydrogen peroxide treatment concentrations included 1% (10,200 mg/L), 2% (20,400 mg/L), 3% (30,600 mg/L) and 4% (40,800 mg/L); each treatment concentration was replicated four times (n=4).

Eggs were transferred to a graduated cylinder, allowed to concentrate on the air/water interface, then transferred to the separatory funnels via pipetting 3x 800 µL volumes of eggs to each funnel. These combined volumes achieved a total of ~750 eggs per funnel as was determined by repeated counts of average number of eggs per 800 µL volume. This process was conducted rapidly in order to minimize exposure to reduced dissolved oxygen which most likely resulted from high concentration of eggs. Addition of the eggs to each funnel constituted initiation of the duration of each sterilization treatment. Each replicate and treatment concentration was conducted with a total contact time of 5 min. Immediately after egg addition and at the 3 min mark, each funnel was swirled in order to better ensure complete contact of the egg surface by hydrogen peroxide. After the 5 minute mark, the funnels were drained as completely as possible before loss of eggs occurred, refilled with 250 mL of disinfected seawater, swirled and allowed to settle for 2 min, drained, and refilled with another 250 mL of disinfected seawater. Staggering the timing of each replicate by one minute eased the ability to manipulate each replicate individually while minimizing total time required per treatment.

Treatments were applied sequentially, starting with the strongest concentration and progressing through successively weaker concentrations. Given the results of other

investigations, it was suspected that stronger concentrations would be more deleterious. Thus, application of the strongest concentration required optimal egg resistance to exposure in order to maximize survival. Initiation of each treatment concentration utilized a fresh batch of eggs collected from the main storage incubator tank. After addition of eggs to each replicate treatment separatory funnel, eggs remaining in the graduated cylinder were discarded.

Upon completion of the sterilization and rinsing procedures, a small sample (10 eggs) were transferred via pipette, which was fitted with a sterilized tip, to a test tube containing 10 mL of Tryptic Soy Broth (TSB) (prepared according to manufacturer's instructions). Within each treatment, for each replicate a corresponding TSB test tube was utilized in order to assess the success of sterilization as a pass/fail response. Broth test tubes were monitored daily for sterilization effectiveness for a total of seven days. Sterilization was considered unsuccessful if the broth developed an opaque nature or deposition of dead microorganisms was observed.

Treated eggs were then stocked into previously disinfected (sodium hypochlorite neutralized with sodium thiosulfate) static water 15 L tanks housed within a flowing water bath. Moderate aeration maintained dissolved oxygen (DO) levels while also providing water mixing. After hatching, expelled egg chorions were siphoned from the tank bottom; thereafter tanks were bottom siphoned daily. The tanks were monitored daily and water quality parameters (water temperature, dissolved oxygen, pH, and salinity) were recorded. Survival was assessed on the evening 2 days post hatch (DPH) via either volumetric sub-samples or direct counts, depending upon larvae concentration.

Statistical analysis was completed using Microsoft Excel (Microsoft, Redmond,

WA, USA) and Stata Release 10 (StataCorp LP, College Station, TX, USA). Before statistical analysis was preformed, survival data relative to differing parameters was plotted in order to investigate trends in the data.

Percent survival data was collected as percentages of numbered initially stocked in each incubation tank assuming 750 eggs per replicate. Percentage data was arcsine transformed in attempt to normalize the binomially distributed percentage data. The resulting values were assessed for normality utilizing Shapiro-Wilk or Shapiro-Francia tests, as necessary, and equivalence of variances were assessed with Bartlett's tests (Zar 2009). Linear regressions were fit to arcsine transformed survival by HPS for each concentration treatment; residuals were then assessed for normality and variance (Zar 2009).

Cases where parametric assumptions were met were analyzed with analysis of variance (ANOVA) tests. These methods were first employed in a two-way ANOVA utilizing hydrogen peroxide (H_2O_2) concentration and HPS, with interaction, as continuous dependent factors. Further investigation of significant differences among all treatments and experiments employed Tukey Honestly Significant Differences (HSD) tests. Multi-factorial ANOVA tests incorporating treatment level, HPS, HPS and treatment interactions, and water quality parameters of incubation tanks recorded immediately after stocking were investigated as well. Non-normally distributed and/or highly heteroscedastic survival data was analyzed utilizing non-parametric Kruskal-Wallis ranked data differences of location tests and differences between means were detected post-hoc via non-parametric multiple means comparisons by simultaneous test procedure (Sokal and Rohlf 1995).

Further ANOVA testing investigated differences within ES2 and ES3, which were found to be statistically similar, thus warranting pooled analysis. ES1 was analyzed separately due to significant interactions of HPS and H_2O_2 concentration. Post-hoc means comparison testing was carried out using either Dunnett's Test (for parametric data) or Dunn's non-parametric means comparison for non-equal sample sizes, depending on the ability of the data to meet the basic assumptions of parametric statistical testing (Zar 2009).

Water quality data was also analyzed for significant differences between treatments on individual days. Individual water quality parameters were first analyzed for normality and homogeneity of variance. Further means testing employed either one way ANOVA or Kruskal-Wallis rank tests as appropriate. When necessary, post-hoc comparison of means tests (either non-parametric or Tukey HSD tests where appropriate) were then employed to detect which treatment means differed.

2.3) Results

Survival sterilization efficacy results are listed in Table 2.1. Table 2.2 presents the water quality data recorded during each trial. The goal of surface sterilization of eggs with 3% solutions of H_2O_2 was not 100% successful (Table 2.1); the one trial which included a 4% solution did yield sterilization, although these results cannot be verified due to lack of repetition in other experiments. Initial investigation for trends in survival data (Table 2.1) revealed readily observable differences among control and treatments (Figure 2.1). Within controls, there were also readily detectable differences in survival between HPS. Plotting of control survival against HPS by treatment (Figure 2.2a)

revealed a trend of increasing survival with increasing HPS. Conversely, decreasing survival trends were observed for 2% and 3% H_2O_2 treatments (Figure 2.2b). As previously noted, lack of replication at differing HPS prevented any conclusions of survival for trends at 4% concentration.

Linear regression of these trends indicated significant fits (Table 2.4) for control ($P < 0.001$) and 2% treatments ($P < 0.001$), yielding increasing and decreasing survival with increasing HPS for control and 2% concentration, respectively. The 3% treatment level did not yield a regression coefficient significantly different from zero ($P = 0.8859$), indicating that observed survival in all three trials was statistically similar irrespective of timing of sterilization treatment. Furthermore, violation of the assumption of normality of residuals discredited utilization of the regression for the 3% concentration treatments. Regressions for control and 2% concentrations were found to not violate regression assumptions.

Given these results, a two-way ANOVA model factoring H_2O_2 concentration, HPS, and their interaction was applied to arcsine transformed survival, yielding a highly significant model fit ($P < 0.001$). Despite detecting highly significant ($P < 0.001$) results for each factor, the highly significant interaction term prevented interpretation of individual factor affects (Table 2.4). Post-hoc means comparisons utilizing a Tukey HSD test revealed significant differences among similar H_2O_2 concentration between trial ES1 and trials ES2 and ES3, though not between similar H_2O_2 concentration between ES2 and from ES2 and ES3 (Figure 2.3). These results indicated the necessity to separate analysis of ES1 from ES2 and ES3 in order eliminate the interaction affect. The latter two trials

were found to not yield significantly different survival respective of treatment and thus were pooled for further analysis (Figure 2.3).

Failure of multiple treatment concentration's arcsine transformed survivals to meet the assumptions of parametric analysis, predominantly those H_2O_2 concentrations which resulted in minimal or no survival, required the utilization of non-parametric methods. Analysis of Trial ES1 detected a moderately significant ($P = 0.015$) difference in survival among treatments (Table 2.5). Further post-hoc pair-wise testing utilizing a one-tailed non-parametric version of a Dunnett's test yielded significant differences between the control and against both 2% and 3% treatments, indicating higher survival in the control treatment (Table 2.6).

Similar non-parametric analysis was conducted on the pooled results from trials ES2 and ES3 yielding highly significant ($P < 0.001$) differences among the treatments (Table 2.7). Further pair-wise analysis required utilization of Dunn's non-parametric multiple comparison test, modified for unequal sample sizes. One-tailed significant differences were detected among control and the 3% and 4% treatments but a marginally non-significant result was detected between the control and 2% treatments (Table 2.8). The latter lack of significance was considered to be an artifact of the power of the non-parametric statistical test utilized, as real differences were suspected to exist. After confirmation of parametric assumptions, an equal variance t-test revealed a highly significant difference ($P < 0.001$) between the control and 2% treatments (Table 2.9).

Analysis of daily water quality parameters between H_2O_2 concentration treatments within trials revealed minimal differences; only those instances yielding significant differences are presented. During trial ES2, DO concentrations on 2 DPH

were found to significantly differ (P = 0.012), and, on 2 DPH during trial ES3, pH readings were found to significantly differ (P = 0.015) (Table 2.10). Multi-factorial ANOVA testing of arcsine transformed survival including the initial water quality parameters recorded immediately after stocking of treated eggs into experimental incubation tanks did not improve model fit, nor were any of these added factors significant among trials (results not presented).

Table 2.1: Summery of survival results and sterilization efficacy from the three different experiments. Mean survival ± standard deviation was computed from 4 replicates per treatment. Percent sterilization is percentage of TSB samples not demonstrating bacterial growth after a 7 day incubation period.

Experiment	H_2O_2 Concentration	Mean Survival (± S.D.)	Survival (arcsine transformed) (± S.D.)	Percent Sterilization*
ES1	0 %	77.40 ± 12.79	62.23 ± 8.63	0
	2 %	0.12 ± 0.23	0.97 ± 1.95	0
	3 %	0.04 ± 0.08	0.56 ± 1.11	100
ES2	0 %	35.50 ± 9.88	36.43 ± 5.96	0
	2 %	6.73 ± 4.65	14.44 ± 5.00	0
	3 %	0 ± 0	0 ± 0	75
	4 %	0 ± 0	0 ± 0	100
ES3	0 %	39.25 ± 7.18	38.74 ± 4.22	0
	2%	3.23 ± 3.23	13.68 ± 4.04	0
	3%	0.25 ± 1.44	0.50 ± 1.44	75

* Percent sterilization calculated from 4 broth test tubes per treatment (1 per replicate within treatments) per experiment

Table 2.2: Summery of mean ± standard deviation daily water quality recorded from the three experiments as computed from 4 replicates per treatment.

Trial	DPH	H_2O_2 Concentration	Temperature (°C)	DO (mg/L)	pH	Salinity (ppt)
ES1	0	0 %	32.8 ± 0.5	5.70 ± 0.04	8.10 ± 0.02	35 ± 0
		2 %	32.5 ± 0.6	5.66 ± 0.12	8.12 ± 0.15	35 ± 0
		3 %	32.8 ± 0.5	5.75 ± 0.03	8.13 ± 0.03	35 ± 0
	1	0 %	30.0 ± 0.0	5.85 ± 0.03	8.15 ± 0.02	35 ± 0
		2 %	30.0 ± 0.0	5.82 ± 0.20	8.16 ± 0.04	35 ± 0
		3 %	30.0 ± 0.0	5.92 ± 0.11	8.16 ± 0.03	35 ± 0
	2	0 %	30.0 ± 0.0	5.87 ± 0.03	8.14 ± 0.02	35 ± 0
		2 %	30.0 ± 0.0	5.86 ± 0.05	8.16 ± 0.03	35 ± 0
		3 %	30.0 ± 0.0	5.84 ± 0.06	8.15 ± 0.03	35 ± 0
ES2	0	0 %	32.8 ± 0.0	5.71 ± 0.03	7.82 ± 0.02	36 ± 0
		2 %	32.8 ± 0.1	5.70 ± 0.00	7.82 ± 0.03	36 ± 0
		3 %	32.8 ± 0.0	5.71 ± 0.02	7.80 ± 0.05	36 ± 0
		4 %	32.7 ± 0.1	5.73 ± 0.10	7.79 ± 0.05	36 ± 0
	1	0 %	31.0 ± 0.1	5.58 ± 0.12	7.91 ± 0.04	36 ± 0
		2 %	30.9 ± 0.0	5.20 ± 0.33	7.83 ± 0.06	36 ± 0
		3 %	30.9 ± 0.0	5.54 ± 0.17	7.91 ± 0.03	36 ± 0
		4 %	30.9 ± 0.1	5.59 ± 0.13	7.86 ± 0.05	36 ± 0
	2	0 %	31.9 ± 0.1	5.53 ± 0.13	7.67 ± 0.07	36 ± 0
		2 %	31.9 ± 0.1	5.74 ± 0.07	7.75 ± 0.14	36 ± 0
		3 %	31.9 ± 0.1	5.62 ± 0.10	7.71 ± 0.04	36 ± 0
		4 %	31.9 ± 0.2	5.28 ± 0.27	7.63 ± 0.68	36 ± 0
ES3	0	0 %	29.1 ± 0.1	6.12 ± 0.04	7.94 ± 0.03	37 ± 0
		2 %	29.1 ± 0.1	6.13 ± 0.03	7.92 ± 0.04	37 ± 0
		3 %	29.1 ± 0.1	6.17 ± 0.04	7.93 ± 0.05	37 ± 0
	1	0 %	30.4 ± 0.1	5.72 ± 0.09	7.90 ± 0.03	37 ± 0
		2 %	30.4 ± 0.1	5.66 ± 0.05	7.88 ± 0.03	37 ± 0
		3 %	30.4 ± 0.1	5.68 ± 0.20	7.77 ± 0.06	37 ± 0
	2	0 %	30.1 ± 0.1	5.55 ± 0.04	7.81 ± 0.07	37 ± 0
		2 %	30.2 ± 0.1	5.59 ± 0.04	7.83 ± 0.03	37 ± 0
		3 %	30.2 ± 0.1	5.62 ± 0.08	7.83 ± 0.06	37 ± 0

Figure 2.1: Percent survival of experiments ES1 (○), ES2 (◊), and ES3 (□) plotted against treatment concentration.

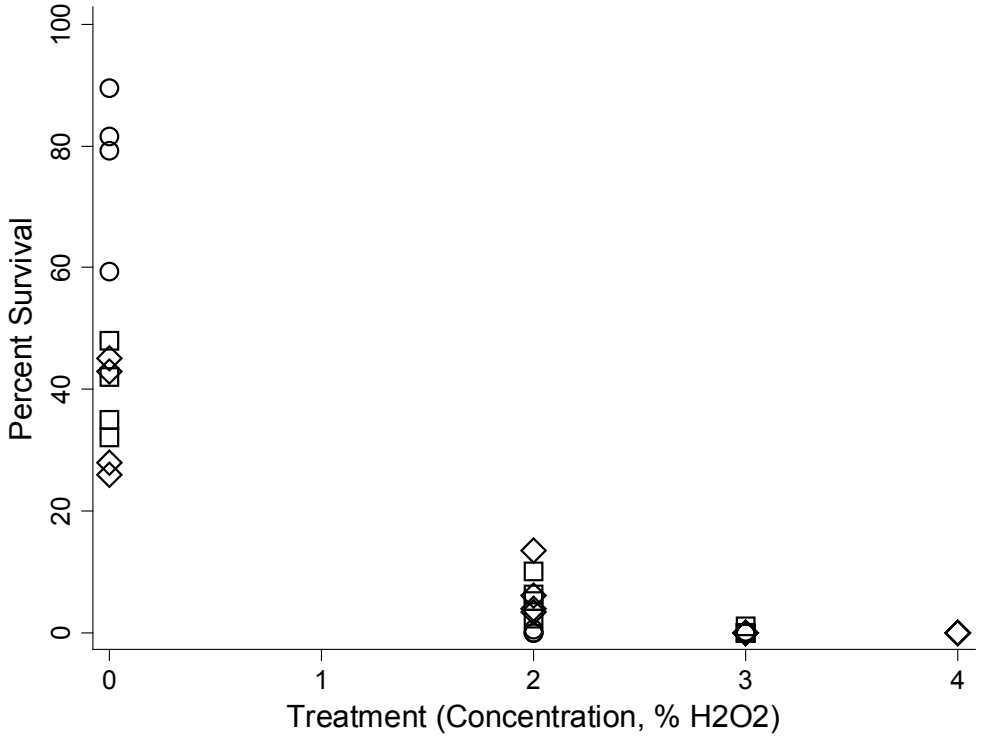
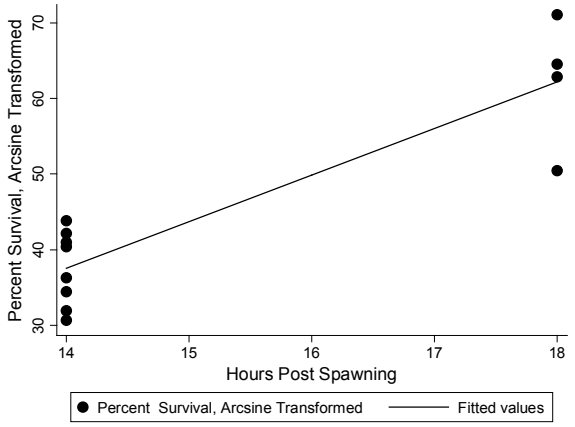
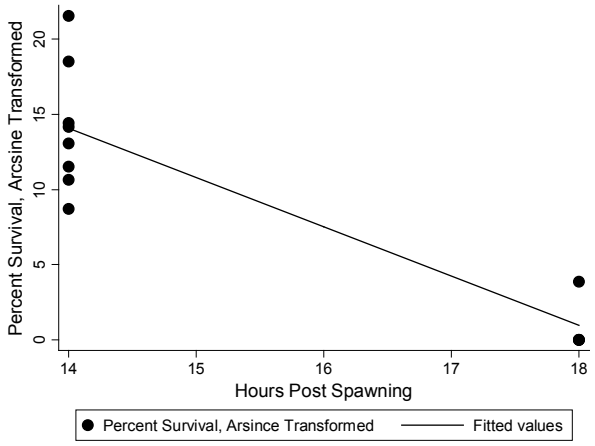


Figure 2.2: Arcsine transformed percent survival of a) control (0% H_2O_2), b) 2% H_2O_2, treatment, and c) 3% H_2O_2 treatment plotted against HPS, a proxy of egg development stage, and resulting trend lines.

a)



b)



c)

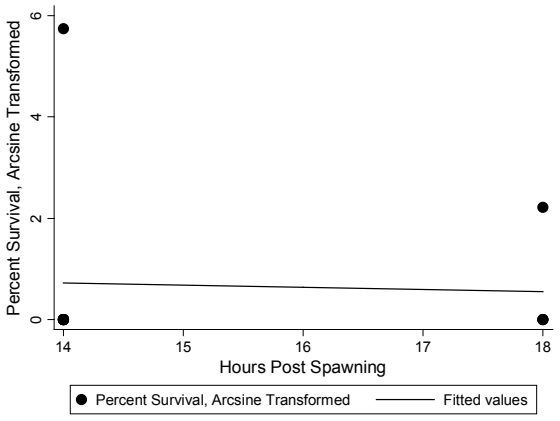


Table 2.3: Results of linear regressions of arcsine transformed survival and HPS within treatment which resulted in significant regressions for control and 2% treatments. A lack of significant regression for the 3% H_2O_2 concentration indicates that treatment survival is similar irrelevant of HPS. Furthermore, this regression should be considered invalid as residuals were found to be non-normally distributed after analysis utilizing a Shapiro-Francia test ($z = 3.48$, $df = 12$, $P < 0.001$).

Treatment	Regression Coefficient ± Std. Err.	Constant ± Std. Err.	Regression F Value	Regression P Value	Adjusted Coefficient of Determination
0%	6.16 ± 0.96	-48.6 ± 14.85	41.06	0.0001	0.785
2%	-3.27 ± 0.57	59.86 ± 8.74	33.46	0.0002	0.747
3%	-0.41 ± 0.28	1.29 ± 4.27	0.022	0.8859	-0.098

Table 2.4: Results from two-way ANOVA testing of ES1, ES2, and ES3 factoring treatment, HPS, and their interaction effects on survival, arcsine transformed.

Source of Variation	df	SS	F Value	P Value
Model	3	15699.36	99.63	< 0.001
Treatment	1	833.23	15.86	< 0.001
HPS	1	1385.10	26.37	< 0.001
Treatment*HPS	1	1806.58	34.39	< 0.001
Residual	36	1806.58		

Figure 2.3: Histogram of percent survival ± standard deviation. Letters denote statistically similar groups as determined by Tukey HSD test performed on arcsine transformed survival (results presented in Table 2.4).

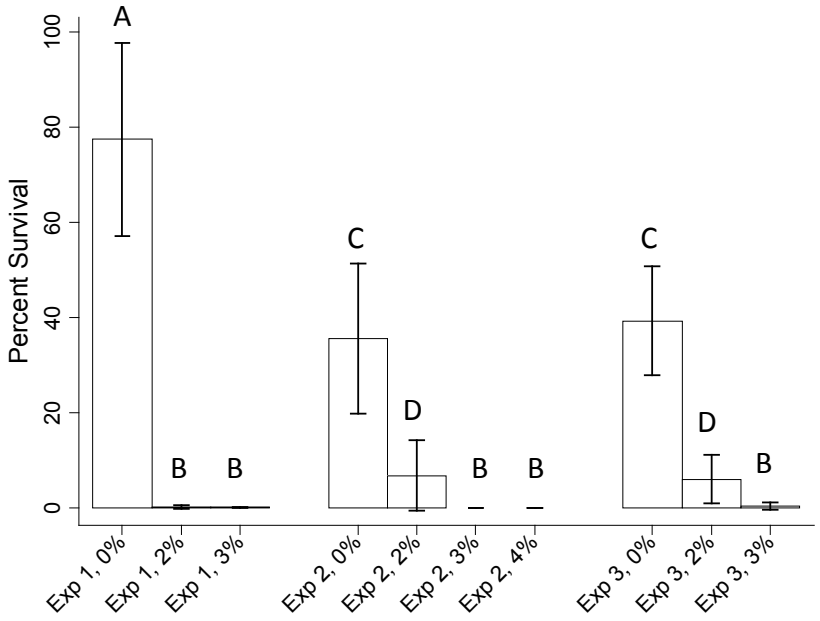


Table 2.5: Results of Kruskal-Wallis rank-sum means comparison, adjusted for tied data, of treatment effect on survival from ES1 (critical $\chi^2_{\alpha = 0.05, 2} = 5.991$). Significance indicated differences in survival among the treatments.

Treatment	n	Rank Sum	χ^2 Computed (w/ ties)	P value
Control	4	42.0	8.425	0.015
2%	4	18.5		
3%	4	17.5		

Table 2.6: Results of Dunnett-style nonparametric equal sample size control and treatment means comparison test (Critical $q_{\alpha = 0.05(1), \infty, 3} = 1.92$) from trial ES1 arsine transformed survival data. Rejection of the null hypothesis indicated significant differences in survival between control and each treatment.

Comparison	Difference of Rank Sums	Standard Error	$q_{computed}$	Conclusion
control vs. 2%	23.5	10.12	2.304365	Reject H_O
control vs. 3%	24.5	10.12	2.402423	Reject H_O

Table 2.7: Results of Kruskal-Wallis rank-sum means comparison, adjusted for tied data, of pooled survival results from trials ES2 and ES3 (critical $\chi^2_{\alpha = 0.05, 3} = 7.815$). Highly significant results indicated differential survival among the treatments.

Treatment	n	Rank Sum	χ^2 Computed (w/ ties)	P value
Control	8	196	25.184	< 0.001
2%	8	132		
3%	8	54		
4%	4	24		

Table 2.8: Results of Dunn's non-parametric post-hoc multiple comparisons test modified for unequal sample size and adjusted for data containing tied values (Critical $Q_{\alpha = 0.05(1),4} = 2.128$). Significant results were detected between control and both 3% and 4% treatments, but not with the 2% treatment.

Comparison	Difference of Mean Ranked Sums	Standard Error	$Q_{computed}$	Conclusion
control vs. 2%	8	3.987248	2.006396	Accept H_O
control vs. 3%	17.75	3.987248	4.451692	Reject H_O
control vs. 4%	18.5	4.883362	3.634791	Reject H_O

Table 2.9: Results of one-tailed t-test between control and 2% H_2O_2 treatment arcsine transformed survival pooled from both ES2 and ES3 ($t_{\alpha(1),14 = 0.001} = 3.787$). Contrary to the results of the post-hoc pair-wise comparisons presented in Table 2.8, parametric testing indicates highly significantly different survival between these treatments.

Treatment	n	Mean Survival	SD	t value	P value
control	8	37.59	4.94	10.24	0.001
2%	8	14.06	2.23		

Table 2.10: Results of a) one-way ANOVA test examining DO from trial ES2, on 2 DPH ($F_{\alpha = 0.05(1), 3, 15} = 3.49$), and b) Kruskal-Wallis test examining pH from trial ES3, 2 DPH ($\chi^2_{\alpha = 0.05, 2} = 5.991$).

a)

Source of Variance	df	Partial SS	Mean Squares	F Value	P Value
Between Treatments	3	0.45	0.15	5.72	0.012
Error	12	0.31	0.026		
Total	15	0.76	0.050		

b)

Treatment	N	χ^2 Value (w/ ties)	P Value
Control	4	8.44	0.015
2%	4		
3%	4		

2.4) Conclusions

The initial goal of consistent surface sterilization of cobia eggs without compromising survival to 2 DPH was not achieved within these trials. These results are similar to other studies which report that this concentration level as not 100% effective (Peck et al. 2004, Verner-Jeffreys et al. 2007). In the current study, solutions of 3% hydrogen peroxide did not completely sterilize as two bacterial cultures exhibited signs of bacterial growth (one each in trials ES2 and ES3. Overall, this concentration was 75% effective for all replicates.

Efficacy of treatment was determined utilizing a growth/no growth criterion, and thus, degree of bacterial disinfection was not investigated. If it had been, perhaps the results would have shown microbial load reduced to a degree that could still be considered sterile for the practical purposes of probiotic inoculation. Even so, incomplete sterilization allows the possibility of pathogen transfer, which should be considered when if this treatment were utilized for shipment of 'sterilized eggs.' The observed incomplete sterilization could be a matter of contamination via airborne microbes or accidental introduction during sampling. However, attempts were made to minimize the possibility of contamination. Further precaution and incorporation of more sophisticated equipment, such as laminar flow hood utilization, could have substantially reduced contamination risk.

The 3% concentrations H_2O_2 necessary for surface sterilization resulted in minimal hatching and survival. Similarly, 2% and 4% concentrations also significantly reduced survival of cobia yolk-sac to 2 DPH, with 4% resulting in immediate embryo mortality and no observed hatching. Two-way ANOVA testing revealed significant

development stage (proxied by HPS) and concentration affects, as well as significant interaction of these factors (Table 2.4). Linear regressions utilized to further investigate this interaction revealed different trends in survival, depending upon treatment concentration. When comparing HPS affects within control treatments, experimental procedures occurring later in development (18 HPS) resulted in increased survival relative to middle stages of development (14 HPS). Conversely, in 2% concentrations, a significant negative trend in survival was detected when between 14 and 18 HPS treatment time. Treatment at 3% concentration did not result in a significant suggesting mortality from this treatment is similar irrespective of treatment timing, nor was the resultant regression was not statistically acceptable, thus precluding further consideration of the results of this statistical test.

Converse to the results of the current study, treatment with H_2O_2 concentrations of 3% were able to successfully surface sterilize red drum (*Sciaenops ocellatus*) eggs without compromising survival (Douillet and Holt 1994). However, yellowtail snapper (*Ocyurus chrysurus*) and spotted seatrout (*Cynoscion nebulosus*) survival were affected by treatments greater than 1% and 2%, respectively (Douillet and Holt 1994). Douillet and Holt (1994) concluded success of H_2O_2 sterilization treatments which do not significantly affect survival would be species, developmental stage, and duration of exposure specific.

Observation of egg developmental stage immediately before treatment revealed that in ES1, embryonic development was nearly complete and the eggs were near hatching. Meanwhile, in experiments ES2 and ES3, the eggs were observed to have only proceeded to the earlier tailbud and early tailbud-free developmental stage, which should

have resulted in maximal survival according to the results of Douillet and Holt (1994). Their study described a temporal 'window' during which germicidal treatment of red drum eggs was least detrimental to hatching success and survival through the yolksac stage (Douillet and Holt 1994). They also noted that this 'window' seems to be wider for hydrogen peroxide than other, more deleterious treatments that were tested (Douillet and Holt 1994).

Converse to the findings of Douillet and Holt (1994), the results of Verner-Jeffreys et al. (2007) suggest investigation of treatment effects on survival when treated at earlier embryonic stages could be warranted. Similar H_2O_2 sterilization methods were utilized to treat amberjack (*Seriola rivoliana*) and Pacific threadfin (*Polydactylus sexfilis*) eggs (Verner-Jeffreys et al. 2007). Concentrations of 0.5% and 1.1% H_2O_2 did not significantly affect Pacific threadfin survival, while concentrations up to 3.4% treatment did not significantly affect survival in amberjack eggs when treated within 3 HPS (Verner-Jeffreys et al. 2007). When treated 24 HPS, amberjack survival was reduced when treated with 3.4% concentrations but not by 1.1% and 0.5% solutions (Verner-Jeffreys et al. 2007). These results further substantiate both species-specific and developmental stage-specific survival responses to differing concentrations of hydrogen peroxide sterilization.

Stage-specific survival due to treatment with hydrogen peroxide has also been observed in Atlantic cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) when treated with 3% hydrogen peroxide (Peck et al. 2004). Treatment during developmental stage III (from first appearance of the germinal ring and embryonic axis to the closure of the blastopore [Laurence and Rogers 1976]) did not significantly reduce

survival (Peck et al. 2004). Cod survival after treatment was not significantly related to developmental stage alone, but an interaction of stage and treatment was significant; for haddock, significant survival effects were detected for both treatment and developmental stage, but not for their interaction (Peck et al. 2004).

Increased survival with increasing HPS within control treatments could be due to the more advanced embryo being more resilient to the handling and mechanical agitation experienced during the experimental process. As an embryo nears hatching, enzymes begin hydrolyze the inner chorion protein structure (Yamagami 1988). Perhaps at this stage physical agitation was less disruptive as the hatching process which would have already begun. However, the preceding logic is speculative as these processes have not been investigated for this species. Similarly, though not quantified, observation of malformed larvae (larvae resulting in a "kink" of the notochord) in 2% and surviving 3% treated yolk-sac larvae further support these survival results. Skeletal abnormalities such as malformations and curvature of the vertebral column are a common effect of sublethal toxin exposure during the egg stage (Von Westernhagen 1988).

Minimal significant differences in water quality parameters were detected within each experiment. These differences were suspected to not have produced a substantial biological impact nor affect survival, as for each significant difference that was detected, minimal actual differences were observed. DO concentrations were detected as significantly different on 2 DPH during experiment ES2. Further pair-wise means comparisons produced unclear results: the control treatment is not significantly different from any other treatment, yet 2 and 3% treatments were significantly different from the 4% treatment. The 4% treatment experienced high mortality, and even with siphoning,

this could have resulted in high biological oxygen demand caused by decaying particulate organic matter and/or dissolved organic matter. Significant differences were also detected among treatments for pH level on 2 DPH during trial ES3. ANOVA testing incorporating water quality parameters as independent factors influencing survival did not detect significant results, further substantiating the conclusion that actual differences did not substantially impact resulting survivals.

Further examination of hydrogen peroxide utilization, at least a disinfection agent for cobia eggs, is warranted. Even though the current results reveal significant reductions of survival even with 2% treatments, the aim of the current study was to produce sterile eggs, and thus 1% solutions were not tested. Perhaps this concentration would not significantly affect survival and would yield a comparable degree of disinfection as formalin treatments (Benetti et al. 2008a, Benetti et al. 2008b), thus yielding comparable treatments which are safer in terms of both the environment and employees. Verner-Jeffreys et al. (2007) noted 1% and 0.5% solutions reduced of CFU egg^{-1} to levels lower than those detected in both controls and mechanical removal of bacteria via rinsing with sterile seawater. Furthermore, not all cobia egg developmental stages were tested in these trials. Further experimentation could reveal that earlier egg stages could be hardier against treatment with hydrogen peroxide, similar to results reported by Peck et al. (2004) and Verner-Jeffreys et al. (2007) under certain conditions.

Further investigations should utilize experimental conditions that more accurately reflect real incubation procedures and equipment utilized by production hatcheries. Static water incubation tanks most likely hindered survival within all trials as water quality, in terms of dissolved oxygen and pH, were noted to degrade during the incubation period.

Although, these parameters stayed within limits that were safe for sub-tropical marine finfish larvae. Dissolved ammonia levels were not investigated and lack of water flow could have allowed concentration of this noxious byproduct.

In summary, for the embryonic developmental stages tested, concentrations of H_2O_2 necessary for surface sterilization (3% or higher) of cobia eggs induced high mortality irrespective of the timing of treatment. Similarly, 2% H_2O_2 concentrations significantly reduced survival, although significant evidence of hardiness of the 'tailbud' stage to sublethal hydrogen peroxide exposure was detected. Another trial not included in the present analysis suggested that 1% solutions could still be useful as a disinfecting treatment as survival and, though not quantified, could have resulted in survival comparable to controls. Since cobia eggs demonstrated differential susceptibility to hydrogen peroxide treatment at different developmental stages, further study of treatment of earlier egg stages could also yield more successful results. Sterilization was not consistently achieved with 3% hydrogen peroxide solutions; as with other studies, this could be a lack of effectiveness of this concentration or this could be due to external contamination to these experiments.

Chapter 3: Incorporation of Probiotics within Yellowfin Tuna *Thunnus albacares* Yolk-sac Larvae Shipping Protocols

3.1) Background

Due to high market demand and value, aquaculture of tuna species has been a subject of interest for the past few decades. Recent criticism of current ranching practices and depletion of wild stocks have intensified research efforts to consistently produce juveniles for growout operations. Japanese researchers have been studying tuna spawning and larviculture since the late 1970's (Kaji et al. 1996, Margulies et al. 2005, Masuma et al. 2008). Recent advances, such as the closing of the life cycle of Pacific bluefin tuna *Thunnus orientalis* (Sawada et al. 2005), have provided further inspiration. Similarly, considerable interest in spawning, larval production, and larval growth of yellowfin tuna (*Thunnus albacares*) has grown, with broodstock facilities in Bali, Japan, and Panama contributing to the knowledge of these processes (Margulies et al. 2005). These broodstock facilities could act as central seed stock suppliers for experimental larviculture trials conducted by other facilities, and thus successful closed-system shipment of yellowfin tuna yolk-sac larvae need to be developed.

Concerns during transport of live fishes include maintenance of high water quality and minimization of stress, which can act synergistically with degrading water quality to cause mortality, either during or post shipment (Harmon 2009). Water quality issues focus upon the maintenance of high dissolved oxygen (DO) concentrations while reducing toxic ammonia accumulation and buffering water from pH shifts. As bacterial probiotic technology develops, continued realization of its applicability to increasing numbers of aquaculture related activities occurs. While protocols and readily available chemical treatments address water quality issues, bacterial probiotics utilization could

improve protocols and provide further safeguards. However, a paucity of studies investigate probiotic utilization during shipping. Furthermore, as transport conditions continue to increase biomass loads in order to maximize profitability (Estudillo and Duray 2003, Benetti et al. 2007, Colburn et al. 2008), increasing safeguards to better ensure transport success are warranted.

Bacterial probiotics have been demonstrated to yield multiple benefits for targeted hosts, including innate immune system stimulation, stress reduction, control of bacterial communities, water quality improvement, and reduction of opportunistic and potentially pathogenic bacteria (Skjermo and Vadstein 1999, Gatesoupe 1999, Verschuere et al. 2000, Vine et al. 2006). Some authors narrowly define probiotics to include live microbial adjuncts, administered orally, which colonize the gastrointestinal tract (GIT) (Gatesoupe 1999, Vine et al. 2006) while others broaden the definition to include external colonization (Skjermo and Vadstien 1999) and/or water quality remediators which improve the host's environment (Verschuere et al. 2000). The present study considers the definition of probiotics to incorporate the latter, broader contexts.

One study has investigated the efficacy of nitrifying bacteria during transport of fishes (Turner and Bower 1982). Studies incorporating more recently developed probiotic blends which potentially provide more benefits than simply ammonia removal are lacking. *Bacillus* spp. are capable of direct ammonia uptake for use as a nitrogen source during amino acid synthesis (Stadtman and Ginsburg 1974, Donohue and Bernlohr 1981). Indeed, studies report *Bacillus* spp. having reduced ammonia concentrations when utilized as probiotic inoculates within open-container shipping protocols (Gomes et al.

2008, 2009) and recirculating aquaculture systems (Chen and Chen 2001, Lalloo et al. 2007).

Bacillus spp. probiotic additions could exhibit other potential beneficial effects on yolk-sac larvae during shipment by reducing stress during transport (Gomes et al. 2008, 2009), stimulation of innate immune response (Rengpipat et al. 2000, Guillan et al. 2004, Ziaei-Nejad et al. 2006, Aly et al. 2008), or reduction of pathogenic bacteria species loads (Moriarty 1998, Vaseeharan and Rasmasamy 2003, Guillan et al. 2004, Lalloo et al. 2007, Hill et al. 2009, Nakayama et al. 2009) and mortality associated when hosts are challenged with pathogens (Rengpipat et al. 1998, 2003; Vaseeharan and Rasmasamy 2003, Aly et al. 2008, Far et al. 2009).

This trial was designed without the utilization of available chemical water quality or temperature modulators to better detect effects on water quality and verify manufacturer's claims when incorporating a commercially available *Bacillus* spp. probiotic blend during shipment. Furthermore, the trial was designed in emulation of shipping conditions which would arise if a shipment lasted longer than anticipated and chemical water quality modulators became compromised.

3.2) Materials and Methods

A mock transport trial was conducted at the Inter-American Tropical Tuna Commission (IATTC) Achotines Laboratory (Las Tablas, Provincia de Los Santos, Panamá) utilizing yellowfin tuna *Thunnus albacares* yolksac larvae hatched from eggs naturally spawned by broodstock held at ambient temperature conditions at the same facility. Fertilized eggs were collected during late evening hours and incubation was

conducted in 300 L conical fiberglass tanks held at ambient temperature conditions (~26°C) as described in Margulies et al. (2005). Incubation systems were of a flow through design with 200%/day exchange; incoming seawater (salinity 31.0 ± 0.1 ppt) was previously filtered to 1 μm and ultraviolet light (UV) sterilized. Yolksac larvae were assumed to be healthy due to observation, though not quantification, of high hatching rate. Similarly, in an unrelated larval rearing trial utilizing larvae from the same spawn, early survival to first feeding was observed to be high; though not constituting as a true negative control, these observations support the assumption of healthy yolksac larvae.

On one day post hatch (dph), yolk-sac larvae were concentrated within incubation tanks and volumetric subsampling was utilized to determine larvae concentration. Transport packing was initiated by addition of 3 L of ambient temperature, 1 μm filtered and UV sterilized seawater to each experimental polyethylene shipping bag. Larvae were then transferred to each shipping bag via 3 L beakers; after larvae additions were complete, further ambient temperature filtered seawater additions yielded a final volume to 20 L per shipping bag. Probiotic treatments (n = 3) consisted of addition of 300 mL of EcoAqua (manufacturer information: *Bacillus subtilis*, *B. licheniformis*, *B. megaterium*, and *B. laterosporous* - Ecomicrobials LLC, Miami, Florida). This volume was assumed to produce a final inoculate in the bag of $1.5x10^6$ colony forming units (CFU)/mL (manufacturer information: 10^8 CFU/mL of EcoAqua). It was assumed the high concentration of inoculate would more rapidly achieve a climax bacterial community during the short duration of the trial. Control treatments (n = 3) did not receive a probiotic addition. Final yolk-sac larvae shipping density was 871 larvae/L.

Oxygen was added to shipping water until DO saturation was above 300%; temperature (°C), salinity (ppt), % DO saturation, and pH were then recorded. Care was taken to eliminate cross contamination while determining water quality parameters by first recording control, and then probiotic replicates. Water quality probes were rinsed between each replicate. Water samples for initial ammonia chemistry were then collected. Air within the shipping bags was expelled and replaced with pure gaseous oxygen. Bags were then secured, placed within an additional shipping bag which was also secured, and packaged individually within styrofoam coolers, which were then packed within cardboard boxes. A total of three replicates were utilized for control and probiotic treatments.

During the trial, transport boxes remained within an air-conditioned room held at 24 °C. Periodically, boxes were lightly shaken for 10 s in order to simulate physical disturbance during shipping. The trial was terminated 24 h after completion of packing. Upon opening of the shipping bags, temperature (°C), salinity (ppt), % DO saturation, and pH were recorded and water samples for final ammonia chemistry analysis were collected. Once water quality sampling was complete, volumetric subsamples of shipping water containing larvae were collected to assess survival. Total ammonia nitrogen (TAN) concentration (mg/L) of individual water samples was obtained via the salicylate method (Hach Company, Loveland, Colorado).

Water quality and survival data were statistically analyzed with Stata Release 10 (Stata Corp., College Station, Texas). Comparisons were made between treatments at similar time periods as well as between time periods within treatments. All data was first analyzed for normality and similarity of variance utilizing Shapiro-Wilk tests and F-tests,

respectively. Distributional analysis revealed multiple instances of violation of parametric assumptions; thus, further analysis of means utilized Wilcoxon rank-sum tests in order to simplify statistical analysis (Zar 2009). Significance was set to $\alpha = 0.05$ for all statistical testing. An 'Increase of TAN' (mg/L) variable was calculated to standardize for differing TAN concentrations (mg/L) observed during initial conditions. Un-ionized ammonia (NH_3) concentration (mg/L) and percent un-ionized ammonia ($\%NH_3$) were computed from TAN concentrations (mg/L) utilizing equations described by Bell et al. (2007, 2008). Percent DO saturation was converted to DO concentration (mg/L) by equations reported by Benson and Krause (1984). All other percentage data was arcsine transformed before further analysis (Zar 2009).

3.3) Results

Survival was high (> 80%) and statistically similar ($P > 0.05$) between control and probiotic treatments (Table 3.1). At initial conditions, no statistical differences were found for temperature (°C), salinity (ppt), DO concentration (mg/L), and pH ($P > 0.05$) between treatments (Table 3.1). Water chemistry within the treated closed shipping bags was differently affected by the addition of probiotics in comparison to controls. While both control ($P = 0.0369$) and probiotic ($P = 0.0463$) treatments experienced significant decreases in pH during the course of the study, final pH condition was significantly higher in the control treatment ($P = 0.0495$) (Table 3.1). Final temperatures were significantly higher in control treatment ($P = 0.0495$); however, both probiotic ($P = 0.0495$) and control ($P = 0.0495$) treatments exhibited significant differences between initial and final mean temperature within treatment (Table 3.1).

