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Kara M. Chuang
University of Maine, kara.chuang@maine.edu

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DEVELOPMENT AND EVALUATION OF NOVEL MICROPARTICULATE DIET TECHNOLOGIES FOR THE DELIVERY OF ESSENTIAL NUTRIENTS TO MARINE FISH LARVAE

By

Kara Chuang

B.A. University of California, Berkeley 2020

A THESIS

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Science

(in Marine Biology)

The Graduate School

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August 2024

Advisory Committee:

Matt Hawkyard, Assistant Extension Professor and Finfish Nutrition Specialist

Deborah Bouchard, Director of the University of Maine Aquaculture Research Institute/ Associate Extension Professor and Aquatic Animal Health Specialist

Michael Habte-Tsion, Assistant Extension Professor and Finfish Nutrition Specialist

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UNIVERSITY OF MAINE GRADUATE SCHOOL LAND ACKNOWLEDGEMENT

The University of Maine recognizes that it is located on Marsh Island in the homeland of Penobscot people, where issues of water and territorial rights, and encroachment upon sacred sites, are ongoing. Penobscot homeland is connected to the other Wabanaki Tribal Nations— the Passamaquoddy, Maliseet, and Micmac—through kinship, alliances, and diplomacy. The University also recognizes that the Penobscot Nation and the other Wabanaki Tribal Nations are distinct, sovereign, legal and political entities with their own powers of self-governance and self-determination.

DEVELOPMENT AND EVALUATION OF NOVEL MICROPARTICULATE DIET TECHNOLOGIES FOR THE DELIVERY OF ESSENTIAL NUTRIENTS TO MARINE FISH LARVAE

By Kara Chuang

Thesis Advisor: Dr. Matt Hawkyard

An Abstract of the Thesis Presented
In Partial Fulfillment of the
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In order to bolster the success of marine finfish aquaculture, improvements must be made in the efficiencies and formulations of microparticulate diets used to feed the early life stages. Marine fish larvae are small and underdeveloped at first-feeding, which presents several challenges in microdiet formulation and delivery. The particles used to feed these life stages are consumed at low rates due to poor acceptability and high sinking rates and must be digestible once consumed. In addition, the particles must be physically stable during suspension in seawater after delivery and be able to deliver a full suite of nutrients to meet the nutritional demands of the fish at this stage. The purpose of this research was to further develop larval microdiets and to evaluate their performance through a series of benchtop trials and performance trials with fish larvae.

Liposomes have shown potential in the aquaculture industry to deliver essential nutrients and water-soluble compounds to fish larvae via liposome-enriched live feeds (i.e. *Artemia* and rotifers). When incorporated into larger alginate-based particles, liposomes may successfully deliver these nutrients directly to marine fish larvae without the need for such intermediates. Here forward known as ‘liposome-containing complex particles’ (LCP), this

novel microdiet has the potential to ameliorate some of the issues inherent to existing microdiets. In chapter 2, we outline the results from a series of benchtop experiments to understand how LCP protein concentrations were affected by factors such as collection bath concentration, payload type and concentration, and liposome inclusion rate. We also developed two methods to manipulate the sink rate of LCP by means of gas-forming agents and novel hollow silica microspheres, both of which achieved a slower sinking particle than commercial-type diets. In addition, particle stability was evaluated to understand how different formulations affect the integrity of these microdiets when suspended in water.

In Chapter 3, LCP were compared to a microextruded marumerized (MEM) diet, a lower-moisture commercial-type microdiet, in growth and acceptability trials with California yellowtail (*Seriola dorsalis*) larvae. Both LCP and MEM diets were made with an open formula, whereby the ingredients and formulations are publicly available to facilitate standardization across institutions. We found that larvae fed LCP did not grow as well with respect to standard lengths, average dry weights, and larval condition factors when compared to those fed MEM and commercial microdiet Otohime. Additionally, ingestion rates of LCP were consistently lower than those fed MEM and Otohime, which was also observed in follow-up acceptability trials. These low ingestion rates contributed to the poor growth observed in LCP treatments in the growth trial and opens avenues for further research and development. Larvae fed MEM diets grew comparably to those fed Otohime and had statistically similar growth and feeding incidence rates. This provides support for the use of this open formula MEM diet as a potential reference diet for marine fish larvae.

The results of this research have provided us with a better understanding of the strengths and limitations of LCP. However, more investigation and development with respect to particle acceptability and digestibility must be conducted before LCP may be considered a viable

microdiet for marine fish larvae. The results of this research also validated the use of the MEM particles produced with the open formula developed for marine fish larvae as a potential open formula reference diet for *Seriola* larvae.

DEDICATION

This work is in dedication to my sweet friend Kalena Kattil-deBrum. Passionate, radiant, and sharp, she embodied what it means to be an unstoppable force in marine science and what it looks like to put love into everything you do.

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I'd like to express gratitude to the folks at the USDA National Cold Water Marine Aquaculture Center for their facilities and infrastructure with respect to proximate analysis and particle size work and for allowing me to grow as a scientist through extra hands-on industry activities. I'd additionally like to thank the folks at Hubbs-SeaWorld Research Institute for their collaboration and infrastructure during feeding trials.

On a personal level, I'd like to give thanks to my family for their endless support in my educational endeavors and for putting up with me living on the complete opposite end of the country yet again. To my support system of friends from afar, thank you for letting me stressfully rant about the complexities of larval nutrition via countless video and phone calls. To my support system of friends nearby thank you for letting me do this, but in perso

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CHAPTER I

GENERAL INTRODUCTION

1.1 The state of finfish aquaculture

In 2022, the global population surpassed a groundbreaking 8 billion people. Coupled with a growth in global per capita consumption[1], there has never been a greater demand for seafood. Presently, more than a third of the world's population depend on seafood for at least 20% of their average protein intake[2] and global consumption of fish is projected to double by 2050[3] due in part to a growing affluent population. Unfortunately, wild fisheries have not been able to meet this demand in decades, leaving a major gap in addressing the global need for sea-derived protein. A dramatic increase in farmed seafood production– specifically fish and shellfish– has been observed in recent decades in an effort to offset these shortcomings and alleviate stress on wild fisheries catch (Tidwell and Allan, 2001). Because of the industry's potential, experts are looking to aquaculture as a solution to meet the global appetite for seafood, with marine finfish aquaculture acting as a major contributor to help meet this demand.

Marine finfish aquaculture has been slow to develop due, in part, to the industry's inability to produce sufficient numbers of larvae and juveniles. This is partially due to inadequate nutrition and rearing protocols during the early larval stages (Hamre et al., 2013). Marine finfish are most vulnerable during the earliest life stages because they are reliant on specific biotic and abiotic conditions that promote development and survival (Hamre et al., 2013). Marine fish hatcheries rely heavily on formulated microparticulate diets for rearing larvae until the fish reach

[1] See SOFIA Report (FAO, 2022).
[2] See 'Blue foods: The role of sustainable fishing in feeding a growing population', a report from the Marine Stewardship Council (2023).
[3] See 'Building blue food futures for the people and the planet: The report of the Blue Food Assessment' (2021).

the juvenile stage. However, early-stage larvae are not equipped with fully developed locomotory, sensory, and digestive capabilities necessary for proper feed uptake and processing of microparticulate diets, hereafter referred to as 'microdiets'. Furthermore, currently available microdiets have technical limitations that result in inadequate nutrient delivery to the larvae. Deficiencies in critical nutrients such as protein, amino acids, and vitamins due to inadequate delivery mechanisms can result in reduced rates of growth and survival and elevated levels of malformations in marine finfish larvae. Comprehensive knowledge and study of nutritional requirements throughout development would help optimize diets and feeding protocols and improve larval and juvenile quality (Hamre et al., 2013). Unfortunately, little can be done to fully define these dietary requirements until effective, formulated diets that can be modified for nutritional studies are established for marine finfish larvae. Thus, the need remains for modifiable and widely applicable microdiet technology that allows the industry to address such issues and improve production outcomes.

1.2 Issues with live feeds

Typically, cultured marine finfish feeding regimes begin by offering live feeds (e.g. rotifers and *Artemia*) to the larvae until they can be weaned onto formulated microparticulate diets as they grow (Stuart et al., 2018). This transition is a critical part of marine finfish culture, yet extremely challenging in terms of production. Unfortunately, large mortality events often occur at the point of weaning. To complicate matters, cultured live feeds are nutritionally deficient in highly unsaturated fatty acids, such as DHA and EPA, required for optimal growth and development of larvae (Samat et al., 2020). Thus, additional steps are usually necessary during live feed production to increase their nutritional value, a process referred to as 'enrichment', before offering them to larvae adding significant operational costs to commercial

hatcheries (Langdon, 2003). Moreover, live feeds are prone to culture crashes due to bacterial or infections, making them an unreliable and risky operation in hatcheries (Langdon, 2003). For these reasons, it is a goal of hatcheries to transition larvae onto formulated microdiets as early as possible.

1.3 Issues with existing larval microparticulate diets

1.3.1 Nutrient leaching

A major challenge surrounding the development of improved microdiets is the difficulty in retaining low molecular weight, water-soluble substances within the particle (Langdon and Barrows, 2011; Langdon et al., 2007; Stuart et al., 2018; Hawkyard et al., 2019). Currently available microdiets are prone to losing their water-soluble nutrients, a process referred to as nutrient 'leaching', when suspended in water (Langdon 2003; Langdon & Barrows, 2011). This phenomenon is due in part to the surface area-to-volume ratios of spheroid objects. The surface area to volume ratio of particles increases with decreasing particle size. In other words, smaller microdiets (<1000 µm in diameter) like those fed to fish larvae, are more prone to leaching their water-soluble nutrients than those used to feed juvenile or adult fish (Guthrie et al., 2000; Langdon and Barrows, 2011; Stuart et al., 2018). This process degrades water quality within the tank by increasing the level of free nutrients and matter which have the potential to be converted to toxic forms of ammonia. This accumulation of leached nutrients and uneaten feed have the potential to increase nitrogen load and bacterial growth within the tank, a potential issue during fish culture (Masser et al., 1992). High levels of ammonia and overall poor water quality are negative stressors for sensitive larvae and may affect the health and growth of the fish if left untreated (Pulsford et al., 1994). Moreover, nutrient leaching results in the ingestion of less nutrients than delivered by larvae from losses over time. Thus, it is necessary to make

improvements within larval microdiet technology to better retain water-soluble nutrients to optimize larval growth and survival outcomes.

1.3.2 Sinking rates

Existing commercial-type microdiets currently fail to supplant inconsistent and expensive cultured live feeds in marine finfish hatcheries for a variety of reasons. As suspension feeders, marine finfish larvae rely on motile prey (live feeds) that essentially act as neutrally buoyant particles. Thus, larvae are used to tracking slow-moving prey that remain suspended in the water column for enough time for capture. Microparticles with high sinking rates do not provide adequate time in suspension for larvae to track and capture them, reducing rates of capture for these slow-swimming larvae. It is estimated that only ~0.5% of all microdiet offered is consumed by larvae though the fish are fed in excess (estimate derived from data reported in Stuart et al., 2018). Once the particles have settled to the tank bottom, it is extremely unlikely that these particles will be eaten. Here, they are likely to degrade water quality, increase CO_2 and bacterial load within the tank, act as a stressor for the larvae, and increase probability of disease outbreaks as a result (Langdon and Barrows, 2011). Additionally, low ingestion rates lead to low feed efficiencies, sub-optimal growth, and negative health outcomes. A solution to this issue is a slow-sinking or neutrally buoyant feed particle that will remain in the water column for a longer duration, providing larvae a greater chance of capture and ingestion while in suspension. Development of a neutrally buoyant microdiet would enhance particle movement in the water column and reduce settlement losses (Langdon, 2003). However, it must be noted that neutrally buoyant particles may only improve feeding outcomes if their particles retain their water-soluble nutrients, which are known to leak at high rates in small microdiets. Neutrally buoyant particles

that leach these nutrients at high rates *and* remain suspended in the water column would be problematic to fish health.

1.3.3 Acceptability and feed ingestion rates

An effective microdiet should possess characteristics that maximize their ingestion by larvae to improve growth outcomes. Feeding success depends on a combination of developmental characteristics (i.e. mouth gape size, swimming ability, etc.), physiological functions (i.e. digestive enzyme activity), and availability of appropriate food items (Rønnestad et al., 2013). Microparticulate diets should have characteristics that facilitate their ingestion, such as being sufficiently sized for detection by larvae and neutrally buoyant to increase rates of capture and reduce settlement losses (Langdon, 2003). Larval feeding behaviors are additionally influenced by a number of physical characteristics and chemosensory responses, including particle smell and taste (Guthrie et al., 2000; Hawkyard et al., 2019). One of the major limitations of existing microdiets is their poor acceptability. This can be partially attributed to the lesser-developed chemosensory systems in larvae and exacerbated by our lack of understanding and application of appropriate chemical attractants (Kasumyan, 2010). Many chemical compounds which stimulate a feed response in larvae are low molecular weight, water-soluble substances (Hawkyard et al., 2019). Within this group, amino acids– specifically glycine, alanine, and betaine– are especially promising in eliciting a feed response when added to feed. Amino acids are thus cited as chemoattractants or chemostimulants and can be used to help facilitate increased rates of ingestion (Carr et al., 1996, Hawkyard et al., 2019). Adding easily digestible low-molecular weight compounds such as peptides and amino acids to feed could potentially increase their digestibility while also increasing their acceptability by larvae (Langdon et al., 2007).

1.4 The need for open-formula diets

Unfortunately, most commercial diets are made with a 'closed formula' meaning that the formulation is proprietary. In other words, the ingredients are listed, but quantitative ingredient formulation and origin is not reported (Barnard et al., 2009). In addition, diet formulations can vary over time and between batches without public disclosure due to least-cost formulation strategies that prioritize minimizing cost over ingredient quality (Barnard et al., 2009). Closed formulation practices make both quality assurance and knowledge transfer between institutions very difficult (Barnard et al., 2009). Ultimately, closed formulation practices inhibit research continuity and slows advancement in creating effective larval microdiets.

A solution to these issues is to promote the use of an open formula, wherein ingredients and formulations are publicly available. Open formula diets provide several advantages to the research community including well-tested, readily available formulations, standardization of research variables, and diet continuity among institutions (Barnard et al., 2009). Moreover, there is simultaneous need for a well-tested standard reference diet. Standard reference diets refer to those which possess appropriate nutrition and can be universally accepted and widely used (Watts and D'Abramo, 2021). An example of a commercial reference diet is Otohime (Marubeni Nisshin Feed Co., Ltd., Tokyo, Japan), which is one of the most commonly used diets for cultured finfish (Fischer, 2022). Unfortunately, Otohime is closed-formula, making comparison very difficult between itself and other microdiets, and may not be commercially available in the future. Thus, there is the need for an open-formula reference diet that should allow researchers to facilitate comparisons across species, systems, institutions, and time periods to promote advancement in this field (Rust et al., 2015). The widespread adoption of open formula practices

would facilitate collaboration and knowledge transfer in industry as well as offer a basic platform for improvement.

1.5 Liposomes and liposome-based complex particles

Traditionally used as a useful tool in biotechnology and the pharmaceutical industries, liposomes have recently been used as a promising method for the delivery of water-soluble substances to marine fish larvae (Barr and Helland, 2007; Monroig, et al., 2003, 2007; Hawkyard et al., 2015, 2016, 2019). In simple terms, liposomes are lipid vesicles that enclose aqueous core materials. Liposomes can be formulated with water-soluble compounds dissolved in the aqueous core (Barr and Helland, 2007; Langdon, 2003; Langdon et al., 2007; Monroig et al., 2003, 2007; Hawkyard et al., 2015, 2016, 2019). For purposes of marine finfish aquaculture, these can include materials like phospholipids, free amino acids, and antibiotics beneficial to larval growth and survival (Barr and Helland, 2007; Langdon et al., 2007; Monroig et al., 2003). Liposomes containing similar substances have been used in research to enrich live feeds, such as rotifers (*Brachionus* sp.) and *Artemia* nauplii. These enriched live feeds have been successfully fed to first-feeding larvae to increase nutrition intake (Langdon et al., 2007; Barr and Helland, 2007; Monroig et al., 2003, 2007; Hawkyard et al., 2015, 2016, 2019). This technology shows promise in its use as a successful delivery method of water-soluble substances to marine fish larvae using a live-feeds intermediate.

"Complex particles" is a term that describes a type of particle created when two or more particle types are combined into a single particle to capitalize on certain characteristics from each (Langdon, 2003; Önal and Langdon, 2005). Liposomes may be incorporated into alginate-bound carrier particles so they may be consumed directly by marine fish larvae without the need for a live feed intermediate (Hawkyard et al., 2019). This particle type will hereafter be referred to as

‘liposome-containing complex particles’ (LCP). Previous research using this particle type has used lipid-walled capsules, lipid spray beads, and liposomes incorporated within microbound particles to deliver water-soluble nutrients to fish larvae and other marine suspension feeders (Villamar and Langdon, 1993; Baskerville-Bridges and Kling, 2000; Langdon, 2003; Hawkyard et al., 2019).

1.6 Research goals and objectives

We hypothesized that liposome-containing complex particles may improve growth and survival outcomes of marine finfish larvae through improved delivery of water-soluble nutrients via the embedded liposomes. The primary focus of this project was to develop these microdiets and evaluate their performance in comparison to other commercial-type microdiets. However, we also aimed to validate the use of the open formula platform to produce microextruded marumerized (MEM) particles and will assess their viability as a potential standard reference diet based on results from performance trials with fish larvae. The primary objectives of this research were as follows:

1) Utilize an open formulation originally developed for marine fish larvae for production of two microparticulate diets (LCP and MEM).
2) Evaluate the effects of various methodologies and formulations (i.e. collection bath, liposome core material, liposome inclusion) on the incorporation of protein-based payload materials in microparticulate diets.
3) Optimize buoyancy of LCP to achieve slow-sinking or neutrally buoyant particles to increase feed uptake rates of marine fish larvae.
4) Compare metrics including growth and survival, of marine fish larvae fed LCP to those fed MEM and other commercial-type microdiets.

5) Evaluate feed ingestion rates of marine fish larvae when offered different microparticulate diets.