A significant difference ($P = 0.0495$) was detected between treatments for final DO concentrations (mg/L), with mean probiotic treatment DO concentration being much higher than the controls (Table 3.1). Expectedly, control treatment final mean DO concentration (mg/L) was significantly lower than initial concentrations ($P = 0.0495$). Interestingly, and converse to the trend observed in controls, probiotic treatment's final mean DO concentration was greater than at initial conditions; this difference was found to be significantly different ($P = 0.0495$) (Table 3.1). Both treatments resulted in non-significant decreases in salinity at final conditions. Although not statistically different, the mean final salinity in control treatments was lower than that of probiotics.

No significant difference was detected between treatments' initial mean TAN (mg/L), though control treatment's mean initial NH_3 concentration was significantly higher than that of probiotics ($P = 0.0463$) (Table 3.2). In order to eliminate potential bias, Increase of TAN (mg/L) was computed in order to standardize final TAN (mg/L) concentrations against differing concentrations present under initial conditions. A significant difference ($P = 0.0431$) in Increase of TAN (mg/L) was detected between treatments, with control treatments yielding the higher mean value (Table 3.2). Similarly, final TAN (mg/L) and final NH_3 (mg/L) concentrations both were significantly higher in control than probiotic treatments ($P = 0.0463$ and $P = 0.0495$, respectively) (Table 3.2). Furthermore, probiotic treatment mean concentrations of TAN (mg/L) did not significantly differ ($P > 0.05$) between initial and final conditions; however, control treatment TAN (mg/L) concentrations significantly increased ($P = 0.0339$). For NH_3 (mg/L), neither treatment significantly differed between initial and final conditions ($P > 0.05$) (Table 3.2). Final $\%NH_3$ was significantly higher ($P = 0.0495$) in the control than

in the probiotic treatment. Within control treatments, initial $\%NH_3$ was significantly greater than at final conditions ($P = 0.0369$), though the same trend was not observed for probiotic treatments ($P > 0.05$) (Table 3.2).

Table 3.1: Mean ± SD temperature (°C), salinity (ppt), DO (mg/L) and % survival for yellowfin tuna yolk-sac larvae mock shipping trial. Letters denote statistically different comparisons within individual columns.

Time Period	Treatment	Temperature (°C)	Salinity (ppt)	DO (mg/L)	pH	Survival
Initial	Control	26.7 ± 0.1 z	31.0 ± 0	20.3 ± 0.1 z	7.93 ± 0.00 z	-
	Probiotic	26.6 ± 0.2 y	31.0 ± 0	20.4 ± 0.2 y	7.93 ± 0.01 y	-
Final	Control	24.5 ± 0.1 z, x	30.3 ± 1.1	16.0 ± 0.7 z, x	7.48 ± 0.35 z, x	87.9 ± 0.05
	Probiotic	24.0 ± 0.1 y, x	30.7 ± 0.6	21.9 ± 1.2 y, x	7.34 ± 0.95 y, x	88.4 ± 0.07

Table 3.2: Mean ± SD ammonia water chemistry parameters TAN (mg/L), Increase in TAN (mg/L), NH_3 (mg/L), and $\%NH_3$. Letters denote statistically different comparisons within individual columns.

Time Period	Treatment	TAN (mg/L)	Increase TAN (mg/L)	NH_3 (mg/L)	$\%NH_3$
Initial	Control	0.3 ± 0.1 z	-	0.009 ± 0.003 z	3.1 ± 0.1 z
	Probiotic	0.1 ± 0.1	-	0.002 ± 0.004 z	1.0 ± 1.8[§]
Final	Control	0.7 ± 0.1 z, y	0.4 ± 0.1 z	0.007 ± 0.0007 y	1.0 ± 0.1 z, y
	Probiotic	0.2 ± 0.0 y	0.1 ± 0.1 z	0.001 ± 0.0003 y	0.7 ± 0.1 y

[§] This value is affected by the lack of detection of TAN in two of the three replicates; these zero values reduce the mean $\%NH_3$ when, for the one replicate which initially contained NH_3, the initial $\%NH_3$ was 3.1 %, the same as control treatments'

3.4) Conclusions

Upon termination of the trial after 24 h, no statistical difference was detected in survival between treatments and overall survival was high for all replicates. Lack of substantial mortality despite degrading water quality parameters was surprising, especially for control treatments. Consideration of interactions between the various water chemistry factors explains this intriguing outcome.

A significant difference in DO concentration (mg/L) between treatments was observed at final conditions. Similarly, higher DO concentration (mg/L) after transport of adult freshwater fishes treated with *Bacillus* spp. probiotics was reported by Gomes et al. (2008). This could have been associated with stress reduction for probiotic treated larvae which reduced oxygen consumption. Observation of increases in DO concentration (mg/L) in probiotic treatments could have been resultant from human error. However, the trends of substantial reduction in control DO and marginal increases in probiotic DO were consistent across all replicates within treatments, suggesting an actual trend.

Gomes et al. (2008, 2009) noted reduced stress, as measured by decreases in cortisol levels and reduced ion flux, with incorporation of *Bacillus* spp. probiotics during transport. In the present study, final mean salinity was reduced from initial conditions in both treatments, with the probiotic treatment remaining more similar to initial values; this is an intriguing result given the closed system nature of the trial. Although not presently investigated, perhaps stress response and ability to maintain hypoosmoregulation were improved in probiotic treated replicates.

The hypothamus-pituitary-interrenal (HPI) axis and related cortisol production of the extensively studied zebrafish *Danio rerio* is developed at hatching (Alsop and Vijayan 2009) and reactive stressors such as seawater exposure at 1 dph (Alderman and Bernier 2009) and handling stress at 2 dph (Alsop and Vijayan 2008). Similarly, cortisol production and an active HPI axis has been demonstrated in Asian seabass *Lates calcarifer* 4hr post hatching (Sampath-Kumar et al. 1995) and exposure to water-soluble crude oil fractions increases cortisol expression in 2 dph turbot *Scophthalmus maximus* yolksac larvae (Stephens et al. 1997). Cortisol is recognized to play important roles in osmoregulation via control of Na+/K+-ATPase activity, ionocyte size and density, and drinking rate (Varsamos et al. 2005) and has been demonstrated as necessary for hypoosmoregulation of larval summer flounder in seawater (Veillete et al. 2007). Thus, previously suggested stress remediation and improved osmoregulatory capabilities at this early developmental stage are plausible.

The observation of significantly different final temperatures between treatments seems implausible as the shipping boxes were stored together in a temperature controlled room. Possible explanations include human error during measurement or location of the shipping boxes in relation to air conditioning vents. Significant differences between initial and final mean temperatures were anticipated as ambient water temperature (~27 °C) lowered to match storage room air temperature (24 °C). Reductions in temperature are commonly found in real shipping situations where ice packs are utilized to cool shipping water temperature to the lower end of the transported organism's viable temperature range.

A significant difference in final pH condition was detected between treatments with the trend for lower pH occurring in probiotic treated shipment bags (Table 3.1). This difference likely resulted from the direct uptake of ammonia by probiotic bacteria resulting in differing H^+ concentrations. In control treatments, increased TAN concentrations could have led to increased uptake of free H^+ by NH_3, thus yielding the observed higher pH condition. *Bacillus* spp. have long been recognized to utilize multiple nitrogen sources, including both NH_3 and NH_4^+, for catabolism of proteins (Stadtman and Ginsburg 1974, Donohue and Bernlohr 1981).

Without buffer utilization, significant differences between initial and final pH condition were anticipated. This is readily explained by the metabolic production of CO_2 and subsequent carbonic acid (H_2CO_3) formation from reaction with water molecules (Millero 2002). Similar reductions in pH are typical of closed transport systems (Estudillo and Duray 2003, Benetti et al. 2007, Colburn et al. 2008). Final pH conditions (minimum observed pH = 7.23) were well above 24 hr LC_{50} values published for other larval fishes (Sparidae = 5.06, Soleidae = 4.51, Gadidae = 4.99) (Brownell 1980). The reduced pH potentially increased physiological stress; for example, carbonic anhydrase activity and related exchange of HCO_3^- as the waste product of respiration is reduced at lower pH (Brauner 2008).

Differences in initial TAN concentrations (mg/L) between treatments was most likely due to the action of bacterial probiotics upon the samples, as TAN analysis on these water samples was not conducted until completion of the shipment packing. Despite a lack of significant difference for initial TAN concentration (mg/L), a significant difference in initial NH_3 concentration (mg/L) was detected. The lack of consistent

significant difference across both variables suggests a marginal actual difference in initial ammonia chemistry between treatments.

Significant differences in final TAN concentrations (mg/L) and Increase in TAN (mg/L) were observed with the inclusion of *Bacillus* spp. probiotics (Table 3.2); similarly, NH_3 concentrations (mg/L) computed at the final conditions were significantly different between treatments. These reductions coincide with other reports of inclusion of *Bacillus* spp. probiotics in open-container transport situations (Gomes et al. 2008, 2009) and within recirculating aquaculture systems (Chen and Chen 2001, Lalloo et al. 2007).

The equilibrium TAN is partially dependent upon pH and temperature and NH_3 increases relative to NH_4^+ with increases in both influential factors (Johansson and Wedborg 1980, Bell et al. 2007, 2008). Within both treatments, NH_3 concentration (mg/L) did not significantly differ between initial and final concentrations. This is less surprising for probiotic treatments, where TAN concentrations (mg/L) did not significantly change between initial and final conditions. This observation must be considered in conjunction with the detection of significantly different $\%NH_3$ between initial and final conditions in the control treatment (Table 3.2). A similar trend would most likely have been observed for probiotic treatments; in the one replicate in which initial TAN was observed the $\%NH_3$ was calculated as 3.1 %, similar to that of control treatments. For both treatments, decreasing pH condition and temperature acted beneficially by reducing $\%NH_3$, and thus NH_3 concentrations (mg/L), despite increased final TAN concentrations (mg/L).

Despite high TAN concentrations (mg/L) in controls at final conditions (Table 3.2), significant differences between estimated survival were not detected (Table 3.1). Information regarding environmental TAN and NH_3 tolerance in early life stages of yellowfin tuna is not available. Wedemeyer (1997) suggested that NH_3 levels remain at or below 0.02 mg/L (1.17 μM) during intensive fish culture. Computed final NH_3 concentrations (control treatment range: 0.001 to 0.007 mg/L [0.06 to 0.4 μM]), were well below the chronic exposure values suggested by Wedemeyer (1997). Furthermore, a study of red drum *Sciaenops ocellatus* larvae exposed to NH_3 concentrations of 0.55 mg/L (32.3 μM) and higher caused 50% mortality within 24 to 48 hrs exposure (Holt and Arnold 1983).

Given the values from the later study being orders of magnitude higher than those calculated here, it is not surprising that survival was not immediately influenced. Physiological stress most likely resulted from the high TAN concentrations, including modification of amino acid metabolism, enzyme induction, impairment of ion exchange, and utilization of more energetically demanding ureoletic nitrogenous excretion (Mommsen and Walsh 1992, Randall and Tsui 2002, Terjesen 2008). Though immediate survival was not negatively impacted, post-trial survival nor growth were not monitored, and thus conclusions regarding observed survival must be made with caution.

It has been demonstrated in coldwater marine species that a peak in ammonia excretion occurs in the time period following hatching (Rønnestad and Fyhn 1993) due to release of ammonia accumulated during the embryonic stage (Terjesen 2008). Despite decreasing pH condition resulting in lowering NH_3 concentrations, lower pH also decreases ammonia toxicity tolerance and in seawater, NH_4^+ remains toxic due to the

enhanced permeability of fishes to this ion (Erickson 1985, Eddy 2005). These conclusions have been confirmed to apply to larval marine finfish (Miller et al. 1990).

This trial suggests a few of the potential benefits of probiotic incorporation during the simulated transport of yellowfin tuna yolk-sac larvae, including reduced TAN concentrations (mg/L), decreased oxygen utilization, and potentially decreased stress as suggested by reduction in mean salinity fluctuation. However, pH shifts related to probiotic utilization, resulting in both potentially positive and negative consequences, should not be ignored. In order to eliminate pH shifts and reduce this source of stress, further study determining proper buffer concentrations when yolk-sac larvae shipment incorporates probiotics is necessary. Investigation of bacterial proliferation and community succession during shipping as well as further study of bacterial colonization in early marine teleost larvae could detect additional benefits from probiotic incorporation during transport of yolk-sac larvae and other life stages. Current shipping protocols are successful without probiotic incorporation, but the marginal increases in cost could yield improvements and further safeguards to transport success and post transport survival and growth.

Chapter 4: Improvement of Rotifer Population Growth via Incorporation of Probiotics

4.1) Background

Traditionally, live feed organisms have been utilized as larval finfish diets during rearing (Watanabe et al. 1983, Lubzens et al. 1989, Lee and Ostrowski 2001). Despite attempts to develop microdiets to replace live feeds, variable success has been achieved, depending upon the species of finfish being cultured; thus, live feeds utilization remains a necessity (Lee 2003). Rotifers (*Brachionus* spp.) remain one of the most common live feed organisms utilized during larval finfish rearing (Watanabe et al. 1983, Lubzens et al. 1989, Lubzens and Zmora 2003). The necessity to continuously culture rotifers and the risk of culture collapse complicates larval finfish hatchery production (Hirayama 1987, Lubzens et al. 1989, Lubzens and Zmora 2003).

Bacterial community composition has long been recognized to influence rotifer culture stability and population growth (Gatesoupe et al. 1989; Yu et al. 1989, Skjermo and Vadstein 1993; Hagiwara et al. 1994, Douillet 2000a, 2000b, Rombaut et al. 2001, Tinh et al. 2006). Community composition influences factors such as water quality remediation, nutritional and vitamin supplementation, and presence of bacterial toxins (Yu et al. 1989, Rombaut et al. 1999, Douillet 2000a, Dhert et al. 2001, Rombaut et al. 2001, Rombaut et al. 2003).

Proactive microbial management, including bacterial probiotics incorporation, is becoming a popular avenue of research for improving rotifer population growth and culture consistency (Skjermo and Vadstein 1993, Rombaut et al. 1999, Douillet 2000a, Dhert et al. 2001, Lubzens et al. 2001, Rombaut et al. 2003, Planas et al. 2004). Bacterial probiotics have been noted to improve population growth rates (PGRs), inhibit

pathogenic bacterial population growth, or improve water quality (Rombaut et al. 1999; Gatesoupe 1999; Douillet 2000a, 2000b, Rombaut et al. 2003, Planas et al. 2004). Verification of commercial product efficacy is necessary, as these products may not always deliver the purported benefits (Douillet 2000a).

Few studies have investigated incorporation of *Bacillus* spp. probiotics within rotifer production cultures; however, the majority of them reported benefits from probiotic utilization (F. J. Gatesoupe, INRA-IFREMER, Unpub. Data; Hirata et al. 1998; Gatesoupe 1999; Douillet 2000a). Contrary to this generalization, Hagiwara et al. (1994) noted a relative reduction in PGR with the addition of a single *Bacillus* sp. to rotifer cultures when compared to axenic controls.

The present trial was conducted to investigate the effects of a commercially available blend of *Bacillus* spp. probiotics on population growth dynamics in rotifer batch cultures. Inoculation of axenic rotifers has been utilized to evaluate the effect of probiotic additions (Hagiwara et al. 1994; Douillet 1998; Rombaut et al. 1999; Douillet 2000a, 2000b; Tinh et al. 2006); however, in the commercial setting, it would be impractical to initiate bacterial probiotic utilization by disposal of current cultures and rebuilding of rotifers stocks. Thus, the present study utilized rotifers harboring bacteria present from previous cultures.

4.2) Materials and Methods

A stock batch of rotifers (*Brachionus plicatilis* sensu lato) utilized in the current study were harvested from cultures maintained at the University of Miami Experimental

Hatchery (UMEH). These cultures had no previous exposure to bacterial probiotics and were assumed to harbor naturally occurring bacterial communities.

Experimental culture tanks consisted of static-flow batch-culture systems maintained within a continuously flowing, ambient temperature saltwater bath. Culture systems and aeration apparatus were disinfected by a 30 min immersion in a 10 ppm sodium hypochlorite solution, followed by a rinse with sodium thiosulfate solution. Municipal tap water and 1 μm, UV sterilized seawater were mixed to obtain a stock culture water of 20 ppt final salinity. The stock culture water was disinfected by addition of sodium hypochlorite at 10 ppm, which was neutralized with sodium thiosulfate after 30 min exposure. Chlorine neutralization was verified by a colorimetric chlorine presence test kit. Final culture volumes of 15 L were achieved by distribution of proper pre-determined volume of concentrated stock rotifers and stock culture water to obtain a target initial stocking density of 100 rotifers/mL.

Individual cultures were aerated with 0.35 μm filtered air using a single airline fitted with a diffusive airstone. Ambient light levels were reduced by 70% with a shade cloth integrated the roof covering the UMEH. Natural daily light cycles were maintained. The trial was conducted for five days.

Probiotic treatment replicates (n=4) were inoculated immediately after culture set-up and once daily thereafter with 0.1 mL/L (manufacturer's information: 10^4colony forming units (CFU)/L EcoAqua, a blend of *Bacillus subtilis*, *B. licheniformis*, *B. megaterium*, and *B. laterosporous* bacteria; Ecomicrobials LLC, Miami, Florida, USA). Control treatment replicates (n=4) did not receive any treatment or additions.

Feed rations consisted of 0.4 g (d.w.) dried baker's yeast *Saccharomyces cerevisiae* and 0.02 g (d.w.) Algamac Protein Plus (Aquafauna Bio-Marine, Inc., Hawthorne, California, USA) per million rotifers blended in 500 mL tap water. Rations were intentionally greater than suggested in the literature (Lubzens et al. 1997) in attempts to reduce influence of food availability on population growth dynamics. Probiotic treatments received 0.1 μL/mL feed mixture (manufacturer's information: 10^4 CFU/L) inoculates added directly to each feed mixture; control feed mixtures received no additional treatment. Care was taken against microbial cross-contamination by utilizing 70% ethanol rinse procedures to disinfect mixing equipment between preparation of differing treatment food mixtures. Further caution was employed by first preparing control, then probiotic, feed mixtures. Feeds were administered twice daily in pulses; upon completion of mixing, half was fed immediately, while the remainder was refrigerated until being fed 12 hr later.

Temperature (°C), dissolved oxygen (DO) concentration (mg/L) (YSI 500A Multi-probe, YSI Inc., Yellow Springs, Ohio, USA), pH (YSI pH100 probe, YSI Inc., Yellow Springs, Ohio, USA), and salinity (via refractometer) were recorded daily during early evening hours. Care was taken to prevent microbial cross-contamination by sequentially recording water quality data in control cultures, followed by the probiotic treated cultures. Water quality probes were rinsed with 70% ethanol and subsequently rinsed with 1 μm, UV sterilized seawater between sampling of individual tanks.

Population estimates for each culture were obtained by taking ~300 mL samples from individual cultures and subsequently analyzing two 1 mL subsamples per culture subsample to determine rotifer and egg densities. Egg ratio was calculated as total

number of eggs per sample divided by total number of females counted per sample. The average of these counts were utilized for determining daily feed rations as well as estimating daily populations and relative reproductive status later utilized for population growth dynamics analysis.

Statistical analysis was conducted utilizing State Release 10 (Stata Corp., College Station, TX, USA); statistical testing was carried out with significance level $P_{\alpha} < 0.05$. All data distributions were first investigated for normality and homogeneity of variance between treatments before further analysis. Water quality parameters, daily populations, and daily egg ratio means comparisons were conducted utilizing Wilkcoxon rank-sum tests. These data were further investigated for temporal trends via analysis of covariance (ANCOVA) testing utilizing treatment and Day of Culture as factors (Zar 2009).

Variability in daily mean populations was investigated via analysis of daily mean population standard deviations (SD_{DMP}) and coefficients of variation (CV_{DMP}). Variance ratio F-tests were utilized to compare daily SD_{DMP} between treatments (Zar 2009). Correlation analysis then was utilized to investigate temporal trends in SDs and Day of Culture (Zar 2009). Coefficients of variation (CV_{DMP}) were calculated via the equation:

$$CV_{DMP} = (1 + 1 / 4n)*(SD_{DMP} / \text{mean})$$

where n is the daily mean population sample size (4) and mean is the daily mean population, which corrects for bias due to small sample sizes ($n \leq 5$) (Sokal and Braumann 1980). Related adjustments were necessary in calculation of standard deviations associated with CV_{DMP} (Sokal and Braumann 1980). Correlation analysis was utilized to investigate temporal trends in CV and Day of Culture (Zar 2009).

Logistic population growth curves were computed following the logistic formula:

$$N_t = N_0 + K/(1+ e^{-r(t-h)})$$

where N_t is rotifer population at time t, t is Day of Culture, N_0 is initial population size, K is the system carrying capacity, r is the intrinsic PGR, and h is the time (days) until the system population reaches half the carrying capacity. Population growth models were fit by nonlinear least squares estimation utilizing the previously mentioned statistical software. Multiple steps were involved in obtaining nonlinear regression models that satisfactorily fit the observed data and met statistical assumptions. This included fitting of models to individual replicates within treatments, fitting one model to pooled replicates within treatments, verifying the necessity of N_0 parameter inclusion within models, and verifying model fits for plausibility of resulting parameter estimates results (Bates and Watts 1988, Kutner et al. 2005, Hardin and Hilbe 2007).

A final model structure was selected which consisted of a single regression fit to the multiple replicates (n=4) of each treatment. Intraclass correlation was addressed via utilization of clustered regression model structure with Day of Culture as the clustering variable (Hardin and Hilbe 2007, Zar 2009). Analysis of overall model significance was investigated by lack of fit F-testing (Zar 2009). Residuals from the resultant regressions were investigated for normality and homoscedasticity utilizing Shapiro-Wilk test and Brown-Forsythe tests, respectively (Kutner et al. 2005, Zar 2009). Further residuals analysis included plotting against the dependent variable in attempts to identify potential outlying data points (Kutner et al. 2005, Zar 2009). Correlation of absolute values of each treatment's residuals with Day of Culture was also computed.

Resultant logistic population growth models for each treatment were then compared by F-test for coincidence of the regressions (Zar 2009). Individual parameter estimates were investigated for satisfaction of large-sample theory, which is necessary to confidently conduct further statistical analysis between treatment parameter estimates. Both consideration of number of iterations necessary to obtain model convergence and bootstrapping of regression models (1000 replications per treatment) were utilized to evaluate acceptability of large-sample theory (Kutner et al. 2005).

4.3) Results

Distributional analysis revealed multiple instances of non-normal or heterogeneous distribution of sample data in question. Thus, comparisons between treatments' water quality parameter daily means, daily mean populations, and daily mean egg ratio were then analyzed by Wilcoxon rank-sum tests in order to simplify data analysis (Zar 2009).

Throughout the trial, water quality remained within acceptable ranges for rotifer batch culture systems (Table 4.1); although, pH and DO concentration (mg/L) tended towards unfavorable conditions as the trial concluded. Highly significant trends ($P_{\alpha(1),2,45} < 0.001$) of decreasing pH, salinity (ppt), and DO concentration (mg/L) with Day of Culture were detected by ANCOVA testing (Table 4.2). These trends were not significantly different between treatments for pH and salinity ($P_{\alpha(1),2,45} > 0.05$). Similarly, pairwise comparison between treatments did not reveal significant differences in daily mean pH or salinity (ppt) (Table 4.2). For DO concentration (mg/L), a significant ($P_{\alpha(1),2,45} < 0.001$) treatment effect was detected by ANCOVA (Table 4.2). Furthermore,

on Day 3 ($z = 2.021$, $P_{\alpha(2),4,4} = 0.043$) and Day 5 ($z = 2.021$, $P_{\alpha(2),4,4} = 0.043$) marginal significant differences in DO concentration (mg/L) were detected between treatments' means (Table 4.1); whereas the DO concentration (mg/L) was lower in the probiotic cultures than in the controls.

Individual culture water temperatures were controlled by water bath utilizing ambient saltwater circulating outside of culture tanks; thus, daily mean temperatures displayed some fluctuation from day to day (Table 4.1). No significant trend was detected by ANCOVA testing for temperature between treatments or for Day of Culture ($P_{\alpha(1),2,45} < 0.05$). However, on Day 0, a significant difference was detected between treatment mean temperatures ($z = -2.428$, $P_{\alpha(2),4,4} = 0.015$) (Table 4.1).

Daily mean populations were found to be significantly different between treatments on Day 2 ($z = -2.309$, $P_{\alpha(2),4,4} = 0.021$), Day 4 ($z = -2.309$, $P_{\alpha(2),4,4} = 0.021$), and Day 5 ($z = -2.309$, $P_{\alpha(2),4,4} = 0.021$) (Table 4.3, Figure 4.1, Figure 4.2). Investigation of daily mean populations via ANCOVA testing revealed a significant treatment effect ($P_{\alpha(2),1,45} < 0.001$) (Table 4.4, Figure 4.1). Similarly, a significant trend was also obtained for Day of Culture ($P_{\alpha(2),1,45} < 0.001$) (Table 4.4).

Previously noted complications with normality of daily mean populations could result in underestimation CV_{DMP} via inflation of the SD_{DMP} (Sokal and Braumann 1980). However, the resultant SD_{DMP} from the only significant non-normal daily mean population (Probiotic Treatment, Day 2, $P = 0.010$) was within the range of other obtained values and thus was deemed not to be negatively influenced (Table 4.3). Variance ratio tests did not detect significant differences ($P > 0.05$) in daily SDs between treatments. As anticipated due to increasing daily population sizes, control SD_{DMP} were

significantly and positively correlated with Day of Culture ($r = 0.85$, $P = 0.032$) (Table 4.3). Interestingly, probiotic SD_{DMP} were not significantly correlated and the resultant trend was only minimally positive ($r = 0.0022$, $P = 0.99$) (Table 4.3). Significant correlations were not detected between CV_{DMP} and Day of Culture for either treatment ($P > 0.05$); however, the resultant control trend was positive ($r = 0.24$) while the probiotic trend was negative ($r = -0.68$) (Table 4.3).

No statistical differences were detected between treatment daily mean egg ratio ($P_{\alpha(2),4,4} > 0.05$). Similarly, ANCOVA testing did not reveal a significant treatment effect in egg ratio ($F = 0.30$, $P_{\alpha(2),1,45} = 0.585$) although the overall model ($F = 18.68$, $P_{\alpha(2),2,45} < 0.001$) and Day of Culture ($F = 37.06$, $P_{\alpha(2),2,45} < 0.001$) were significant.

Logistic population growth model fitting to individual replicates within treatments yielded multiple unlikely solutions. Thus, daily population data was pooled by treatment for further regression modeling. Intraclass correlations computed for each treatment ($F = 72.8$, $P_{\alpha(2),5,18} <0.001$ and $F = 302.3$, $P_{\alpha(2),5,18} <0.001$ for control and probiotic treatments, respectively) indicated non-independence of daily population data within treatment after pooling. This necessitated application of clustered, robust regression techniques to the model building (Liang and Zeger 1993, Hardin and Hilbe 2007, Zar 2009). In comparison to non-clustered regression results, clustered robust least squares regression resulted in substantially smaller parameter standard deviations in both treatments, suggesting overall improvement of the individual models' fit.

Further model structure considerations included investigation of the necessity for N_0 parameter inclusion. Likelihood ratio tests conducted for each treatment ($F = 78.98$, $P_{\alpha(2),1,20} < 0.001$ and $F = 70.33$, $P_{\alpha(2),1,20} < 0.001$ for control and probiotic treatments,

respectively) indicated a significant increase in explanation of observed data. Thus, inclusion of the N_0 parameter was statistically justified (Bates and Watts 1988, Kutner et al. 2005).

Resulting population growth models and residuals were investigated for model fit. Non-significant results from lack-of-fit F-testing indicated that obtained models adequately fit the observed data ($F = 5.95*10^{-5}$, $P_{\alpha(2),3,5} = 1.00$ and $F = 0.46$, $P_{\alpha(2),3,5} = 0.765$ for control and probiotic treatments, respectively) (Table 4.5). Shapiro-Wilk testing of residuals detected non-normally distributed control and probiotic residuals on Day 2 ($P = 0.050$ and $P = 0.010$, respectively). All other residuals' normality tests were all found to be highly non-significant ($P >> 0.05$).

Within treatments, residuals were generally found to meet the assumptions of regression analysis. However, non-normality of residuals was detected on Day 2 for both treatments. Absolute values of control residuals were significantly and positively correlated to Day of Culture ($r = 0.50$, $P = 0.013$) while similar investigation of absolute values of probiotic residuals produced a negative, but non-significant trend ($r = -0.14$, $P = 0.519$) (Figure 4.3).

Resultant population growth models were found to be statistically non-coincident ($F = 3.64$, $P_{\alpha(2),5,10} = 0.039$), suggesting at least one of the obtained parameters were dissimilar between treatment models (Table 4.6, Figure 4.2). Further inferences regarding individual parameter estimates required satisfaction of large-sample theory. One indicator can be quick convergence of the iterative procedure (Kutner et al. 2005), which occurred after 3 and 5 iterations during control and probiotic regression modeling, respectively. However, bootstrapped regressions (1000 replications) yielded highly

inflated standard errors about the parameter estimates, suggesting the large-sample theory estimates were not reliable for further comparisons (Kutner et al. 2005).

Although statistical inferences regarding estimated population growth model parameters could not confidently be made, simple comparisons were conducted to note important difference between the resultant estimates (Table 4.6). The computed probiotic treatment logistic PGR ($r_p = 1.01$) was 7.45% greater than that of control's ($r_c = 0.94$) and the computed probiotic carrying capacity ($K_p = 7.80*10^6$) was 29.4% greater than that of controls ($K_c = 6.03*10^6$) (Table 4.6, Figure 4.2). However, probiotic time to half system carrying capacity ($h_p = 3.94$ days) was 3.96% greater than that of the control treatment's ($h_c = 3.79$ days) (Table 4.5).

Table 4.1: Mean water quality parameters ± SD per treatment; letters denote statistically different pairs between treatment daily values ($P_{\alpha(2),4,4} < 0.05$).

Day of Culture	Treatment	Temperature (°C)	DO (mg/L)	pH	Salinity (ppt)
0	Control	27.7 ± 0.05 z	6.3 ± 0.2	7.95 ± 0.0082	21 ± 1
	Probiotic	27.8 ± 0.05 z	6.1 ± 0.1	7.95 ± 0.015	21 ± 1
1	Control	29.3 ± 0.08	5.9 ± 0.09	7.80 ± 0.017	20 ± 0
	Probiotic	29.3 ± 0.05	5.8 ± 0.06	7.81 ± 0.02	20 ± 0
2	Control	30.3 ± 0.05	6.0 ± 0.1	7.74 ± 0.043	20 ± 0
	Probiotic	30.3 ± 0.05	5.8 ± 0.1	7.69 ± 0.051	20 ± 0
3	Control	30.4 ± 0.05	5.8 ± 0.1 y	7.61 ± 0.045	20 ± 0
	Probiotic	30.4 ± 0.05	5.6 ± 0.2 y	7.60 ± 0.053	20 ± 0
4	Control	28.5 ± 0.2	5.9 ± 0.2	7.58 ± 0.16	20 ± 0
	Probiotic	28.5 ± 0.0	5.6 ± 0.3	7.65 ± 0.061	20 ± 0
5	Control	29.1 ± 0.2	5.5 ± 0.2 x	7.57 ± 0.43	20 ± 0
	Probiotic	29.1 ± 0.1	5.2 ± 0.2 x	7.45 ± 0.11	20 ± 0

Table 4.2: Results of ANCOVA water quality parameter testing with treatment as a discrete and Day of Culture as a continuous factor; water temperature (°C) testing is not reported as the model was found to be non-significant ($P_{\alpha(2),2,45} > 0.05$).

Water Quality Parameter	Model Adjusted R^2		SS	Df	F	$P_{\alpha(2)}$
DO (mg/L)	0.672	Model	3.1481	2	49.1	< 0.001
		Treatment	0.5355	1	16.7	< 0.001
		Day of Culture	2.6126	1	81.5	< 0.001
		Residual	4.591	45		
pH	0.766	Model	0.9332	2	77.9	< 0.001
		Treatment	0.0033	1	0.56	0.46
		Day of Culture	0.93	1	155.3	< 0.001
		Residual	0.2695	45		
Salinity (ppt)	0.150	Model	0.4226	2	3.98	0.03
		Treatment	0.0208	1	0.39	0.53
		Day of Culture	0.4018	1	7.57	0.001
		Residual	2.8125	45		

Table 4.3: Mean ± SD daily mean population ($x10^6$), coefficient of variation (CV), and egg ratio per treatment. Letters denote statistically different pairs between treatment daily values ($P_{\alpha(2),4,4} < 0.05$); for daily mean population, letters coincide with those utilized in Fig. 1 and Fig. 2 to denote statistical differences.

Day of Culture	Treatment	Population ($x10^6$)	CV	Egg Ratio
0	Control	1.44 ± 0.16	0.12 ± 0.052	0.11± 0.026
	Probiotic	1.68 ± 0.17	0.11 ± 0.049	0.11 ± 0.0063
1	Control	1.70 ± 0.16	0.097 ± 0.042	0.050 ± 0.0073
	Probiotic	2.27 ± 0.24	0.22 ± 0.096	0.04 ± 0.016
2	Control	2.26 ± 0.16 z	0.074 ± 0.032	0.27 ± 0.011
	Probiotic	2.53 ± 0.13 z	0.056 ±0.024	0.32 ± 0.043
3	Control	3.29 ± 0.37	0.12 ± 0.052	0.20 ± 0.039
	Probiotic	3.85 ± 0.31	0.087 ± 0.038	0.25 ± 0.38
4	Control	4.66 ± 0.72 y	0.16 ± 0.073	0.24 ± 0.0088
	Probiotic	5.71 ± 0.19 y	0.036 ± 0.016	0.25 ± 0.056
5	Control	5.89 ± 0.54 x	0.097 ± 0.042	0.27 ± 0.0036
	Probiotic	7.47 ± 0.038 x	0.041 ± 0.018	0.25 ± 0.024

Table 4.4: Results of ANCOVA testing of daily average population with treatment as a discrete and Day of Culture as a continuous factor ($R^2_{adj.} = 0.895$).

	SS	Df	F	$P_{\alpha(2)}$
Model	$1.58*10^{14}$	2	201.4	< 0.001
Treatment	$5.69*10^{12}$	1	14.5	< 0.001
Day of Culture	$1.53*10^{14}$	1	388.4	< 0.001
Residual	$1.77*10^{13}$	45		

Table 4.5: Results of F-testing for lack-of-fit of logistic population growth models of each treatment.

Treatment	Model Fit Diagnostics					
	SS_{LF}	Df_{LF}	SS_{PE}	Df_{PE}	F_{LF}	P Value
Control	$4.08*10^{7}$	3	$3.09e*10^{12}$	5	$5.95*10^{-5}$	1.00
Probiotic	$1.40*10^{11}$	3	$1.37e*10^{12}$	5	0.46	0.765

Table 4.6: Resulting parameter estimates ± SD from logistic population growth modeling where N_0 = initial population size, K = carrying capacity, r = logistic population growth rate (day^{-1}), and h = time to reach half carrying capacity (days). Although SDs are reported for individual parameters, lack of validation of large-sample theory precluded further statistical testing leading to inferences regarding potential differences between the parameter estimates.

Treatment	Model Parameter Estimates			
	N_0	K	r	h
Control	$1.26*10^6 \pm 0.020$	$6.03*10^6 \pm 0.010$	0.94 ± 0.0024	3.73 ± 0.0030
Probiotic	$1.61*10^6 \pm 0.13$	$7.82*10^6 \pm 0.50$	1.01 ± 0.089	3.91 ± 0.10

Figure 4.1: Mean ± SD daily populations and significantly different ($P_{\alpha(2),1,45} < 0.001$) linear trends as detected by ANCOVA testing. Significantly different ($P_{\alpha(2),4,4} < 0.05$) daily mean populations are marked by letters which correspond to those utilized in Table 1 and Fig. 2.

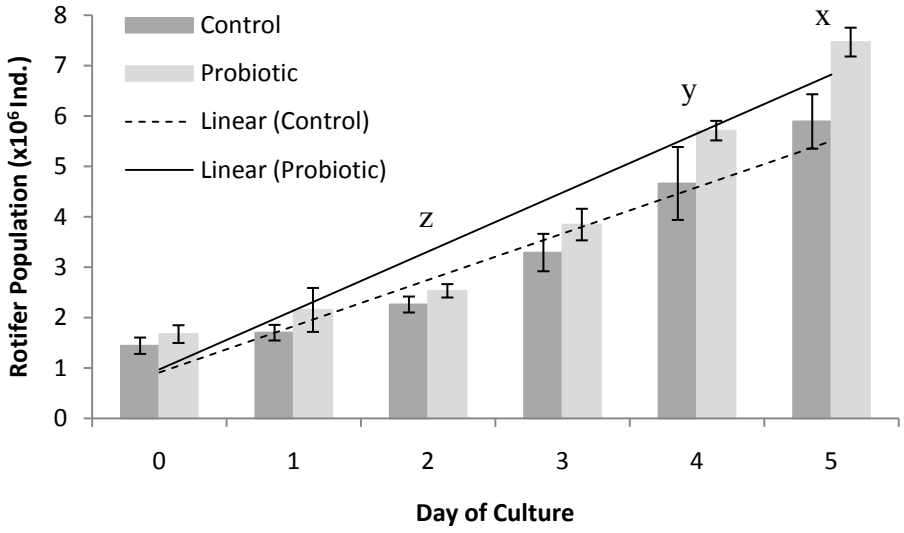


Fig. 4.2: Mean ± SD daily populations and logistic population growth models for daily population pooled within treatment for control (○, - -) and probiotic (◊, ——) treatments. Significantly different ($P_{\alpha(2),4,4} < 0.05$) daily mean populations are marked by letters which correspond to those utilized in Table 1 and Fig. 1. F-testing of coincidence of the curves yielded a significant result ($F = 3.64$, $P_{\alpha(2),5,10} = 0.039$).

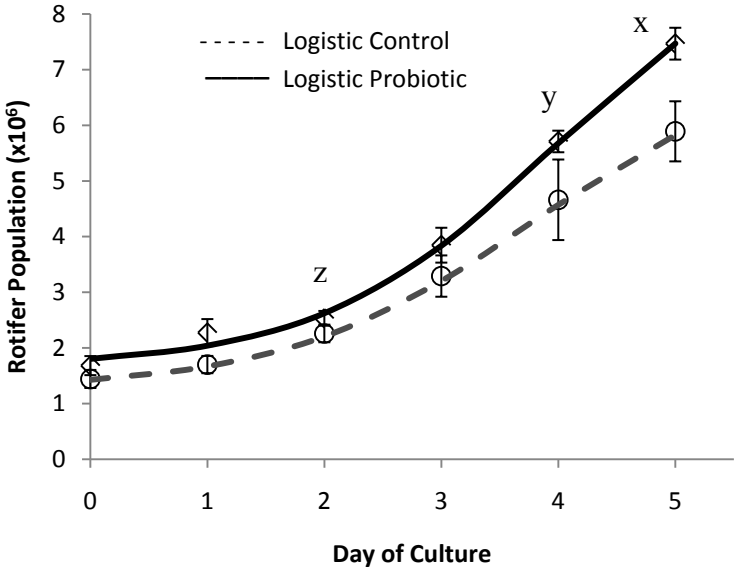
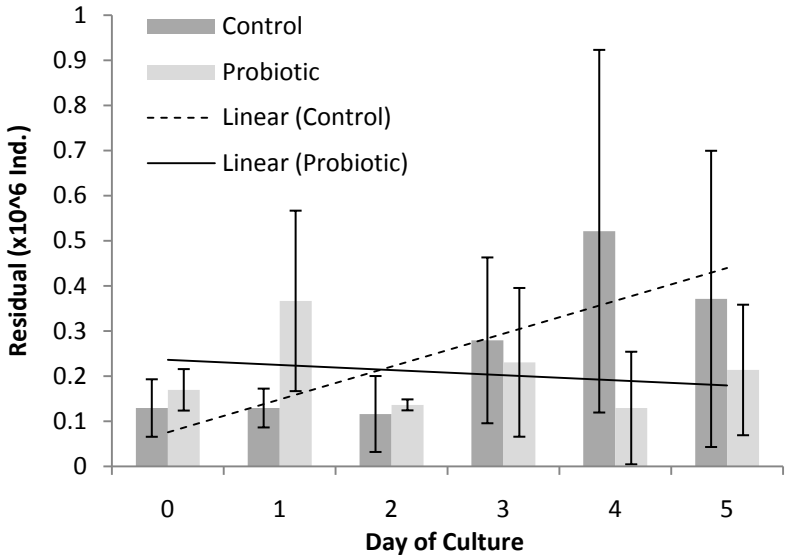


Fig. 4.3: Absolute values of logistic regression residuals ± SD for each Day of Culture and linear trends. Control residuals were significantly and positively correlated with Day of Culture (r = 0.50, P = 0.013) while probiotics' were non-significantly, negatively correlated (r = -0.14, P = 0.519).



4.4) Conclusions

Individual rotifer cultures performed reasonably well with none experiencing a culture collapse. Monitored water quality parameters were satisfactorily maintained within ranges for maximal growth for *B. plicatilis* (~20 ppt or less, ~ 30 °C, DO > 4.0 mg/L, pH between ~7.5 and 8.5) (Hagiwara et al. 1993, Hagiwara et al. 1995, Dhert et al. 2001). Although some differences were detected in daily means of certain water quality parameters between treatments, none were suspected of influencing experimental outcomes. For example, actual daily mean DO concentration (mg/L) differences between treatments in were not substantially different (0.2 and 0.3 mg/L, respectively) despite detecting statistical differences (Table 4.1). Trends in decreasing pH and DO concentration (mg/L) during the course of the trial were anticipated as rotifer populations grew and continued to degrade water quality (Table 1) (Hagiwara et al. 1993). More importantly, the significant trend of more rapid decrease in DO concentration (mg/L) in probiotic treatments is explained by the higher rotifer populations in probiotic treated replicates (Table 4.2).