We hypothesized that optimized liposome-containing complex particles would be able to successfully deliver complete nutrition to marine finfish larvae without leaching critical nutrients, resulting in increased larval growth and performance. If successful, this technology could transform the way marine finfish larvae are fed in commercial hatcheries and enhance overall output as well as larval quality from production systems. Moreover, this diet type has the potential to reduce the need for live feeds, a sought-after goal of the industry that would spare hatcheries labor and resources. Lastly, liposome-based complex particles may have broader impacts beyond the scope of larval nutrition and act as a delivery method for vaccines, antibiotics and other bioactive compounds. This could broaden the impact and scope of this technology and make this novel particle type relevant to multiple applications.

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CHAPTER II

DEVELOPMENT AND EVALUATION OF LIPOSOME-CONTAINING COMPLEX PARTICLES (LCP) FOR THE IMPROVED DELIVERY OF NUTRIENTS TO MARINE FISH LARVAE

2.1 Abstract

To improve growth and survival outcomes in marine finfish hatcheries, larval microdiet technology must be improved upon. Existing commercial microdiets are imperfect and possess a suite of issues that make nutrient delivery to marine fish larvae very difficult. These diets are prone to high sink rates and rapid losses of water-soluble nutrients when delivered in the water column, to name a few. These factors contribute to low ingestion rates, insufficient nutrient delivery, and suboptimal growth outcomes. Previous research has shown liposomes are effective means to elevate key water-soluble nutrient concentrations in live feeds, such as rotifers and *Artemia*, and that liposomes can also be embedded within larger alginate-based particles in order to more directly deliver water-soluble nutrients to finfish larvae without the need for live feeds intermediates. The purpose of the present study was to further develop these liposome-based complex particles (LCP) to achieve a more nutritionally complete diet for marine fish larvae and to optimize particles characteristics. Specifically, this study was focused on modifying particle buoyancy and optimizing the inclusion of water-soluble core materials, particularly hydrolyzed protein and free amino acids, and increasing particle stability without compromising other aspects of particle performance. We identified two methods that were effective for modifying particle buoyancy both of which resulted in slower sinking particles when compared to non-modified particles. We also found that several formulations and production parameters, particularly the $CaCl_2$ concentration of the collection bath, core material type and liposome inclusion rates influenced the inclusion rates of free amino acids and protein hydrolysates within

LCP. Lastly, we found that the inclusion of warm-water fish gelatin to formulation can be used as a tool to enhance particle stability. Ultimately, the results of this study provide guidance for improved formulation and production methods of LCP as well as our understanding of the current limitations of this particle type.

2.2 Introduction

There are many difficulties in successfully rearing early-stage marine finfish larvae, many of which can be attributed to inadequate artificial diets. In order to be successfully used at marine finfish hatcheries, larval microparticulate diets must be: 1) visible and catchable for slow-swimming larvae, 2) attractive and palatable, 3) easy for larvae to digest and assimilate the provided nutrients, and 4) have and retain a nutrient composition that meets the requirements of the larvae (Kvåle et al., 2006). However, deficiencies in one or more of these areas may lead to suboptimal health outcomes of the larvae and bottlenecks in production (Langdon, 2003).

Most commercially available larval microdiets are not effective in retaining their water-soluble nutrients when suspended in water, a process referred to as 'nutrient leaching' (Langdon and Barrows, 2011; Langdon et al., 2007; Stuart et al., 2018; Hawkyard et al., 2019). One of the major factors that promotes leaching is their surface area-to-volume ratio. As particles decrease in size their surface area-to-volume ratios increase, leading to larger relative nutrient losses (Guthrie et al., 2000; Langdon and Barrows, 2011; Stuart et al., 2018). Nutrient leaching may result in lower effective nutrient concentrations in the diet, i.e. those actually experienced by larvae, when compared to formulated values (Stuart et al., 2018). Leached nutrients may also degrade water quality and promote microbial growth, both of which can act as stressors that can affect the growth and health of vulnerable larvae (Pulsford et al., 1994). Thus, it is critical that

larval microdiets are either developed to better retain water-soluble nutrients or should be formulated with ample nutrient levels to account for losses that may occur during leaching.

Liposomes consist of water-soluble nutrients, such as free amino acids and antibiotics, encapsulated within a hydrophobic outer lamella typically made of phospholipid. Approximately 0.5 to 10 µm in diameter, liposomes are too small to be directly ingested by most fish larvae but may be first fed live feeds, a process referred to as enrichment, and can be subsequently fed to the larvae. In a study published by Hawkyard et al. (2015), taurine liposomes were successfully used to enrich rotifers (*Brachionus plicatilis*) with taurine and resulted in increased growth rates of northern rock sole (*Lepidopsetta polyxystra*) larvae when compared to those fed unenriched live feeds. In a subsequent study, liposomes containing free amino acids and amino acid derivatives (glycine, alanine, and betaine) were incorporated into larger alginate-based complex particles so that they could be fed directly to marine fish larvae (Hawkyard et al. 2019). The term "complex particle" describes a particle type wherein one or more particle types are combined to capitalize on the unique characteristics of each (Langdon, 2003; Önal and Langdon, 2005; Hawkyard et al., 2019). For example, Önal and Langdon (2005) incorporated lipid spray beads containing free amino acids into larger zein-bound particles resulting in complex particles that were successfully ingested and broken down by clownfish, showing promise for similar complex particle models. More recently, it was found that liposome-based complex particles retained more than 70% of their encapsulated amino acids after 1 h of suspension in seawater and that these particles were ingested by California yellowtail (*Seriola dorsalis*) and White seabass (*Atractoscion nobilis*) larvae (Hawkyard et al., 2019).

The focus of this research was to build upon the early successes of liposome-based complex particles to develop particles that can provide complete nutrition to early-stage marine

finfish larvae. Our approach was to develop liposome-containing complex particles (LCP) that contained an open formula ingredient mixture, containing squid and krill meals as the primary protein sources and attractants, previously developed for marine fish larvae (Rust et al. 2015; Stuart et al., 2018). Using an open formula ingredient mixture allows for more direct comparison with other studies and particle types that utilize this formulation.

Most existing larval microdiets have rapid sink rates, leading to low rates of capture for slow-swimming larvae that are adapted to eating more neutrally buoyant and motile live feeds (Takeuchi and Haga, 2015). Sink rates of microparticulate diets can be adjusted in several ways, such as adjusting production methods or formulation. For example, a study by Barrows and Lellis (2006) showed that diets produced by microextrusion (MEM diets) were denser than those produced via particle-assisted rotational agglomeration (PARA), and this was directly correlated to the observed sinking rate of these particles. Sink rates can additionally be manipulated through changes in formulation (i.e. lipid additions), changes in moisture content, and by the formation of air pockets in the particle matrix from gas-forming agents (Choi et al., 2002; Orire and Salihu, 2020). For example, it has been shown that the inclusion of sodium bicarbonate ($NaHCO_3$) in the particle mash results in chemical reactions when alginate particles are sprayed into collection baths containing acid, producing air (CO_2) pockets within the particle matrix that cause them to be more buoyant in water (Choi et al., 2002). This method can be applied to complex particles by adding sodium bicarbonate into the particle mash and spraying into a collection bath containing a small amount of acetic acid.

The goal of this research was to further develop LCP in order to provide complete nutrition to marine fish larvae. This was achieved by: 1) evaluating two methods for manipulating particle sink rate, 2) manipulating the type and concentrations of payload materials

incorporated into liposomes (free amino acids and protein hydrolysates), 3) optimizing liposome content to maximize the quantities of free amino acids and polypeptides without exceeding the dietary lipid concentrations typically recommended for marine fish larvae, and 4) evaluating the effects of particle formulations on particle stability. Ideally, this study would result in particles that are slow-sinking, water stable, and able to deliver a full suite of macro and micronutrients to marine fish larvae.

2.3 Methods

2.3.1 Production of liposomes

Liposomes were produced based on methods originally described by Barr and Helland (2008) and further modified by Hawkyard et al. (2015, 2016, 2019). Liposomes were produced with core solutions comprised of: 1) 13% w/v glycine [(CAS #56-40-6, Sigma-Aldrich, St. Louis, MO); "13% glycine liposomes"]; 2) 13% essential amino acids (see Table 2.2 for full ingredient list); "13% EAA liposomes"]; 3) 20% casein hydrolysate [(Peptone from casein, enzymatic digest, CAS #91079-40-2, Sigma-Aldrich, St. Louis, MO); "20% casein hydrolysate liposomes"]; or 4) distilled water only ("empty liposomes"). All liposomes were produced using PL-90H (Phospholipon 90H, CAS #97281-48-6, Lipoid GmbH, Ludwigshafen, Germany) as the phospholipid source.

2.3.2 Production of liposome-containing complex particles (LCP)

LCP were produced using either 1) an open formula (OF) ingredient mixture initially published in Stuart et al. (2018) and further modified to fit our research needs (Table 2.1; "OF-based LCP"), 2) wet-milled commercial diet Otohime (Marubeni Nisshin Feed Co., Ltd., Tokyo, Japan; "MCD-based LCP"), or 3) rice flour (Bob's Red Mill, Milwaukie, OR; "rice flour-based LCP"). Rice flour contains very minimal protein concentrations (2 g per 40 g serving). It was

occasionally used as a base for LCP to more resolutely observe the contribution of protein by liposomes with lower background protein levels when compared to other ingredients, like squid meal and krill meal present in the open formula or milled commercial diet bases.

LCP diets were formulated using Excel (Microsoft Excel 2024 Version 16.85, Microsoft Corporation, Redmond, WA). Liposome suspensions were added to LCP formulations at concentrations generally between 10% to 50% (w/w) on an as-is basis. In all LCP formulations, a 10% w/v alginate (Alginic acid sodium salt from brown algae, low-viscosity A-2158, CAS #9005-38-3, Sigma Aldrich, St. Louis, MO) stock solution was produced and then added to the LCP mixture to achieve a final alginate concentration of ~1% w/v (as-is) in the LCP. Distilled water was added to the formulation to achieve a final moisture content of between 70% to 75%. This pre-spray mixture of open formula ingredients, rice flour, or wet-milled commercial diets in combination with the liposomes, alginate, buoyancy-adjusting agents, and water will here forward be referred to as 'mash'.

LCP were made using a modified apparatus described in Hawkyard et al. (2019). A diagram of the apparatus is shown in Figure 2.1. For large batches (>500 g) the mash was loaded into a pressure pot and extruded using low pressure nitrogen gas. Smaller batches utilized a 60 mL syringe and syringe pump with a pump rate of ~ 20.2 mL h^{-1}. The mash was pushed through an atomizing nozzle (1/4 JBCJ-SS, Spray Systems Inc., Pomona, CA) fitted with a fluid cap (PF60100 brass fluid cap for air atomizing spray performance setup, Spray Systems Inc., Pomona, CA) using compressed nitrogen gas (~10 to 15 psi) as a source of atomizing air. The resultant droplets were captured in an aqueous bath of $CaCl_2$ (0.5 to 4% $CaCl_2$ w/v; (≤7.0 mm, ≥93.0%, CAS #10043-52-4, Sigma-Aldrich, St. Louis, MO) which was used to cross-link the alginate binder. $CaCl_2$ is a commonly used compound to cross-link alginate hydrogels, such as

those used as binders in aquatic feeds (Malektai et al., 2023). When producing LCP containing gas-forming $NaHCO_3$, 10% v/v acetic acid (glacial ≥99%, CAS #64-19-7, Sigma-Aldrich, St. Louis, MO) was additionally added to the $CaCl_2$ collection bath in order to facilitate the CO_2-forming chemical reaction. Particles were allowed to cure for ~2 to 15 minutes in the bath (trial-dependent) and then filtered over stacked 12-inch stainless test sieves to isolate the desired particle size range. This is typically in the range of 600 μm to 1.2 mm for the purposes of our larval nutrition studies because it reflects the average mouth gape of larval marine finfish during the developmental stage of interest (approximately 25 to 50 days post-hatch). LCP were then stored in a conventional refrigerator (4°C) until use.

2.3.3 Proximate analysis

Proximate analysis was conducted using AOAC methods (1990). All samples were freeze dried until stable weight was achieved before analysis. Crude protein was measured in select samples using a LECO FP-828 Nitrogen/Protein Analyzer (LECO Corp., St. Joseph, MI). Protein concentrations listed are on a percent dry-matter basis (% w/w DM). Lipid analysis was done using an ether extractor (ANKOM XT15 Extraction System, Macedon, NY) using AOCS crude fat extraction methods. All analysis was done using facilities and support from the National Cold Water Marine Aquaculture Center (Franklin, ME).

2.3.4 Buoyancy adjustment and evaluation

In theory, slow-sinking or neutrally buoyant particles should facilitate higher rates of particle ingestion by fish larvae. To achieve this in LCP, two types of buoyancy-altering agents were evaluated. The first was sodium bicarbonate ($NaHCO_3$), a gas-forming agent that has been used to adjust the buoyancy of simple-alginate particles when added to the particle matrix (Choi et al., 2002). In this method, LCP were sprayed into a bath containing both $CaCl_2$ and acetic acid

(10% v/v), facilitating a chemical reaction between the sodium bicarbonate and acetic acid that results in the production of CO_2 bubbles in the particle matrix. The second method was to add hollow silica microspheres (Hollow glass spheres, #OL-GL0237B/1-140, 1 to 140 µm, Mo-Sci Online, Rolla, MO) to the LCP mash. Used in the biomedical industry as an organic floating drug delivery system, hollow silica microspheres are presented here as a novel buoyancy-adjusting agent in larval microdiets (Jain et al., 2008). Silica microspheres were chosen to adjust buoyancy because silica is nutritionally inert, naturally occurs in marine food webs, and reflects the natural prey of finfish larvae (Drillet et al., 2011). Microspheres were sieved so that they were <75 µm in diameter and were added to the ingredient mixture in concentrations between 0.1% and 1.5% w/v.

Sinking rate trials were conducted in a 17-inch tall 2000-mL graduated cylinder filled with 35 ppt saltwater. One gram of each respective LCP diet was suspended in 10 mL of seawater and added to the cylinder. For each trial, a timer began at the point at which the suspended particles touched the surface of the water and stopped once the first particle reached the bottom of the cylinder. These times were divided by the depth of the cylinder to establish a sinking rate (cm sec^{-1}) which were statistically compared across treatments.

2.3.5 Protein content optimization

2.3.5.1 Effect of collection bath concentration on LCP protein content

An experiment was conducted to test the effects of $CaCl_2$ collection bath concentration on LCP protein content. Initial experimentation was conducted to see how the protein concentrations of MCD-based LCP changed in response to increasing concentrations of $CaCl_2$. LCP were sprayed into baths containing either 0.2%, 1%, 1.8%, 2%, 3%, 3.7%, 4%, 5.5%, 6%,

7%, 7.3%, or 8% w/v $CaCl_2$. Protein concentrations in dried LCP samples were analyzed using methodology outlined in Section 2.3.3.

Once a suitable range of concentrations was found, a second trial was conducted using a more narrow range of concentrations. In this second trial, 20% casein hydrolysate liposomes were made and incorporated into MCD-based LCP mash at an inclusion rate of 20% (w/v). A parallel treatment consisting of empty liposomes incorporated into a similarly formulated MCD-based LCP so that the protein contents of these two particle formulations could be directly compared and differences attributed to the liposome core materials (protein hydrolysates). These were sprayed into baths containing either 0.25%, 0.5%, 1%, 1.5%, or 2% $CaCl_2$ (w/v). All LCP particles were collected post-production, freeze dried (Labcono Freezone 12L, -84ºC; Labcono, Kansas City, MO) until stable weight was achieved, and ground with a mortar and pestle. Samples were analyzed for crude protein using the methods outlined in Section 2.3.3.

2.3.5.2 Effect of increasing liposome concentrations within LCP

Liposomes are composed of both lipids (in the form of phospholipids), water, and aqueous payload materials. The purpose of this trial was to evaluate the effects of increasing liposome concentrations in LCP on the protein (payload) and lipid concentrations in the resultant LCP. Two different liposome types were used, 20% casein hydrolysate and empty liposomes, and were added at increasing concentrations to LCP mash. Ideally, the protein concentrations in LCP could be substantially elevated without obtaining lipid concentrations in excess of the dietary needs of the larvae. LCP were produced using a rice flour base to minimize background protein levels from other ingredients like squid meal and krill meal that are major components of the open-formula or wet-milled commercial diet mixtures. Either empty liposomes or liposomes

containing 20% casein hydrolysate were added to each individual formulation. Liposomes were added to the mash to obtain concentrations of 20%, 30%, 40%, and 50% (w/v on an as-is basis) of the LCP mash. Particles were sprayed into a 0.5% $CaCl_2$ bath and then collected, freeze dried, and ground as previously described. Crude protein and lipid concentrations were analyzed using methods described in Section 2.3.3.

2.3.5.3 Effect of dialysis on liposome and resultant LCP protein concentration

When initially produced, liposomes are formed within a solution of aqueous materials and only a fraction of this core solution is encapsulated. We conducted an experiment to evaluate the contribution of unencapsulated liposome core materials (protein hydrolysate) to the payload concentrations of LCP. This was to determine if dialysis was necessary or if these materials were removed during the period in which LCP were suspended in the $CaCl_2$ production bath. In order to assess this, LCP were produced with either dialyzed liposomes or non-dialyzed and the resultant protein concentrations measured and compared in both liposomes and LCP.

Three 200-mL batches of 13% essential amino acid liposomes were produced as described in general methodology. Each batch of liposomes was split into two, wherein half of the batch underwent a five-day dialysis, and the other did not. Dialysis of liposomes was performed as follows: One hundred milliliters of each liposome suspension was dialyzed in a membrane bag (Spectra/Por 5 RC Dialysis Membrane Tubing, 12 to 14 kDa, Spectrum Chemical Manufacturing Corp., New Brunswick, NJ) in 2L of artificial seawater (35 ppt) for five days with water changes twice per day. Either dialyzed liposomes or non-dialyzed liposomes were then incorporated into open formula-based LCP (30% inclusion rate) and sprayed into a 0.5% $CaCl_2$ collection bath. This was repeated for each replicate batch. Open formula LCP containing empty liposomes were also produced and compared with the LCP produced with 13% glycine

liposomes. Liposomes and LCP were collected from both dialyzed and unwashed treatments. These samples were then frozen, freeze dried and then subjected to crude protein and lipid analyses described in Section 2.3.3.