Direct comparisons of population dynamics among rotifer studies may be complicated by utilization of differing strains or previous culture conditions (Scott and Baynes 1978, Snell et al. 1983). Recent genetically-oriented investigation has led to consideration of *B. plicatilis* sensu lato as a cryptic complex of species (Gomez et al. 2002, Suatoni et al. 2006). Differing population growth dynamics, reproduction rates, (Kotani et al. 2006, Hagiwara et al. 2007, Kostopoulou and Vadstein 2007), and even associated microbial communities (Qi et al. 2009) have been demonstrated between biotypes. The lack of screening of the exact biotype utilized in this study complicates

comparison to others; however, such comparisons are necessary to place the current results in a greater context.

Significant differences in daily mean populations on differing Day of Culture indicate benefits from the inclusion of the tested *Bacillus* spp. probiotics within rotifer batch cultures. This conclusion was strengthened by detection of a significant treatment effect in ANCOVA testing of daily populations. Upon termination of the trial (Day 5), probiotic rotifer densities were ~500 rotifers/mL, which are comparable to or higher than maximal densities attained during other small scale probiotics investigations maintained for similar time periods (Rombaut et al. 1999, Yoshinaga et al. 2001, Rombaut et al. 2003). The present study was terminated before systems reached carrying capacity, thus precluding the ability to draw conclusions concerning final population densities that could have been attained.

Probiotic inclusion reduced variability in daily populations as culture age increased. Daily SD_{DMP} would be anticipated to positively correlate in proportion with increases of daily mean populations (Zar 2009). However, probiotic treatment's SD_{DMP} did not produce a significant trend, nor was the trend highly positive. Furthermore, analysis of temporal trends in CV_{DMP} produced a positive trend for control and a negative trend in probiotic treatments. However, in depth conclusions drawn from CV_{DMP} trends is cautioned as the correlations were found to be non-significant. Similar stabilization of rotifer cultures resulting from manipulation of bacterial communities resulting has been demonstrated in other studies (Douillet 2000a, Rombaut et al. 1999).

Daily egg ratios, ranging from 0.025 to 0.37 (Table 4.3), suggested a low reproductive rate compared to values observed during the exponential population growth

phase under similar physical conditions (0.5 to 2.0, depending upon food source, Korstad et al. 1995) and under unlimited feed availability (0.6, Yúfera and Navarro 1995). This was most likely due to the timing of population estimate sampling as egg ratio can rapidly decrease in response to exhaustion of food supply (Scott and Baynes 1978). Pulse feeding twice daily may have induced a cyclic response in reproduction output, resulting in decreased reproduction rates at the end of a pulse. Indeed, the observed egg ratio range (0.04 to 0.27, Table 4.3) was similar to values reported under starvation conditions (ranging from 0.552 to 0.017 as starved time increases) (Scott and Baynes 1978).

Various methods for computation of rotifer PGRs exist in the literature. Traditionally, rotifer PGRs (r) have been computed utilizing a linearized exponential approximation which incorporates initial and final rotifer density (Lubzens and Zmora 2003, Planas et al. 2004). Initial and final density calculation can be modified to combine egg and rotifer densities (Hagiwara et al. 1989, Lubzens and Zmora 2003) which can substantially change obtained PGR values. More recent studies continue to utilize the exponential approximation (Tinh et al. 2006, Qi et al. 2009). In the present study, computation of exponential approximations would have resulted in underestimates of PGR as system carrying capacities were not attained, and thus final density would have affected the computed PGRs.

However, population growth with negative environmental feedback or competition has long been known to follow a sigmoid curve (Pearl 1926 as cited in Nicholson 1933, Brown and Rothery 1993, Kot 2001). Sigmoid population growth is well known to occur in rotifer populations (Yúfera and Navarro 1995, Yoshinaga et al.

2001) as demonstrated by plots of rotifer population against time (Hagiwara et al. 1989, Yúfera and Navarro 1995, Hagiwara et al. 1997, Yoshinaga et al. 2001, Rombaut et al. 2001, Luna-Andrade et al. 2002, Rombaut et al. 2003). Some recent studies apply logistic and other modified sigmoid population growth curves to model rotifer population growth (Rombaut et al. 1999, Yoshinaga et al. 2001, Planas et al. 2004, Serra et al. 2005), which have been demonstrated to better model observed population data (Yoshinaga et al. 2001, Planas et al. 2004). Furthermore, utilizing logistic equations yields descriptive functions which more completely describe rotifer population growth dynamics (Brown and Rothery 1993, Yoshinaga et al. 2001, Planas et al. 2004). Similar to other PGR calculation methods, comparison of obtained PGRs can be complicated by variations in values obtained from differing population growth equation formulations.

Pooling of replicate daily populations within treatments was necessitated based upon the observation unlikely parameter estimates from individual replicates' regressions. Most notably, one model for a poorly performing control replicate resulted in extremely low PGR ($r = 0.40$), as well as extremely high carrying capacity ($K = 3.34*10^9$) and time to half carrying capacity ($h = 21.4$) parameter estimates. Clustered regressions were necessitated in order to better model underlying patterns in the data, as verified by significance of intraclass correlations for both treatments.

In general, resultant parameterizations met the assumptions of regression analysis. Adverse effects from residuals' non-normality on Day 2 for both treatments were considered minimal due to equal sample sizes and homoscedasticity of variances (Zar 2009). Statistical comparison of estimated parameters between treatments could not be conducted due to lack of confidence in meeting the assumptions of large-sample theory

(Kutner et al. 2005). However, treatment regressions were found to be statistically non-coincident curves, suggesting at least one of the estimated parameters was different between treatments. This confirms notions previously discussed regarding testing of daily mean populations resulting in differing population dynamics between treatments.

Investigation of regression residuals resulted in trends which confirm previously noted suggestions of decreases in population variability with time in probiotic treated rotifer cultures. Correlation of the absolute value of control residuals against Day of Culture resulted in a significant, positive trend for the control treatment (Fig. 4.3). This trend exemplifies the previously discussed necessity to construct pooled regressions. Conversely, a non-significant, negative trend was found for probiotic residuals (Fig. 4.3).

The probiotic estimated r and K values were higher than those estimated for control regressions (Table 4.6). Similar increases in PGR (Hagiwara et al. 1994; Doulliet 2000a, 2000b; Rombaut et al. 1999; Planas et al. 2004) and carrying capacity (Planas et al. 2004) due to microbial community manipulation or synxenic culture with selected bacteria has been demonstrated in other studies. The small increase in probiotic PGR did not sufficiently augment the relatively greater increase of K in comparison to control parameter estimates, as the probiotic h was greater than the control estimate (Table 4.6). However, this represents a minimal actual increase of approximately one half culture day to reach carrying capacity.

The PGRs of the current study were greater than those reported from other studies incorporating logistic rotifer population growth models (r_{max} = 0.664, Rombaut et al. 1999; r = b – d = 0.8, Serra et al. 2005; r_{max} = 0.24, Yoshinaga et al. 2001). Discrepancies may be a result product of logistic model formulation. Conversely, the

present PGRs do agree with the upper range of those obtained by Planas et al. (2004) (0.209 to 1.055). Perhaps this similarity was due to utilization of rotifers already harboring microbial flora from previous cultures; mixtures of bacterial additives rather than utilization of a single species can result in higher PGR (Gatesoupe 1991, Douillet 2000b). However, comparison to the Planas et al. (2004) study warrants caution due differing logistic population growth model formulation as previously discussed.

PGRs represent the difference between birth and death rates (Brown and Rothbury 1993, Kot 2001, Yoshinaga et al. 2001). After investigation of sigmoidal population growth in *B. plicatilis* batch cultures, Yoshinaga et al. (2001) concluded death rates were nearly constant and initially higher birth rates were the density-dependent factor causing logistic population growth. Further investigation of whether probiotic utilization increases reproduction rates or reduces mortality, thus increases reproductive output per individual, should be conducted in order to better understand their influence on PGRs.

Previous studies have also demonstrated the capability of *Bacillus* spp. probiotic blends to improve rotifer culture population growth in comparison to ambient microbial community conditions (Hirata et al. 1998, Douillet 2000a). Conversely, Hagiwara et al. (1994) noted reduced PGRs when a *Bacillus* sp. was utilized to produce synxenic rotifer cultures compared to axenic control cultures. Perhaps this discrepancy highlights the subtle differences similar bacterial species, or even strains within a species, can produce.

Mechanisms for either reducing rotifer mortality or increasing rotifer reproduction could include reduction of potentially pathogenic bacteria, improvement in nutrition, or improvement of water quality. Studies have demonstrated a ~78% reduction of

opportunistically pathogenic *Vibrio* spp. associated with rotifers with the addition of a *Bacillus* sp. (F. J. Gatesoupe, INRA-IFREMER, Unpub. Data). It was later suggested that natural antimicrobial production by the *Bacillus* sp. caused the reduction in *Vibrio* spp. (Gatesoupe 1999). Similarly, the current blend of *Bacillus* spp. has been demonstrated to inhibit *Vibrio* spp. growth in previous investigations (A. Didoha, Florida Institute of Technology, Unpub. Data). *Bacillus* spp. could also be beneficial to rotifer population growth by providing an alternative food source, reduced production of pathogenic bacteria toxins, or improving nutrition such as production of Vitamin B_{12} (Yu et al. 1989). *Bacillus* spp. probiotic blends, including the presently tested blend (Zink, In Press), have been demonstrated to reduce ammonia concentrations (Chen and Chen 2001, Lalloo et al. 2007, Gomes et al. 2008, Gomes et al. 2009), which is known to inhibit rotifer population growth (Schülter and Groeneweg 1985).

Despite gaps in causal understanding, the tested *Bacillus* spp. probiotic blend was observed to significantly increase, and reduce variability in, daily mean populations. Other measures of rotifer batch culture population growth dynamics, such as PGR and carrying capacity, were also improved. Further study confirming these results and identifying direct causes of observed benefits in *B. plicatilis* population dynamics with *Bacillus* spp. probiotic additions are warranted.

Chapter 5: Investigation of Determinants of Cobia Egg Diameter Variability

5.1) Background

Broodstock management aims to provide husbandry and nutritional conditions which maximize spawning output in terms of both quantity and quality (Bromage 1995). Maximizing spawning events is desirable in order to counter the inherent risk of mass mortality events during larviculture, and thus momentary setbacks in hatchery production (see production outcomes discussed in Chapter 6). Maximal quality of oocytes per spawning event would maximize potential production efficiency via maximizing available tank space, material input, and labor due to improvement of larval survival.

Although discrepancies appear in the literature, the general consensus is that high egg quality correlates to increased survival and growth in larval fishes (Bromage 1995, Brooks et al. 1997). Consequently, some method of determining egg quality is often practiced when assessing a collected spawn. Egg diameter is often utilized as a proxy due to ease in obtaining these values (Bromage 1995, Brooks et al. 1997). Studies indicate variation in the size of cobia eggs (1.25 mm, Ryder 1885; 1.16 to 1.42 mm, Joesph et al. 1964; 1.15 to 1.3 mm, Ditty and Shaw 1992, Holt et al. 2007b, Faulk and Holt 2008), although a majority of these are field observations which cannot lead to determination of factors influencing the observed variation.

Within fishes, intraspecific absolute and batch fecundity are well known to increase with increasing body size (both length and weight) (Hislop 1984, Hislop 1988, Bromage 1995, Trippel et al. 1997). Although not as distinct, this trend is evident interspecifically as well (Durante and Acaraz 1989). Intraspecifically, increasing oocyte diameter has also been shown to correlate to increasing fish size (Blaxter 1988, Hislop

1988, Bromage 1995, Brooks et al. 1997, Trippel et al. 1997, Berkeley et al. 2004). Generally, female size and fecundity are known to relate in an asymptotic manner; therefore, larger females nearing L_∞ actually have reduced relative fecundity (Hislop 1988, Bromage 1995).

Though in practice, fish size is considered the primary indicator of fecundity; applying this metric to egg size and quality may be inherently flawed, as age has been suggested as a better indicator of egg quality and survival in numerous studies (Nikolskii 1969, Hislop 1988, Trippel et al. 1997, Berkeley et al. 2004, Sahin et al. 2007). As reviewed by Nikolskii (1969), a number of studies indicate qualities such as egg size, embryo size, and egg viability are markedly dependent on age resulting in a bell-shaped relationship of age and egg quality. Thus, moderate aged females would produce maximal egg quality, as determined by egg diameter (Nikolskii 1969), while exhibiting higher relative fecundities than older females (Bromage 1995).

Brooks et al. (1997), in review of egg quality and the determinants thereof, conclude older females are associated with increased survival of eggs or offspring. Another review stresses the importance of maternal age influences on egg quality and strongly suggest fisheries management and recruitment models take these influential factors, and related factors such as age truncation, into account when considering reproduction potential of wild stocks (Birkeland and Dayton 2005). Other works have demonstrated more prominent relationship between spawning age and egg size, egg quality, and/or subsequent larval survivorship (Hislop 1988, Berkeley et al.. 2004, Sahin et al. 2007, Sogard et al. 2008).

Hislop (1988) investigated the relationships of egg size and weight to female length and age of haddock (*Melanogrammus aeglefinus*) of the North Sea, reporting a significant correlation of female length to egg diameter and egg dry weight. However, the observed correlation may largely be attributed to the fact that many of the small fish sampled in the study were within the 2-year-old (recruitment spawning) age class (Hislop 1988). Over the remaining age classes (3-8 year classes), the correlation was less significant; with exclusion of the 3rd year class, the correlations were non-significant (Hislop 1988). The author also concluded mean relative fecundity was found to increase asymptotically as age increased (Hislop 1988).

In an elegant study conducted by Berkeley et al. (2004), female age was found to correlate with increased egg oil globule volume, larval growth, time to 50% mortality in unfed larval cultures, and provisioning of triacylglycerol lipids within the black rockfish (*Sebastes melanops*). Stepwise multiple regressions for each of the above parameters were all highly significant with maternal age as a factor explaining the majority of the variability (Berkeley et al. 2004). In the case of larval growth in terms of weight, maternal age was the only significant predictor (Berkeley et al. 2004). The investigation also revealed that oil globule volume at parturition was the only significant predictor later improvement in larval quality (Berkeley et al. 2004). A related study of other *Sebastes* spp. revealed that five other species of rockfishes exhibited similarly increased lipid provisioning and weight-specific fecundity with increasing maternal age and size (Sogard et al. 2008).

Sahin et al. (2007) investigated the differences of egg diameter, fertilization, hatching rates, and fecundity of 4-5 year old wild broodstock and 3 year old hatchery

reared broodstock of Black Sea salmon (*Salmo trutta labrax*). Their results indicate total fecundity differed significantly between these two stocks and was correlated to body mass within each spawning group. Egg diameters between the spawning groups also differed significantly, with the older, higher weight spawning group producing larger eggs (Sahin et al. 2007). Despite significant differences between the groups, there was a trend towards higher fertilization and hatching rates from the larger eggs, which were spawned by the larger and older females (Sahin et al. 2007).

Furthermore, Kjørsvik et al. (2003) demonstrated significant positive correlations of hatching rates, larval fish tolerance to acute salinity stress, survival, timing of metamorphosis, and juvenile pigmentation to improved egg quality as determined by fertilization rate and blastomere development rates in turbot (*Scophthalmus maximus*).

It is generally accepted that larger eggs beget larger larvae at first feeding, all other factors being equal (Blaxter 1988). These factors could translate to higher survival rates if one considers the ability of larger larval fish to pass more rapidly through vulnerable early life stages and develop physiological and physical features that allow for prey detection and capture, predator avoidance, and resistance to environmental changes at faster rates than less advanced larvae of the same species (Fuiman and Cowan 2003). Initially larger individuals also have the potential to remain more developmentally advanced and relatively larger at later stages of development (Blaxter 1988, Chambers 1997); however, other studies indicate these advantages could be less observable during later larval and juvenile stages (Blaxter 1988). Higher initial survival from during the yolk-sac and early larval stages could translate to increased survival and juvenile hatchery production.

Gross nutrient availability is noted to be intrinsically important in various factors of broodstock reproductive physiology and can influence egg quality (Bromage 1995). Micronutrient and individual components of broodstock diets are increasingly being investigated to ensure proper nutritional needs are being met (Bromage 1995). For instance, a study of cobia egg quality over two consecutive years found significant differences in fatty acid composition between spawning seasons which was attributed to variable quality of broodstock diet (Faulk and Holt 2008). With better understanding of these processes leading to improved feeds formulation, meeting these requirements can be achieved.

Other factors are known also to affect initial egg size, including ambient temperature, ambient salinity, seasonal variation, and geographic variation (which can be but not necessarily related to genetic variation) (Bagenal 1971, Blaxter 1988, Bromage 1995, Chambers 1997).

Seasonality causes variation in numerous environmental characteristics which may contribute to differences in observed egg size within species, stock, and/or spawning season. Seasonality induces mobilization of energy reserves and reduction of available energy reserves for oocyte maturation at the beginning and end of the spawning season, respectively (Bagenal 1971, Blaxter 1988, Chambers 1997). Within a population or a given species as a whole, spawning output generally follows a peaked distribution of egg size during the spawning season (Chambers and Waiwood 1996). These observed changes in egg size have been attributed to various other factors, such as age and size of the female spawners, order in which females of varying ages spawn during the season, condition of the female before and after spawning, and temperature and salinity regimes

experienced during oogenesis and at the moment of spawning (Bagenal 1971, Blaxter 1988, Chambers 1997).

The cobia's protracted spawning season begins in April and continues through September (Lotz et al. 1996, Brown-Peterson et al. 2001), providing ample opportunity for seasonal influence. In a study of cobia broodstock, no significant relationships were found between spawning date and egg diameter or the ratio of vitamin E to *n*-3 highly unsaturated fatty acids (HUFAs) (Faulk and Holt 2008). The authors attributed this lack of seasonality in egg quality to the fact that cobia held in captivity are fed continuously and thus reductions in energy reserves for allocation to egg production are consistently replenished (Faulk and Holt 2008).

Intraspecific geographic variability in egg size has been demonstrated; however, variability from this factor is more often related to local environmental conditions, or genetic variation resultant from local population adaptation to those conditions, rather than a true geographic causation (Chambers and Waiwood 1996, Chambers 1997). Tagging studies conducted by Franks et al. (1992) and mitochondrial DNA studies conducted by Hrincevich (1993) suggest adequate mixing of the southeastern United States cobia occurs so that geographically oriented sub-populations cannot be distinguished. However, Brown-Peterson et al. (2001) described slightly different periods of initial maturation of ovaries, indicative of geographical differences in spawning characteristics. These differences are most likely the artifact of local environmental conditions acting upon reproductive behavior and physiology as has been noted for other species (Bagenal 1971, Blaxter 1988, Chambers 1997, Brown-Peterson et al. 2001).

Regrettably, the Brown-Peterson et al. (2001) study was limited to investigations of immature oocytes and does not include any investigation of post-spawned egg characteristics such as egg diameter. The authors utilized regression analysis to describe the fecundity relationship to female size, both in terms of both length and weight. Both regressions were statistically significant, however corresponding R^2 values were relatively low (0.132 for fork length and 0.143 for ovary-free body weight) (Brown-Peterson et al. 2001). One can only speculate whether regression analysis would find age as a better indicator of these, and other indices of egg quality, as in Berkeley et al. (2004).

Temperature, photoperiod, and salinity are environmental factors which can cause variation of egg size and may be related to seasonality. Temperature and photoperiod are the two main environmental cues which trigger spawning behavior. Temperature can affect spawned egg diameter both during oogenesis and during incubation after spawning (Chambers 1997). Salinity can be seasonally correlated, although differences are more often associated to localized conditions encountered by fishes when spawning at various estuary and open-ocean locations (Chambers 1997). Salinity seems to influence egg size most prominently during oogenesis due to the permeable nature of developing oocytes allowing salt and water content to equalize osmotic balance with the female's body fluids, which in turn will vary with ambient salinities (Holliday 1969).

Indeed, egg diameter has been noted to vary in relation to salinity when cobia broodstock maintained in systems where salinity varied from 28-35 g/L (Holt et al. 2007b). Linear regression revealed a significant relationship of decreasing egg diameters with increasing salinity (Holt et al. 2007b). The authors conclude modification of water

content effectively modifies egg specific gravity and surface area to volume ratios, allowing for egg buoyancy characteristics to match ambient salinity experienced by the spawning female (Holliday 1969, Holt et al. 2007b). The authors (2007b) further noted these variations did not affect initial size of yolk-sac larvae nor growth rate through 9 DPH.

Although few studies investigate the relationship between female age and egg quality, those that have provide evidence which suggests age should be considered as a prominent factor in broodstock management. The following investigation was conducted to determine whether age-related differences in egg diameters of cobia exist.

5.2) Materials and Methods

Cobia broodstock groups were maintained in conditions presently described by (Benetti et al. (2008a). These systems included two independent RAS with a total volume of 80,000 L. Water replacement of ~10% water per day occurred with seawater was pumped from Bear Cut, Virginia Key. Replacement water was mixed with recirculating tank water in system sumps, filtered to 10 μm, and UV sterilized before entering broodstock systems. Salinity variation mirrored ambient water conditions, though variation was minimal due the location of the UMEH near relatively stable, oceanic water source. However, variation was observed between 34 and 36 ppt. Temperature was maintained by heat pumps utilized to thermally induce reproductive maturation and thus daily fluctuation was negligible.

The broodstock were fed a diet consisting of previously frozen sardines, goggle eyes, or squid. Each tank received feed six days a week at a rate of ~3 % tank biomass

per day. Broodstock diets were supplemented once weekly with encapsulated vitamin premix which was hidden within thawed feeds. Broodstock Group 1 (BG1) were wild-caught broodstock held in captivity for two years at the time of data collection. Given this and age of cobia sexually maturity in the wild (2 years, Brown –Peterson et al. 2001), BG1 spawning females were assumed to be least four years of age, although possibly the spawning female was older. Broodstock Group 2 (BG2) and Broodstock Group 3 (BG3) were comprised of individuals raised in captivity from egg at the UMEH. Both of these groups reached sexual maturity at an age of two years.

During the summer of 2007, a female individual of BG1 was induced to spawn utilizing water temperature conditioning complemented by injection of 1,000 IU/kg body weight of human chorionic gonadotropin (HCG) (Sigma-Aldrich Co., St. Louis, MO, USA). Immediately prior to injection, observation of sampled oocytes of ~800 μm diameter indicated the female was nearing final oocyte maturation (Biesiot et al. 1994, Lotz et al. 1996, Brown-Peterson et al. 2001). Spawning commenced approximately 36 hours post injection. Upon egg collection, egg diameters were measured on the morning of May 17th. This represents the only spawn from BG1 analyzed in the present study. BG2 was induced to spawn utilizing water temperature conditioning alone. Eggs from this group were collected and egg diameters measured during the mornings of May 17th ($BG2_{SP1}$) and May 29th ($BG2_{SP2}$).

During the summer of 2008, BG2 were again conditioned to spawn utilizing water temperature alone. Although courtship behavior was evident before, the first spawning event of the season occurred on April 7th. Spawned eggs were collected the same night and egg diameter were subsequently measured ($BG2_{SP3}$). During this reproductive

season, BG3 reached sexual maturity and were also thermally conditioned to reproductive maturity. On April 14th, Broodstock Group 3 (BG3) spawned for the first time ($BG3_{SP1}$). Eggs were subsequently collected and egg diameters were measured the following morning. Spawns $BG2_{SP4}$ and $BG3_{SP2}$ were collected on May 5th and 6th, respectively, and respective egg diameters were measured the following mornings.

Upon collection, fertilized, positively buoyant eggs were separated from unfertilized eggs via flotation in a 1 L graduated cylinder. Either immediately after collection or early the following morning, diameters of viable eggs were measured with an ocular micrometer within a dissecting microscope at 25x magnification (Motic model SMZ-168).

Egg diameter samples from each spawning were first analyzed for normality with Shapiro-Francia *W'* tests; although the Shapiro-Wilk test is considered more powerful, it can be adversely affected by tied data (Zar 2009). Variance between groups was analyzed utilizing Brown and Forsythe's (1974) modification of the Levene Variance Test. Due to unequal replication of the each factor, individual ANOVA tests were utilized to investigate the influence broodstock tank temperature (°C), broodstock tank salinity (ppt), and broodstock age on mean egg diameters (mm). Further post-hoc pair-wise comparisons were made via the Tukey-Kramer method as modified by Games and Howell. The Tukey-Kramer procedure more accurately confines to the intended α level among unequal sample sizes than other post-hoc pair-wise comparison tests while the Games and Howell modification integrates the individual group variances, rather than computing a single variance from pooled data, when individual sample variances are suspected of being dissimilar (Zar 2009).

Robust regression analysis was utilized to further investigate relationships between egg diameter, salinity, and age. Although investigation of normality provided acceptable results, concerns regarding differing variances necessitated robust regression methods which utilize weighting in order to reduce the influence of data points with high leverage and/or outliers (Zar 2009, Stata…Robust Regression). Pairwise correlation was first utilized to determine the primary explanatory variable, and thus first entry into the regression. Partial F testing was also utilized to ensure validity of independent variable addition to the model. Residuals analysis was utilized to verify regression assumptions.

5.3) Results

Mean egg diameters, sample size, and age of spawning female are summarized in Table 5.1. All individual measured values, were in agreement with values published in the literature (1.25 mm, Ryder 1885; 1.16 to 1.42 mm, Joesph et al. 1964; 1.15 to 1.3 mm, Ditty and Shaw 1992; 1.40 mm mean value, Arnold et al. 2002; 1.28 to 1.38 mm mean values, Holt et al. 2007b). The mean egg diameters, grouped by salinity (1.29 ± 0.02, 1.28 ± 0.05, and 1.27 ± 0.03 mm for 34, 35, and 36 ppt, respectively), were also in agreement with anticipated egg diameters spawned at similar salinities (1.29, 1.28, and 1.27 mm for 34, 35, and 36 ppt, respectively) computed from the egg diameter and salinity relationship published by Holt et al. (2007).

Normality testing indicated no significant deviations when egg diameters were grouped by temperature, by age, or by salinity. Variance homoscedasticity analysis revealed differences for all the above listed groupings. Since ANOVA analysis is robust

to departures of homoscedasticity, further analysis was continued (Sokal and Rohlf 1995, Zar 2009).

Comparison of all egg diameter means utilizing ANOVA revealed broodstock age ($P < 0.0001$) and spawning salinity ($P = 0.0001$) as highly significant factors. Temperature was not found to be a significant factor ($P = 0.3184$) (Table 5.2). Post-hoc means comparisons indicated highly significant differences between mean egg diameter between each age group (Table 5.3a, Fig 5.1). However, significant differences were not detected between all salinity group comparisons (Table 5.3b).

Investigation of correlations between egg diameter and salinity and age revealed age ($r = 0.3754$, $P < 0.0001$) should be entered into the model before salinity ($r = -0.2634$, $P = 0.0001$). Upon entry of both factors into a robust regression model, both were found to significantly contribute to explanation of egg diameter variability via partial F testing (Table 5.4). The resultant regression was found to be highly significant ($P < 0.0001$) although overall variability in egg diameter was not well explained (adjusted $R^2 = 0.1457$). The computed coefficient for salinity (-0.0071) was slightly different from that computed by Holt et al. (2007b); however, the Holt et al. (2007b) value falls within the standard deviation range of the coefficient computed here.

Table 5.1: Female age, mean egg diameter ± standard deviation, sample size, temperature, and salinity observed during spawning events of 2007 and 2008.

Spawn	Mean Egg Diameter (mm) ± SD	n	Age (yrs) at Time of Spawn	Broodstock Water Temp (°C)	Broodstock Water Salinity (ppt)
BG1	1.33 ± 0.026	12	≥ 4	26	35
$BG2_{SP1}$	1.24 ± 0.021	15	2	28	36
$BG2_{SP2}$	1.25 ± 0.023	20	2	26	36
$BG2_{SP3}$	1.29 ± 0.020	40	3	29	34
$BG3_{SP1}$	1.26 ± 0.041	40	2	26	35
$BG2_{SP4}$	1.27 ± 0.028	40	3	27	36
$BG3_{SP2}$	1.29 ± 0.024	40	2	26	36

Table 5.2: Results of multi-factorial ANOVA analysis of egg diameter utilizing broodstock age, temperature (°C), and salinity (ppt) as factors.

a)

Source of Variation	df	Partial SS	Mean Squares	F Value	P Value
Age	2	0.04111	0.02056	19.98	<0.0001
Residual	202	0.20990	0.00103		
Total	206	0.25101	0.00122		

b)

Source of Variation	df	Partial SS	Mean Squares	F Value	P Value
Salinity	1	0.01742	0.01742	15.28	0.0001
Residual	205	0.23359	0.00114		
Total	206	0.25101	0.00122		

c)

Source of Variation	df	Partial SS	Mean Squares	F Value	P Value
Temperature	1	0.00122	0.00122	1.00	0.3184
Residual	202	0.24979	0.00122		
Total	206	0.25101	0.00122		

Table 5.3: Results of Tukey-Kramer procedure, modified by Games and Howell for unequal variances, pairwise testing of mean egg diameters grouped by a) age, and by b) salinity.

a)

Age Comparison	n	Group Means		Mean Difference	TK-value Computed	df	Significance Level
2, 3	115	1.2668	1.2816	0.0148	4.64	191	0.0042
2, 4	80	1.2668	1.3250	0.0582	10.00	16	< 0.001
3, 4	12	1.2942	1.3250	0.0434	7.53	15	< 0.001

b)

Salinity Comparison	n	Group Means		Mean Difference	TK-value Computed	df	Significance Level
34, 35	40	1.2668	1.2816	0.0148	3.44	78	0.047
34, 36	52	1.2668	1.3250	0.0582	9.60	44	< 0.001
35, 36	115	1.2942	1.3250	0.0434	1.53	71	> 0.50

Figure 5.1: Mean egg diameter of each spawning event plotted with standard deviation bars. Letters denominate groups of significantly similar size when pooled by age of broodstock.

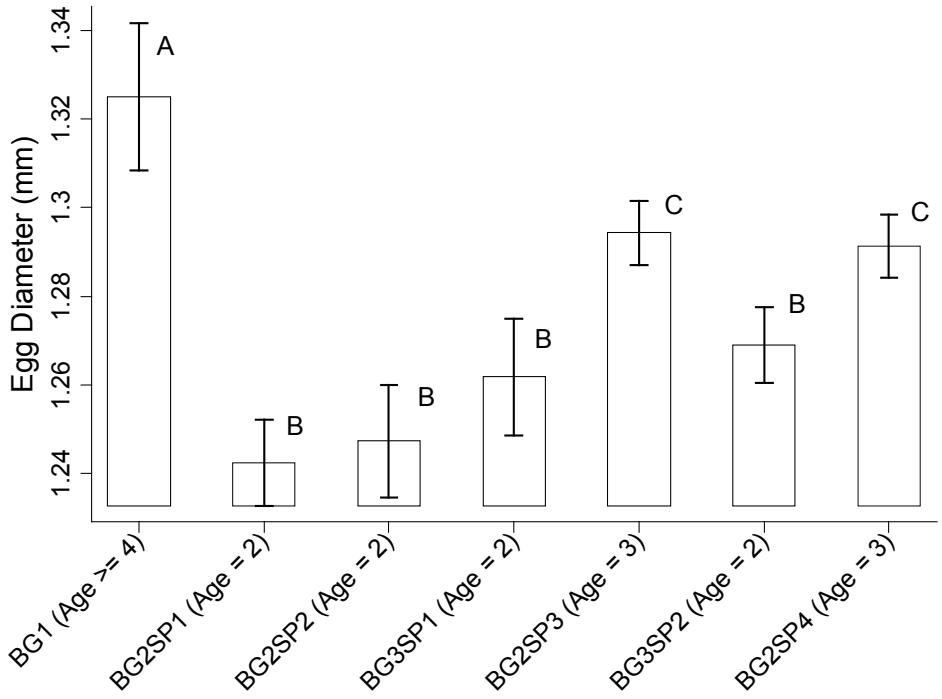


Table 5.4: Results of egg diameter robust regression analysis with age and salinity utilized as independent factors.

Dependent Variable	Independent Variable	Indep. Variable Coefficient	Standard Error of Coefficient	P Value Indep. Variable	Adjusted R^2	Regression Computed F Value	df	P Value of Regression
Egg Diameter					0.1457	17.39	2	< 0.0001
	Age	0.0173	0.0041	< 0.001				
	Salinity	-0.0071	0.0032	0.027				
	Constant	1.4832	0.1165	<0.001				

5.4) Conclusions

Broodstock management often aims to maximize spawning output. Since husbandry conditions ultimately lead to reduction in final size due to tank size, the issue of relative fecundity becomes an important factor to consider. Similar to other fishes, increasing absolute fecundity with size has been observed in wild cobia specimens (Brown-Peterson et al. 2001) and has been confirmed with cobia held in captivity (Faulk and Holt 2008, I. Zink Pers. Obs.). As females age, egg diameter would continue to increase, further impacting relative fecundity in growth-limited situations. Thus, better understanding of the tradeoffs between female age and size which maximize both egg quality and quantity could lead to maximization of larval quality and survival, and thus production efficiency. However, determination that cobia female age influences egg quality, as proxied by diameter, must occur before it is further considered as a factor in broodstock management.

Significant differences between mean egg diameters and of differing age groups were observed (Table 5.2a, Table 5.3a, Fig. 5.1). This observation coincides with multiple studies considering the age of spawning females (Nikolskii 1969, Hislop 1988, Trippel et al. 1997, Berkeley et al. 2004, Sahin et al. 2007). However, this is contrary to other studies of cobia which did not detect age influence (Holt et al. 2007b, Faulk and Holt 2008, C. Faulk Pers. Comm.). The elegant study conducted by Berkeley et al. (2004) demonstrates the potential implications of increasing egg quality with female age, not only for wild fisheries management, but to broodstock management and hatchery production as well. Older females produce larger eggs due to increased relative

allocation lipid stores per egg which results in faster initial growth and better survival characteristics in the resultant larvae (Berkeley et al. 2004).

ANOVA investigation also revealed the significance of spawning salinity on egg size (Table 5.2b, Table 5.3b). Computed mean values from the present study are similar to those computed from the regression reported by Holt et al. (2007b). These observations further substantiate the trend of increasing diameter with decreasing salinity as is known to occur in many other fishes (Chambers 1997). Furthermore, regression analysis yielded a salinity coefficient very similar to that computed by Holt et al. (2007b) ($y = -0.0098 x + 1.6249$) after influence of age on egg diameter variation was accounted for in the model. However, comparison of the present study to that of Holt et al. (2007b) is hindered by reduced salinity range observed during the current study.

Although differences in mean egg diameter do not seem relatively large, their impact is better put in perspective with consideration of resultant egg volumes: if one assumes a spherical egg, the largest mean egg diameter from BG1 produced a volume 23.4% greater than the smallest mean value observed in this study. Under the same assumptions, the largest published cobia egg diameter (1.42 mm) would result in an egg volume 50.2% larger than that of the smallest published value (Joesph 1964).

Adjustment of egg buoyancy characteristics by spawning females to ensure egg floatation at differing spawning salinities was speculated as explanation for observed egg diameter variability reported by Holt et al. (2007b). Although salinity was detected as a significant factor in the present study, its influence was less than that of maternal age, as revealed by correlation analysis. Thus, changes in oocyte water content cannot logically, nor statistically, describe the total variation observed in the present study. Greater stores

of yolk and/or a larger oil globule size, in relation to female spawning age, could have been the source of the observed size variation, as is known to occur for other fishes (Bagenal 1971, Berkeley et al. 2004). In the present study, due to lack of biochemical investigation of egg composition and measurement of oil globule diameter, no concrete conclusions should be made.

Faulk and Holt (2008) investigated differences in biochemical, subsequent larval growth and survival, and other factors associated to cobia egg quality during two consecutive spawning seasons. The authors observed no differences in growth or survival between any of the spawning events investigated despite detecting differences in fatty acid composition and total lipid content. Furthermore, despite differences in total lipid content, no difference in egg diameter was detected (Faulk and Holt 2008). Fatty acid and total lipid content differences were attributed to differences in broodstock food quality over the course of the study (Faulk and Holt 2008). Both Faulk and Holt (2008) and the present study utilized similar broodstock diets consisting of frozen bait fishes/squids supplemented with vitamin additions. Thus, similar affects could have influenced the present study. However, their study does not preclude the potential for differences in larval quality to result from maternal age influences.

Hormonal implants are often employed in order to force spawns from fishes that either in are in season and/or are observed to be reproductively mature and are not spawning voluntarily. Hormonal treatments have been observed to negatively affect egg quality, presumably by disrupting the timing of final oocyte maturation and ovulation (Mylonas et al. 1992, Tucker 1994, Brooks et al. 1997). Utilization of HCG to elicit the BS1 spawn could explain the reduced fertilization and hatching rates associated with this

spawning (data not presented). Relative to other treatments with protracted effects, such as gonadotropin releasing hormone analogues, HCG utilization is less likely to reduce egg quality during vitellogenesis because of its direct and rapid action on final oocyte maturation. Vitellogenesis is a distinct process that is complete before the initiation of final maturation and ovulation occurs; thus, lipid content would not have been affected (Hoar 1969, Stacey 1984). Converse to vitellogenesis, the hydration process could have been impacted by HCG utilization. Final egg volume is influenced during the hydration process (Greeley Jr. et al. 1991), which is a major component of final oocyte maturation (Hoar 1969, Stacey 1984).

Though HCG utilization could have impacted final oocyte size, and thus the magnitude of the difference between mean egg sizes for the 4 year old sample relative to the others, the dataset as a whole yet suggests that maternal age influenced spawned egg diameter. However, this could explain the substantial increase in egg diameter between 3 year old and 4 year old fishes observed in the present study. Since only one spawn from BG1 was analyzed in the present study, further conclusions regarding the influences of HCG cannot be drawn.

Seasonality most likely did not play a role in observed differences in egg sizes due to all data collection taking place early in the spawning season. Furthermore, seasonality was not detected in the study conducted by, which investigated spawns from throughout the spawning season (Faulk and Holt (2008). Similar to protocols followed in this study, the authors concluded a consistent and constant diet the cobia broodstock received over the course of their protracted spawning allowed for nutritional replenishment between spawning events (Faulk and Holt 2008).

Temperature is often considered a causative factor of seasonal variation, although this may not be true for all situations. The general intraspecific trend is reduction of egg diameter at higher spawning temperatures (Chambers 1997). Temperatures of broodstock tanks did vary from spawn to spawn, although multi-factorial ANOVA analysis did not reveal any significant affects. However, temperature variation was not as substantial as is often experienced in the wild due to utilization of temperature control heat pumps in broodstock RAS systems. Similarly, temperature induced effects during oogenesis on cobia spawned egg diameter have not been reported in other studies (Holt et al. 2007b, Faulk and Holt 2008).

Further studies of the causes and effects of these egg diameter differences, which include greater numbers of spawns collected from older female cobia and older female cobia as well, could provide more conclusive evidence for the hypothesis of female age relation to egg diameter and quality characteristics. These studies should incorporate factors; such as oil globule diameters, total lipid content comparisons, ratios of individual fatty acids; which would influence later larval performance. Ergo, these studies would require investigation of subsequent larval performance; such as starvation studies and early growth rates.

Few studies investigate broodstock management; though, informal discussions have indicated other researchers agree with the potential need of larger broodstock tanks in order to keep larger cobia voluntarily spawning (C. Faulk, Pers. Comm.). Indeed, Taiwanese research laboratories, who hold the most experience working with this species, maintain larger cobia broodstock in tanks 10 m in diameter resulting in tank volumes of approximately 100 m^3 (Orhun, pers. com). These tanks are substantially

larger than those currently utilized in the United States. The inverse relationship between egg size and fecundity (Blaxter 1988), as well as limitations to growth due to confinement, suggest in order to effectively optimize both quality and quantity of reproductive output, optimization of tank size and stocking density would be necessary. Although size has long been considered a determinant of absolute fecundity, consideration of female age and resultant influences on egg quality, could improve broodstock management and maximize not only reproductive output, but later larval survival and growth as well, and thus overall hatchery output.

Chapter 6: Towards Optimization of Cobia Larviculture Production Outcomes with Improvements in Growth Analysis

6.1) Background

The ultimate goal of aquaculture hatchery production is similar to any type of production scenario: maximization of production while minimizing costs. At times, minor cost increases are acceptable if justified by substantial production gains. Understanding of the processes and interacting factors which influence production outcomes can lead to improved production maximization. In relation to larval rearing and production of juvenile cobia, some rudimentary gaps in knowledge of influential factors yet exist, which, when filled, could yield improved production. Schwarz et al. (2007) pointed out that basic nutritional and environmental requirements for the larval stage of this species have yet to be established, and further stated "for cobia to realize its latent potential as an aquaculture species, it is clear that much more information must be gathered upon its basic environmental requirements."

Many factors can contribute to growth variation. Determining which of these factors most greatly influence, as well as relative contributions of each factor, variation in larval growth, survival, and recruitment in an ecological sense has been a driving force behind early marine finfish larval culture studies. In relation to more recent aquaculture industry expansion, these studies take on a difference focus; however, the underlying principles are similar. In a simple irony, aquaculturists should return to the historical works of their fellow ecological fish biologists, and related culture experiments, to develop a fundamental basis of understanding how behavior, life history, and ecology of

a species influences growth and survival. Such a basis would provide the initial stepping stones for maximizing production in the laboratory or larviculture setting.

In Chapter 5, the major determinants affecting egg size and subsequent larval fish growth were discussed. Effects of captivity on larval growth and distributions are similarly recognized: 'size hierarchies;' shifts in size proportions; relative increases in abnormalities; tank shape, volume, and color; crowding and density dependency of growth; and related influences upon biochemical composition of tissues (Blaxter 1988). Rates of development are recognized to be most strongly influenced by temperature regimes, influencing Q_{10} physiological forcing of size-at-hatching, efficiency of yolk utilization, growth, feeding rate, time to metamorphosis, behavior, swimming speeds, digestion and gut evacuation rates, and metabolic demand (Blaxter 1988).

Other conditions, including physical water conditions such as water oxygen capacities, water viscosity (Blaxter 1988), pH and salinity and their influences on osmotic and ionic regulation (Alderdice 1988), and pollutants, noxious chemicals, and the like (including chemotherapeutants) (Von Westernhagen 1988) will also influence growth. Still yet other factors include critical periods in development and factors affecting survival at critical periods, including first feeding and prey availability, respiration, and metamorphosis (Blaxter 1988).