2.3.6 Particle size and stability evaluation

An experiment was conducted to evaluate the effects of warm-water fish gelatin inclusion and two different $CaCl_2$ collection bath concentrations on particle stability of OF-based LCP. Gelatins are commonly used as stabilizers in the food industry, as well as in many aquaculture diets, due to their binding ability and high digestibility (Lin et al., 2017). We used warm water fish gelatin (Fish Gelatin Powder, 250 Bloom, Modernist Pantry, LLC, Eliot, ME) as a binding agent because of its melting (25° to 35° C) and gelling temperature range (15° to 25° C) were well matched to the culture temperatures used when rearing California yellowtail, but below the phase transition point of liposomes produced with saturated soy-derived phospholipids. A complete-block design experiment was conducted wherein OF-based LCP containing 13% glycine liposomes were produced with eitherd 1) a solution of 20% warm water fish gelatin (approximately 1.85% dry weight inclusion) or 2) no gelatin. Each of these LCP mashes were split and sprayed into either a a) 0.5% or b) 2% $CaCl_2$ collection bath. LCP were captured on a 600 μm sieve after passing through a 1.2 mm test sieve and saved for particle size analysis.

LCP were analyzed in quadruplicate using a laser diffraction particle size analyzer (Mastersizer 3000, Malvern Panalytical, Worcestershire, UK) before and after one week of storage. The machine's mechanical stirrer was set to 500 rpm to determine initial particle size and 1600 rpm when tested following one week of storage. The mean particle size at the 10^{th} (Dx[10]), 50^{th} (Dx[50]), and 90^{th} (Dx[90]) percentiles (aggregated over 5 measurements) were recorded for each sample and used for statistical analysis. Particle Stability Index (PSI), or the

proportion of particles that retained their size after vigorous agitation, was used to evaluate the proportion of particles that retained their size dispersion after vigorous agitation. PSI was calculated as follows:

$$PSI = \frac{Particle\ size\ Dx(50) at\ 500\ rpm}{Particle\ size\ Dx(50) at\ 1600\ rpm} \times 100$$

Where PSI is a statistical index used to assess particle stability of LCP after agitation, 'Particle size Dx(50) at 500 rpm' represents the median initial particle size of 50% or less of sample particles, as detected by Mastersizer 3000 at 500 rpm (low agitation), and 'Particle size Dx(50) at 1600 rpm represents the median particle size of 50% or less of sample particles after vigorous agitation at 1600 rpm (vigorous agitation). PSI was calculated for all four treatments and statistically compared.

2.3.7 Statistical analysis

Statistical analyses were performed using JMP software Pro Version 17.1.0. T-tests were used to compare the protein concentrations between dialyzed and non-dialyzed liposomes as well as the resulting LCP. A two-way ANOVA with interaction was used to compare particle sizes of LCP made with different formulations (with and without gelatin) and bath concentrations (0.5 or 2%). Assumptions of normality and equal variance were adhered to during ANOVA analyses and were verified where necessary. Tukey's HSD was used as a post-hoc test for ANOVA analyses and significant differences in treatments were depicted graphically via connecting letters reports. Regression analyses were used to detect differences in protein and lipid content between LCP made with 20% casein hydrolysate and empty liposomes at increasing concentrations of $CaCl_2$ and particle sink rates as a function of increasing buoyancy-altering

agents. Assumptions of normal distribution and equal variance were assessed and significant effects impacting observed outcomes were noted.

2.4 Results

2.4.1 Sinking rate trial results

The goal of this trial set was to obtain LCP with a sinking rate of approximately 2 cm sec^{-1} which was chosen because it is less than that of commercial diet Otohime (C1; sink rate ~4 cm sec^{-1}) and this sinking rate should provide larvae with adequate opportunity to consume the particles. Increasing concentrations of sodium bicarbonate or hollow microspheres resulted in decreasing sinking rates of LCP (Multiple regression analysis, main effect 'Concentration of BAA' $p<0.0001$; Figure 2.2). The main effect 'Buoyancy-altering agent (BAA)' (i.e. either hollow silica microspheres or $NaHCO_3$) was also significant ($p=0.0118$) whereby the sink rates of LCP produced with hollow silica microspheres were lower across all concentrations of BAA when compared to those produced with $NaHCO_3$. The interaction between the buoyancy-altering agent and their respective concentration was not significant ($p=0.6396$).

2.4.2 Protein content optimization

2.4.2.1 Effect of collection bath concentration on LCP protein content

From our initial range-finding experiment, we found that the protein concentration of MCD-based LCP decreased as $CaCl_2$ concentration increased from 0.25% to 8% w/v of the bath (Figure 2.3; $R^2=0.753$). These results are also visualized in Figure 2.4 with $CaCl_2$ collection bath concentration expressed in terms of osmolality (mOsm kg^{-1}) instead. In both empty liposome and 20% casein hydrolysate liposome treatments, MCD-based LCP contained the most protein at 0.25% $CaCl_2$ concentration within the collection bath (Figure 2.5). Both treatments showed a decrease in overall protein content due to increasing $CaCl_2$ concentration within the collection

bath from 0.25% to 2% w/v $CaCl_2$. Based on the results of a multiple regression analysis, the main effect '$CaCl_2$ concentration' was significant (p=0.0118) with increasing concentrations of $CaCl_2$ resulting in decreasing protein concentrations measured in LCP. The main effect 'Liposome type' was also significant (p=0.0333) whereby LCP produced with liposomes that contained protein hydrolysates had higher protein concentrations than those produced with empty liposomes. The interaction between '$CaCl_2$ concentration' and 'Liposome type' was not significant (p=0.2421).

2.4.2.2 Effect of increasing liposome concentrations within LCP

To determine what concentration of liposomes to include in LCP without compromising nutritional composition, lipid concentrations, and particle stability, rice flour-based LCP diets were made with increasing concentrations (20%, 30%, 40%, or 50% w/v of overall particle) of either empty liposomes or 20% casein hydrolysate liposomes (Figure 2.6). Protein concentrations of LCP were positively and linearly correlated with the concentration of liposomes used to produce the LCP (Multiple regression analysis, main effect 'Liposome concentration', p=0.0030). Protein concentrations were higher in LCP produced with liposomes containing protein hydrolysates when compared to those produced with empty liposomes (main effect 'Liposome type', p=0.0019). The interaction effect between 'Liposome concentration' and 'Liposome type' was also significant (p=0.0329).

With respect to lipid concentration (%), multiple regression analysis revealed that the main effect 'Liposome concentration' was significant (Multiple regression analysis, p=0.0002) whereby increasing liposome concentrations resulted in increasing lipids concentrations measured in LCP. However, the main effect 'Liposome type' was not significant (p=0.1441), nor was the interaction between 'Liposome concentration' and 'Liposome type' (p=0.7072).

2.4.2.3 Effect of dialysis on liposome and resultant LCP protein concentration

Protein concentrations (% w/w DM) were significantly different between 13% EAA liposomes that underwent dialysis when compared to those produced with non-dialyzed 13% EAA liposomes (T-test, p=0.0012; Figure 2.7). On average, protein content (mean $\pm$ 1 SD) was higher in non-dialyzed liposomes (58.77 $\pm$ 1.30%) when compared to liposomes that underwent dialysis (27.83 $\pm$ 3.23%). Lipid concentrations (% w/w DM) of 13% EAA liposomes were significantly different between treatments as well (T-test, 0.0100), with higher lipid concentrations in dialyzed liposomes in contrast to the non-dialyzed.

Protein content of OF-based LCP produced with either non-dialyzed or dialyzed liposomes were significantly different as well (T-test, p=0.0090). Protein concentrations (%) of OF-based LCP with non-dialyzed 13% EAA liposomes were 53.2 $\pm$ 0.92%, while LCP made with dialyzed liposomes had protein concentrations of 56.5 $\pm$ 0.74%. However, there was no significant difference in resultant lipid concentration between LCP treatments (T-test, p=0.0653), despite having different protein concentrations.

2.4.3 Particle size and stability evaluation

Initial particles sizes of LCP were not significantly affected by '$CaCl_2$ concentration' nor 'Gelatin inclusion' (two-way ANOVA, p=0.2103 and 0.0199, respectively; Appendix B) nor was there a significant interaction between '$CaCl_2$ concentration' and 'Gelatin inclusion' (p=0.3841). Particle stability index (PSI) was not significantly affected by the main effect '$CaCl_2$ concentration' (two-way ANOVA, p=0.0646) but it was affected by the main effect 'Gelatin inclusion" (p<0.001). The interaction between '$CaCl_2$ concentration' and 'Gelatin inclusion' on

resulting PSI was also significant ($p<0.001$). Specific treatment comparisons based on Tukey's HSD are shown in Figure 2.8.

2.5 Discussion

Our results show that washed liposomes were, on-average, 2/3 lipid and 1/3 protein on a dry-weight basis, which must be considered during LCP formulation. As a result, increasing the liposome concentrations in LCP resulted in larger increases in lipid concentrations when compared to protein concentrations of LCP at any given inclusion rate. This limits the total concentration of liposomes that can be included in LCP before lipid levels above those needed by marine fish larvae are achieved. Juvenile *Seriola* (45 to 80 g) were noted to have a dietary lipid requirement of 9%, while it is expected that the dietary requirement for larvae is to be much higher (Masumoto, 2002). For reference, practical diets of CYT have been reported to include 40 to 45% protein (Oladipupo et al., 2024; Miller, 2023; Booth et al., 2010). *Seriola sp*. were reported to perform best on a diet of approximately 25% lipid (Stone et al., 2022; Miller et al., 2023). Larvae grow extremely rapidly and thus the total ingestion of nutrients should be very high and differ from those of juvenile and adult stages (Hamre et al., 2013). Our results suggest that a 30% (w/w as is) inclusion rate of washed liposome suspension can be used without greatly exceeding this threshold. Additionally, our data suggests that approximately 2.6% of the overall particle is made of the encapsulated protein hydrolysate on a dry-matter basis at a 30% liposome inclusion rate as well. It is important that larval feeds are not supplemented with nutrients at levels that far exceed their dietary requirements at the given life stage, as this may detrimentally affect the growth and health of the organism. Fish fed in excess of their dietary requirements of lipid can have a negative impact on qualities like feed ingestion, survival, growth, disease resistance, stress response, and metabolism, so this must be kept in mind when delivering

nutrients to larvae (Fan et al., 2021). Moreover, adding too much of one ingredient (i.e. liposomes) compromises room in formulation for other ingredients like protein sources, vitamins, and minerals which are also essential to fish health and growth. Due to this limitation caused by high lipid:payload ratios, liposomes may be more efficiently used to deliver water-soluble compounds that are highly beneficial, but needed in smaller quantities by marine fish larvae. This may include conditionally essential amino acids and/or water-soluble microminerals and vitamins which are essential to fish and benefit growth, but are needed in smaller quantities. It may also be possible to either select alternative inclusion particles, rather than liposomes, or develop liposomes that have lower ratios of lipid to core materials. For example, other inclusion particles (i.e. lipid spray beads) have been shown to have a 1:2 protein to lipid delivery ratio, showing there is room for improvement with respect to encapsulation efficiency for our particles (Langdon et al., 2006). The amount of core materials that can be encapsulated within liposomes is, in part, limited by the solubility of the compounds that are dissolved in the core solution. In our study, we were able to dissolve 20% (w/v) casein hydrolysate and high concentrations (13% to 20% w/v) of the free amino acid glycine in the aqueous core and this likely represents the upper end of what can be achieved. Future research should focus on reducing the amount of phospholipids through more efficient encapsulation methods. It would also be important that any improved form of liposome be cost-effective and scalable for aquaculture purposes.

Based on the known lipid:protein ratios for liposomes, the measured changes in protein observed were far less than expected at any given liposome inclusion. On average, the lipid:protein ratio (on a dry weight basis) for our liposomes seems to be 2.5:1, where in liposomes deliver 2.5x the amount of lipid as protein for any given liposome inclusion. At a 30% inclusion rate of 20% casein hydrolysate liposomes, we observed a ~25% change in lipid in rice

flour-based LCP. From this obtained lipid value and with our known lipid:protein ratio, we would have expected to see a 10% increase in protein concentration in LCP at this inclusion rate. However, we actually observed only a 2.6% increase in protein at this inclusion rate, predicted to be due to the encapsulated casein hydrolysate. This gives us a protein delivery efficiency of only 26%, far less than expected at that inclusion rate. This indicates that there is a good deal of protein loss during production, which reveals a potential area for improvement.

Both gas-forming $NaHCO_3$ and hollow silica microspheres were effective for manipulating the buoyancy of LCP. Gas-forming $NaHCO_3$ would be the more preferrable method due to its cost-effectiveness and ease of handling. However, this agent requires the addition of acetic acid in the bath which is known to be a noxious stimulus to larval zebrafish (Lopez-Luna et al., 2017), and may reduce palatability and feed ingestion rates in larvae. In contrast, hollow silica microspheres were able to manipulate buoyancy just as well; however, adding another inclusion particle into the LCP matrix introduces another variable to the system. Hollow silica microspheres have many uses in material science and have been used as a buoyancy-assisted separator for infectious pathogens in bodily fluids (Weigum et al., 2016). To our knowledge, hollow silica microspheres had never been used to adjust buoyancy in marine finfish feeds and their performance had not been previously tested for this manner. However, due to the disproportionate change in buoyancy they caused in LCP at only 0.5% w/v inclusion, we decided they may be an effective buoyancy-altering agent for our needs. For these reasons, hollow silica microspheres were chosen as the preferred method because they should be nutritionally inert and have minimal, if any, impact on particle taste. Hollow silica microspheres were used in later iterations of the LCP particle and included as a staple in formulation.

It was first assumed that osmolality may be a significant force influencing final LCP protein concentrations. From initial data observing the relationship between collection bath concentration and osmolality (R^2=0.997, Appendix A), it was found that there is a direct linear relationship between increasing concentrations of $CaCl_2$ and osmolality. Our initial assumption was that LCP would better retain their protein in an iso-osmotic environment with the external media. In an iso-osmotic environment, there would be no osmotic gradient and thus no flux of water and solutes in and out of the liposomes embedded in LCP, which seemingly reduces nutrient leaching and better retain protein. In the case of tank culture, the external delivery medium and final destination for these diets would be seawater, which has an approximate osmolality of 1000 mOsm kg^{-1}. Thus, we attempted to produce both the aqueous $CaCl_2$ collection bath and the liposome core material with similar osmotic values to each other and to seawater. Based on this hypothesis, we expected that LCP sprayed into a collection bath of approximately 3.4 % $CaCl_2$ would have the highest protein retention post-production and we would observe a spike in protein content at as $CaCl_2$ bath concentrations increased. Instead, we observed a linear reduction protein concentrations in LCP as $CaCl_2$ concentrations, and thus osmolality, increased. This occurred both over a wide range of $CaCl_2$ concentrations in the first trial (Figure 2.3) and was consistent between both LCP with and without casein-hydrolysates in the second trial (Figure 2.4). These results did not appear to be related to differences in osmolality between the liposome core solution and collection bath water, as demonstrated by the consistent linear decrease in protein content as iso-osmotic conditions (i.e. 3.4% $CaCl_2$ or 1000 mOsm kg^{-1}) were both approached and exceeded this estimated level (Figure 2.5). It is more likely that Ca^{2+} and Cl^- ions diffused into the LCP during immersion in the bath and diluted the protein

concentrations in the dried LCP. This lends evidence to the fact that $CaCl_2$ concentration, not osmolality, may be a driving force influencing LCP protein concentration.

We found that the inclusion of warm-water fish gelatin led to greater particle stability in LCP when sprayed into baths containing 2% $CaCl_2$. It is unclear why particle stability was not affected by the inclusion of gelatin when the LCP were sprayed into 0.5% $CaCl_2$ baths. Warm water fish gelatin (from tilapia) has a gel point (25-35 ºC) complimentary to the temperatures used to grow a wide range of warm-water marine species. However, it also has a melting point below the phase transition point of saturated-soy phospholipid meaning that LCP production temperatures can be high enough to keep it from setting and yet not disrupt the liposome integrity. Future formulations should utilize warm-water fish gelatin or similar gelatins when enhanced particle stability is desired. The disadvantage to the use of gelatin in this manner is that it competes for space with additional feed ingredients. However, there could be an opportunity to use gelatin to partially replace alginate as a binder in future formulations.

Protein concentrations of casein-hydrolysate liposomes were significantly reduced by dialysis showing that this was an effective means to remove the non-encapsulated fraction of the core solution that remained in the liposome suspension following extrusion. However, since LCP are immersed in $CaCl_2$ baths during production, we hypothesized that pre-washing (via dialysis) the liposomes may be an unnecessary step since these unencapsulated materials were potentially pre-leached during production of LCP. Dialysis of large quantities of liposomes is a labor-intensive processes, adds an additional step that increases risk of contamination and is likely uneconomic at commercial scales. We found that dialysis of 13% EAA liposomes resulted in statistically higher protein concentrations when compared to non-dialyzed liposomes, however the numerical differences were minor (Figure 2.7). The most likely explanation for the observed

difference is that free phospholipids from liposomes diffused through the membrane along with non-encapsulated core materials (13% EAA) during dialysis. This theory was supported by the lower lipid concentration in LCP with dialyzed liposomes (24.76 $\pm$ 0.82%) when compared to those containing non-dialyzed liposomes (28.5 $\pm$ 1.06%). Free phospholipids have a molecular weight of approximately 7.5 kDa[4] (estimated based on soy lecithin), which is approximately half the pore size of the dialysis membrane used (12 to 14 kDa) supporting this hypothesis. In addition, we observed an opaque cloudiness in dialyzed bath water which had an appearance similar to dilute suspended phospholipids. Ultimately, the dialysis of liposomes appears to be an unnecessary, though probably not injurious, step during the production of LCP and results in numerically similar final concentrations of water-soluble payload materials.

2.6 Conclusion

This study has resulted in improvements and a better understanding of the limitations of liposome-based complex particles as a microparticulate diet for finfish larvae. Specifically, we found that no more than 30% w/w of the LCP formulation should be comprised of liposome suspension and at this inclusion rate the contribution of the liposome-encapsulated protein hydrolysates was only ~2.6% of the particles dry matter. These findings suggest that: 1) LCP in their current form should focus on either conditionally essential amino acids or water-soluble micronutrients that are highly important but only needed in small quantities and 2) that reduction of the lipid levels of liposomes and other improvements to retention efficiencies are needed to avoid this limitation. This work has also shown that low levels of $CaCl_2$, down to 0.5% w/v,

[4] Molecular size of phospholipid based off size of soybean phospholipid in lecithin (National Institute of Health). https://pubchem.ncbi.nlm.nih.gov/compound/Lecithin

resulted in greater payload concentrations and that warm-water fish gelatin appears to improve particle stability. Two methods, gas-forming agent $NaHCO_3$ and hollow silica microspheres, were effective means of altering LCP buoyancy to achieve slow-sinking particles which should result in higher ingestion rates of these particles by marine fish larvae. And finally, we found that dialyzing liposomes before incorporating them into LCP was an effective means of removing unencapsulated core materials (measured as protein) but that this resulted in only minor differences in the final payload concentration of the resultant LCP. Future investigations should evaluate the effects of LCP on marine finfish larvae to assess their palatability, digestibility, and effect on larval growth to assess viability as a potential microdiet for marine fish larvae.