Early studies of cobia are sparse, most likely due to the lack of commercial fisheries importance of the species. The earliest study on early life stages of cobia was conducted by Ryder (1887), who strip spawned reproductively active adults collected from the Chesapeake Bay in study of embryonic development. Joseph et al. (1964) presented a study of cobia spawning activity via collection of egg, larval, and juvenile

specimens immediately offshore and in the mouth of the Chesapeake Bay. The authors noted a June and July peak of spawning with attenuation into August based upon their observations of egg dispersal and numbers collected (Joseph et al. 1964). It was concluded that the lower Chesapeake Bay is an important spawning area, based on distributions of eggs collected in lower-salinity (between 23 and 30 ppt) estuarine plumes being advected from the bay; unfortunately, temperature data of the water masses was not provided (Joseph et al. 1964). Included in the study were observations of three juvenile cobia; the capture locations of these juveniles, one nearshore on the eastern, Atlantic shore of Virginia and two near the mouth of the York River, suggested cobia early life stages and juveniles inhabit estuarine waters (Joseph et al. 1964).

Richards (1967) also reported on collection of reproductively active female cobia well within the Chesapeake Bay. Dawson (1971) reported collecting prejuvenile and early juveniles (12.6 – 27.0 mm standard length [SL]) in immediate offshore waters of Mississippi in temperatures ranging from 25.9 to 32.0 °C and salinities from 27.8 to 37.7 ppt. Other collected individuals included 14 to 23 mm SL individuals collected 925 km offshore of Delaware (Dawson 1971). Dawson (1971) concluded larval and juvenile cobia occur in both offshore and estuarine areas, with juveniles more likely in coastal areas; however, the author notes being unable definitively conclude whether this truly represents an offshore-inshore size relationship or was an artifact of sampling.

Hassler and Rainville (1975) report upon the first known culture of cobia which was achieved with eggs collected from the Gulf Stream approximately 15 to 30 miles offshore of Hatteras, North Carolina. An early review of cobia early life stages mentions

a 7.0 mm larvae collected in ‘brackish’ waters of a coastal lagoon of India (Hardy Jr. 1978).

Shaffer and Nakamura (1989), in their synopsis of cobia biology, list temperature ranges from 16.8 to 32.0 °C and salinities ranging from 22.5 to 37.7 ppt associated with collection of wild larval and early juvenile cobia. The authors conclude that most cobia eggs and larvae are found offshore and juveniles move inshore to coastal areas (Shaffer and Nakamura 1989). Ditty and Shaw (1992), while both actively investigating and reviewing larval cobia development and distribution in the Gulf of Mexico, discuss sampling the Crystal River Estuary, Florida, where eggs and yolk-sac larvae were collected in waters ranging from 28.1 to 29.7 °C and 30.5 to 34.1 ppt. This study also mentions collection of a single cobia egg from a power plant discharge canal at 36.7 °C and 25.2 ppt, but do not indicate whether it was still viable (Ditty and Shaw 1992).

It is both plausible that the larger size larvae collected offshore may have been individuals originally spawned in offshore waters or individuals that were advected from inshore waters. Taken as a whole, these ecological and distributional studies and reports confirm that cobia spawning and early life stages occur in both estuarine and offshore conditions, where they face broadly varying physical parameters, such as temperature and salinity regimes. To better understand and maximize growth, survival, and other important production characteristics, further investigation over the range of these parameters is warranted.

A number of experiments have demonstrated the short term hardiness, of cobia juveniles maintained at a broad range of salinities (Atwood et al. 2004, Resley et al. 2006, Burkey et al. 2007) although some also report deleterious effects on plasma

osmolarity, hematocrit, specific growth rate, and food conversion efficiency (Denson et al. 2003, Burkey et al. 2007, and Chen et al. 2009). Cobia larvae have been demonstrated to be relatively resistant to abrupt and gradual salinity changes, with increasing survival observed in relation to increasing larval age (Faulk and Holt 2006). Spawning cobia held in captivity have also been observed to modify egg diameter and water content in relation to salinity (Holt et al. 2007b, Chapter 5). It was suspected that this plasticity, which has also been reported for the estuarine/coastal species spotted seatrout *Cynoscion nebulosus*, is conducted in efforts to decrease egg specific gravity and produce neutrally buoyant eggs (Holt et al. 2007b). These studies further substantiate the distributional hardiness of cobia early life history stages in relation to salinity variation. Though verification is always warranted, this plasticity is not surprising given previous ecological studies.

Early life stages of cobia are recognized to withstand a wide range of temperatures due to their subtropical and tropical distribution (Hassler and Rainville 1975, Shaffer and Nakamura 1989, Schwarz et al. 2007b). Similar to salinity tolerance observations, the previously discussed ecological studies both suggest and verify this conclusion. For juvenile cobia, median lethal low temperature has been reported as 12.1 °C (Atwood et al. 2004) while lethal high temperature is reported to be 37.7 °C (Hassler and Rainville 1975). Unfortunately, similar lethal ranges are not readily available for cobia larvae. Despite this, environmental data associated with wild collection of larvae suggest typical temperatures experienced range from 24.2-32.0 °C (Ditty and Shaw 1992).

Few investigations study temperature influences on growth during cobia early life stages. A majority of those which do so relate to juvenile, rather than earlier, life stages. One study of juvenile cobia growth and energy budget found peaks of feed absorption efficiency, maximal ration level, and fecal production at 31 °C; furthermore, specific growth rates (wet weight, dry weight, protein, and energy) did not statistically differ between 27 and 31 °C (Sun et al. 2006). However, feed conversion efficiency and proportion of food energy allocation to growth peaked at 27 °C (Sun et al. 2006).

A follow-up study of ration and temperature effects on energy budget and growth of juvenile cobia found that when fed to satiation, juvenile specific growth rate (wet weight, dry weight, protein, and energy) relationships maximized at 31.0-33.0 °C and feed conversion efficiency (wet weight, dry weight, protein and energy) relationships maximized at 29.5-31.3 °C (Sun and Chen 2009). The authors, pointing out aquaculturists generally aim for maximization growth, conclude that temperatures of 31-33 °C would best achieve these results, but also caution against potential increased relative cost of production per individual caused by increased disease incidence and feeds consumption. Investigation of reduced temperatures on juvenile cobia growth yielded no significant difference in feed efficiency between 23 and 29 °C; though, specific growth rate of weight and length were found to significantly differ (Schwarz et al. 2007b).

Presently, there are no analogous studies of temperature affects on larval cobia growth or feed conversion efficiencies. Conclusions on differences in growth can only be drawn by comparison of physical parameters, other related factors, and growth rates as reported in various larviculture studies. Reported coefficients of instantaneous growth in length range from 0.063 to 0.1081 (see Table 6.22) (Faulk et al. 2007a, Holt et al. 2007b,

Benetti et al. 2008b). However, direct comparisons of this nature are difficult to interpret due to variation in factors such as initial stocking density, final density, temperature regimes, nutritional value of live feeds, and other factors which influence growth. The highest larval growth rate reported in the literature (0.1081) was obtained from a low density larviculture (initial stocking density 4 eggs L^{-1}) and at culture temperatures ranging from 25 to 26 °C (Holt et al. 2007b). Thus, this instantaneous growth rate could not be expected to represent the potential upper bound as collection of wild cobia larvae suggests it can tolerate higher temperature regimes.

As previously noted, paternal affects and other factors influencing egg quality and subsequent larval survival and growth are numerous (previously discussed in Chapter 5). Faulk and Holt (2008) examined egg biochemical composition and quality spawned from captive cobia broodstock and noted significant increases and decreases of individual fatty acids between the two consecutive spawning seasons encompassing the study. The authors attributed these differences to variation in broodstock diet quality. Changes in total amount of n-3 PUFAs were correlated with egg quality, as increasing numbers of floating eggs were observed with decreasing absolute levels of these fatty acids (Faulk and Holt 2008). Further relationships concerning larval growth and survival were not found to associate with egg quality (Faulk and Holt 2008). Seasonality was not detected in egg composition or quality, the lack thereof being attributed to continuous and consistent feeding throughout the spawning season (Faulk and Holt 2008).

Differences in salinity at the time of final oocyte maturation and spawning have been observed to produce changes in egg diameter (Holt et al. 2007b, Chapter 5). Although, this factor was not found to affect growth up to 9 DPH (Holt et al. 2007b).

Spawning female age may contribute to egg diameter and subsequent larval quality as elegantly demonstrated by Berkeley et al. (2004). Significant differences between cobia egg diameters spawned from females of different ages have been detected (Chapter 5), and thus broodstock age could influence later larval growth and survival in this species as well.

Ensuring proper nutritional requirements are met is essential to maximizing growth and survival, especially for early life stages which exhibit more rapid growth and development. While numerous studies have investigated juvenile nutritional requirements, fewer have done so for larval cobia. Those that have focus upon fatty acid enrichment profiles of live feeds and resulting profiles within larvae (Faulk and Holt 2003, Turner and Rooker 2005, Faulk and Holt 2005). Niu et al. (2008) claims to investigate dietary phospholipid levels for larval cobia, but since this study was initiated with individuals aged 20 DPH, it can be argued that this study really was of cobia juvenile nutrition.

Faulk and Holt (2003) concluded temperature independent larval cobia growth (growth in Degree Days) in length was comparable whether cobia larvae were fed enriched, maintained live feeds or wild zooplankton consisting primarily of copepod nauplii and copepidids (Hassler and Rainville 1975). Fatty acid profiles observed in endogenous feeding larvae are thought to reflect upon later exogenous nutritional requirements; thus, their study of cobia egg and early larval fatty acid composition has served as a baseline to later test the affects of differing enrichment diets as the (Faulk and Holt 2003). The study confirmed high poly-unsaturated fatty acid (PUFA) and individual essential fatty acid levels for cobia and notes an interesting reduced ratio of

eicosapentaenoic acid (EPA) to arachidonic acid (ARA), due to increases in absolute ARA levels, in comparison averages of other marine finfish species (Faulk and Holt 2003).

Turner and Rooker (2005) demonstrated that larval cobia PUFA profiles significantly reflect changes in diet and observed PUFA signature changes within 3 days of changes in diet source. Contrary to these findings, Faulk and Holt (2005) found that fatty acid profiles of 7 DPH cobia larvae more closely resembled those of yolk-sac larvae prior to the onset of exogenous feeding rather those of the rotifers the larvae had been consuming over the previous 4 days.

Faulk and Holt (2005) did not find increased levels of ARA in enriched *Artemia* until 20% of Algamac2000 diets were replaced with an ARA supplement; these levels were similar to those observed when utilizing Algamac3050 enrichment diet alone (Faulk and Holt 2005). This suggests that Algamac2000 alone, utilized by the study conducted by Turner and Rooker (2005), is not sufficient to boost ARA levels in enriched live feeds, and therefore in larvae preying upon these live feeds (Faulk and Holt 2005). Despite increasing levels of ARA supplementation in live feed enrichment diets, no significant difference in larval 16 DPH whole body tissue ARA was detected nor were significant differences in growth or survival detected (Faulk and Holt 2005). Despite a lack of significance, the authors a did note an increasing trend in survival with increasing levels of ARA (Faulk and Holt 2005). Furthermore, significant increases of docosahexaenoic acid (DHA) levels in live feeds, which more closely matched levels observed in endogenous feeding larvae, also did not significantly increase survival or growth in this

study, suggesting DHA levels at the lower levels observed during the study are sufficient for cobia larvae (Faulk and Holt 2005).

Other nutritional requirements have not been investigated for cobia larvae. While Faulk and Holt (2005) discussed a lack of difference between protein, carbohydrate, and total lipid levels between commercial enrichment diets and live microalgae as well as lack of free amino acid (FAA) differences between diets from various other studies, they do not discuss whether these diets match amino acid (AA) requirements of larval cobia. A later study by Faulk and Holt (2008) investigated protein and individual total amino acid (TAA) content in cobia fish eggs, providing a baseline for further study which suggests early larval requirements, similar to as discussed in relation to PUFAs. The authors reported 25.5% of egg dry weight consist of protein and 51.1% of dry weight was comprised of TAA, indicating ~50% of TAA was FAA (Faulk and Holt 2008). Earlier ovarian developmental studies within wild cobia found that protein content did not greatly fluctuate during the course of the spawning season and ranged between 49-55% (Biesiot et al. 1994).

Since larval fish are generally comprised of 50% or more protein, it is argued that more attention should be given to matching of TAA requirements (Faulk and Holt 2008). Larval indispensable amino acid (IAA) profiles are noted as indicative of FAA requirements, although, this relationship can be complicated by relative bioavailabilities of an individual AA and its absorption and catabolism (Conceição et al. 2003). When studying live feeds enrichment and IAA profiles of larval turbot (*Scopthalmus maximus*), the enrichments seemed to be deficient in some IAA (Conceição et al. 2003). More recently developed techniques can be utilized to study bioavailabilities of AA and, in

rotifers, a deficiency of threonine and leucine required by gilthead seabream (*Sparus aurata*) was noted (Conceição et al. 2003). Studies such as these would similarly improve the understanding of nutritional and dietary requirements for larval cobia.

Related to nutritional studies, investigation of the ontogenetic development of the gastrointestinal tract and enzymatic activity can reveal insights relating to digestion capabilities at various early life stages and the development of nutrient absorption. Faulk et al. (2007b) describe these topics for cobia larvae reared at 26.5 °C and stocked within 150 L tanks at an initial density of 1000 eggs $tank^{-1}$. The study notes that gastrointestinal and enzymatic development were more closely related with SL rather than age. Luminal digestion of protein was initiated approximately 10-12 DPH; gastric cells first appeared 10 DPH, although surface mucous cells did not stain positively for neutral mucosubstances until 12 DPH, suggesting prior non-functionality in gastric cells (Faulk et al. 2007b). Also beginning10 DPH, large supranuclear vacuoles had completely disappeared, coinciding with marked increases in trypsin activity; together, these events suggest the end of intracellular and beginning of extracellular protein digestion (Faulk et al. 2007b). The authors suggest that the digestion and assimilation of compound commercially prepared diets would be possible at this time, and further suggest increasing study of palatability of these feeds, as taste buds begin to appear as early as 3 DPH and feeding trials suggested starvation-related mortality was related to formulated feed attractiveness and not digestibility (Faulk et al. 2007b).

Influences of timing of feeding regimes on growth and survival have not been directly studied; although, a recent cobia larviculture review suggests higher survival and growth rates may be attainable if *Artemia* feeding period duration could be reduced,

noting that cobia larvae could ingest larger food items as early as 14 DPH (Holt et al. 2007b). Indeed, Benetti et al. (2008b) concluded that rearing temperatures ranging from 29.4-31.8 °C accelerated development and metamorphosis, thus accelerating weaning onto commercial diets. Increased food intake was deemed crucial to meet the demands of rapid development and high metabolic demands, as manifested by earlier development of gills and red blood cell formation (Benetti et al. 2008b). Increased food intake requirements were met by increasing the amount of dry commercial diet offered throughout the weaning process while offering *Artemia* at relatively static densities (Benetti et al. 2008b).

Potential benefits of greenwater incorporation within larviculture protocols include reduction of larval fish interaction, which should reduce cannibalism and increase feeding success. Potential digestion and nutritional benefits for larvae; continued enrichment of live feeds after stocking within larval rearing culture tanks; reduction in ambient light intensity, especially in relation to damaging ultraviolet radiation; water quality remediation; and microbial community influences are all potential benefits from greenwater utilization (Reitan et al. 1997, Lee 2003). Faulk and Holt (2005) reported significant increases in survival with greenwater cultures relative to clear water cultures, although no significant difference in growth in length was detected at 7 or 16 DPH. Algal pastes have been proposed as a replacement for live algal greenwater cultures that would yield some of benefits of live algae utilization. Financial savings in terms of labor necessitated for live algae culture, as well as reliability when compared to potential and unpredictable algal culture crashes, are two aspects of hatchery production which algal paste replacement of live algal cultures can improve. Schwarz et al. (2008) found no

significant differences in mean final weight (28 DPH), percent survival, or final densities when larval cultures incorporated algal pastes or live microalgae.

Live algae utilization has been observed to stimulate pancreatic and digestive functions and development in larval sea bass *Dicentrachus labrax* (Cahu et al. 1998). Whether these affects were related to the mere presence of algae or due to extracellular compounds released by live algae is not specified was speculated upon within the Schwarz et al. (2008) study. Thus, the authors suggest there may be other benefits foregone when utilizing algal paste that their study did not investigate (Schwarz et al. 2008). However, they further suggest bacterial community modification benefits realized with incorporation of live greenwater may not necessarily apply or be as crucial in RAS systems, which generally harbor reduced numbers of opportunistic and pathogenic bacteria (Schwarz et al. 2008).

Disease and parasites cause stress and reductions in growth as metabolism is diverted to immunological related needs. As water temperatures increase, bacterial communities could be more volatile and, synergistically with the increased water temperatures, could cause even greater stress on growing cobia. A number of bacterial infections are known to infect various life stages of cobia, including *Photobacterium* spp., *Vibrio* spp., *Mycobacterium* spp., *Streptococcus* spp., *Aeromonas* spp., and *Citrobacter* spp. (Lopez et al. 2002, Liu et al. 2004, Liao et al. 2004, Lowry and Smith 2006, Benetti et al. 2008b). Other known diseases and parasites include *Amyloodinium ocellatum* infestations (Liao et al. 2004, Benetti et al. 2008b), *Epistylis* and *Trichodina* ciliate and *Nitzchia* phytoplankton infestations (Liao et al. 2004), viruses (Chi et al. 2003, Liao et al. 2004), *Ichthyobodo* spp. flagellates (Bunkley-Williams and Williams 2006),

Cryptocaryon irritans (Bunkley-Williams and Williams 2006), *Brooklynella hostiles* slime-blotch disease (Bunkley-Williams and Williams 2006), monogeneans (Lopez et al. 2002), myxosporeans (Lopez et al. 2002, Liao et al. 2004), digeneans (Bray and Cribb 2003, Bullard and Overstreet 2006), and *Neobenedenia* sp. flukes (Liao et al. 2004). Of these, bacterial infections and *A. ocellatum* infestations are the greatest concerns during the larval and early-juvenile stages (Benetti et al. 2008b).

Prophylactic chemotherapeutant treatments, such as periodic formalin treatment (Benetti et al. 2008b), have been developed and demonstrate success; although, further investigation of such treatments is still warranted, especially in regards to bacterial community perturbations and in conjunction with probiotic efficacy. Even so, 75 ppm formalin treatment success suggests that any direct negative impacts on survival and growth are most likely minimal and are potentially counterbalanced by reductions in losses relating to diseases and parasites. Juvenile cobia have been successfully vaccinated against three bacterial spp. which commonly cause disease (Lin et al. 2006). Further studies are necessary in order to further develop vaccines in order to limit production losses and reduce reliance on antibiotics to counteract bacterial infection.

The potential deleterious effects on growth and survival of differing chemicals and chemotherapeutants remain largely unstudied for cobia larvae. Dung et al. (2005) studied the effects of cyanide, zinc, and copper sulfate to various early stages of cobia; for copper sulfate concentrations, 1 DPH larvae 24hr LC_{50} was 0.091 mg L^{-1}, 1 DPH larvae 96 hr LC_{50} was 0.060 mg L^{-1}, and 20 DPH juvenile 96 hr LC_{50} was 0.087 mg L^{-1}. Long term affects on growth at lower concentrations was not investigated, nor were temporary exposure to higher concentrations.

Other chemotherapeutants and additives to cultures could yield positive influences on production characteristics. The utilization of antibiotics in other aquaculture industries has yielded higher growth and increased survival, yet these results have not been investigated in relation to cobia. Probiotic incorporation within production protocols has produced beneficial results in other aquaculture industries (see Chapter 1), although this topic also has not been investigated in relation to cobia larviculture. Inclusion of mannan oligosaccharide within live feeds enrichment protocols was found to improve larval cobia survival when challenged with low salinity stresses; further benefits included stimulated gastrointestinal development in terms of increased microvilli height and reduction of supranuclear vacuoles, suggesting more rapid development towards complete lipid and protein digestion and absorption (Salze et al. 2008). While the study demonstrated oligosaccharide modification of gut microflora, these compounds can also work as prebiotics. Further study of these short-chain carbohydrates and probiotics inclusion within live feeds cultures should incorporate microbial community analysis as well.

Water quality stressors can have deleterious effects on growth and survival. The high metabolic demands of rapidly growing and developing cobia demand high oxygen concentrations (Feeley et al. 2008). This is especially true as metabolic demands and food intake increases with temperature (Sun and Chen 2009). Indeed, Benetti et al. (2008b) noted high mortalities as a result of rapid drops in DO concentrations to <4.0 mg L^{-1}. This event occurred immediately after a feeding event when metabolic demand within the larviculture peaked and included both larvae and recently stocked live feeds (Benetti et al. 2008b). Toxicity and sublethal effects of ammonia and nitrite for juvenile

cobia have been studied (Rodrigues et al. 2007); although these findings most likely do not readily apply to less hardy larval stages.

Stocking density and its effects on cobia larval growth and survival were studied by Hitzfelder et al. (2006) in attempts to find proper initial densities to achieve maximal production characteristics (growth in length, survival, and final density). The authors reported no difference in survival between treatments initial stocked at densities of 1, 5, and 10 larvae L^{-1}, although, in the 1 larvae L^{-1} treatment, final density was significantly lower and SL was significantly greater than the other two treatments (Hitzfelder et al. 2006). The study also found relatively high initial stocking densities (> 10 larvae L^{-1}) resulted in increased 'startle response' behavior, which negatively impacted feeding success. These behaviors have been confirmed at stocking densities of ~15 yolk-sac larvae L^{-1} in trials conducted at the Cape Eluethera Institute (Zink Pers. Obs.).

Converse to the findings of Hitzfelder et al. (2006), Benetti et al. (2008b) detected significant differences in length, at both 15 and 21 DPH, and survivorship between initial stocking densities of 5 and 10 larvae L^{-1} when reared at commercial scales. Despite these differences, final yield between the treatments was not significantly different, although a trend towards higher densities was observed with higher initial stocking densities (Benetti et al. 2008b), similar to Hitzfelder et al. (2006). Though not studied in relation to cobia larvae, larger culture vessels or tanks are recognized to improve growth and survival outcomes (Blaxter 1988). Perhaps culture tank size can explain the differing findings between Benetti et al. (2008b) and Hitzfelder et al. (2006).

The following analysis of cobia commercial scale production data was conducted in attempts to determine influential factors on production outcomes (growth rates,

survival, and total number of juveniles produced). By better understanding some of the basic factors influencing early cobia growth, more efficient production can be realized. Through analysis of data variability, improvement in methodologies utilized to model cobia larval growth and improved future data collection can be achieved. These investigations were carried out at larviculture scales which better emulate those utilized during the commercial production of cobia juveniles; thus, results and conclusions drawn from them would readily apply to the majority of commercial cobia larviculture practices.

6.2) Materials and Methods

6.2.1) Trials and Experiments within Trials

The following analysis incorporates larval rearing data collected over a number of rearing seasons spanning from the fall of 2005 to the spring of 2008. Many differences exist between the successive larviculture protocols. Some differences were resultant from deliberate manipulations or changes and improvements in protocols and methods while others represent variable ambient conditions which were not controlled, such as temperature regimes. Similarly, further differences occur within individual treatments. These also represent deliberate treatments or manipulations, such as initial stocking density, while others, such as final density of juveniles, could not be controlled for.

These variations between trials were utilized as independent variables for the construction of regressions investigating the optimization of growth in length, survival, and number of juveniles produced. Through the remainder of this study, each individual rearing tank was treated as a separate outcome, although for some analysis it was more fitting to average rearing tanks within a single larval rearing due to non-variation within a

larval rearing of a particular variable under analysis. Thus, this study is comprised of 5 Trials, each of which comprise a total of 13 experimental units (Experiments). Experiments 1 and 2 are associated with Trial 1 while Experiment 3 is the only larval rearing tank data analyzed from Trial 2. Trial 3 consists of Experiments 4 through 7, while Trial 4 consists of Experiments 8 through 11. Finally, Trial 5 consists of Experiments 12 and 13. Naming will henceforth refer to individual Trials (T1, T2, T3, etc.) or individual Experiments both within and between Trials (such as T1E1, T1E2, and T4E8).

6.2.2) General Methodology for All Trials

In general, broad protocols for all larvicultures were similar between Trials; factors or treatments that were different between Trials are discussed in subsequent sections. The majority of eggs utilized in these Trials were obtained from spawning of either wild, F1, or F2 generation broodstock maintained at the University of Miami Experimental Hatchery (UMEH) facilities. However, Trial 1 utilized eggs obtained from the Marine Science Institute, University of Texas, Port Aransas, Texas, as described in a subsequent section. At the UMEH, fertilized eggs were collected either approximately 2 to 4 hours or 10 to 12 hours after spawning, depending whether collection was conducted the same evening or the morning following a spawning event. Collected eggs were concentrated and settled in 20 L buckets to separate unviable or unfertilized eggs from viable eggs. Total number of eggs, number of viable eggs, and number of non-viable eggs were then counted using volumetric samples in large graduated cylinders (420 eggs

mL^{-1}; Arnold et al. 2001), and stocked within 1,000 L flow through conical bottom incubators which had been previously disinfected utilizing sodium hypochlorite.

Incoming ambient seawater (salinity variation from 34 to 36 ppt) of incubators was filtered via glass media filters capable of removing particulates ≥5 μm. Water flow exchange rate was set to 100% day^{-1}. Moderate aeration provided mixing of the tank water and maintained the developing eggs in suspension, while use of supplemental pure oxygen bubbled within the incubators ensured DO concentrations remained near or above saturation. Surface skimmers were utilized to collect surface films and oils that would develop, chorions once hatching was complete, and particulates that settled on the water surface. Removal of the collected film and debris was achieved utilizing paper towels and surface skimming with small beakers. Center stand pipes were fitted with 500 μm mesh, which allowed smaller debris to flush from the system and facilitated water exchange via reduced clogging. Photoperiods were maintained at ambient conditions. Illumination was reduced to 80% of ambient levels via shade cloth incorporated into the hatchery roof structure.

Approximately 12 to 14 hours post spawning, a 100 ppm formalin treatment was applied to the incubators as an egg surface disinfectant. During this 1 hour treatment period, water exchange was suspended. Upon completion of the treatment, flow was restored at 500% day^{-1}exchange for 1 hour in order to flush formalin from incubator systems. After flushing, water exchange was returned to pre-treatment exchange rate.

Depending upon ambient water temperature, hatching began between 18 and 30 hours post spawning. Once hatching was complete, water flow and aeration were temporarily suspended allowing positively buoyant yolk-sac larvae to float close to the

tank water surface and negatively buoyant expelled chorions to sink. A 'paddle' was utilized to create a mild vortex current within the tank to further assist the settling process and concentrate chorions within the conical bottom of the incubator tank. Moderate pure oxygen was maintained to ensure DO levels did not drastically plummet during this settling process. Once settling was complete, the debris was rapidly siphoned from the tank bottom during which care was taken to minimize removal of yolk-sac larvae. Water flow and full aeration were then restored and set to previous levels.

Larvae were maintained within the hatching incubators throughout the yolk-sac stage. During this period, water exchange rates, aeration, and pure oxygen were maintained as previously described. Previously described bottom siphoning procedures were repeated as necessary once or twice daily. On either 2 DPH or 3 DPH, yolk-sac larvae survival and density were determined utilizing volumetric subsampling. Once these estimates were known, the yolk-sac larvae were concentrated and distributed among larval rearing tanks until target initial stocking density was achieved.

Larval rearing was conducted within 12,000 L cylindrical tanks with a slightly sloping bottom. These larviculture systems were of a single-pass, flow-through design. Incoming seawater treatment involved filtering through glass media filters, as previously described, with no modification of temperature from ambient conditions. Individual tank water inlet pipes were also fitted with 10 µm sock filters which were cleaned twice daily or more often as necessary. Throughout all Trials, ambient photoperiod was utilized, although illumination was reduced to 80% of ambient outdoor levels via shade cloth incorporated into the hatchery roof structure.

Generally, seawater exchange rates, center standpipe mesh size, aeration, and supplemental oxygen were increased in stages throughout the larvicultures in order to match larviculture metabolic oxygen demands, waste removal, and the physical integrity and swimming capabilities of the developing larvae. These efforts were also necessary to balance successful feeding in weaker, younger larvae while attempting to reduce cannibalistic behavior of larger larvae at older stages. Initially standpipe screens consist of 150 μm mesh, this is later changed to 350 μm, 500 μm, and finally 1 mm mesh sizes as prey item size and swimming capabilities of the larvae changed. The largest mesh size of 1 mm was necessary during weaning, as larger sized weaning diets were found to clog smaller screen sizes. Water exchange rate was initially set to 100 % day^{-1} during daylight hours and 200 % day^{-1} during nighttime hours. This was increased to 200% day^{-1} during the day and 400% day^{-1} at night and finally 500% day^{-1} through both day and nighttime hours.

Aeration protocols remained the same during all Trials. Tank aeration systems consisted of an air ring secured to the base of center stand pipes and one air stone suspended in each of the four quadrants of the larviculture tank. Aeration strength was initially weak to moderate, as to minimize disruption of the larval ability to swim, track, strike, and consume prey items. As the larvae grew, aeration strength was increased to assist in maintaining more homogenous larvae distribution and minimize cannibalism. Supplemental pure oxygen bubbled within larviculture systems was also found to be indispensible. Initially supplemental oxygen was utilized only when necessary, which was generally during later stages of the rearing. Later Trials incorporated oxygen supplementation from initial stocking until termination of the rearing.

Greenwater systems where utilized by direct addition of microalgae culture water to larviculture systems. These protocols also evolved during the different Trials and are subsequently described. At times, changes in protocols reflected shortfalls in microalgae production at the UMEH. Generally, microalgae was added in pulses multiple times a day to achieve cell densities between 2,000 to 35,000 cells mL^{-1}. Microalgae species utilized included *Isochrysis galbana*, *Nannochloropsis oculata*, and *Tetraselmis chuii*. At times, concentrated algal paste (Instant Algae [IA], Reed Mariculture, Campbell, CA, USA) was utilized as a substitute when live microalgae was not available.

Timing of feeding regime schedules was variable between Trials. These variations were partially the result of the subjective nature of the decision making process for transition onto new prey items which was based upon observation of larval development, although unintended differences were also related to variation in live feeds production. Feeding with enriched rotifers (*Brachionus plicatilis*), cultured at UMEH facilities, began on either the 2nd or 3rd DPH, depending upon observation development of larval buccal, digestive, visual apparatus, and hunting behavior. Co-feeding with Instar-1 *Artemia* (*Artemia franciscana* GSL strain) varied between trials and is explained in subsequent sections. Co-feeding with Instar-1 *Artemia* nauplii generally lasted from 1 to 3 days, at which time feeding with enriched Instar-2 *Artemia* began. At times, co-feeding was carried out for a day or two utilizing AF-strain *Artemia* (INVE Aquaculture, Dendermore, Belgium). This *Artemia* averages ±428 µm as opposed to the larger average size of ±486 µm (both sizes of 1st Instar nauplii) for GSL-strain *Artemia* utilized throughout the remainder of the live feeds phase of larviculture. Live feeds were pulse fed to larval cobia and commenced daily approximately at sunrise. Pulses of live feeds

were added to larviculture tanks throughout the day as necessary to maintain prey densities. Final feeding occurred approximately 1 hour before sunset.

Enriched Instar-2 *Artemia* feeding was utilized until weaning of the larvae onto dry feeds was achieved. As explained above, the timing of these events varied between Trials. Weaning utilized Otohime weaning diets (Marubeni Nisshin Feed Co., Ltd., Tokyo, Japan) beginning with size B1 (200-360 μm) and increasing to sizes B2 (360-620 μm) and C1 (920-1410 μm). Transitions between different food sizes involved feeding with mixtures of smaller and larger size grades.

Culture and enrichment diet protocols for both rotifers and *Artemia* evolved during the course of these Trials. For rotifers, culture maintenance diets first utilized Culture Selco Plus (INVE Aquaculture, Dendermore, Belgium) (Rotifer Maintenance Diet 1: RMD1). In subsequent Trials, culture maintenance diet (Rotifer Maintenance Diet 2: RMD2) consisted of 90% dry baker's yeast combined with 10% concentrated microalgae in either dry (Algamac Protein Plus, Aquafauna Biomarine, Inc., Hawthorne, CA, USA) or liquid (IA, *Nannochloropsis*, *Isochrysis*, and/or *Pavlova* spp.) (Reed Mariculture, Campbell, CA, USA) form. Enrichment of rotifers was achieved by substitution of culture maintenance diet with enrichment diet for the 24 hr period before harvesting and stocking within larvicultures. Enrichment diets initially consisted of 50% RMD1 supplemented with 50% Easy Super Selco (INVE Aquaculture, Dendermore, Belgium) (Rotifer Enrichment Diet 1: RED1). Later trials utilized an enrichment diet consisting of a mixture of RMD2 and commercial products designed to boost highly- and poly-unsaturated fatty acid (HUFA/PUFA) levels (Rotifer Enrichment Diet 2: RED2). The RED2 final mixture of this diet consisted of 45% baker's yeast, 2.5% Algamac

Protein Plus, 42.5% Algamac 3050, and 5% Algamac ARA (Aquafauna Biomarine, Inc., Hawthorne, CA, USA) and 2.5% Naturose Astaxanthin (Cyanotech, Kailua-Kona, HI, USA) and 2.5% *Nannochloropsis* IA (Reed Mariculture, Campbell, CA, USA). Both culture and enrichment diet ingredients were blended in 15 L freshwater, and delivered continuously via peristaltic pump over a 24 hr period. Feeding rates were between 0.3 to 0.5 g 10^6 rotifers^{-1} day^{-1}.

Artemia enrichment diets initially consisted of Easy Super Selco (INVE Aquaculture, Dendermore, Belgium) which was utilized following manufacturer's instructions (0.6 g Easy Super Selco L^{-1} of enrichment tank water, not to exceed 300,000 nauplii L^{-1}) (AED1). Later, a second *Artemia* enrichment diet (AED2) was developed which also aimed to maximize HUFA/PUFA concentrations but included other enrichments as well. This diet consisted of 85% Algamac 3050, 10% Algamac ARA (Reed Mariculture, Campbell, CA, USA), and 5% Naturose Astaxanthin (Cyanotech, Kailua-Kona, HI, USA). The enrichment diet was mixed within 5 L of freshwater then added directly to enrichment culture tanks; enrichment occurred for the 12 and 14 hour period before harvest of the enriched Instar-2 *Artemia* nauplii.

Harvesting of rotifer cultures involved concentrating and filtering rotifer culture water within a washing sink fitted with a 64 µm screen and further rinsing with seawater, filtered to 1 µm and subsequently UV sterilized, for 30 to 45 min. or until culture water was flushed and water within the washing sink was clear. Similarly, *Artemia* harvesting consisted of concentrating and filtering in washing sinks fitted with 150 µm screens and subsequent rinsing of culture water followed the same procedures and endpoints as rotifer culture washing. Later, larger washing barrels of approximately 200 L capacity were

developed and utilized in order to more easily harvest the large volumes of *Artemia*. Rotifers and *Artemia* ready for feeding to larvae were stored in respective previously cleaned and disinfected coolers. Enriched live feed storage water was chilled to approximately 10°C via frozen water bottles while pure oxygen supplementation maintained high DO concentrations. These storage procedures assisted with maintaining heightened nutritional enrichment by lowering metabolic activity of the live feed organisms while allowing fairly precise, on demand feeding by calculation of sub-volumes of required during each feeding event.

Development of larval rearing and live feed production protocols which incorporated probiotics occurred during the course of these Trials. Incorporation of probiotics into individual Trials is later described. Probiotic inoculates consisting of four species of *Bacillus* bacteria (EcoAqua commercial blend: *B. subtilis*, *B. licheniformis*, *B. megaterium*, and *B. laterosporous*; EcoMicrobials LLC, Miami, FL, USA, www.ecomicrobials.com) were incorporated within live feed culture and enrichment regimes during later trials. In rotifer cultures, probiotic inoculates consisted of daily direct additions to culture tanks of 10^3 CFU ml^{-1} final concentration (1 ml EcoAqua per 100 L of culture water); probiotics were also added to rotifer feed mixture vessels at 10^5 CFU mL^{-1} final concentration (1 mL EcoAqua per L of blended diet). *Artemia* hatching and enrichment cultures received direct probiotic inoculates of 10^4 CFU ml^{-1} final concentration (0.1 mL EcoAqua per L of culture water) subsequent to initial culture setup and enrichment feed addition, where applicable.

Later larval rearing Trials incorporated periodic prophylactic formalin treatments beginning the day after stocking of larviculture systems and every three days thereafter.

Incorporation of these protocols within individual Trials is later described. Treatments consisted of suspension of water exchange and formalin addition to larviculture tank water in a multi-stage process. Formalin was first added at final concentration of 50 ppm and after 30 min. a second, boosting dose of 25 ppm final concentration was added. Each formalin addition was mixed in approximately 10 L of seawater before addition, which eased distribution about the tank and reduced localized concentrations before mixing via aeration could sufficiently dilute the addition to the entire tank. Total treatment time was 1 hour in duration. At the termination of the treatment period, water exchange was set to 500% day^{-1} for 2 hours in order to flush the larvicultures of the formalin solution.

Individual Trials incorporated additional treatments that were utilized on an as-needed basis. These were reactionary treatments which were enacted in order to combat disease and parasite outbreaks as they occurred. Direct observation of *A. ocellatum* trophonts attached to larval gill structures and primary lamellae and concurrent substantial increases in mortality prompted utilization of copper sulfate treatments, mixed in a 1:1 ratio with citric acid acting as a sequestering agent, in order to combat the disease and reduce mortality. These treatments consisted of an initial addition of 0.3 mg L^{-1} final concentration of a previously prepared stock solution to larviculture tank waters. Daily monitoring of copper sulfate concentrations and additions of stock solution to increase tank concentrations to 0.3 mg L^{-1} were conducted over the following days. Similar to formalin treatments, copper sulfate treatment additions were first diluted in approximately 10 L seawater before addition to larviculture tanks. Copper sulfate treatments did not consist of suspension of tank water exchange.

During Trial 2, observation of distended abdomens and hypertrophic gill primary lamellae as well as concurrent increases in mortality led to the conclusion that bacterial enteric disease was infecting the larvae. Treatments consisting of additions of 20 mg L^{-1} (20 ppm) oxytetracycline were utilized in efforts to reduce mortalities. These treatments included temporary suspension of tank water exchange 2 hrs in duration. Similar to other treatments, oxytetracycline additions were first diluted in approximately 10 L seawater before being added to larviculture tanks. Further attempts to deliver this antibiotic treatment to the infected larvae included bioencapsulation in live *Artemia.* This treatment consisted of maintaining concentrated *Artemia* in oxytetracycline solutions at a concentration of 100 mg L^{-1} (100 ppm) for 30 min before being stocked within larviculture tanks.

Earlier larviculture sanitation maintenance protocols involved periodic cleaning of standpipes, periodic cleaning, as needed, of surface skimmers throughout the day, and wiping of tanks walls at the water/air interface as necessary. Initially, siphoning of tank bottoms was conducted sporadically and as necessary. During the middle stages of larvicultures, siphoning occurred daily, and by approximately 10 DPH siphoning was conducted twice daily. Later protocols involved more careful attention to tank maintenance; tank bottom siphoning was conducted daily post stocking and by 10 DPH was conducted twice daily. Initially, center standpipes utilized were constructed with fixed screens. Standpipe cleaning consisted of standpipe removal, cleaning, and replacement. During later Trials, center standpipes with easily removed screens were constructed. Thus, soiled screens could then be cleaned, sanitized, and stored for later use while standpipes themselves were cleaned and immediately replaced with previously

cleaned and sanitized screens. This screen cleaning process was conducted daily immediately after stocking of larvae. By 10 DPH, it was necessary to repeat the process twice daily. Periodic cleaning of surface skimmers was conducted multiple times a day, as was cleaning of tank walls at the water/air interface, similar to earlier trials.

6.2.3) Trial 1

Trial 1 was initiated on October 13th, 2005, with cobia eggs obtained from the Marine Science Institute of the University of Texas (Port Aransas, Texas). The Trial was terminated and survival was assessed 38 DPH on November 18th, 2005. Upon arrival, the 2 DPH yolk-sac larvae were acclimated to ambient water conditions at UMEH. Once acclimation was complete, the larvae were stocked within two 12,000 L tanks (T1E1and T1E2), resulting in stocking densities of 0.875 and 1.025 larvae L^{-1}, respectively. Although only two tanks were initially stocked, this trial later incorporated 2 other 12,000 L tanks as well as multiple 1200 L tanks for grading purposes. Grading was initiated 20 DPH and was continued throughout the rest of the Trial.

Water exchange early during the trial began with exchange rates of 100% day^{-1}, increased on 8 DPH to 250% day^{-1} and to 300% day^{-1} from 10 DPH until the end of the trial. Due to high mortalities throughout the Trial, higher exchange rates were not necessary to maintain water quality and lower flow rates were utilized to conserve live feed organisms within the larvicultures. Sporadic supplemental pure oxygen utilization was employed to maintain high DO concentrations as needed, although efforts were complicated due to shortages and delivery delays in replacement oxygen cylinders. Center stand pipes in which screen material was permanently affixed to the stand pipe

were utilized. Larviculture tank maintenance follow previously described early Trial protocols.

Greenwater treatment originally consisted of live *Isochrysis galbana* additions from 2 to 6 DPH. Due to shortcomings in microalgae production, it was no longer available for use after 6 DPH. *Nannochloropsis* IA (Reed Mariculture, Campbell, CA, USA) was utilized as a substitute for live algae in order to create 'artificial' green water cultures (McLean et al. 2005). The IA green water technique was terminated on 13 DPH. Rotifers, enriched following enrichment protocol RED1, were introduced 3 DPH. Densities were maintained between 0.5 and 3.7 rotifer mL^{-1} until 11 DPH. Co-feeding with Instar-2, enriched with AED1, *Artemia* nauplii began on 7 DPH. Co-feeding utilizing Instar-1 *Artemia* nauplii was not utilized in this Trial. *Artemia* feeding continued until 34 DPH when larvae were completely weaned onto dry feeds. *Artemia* densities were maintained between 0.23 and 0.47 mL^{-1}. This trial is unique from all others in the fact that a different *Artemia* product, a pre-decapsulated *Artemia* paste (Embryon; INVE Aquaculture, Dendermore, Belgium) was utilized. Weaning, using previously described protocols, was initiated on 24 DPH and continued until 34 DPH when *Artemia* feeding was suspended.