2.7 Acknowledgements

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TABLES

Table 2.1 Open formulation published in Stuart et al. (2018) in comparison to modified open formula LCP developed in this research.

Percent of ingredient mixture by weight (% as-is, excluding water)		
Ingredient	**Open formula (Stuart et al., 2018)**	**Open formula LCP (2023)**
Squid meal	39.20	15.68
Krill meal	25.60	10.24
Fish oil	10.50	5.18
Lecithin	10	0
Wheat gluten meal	9	3.59
Vitamin premix	2	0.91
Dicalcium phosphate	2	0.73
Taurine	0	0
Vitamin C	0.20	0.07
Astaxanthin	0.50	0.10
Alginate suspension	0	14
Gelatin	0	0
Liposomes	0	42
Hollow silica microspheres	0	0.50
SUM INGREDIENTS	**100**	**100**

Table 2.2 List of essential amino acids commonly present in fish diets. Percentages contributed by each amino acid are based on amino acid estimates present in major protein-contributing sources in the open formula diet.

Amino Acid	Chemical information	Amount contributed to EAA dry mixture (% w/w)
Arginine	L-Arginine (CAS #74-79-3, Sigma-Aldrich, St. Louis, MO)	15.88%
Histidine	Histidine (CAS #71-00-1, Sigma-Aldrich, St. Louis, MO)	5.56%
Isoleucine	Isoleucine (CAS #73-32-5, Sigma-Aldrich, St. Louis, MO)	10.17%
Leucine	Leucine (CAS #61-90-5, Sigma-Aldrich, St. Louis, MO)	18.66%
Lysine	Lysine (CAS #657-27-2, Sigma-Aldrich, St. Louis, MO)	14.25%
Methionine	Methionine (CAS #63-68-3, Sigma-Aldrich, St. Louis, MO)	6.54%
Phenylalanine	Phenylalanine (CAS #63-91-2, Sigma-Aldrich, St. Louis, MO)	6.06%
Threonine	L-Threonine (CAS #72-19-5, Sigma-Aldrich, St. Louis, MO)	10.42%
Tryptophan	Tryptophan (CAS #73-22-3, Sigma-Aldrich, St. Louis, MO)	2.44%
Valine	L-Valine (CAS #72-18-4, Sigma-Aldrich, St. Louis, MO)	10.02%
TOTAL	-	**100%**

FIGURES

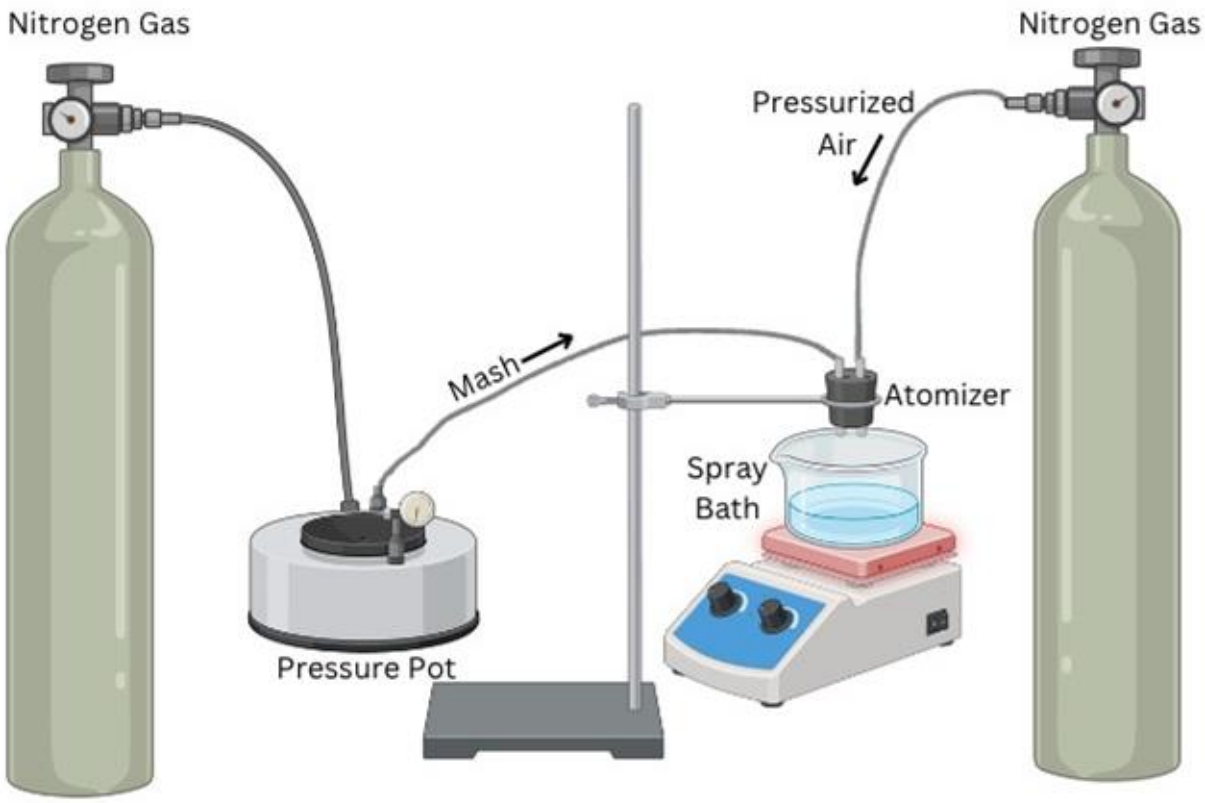

Figure 2.1 Illustration of system used for producing LCP in batches >500 g. Small batches were produced with a similar system but utilized a syringe and syringe pump in the place of the pressure pot.

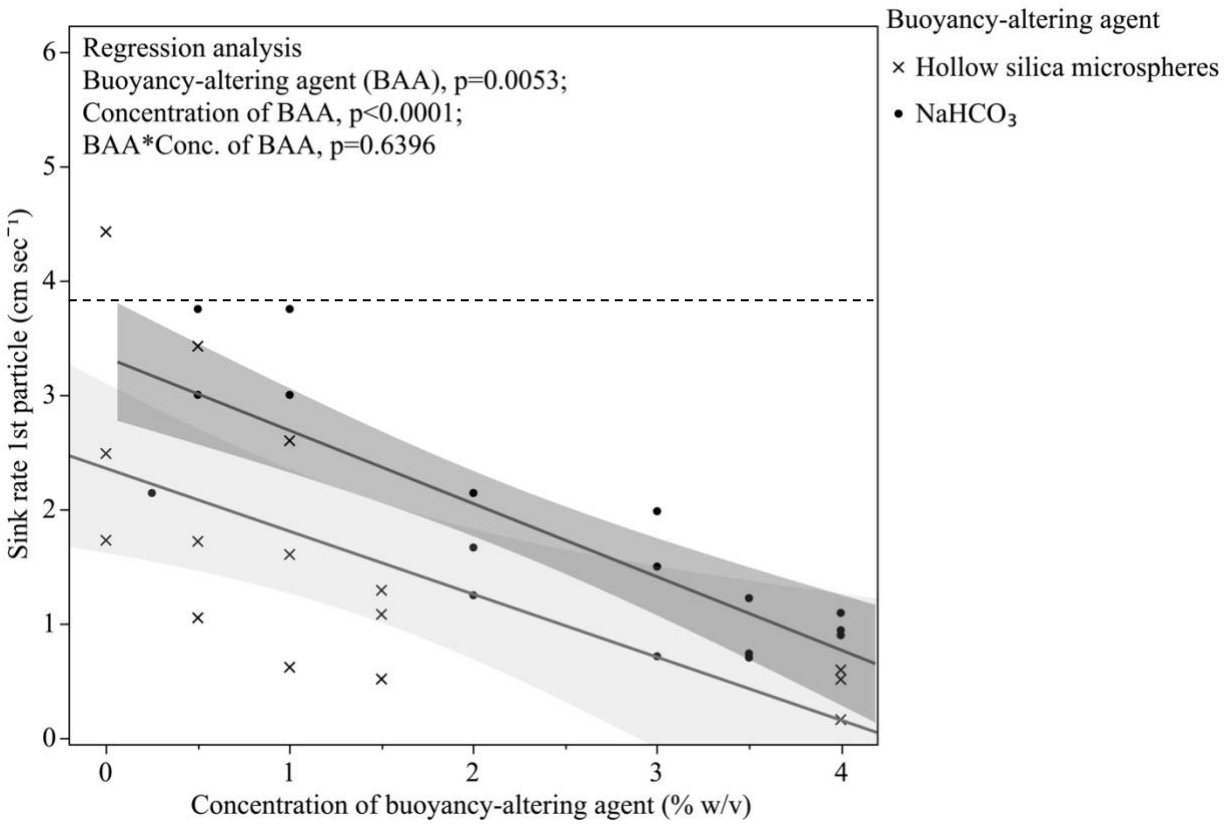

Figure 2.2 Sink rate (cm sec^{-1}) of LCP (first particle) produced with increasing concentrations of either 1) gas-forming agents (NaHCO3; filled circles) or 2) hollow silica microspheres (x's). The dashed vertical line represents the average sink rate of Otohime C1 (3.92 cm sec^{-1}) and is shown for reference. Results of the multiple regression analysis are show in the top left corner.