Reduced water quality (high turbidity and demand on hatchery filtration systems) resulting from the passing of Hurricane Wilma began 13 DPH. By 16 DPH, increasing mortality was observed in the larviculture systems. Observations of distended larval abdomens and epidermal sloughing led to the conclusion that bacterial infections/bacterial enteritis was causing the increased mortalities. Formalin treatments of 100 ppm with static water flow for one hour were utilized in T1E1 and T1E2 on 18

and 19 DPH, respectively. These formalin treatments represented one time treatments and thus this Trial is not considered to employ periodic formalin treatments as utilized in later Trials. Probiotics experimentation was also attempted by addition of 300 mL of EcoAqua probiotics (representing an inoculation of ~8.33 $x10^2$ CFU mL^{-1}) to tank T1E1 while water flow was suspended for 2 hrs. It was intended that probiotic bacteria may be able to combat the pathogenic bacteria present although later discussion with microbiological experts suggested that this treatment would not have had much, if any, impact and thus this treatment is later ignored in relation to 'probiotic' treated Experiments.

During later stages of this Trial, another period of increased mortality was observed beginning on 25 DPH. Observation of *A. ocellatum* trophonts attached to gill primary lamellae was suspected to be the cause. Copper sulfate ($CuSO_4$) treatments, as previously described, were utilized in order to combat the infestation. Treatments continued for 8 days (27 DPH to 34 DPH) and were suspended when *A. ocellatum* trophonts were no longer observed on dissected individuals.

6.2.4) Trial 2

Trial 2 was initiated on June 12th, 2006 utilizing eggs collected from a natural spawning event occurring at UMEH facilities on June 8th. The Trial was terminated on July 15th (37 DPH) when survival was assessed. Larvae were stocked during early hours 3 DPH within one 12,000 L larval rearing tank (T2E3) at an initial density of 8 larvae L^{-1}. Stocking into a single tank was employed due to shortfalls in rotifer production and desire to maximize rotifer utilization between as many larvae as possible. Tank T2E3

was later split into another 12,000 L tank on 7 DPH in order to reduce stocking density. It was estimated that approximately 1/3 of larvae in T2E3 were transferred. Further transfers initiated for size grading began 12 DPH although these efforts began in earnest 20 DPH. Monitoring of growth was conducted only for one tank (T2E3); thus, this is the only larviculture system that is included in this analysis for this Trial.

Water exchange rates were initially set to 100% day^{-1}, increased to approximately 200% day^{-1}, and varied between 300 to 500% day^{-1} during later stages of the Trial. Supplemental pure oxygen was utilized sporadically when needed. Center stand pipes with permanently fixed screens were utilized. Siphoning was initiated 6 DPH and was repeated once every other day until 14 DPH. Thereafter, siphoning was conducted daily. Tank bottom 'spot' siphoning was initiated early during the Trial on 4 DPH. During later stages of the Trial, siphoning was completed twice daily as needed.

Greenwater protocols utilized additions of live *I. galbana* on a near daily basis from 3 DPH until 14 DPH, depending on availability. Rotifers were stocked within larvicultures immediately after being stocked with the larvae. Rotifers, enriched with the RED2 regime, were fed from 3 until 6 DPH at a density of 2.0 to 5.0 rotifer mL^{-1}. Co-feeding with AF-strain *Artemia* occurred on 4 and 5 DPH. On 7 DPH, the larvae were fed a mixture of Instar-1 AF-strain *Artemia* and Instar-2 enriched GSL-strain *Artemia* in an approximately 1:1 ratio. Enrichment protocols utilized the AED2 regime. *Artemia* densities were maintained between initial concentrations of 0.3 *Artemia* mL^{-1} and soon averaged ~1.0 to 1.5 mL^{-1}. These higher density pulse feedings of *Artemia* resulted in reduced maintenance in terms of monitoring prey densities in larvicultures and reduced number of feed pulses per day. Weaning was initiated 17 DPH utilizing protocols similar

to those previously described. The larvae were considered fully weaned on 25 DPH, which constituted the day of last feeding with *Artemia*.

Bacterial infections were again problematic during this Trial. Hyperplastic gill primary lamellae were observed on 8 DPH. It was suspected that physiological stress brought on by metamorphosis and gill development allowed for secondary bacterial infections to establish themselves. At 16 DPH, observation of high mortalities and continued observation hyperplastic gill primary lamellae resulted in the decision to utilize antibiotic and chemotherapeutant treatments in efforts to reduce mortalities. On 17 DPH, a treatment of 1 mg L^{-1} $CuSO_4$ was administered to larviculture tanks. A treatment of 20 mg L^{-1} oxytetracycline with static flow for 2 hours was also administered. Later that same afternoon, $CuSO_4$ was again added to the larvicultures restore concentration to 0.75 mg L^{-1}. Oxytetracycline was also administered to *Artemia* as a treatment of 100 mg L^{-1} for 30 minutes previous to stocking in larviculture tanks.

Oxytetracycline treatments continued until 21 DPH for a total treatment period of 5 days. During this period bioencapsulation of *Artemia* with oxytetracycline was also continued. Starting on 22 DPH and terminating on 26 DPH, $CuSO_4$ treatments of 1 mg L^{-1} were again employed. Another series of $CuSO_4$ treatments were employed on 30 and 31 DPH. On 31 DPH, a static-flow 100 ppm formalin treatment also was applied to the larviculture. All of these various treatments were employed in attempts to limit severe mortalities that were occurring. The culmination of these treatments evidently brought about positive results, daily mortality dropped to minimal rates by 33 DPH. No observation of *A. ocellatum* trophonts occurred and thus bacterial infections alone were suspected of causing these mortalities.

6.2.5) Trial 3

Trial 3 was initiated on May 18th, 2007 utilizing eggs obtained from a UMEH broodstock spawning event utilizing Human Chorionic Gonadotropin (HCG) to induce final oocyte maturation. On May 15th, broodstock reproductive status investigation revealed one female with oocytes of approximately 800 μm diameter. This female was injected with approximately 1,000 IU kg^{-1} of HCG. On the evening May 16th, a spawning event occurred in this broodstock group. Upon collection of eggs the following morning, fertilization ratio was determined to be 48.3%, and volumetric subsampling of eggs from incubators indicated 49.2% of the eggs collected were unviable. Observation of unviable eggs revealed opaque, tan-yellow discolored eggs that did not exhibit signs of development into embryos. The hatch ratio for this spawning event was later estimated to be 54.7%, much lower than typically observed.

Yolk-sac larvae were transferred 1 DPH between two 12,000 L larval rearing tanks (T3E4 and T3E5). The previously discussed problems regarding egg quality resulted in low numbers of yolk-sac larvae and hence more tanks could not be stocked. The initial stocking density was 10 larvae L^{-1}. Each tank was later split into another tank of 12,000 L on 12 DPH (larvicultures T3E6 and T3E7). The decision to split the larvae was based on the high survival thus far realized and concerns regarding crowding. This Trial was initiated as an experiment intending to investigate differences in growth and survival with the incorporation of probiotics within live feeds production protocols. Thus, T3E4 and T3E6 utilized live feeds produced with probiotic enrichments incorporated within production procedures.

Respective to previous trials, stand pipes of a modified design were utilized in this Trial. Mesh screens made in the form of a giant sock were fitted over standpipes, facilitating stand pipe cleaning procedures. As previously described, mesh screens increased in size in order to accommodate changing needs of particulate waste removal and retention of live prey items. Water exchange rate protocols followed similar schedules as those previously described. Supplemental pure oxygen was at first not utilized. However, during later stages of the rearing its necessity was realized and utilization continued from 16 DPH until the end of the rearing.

Greenwater larviculture protocols were utilized during this trial; beginning on 2 DPH and terminating on 20 DPH, once or twice daily live *I. galbana* and/or *N. oculata* was added to larvicultures at densities ranging from 1,233 to 15,000 cells mL^{-1}. Shortcomings in microalgae production prevented its use on days 2, 16, and 19 DPH. Rotifers were added to larvicultures from 2 to 9 DPH at densities between 1 to 5 rotifers mL^{-1}. Rotifer enrichment followed the RED2 protocol. *Artemia* co-feeding began 8 DPH with the introduction of Instar-1 nauplii and on 9 DPH enriched (AED2 protocol) Instar-2 *Artemia* were offered. *Artemia* feeding was terminated 25 DPH. As mentioned, previously described probiotic additions were utilized in live feed cultures utilized to feed larviculture T3E4 and T3E6. Care was taken to not cross contaminate cultures, both during live feeds production and feeding to probiotic treatment larvicultures. Weaning was initiated 18 DPH and the larvae were considered weaned 25 DPH and followed protocols as previously discussed.

Periodic prophylactic formalin treatments became the integrated as standard procedures during the entire larval rearing; treatment procedures followed previously

described protocols. No further chemotherapeutant treatments were found to be necessary during this Trial.

6.2.6) Trial 4

Trial 4 was initiated on July 6, 2007 with eggs obtained from a natural spawning event from UMEH broodstock. In respect to the previous Trial, egg quality was considered much higher: fertilization/egg viable was estimated to be 95.02%. Similarly, volumetric subsampling of incubators revealed an estimated hatching of 74.97% of the viable eggs originally stocked. This relatively lower than anticipated hatching percentage was most likely the result of lower counts in one of the two incubators utilized. A small tare was later observed in the center stand pipe and was suspected in causing the loss of many eggs and yolk-sac larvae. This Trial was initiated to investigate the performance characteristics of two different initial stocking densities in commercial scale cobia larviculture. Stocking of 12,000 L larviculture tanks was conducted on 2 DPH. Two tanks were stocked with 10 yolk-sac larvae L^{-1} (T4E8 and T4E9) while another two were stocked at 5 yolk-sac larvae L^{-1} (T4E10 and T4E11).

Recently developed protocols for stand pipe cleaning and maintenance with removable standpipe screens were utilized during this Trial. Water exchange protocols followed those previously described. By the end of the trial, it was observed that high exchange rates (500% day^{-1}) were necessary. Tank bottom 'spot' siphoning was initiated early during the trial on 4 DPH, by 5 DPH it was recognized that daily siphoning was necessary. During later stages of the Trial siphoning was completed twice daily,

beginning 18 DPH. Utilization of supplemental pure oxygen was employed throughout the entire Trial.

Greenwater protocols were utilized during Trial 4, though shortcomings in algae production limited its utilization from 2 to 12 DPH. Algae concentrations were also reduced compared to those utilized during previous larvicultures. Once or twice daily additions of *I. galbana* and/or *N. oculata*, resulting in final concentrations between 5,000 and 10,000 cell mL^{-1}, were added to larvicultures on a near-daily basis during this period. Live feeds utilization was adjusted to account for differing larval densities between the treatments, thus higher numbers of prey items were offered to higher initial stocking density tanks. Rotifers were introduced on the evening 2 DPH and their use continued until 8 DPH. *Artemia* co-feeding was initiated 7 DPH with AF-strain Instar-1 nauplii. From 8 DPH until 20 DPH *Artemia* feeding consisted of enriched GSL-strain Instar-2 nauplii. Rotifer and *Artemia* enrichment protocols followed RED2 and AED2, respectively. Probiotics incorporation to live feeds cultures were included in live feeds cultures and followed previously described protocols. Weaning was initiated 11 DPH and was considered complete at 20 DPH. Periodic formalin treatments were utilized throughout the Trial following previously described protocols. No other chemotherapeutant treatments were necessary during the course of the Trial.

6.2.7) Trial 5

Trial 5 was initiated on April 10th, 2008 with eggs obtained from natural spawning of UMEH broodstock. Stocking of four 12,000 L larval rearing tanks was conducted 2 DPH. Each tank was initially stocked at 5 larvae L^{-1}. For reasons to be

described, only two of these tanks (T5E12 and T5E13) are described and analyzed in this study. This Trial was conducted for production rather than experimental purposes, and thus no new protocols, treatments or other factors under control of hatchery staff were different or manipulated from protocols described in Trial 4.

Water exchange rates during this Trial were higher than previously discussed. By 7 DPH, exchange rates were 200% day^{-1} during daylight hours and 250% day^{-1} during nighttime hours. By 10 DPH, these had increased to 300% day^{-1} and 400% day^{-1} for daylight and nighttime hours, respectively, and by 12 DPH they had been increased to 600 % day^{-1} and 700% $night^{-1}$. From 14 DPH through the remainder of the Trial, exchange rates of 700 to 800% day^{-1} were utilized at all times. Center stand pipes with removable screens were maintained as previously described. Supplemental pure oxygen was utilized at all times throughout this Trial.

Greenwater protocols were not utilized during this Trial. Rotifer feeding was initiated 3 DPH and was continued until 11 DPH. Co-feeding with *Artemia* began on 8 DPH with AF-strain Instar-1 nauplii while Instar-1 GSL-strain *Artemia* were co-fed on 9 and 10 DPH. Enriched *Artemia* Instar-2 nauplii were not offered until 11 DPH and the final *Artemia* feeding occurred on 28 DPH. Rotifer and *Artemia* enrichment protocols followed RED2 and AED2 protocols, respectively. Probiotic incorporation into live feeds culture protocols were followed as previously described. Weaning was initiated 18 DPH and the larvae were not considered weaned until 28 DPH. Periodic formalin treatments were again utilized during this Trial following protocols as previously described. No other chemotherapeutant treatments were utilized during this Trial.

6.2.8) Data Collection

Temperature and DO were measured using a YSI 500A submersible probe (YSI Inc., Yellow Springs, OH, USA) and pH was monitored using a YSI 100 (YSI, Inc., Yellow Springs, OH, USA) probe. TAN concentrations were analyzed via colorimetric analysis utilizing the salicylate method with a Hach DR890 Pocket Colorimeter (Hach Company, Loveland, CO, USA).

Larval total length (TL) data was collected utilizing a Motic Model SMZ-168 dissecting microscope in conjunction with either an ocular micrometer or a digital caliper, depending upon size of the larvae being measured. Measurements taken via ocular micrometer were recorded as unitless numbers and converted to mm via calibration of the ocular micrometer with a standard slide. Length measurement sampling was conducted without replacement as larvae of all ages were found to most likely not survive this process. Those that did were visibly weakened once returned to larval rearing tanks and at later developmental stages during a rearing, these individuals were observed to be targets of cannibalism, potentially resulting in the death of both larvae. Thus, these weakened individuals utilized for length measurements were euthanized.

Among Trials, survival data was collected on different days. This was most often due to differences in growth between Trials and necessity to wait until larval body integrity was able to physically withstand capture with nets and transfer. Survival was assessed by hand counting netted individuals and transferring them to holding tanks before shipment to buyers or growout operations. Careful notes of individuals graded

from the main larval rearing tanks before final assessment of survival were combined with shipping counts in order to accurately reflect true production per larval rearing tank.

6.2.9) Data Investigation and Statistical Analysis

All data computation and analysis was carried out using Microsoft Excel ver. 2007 (Microsoft, Redmond, WA, USA) and Stata Release 10 (StataCorp LP, College Station, TX, USA). Percent survival data was arcsine transformed in order to normalize the raw distribution of this proportional data (Sokal and Rohlf 1995). A number of variables were included in the resulting analyses and investigations of growth, survival, and production optimization. An exhaustive list of the variables considered include month of start of rearing, initial stocking density of yolk-sac larvae (#/L), computed constant of growth coefficients from linear regressions of length-at-age, computed intercept from linear regressions of growth, arcsine transformed survival, number of juveniles produced, final density of juveniles (#/L), average temperature (°C), maximum temperature (°C), minimum temperature (°C), median temperature (°C), average dissolved oxygen (DO, mg L^{-1}), average pH, initial day post hatch (DPH) of rotifer feeding, final DPH of rotifer feeding, initial DPH of *Artemia* feeding, final DPH of *Artemia* feeding, total rotifer utilization, total *Artemia* utilization, rotifer enrichment protocol (categorical variable), *Artemia* enrichment protocol (categorical variable), green water utilization (categorical variable), initial DPH of green water utilization, final DPH of green water utilization, initial DPH of weaning, final DPH of weaning, probiotics utilization (categorical variable), formalin utilization (categorical variable), antibiotics utilization (categorical variable), and copper sulfate utilization (categorical variable).

The unfortunate loss of some datasheets soon after the completion of T3 resulted in the loss of water quality data associated with the first portion of the trial (data from the month of May). A substitution of temperature data collected from inflow of ambient water to UMEH cobia broodstock tank systems which had passed through the hatchery's main filtration systems could rectify this issue in regards to recovering temperature data. In order to validate this assumption, the means and variances of two different periods of water temperature data concurrently collected from the same indoor tanks utilized for larval rearing and the ambient temperature water broodstock system inflow were compared.

The first validation test (VALID1) utilized temperatures recorded from the two different indoor tanks and ambient broodstock water temperature recorded during the same period of October 13, 2005, to November 14, 2005 and was partially comprised of data collected concurrent to T1. The second validation test (VALID2) consisted of water temperature data collected from an indoor tank and the ambient broodstock water temperature from 4/1/2007 to 4/25/2007. This represents data collected the month prior to T3. VALID1 variance was investigated using Levene's robust variance test statistic and mean comparison utilized ANOVA testing while VALID2 variance was investigated using a standard F-test for differences in variance and mean testing was carried out via a 2 tailed t-test (Zar 2009).

Growth analysis was first initiated by constructing linear growth regressions of individual experiments. Growth regressions were constructed from the exponential growth equation:

$$L_{(\tau)} = L_0 e^{G_L (\tau)} \quad \text{Eq. 6.1}$$

Where $L(\tau)$ is length at time τ, τ is age (DPH), G_L is the instantaneous growth coefficient, and L_0 is a constant which describes initial length, or length at hatching. In order to linearize the resulting regressions, this equation was first logarithm transformed, yielding:

$$\text{Ln}\,(L_{(\tau)}) = G_L(\tau) + \text{Ln}\,(L_0) \quad \text{Eq. 6.2}$$

Thus, individual TL (mm) measurements were natural log transformed before being utilized to construct linear regression models. Size-at-hatching data was not collected during each individual Experiment/Trial. Thus, an average size-at-hatching (TL = 3.67 mm) was computed from data listed in the literature and unpublished results from investigations conducted at UMEH (3.5 mm, Hitzfelder et al. 2006; 3.52 mm, Kilduff et al. 2002; 3.58 mm, I. Zink Unpub. Data; 4.08 mm, I. Zink Unpub. Data).

After building of individual Experiment linear regressions of growth in length, the resulting distributions of coefficient and constant sets computed from regressions constructed with and without the computed average size-at-hatching data point were investigated. Percent change in both coefficients and constants between regressions constructed with and without the size-at-hatching was computed. Further analysis was conducted by analyzing the distributions of computed coefficients and intercepts, considering utilization of the average size-at-hatching during regression fitting as treatments, for departures from normality, equality of variance by F tests (both 1- and 2-tailed), and equality of means by t-test (2-tailed). Utilizing F- and t-tests for computed

coefficient and intercept testing, respectively, linear regressions were directly compared in a pairwise fashion (with and without average size-at-hatching) (Zar 2009). Further analysis was conducted utilizing linear growth regressions computed with inclusion of the average size-at-hatching data point.

Residual analysis was conducted on the resulting regressions in order to validate linear regression assumptions and identify any problematic data points. This included qualitative graphical investigation of data dispersion, residual dispersion, and linearity. Quantitative and statistical verification of linear regression assumptions included investigation of variance of data from each sampling event within experiments, calculation of Cook's D values (Kutner et al. 2005, Stata Web Books: Regression with Stata).

Those Experiments in which post-regression model fit analysis was not satisfactory were further investigated by removal of individual data points identified as outliers or to exhibit high leverage on the regression. At times, this process became a repetitive, positive feedback of sequential data point removal. Upon further deliberation and iterations of this process, it was observed that data point removal most often involved outliers of the oldest sampling age. Further experimentation with regression construction utilizing iteratively reweighted least squares regression could not rectify these issues either. Similarly, utilization of Box-Cox transformations to data also did not yield satisfactory results.

These issues were attributed to be the manifestation of the commonly observed phenomena of increasing range (variance) in size-at-age (size hierarchy or depensation of growth) for larval cohorts raised in captivity in mesocosms (Blaxter 1976 as cited in

Blaxter 1986), also known as depensatory growth (Blaxter 1988). This, taken in conjunction with advice concerning 'leptokurosiphobia' as discussed by Sall and Jones (2008), suggest that concerns with strictly adhering to assumptions of normal residuals may not be warranted and thus the computed growth coefficients and constants were utilized for further analysis without manipulation of outlying or influential data points.

Linearity of the natural log transformed growth in length data between successive sampling events was investigated by fitting linear regressions between sampling events within Experiments and subsequently applying F-tests to examine differences between the resulting regression coefficients (Zar 2009). Investigation of temperature differences between growth periods within Experiments was conducted by employing t-tests and Kruskal-Wallis tests, where appropriate, for differences between mean temperatures for periods between growth sampling events. Necessity was dictated by first applying Shapiro-Wilk normality tests, equal variance tests, and number of groups being compared (Sokal and Rohlf 1995, Zar 2009). These investigations suggested that varying temperatures may have influenced differing growth rates within individual Experiments. Although statistically significant differences in mean temperature for each successive sampling event within each Experiment did not match the pattern of statistical differences in growth rates within each Experiment, detection of differences did lend credibility that temperature changes may be causing an influence.

Attempts to rectify temperature influences on growth led to investigation of Degree Day utilization. Degree Day, a measure of timing of development events which attempts to standardize for changes in temperature, has been found to not be truly independent of temperature, even over relatively small changes in temperature (Kamler

1992). Furthermore, Degree Day has been recognized to remain more temperature dependent within optimal temperature ranges for warm-water species (Kamler 1992). Effective Degree Day (D°_{eff}), a modified time to development measure incorporating rearing temperature standardized relative to Effective Temperature (t_{eff}) , a theoretical temperature at which development stops, has been considered truly independent of temperature, and thus a more suitable option (Kamler 1992).

In order to standardize for temperature effects upon growth, Eq. 6.2 was modified to incorporate t_{eff} and D°_{eff} instead of utilization of age (DPH) as the independent variable during modeling of individual Experiment growth regressions. Effective Temperature was computed by compiling time to hatching and temperature data from the literature and also utilizing data collected at the UMEH. Development rate, V, was first computed as the inverse of incubation time. Incubation time (hours to hatching) can be considered a standard measure of differing development rate at different temperatures (Kamler 1992). The development rate V was linearly regressed against temperature to determine the relationship between development rate and temperature.

Investigation of normally of residuals resulted in the decision to apply a Box-Cox transformation to the dependent variable development rate V. The resulting transformation was incorporated within a linear regression which satisfactorily fulfilled assumptions of regression modeling. The resulting regression was then solved for y = 0 in order to determine the Temperature Effective:

$$(y \text{ ^ } 2.685193 - 1) / 2.685193 = 8.40*10\text{^-6 x } -0.3725689 \qquad \text{Eq. 6.3}$$

The resulting t_{eff} value (18.6 °C) was then utilized in the equation:

$$D^{\circ}_{eff} = \tau\,(t - t_{eff}) \qquad \text{Eq. 6.6}$$

in which D°_{eff} is Effective Degree Days, τ is age (DPH), t is mean temperature from individual Experiments, and t_{eff} is effective temperature. Solving of this equation was necessary in order to compute D°_{eff} to be utilized as the independent variable for linear growth in length regressions. This resulted in a set of D°_{eff} values, one for each length measurement. As previously mentioned, the resulting new set of growth regressions utilized a modified form of Eq. 6.2:

$$\text{Ln}\,(L_{(\tau)}) = G_L(\tau(t-t_{eff})) + \text{Ln}\,(L_0) \qquad \text{Eq. 6.7}$$

Before continuing with D°_{eff} growth regressions for each individual experiment; further analysis investigated whether the incorporation of D°_{eff} could improve the linearity (i.e. the similarity of slopes) of growth regressions computed for growth periods within Experiments, which, in some instances, were found to significantly differ within Experiments without its utilization. Computed D°_{eff} growth regressions for each growth period incorporated mean temperature for the particular growth period being regressed. Statistical comparisons of slopes of growth periods within Experiments computed utilizing D°_{eff} were conducted similar to slopes comparisons as previously described. The results of these investigations let to the decision to include D°_{eff} as a means to standardize for differences in temperature between individual Experiments;. These regressions incorporated D°_{eff} which was computed utilizing the mean temperature computed for each

Experiment. Residuals analysis was applied to this new linear regressions fit from the D°_{eff} formulation, unsatisfactory regressions were again encountered. Similar to complications encountered when modeling growth regressions with standard age as the independent variable, no attempts or modifications were utilized to correct for violations encountered during residuals analysis.

General linearized modeling (GLM) of computed growth coefficients (both 'standard,' i.e. age, computations and D°_{eff} computations), growth constants, arcsine transformed survival, and number of juveniles produced were utilized individually as dependent variables as a method to determining variables which significantly influence and explain these production outcomes. GLMs were deemed the more appropriate method use when modeling these functions due to the relaxation of assumptions concerning distributions of dependent variables and residuals. Preliminary investigations of residuals revealed heterogeneous variance distributions of independent variables, necessitating GLMs rather than multivariate regressions (Hardin and Hilbe 2007). The dependent variables would best be considered as continuous response variables and were found to be normally distributed, thus meeting assumptions of the Gaussian distribution.

GLM modeling was initiated by computing global pairwise correlations between each dependent variable and the set of previously listed independent variables. In order to investigate a broader suite of correlation relationships, those variables which were significantly correlated with an α level set to ≤ 0.10 were further considered. For further GLM model construction, the variable with the highest correlation for each of the above listed dependent variables was selected for the initial entry variable into GLM models (Zar 2009).

The Newton-Raphson Maximum Likelihood (ML) Estimation Algorithm was utilized during GLM parameter estimation. ML is considered to produce more accurate standard errors, which becomes increasingly noticeable in datasets with fewer observations (Hardin and Hilbe 2007), as was the case in this analysis (n = 13). Model estimated variance matrices were computed utilizing modified sandwich (robust clustered) standard error estimates. The overall small sample size would most safely be analyzed using conservative robust estimates and data from each Experiment within Trials (Y data) was suspected to not be completely independent (Hardin and Hilbe 2007, Stata…(Clustered) Data). In other terms, the dependent variables (from individual Experiments) were suspected to be correlated in regards to timing of when the individual Trials were carried out. This was verified statistically by computation of intraclass correlation coefficients for each dependent variable and subsequent F-testing to determine their significance (Zar 2009).

Previously obtained patterns in significance from pairwise F-testing of computed growth coefficients between Experiment growth regressions provided further, if superfluous, evidence for the necessity of clustered modeling. Selection between utilization of identity or log link functions for GLMs was contingent upon that link function which produced the greatest reduction in deviance (Harbin and Hilbe 2007). All models were constructed utilizing an identity link.

Construction of GLMs was carried out utilizing forward selection procedures. As previously discussed, initial independent variables were selected in regards to degree of correlation to the dependent variable in question (Zar 2009). Once this initial model was constructed, all other independent variables were individually added to the model.

Selection of which new independent variable would stay in the model was determined by the degree of significance of partial F-tests comparing each new model with the previous, more parsimonious model (Harbin and Hilbe 2007, Zar 2009). Partial F-testing employed critical values of α/k, where k = number of independent variables included in that particular level of model, in order to correct for increasing probability of finding significant explanatory variables with increasing numbers of independent variables being incorporated within the model (Hardin and Hilbe 2007).

Once models were constructed with single order independent variable sets, higher order functions and interaction terms of the independent variables were analyzed in order to determine any complicating affects and model fit using partial F-tests (Zar 2009). For the independent variable 'total *Artemia* utilization,' missing values from Trial 1 were corrected and accounted for by utilizing multiple imputation in order to estimate their values. Multiple imputation has been considered a more unbiased method for addressing the issue of missing values than traditional approaches, such as mean substitution or case deletion (Acock 2005).

Changes in feeding regime schedule could be considered as an indicator of developmental growth. Faster development would yield earlier switches from rotifers to *Artemia* and weaning onto formulated dry feeds. A unitless variable, Feeding Stage Progression, was created. Its values increased by one unit for each change in feeding regime. The quantities start of rotifer feeding, start of *Artemia* feeding, end of rotifer feeding, start of weaning, and end of *weaning* (exactly coinciding with end of *Artemia* feeding), all measured in DPH, were utilized as the independent variable in this analysis. The previously listed order incorporates each quantity as each one was temporally

realized during the course of a larval rearing. Within each Trial, these changes in feeding regime schedule were conducted on the same day. Thus, analysis was conducted at the Trial, rather than Experiment, level.

Regressions were computed for each Trial, resulting in the formation of a Feeding Stage Development Coefficient. Post modeling, each regression was checked for normality and distribution of residuals. Individual regressions were then compared pairwise in order to determine differences among the regression coefficients. Normality of the distribution of resulting regression coefficients from each Trial was analyzed. They were then incorporated as the dependent variable in regressions with final density of juveniles (fish/L) and average temperature (°C), averaged within Trials, as independent variables. Post regression fitting, each was checked for normality and distribution of residuals in order to validate regression assumptions.

In order to investigate a case study in proper sample size requirements for ANOVA testing of size distributions at differing sampling ages, scenarios were constructed using data observed during the Experiments 9 and 10. These experiments were similarly tested in a previous cobia larval growth analysis (Benetti et al. 2008b) and thus serve as an example of real-world scenarios of data analysis complications. Standard deviation values, observed mean values, anticipated percent difference in means of the length distributions, and a probability of detection estimate were utilized to tabulate necessary samples sizes under these conditions (Sokal and Rohlf 1995). Complementary calculations were made to ascertain a relative degree of impact on these sampling schemes on other important experimental outcomes such as survival.

6.3) Results

As a basis for the analyzation of growth and factors which influence differential growth between individual Trials and Experiments, it is crucial to first observe differences between methods, production results, and environmental parameters which could have influenced growth differences. These differences often times are the basis for further analysis conducted herein.

6.3.1) Trial 1

This rearing was initiated late during the spawning season well into autumn from a spawning achieved by photothermal manipulation of broodstock. Thus, ambient temperature regimes were relatively low. Mean temperature over the course of the rearing was 25.7 °C for both Experiments while T1E1 and T1E2 daily temperature measurements ranged from 22.0 to 29.6 °C and 21.9 to 29.7 °C, respectively. Median temperatures were 25.6 and 25.4 °C for tanks T1E1 and T1E2, respectively. The median temperatures indicate a substantial portion of the rearing period was within acceptable temperature ranges for cobia, but brief periods of the previously mentioned low temperatures were reason for concern. On Oct. 24th (13 DPH), the passing of Hurricane Wilma caused inshore transport of cooler, upwelled seawater. On Oct 25th, this caused ambient temperature to drop approximately 4 °C from the previous day. Temperatures remained at 24 ± 2 °C for the remainder of the trial, much lower than the initial temperatures, which were approximately 29 °C. Although not quantified in any manner, the passing of Hurricane Wilma also caused a noticeable reduction in water quality. Violent mixing of nearshore waters substantially increased turbidity (and presumably

nutrient and bacterial loads as well), which subsequently reduced the hatchery filtration system's efficiency and increased filter maintenance. Despite these adjustments, particulate deposits and more turbid seawater were observed in the larviculture tank. This in turn required increased sanitation (standpipe and siphoning) maintenance.

Average DO over the course of the trial was below saturation levels (5.4 and 5.9 mg L^{-1} for T1E1 and T1E2, respectively) despite periodic use of pure oxygen supplementation when it was available. Both tanks average pH was 8.12 over the course of the rearing, representing satisfactory values normally associated with seawater. Sporadic monitoring of total nitrogen-ammonia (TAN) measurements revealed did not exceed 0.02 mg L^{-1} in either tank during the trial.

Total rotifer utilization was 168.48 $x10^6$ and 137.16 $x10^6$ for tanks T1E1 and T1E2, respectively; total *Artemia* utilization could not be computed as records from the Trial were not complete in respect to this variable. Slower development caused changes in food regime, and the length of the rearing itself, to be longer than in most other Trials. Ultimately, 1010 fish were produced from T1E1 and 532 from tank T1E2, equating to 9.62% and 4.25% survival, respectively.

6.3.2) Trial 2

T2 spanned late spring and early summer seasons; thus, relatively warmer water temperature regimes occurred during this rearing. Average temperature was 29.4 °C and ranged from 28.0 to 30.4 °C while the median temperature was 29.5 °C. These data, taken in conjunction, indicate very little variability in the temperature regimes of this trial. Sporadic use of supplemental oxygen maintained high levels of oxygen (~8.0 mg L^{-}

^{1}) when in use. Although, the majority of the trial did not utilize supplemental oxygen and the average DO for the entire trial was 5.5 mg L^{-1}. Other water quality parameters, such as pH (trial average of 8.08) and TAN (remaining below 0.1 mg L^{-1} during the Trial), remained within acceptable ranges.

Within T2E3, total rotifer utilization was $162x10^{6}$. This low rotifer utilization was resultant from the rotifer feeding period occurring before larvae were split from the one tank initially stocked. Shortcomings in rotifer production and adaptive management to this situation influenced these events. Total *Artemia* utilization for tank T2E3 throughout the Trial was $239.88x10^{6}$. More rapid development allowed for earlier transitions within the food regime schedule. Survival was unsatisfactory at 3.6%, resulting in the production of 3,500 fingerlings from larviculture T2E3.

6.3.3) Trial 3

This Trial occurred completely during the late spring season and thus water temperatures were moderate. Although extremes in either direction did not occur, initially, temperatures were relatively lower. The average temperatures were 26.5, 26.6, 26.4, and 26.4 °C for tanks T3E4, T3E5, T3E6, and T3E7, respectively. Daily temperatures ranged from 24.5 to 28.8 °C for all larviculture systems combined. Median temperatures ranged from 26.0 to 26.5 °C, indicating a majority of the rearing period experienced relatively slightly lower than moderate temperatures, despite brief initial periods at much lower temperatures.

Initially, supplemental oxygen was not utilized in the larvicultures. Monitoring of DO revealed that levels remained acceptable (above 6.0 mg L^{-1}) until 16 DPH. On 16

DPH, morning DO measurements had were near 5.0 mg L^{-1}; an overfeeding event in the afternoon of that day resulted in substantial decreases in DO to between 3.0 and 5.0 mg L^{-1} in all larval rearing tanks. Immediate supplemental oxygen was bubbled within the larvicultures. Despite these response efforts, severe mortalities resulted (observational estimate of density reduced by 50% in one case). For the remainder of the trial, use of supplemental oxygen maintained DO level at ≥ 7.0 mg L^{-1}. Other water quality parameters remained within satisfactory ranges. Sporadic measurements of TAN concentrations never exceeded 0.1 mg L-1 and average pH ranged between 8.06 and 8.11 for all tanks.

Total rotifer utilization was ~657 $x10^6$ per tank (T3E4 and T3E5). Only two tank values are presented as the rotifer feeding stage occurred before the later splitting of each of these two tanks into one other tank. Total *Artemia* utilization was 276.24, 261.84, 248.64, and 247.44$x10^6$ per tanks T3E4, T3E5, T3E6, and T3E7, respectively. Final survival was determined to be 17.7, 20.0, 16.4, and 14.9% for tanks T3E4, T3E5, T3E6, and T3E7, respectively. Resultant production was a total of 10,621; 12,027; 9,858; and 8,966 fingerlings from T3E4, T3E5, T3E6, and T3E7, respectively.

6.3.4) Trial 4

T4 was conducted during the summer season, thus, temperature regimes remained relatively higher than previously experienced during larviculture trials at UMEH. Average temperatures were 30.0, 30.1, 30.1, and 30.2 °C for tanks T4E8, T4E9, T4E10, and T4E11, respectively, with temperatures ranging from 29.0 to 31.4 °C for all tanks combined during this Trial. Median temperatures were slightly higher than averages

(30.1 to 30.4 °C), indicating a greater portion of the Trial experienced temperatures above 30°C. Utilization of supplemental oxygen was employed throughout the entire rearing, resulting in average DO measurements of 7.7-7.8 mg L^{-1} overall. Careful monitoring of oxygen supplementation and DO measurements were utilized to maintain DO above 6.63 mg L^{-1} throughout the entire Trial. Monitoring of pH levels was not conducted due to lack of functioning equipment. Sporadic TAN sampling revealed concentrations remained $\leq$ 0.18 mg L^{-1}.

Total rotifer utilization was 876, 882, 744, and 750 $x10^6$ per tanks T4E8, T4E9, T4E10, and T4E11, respectively. *Artemia* utilization totaled 348.12, 348.12, 248.02, and 248.02 $x10^6$ for tanks T4E8, T4E9, T4E10, and T4E11, respectively. More rapid larval development rates allowed for more rapid transitions within the food regime schedule. Thus, weaning was completed by 21 DPH, which was earlier than any other previous Trial. A total of 23,061; 21,001; 18,836; and 20,960 fingerlings were produced representing survival of 19.2, 17.5, 31.4, and 34.9% for tanks T4E8, T4E9, T4E10, and T4E11, respectively.

6.3.5) Trial 5

Water quality remained high during the majority of Trial5. However, problems were encountered with temperature regimes experienced during this Trial due to the passage of a late season cold front influencing ambient water temperatures. Average temperature was 25.2 °C for both T5E12 and T5E13 while temperatures ranged from 21.5 to 27.5 °C for both larvicultures combined. Median temperature was 25.0 °C for both larvicultures, suggesting fluctuations about the average temperature did not occur

for extended periods and were balanced by fluctuations as both higher and lower than the average temperatures.

As previously mentioned, on 7 DPH a late season cold front passed through south Florida and caused dramatic reductions in ambient seawater temperatures. On 8 DPH the daily average temperature was a relatively low of 21.5 °C. It was not until 13 DPH that daily average temperatures returned to levels similar to those experienced before the passing of this cold front. Reduced feeding behavior and higher mortalities were observed during this period of reduced water temperatures.

DO concentrations remained near saturation throughout the trial via use of supplemental oxygen. Average DO for tanks T5E12 and T5E13 were 8.7 and 8.1 mg L^{-1}, respectively. TAN concentrations and pH levels were not monitored during this trial.

A total of 835.2 and 817.2 $x10^6$ rotifers were utilized for tanks T5E12 and T5E13, respectively. *Artemia* utilization totaled 338.4 and 331.8 $X10^6$ for T5E12 and T5E13, respectively. Relatively slower development rates extended the length of this larval rearing, as well as the timing of the feeding regime schedule. A total of 3,840 and 5,455 fingerlings were produced representing survival of 6.98 and 9.92% for T5E12 and T5E13, respectively.

6.3.6) Data Investigation and Statistical Analysis

Statistical testing validating the substitution of temperature data recorded from the ambient water inflow to broodstock tanks at UMEH for indoor larval rearing tank temperatures verified acceptability of this procedure (Table 5.1). Shapiro-Wilk tests indicated all sample sets were distributed normally (VALID1: z = -0.043, n = 32, P =

0.517; z = 0.281, n = 33, P = 0.38918; z = 0.457, n = 33, P = 0.324; VALID2: z = -0.807, n = 25, P = 0.790; z = -1.223, n = 23, P = 0.889). VALID1 compared temperature distributions from two different indoor larval rearing tanks and the corresponding broodstock water temperature data. Further statistical investigation did not detect significant differences between variance distributions ($F_{computed}$ = 0.251, df = 2, 95, P = 0.778) or means ($F_{computed}$ = 0.18, df = 3, 95, P = 0.833) in these three datasets (Table 6.1). VALID2 utilized temperature data collected from one indoor larval rearing tank and compared it with the concurrent dataset collected from the broodstock ambient replacement water. Again, statistical testing did not reveal significantly differing variances ($F_{computed}$ = 0.899, df = 24, 22, P = 0.7961) or means ($t_{computed}$ = 0.224, df = 46, P = 0.824) (Table 6.1).

As previously discussed, an average size-at-hatching (TL = 3.67 mm) was obtained by combining unpublished mean sizes observed by the author and other values reported in the peer reviewed literature. Linear regressions were then fit both with and without utilization of this average size-at-hatching value. Both growth coefficients and growth constants of regressions computed with and without the average size at hatching were found to change substantially in some instances (Table 6.2). Changes were recognized to be both positive and negative with no readily observable trend in direction nor in magnitude in either a positive or negative direction. Absolute differences, computed by percent change, of not using the size-at-hatching data point were as high as 14.94% for growth coefficients and 19.14% for growth constants (Table 6.2). When all percent change in coefficients were summed, a total difference of -1.85% was observed. For growth constants, the sum percent change was 26.23%.

Shapiro-Wilk testing revealed that each corresponding set of growth coefficients (with 0 DPH z = -0.266, n = 13, P = 0.605l; without 0 DPH z = -0.127, n = 13, P = 0.551) and constants (with 0 DPH z = 0.587, n = 13, P = 0.279; without 0 DPH z = -0.641, n = 13, P = 0.739) were normally distributed. Two tailed F-test comparing homogeneity of variances and t-tests comparing homogeneity of means resulted in no significant differences between the distributions of coefficients ($F_{computed}$ = 0.513, df = 12, 12, P = 0.262; $t_{computed}$ = -0.171, df = 24, P = 0.866) or constants ($F_{computed}$ = 0.371, df = 12, 12, P = 0.099; $t_{computed}$ = 0.120, df = 24, P = 0.905) obtained by either method (Table 6.3). Significance was detected for one-tailed homogeneity of variance F-testing between distributions of constants computed with or without a size-at-hatching data point ($F_{computed}$ = 0.371, df = 12, 12, P = 0.0495) (Table 6.4), representing a reduction in computed intercept variance about the mean with inclusion of the average size-at-hatching data point.

Investigation of back transformation of computed constants revealed highly implausible values for those computed without the size-at-hatching data point (range of 2.53 to 5.28 mm). The range of back-transformed constants computed when the size-at-hatching data point was included in regression (2.67 to 4.26 mm) was closer to reported and unreported values (Hitzfelder et al. 2006, Kilduff et al. 2002, I. Zink Unpub. Data). Pairwise testing for differences between regressions computed with or without the size-at-hatching data point did not yield any significant differences between the pairs of coefficients or constants (Table 6.5).

Further analysis thus included linear regressions of natural log transformed length (TL) data against age (DPH) computed utilizing the average size-at-hatching (0 DPH data

point). All regression models were highly significant ($P << 0.001$) and coefficient of determination (R^2) values were also relatively high (ranging from 0.6563 to 0.9843) (Table 6.6). Significant departures from the assumptions of regression analysis (normally distributed and homoscedastic residuals) were detected for some models (nonnormal residuals: Experiments T1E2, T3E7, T4E11, T5E12, T5E13; heteroscedastic residuals: Experiment T3E5). Testing for model specification also revealed a number of models that seemed lack explanatory variables due to significant results of RESET analysis (Experiments T1E1, T1E2, T2E3, T3E4, T5E12, and T5E13).

In attempts to improve growth regression fit, data points which were identified as outliers were removed from regression modeling one at a time. For some regressions, these procedures yielded models which met the assumptions of linear regression after manipulation of one or a few data points. In other situations, multiple iterations of removal of individual data points were necessary in order to meet linear regression assumptions, thus discrediting these efforts.

The observation of multiple misspecification errors of linear growth regressions modeled with only age as an independent variable, in conjunction with qualitative visual analysis of plots of raw data points for each Experiment and observation of sigmoid shaped distributions, led to investigation of other factors could have influenced growth. Linear regressions were fit for each period between consecutive sampling events within each Experiment. Analysis of resulting regression slopes revealed growth rates which significantly differed between periods within Experiments T1E1, T1E2, T2E3, T3E5, T5E12, and T5E13 (Table 6.7).

Changes in two factors were suspected of causing the changes in growth coefficients between periods: temperature and biomass/ larval density. Average temperatures computed for individual growth periods between sampling events within Experiments yielded significant differences for all Experiments except T5E12 and T5E13 (Table 6.8). This pattern in temperature differences did not match the pattern of Experiments which exhibited differing growth rates between periods. Further investigation of biomass influences on growth could not be conducted as weight data was not sampled and length data could not be converted into weight data for lack of a reliable relationship between these measures. Thus, these investigations were not considered further, and the resultant regressions, as previously described, were utilized in for further analysis.

Modeling of individual Experiment regressions with $D°_{eff}$ was conducted in order to ascertain whether utilization of this measure as an independent variable could improve the linearity of regression slopes between growth sampling periods within Experiments. Each growth period $D°_{eff}$ was computed utilizing the average temperature for the particular growth period regression being computed. In general, these regression methods resulted in reductions in significant differences in slopes between growth sampling periods within Experiments (Table 6.9). Only for Experiments T1E1 ($F_{computed}$ = 37.08, df = 3, 28, $P < 0.0001$) and T1E2 ($F_{computed}$ = 64.97, df = 3, 28, $P < 0.0001$) were significant differences detected (Table 6.9). During this Trial, substantial reductions in temperature during later stages of the rearing resulted in computation of negative growth rates between growth sampling periods, most likely causing the resulting significant difference in slopes within this trial.

The culmination of these investigations resulted in two sets of linear regressions utilized to further investigate growth differences between Experiments and Trials: one set which utilized age as the independent variable and one utilizing $D°_{eff}$ as the independent variable. Both sets of regressions utilized the average size-at-hatching data point, as previously discussed. Similar to regressions computed utilizing age as the independent variable, regressions utilizing $D°_{eff}$ were found to violate assumptions of linear regression modeling, namely normally distributed and homogeneity of variance distributions of residuals. Given the results of similar investigations when age was the independent variable, these complications were ignored for further analysis. As utilization of $D°_{eff}$ does not change the distribution of Y values (natural log transformed length samples) within a sampling event, model fit parameters are not changed by utilization of this independent variable. Thus, model fit parameters such as significance of each regression and coefficient of determination are the same as those computed for regressions utilizing age as the independent variable (Table 6.6).

Within each set of regressions, pairwise comparison of regression coefficients was utilized to investigate differences in growth. For regressions modeled with age, relatively few significant differences were detected and all of these all involved differences between individual Experiments and T5E12 or T5E13 (Table 6.10). Many more significant differences between comparisons of growth coefficients were detected when $D°_{eff}$ was utilized as the independent variable (Table 6.11). Patterns of significance within both investigations, and especially the later utilizing $D°_{eff}$, indicated clustering of statistically similar growth coefficients within Trials.

Further analysis involved GLM modeling of production outcomes. The resulting suite of growth coefficients, both computed with age and $D°_{eff}$ as independent variables, and constants were added to a large table of factors which included environmental and water quality parameters; stocking densities; survival and production outcomes; live feeds utilization, enrichment protocols, and schedules; and chemotherapeutant and probiotic treatments. These variables represent potential factors influencing the growth coefficients and constants computed from individual experiments. Tables 6.12, 6.13, and 6.14 summarize their values. Results of pairwise correlation of all influential variables with respective dependent variables are summarized in the following tables: growth coefficients and constants (Table 6.15), $D°_{eff}$ growth coefficients (Table 6.15), arcsine transformed survival (Table 6.16), and total number of juveniles produced (Table 6.17).

Few independent variables were found to significantly correlate with computed growth coefficients. Those that did correlate (final density, total number of juveniles produced, and arcsine transformed survival) can be considered to represent density-dependent growth (Table 6.15). Many factors were found to correlate to growth constants with the most highly significant correlates including temperature variables, one feeding regime schedule variable, and variables relating to density-dependent growth (Table 6.15). Multiple variables were found to correlate with $D°_{eff}$ growth coefficients including temperature factors, production and density-dependent growth factors, and feeding regime schedule variables (Table 6.15). Factors highly correlated with arcsine transformed survival included density-related variables, which can be considered collinear and thus excluded from further consideration in this case, feeding regime schedule factors, most chemotherapeutant treatments, and finally temperature variables

(Table 6.16). Total number of juveniles produced was found to be most highly correlated with feeding regime schedule variables, temperature variables, and various chemotherapeutant treatments (Table 6.18).

Data collected or computed from individual Experiments occurring within the same Trial were suspected of being correlated to each other due to clustering of data points by Trial observed in constructed scatter plots. Furthermore, pairwise statistical testing of growth coefficients generally were non-significant within Trials, but significant differences were often detected when Experiments from differing Trials were compared (Table 6.10, Table 6.11). Before commencing with GLM modeling, these suspicions were investigated via intraclass correlations. Significance was detected in all dependent variables that were to be modeled with GLMs: age growth coefficients ($F_{computed} = 6.41$, df = 4, 8, P = 0.013), $D°_{eff}$ growth coefficients ($F_{computed} = 74.15$, df = 4, 8, $P < 0.0001$), growth constants ($F_{computed} = 12.23$, df = 4, 8, P = 0.002), arcsine transformed survival ($F_{computed} = 9.74$, df = 4, 8, P = 0.004), and total number of juveniles produced ($F_{computed} = 96.72$, df = 4, 8, $P < 0.0001$) (Table 6.18).

Due to significant intraclass correlations, GLM modeling for each dependent variable commenced following the previously described procedures utilizing clustered analysis of data. Highly significant models were fit for all dependent variables modeled with instances of multiple independent variables being fit for each model (Table 6.19). Growth coefficients computed from age were best explained by the final density of juveniles produced and the start of weaning (Adj. $R^2 = 0.6087$, $F_{computed} = 21.704$, df = 3, 10, $P < 0.001$) (Table 6.19). Growth constant variation was explained only by highest temperature recorded for each Experiment (Adj. $R^2 = 0.6156$, $F_{computed} = 17.6145$, df = 2,

11, $P < 0.001$) (Table 6.19). $D°_{eff}$ growth coefficient variation was found to be significantly explained by low temperature recorded for each Experiment (Adj. $R^2 = 0.8486$, $F_{computed} = 68.2677$, df = 2, 11, $P < 0.001$) (Table 6.19). Survival (arcsine transformed) variation was found to be best explained by the start of weaning and initial stocking density (Adj. $R^2 = 0.6385$, $F_{computed} = 24.3500$, df = 3, 10, $P < 0.001$) (Table 6.19). Further investigation suggested start of *Artemia* feeding was also a valid entry into this model based upon partial F tests, but investigation of collinearity with start of weaning suggested its exclusion. Variation in the total number of juveniles produced was significantly explained by the start of weaning (Adj. $R^2 = 0.8478$, $F_{computed} = 61.2535$, df = 2, 11, $P < 0.001$) (Table 6.19). In this model, antibiotic use was also found to be a valid entry as an explanatory variable. It was decided to exclude this variable, as its use occurred during only one larviculture and thus most likely does not provide much explanatory power despite significance.

Investigation of scatter plots and linear model fits of each independent and dependent variable modeled assisted with visualizing these relationships, as trends were readily apparent in the figures (Figures 6.1 through 6.7). Residuals analysis did not identify any models which violated normality or homogeneity in residual distributions. Thus, these models as constructed were utilized for further this analysis and interpretation.

Correlations between variables significantly ($P \leq 0.10$) describing changes in feeding regime schedule are presented in Table 6.20. Aside from collinearity with other feeding regime schedule variables, temperature variables often rank within the most significant of correlated variables. Correlations between temperature variables and

feeding schedule variables were found to always be negatively correlated (Table 6.20). A number of treatment and chemotherapeutant variables, such as probiotic, antibiotic, and formalin utilization, were also found to be significantly correlated to feeding schedule variables (Table 6.20).

All regressions of Feeding Stage Progression (unitless variable) and age (DPH) at which each change in feeding regime schedule took place were highly significant (Table 6.21). The resultant coefficients did not differ greatly and only slopes of regressions from Trial 1 and Trial 4 were found to significantly differ ($F_{computed} = 5.923$, df = 1, 3, P = 0.020) (Figure 6.8). Resulting Feeding Stage Development Coefficients were regressed against average temperature (°C) and final juvenile density (fish/L) (averaged within each Trial) as independent variables. Final juvenile density was found to significantly explain changes in Feeding Stage Development Coefficients ($R^2 = 0.744$, $F_{computed} = 12.604$, df = 1, 4, P = 0.038) while the average temperature regression was not significant ($R^2 = 0.523$, $F_{computed} = 5.38$, P = 0.103). Furthermore, residuals analysis revealed that when regressing Feeding Stage Development Coefficient against average temperature, violation of normality of residuals occurred. Conversely, no violation was detected when final density of juveniles was utilized as the regression independent variable. Even so, both regressions resulted in readily visible positive trends with increasing average temperature (Figure 6.9a) and increasing final density (Figure 6.9b).

In order to compare growth rates computed in this analysis with those published in the literature, available growth rates, as well as factors potentially influencing growth rates such as mean temperature and initial stocking densities, were compiled into a single

table (Table 6.23). Final average length of juveniles varied widely, at times by an order of magnitude when comparing results from very different production methods.

As an exercise for improving future sampling design for detecting differences in length at various ages, computation of sample size required to detect differences by ANOVA testing at different sampling ages were investigated utilizing data from T4E9 and T4E10 (Table 6.24). These computations reveal that at the first sampling event (15 DPH), smaller sample sizes of $n \leq 10$ could have been utilized and yet detection of potential significant difference would have occurred, if there was one. In this case, the percent difference between the mean TL was a considerably large at 19.2% (Table 6.23). At 21 DPH, this difference in mean TL had reduced to 11.4%. Thus, larger sample sizes of $n > 20$ were computed to be necessary for the detection of significant differences (Table 6.23).

Table 6.1: Results of investigation to confirm acceptability of utilization of ambient broodstock water temperature data as a substitute for ambient indoor larval rearing temperature data utilizing two different time periods (VALID1 and VALID2).

	Variance Testing		
	Test	df	P Value
VALID1	Levene's Robust Variance	2, 95	0.778
VALID2	F test, variance comparison	24, 22	0.796
	Means Testing		
	Test	df	P Value
VALID1	ANOVA	98	0.833
VALID2	t test, 2 tailed	46	0.824

Table 6.2: Parameter estimates computed for linear growth regressions with and without average size-at-hatching data point (constants reported in their natural log form as computed by regression analysis) and percent change in each value when average size-at-hatching data point is included in regression modeling. Models were constructed utilizing age (DPH) as the independent variable.

Experiment	Without Age Zero		With Age Zero		Percent Change	
	Coefficient	Constant	Coefficient	Constant	Coefficient	Constant
T1E1	0.089	1.311	0.089	1.310	0.09	-0.08
T1E2	0.090	1.322	0.090	1.317	0.23	-0.36
T2E3	0.107	1.102	0.100	1.194	-7.01	8.30
T3E4	0.098	0.977	0.088	1.168	-10.20	19.60
T3E5	0.091	1.071	0.083	1.207	-7.85	12.64
T3E6	0.075	1.374	0.078	1.326	3.45	-3.47
T3E7	0.084	1.252	0.082	1.281	-1.80	2.27
T4E8	0.068	1.489	0.073	1.378	8.27	-7.45
T4E9	0.087	1.222	0.085	1.268	-2.64	3.77
T4E10	0.072	1.663	0.083	1.449	14.94	-12.87
T4E11	0.087	1.470	0.092	1.370	5.82	-6.80
T5E12	0.105	0.930	0.102	0.982	-2.66	5.61
T5E13	0.104	0.956	0.102	1.005	-2.49	5.07

Table 6.3: Results of 2-tailed F-tests of homogeneity of variances and t-tests of homogeneity of means of regression coefficients and constants computed with and without average size-at-hatching (presented in Table 6.2). Statistical testing was conducted on constants in their computed natural logarithm form.

		F-test Homogeneity of Variances		t-test Equality of Means	
	n	F Computed	P Value	t Computed	P Value
Coefficients from Regressions	13	0.5132	0.262	-0.1712	0.8655
Constants from Regressions	13	0.3712	0.0991	0.1202	0.9053

Table 6.4: Results of 1-tailed F-test of homogeneity of variances between distributions of growth constants computed with and without average size-at-hatching data point (presented in Table 6.2). Means and standard deviations of growth constants presented in their computed natural logarithm form.

	n	Mean	Std. Dev.	F Value	P Value
With 0 DPH	13	1.25029	0.13819	0.3712	0.0495
Without 0 DPH	13	1.24143	0.22682		

Table 6.5: Results of pairwise testing within Experiments of growth coefficients and constants of regressions between those computed with and without average size-at-hatching data point.

Experiment	Coefficients			Constants		
	F Value	df	P value	t Value	df	P Value
T1E1	0.000	1, 37	0.984	0.010	1, 38	0.992
T1E2	0.002	1, 37	0.966	0.022	1, 38	0.983
T2E3	1.751	1, 17	0.203	1.231	1, 18	0.234
T3E4	0.408	1, 37	0.527	1.121	1, 38	0.269
T3E5	0.211	1, 37	0.649	0.105	1, 38	0.917
T3E6	0.198	1, 37	0.659	0.101	1, 38	0.920
T3E7	0.015	1, 37	0.905	0.028	1, 38	0.978
T4E8	0.128	1, 77	0.722	0.057	1, 78	0.955
T4E9	0.016	1, 77	0.898	0.020	1, 78	0.984
T4E10	0.486	1, 77	0.488	0.111	1, 78	0.912
T4E11	0.130	1, 77	0.720	0.057	1, 78	0.954
T5E12	0.207	1, 117	0.650	0.159	1, 118	0.874
T5E13	0.285	1, 117	0.595	0.187	1, 118	0.852

Table 6.6: Results of linear regression fitting of natural log transformed length (TL) data and age (DPH). Within Experiments, data distributions were shifted consistently along the x-axis by utilization of $D°_{eff}$, hence the same model fit statistics apply to those regressions as well.

	Model Fit Statistics			
Experiment	df	F Computed	P Value	Adjusted R^2
T1E1	1, 19	328.71	< 0.001	0.9425
T1E2	1, 19	534.89	< 0.001	0.9639
T2E3	1,9	627.99	< 0.001	0.9843
T3E4	1, 19	105.4	< 0.001	0.8392
T3E5	1, 19	99.08	< 0.001	0.8306
T3E6	1, 19	67.6	< 0.001	0.7806
T3E7	1, 19	151.98	< 0.001	0.8830
T4E8	1, 39	74.69	< 0.001	0.6570
T4E9	1, 39	74.49	< 0.001	0.6563
T4E10	1, 39	97.24	< 0.001	0.7064
T4E11	1, 39	147.15	< 0.001	0.7851
T5E12	1, 59	601.35	< 0.001	0.9091
T5E13	1, 59	938.55	< 0.001	0.9399

Table 6.7: Results of statistical comparison of linear regression slopes computed between sampling periods within Experiments utilizing age (DPH).

Experiment	Period	Growth Coefficient	Results of Coefficient Similarity Testing		
			df	F Value	P Value
T1E1	1	0.066	3, 28	15.09	< 0.001
	2	0.142			
	3	0.082			
	4	0.054			
T1E2	1	0.073	3, 28	19.61	< 0.001
	2	0.132			
	3	0.082			
	4	0.063			
T2E3	1	0.059	1, 11	12.52	0.005
	2	0.118			
T3E4	1	0.076	1, 27	1.57	0.221
	2	0.098			
T3E5	1	0.075	1, 27	16.42	0.000
	2	0.090			
T3E6	1	0.080	1, 27	0.77	0.389
	2	0.065			
T3E7	1	0.080	1, 27	0.05	0.832
	2	0.084			
T4E8	1	0.080	1, 47	0.45	0.505
	2	0.068			
T4E9	1	0.082	1, 47	0.06	0.814
	2	0.087			
T4E10	1	0.097	1, 47	1.72	0.196
	2	0.072			
T4E11	1	0.098	1, 47	0.46	0.502
	2	0.087			
T5E12	1	0.072	2, 95	5.23	0.007
	2	0.089			
	3	0.121			
T5E13	1	0.073	2, 95	7.37	0.001
	2	0.090			
	3	0.119			

Table 6.8: Results of statistical testing of differences in mean temperature between growth sampling periods within Experiments.

Experiment	Test	Statistical Testing Results		
		Chi^2 Computed	df	P Value
T1E1	Kruskal-Wallis w/ ties	15.92	3	0.001
T1E2	Kruskal-Wallis w/ ties	14.21	3	0.003
T5E12	Kruskal-Wallis w/ ties	3.29	2	0.193
T5E13	Kruskal-Wallis w/ ties	4.14	2	0.126
		t Computed	df	P Value
T2E3	T Test, 2-tailed	-4.43	10	0.001
T3E4	T Test, 2-tailed	-2.90	19	0.009
T3E5	T Test, 2-tailed	-2.68	19	0.015
T3E6	T Test, 2-tailed	-2.45	19	0.024
T3E7	T Test, 2-tailed	-2.13	19	0.047
T4E8	T Test, 2-tailed	2.32	19	0.032
T4E9	T Test, 2-tailed	2.87	19	0.010
T4E10	T Test, 2-tailed	2.87	19	0.010
T4E11	T Test, 2-tailed	2.78	19	0.012

Table 6.9: Results of statistical comparison of linear regression slopes computed between sampling periods within Experiments utilizing temperature independent ages ($D°_{eff}$).

Experiment	Period	Growth Coefficient	Results of Coefficient Similarity Testing		
			df	F Value	P Value
T1E1	1	0.0067	3, 28	37.08	< 0.001
	2	0.0169			
	3	-0.0900			
	4	0.0101			
T1E2	1	0.0076	3, 28	64.97	< 0.001
	2	0.0157			
	3	-0.1168			
	4	0.0124			
T2E3	1	0.0057	1, 11	4.19	0.065
	2	0.0098			
T3E4	1	0.0109	1, 27	0.04	0.843
	2	0.0104			
T3E5	1	0.0107	1, 27	0.22	0.646
	2	0.0097			
T3E6	1	0.0114	1, 27	3.22	0.084
	2	0.0073			
T3E7	1	0.0113	1, 27	0.74	0.397
	2	0.0096			
T4E8	1	0.0068	1, 47	0.00	0.983
	2	0.0067			
T4E9	1	0.0068	1, 47	1.10	0.299
	2	0.0090			
T4E10	1	0.0081	1, 47	0.20	0.659
	2	0.0073			
T4E11	1	0.0081	1, 47	0.24	0.623
	2	0.0089			
T5E12	1	0.0127	2, 95	0.17	0.844
	2	0.0146			
	3	0.0141			
T5E13	1	0.0133	2, 95	0.05	0.947
	2	0.0142			
	3	0.0141			

Table 6.10: Results of pairwise testing for significantly different slopes between individual Experiments computed using standard procedures (age (DPH) as independent variable). Only statistically significant results are presented.

Experiment Pair	F Value	df	P Value
T1E1, T5E13	4.668	1, 76	0.034
T1E2, T5E13	4.752	1, 76	0.032
T3E5, T5E12	4.035	1, 76	0.048
T3E5, T5E13	5.267	1, 76	0.024
T3E6, T5E12	10.196	1, 76	0.002
T3E6, T5E13	13.445	1, 76	<0.001
T3E7, T5E12	5.120	1, 76	0.027
T3E7, T5E13	6.978	1, 76	0.010
T4E8, T5E12	9.861	1, 96	0.002
T4E8, T5E13	11.980	1, 96	<0.001
T4E10, T5E12	4.344	1, 96	0.040
T4E10, T5E13	5.189	1, 96	0.025

Table 6.11: Results of pairwise testing for significantly different slopes between individual Experiments computed with as $D°_{eff.}$ Only statistically significant results are presented.

Experiment Pair	df	F Value	P Value	Experiment Pair	df	F Value	P Value
T1E1, T2E3	1, 26	9.995	0.004	T3E4, T5E12	1, 76	12.190	< 0.001
T1E1, T3E6	1, 36	6.396	0.016	T3E4, T5E13	1, 76	16.148	< 0.001
T1E1, T4E8	1, 56	31.701	< 0.001	T3E5, T4E8	1, 56	9.100	0.004
T1E1, T4E9	1, 56	18.195	< 0.001	T3E5, T4E9	1, 56	4.363	0.0413
T1E1, T4E10	1, 56	24.398	< 0.001	T3E5, T4E10	1, 56	5.955	0.0179
T1E1, T4E11	1, 56	21.813	< 0.001	T3E5, T4E11	1, 56	4.292	0.043
T1E1, T5E12	1, 76	8.585	0.004	T3E5, T5E12	1, 76	16.441	< 0.001
T1E1, T5E13	1, 76	11.530	0.001	T3E5, T5E13	1, 76	21.978	< 0.001
T1E2,T2E3	1, 26	16.224	< 0.001	T3E6, T5E12	1, 76	24.451	< 0.001
T1E2, T3E6	1, 36	7.882	0.008	T3E6, T5E13	1, 76	32.535	< 0.001
T1E2, T3E7	1, 36	4.436	0.042	T3E7,T4E8	1, 56	10.083	0.002
T1E2, T4E8	1, 56	35.736	< 0.001	T3E7, T4E9	1, 56	4.735	0.034
T1E2, T4E9	1, 56	20.196	< 0.001	T3E7, T4E10	1, 56	6.622	0.013
T1E2, T4E10	1, 56	27.635	< 0.001	T3E7, T4E11	1, 56	4.886	0.031
T1E2, T4E11	1, 56	25.247	< 0.001	T3E7, T5E12	1, 76	17.470	< 0.001
T1E2, T5E12	1, 76	8.770	0.004	T3E7, T5E13	1, 76	24.290	< 0.001
T1E2, T5E13	1, 76	12.165	0.001	T4E8, T5E12	1, 96	86.752	< 0.001
T2E3, T4E8	1, 48	4.316	0.043	T4E8, T5E13	1, 96	107.341	< 0.001
T2E3, T5E12	1, 66	25.819	< 0.001	T4E9, T5E12	1, 96	61.250	< 0.001
T2E3, T5E13	1, 66	39.35131	< 0.001	T4E9, T5E13	1, 96	73.180	< 0.001
T3E4, T4E8	1, 56	11.978	0.001	T4E10, T5E12	1, 96	73.226	< 0.001
T3E4, T4E9	1, 56	6.246	0.015	T4E10, T5E13	1, 96	90.578	< 0.001
T3E4, T4E10	1, 56	8.376	0.005	T4E11, T5E12	1, 96	67.726	< 0.001
T3E4, T4E11	1, 56	6.576	0.013	T4E11, T5E13	1, 96	85.669	< 0.001

Table 6.12: Physical and chemical water quality parameters, chemotherapeutant utilization, other treatment utilization in growth and production analysis. Physical water parameters represent averages of the entire rearing. Treatments are binary coded (0) for non-utilization and (1) for utilization.

Experiment	Average Temp. (°C)	Max. Temp. (°C)	Min. Temp. (°C)	Median Temp. (°C)	Average DO (mg/L)	Average pH	Probiotics	Formalin	Antibiotics	Copper Sulfate
T1E1	25.7	29.6	22.0	25.6	5.4	8.12	0	0	0	1
T1E2	25.7	29.7	21.9	25.4	5.9	8.12	0	0	0	1
T2E3	29.4	30.4	28	29.5	5.5	8.08	0	0	1	1
T3E4	26.5	28.8	24.6	26.3	6.94	8.07	1	1	0	0
T3E5	26.6	28.6	24.6	26.5	7.36	8.11	0	1	0	0
T3E6	26.4	28.7	24.5	26.3	7.14	8.06	1	1	0	0
T3E7	26.4	28.5	24.6	26.0	7.55	8.09	0	1	0	0
T4E8	30.0	31.2	29.0	30.1	7.7	-	1	1	0	0
T4E9	30.1	31.4	29.0	30.2	7.8	-	1	1	0	0
T4E10	30.1	31.2	29.0	30.3	7.7	-	1	1	0	0
T4E11	30.2	31.3	29.0	30.4	7.7	-	1	1	0	0
T5E12	25.2	27.0	21.5	25.0	8.7	-	1	1	0	0
T5E13	25.2	27.5	21.5	25.0	8.1	-	1	1	0	0

Table 6.13: Initial and final densities, survival characteristics, production, and growth parameters utilized in analysis of growth and production.

Experiment	Initial Density (larvae/L)	Final Density (fish/L)	Percent Survival	Survival, Arcsine Transformed	Number of Juveniles Produced	Final Day of Growth Measurements (DPH)	Calculated Growth Coefficient	Calculated $D_{\circ eff}$ Growth Coefficient	Calculated Growth Constant	Average Final Length (TL, mm)
T1E1	0.875	0.092	9.62	18.05	1010	25	0.0892	0.0126	1.3102	30.6
T1E2	1.025	0.048	4.25	11.97	532	25	0.0898	0.0127	1.3168	32.2
T2E3	8	0.318	3.6	10.94	3500	14	0.0995	0.0092	1.1937	13.5
T3E4	5	0.966	17.7	24.83	10621	22	0.088	0.0111	1.1679	23.60
T3E5	5	1.093	20.0	26.57	12027	22	0.0834	0.0104	1.2067	22.17
T3E6	5	0.896	16.4	23.89	9858	22	0.0779	0.0092	1.326	23.38
T3E7	5	0.815	14.9	22.71	8966	22	0.082	0.0105	1.2805	22.28
T4E8	10	2.096	19.2	25.99	23061	21	0.0733	0.0064	1.3776	18.85
T4E9	10	1.909	17.5	25.73	21001	21	0.0847	0.0074	1.268	21.89
T4E10	5	1.712	31.4	34.08	18836	21	0.0831	0.0072	1.4494	24.72
T4E11	5	1.905	34.9	36.21	20960	21	0.09185	0.0079	1.3699	27.48
T5E12	5	0.349	6.98	15.34	3840	24	0.1023	0.0155	0.9822	33.42
T5E13	5	0.496	9.92	18.34	5455	24	0.1018	0.0154	1.0048	33.4

Table 6.14: Feeding regime schedules and total live food utilization by type utilized in of growth and production. Experiments 1 and 2 were missing total *Artemia* utilization numbers due to lack of careful record keeping and inability to retrospectively determine reliable estimates. This was remedied via application of multiple imputation techniques during further analysis.

Experiment	Start of Rotifer Feeding (DPH)	End of Rotifer Feeding (DPH)	Start of *Artemia* Feeding (DPH)	End of *Artemia* Feeding (DPH)	Start of Weaning (DPH)	End of Weaning (DPH)	Total Rotifer Utilization ($x10^6$)	Total *Artemia* Utilization ($x10^6$)
T1E1	3	11	8	34	24	34	168.48	-
T1E2	3	11	8	34	24	34	137.16	-
T2E3	3	6	5	22	17	22	162	239.88
T3E4	3	9	8	25	18	25	328.5	276.24
T3E5	3	9	8	25	18	25	328.5	261.84
T3E6	3	9	8	25	18	25	328.5	248.64
T3E7	3	9	8	25	18	25	328.5	247.44
T4E8	2	8	7	20	11	20	876	348.12
T4E9	2	8	7	20	11	20	882	348.12
T4E10	2	8	7	20	11	20	744	248.04
T4E11	2	8	7	20	11	20	750	248.04
T5E12	3	11	8	28	18	28	835.2	338.4
T5E13	3	11	8	28	18	28	817.2	331.8

Table 6.15: Variables significantly correlated to modeled growth parameters.

Growth Parameter	Correlated Factor	Correlation Coefficient	df	P Value
Growth Coef., Age	Final Density	-0.5710	12	0.0415
Growth Coef., Age	Number Produced	-0.5710	12	0.0415
Growth Coef., Age	Arcsine Survival	-0.5098	12	0.0751
Growth Coef., $D°_{eff}$	Minimum Temperature	-0.9326	12	< 0.0001
Growth Coef., $D°_{eff}$	Average Temperature	-0.8889	12	< 0.0001
Growth Coef., $D°_{eff}$	Median Temperature	-0.8825	12	0.0001
Growth Coef., $D°_{eff}$	Maximum Temperature	-0.8537	12	0.0002
Growth Coef., $D°_{eff}$	Start of *Artemia* Feeding	-0.8461	12	0.0003
Growth Coef., $D°_{eff}$	Greenwater Utilization	-0.7531	12	0.003
Growth Coef., $D°_{eff}$	Number Produced	-0.8030	12	0.0009
Growth Coef., $D°_{eff}$	Final Density	-0.8030	12	0.0009
Growth Coef., $D°_{eff}$	Arcsine Survival	-0.6291	12	0.0212
Growth Coef., $D°_{eff}$	End of Rotifer Feeding	-0.5888	12	0.0342
Growth Coef., $D°_{eff}$	Initial Density	-0.5806	12	0.0375
Growth Coef., $D°_{eff}$	Average pH	0.7651	12	0.0451
Growth Coef., $D°_{eff}$	End of *Artemia* Feeding	-0.4979	12	0.0834
Growth Coef., $D°_{eff}$	End of Weaning	-0.4979	12	0.0834
Growth Constant	Maximum Temperature	0.7846	12	0.0015
Growth Constant	Growth Coefficient	-0.7344	12	0.0043
Growth Constant	Greenwater Start	-0.6947	12	0.0177
Growth Constant	Minimum Temperature	0.6021	12	0.0295
Growth Constant	Average Temperature	0.5961	12	0.0316
Growth Constant	Median Temperature	0.5881	12	0.0345
Growth Constant	Start of Rotifer Feeding	-0.5822	12	0.0368
Growth Constant	Arcsine Survival	0.5378	12	0.0580
Growth Constant	Number Produced	0.5088	12	0.0758
Growth Constant	Final Density	0.5088	12	0.0758

Table 6.16: Variables significantly correlated to arcsine transformed percent survival.

Correlated Factor	Correlation Coefficient	df	P Value
Number Produced	0.8485	12	0.0002
Final Density	0.8485	12	0.0002
Start of Weaning	-0.7202	12	0.0055
Start of Rotifer Feeding	-0.7114	12	0.0064
Formalin Utilization	0.6721	12	0.0119
Copper Utilization	-0.6721	12	0.0119
End of *Artemia* Feeding	-0.6539	12	0.0153
End of Weaning	-0.6539	12	0.0153
Minimum Temperature	0.6148	12	0.0254
Total Rotifer Use	0.6071	12	0.0476
Average Temperature	0.5634	12	0.0450
Growth Constant	0.5571	12	0.0480
Median Temperature	0.5561	12	0.0484
Probiotic Utilization	0.4970	12	0.0840

Table 6.17: Variables significantly correlated to total number of juveniles produced.

Correlated Factor	Correlation Coefficient	df	P Value
Start of Weaning Period	-0.9207	12	< 0.0001
Start of Rotifer Feeding	-0.8915	12	< 0.0001
End of *Artemia* Feeding	-0.8513	12	0.0002
End of Weaning Period	-0.8513	12	0.0002
Arcsine Transformed Survival	0.8485	12	0.0002
Minimum Temperature	0.8307	12	0.0004
Average Temperature	0.7962	12	0.0011
Median Temperature	0.7930	12	0.0012
Initial Stocking Density	0.6575	12	0.0146
Total Rotifer Utilization	0.6499	12	0.0162
Formalin Utilization	0.6494	12	0.0163
Copper Utilization	-0.6494	12	0.0163
Maximum Temperature	0.6485	12	0.0165
Probiotic Utilization	0.5726	12	0.0408
End of Rotifer Feeding	-0.5629	12	0.0452
Rotifer Enrichment	0.5563	12	0.0483
Artemia Enrichment	0.5563	12	0.0483

Table 6.18: Results of statistical testing of intraclass correlation within Trials for each production outcome variable.

Dependent Variable	Intraclass Correlation Coefficient	F Value	df	P Value
Growth Coefficient	0.599	6.41	4, 8	0.013
Growth Coefficient, $D_{\circ eff}$	0.953	74.15	4, 8	< 0.001
Growth Constant	0.756	12.23	4, 8	0.002
Survival, Arcsine Transformed	0.707	9.74	4, 8	0.004
Number of Juveniles Produced	0.964	96.72	4, 8	< 0.001

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Table 6.19: Results of generalized linear modeling of selected production outcomes of larval rearing Trials.

Dependent Variable	Independent Variable	Indep. Variable Coefficient	Standard Error of Coefficient	P Value Indep. Variable	Adjusted R^2	Regression Computed F Value	df	P Value of Regression
Growth Coefficient					0.6087	21.7036	3, 10	<0.001
	Final Density	-0.0242	0.0043	<0.001				
	Start of Weaning	-0.0030	0.0006	<0.001				
	Constant	0.1616	0.0151	<0.001				
Growth Coefficient, $D°_{eff}$					0.8486	68.2677	2, 11	<0.001
	Minimum Temperature	-0.0009	0.0001	<0.001				
	Constant	0.0330	0.0038	<0.001				
Growth Constant					0.6156	17.6145	2, 11	<0.001
	Maximum Temperature	0.0727	0.0205	<0.001				
	Constant	-0.8975	0.6251	0.151				

Table 6.19, cont.d: Results of generalized linear modeling of selected production outcomes of larval rearing Trials.

Dependent Variable	Independent Variable	Indep. Variable Coefficient	Standard Error of Coefficient	P Value Indep. Variable	Adjusted R^2	Regression Computed F Value	df	P Value of Regression
Survival, Arcsine Transformed					0.6385	24.3500	3, 10	<0.001
	Start of Weaning	-2.2653	0.3245	<0.001				
	Initial Density	-2.2521	0.5611	<0.001				
	Constant	72.5880	7.5203	<0.001				
Number of Juveniles Produced					0.8478	61.2535	2, 11	<0.001
	Start of Weaning	-1615.3250	172.9849	<0.001				
	Constant	37707.1200	2759.1760	<0.001				

Figure 6.1: Scatter plot of calculated growth coefficients vs. final fish density (fish L^{-1}) and the linear fit of their relationship. Data point labels denote Experiment.

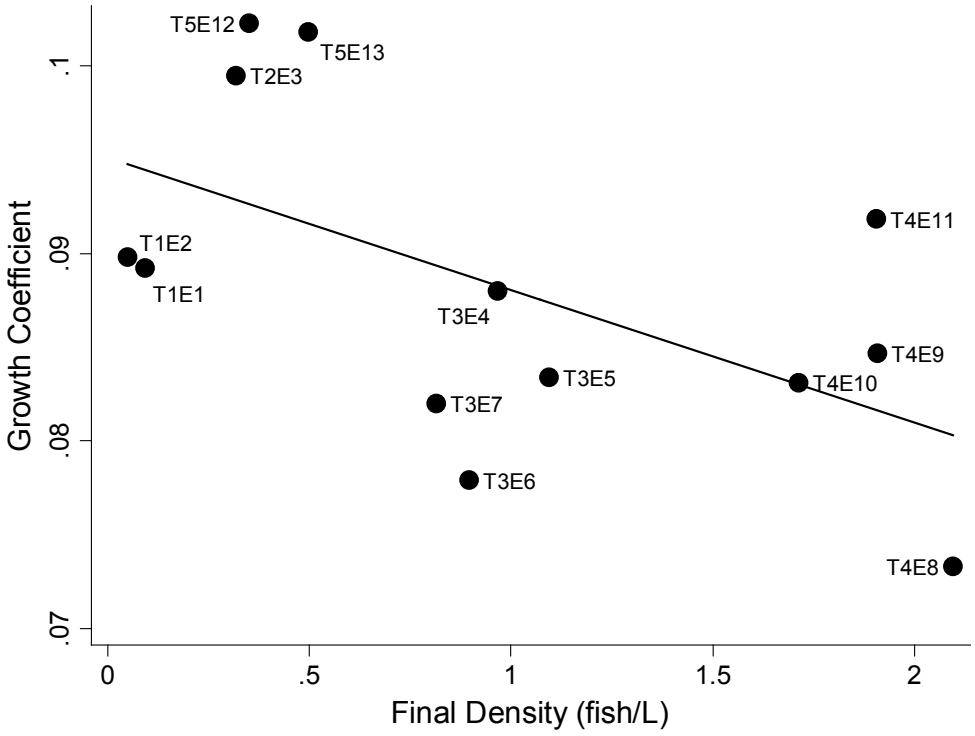


Figure 6.2: Scatter plot of calculated growth coefficients residuals (after modeling with final density as the first entry independent variable) vs. start of weaning period (DPH) and the linear fit of their relationship. Data point labels denote Experiment.

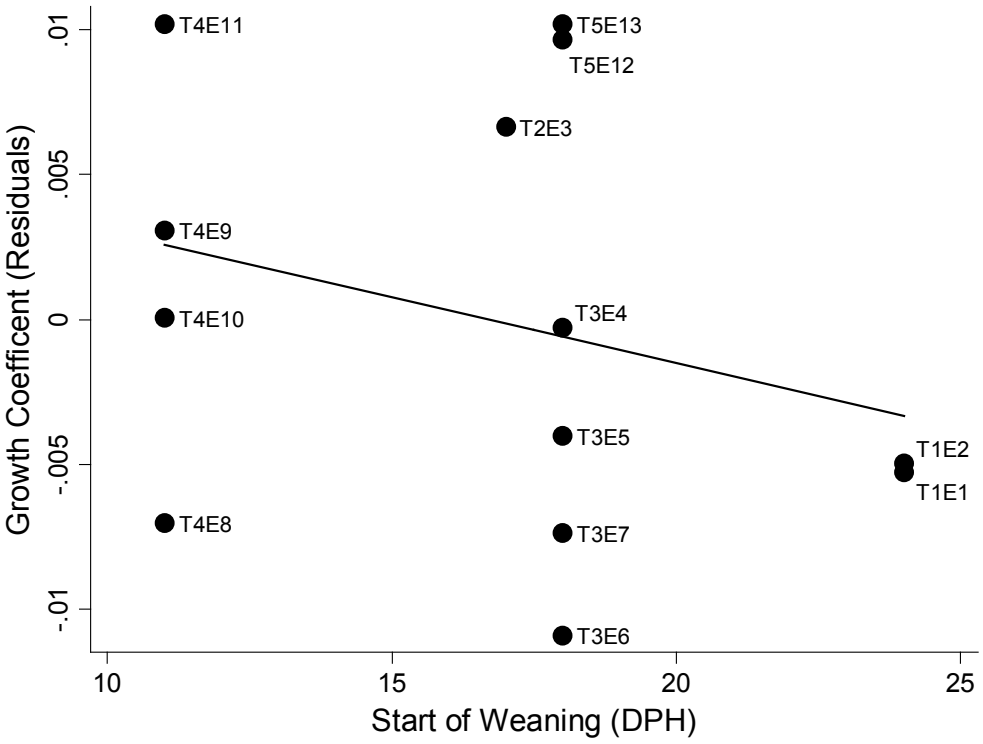


Figure 6.3: Scatter plot of calculated growth constants vs. high temperature (°C) and the linear fit of their relationship. Data point labels denote Experiment.

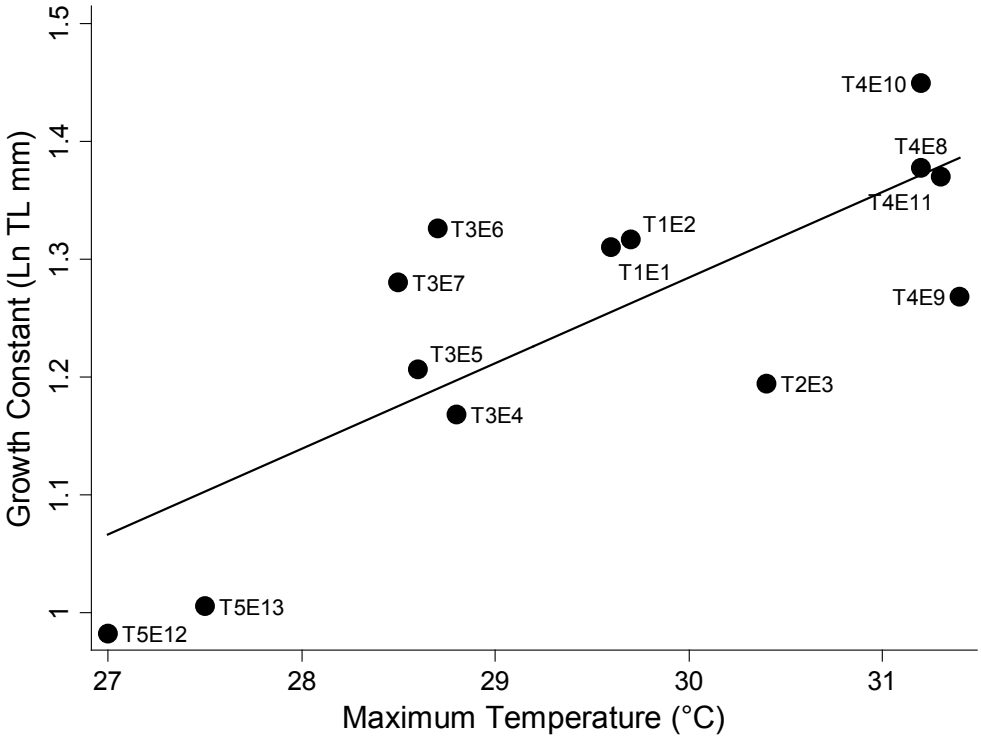


Figure 6.4: Scatter plot of calculated $D°_{eff}$ growth coefficients vs. low temperature (°C) and the linear fit of their relationship. Data point labels denote Experiment.

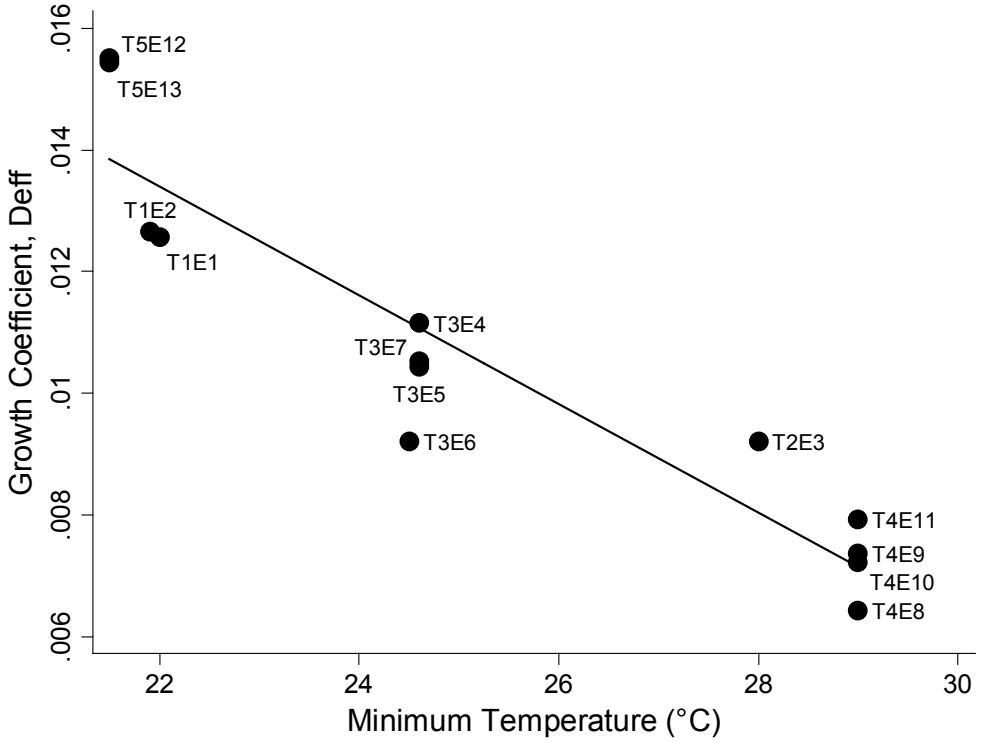


Figure 6.5: Scatter plot of observed survival (arcsine transformed) vs. start of weaning period (DPH) and the linear fit of their relationship. Data point labels denote Experiment.

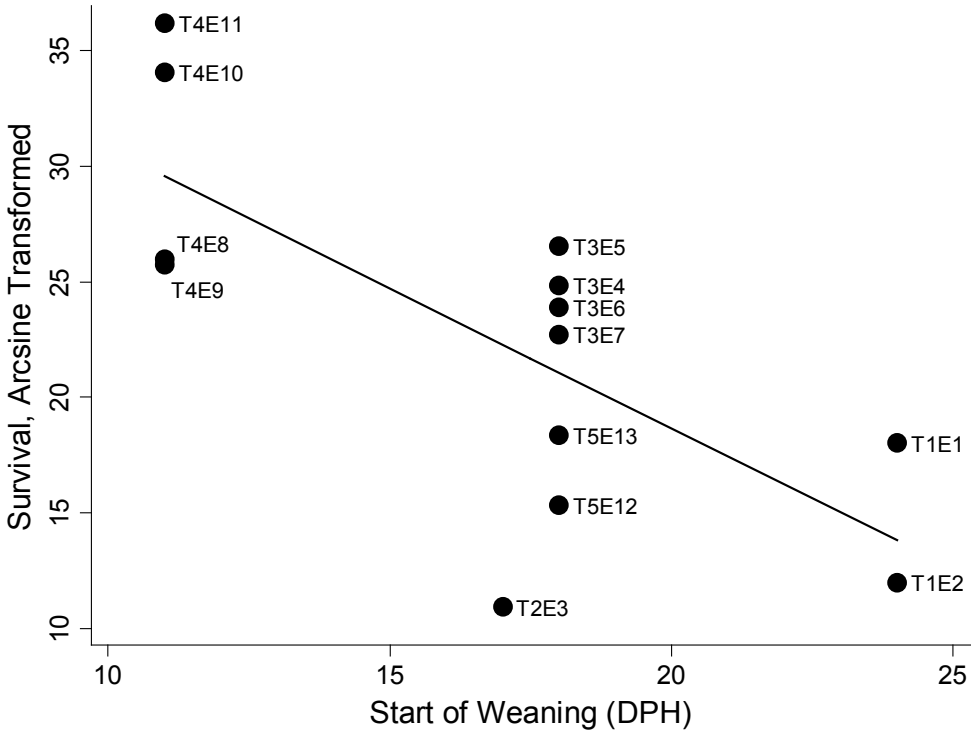


Figure 6.6: Scatter plot of arcsine transformed percent survival (after modeling with start of weaning as the first entry independent variable) vs. initial density (Larvae L^{-1}) and the linear fit of their relationship. Data point labels denote Experiment.

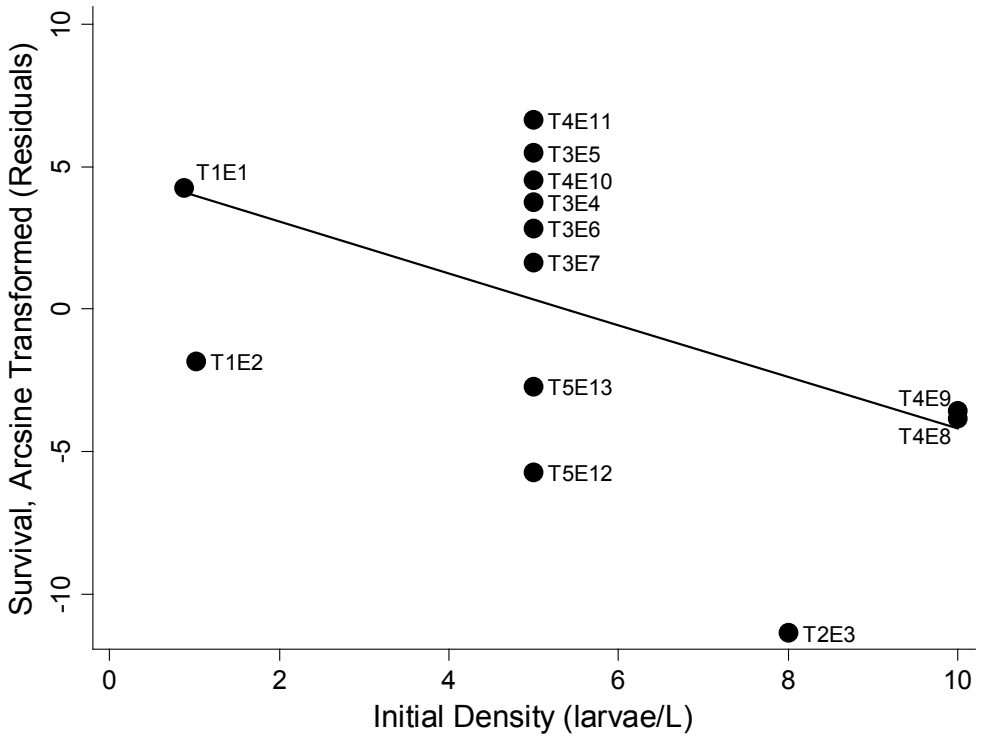


Figure 6.7: Scatter plot of observed number of juveniles produced vs. start of weaning (DPH) and the linear fit of their relationship. Data point labels denote Experiment.

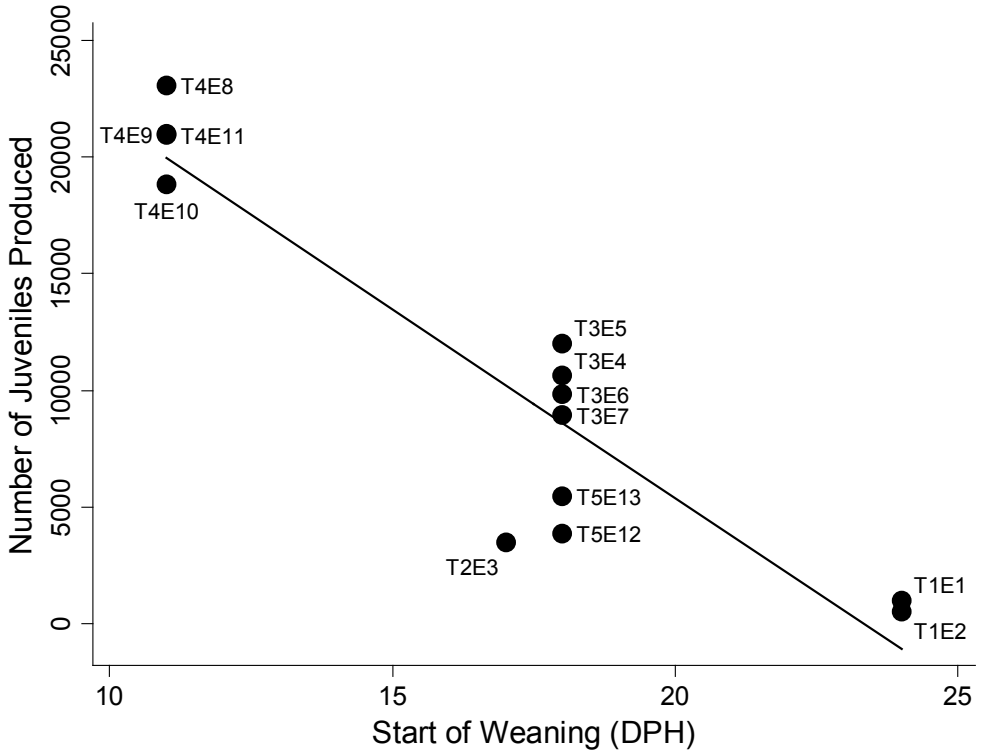


Table 6.20: Variables significantly correlated to timing of feeding regime.

Factor	Correlated Factor	Correlation Coefficient	df	P Value
Start of Rotifer Feeding	Median Temperature	-0.8685	12	0.0001
Start of Rotifer Feeding	Average Temperature	-0.8670	12	0.0001
Start of Rotifer Feeding	Start of Weaning Period	0.8710	12	0.0001
Start of Rotifer Feeding	Minimum Temperature	-0.8224	12	0.0006
Start of Rotifer Feeding	Maximum Temperature	-0.8119	12	0.0007
Start of Rotifer Feeding	End of *Artemia* Feeding	0.7179	12	0.0057
Start of Rotifer Feeding	End of Weaning Period	0.7179	12	0.0057
Start of Rotifer Feeding	Start of Green Water Utilization	0.6901	12	0.0188
Start of Rotifer Feeding	End of Green Water Utilization	0.6226	12	0.0408
Start of Rotifer Feeding	Total Rotifer Utilization	-0.5424	12	0.0848
Start of Rotifer Feeding	Probiotic Utilization	-0.6867	12	0.0095
Start of Rotifer Feeding	End of Rotifer Feeding	0.4813	12	0.0959
End of Rotifer Feeding	Minimum Temperature	-0.8915	12	0.0000
End of Rotifer Feeding	End of *Artemia* Feeding	0.8415	12	0.0003
End of Rotifer Feeding	End of Weaning Period	0.8415	12	0.0003
End of Rotifer Feeding	Average Temperature	-0.8384	12	0.0003
End of Rotifer Feeding	Start of *Artemia* Feeding	0.8286	12	0.0005
End of Rotifer Feeding	Median Temperature	-0.8324	12	0.0004
End of Rotifer Feeding	End of Rotifer Feeding	0.6665	12	0.0129
End of Rotifer Feeding	Maximum Temperature	-0.6564	12	0.0148
End of Rotifer Feeding	Antibiotic Utilization	-0.5955	12	0.0318
End of Rotifer Feeding	Rotifer Enrichment	-0.5497	12	0.0516
End of Rotifer Feeding	*Artemia* Enrichment	-0.5497	12	0.0516
End of Rotifer Feeding	Green Water Utilization	-0.5497	12	0.0516
Start of *Artemia* Feeding	Antibiotic Utilization	-0.8433	12	0.0003
Start of *Artemia* Feeding	Median Temperature	-0.7375	12	0.0040
Start of *Artemia* Feeding	Average Temperature	-0.7351	12	0.0042
Start of *Artemia* Feeding	Minimum Temperature	-0.6964	12	0.0082
Start of *Artemia* Feeding	Maximum Temperature	-0.6109	12	0.0266
Start of *Artemia* Feeding	End of *Artemia* Feeding	0.5720	12	0.0411
Start of *Artemia* Feeding	End of Weaning Period	0.5720	12	0.0411

Table 6.20, cont'd: Variables significantly correlated to timing of feeding regime.

Factor	Correlated Factor	Correlation Coefficient	df	P Value
End of *Artemia* Feeding	End of Weaning Period	1.0000	12	0.0000
End of *Artemia* Feeding	Minimum Temperature	-0.9045	12	0.0000
End of *Artemia* Feeding	Average Temperature	-0.8464	12	0.0003
End of *Artemia* Feeding	Median Temperature	-0.8377	12	0.0004
End of *Artemia* Feeding	Rotifer Enrichment	-0.8071	12	0.0009
End of *Artemia* Feeding	*Artemia* Enrichment	-0.8071	12	0.0009
End of *Artemia* Feeding	Start of Weaning Period	0.9449	12	0.0000
End of *Artemia* Feeding	Total Rotifer Use	-0.5983	12	0.0518
End of *Artemia* Feeding	Formalin Utilization	-0.5720	12	0.0411
End of *Artemia* Feeding	Copper Utilization	0.5720	12	0.0411
End of *Artemia* Feeding	Maximum Temperature	-0.5586	12	0.0472
End of *Artemia* Feeding	Total Rotifer Utilization	-0.5375	12	0.0582
End of *Artemia* Feeding	Probiotic Utilization	-0.4902	12	0.0890
Start of Weaning Period	End of Weaning Period	0.9449	12	0.0000
Start of Weaning Period	Minimum Temperature	-0.8655	12	0.0001
Start of Weaning Period	Average Temperature	-0.8504	12	0.0002
Start of Weaning Period	Median Temperature	-0.8499	12	0.0002
Start of Weaning Period	Total Rotifer Utilization	-0.7519	12	0.0030
Start of Weaning Period	Rotifer Enrichment	-0.7152	12	0.0060
Start of Weaning Period	*Artemia* Enrichment	-0.7152	12	0.0060
Start of Weaning Period	Probiotic Utilization	-0.6365	12	0.0193
Start of Weaning Period	Formalin Utilization	-0.6254	12	0.0223
Start of Weaning Period	Copper Utilization	0.6254	12	0.0233
Start of Weaning Period	Maximum Temperature	-0.5951	12	0.0319
Start of Weaning Period	Initial Stocking Density	-0.5820	12	0.0369
Start of Weaning Period	Average DO	-0.5762	12	0.0393
Start of Weaning Period	Average pH	0.7621	12	0.0464
End of Weaning Period	Minimum Temperature	-0.9045	12	0.0000
End of Weaning Period	Average Temperature	-0.8480	12	0.0002
End of Weaning Period	Median Temperature	-0.8439	12	0.0003
End of Weaning Period	Formalin Utilization	-0.5720	12	0.0411
End of Weaning Period	Copper Utilization	0.5720	12	0.0411
End of Weaning Period	Maximum Temperature	-0.5586	12	0.0472
End of Weaning Period	Total Rotifer Utilization	-0.5375	12	0.0582
End of Weaning Period	Probiotic Utilization	-0.4902	12	0.0890

Table 6.21: Results of regression model fitting of changes in feeding regime (DPH) within individual Trials.

Independent Variable	Adjusted R^2	F Value	df	P Value
Trial 1	0.922	48.543	1, 4	0.006
Trial 2	0.852	24.038	1, 4	0.016
Trial 3	0.924	49.705	1, 4	0.006
Trial 4	0.871	27.907	1, 4	0.013
Trial 5	0.939	62.791	1, 4	0.004

Figure 6.8: Plot of computed regressions modeling changes in feeding regime schedule for each Trial. Only one pairwise comparison of regressions was found to be significantly different: Trial 1 and Trial 4 ($F_{1,3} = 5.923$, $P = 0.020$). Letters denote groupings of significantly similar regressions.

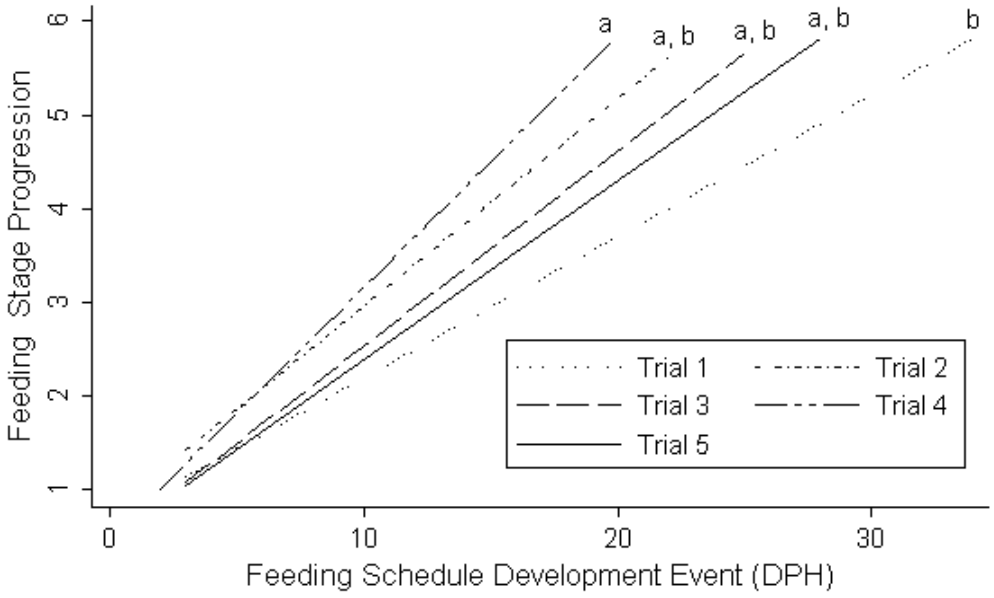
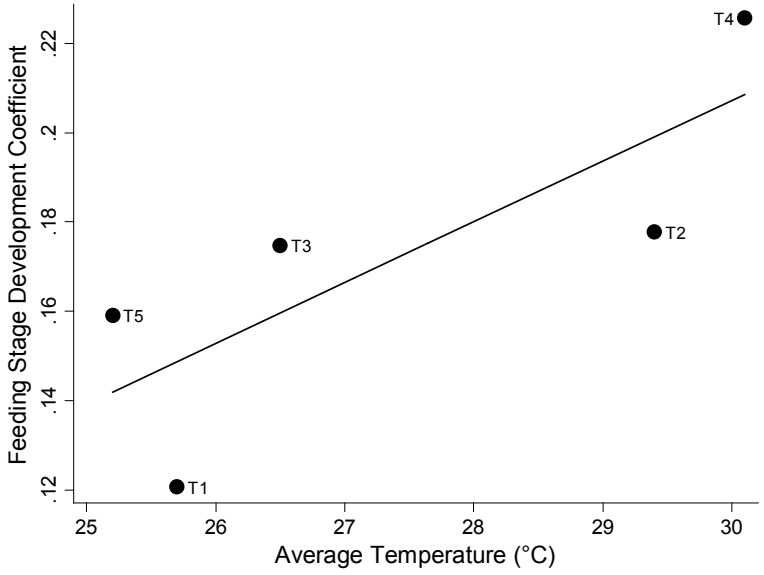


Table 6.22: Results of regression analysis of feeding stage development coefficient and factors found to influence growth coefficients computed from individual Experiments.

Independent Variable	Adjusted R^2	F Value	df	P Value
Average Temperature	0.523	5.380	1, 4	0.103
Final Density	0.744	12.604	1, 4	0.038

Figure 6.9: Scatter plots and fitted regressions from analysis of feeding stage development coefficient and a) average temperature (°C) ($R^2 = 0.523$, P = 0.103) and b) final juvenile density (fish/L) ($R^2 = 0.744$, P = 0.038). Data points represent averages within Trials and data point labels denoted Trial Number.

a)



b)

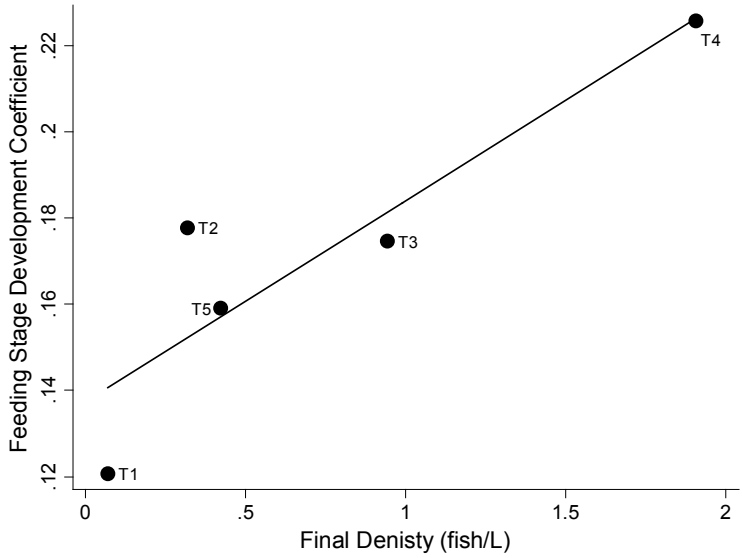


Table 6.23: Comparison of growth data from this study and that available in the literature, including various factors affecting growth (Sources: 1) Holt et l. 2007, 2) Faulk et al. 2007a, 3) Faulk et al. 2007b, 4) Salze et al. 2008, 5) Weirich et al. 2004, 6) Liao et al. 2004 7) Sardanberg et al. 2007 8) Hitzfelder et al. 2006, 9) Benetti et al. 2008b).

Culture System	Avg. Temp. (°C)	Tank Size (m^3)	Initial Density (larvae/L)	Survival (%)	Final Density (fish/L)	Final Measurement (DPH)	Avg. Final size (cm)	Growth Coefficient	Source
Int. Recirc. Tank	25.5	1	4 eggs/L	N/A	N/A	30	4.5	0.1081	1
Int. Recirc. Tank	28.5	1.1	N/A	N/A	N/A	32	6.7	N/A	1
Int. Recirc. Tank	27.4	1.34	8.7	13.2 (29 DPH)	0.9	22	1.47	0.063	2
Int. Recirc. Tank	27.5	1.34	14.7	10.4 (43 DPH)	1.2	22	1.46	0.064	2
Int. Recirc. Tank	25.9	0.15	6.7 eggs/L	N/A	N/A	22	2.01	0.08	3
Int. Recirc. Tank	28.5	0.3	10	24.4 (28 DPH)	2.5	14	~2.0	N/A	4
Ext. Pond	28.9	3750	0.05	5.3 (~35 DPH)	N/A	~35	12.93	N/A	5
Ext. Pond	22 – 27	5000	N/A	5- 10 (45 DPH)	N/A	45	8 – 10	N/A	6
Ext. Pond	24-30	1000	0.5	3 (28 DPH)	N/A	22	4.48	N/A	7
Int. Recirc. Tank	27.4	0.4	0.5	13	0.1	21	2.48	N/A	8
Int. Recirc. Tank	27.4	0.4	1.1	12.7	0.1	21	2.33	N/A	8
Int. Recirc. Tank	27.4	0.4	2	8.8	0.2	21	2.08	N/A	8
Int. Recirc. Tank	27.4	0.4	1.4	14.4	0.2	21	1.8	N/A	8
Int. Recirc. Tank	27.4	0.4	5.9	12.7	0.7	21	1.29	N/A	8
Int. Recirc. Tank	27.4	0.4	11.6	6.9	0.8	21	1.41	N/A	8
Int. Recirc. Tank	27.4	0.4	12	9.4	1.1	21	1.41	N/A	8
Int. Recirc. Tank	27.4	0.4	23.1	3.7	0.9	21	1.3	N/A	8
Int. Recirc. Tank	27.4	0.4	34.3	1.9	0.6	21	1.37	N/A	8
Int. Flow Through Tank	25.7	12	0.875	9.62	0.09	25	3.06	0.0892	T1E1
Int. Flow Through Tank	25.7	12	1.025	4.25	0.05	25	3.22	0.0898	T1E2
Int. Flow Through Tank	29.4	12	8	3.6	0.32	14	1.34	0.0995	T2E3
Int. Flow Through Tank	27.2	12	5	17.7	0.97	22	2.36	0.088	9, T3E4

Table 6.23, cont'd: Comparison of growth data from this study and that available in the literature, including various factors affecting growth (Sources: 1) Holt et l. 2007, 2) a2007b, 3) Faulk et al. 2007b, 4) Salze et al. 2008, 5) Weirich et al. 2004, 6) Liao et al. 2004 7) Sardanberg et al. 2007 8) Hitzfelder et al. 2006, 9) Benetti et al. 2008b).

Culture System	Avg. Temp. (°C)	Tank Size (m^3)	Initial Density (larvae/L)	Survival (%)	Final Density (fish/L)	Final Measurement (DPH)	Avg. Final size (cm)	Growth Coefficient	Source
Int. Flow Through Tank	27.2	12	5	20.0	1.09	22	2.22	0.0834	9, T3E5
Int. Flow Through Tank	27.0	12	5	16.4	0.90	22	1.99	0.0779	9, T3E6
Int. Flow Through Tank	27.0	12	5	14.9	0.82	22	2.23	0.082	9, T3E7
Int. Flow Through Tank	30.0	12	10	19.2	2.10	21	1.89	0.0733	9, T4E8
Int. Flow Through Tank	30.1	12	10	17.5	1.91	21	2.19	0.0847	9, T4Ex9
Int. Flow Through Tank	30.1	12	5	31.4	1.71	21	2.47	0.0831	9, T4E10
Int. Flow Through Tank	30.2	12	5	34.9	1.91	21	2.75	0.09185	9, T4E11
Int. Flow Through Tank	25.2	12	5	6.98	0.35	24	3.34	0.1023	T5E12
Int. Flow Through Tank	25.2	12	5	9.92	0.50	24	3.34	0.1018	T5E13

Table 6.24: Results of investigation of sample size requirements to confidently detect differing percent differences between means of TL (mm) of cobia larvae at differing ages via ANOVA testing.

	% Difference Between Higher Mean and Lower Mean		
	5 % Difference	10 % Difference	Actual % Difference
T4E10 vs. T4E9; 15 DPH (Actual difference = 19.2%)			
Actual Mean (mm)	15.72	15.72	15.72
Actual Standard Deviation	1.82	1.82	1.82
Detection Difference (mm)	0.786	1.57	3.03
Number of ANOVA Groups	4	4	4
Probability of Detection	0.8	0.8	0.8
Needed Sample Size	30.13	8.06	3.09
T4E10 vs.T4E9; 21 DPH (Actual difference = 11.4%)			
Actual Mean (mm)	24.72	24.72	24.72
Actual Standard Deviation	5.60	5.60	5.60
Detection Difference (mm)	1.24	2.47	2.81
Number of ANOVA Groups	4	4	4
Probability of Detection	0.8	0.8	0.8
Needed Sample Size	113.53	28.86	22.48

6.4) Conclusions

Rigorous adherence to the scientific methodology is the basis of any investigatory endeavor. Understanding and application of the basic assumptions of statistical inference is crucial to confidently obtaining results from statistical analysis. Random sampling is a critical tenant of statistical inference. Not only should each potential measurement have an equal and independent chance of being selected as a member of the sample, but the selection of an individual measurement should also not influence the selection of any other (Zar 2009). Therefore, sample sizes should not be curtailed in conjunction with directed sampling with intentions of drawing a small yet representative sample of the underlying population distribution. The underlying population distribution should be estimated utilizing unbiased sampling with large enough sample sizes to be representative of the variation at the population level.

Investigation of proper sample size requirements in order to confidently detect differences in mean size by ANOVA are beneficial to improving experimental design and can assist with reduction of committing a Type II error. Evaluation of samples sizes required for ANOVA testing revealed a necessary increase in sampling intensity as cobia larvae increase in age and size variability (Table 6.24). Within individual Experiments, significant differences in variance of TL between consecutive sampling events were often detected (data not presented). This was not unexpected and supports the well known trend of depensation of growth, or 'size hierarchies,' in captive reared cohorts of marine fish larvae (Blaxter 1976, as cited in Blaxter 1988).

In relation to statistical analysis of mean sizes of larval 'cohorts,' depensatory growth complicates sampling regimes by necessitating increased sampling as larval age

increases. This realization can complicate other experimental outcomes, such as survival, when working with fragile species that do not survive the sampling process (i.e. sampling without replacement). One could minimize influences on later survival by adjusting sample size as larval cobia increase with age, although this would complicate statistical analysis. In order to simplify the calculation of regressions and later comparisons, sample sizes should remain constant throughout a rearing (Sokal and Rohlf 1995). Although this is less of a problem due to advances in computing power, simplistic and experimental design still dictates this value should be upheld. More importantly, in order to maintain equal 'weight' in regression computations, data should be as equally distributed across the range of *X* values as possible. In other words, sample sizes should remain consistent in order to maintain equal weight throughout the range of the regression.

The present study demonstrates a substantial advantage to experimenting with large numbers of individuals within a single sampling unit. Larvicultures employing large volume tanks allow for repeated sampling, without replacement, at the larger sample sizes required to confidently detect differences between treatments without substantially affecting other variables, such as final density or survival. For instance, in the present example of sample sizes required to compare mean sizes between T4E9 and T4E10 (Table 5.24), ideal sampling of 30 larvae per sampling event with 4 sampling events (size at stocking, 7 DPH, 14 DPH, 21 DPH) during the rearing would yield a total of 120 larvae sampled without replacement (Table 6.24). This total represents a miniscule reduction 0.64% of the 18,836 total juveniles produced from the single larviculture tank, assuming that all sampled larvae would survive to the termination of the

experiment. This assumption is highly unlikely as other mortality processes would act similarly on sampled larvae and the main cohort of larvae. Utilizing this line of reasoning, had the sacrificed research subject larvae not been sampled, production would have increased by 37 juveniles and percent survival by 0.2%. In other scenarios, where lower levels of survival are realized, this necessary levels of sampling could exert greater influence on other production outcomes, such as final density and survival. In a worst-case scenario from data presented in this study, sampling of 120 larvae from the lowest number produced from an experiment would yield a 22.6% loss in final number of juveniles produced.

Research is conducted in a real-world where accidents can happen and thus problems and complications must at times be overcome by the creative use of all data available to a researcher so long as application is justified. The mis-location of water quality and daily notes from the beginning of rearing T3 necessitated verification of the acceptability of replacement with temperature data recorded from a different UMEH hatchery system. Comparison of variance distribution and means between ambient water addition to broodstock RAS at UMEH and indoor larval rearing tanks from consecutive periods were found not significantly differ and thus providing satisfactory justification for substitution. This outcome was anticipated, as the source water for both systems is the one in the same.

Whenever computation or measurement of numbers is utilized, it is important to reflect upon the plausibility of the obtained result. In the present case, growth regressions resulted in a broad range of intercepts (2.53 to 5.28 mm when back-transformed) which include implausible size-at-hatching values for larval cobia. Later growth can be

influenced by such factors, such as differing temperature regimes experienced during embryonic development, which result in differing size-at-hatching. Inclusion of as much information as possible in attempts to so model growth would provide better models which more thoroughly describe the phenomena under investigation. Further factors causing variability, such as temperature affects of broodstock temperature regimes, would be more difficult to consider during routine growth investigations, but size-at-hatching information could easily be incorporated. It is well known that size-at-hatching varies with incubation temperature in generally a linear fashion (Chambers 1997). Incubation temperature regimes have resulted in significantly differing size-at-hatching in cobia (Zink Unpub. Data).

Jordaan et al. (2006), studying temperature influences on the stage at hatch of reared Atlantic cod *Gadus morhua* larvae, found that developmental stage at hatch was different when eggs were incubated under different temperatures. Furthermore, though much of the initial size difference variability was soon reduced, lasting effects were detected from initial size and developmental state differences (Jordaan et al. 2006). The authors conclude that developmental stage of larvae at hatching should be reported when later comparisons of length and morphology are to be employed (Jordaan et al. 2006). A similar study noted lasting effects of differing developmental state, even though growth in length resulting from different egg incubation temperatures was soon minimized, between groups of haddock *Melanogrammus aeglefinus* when further reared at the same temperature (Martell et al. 2005).

Unfortunately, sampling of size-at-hatching was not conducted in the present study. Investigation of regression results when an average size-at-hatching (3.67 mm

TL) variable was included within the individual regressions resulted in multiple satisfactory results. Its inclusion was found to not significantly change instantaneous growth rates nor regression y-intercepts (Table 6.3). However, a one-tailed F-test revealed a significant reduction of regression y-intercept variance (P = 0.0495) (Table 6.4). Effectively, though regressions themselves were not significantly altered, growth constant variation was significantly reduced and the resulting range of back transformed values (2.67 to 4.26 mm TL) were more biologically meaningful. Although the resulting range of growth constants (y-intercepts) can still be considered outside of the range of actual values (3.5 mm, Kilduff et al. 2002; 4.08 mm Zink Unpub. Data), an obvious improvement was achieved. If growth models yield y-intercepts that are biologically implausible, then the resultant regressions do not accurately the phenomena under investigation, i.e. growth over the entire larval period including the initial starting point of size-at-hatching.

Previously discussed issues concerning uneven sample sizes for sampling events throughout a regression can also be applied in this situation. In T5E12 and T5E13, faster growth was achieved towards the end of the Trial which most likely resulted from high mortality without disease and thus a release of density dependent growth. This resulted in much higher values which can be considered to effectively 'pull' regression slopes towards higher positive values, which results in a concurrent decrease in fitting of temporally earlier values in the regression. This effectively results in lowering of the regression intercept value. The resulting y-intercepts (back transformed: 2.67 and 2.73 mm TL for T5E12 and T5E13, respectively) were the most illogical and least biologically relevant y-intercepts computed, falling well outside the range of expected values. Again,

perhaps having an equal sample-sized sampling event for size-at-hatching would have resulted in regressions which remained more biologically relevant.

A number of factors can influence larval finfish growth. Ostensively, growth would vary if those influencing factors varied during the course of an experiment or larviculture. Inclusion of size-at-hatching data point effectively increased numbers of 'growth periods,' or number of sampling events, within individual Experiments. By increasing the number of growth periods within an Experiment, variation in growth rate within individual Experiments could be investigated. Thus, inclusion of the average size-at-hatching data point was especially useful in situations where growth data was limited to only two data sampling events. Investigation of growth regression coefficients modeled for these individual periods within an Experiment revealed significant differences (Table 5.7). Changing growth rates between growth sampling periods could have been related to timing of data sampling and metamorphic and developmental ontogenetic changes in energy allocation from somatic growth. These events are recognized to represent rapid changes in structure and function and temporary reductions in somatic growth disbursed between relatively prolonged intervals of predominantly somatic growth (Balon 1981, Blaxter 1988).

Although slight variations in larval finfish growth can be anticipated when investigating relatively short time periods (i.e. day to day), especially when these investigations are carried out during ontogenic developmental events such as the switch from endogenous to exogenous feeding or from cutaneous to gill respiration (Kamler 1992, Chambers 1997), variations in growth over the relatively longer time periods, such as those separating sampling events in the current study, were suspected to be influenced

by other factors. Varying density and temperature affects were suspected to most likely cause the changes in growth rates following well recognized trends in the literature (Blaxter 1988) and previous studies of cobia larviculture (Hitzfelder et al. 2006). Investigations of changes in mean temperatures within each growth period within Experiments revealed significant differences (Table 6.8). However, the pattern significantly differing temperatures did not coincide with those Experiments where significant changes in growth were detected. Further investigation of variation density affects within a larviculture tank during the course of a rearing could clarify these issues and investigation of these influences may yield interesting results.

Despite these unsatisfying results, concerns over temperature differences were not ignored altogether. Although temperature variation between Trials was one influential factor intended to be investigated during further analysis of cobia larval growth in production systems, temperature standardization could also be useful in highlighting other influential factors. Neuheimer and Taggart (2007) promoted greater utilization of the Degree-Day in growth analyses, concluding that its utilization can eliminate temperature affects for comparisons of growth between different constant temperatures but also when temperature varies with time. Kamler (1992) reviewed historical attempts and theoretical formulations of growth equations in larval finfish, concluding that the $D°_{eff}$ is a better metric with which to standardize growth across varying species and temperatures.

Degree-Day was originally thought to be independent of temperature; however, multiple cases of decreases in Degree-Day with increasing temperature have been observed (Kamler 1992). This phenomena has been more recently verified by study of

development, development, and growth rate calculations conducted by Weltzien et al. (1999). Furthermore, warm-water species Degree-Day estimates are acknowledged to be more dependent upon temperature than for cold-water species (Kamler 1992). Thus, further investigation into the standardization of temperature influences on growth resulted in computation of $D°_{eff}$ and its incorporation into growth regression coefficients testing.

A review of the literature as well as data collected at the UMEH provided time-to-hatch values utilized to calculate $D°_{eff}$ following the methods outlined in Weltzien et al. (1999) and Kamler (1992). Time-to-hatch was found to range from 39.5 hrs at 21 °C (Zink Unpub. Data) to 21 hrs at 31 °C (Liao et al. 2004). Attempts to regress the calculated development rates resulted in detection of non-normal distribution of development rates; after application of a Box-Cox transformation, satisfactory results were realized and the resulting of t_{eff} value of 18.6 °C was calculated. This value seemed more reasonable than the value obtained without Box-Cox transformation (8.6 °C). However, oftentimes calculated t_{eff} values are lower, by a few degrees, than lethal low temperatures (Kamler 1992).

Kamler (1992) discusses that t_{eff} should approximately coincide with lethal mortality inducing low temperature. Observations of suppressed feeding behavior and mortality during T5 during a period of the larviculture when rearing temperatures dropped to ~21.5 °C. Similarly, high mortalities were also observed during T1 when low temperatures reached 21.9 °C, although these high mortalities events were complicated by reduced water quality and disease experienced associated with the passing of Hurricane Wilma. Conversely, Milstein and Thomas (1976) report collection of juvenile cobia in temperatures of 16.8 °C off the New Jersey coast, calling then uncommon to the

region. Furthermore, experimental lethal low temperatures of 12.1 °C have been reported (Atwood et al. 2004) and cessation of juvenile feeding behavior is reported to occur at 18.3 °C (Hassler and Rainville 1975). Perhaps further investigation of utilizing this metric would incorporate a robust regression, rather than a Box-Cox transformation, which results in the more plausible t_{eff} value of 9.82 °C given the knowledge of juvenile lethal low temperatures.

The above cited discrepancies between tolerable temperatures could be due to differing tolerances between larval and juvenile life stages of cobia. No readily available study currently exists which investigates the true lethal low temperature for cobia larvae. Variability in calculated development rates yielded a regression coefficient of determination of 0.770, substantially lower than the multiple coefficient of determination values for varying species reported in Kamler (1992) and the value reported by Weltzien et al. (1999). Perhaps the variability present in development rate (time-to-hatching) was influenced by lack of careful measure and reporting of time-to-hatch with 50% hatch ratio being the theoretical values that should be utilized in these calculations. In practice, reported values for time-to-hatch did not indicate whether the time was to 50% hatch nor was this procedure followed with sampling of time-to-hatch for data obtained at the UMEH; thus, utilized values were most likely more ambiguous than those utilized in the previously mentioned investigations of t_{eff} and development rate variation with temperature.

Before application of the $D°_{eff}$ to growth regressions for individual Experiments, properties of utilization of this standard's ability to linearize growth functions between growth sampling events was investigated. If $D°_{eff}$ could produce statistically similar

regression slopes between growth periods then its further utilization would be better justified. Comparison of slopes of growth periods utilizing $D°_{eff}$ values computed by the average temperature of each growth period where found to not statistically differ in all cases exempt for T1E1 and T1E2. Computed slopes for growth periods in these two Experiments actually resulted in one case each where growth was negative according to $D°_{eff}$ formulation (Table 6.9). Although true negative growth was not observed in these Experiments, it appears theoretically due to rapid decrease in temperature, which caused the growth sampling event which ends period 3 to relatively occur before the sampling event marking the beginning period 3 according to $D°_{eff}$ formulation of growth. Due to changes in patterns of significance between the standard method of regressing growth in length against age and regressing growth in length against $D°_{eff}$, it can be deduced that temperature influences caused some of the changes in growth during the course of a larviculture. After application of $D°_{eff}$, it was easier to investigate other factors which could have affected growth during the course of each larviculture.

As previously stated, Neuheimer and Taggart (2007) advocated the application of standardization of growth relative to temperature changes when temperature varies with time. In the present study, application of the $D°_{eff}$ formulation to growth regressions between growth periods resulted in a unsatisfactory results for Experiments T1E1 and T1E2 (Table 6.9). Due to these complications, and to better assess average temperature influences between Experiments, an average temperature over the course of an Experiment was utilized for $D°_{eff}$ computations within Experiments. Linear regressions were then fit for each Experiment utilizing both age-based and $D°_{eff}$ as dependent variables. The resulting growth coefficients were then compared pairwise within

computational method in order to detect significantly differing growth between Experiments. Fewer significant differences were found between age-based regressions (Table 6.10) than $D°_{eff}$ based regressions (Table 6.11). In regards to age-based regressions, significantly differing slopes were detected between Experiments T5E12 or T5E13 and other individual Experiments (Table 6.9).

Significantly differing pairwise comparisons of slopes computed utilizing $D°_{eff}$ modified ages always occurred between Experiments not within the same Trial. Significant differences of pairings of Experiments within Trials were at anticipated in some instances. With the sample data measured during Trial 4, which was intended to investigate the differences between initial stocking densities of 5 and 10 larvae L^{-1}, Benetti et al. (2008b) detected significant differences in distributions of mean lengths between these treatments although in the present study no significant difference in growth rates was detected. This suggests that focusing on growth rates alone in order to detect treatment differences may not provide adequate information as to impacts of treatments.

Substantially greater numbers of significant differences were detected in pairwise comparisons of slopes of growth regressions utilizing $D°_{eff}$ than those computed utilizing standard procedures (i.e. age as the independent variable in regression). This was most likely due to inflation of X_i values with utilization of $D°_{eff}$; this in turn inflates the sum of squares of X_i. Substantially greater increases in sum of squares of X_i relative to inflation of the sum of the cross product (Σxy) rapidly decreased the absolute value of computed regression coefficients, despite inflation of the sum of the cross product. Comparison of growth coefficients from the same Experiment but computed by these two methods reveals this decrease in absolute value. Similarly, when computing the test statistic for

comparison of multiple slopes, the inflation of the sum of squares of X_i for each regression substantially deflates the standard error of the difference between regression coefficients, which are located in the denominator of the test statistic. However, trends in differences in the computation of individual regression coefficients utilized in computation of the numerator of this test statistic can complicate these changes. Comparison of changes in the $F_{computed}$ values of instances where significant differences were found between the same Experiments under both computations reveals this pattern (Table 6.10 and 6.11).

The inflation of the sum of squares of X_i does not change the values of the total sum of squares ($\Sigma(Y_i - Y_{mean})^2$) nor the regression sum of squares ($\Sigma(\hat{Y}_i - Y_{mean})^2$), and thus not the residual sum of squares. Therefore, regression fit parameters such as the coefficient of determination, the regression $F_{computed}$, and the resulting significance value are not affected by the inflations of X_i values. For this reason, the later values can summarize regression fit characteristics for both standard (age) regressions and $D°_{eff}$ (Table 6.6). Not only does $D°_{eff}$ effectively eliminate differences in growth due to differences in temperature, it also increases the probability of detecting significant differences between regressions. In the present study, this property was useful in detecting differences between Experiments from differing Trials but did not detect significant differences between Experiments within Trials.

An understanding of the events occurring during the course of each larviculture and how they would be expected to influence growth can provide improved understanding of these conclusions. For example, during T2E3, bacterial disease was suspected of causing major mortalities, which would have thus produced a release in

terms of density dependent growth once the direct reductions in growth due to the diseased state had passed, was coupled with increases in temperature during the later period of the Trial. Despite significantly different slopes between under standard growth regression methods (Table 6.7), the slope differences are still positive yet not significantly so when the regressions are carried out utilizing $D°_{eff}$ (Table 6.9). The case of Experiment T3E7 demonstrates a non-significant increase in growth under standard regression procedures, but this trend switches to a non-significant decrease in growth rate under $D°_{eff}$ formulation. In general, with utilization of $D°_{eff}$ regression formulations for individual growth periods, a majority of Experiments demonstrated trends for reduction in growth rate as the larviculture progresses (Table 6.9). Thus, the assumption that density dependent growth has been influencing these later stages of growth is given merit.

Another explanation for this could be achievement of ontogenic developmental stages where growth is reduced. It is well known that larval finfish growth slows once metamorphosis to the juvenile life stage has been achieved (Blaxter 1988, Kamler 1992, Chambers 1997). Further investigation of relationships of larviculture biomass and densities to changes in growth rates could provide evidence of whether density dependent or metamorphic changes are more important drivers of the reductions in growth rate.

Similarly, significant difference differences were detected between age-based growth coefficients of T1 and T5, with those Experiments in T5 resulting in higher growth coefficients (Table 6.13). This is despite similar low, average, and median temperatures between these Trials, and even slightly greater high temperatures observed during T1. Furthermore, final densities and production numbers were lower in T1, which

theoretically would have allowed for higher growth rates. The influences of other compounding factors most likely explain these seemingly contradictory results. Disease most likely further reduced growth during T1 while the order of changing temperature regimes shifted could have also influenced these outcomes. During T1, lower temperatures occurred during the later period of the larviculture, which would have further suppressed growth despite the release in density depression of growth resulting from disease related mortality. During T5, however, lower temperatures were observed during the early to middle period of the rearing and higher temperatures occurred towards the later period, allowing larval growth to take further advantage of the release of density-dependent growth caused by high mortality during low temperatures experienced in the middle period of the rearing.

Resultant age-based growth coefficients were within the range of values published within various sources within the literature (Table 6.22). The highest age-based growth rates computed in this study (0.1023 and 0.1018 for T5E12 and T5E13 respectively, Table 6.13) are comparable to the next highest growth rate reported for cobia larval growth under similar rearing conditions (0.1081, Holt et al. 2007b) (Table 6.23). While average temperatures between the two studies were similar, other factors, such as initial stocking densities, changing temperature regimes, and tank size, differed between the two studies. Average temperatures for T5E12 and T5E13 were both 25.2 °C and in the above mentioned Holt et al. (2007) study, average temperature was reported as 25.5 °C. Despite the relatively low temperature regimes of both studies, it is conceivable to achieve higher growth rates due to utilization of relatively low stocking densities. Currently, no study investigates the true maximal cobia larval growth rates possible in

tank culture initiated with densities low enough as to not induce density-dependent growth influences. Indeed, though growth rates were not reported, under extensive pond culture rearing conditions and average temperature of 28.9 °C, larval cobia have been observed to reach juvenile lengths of ~12.93 cm 35 DPH (Weirich et al. 2004). Juvenile lengths of 4.5 cm at 30 DPH and 3.34 cm at 24 DPH were observed from the previously discussed growth rates reported by Holt et al. (2007) and those of T5 in this study, which are much smaller than those reported by Weirich et al. 2004).

Computed regression coefficients, both age- and $D°_{eff}$-formulated, and constants were added to a table incorporating a multitude of factors that varied between Experiments in order to construct GLMs to investigate the most influential factors on production outcomes of concern for commercial aquaculture hatcheries (Tables 6.11, 6.12, 6.13, and 6.14). With so many factors included, it was anticipated that many would become ancillary to the investigation and not provide much explanatory power. This conclusion was further substantiated by the at times subjective nature of management decisions applied to the larvicultures. For example, the beginning of greenwater utilization was often associated with stocking of larviculture tanks, but its termination, though influenced by development of the larvae, was also influenced by short comings in on site production. Similarly, short comings in rotifer production at times limited stocking densities and total utilization. Furthermore, collinearity was suspected in a number of factors and was indeed later detected statistically (results not presented).

Co-variation of variables such as differing measures of temperature regimes must be kept in mind when conducting similar analyses. Correlations between production outcome variables and other factors were first investigated as preliminary steps to in

order to build GLM models. In order to build a broader understanding of related factors, correlation α levels were relaxed to $\alpha = 0.10$.

The variables final density, number of juveniles produced, and arcsine transformed survival were found to negatively correlate with age-based growth coefficients. These co-variates could be interpreted to be associated or influential in terms of density-dependent growth. Construction of a GLM model utilizing age-based growth coefficients as the dependent variable resulted in fitting of final density and start of weaning as independent variables; despite explaining only a moderate ~61% of the variability in growth coefficients, further testing did not detect other variables which furthered explained variation and pass partial F-tests validating entry into the model.

The finding that final density was the most influential variable and resulted a in negative coefficient was not surprising as density dependent growth is readily recognized as a major determining factor of growth (Blaxter 1988). In the literature, a plethora of studies exist which demonstrate reduced growth as stocking densities increase (Houde 1975, Houde 1977, Gomes et al. 2000). Final density, and density-dependency of growth, can be influenced directly by the effects of crowding upon individual larvae's behavior. For instance, Barton and Iwana (1991), in review of fish stress in relation to aquaculture, note how crowding can reduce growth by increasing individual's metabolic activity or increasing stress, which reduces oxygen availability for other growth processes. Indeed, stress related to crowding can reduce appetite and feeding rate, feed assimilation efficiency, and increase metabolic rate which reduces energy availability for somatic growth (Wendelaar Bonga 1997).

Hitzfelder et al. (2006) noted that at extremely high initial stocking densities, cobia larval feeding behavior was observed to be reduced and initiation of feeding strikes were repeatedly aborted due to initiation of startle response among individuals. Although the initial stocking densities of the current study do not approach these levels of 20 and 30 larvae L^{-1}, this sort of behavior has been observed in larvicultures initiated at densities of approximately 18 larvae L-1 (Zink Pers. Obs.). Unlike findings by Hitzfelder et al. (2006), initial stocking densities were not found to correlate with growth. This was most likely due to complications within the present analysis of high mortality events associated with disease, environmental influences, or both, which allowed for a 'release' of density dependent growth in certain larvicultures. In the Hitzfelder et al. (2006) study final density was not specifically investigated statistically, a dome shaped response between initial and final stocking densities can be envisioned utilizing the data provided in Table 1 of that study. In the current study, initial stocking densities only represent the first half of this dome (up to 10 larvae L^{-1} initial stocking density) but trace the same increase in final density as initial density increases within the range utilized.

Hitzfelder et al. (2006) further suggest that final densities are important for determining density effects on growth. The current results verify these conclusions. Final and initial stocking densities are positively correlated to each other ($r = 0.6575$, $df = 12$, $P = 0.0146$), suggesting a connection between initial and final stocking densities that was not apparent in other areas of the present analysis, but also highlights complications with association of growth with initial stocking densities due to confounding influences experienced during the course of a larviculture. Experimentation with European sea bass (*Dicentrarchus labrax*) initial stocking densities did not reveal differences in growth

during early larval stages even at relatively high stocking densities (200 larvae L^{-1}) (Hatziathansiou et al. 2002). Only with post-larval stages were differences in growth realized between differing initial stocking densities (Hatziathansiou et al. 2002), which lends greater credibility to more important influences of final density rather than initial density. In the current study, as well as the study conducted by Hitzfelder et al. (2006), production outcomes were considered once larvae had metamorphosed. Thus, influences between larval vs. post-larval stage cannot be differentiated in these studies.

Trial T5 exemplifies the complications between initial and final stocking densities and further factors occurring during a larviculture which can muddle the relationships previously discussed; despite reduced temperature regimes and similar initial stocking densities relative to other Trials such as T2, T3, T4E10, T4E11, the highest growth rates computed in this study were realized in T5. Temperature variables were anticipated to directly influence resulting growth coefficients, but no significant direct signature was detected in pairwise correlations with these factors nor was inclusion of temperature related variables during the course of GLM modeling warranted.

This finding of final density as the most important factor affecting instantaneous growth in length wields strong implications on the investigation of other factors contributing to growth. Other influences, such as enrichment regimes or chemotherapeutant utilization, are most likely masked by density affects, especially in this analysis of a larger dataset. Despite a doubling in initial stocking density from 5 to 10 yolk-sac larvae L^{-1} between Experiments T3E10 and T3E11 and Experiments T3E8 and T3E9, no significant differences were detected between the age-based growth rates between these Experiments. Variations in final density, along perhaps with previously

discussed issues regarding inadequate sample sizes, most likely influenced this lack of detectable difference. Indeed, Benetti et al. (2008b), which analyzed this same data subset, found significant differences in size distributions at the final sampling event although no significant difference was found in final numbers produced. Even within individual experiments investigating the effects of treatments other than density, lower stocking densities are most likely crucial in order to limit the effects of density limitation upon growth if it is anticipated to be influenced by the treatment applied.

Start of weaning was also found to significantly explain residual variance in age-based growth coefficients once final density was incorporated within GLM models. The resulting coefficient for this independent variable was negative. Holt et al. (2007) anticipated increased growth rates with earlier weaning onto commercial diets. The current finding supports this prediction. Benetti et al. (2008b) suggested that weaning onto compound feeds would be related to water temperature: as larvae grew faster and metamorphosed earlier they would be able to feed upon weaning diets earlier. Presumably, temperature influences were not detected in the present investigation of age-based growth rates most likely due to heavy influence of growth from density dependent growth. Understanding of cobia gastrointestinal development in light of this finding could shed further light upon the observation of start of weaning influences upon growth rates.

Timing of the ontogenetic development of the gastrointestinal digestive tract of cobia larvae, even at lower rearing temperatures, suggests that larval cobia can begin at least rudimentary digestion of compound feeds beginning from 8 DPH, with much more advanced and capable development achieved by 12 DPH (Faulk et al. 2007b). Caution is

suggested if trying to wean larvae onto formulated diets too early and before more complete digestive tract development has been achieved (Kolkovski 2001). Indeed, cobia larvae have been observed to accept formulated feeds as early as 10 DPH when reared under extensive conditions and high temperatures of 28.9 ± 1.0 °C, which could have acted synergistically to increase growth and development rates under these conditions (Weirich et al. 2004).

Indeed, diet type has been found to influence both growth and development rate in other species (Kamler et al. 1990). Furthermore, the matching of larger food items to increasing body size, mouth width, and mouth gape, along with the high metabolic requirements of cobia early life stages would maximize energy intake versus energy expended while feeding (foraging for live prey items) (Feeley et al. 2008, Benetti et al. 2008b). Weaning within these Trials was initiated with crumble diets ranging in size from 200 to 360 µm, which is smaller than average GSL-strain *Artemia* larvae (~460 µm in length) but soon advanced to include a mix of this size and larger crumble diet ranging from 360 to 620 µm. Thus, during weaning, 'prey' size could be considered as better matched to meet the growth requirements of rapidly developing cobia larvae.

Commercial diets can provide substantially greater amounts of protein necessary for the somatic growth which these instantaneous growth in length coefficients is modeling (Benetti et al. 2008b). Indeed, the loss of protein content during *Artemia* enrichment has been documented (Aragão et al. 2004), and live feeds enrichment manufacturers are investigating and formulating enrichment additives which boost protein levels and yield more complete nutritional requirements as needed by fish larvae. Protein content of *Artemia* Instar II nauplii enriched with lipid-based feeds was found to

average 36.7 ± 1.1 % (Aragão et al. 2004), while weaning diets utilized in this study are reported to contain 50.0% minimum protein (http://www.reed-mariculture.com/otohime /details.asp). If conclusions concerning egg and early larval protein, FAA, and lipid levels and ratios as indicative of nutritional requirements for later larval stages are correct (Faulk and Holt 2005), then the connection between the protein levels provided in the formulated feeds and ovarian protein levels (49-55%, Biesiot et al. 1994) and eggs (25.5% dry weight protein and 51.1% AA, Faulk and Holt 2005) are readily realized. Perhaps the relation of initiation of start of weaning earlier to increased growth relates better meeting of the nutritional needs of the rapidly development and somatic growth of cobia larvae.

Alternatively, although start of weaning obviously co-varies with age-based growth coefficients, this notion of covariance could be extended further leaving start of weaning as not a good theoretical predictor of growth coefficients. As growth in length increases, other advances in development of individuals occur in a relative manner; investigations of variability of different measures of growth and development find that size at which a development event occurs is much less variable than age (Fuiman et al. 1998). Development of gastrointestinal capabilities needed for digestion of formulated feeds are most likely influenced by other factors limiting growth in size, such as density or temperature influences. Though, once these events have occurred, the utilization of a more protein rich diet may then be able to contribute to faster growth in size. The age-based growth coefficients utilized in this GLM still contain elements of temperature influences of growth. Therefore, their values are relatively higher for those Experiments conducted at higher temperatures despite density dependent influences on growth. As

larval development, such as ability to wean onto formulated commercial feeds better linked to growth in length metrics than age, such developmental steps occur earlier at higher temperatures, despite high density impacting length in growth.

Investigation of the changes in metabolism over these temperature ranges would be interesting; feeding rates and total energy demand would most likely increase with increasing temperature, but metabolic demands may actually become more efficient with increasing temperature. For example, basal metabolism could become more efficient as protein turnover and repair become relatively less energy consuming. Although, other maintenance physiological processes, such as ion transport, may become more energy intensive. At higher temperatures active metabolism should actual decrease in relation to energy expended while swimming (Fuiman and Batty 1997). Furthermore, in terms of protein synthesis, higher growth rates are related to lower aerobic costs in vitro. In polkiotherms such as larval fish, studies indicate there in not a substantial increase in oxygen consumption with growth rate (Ruzicka and Gallager 2006). Protein synthesis is suspected to achieve a theoretical minimal cost as temperature increases (Pedersen 1997, Ruzicka and Gallager 2006), thus further increases in temperature can improve energy allocation to somatic growth.

Efforts to compare growth rates once temperature influences were effectively eliminated yielded substantially greater numbers of significant differences in Experiments' slopes compared between Trials, as previously discussed. Investigation of $D°_{eff}$ growth rate variability in relation to other factors and their correlation structure also provided very different results. Interestingly, $D°_{eff}$ formulated growth coefficients were most highly correlated with temperature variables. Even more interesting is the negative

nature of these correlations. GLM modeling of $D°_{eff}$ growth rates resulted in only minimal temperature being fit to the model; the resulting negative relationship resulted in explaining approximately 85% of the variability in $D°_{eff}$ growth rates. This was suspected to be due to influences of reduced rearing temperatures causing increased mortality, and thus allowing faster growth due to release from density dependence which had proven to be a strongly influential factor in explaining the variability in age-based growth coefficients.

The fact that maximal temperatures (~37 °C) that would be anticipated to negatively impact survival were not encountered during these Trials allows this correlation to exist. If such high temperatures were encountered, then the growth relationship between $D°_{eff}$ growth coefficients and temperature would most likely have taken on a more parabolic distribution due increased growth rates at higher temperatures related to release of density dependent growth as increased mortality levels are realized, in conjunction with high temperatures influencing physiological response and increasing growth. Indeed, while not studied for larval cobia, juvenile lethal high temperatures have been reported to be 37.7 °C (Hassler and Rainville 1975), well above the temperature ranges observed during these Trials.

These conclusions insinuate that maximal temperature ranges for larvicultures studied herein (29.0 to 31.4) are acceptable, and could even be considered beneficial by maximizing both growth (age-based coefficients are only slightly lower than maximal values investigated here) and survival, as was realized for T4. For this same Trial, temperature-independent growth coefficients are the lowest for the larvicultures analyzed herein (Table 6.13). Though at first seeming counterintuitive, these observations again

relate to density dependent growth. Those Experiments were temperature-standardized growth was the fastest were also those same Experiments were cold-water temperatures, either with or without the conjunction of disease events, caused extensive mortalities and a density dependent release of growth. The act of standardizing growth for temperature further extenuates the density related effects. At higher temperatures, higher final densities (related to higher survival rates) reduced growth rates; indeed, among temperature-standardized growth rates, the lowest growth rates were those which resulted in the highest final densities, highest survival, and the highest average temperatures.

Growth constants were found to co-vary positively with predominately temperature variables. However, an interesting significant negative correlation with age-based growth coefficients was also detected. Temperature relationships are readily explained as temperature affects on growth and Q_{10} physiological processes are well recognized, with higher temperatures resulting in higher growth (Blaxter 1988, Kamler 1992, Chambers 1997).

Although growth constants are biologically representative of size-at-hatching, they also represent the elevation of growth regressions. As growth coefficients, and thus computed slopes from the individual regressions, increase, their intercepts will fall vertically lower on the y-axis. This is mostly likely related to high growth rates attained at lower densities, which occurred at lower temperatures, and the previously discussed weight of later sampling events which contained higher sample sizes. Thus, all other factors being equal, as temperature increases, positively correlation with growth constants translates to an increase in their values as well, resulting in increased sizes of larvae for a

given age; in other words, increased growth in size with increases in temperature at a given age about the whole length of the regression.

These conclusions are counterintuitive if one considers growth constants as representative of size-at-hatching; well established parabolic relationships of temperature and size-at-hatching dictate maximal size at moderate temperatures (Blaxter 1988, Chambers 1997, Kamler 1992). Significantly greater size-at-hatching at significantly lower incubation temperatures has been observed for cobia larvae as well (Zink Unpub. Data). Perhaps the positive correlation of temperature with growth constants is also complicated by size-at-hatching being represented by a single data point in the growth regressions and thus increased weight in computation of the least-squares regression lying within older aged sampling events, which would be tend towards higher growth at higher temperatures independent of other complicating factors. Indeed, the regression constants computed for T5 represent the most non-biologically relevant values (Table 6.13, Fig. 6.3); the T5 Experiments also exhibited the highest growth rates, irrespective of age or $D°_{eff}$ formulation of the regressions (Table 6.13). These two observations lend further credibility to the notion of later growth stages, and increased average size at those sampling events, as more heavily influencing computed growth constant values.

The ostensively co-varying production variables of number of juveniles produced and final density yielded the most significant correlations with arsine transformed survival. Beyond these variables, variables representing timing of changes in feeding schedule regimes were most significantly, and positively, correlated. Of these, start of weaning was the most highly correlated. Once start of weaning, yielding a negative relationship with survival, had been incorporated during GLM modeling; subsequent

testing warranted addition of the variable initial density to the model, which also yielded a negative relationship.

Previous discussion of start of weaning and improved nutrition and availability of protein for the rapidly growing cobia larvae are certainly applicable to improving survival. As previously discussed, nutritional requirements of rapidly developing and growing cobia larvae could have been better met by transitioning earlier onto compound feeds. Earlier transitioning onto compound diets of larger sizes could have also reduced cannibalism; it can be reasoned that if a larvae consumes large enough prey and is satiated both in terms of total food volume and nutritional requirements, then perhaps there would be a lower incidence of cannibalism.

Liao et al. (2001) suggested further work on optimization of larval feeds size and kind in order to reduce cannibalism while Holt et al. (2007) have suggested faster weaning and offering of commercially prepared diets earlier in order to circumvent cannibalism and increase survival during cobia larviculture. While this study does not provide clear understanding of these relationships, the results support these suggestions that survival can be improved by more rapid feeding schedules and earlier weaning onto prepared diets. First incidence of cannibalism in cobia larvae has been observed to fluctuate based upon either age and/or size (Zink Pers. Obs.). Ages that coincide with first observations of cannibalism from this study coincide with ages of gastrointestinal development in relation to increases in efficiency and protein uptake (Faulk and Holt 2007). Certainly, piscivory is behavior trait of older larvae transitioning onto more nutritious food sources. If nutritional needs are better met when these developmental changes occur, then perhaps ‘cannibalistic desire’ can at least be reduced.

In review of cannibalism as it relates to aquaculture, Hecht and Pienaar (1993) note that availability of alternative prey, nutritional composition of food items, and prey items not meeting energetic requirements all have impacts upon cannibalism. The authors also discuss how offering of live prey items along with dry feeds reduced cannibalistic and territorial aggression in juvenile African catfish, although compounding this conclusion is the observation of increased territorial behavior when a variety of live feed prey sizes was available (Hecht and Pienaar (1993). During rearing of pikeperch *Sander lucioperca* larvae, lower cannibalism rates were observed when larval groups were weaned earlier onto artificial diets; although, in contrary to the findings of this study, groups with earlier initiation of weaning also expressed the lowest growth and highest mortality (Kestemont et al. 2007).

In the present study, the compounding influence of increased temperature allowing for more rapid development and earlier weaning most likely contribute to these differences, as the studies of Kestemont et al. (2007) were all conducted in temperatures ranging from 16-17 °C. Cannibalism has also been recognized as a major source of mortality in later stages of cobia larvicultures (Holt et al. 2007b, Benetti et al. 2008b). Methodologies which potentially reduce cannibalism, such as earlier weaning onto formulated diets, should enhance survivorship and thus production yields in terms of this variable.

Previous studies have investigated influences of initial stocking densities upon production outcomes of cobia larvicultures. Hitzfelder et al. (2006), working with small recirculating aquaculture systems, found no significant differences in survival among moderate cobia larvae initial stocking densities (5 and 10 larvae L^{-1}). Similarly,

comparisons of survival at low stocking densities (0.5, 1.1, and 2.0 larvae L^{-1}) did not detect significantly differing final densities. Benetti et al. (2008b) also did detect significant differences in survival at moderate initial stocking densities (5 and 10 larvae L^{-1}) when reared in commercial sized flow through tanks, although the trend was for higher survival in lower initial stocking densities. Although not analyzed across individual experiments and not exactly clear, a rough trend of reduced survival with increasing initial stocking density was presented in Table 1 of the Hitzfelder et al. (2006). That observation is similar to the current finding that initial density significantly influences survival with a negative relationship, although trends in the current study are also complicated by disease and reductions in optimal rearing conditions. As previously discussed, Haziathanasiou et al. (2002) concluded that cannibalism was the main source of mortality during experimentation with initial stocking densities of post-larval European sea bass (*Diccentrarchus labrax*), suggesting the interrelations between densities and a major source of mortality during this phase of larvicultures.

The present study, as well as previous studies relating to initial stocking density of cobia larvicultures, analyzes survival only at the termination of the rearing. Thus, factors affecting larval and post-larval stages cannot be addressed independently without further analysis of mortality during the course of a larviculture. In the present study, a significant positive correlation between initial and final stocking densities ($r = 0.6575$, $df = 12$, $P = 0.0146$) and, while anticipated to co-vary, final density significantly and positively correlated with arcsine transformed survival ($r = 0.8485$, $df = 12$, $P = 0.0002$, Table 6.16). These correlations further demonstrate the linkages between survival, initial and final stocking densities, and the linkages between these factors and production

outcomes. These linkages between initial and final stocking densities and survival can also be interpreted in terms of stress and crowding in larviculture systems. As previously discussed, crowding can increase metabolic demand, reduce energy allocated towards growth, feed assimilation efficiency, and impact feeding behavior (Barton and Iwana 1991, Hitzfelder et al. 2006, Wendelaar Bonga 1997). These factors can also influence not only growth but survival as well.

Total number of juveniles produced was found to most significantly correlate with timing of feeding regimes, followed by temperature measures and then, interestingly, by various chemotherapeutant treatments. GLM modeling of total number of juveniles produced resulted in a model with only start of weaning as an explanatory variable; although, the negative association with this single variable explained over 84% of the variability in the number of juveniles produced. Number of juveniles produced and final density are exactly correlated in a one to one fashion due to the former being the product of the later and the larviculture tank volume; therefore, discussion of factors influencing final density similarly apply to number of juveniles produced. Potential nutritional benefits and reductions in cannibalism can again explain this relationship as start of weaning is carried out earlier during a rearing could potentially increase survival, and thus number of juveniles produced.

It is both intriguing and yet anticipated that no chemotherapeutant or other treatments were incorporated during GLM modeling of production outcomes; despite this, formalin utilization was found to positively and significantly correlate with number of juveniles produced (Table 6.17) and survival (Table 6.16). Similarly, a marginally significant positive correlation between probiotic utilization and number of juveniles

produced was also observed (Table 6.17). Only by increasing α level to 0.10 does probiotic utilization becomes significantly correlated with survival. Although formaldehyde has been found to act preferentially in a stronger manner against gram-negative bacteria (Spicher and Peters 1976 as cited in Gatesoupe 2002), formalin utilization may have also inhibited efficacy of probiotics and their extended viability within digestive larval digestive tracts (Gatesoupe 2002). Further study of how prophylactic formalin treatments disrupt and alter bacterial communities of larval fish and larviculture water are certainly warranted, especially when utilized in conjunction with probiotics, in order to ensure these prophylactic treatments are not counter-productive. Concern over rapid capabilities of bacteria to develop resistance to this treatment when utilized as a disinfecting treatment during *Artemia* hatching suggests further investigation of microbial community development and its utilization during larvicultures (Gatesoupe 2002)

It is not surprising that other chemotherapeutants did not contribute to production outcomes. Although antibiotic utilization was warranted addition to the number of juveniles produced GLM, it was decided not to include it due to the lack of logical explanatory power of this variable due to unbalanced sample size, as it was only utilized in one Experiment. Furthermore, the utilization of antibiotic and copper sulfate treatments were reactive measures towards active infestations or bacterial outbreaks; therefore, though their utilization may statistically contribute to explanation of production outcomes, it is not surprising to see negative correlations with production outcomes such as survival (Table 6.16) and number of juveniles produced (Table 6.17). Copper sulfate 24 hr LC_{50} concentrations were detected to be 0.091 mg L^{-1} (Dung et al. 2005). Given

this value and the knowledge that copper sulfate treatments on larvicultures at UMEH attempted to maintain concentrations of ranged between 0.0 and 0.5 mg L^{-1}, one must consider which caused greater mortality: infestation by *A. ocellatum* or the copper treatments utilized to combat this parasite.

In order to better ascertain temperature affects on growth, Q_{10} values computed where other experimental conditions would potentially allow a proper comparison. Although similar initial conditions which could have led to computation of Q_{10} values between T4E10 and T4E11 and T5E12 and T5E13, Q_{10} values of 0.657 and 0.814 comparing T4E10 with T5E12 and T4E11 with T5E13, respectively, were deemed too small in magnitude to portray meaningful values. Computed Q_{10} values for cobia were suspected to be complicated by differences in final densities and its significant and greater influence on growth in length than temperature. Q_{10} values greater than one indicate the biological process under study is temperature dependent and its rate increases with an increase of temperature. Therefore, these values would indicate there is not a Q_{10} related change in growth in terms of TL with increases of temperature and, in fact, suggests a decreasing growth over with increasing temperature within this range.

The repeated finding of the importance of changes in feeding regime schedule, namely start of weaning, in earlier portions of this analysis led to further investigation of factors influencing changes in feeding regime schedules. Pairwise correlations revealed a majority of the most significant pairings were with differing measures of temperature; these correlations were all negative (Table 6.20). Pairwise comparisons regressions of feeding stage progression and age were, for the most part non-significant (Fig. 6.8) although a trend is discernable. T4, during which the highest temperatures of the analysis

were observed, yielded the steepest feeding stage progression coefficient. Conversely, for Trial T1, which experienced some of the lowest recorded temperatures in this study and which these temperatures occurred towards the later stages of the rearing, and for T5, which exhibited the lowest recorded temperature in this analysis during earlier stages of the rearing, both were the lowest and second lowest value slopes, respectively (Table 6.21).

Since both temperature and density-dependent influences on growth were repeatedly indicated as the most important drivers of growth in previous portions of this analysis, the relationship between the feeding stage development coefficients and these factors were investigated. Trial feeding stage coefficient regressed against average temperature did not yield a significant regression (Table 6.22), though a readily recognizable trend was evident (Fig. 6.9a). This is converse to results with North-east Atlantic mackerel (*Scomber scombrus*), which were observed to switch prey types faster at incremental higher temperatures, further coinciding with the most rapid growth in terms of length (Mendiola et al. 2007). Regression of Trial feeding stage progression coefficients against average final densities did yield a significant regression (Table 6.22) and an easily discernible trend (Fig. 6.9b). These observations, along with the finding that final density was the most highly correlated variable with age-based growth coefficients, confirms findings by Fuiman et al. (1998) that development rate is best associated with larval size and not age.

This study investigated somatic growth in terms of TL (mm). Suspected temperature linkages of increased growth rate with increasing temperature were muddled by density dependent growth affects, though changes in individual Experiment's growth

coefficient ordering relative to each other between age-based and $D°_{eff}$ indicate the influences of temperature regime influencing growth rates. The highest instantaneous growth rate from T4 was ~90% of the maximal instantaneous growth rate computed in this study (T4E11: 0.0919 and T5E12: 0.1023); the growth rate of T4E11 is only ~51% of T5E12 when $D°_{eff}$ growth rates are compared. Growth was actually negatively correlated with average temperature (-0.3606), though not significantly (P = 0.2261); this negative relationship is reinforced by the above Q_{10} values < 1, which suggest decreasing growth with increasing temperature. These observations are most likely due to high mortality occurring during periods of low temperature, allowing a release from the more dominant density dependence on growth. The example of T4 exemplifies moderately high growth while concurrently realizing the highest survival and production within this study. Final density, in terms of total number of juveniles produced, was found to be positively and highly significantly correlated to average temperature (0.7941, P = 0.0012).

This same T4 highest growth rate is ~85% of the highest growth rate reported for tank culture of cobia larvae despite substantially higher average temperature regimes, initial stocking density, and final density in T4 (Table 6.23) (Holt et al. 2007b). These conditions also resulted in final larval lengths substantially greater than those reported by Hitzfelder et al. (2006) for cobia reared from similar initial densities (5 and 10 larvae L^{-1}) but lower temperatures (Hitzfelder et al. 2006: 27.4 ± 0.5 °C, T4: 30.1 ± 0.7 °C averaged across the four Experiments in the trial) (Table 6.23). Despite what has already been discovered concerning density dependence in this analysis, these results were attained at final densities two times greater than those reported by Hitzfelder et al. (2006). Marginal

reductions of instantaneous growth rates should be anticipated and accepted when, more importantly, production numbers are vastly increased. Under the higher temperature conditions observed in this analysis, maximal growth and total number of juvenile production were achieved, though further analysis would be necessary to verify this observation.

Despite lack of a direct linkage between growth rates and temperature, significant effects were observed relating to the vertical location of individual growth regression intercepts. As larval growth was more rapid throughout each Trial, larval size distributions would have been higher in elevation earlier on and increased survival would have reduced later vertical elevations. Thus, this shifting of the growth model slope, and thus also the intercept, in relation to modeling growth over the entire larval period can be visualized as an indicator of faster growth and higher survival at higher temperatures.

The timing of feeding regime schedule and more rapid changes between prey types, and thus more rapid ontogenetic development of larvae themselves, was a repeated theme in improving survival, growth, and total production numbers within this study. Changes in timing of feeding regime schedule can be considered as a reflection of faster developmental growth, and thus reflective of those factors which affect growth. Furthermore, developmental progress has been found to more closely coincide with larval size rather than age (Fuiman et al. 1998). In the present study, crowding depressed growth in length, but higher temperature still allowed for trends in more rapid rates of transition onto successive prey types.

In review of temperate species larval development, growth, and mortality, Pepin (1991) concludes that temperature increases rates of development in larval fishes.

Besides previously discussed changes in timing of feeding schedule, other ontogenetic changes were affected by temperature and density dependent growth. In the current study, increased rates of developmental change were similarly observed for structures and behaviors, such as the completion of gill lamellae development at 9 DPH (T4) versus 14 DPH (T5) and first incidences of cannibalism 8 DPH (T4) versus 10 DPH (T2). Fuiman et al. (1998) note that in general, temperature alone caused developmental events to occur between 4.7 and 8.1% earlier. In this study, gill lamellae formation occurred 36% earlier in T4 than T5. Despite density dependent growth generally overwhelming temperature related increases in growth, increased temperatures still allowed for earlier development.

Perhaps, as was observed by Hatziathanasiou et al. (2002) for European sea bass *Dicentrarchus labrax*), these early stages in larval growth are less influenced by density dependency and thus gill lamellae, and growth and development in general, were more heavily influenced by temperature. Furthermore, T4 average size of larvae from the differing Experiments at 9 DPH ranged from 7.67 to 9.00 mm, while in T5 average size of larvae from the differing Experiments ranged from 11.20 to 11.35 mm at 14 DPH. Though there was a 36% age difference in the timing of this event between the two trials, size ranges between pair of maximal and minimal size differences between the Trials was 20 to 32%, respectively. The reduction in variability in size over age at the timing of gill lamellae formation supports the findings of Fuiman et al. (1998) that size-at-development stage rather than age-at-development stage is more closely related and with minimal variation in comparisons.

In this study, density dependent growth generally overwhelms temperature effects, although it is evident how these two factors interacted and complicated growth in

length. GLM modeling revealed that the most important driver of growth, as significance to age-based growth coefficients, was final density; this parameter is also more significant than temperature in regressions computed for rate of food schedule development. Meanwhile, when growth rates are standardized for temperature differences between Experiments, minimal temperature becomes the most important explanatory variable; this seems to contradict the previous observations, but correlation between minimal temperature and final density ($r = 0.8407$, $df = 12$, $P = 0.0004$) helps clarify these interactions. This indicates that those factors which drive growth in size will also drive development and the greater dependence on size-at-development stage rather than age-at-development stage. Developmental studies from captive and laboratory settings should include descriptions of not only original stocking density, but measures of survival and final stocking density so that later investigation can at least qualitatively ascertain these impacts upon the observed growth.

Though not investigated in this study, increases in temperature can also have many other beneficial effects for larval growth, development, and energy utilization. Higher temperatures have been shown to increase muscle contraction speed when initiating strikes and increase escape speeds, which would affect prey capture capabilities as well as predator (i.e. cannibalism) avoidance. However, tail beat frequencies are also increased, which could slightly increase energy utilization (Batty and Blaxter 1992). Increasing size improves absolute escape swimming speed (Batty et al. 1993). Perhaps within a single larval rearing (cohort), as relative lengths of individuals increase faster at higher temperatures, there are better chances that a larger percentage would survive due to decreased successful cannibalistic encounters with larger individuals. In relation to

cannibalism, it would be interesting to determine the relationships between temperature and density depression of growth on survival and variability of size.

Higher temperatures would also allow cohorts to attain larger sizes faster given when other factors, such as density, limit growth. This could aid swimming efficiency via changing Reynolds numbers similar to as demonstrated between larger sizes of cod *Gadus morhua* relative to smaller individuals (Ruzicka and Gallager 2006). Temperature alters viscosities of seawater and causes, over certain temperature ranges, reductions in swimming speed in relation to increasing viscosities at lower temperatures, as demonstrated for Atlantic herring *Clupea harengus* (Fuiman and Batty 1997). In the current study, perhaps increased temperatures reduced viscosity enough to represent savings in terms of swimming energetics, and thus allowed greater allocation of energy to growth. Further investigation would be warranted to determine the precise nature of these interactions at these temperatures and for this species. Hunt von Herbing (2002) found that in warmer water, temperature induced Q_{10} physiological effects are more important than temperature induced changes in swimming performance.

Although not fully investigated in this analysis, conclusions about temperature minimums for larval cobia growth and survival can also be ascertained. A large mortality event was observed during T5, most likely resulting from the observed depressed feeding behavior during a period of low temperatures (~21 to 23 °C). Although a complete cession of feeding was not observed, reductions of feeding behavior exhibited by the larvae occurred and, potentially, cessation of feeding occurs at a higher temperature than the reported 18 °C for older individuals (Hassler and Rainville 1975). Although lethal temperature ranges for cobia larvae are not readily available, the collection of most wild

specimens has been reported to occur from waters between ~ 24 and 32 °C (Ditty and Shaw 1992), suggesting that at lower temperatures either spawning does not occur or spawned larvae do not survive. Indeed, spawning behavior in captivity is initiated at temperatures ranging from 24 to 26 °C and continues through temperatures < 32 °C (Benetti et al. 2008a). Furthermore, computation of t_{eff} yielded a value of 18.6 °C; while improvements in the computation and true value of t_{eff} could be realized with more careful investigation and reporting of observations, this value confirms with above cited information and insights regarding temperature minimums for larval cobia.

Other factors not included in this analysis could have contributed to variability in production. Broodstock nutritional status and diet quality has long been considered an important component of egg composition and later larval survival and growth (Blaxter 1988, Bromage 1995). Faulk and Holt (2008) report no correlation of cobia egg composition (lipid content, AA composition, protein content, carbohydrate content, and Vitamin E content) or egg dry weight to measures of egg quality (proportion of floating eggs, hatch rate, larval growth, and larval survival). Fatty acid profiles were found to differ between spawning seasons and decreasing numbers of floating eggs significantly correlated to increasing total n-3 HUFAs (Faulk and Holt 2008). Similar to the conditions described in Faulk and Holt (2008), broodstock diet quality and composition could have varied; in the present study, broodstock diet was comprised of frozen, wild caught prey items (sardines, goggle eyes, squid, and shrimp) supplemented with vitamin mix.

Other egg quality variation could have been introduced by the use of hormone injections in order to stimulate some of the spawns utilized in the current study. Though

the literature is convoluted, there is a general consensus that hormonal induction of spawning may decrease egg quality, and thus, later aspects of larval survival and growth (Mylonas et al. 1992, Tucker 1994, Brooks et al. 1997).

Temperature regimes experienced during early life stages (incubation and yolk-sac larval stage) could have affected later larval growth, development, and survival. In review of temperature and size affects on growth, survival, and development, Pepin (1991) concludes that temperature seems to affect one stage of larval survival may be equal and opposite than affects at other stages, and thus no net positive or negative affect could be ascertained. Thus, initial size did not seem to significantly influence survival in the egg, yolk-sac, or postlarval stages, although average size per se was found to influence survival of postlarval fishes. Pepin (1991) further concludes that initial size has no significant affect on later development rates of either yolk-sac or postlarval stages, but larger average size does impact later growth by 'jump-starting' larvae capabilities to search greater areas and more effective foragers.

Lower temperatures during incubation have been demonstrated to produce larger haddock *Melanogrammus aeglefinus* (Martell et al. 2005) and cod *Gadus morhua* (Jordaan et al. 2006) larvae at hatching. Both of these studies conclude that the higher developmental stages led to lasting differences in development rate despite size differences being soon eliminated. Cobia larvae size-at-hatching has been observed to significantly increase at lower temperatures (Zink Unpub. Data). Confounding interactions of temperature regimes and density dependent growth and their affects on growth and development rates most likely would have masked any minimal benefits that could have been realized by increased developmental stage or size-at-hatching.

Day length could also have affected these results. During larvicultures conducted at maximal temperatures, general Q_{10} physiological implications suggest that greater amounts of energy ingestion would be required to maintain growth and physiological demand. Since these larval rearing procedures utilized ambient light cycles, maximal temperatures more or less coincided with maximal day length (summer solstice June 21, maximal temperatures occurred during T4, 7/6/2007 to 7/31/2007). Thus, in this study, Trials conducted during maximal temperature regimes and energetic requirements were greatest coincided with relatively longer periods in which feeding was capable.

In conclusion, though no current study specifically focuses on determining temperatures which maximize outcomes (growth, survival, and total production) for cobia larval rearing, this study detects the a potential maximization at approximately 31 °C. This temperature coincides with maximal temperatures reported during collections of wild larvae (Ditty and Shaw 1992) and feeding and growth efficiency studies for juveniles (Sun et al. 2006, Sun and Chen 2009). The interactions of temperature and density must be taken into account in order to truly understand maximizing production as it relates to scales equitable to those generally utilized in aquaculture production. Growth rates in terms of length, although an important metric to report, is not necessarily the best metric for comparison as density dependent growth can limit growth rates or allow them to increase under conditions which do not maximize juvenile production. The pattern in significance also suggested that majority of the growth rates, those falling within the middle areas of the rate distribution presented herein, did not significantly differ from each other; thus, minimal changes in growth rate should not be as much of a concern or stressed overly as a result.

Standardizing compounding factors of growth can yield regressions which can improve Type I error, while also resulting in regressions that are more readily comparable between differing studies and laboratory conditions. Further improvement of regression and growth analysis in rates of development may actually be more important for increasing survival and production where density effects limit somatic growth. Even development may be hampered by density dependent growth, as there seems to be a greater association of ontogenetic development to larval fish size rather than age (Fuiman et al. 1998). Development in relation to the weaning onto prepared diets may be especially crucial in order to provide better nutrition, match prey size to larger larvae, and reduce cannibalism, in order maximize the desired production outcomes growth, survival, and numbers of juveniles produced.

Careful notes of ontogenetic development should be noted, as they can be utilized as a means to assess growth in means other than typical measures which may be more highly influenced by other factors, for instance larval fish length and its dependence on final densities within the rearing system. Sampling and modeling of growth should incorporate sample sizes which will achieve desired results of describing growth of the entire course of a rearing; sample sizes of 20 to 30 larvae should be adequate to detect differences in final size and reducing confidence interval in growth regressions. Sampling should occur immediately at the beginning of the rearing, or even include size-at-hatching, in order for growth regressions to better reflect the complete growth history. Besides direct applicability to the commercial industry, one of the advantages of conducting larvicultures within large, commercial scale systems is the ability to minimally impact survival and production outcomes when sampling larval fish without

replacement; although, the resources necessary for utilizing larger culture systems hampers increasing replication as would be desired to improve other aspects of statistical design and analysis.

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