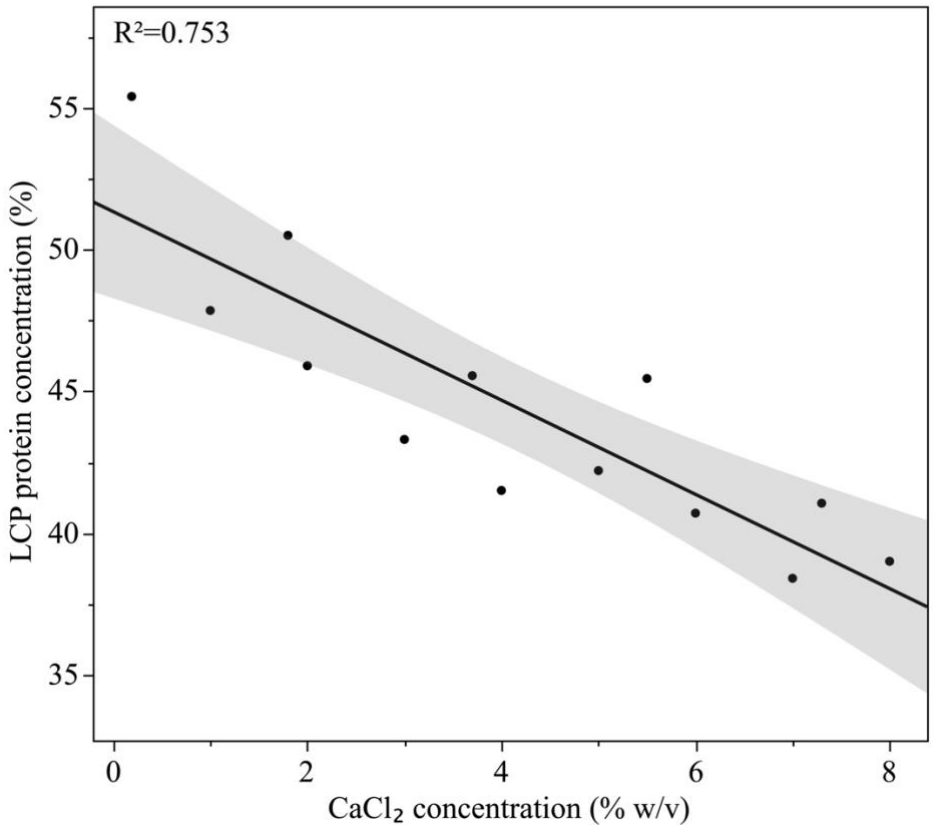

Figure 2.3 Protein concentrations (% w/w DM) of MCD-based LCP that contained 20% casein hydrolysate liposomes (10% liposome inclusion rate) with respect to increasing concentrations of $CaCl_2$ (% w/v) in the collection baths.

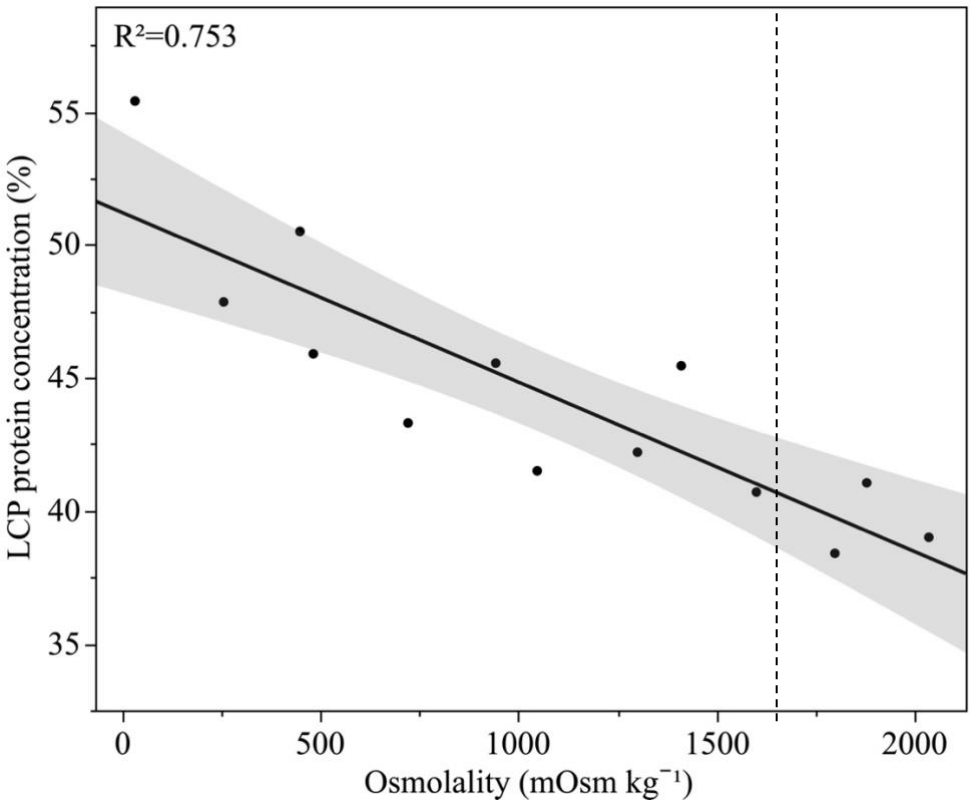

Figure 2.4 Protein concentrations (% w/w DM) of MCD-based LCP that contained 20% casein hydrolysate liposomes (10% liposome inclusion rate) with respect to increasing osmolalities mOsm kg^{-1}) in the collection baths. The dashed vertical line represents the measured osmolality of the 20% casein hydrolysate liposome core solution (1565 mOsm kg^{-1}).

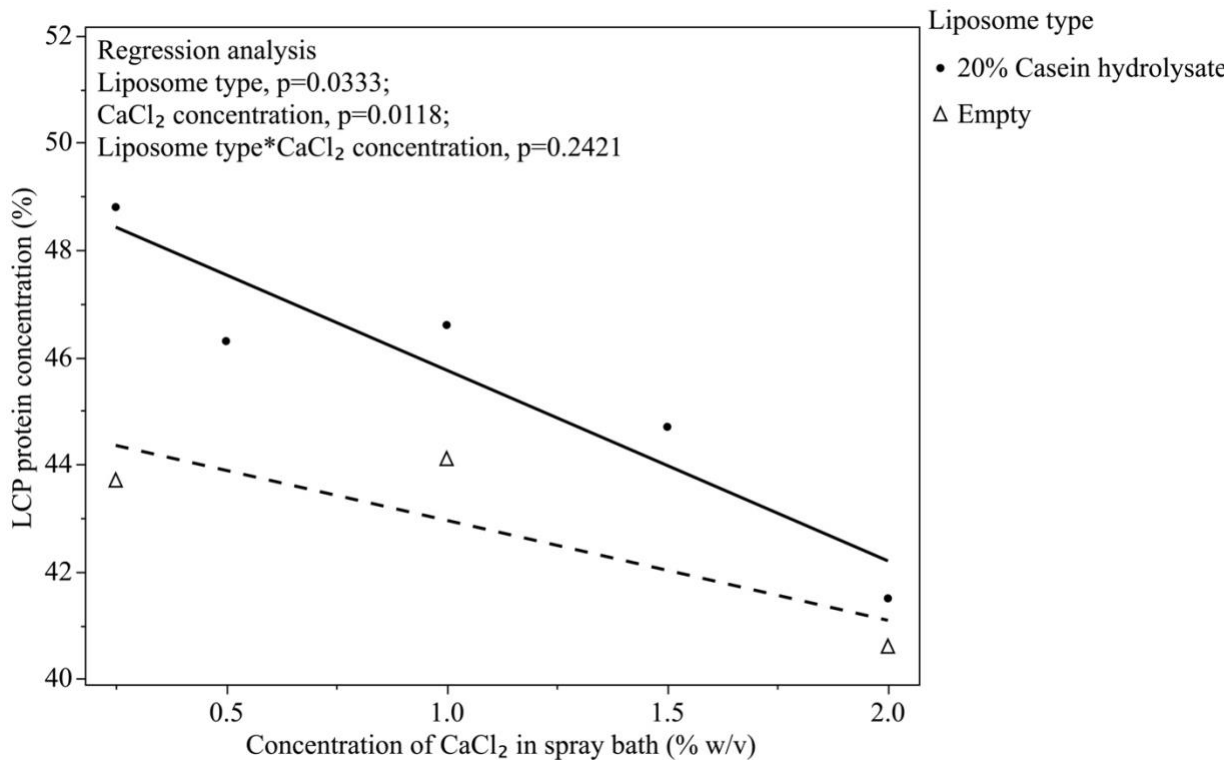

Figure 2.5 Protein concentrations (% w/w DM) of MCD-based LCP prepared with liposomes that contained either 1) 20% casein hydrolysate or 2) distilled water (empty) as a result of increasing concentrations of calcium chloride (% w/v) in collection bath. Results of the multiple regression analysis are show in the top left corner.

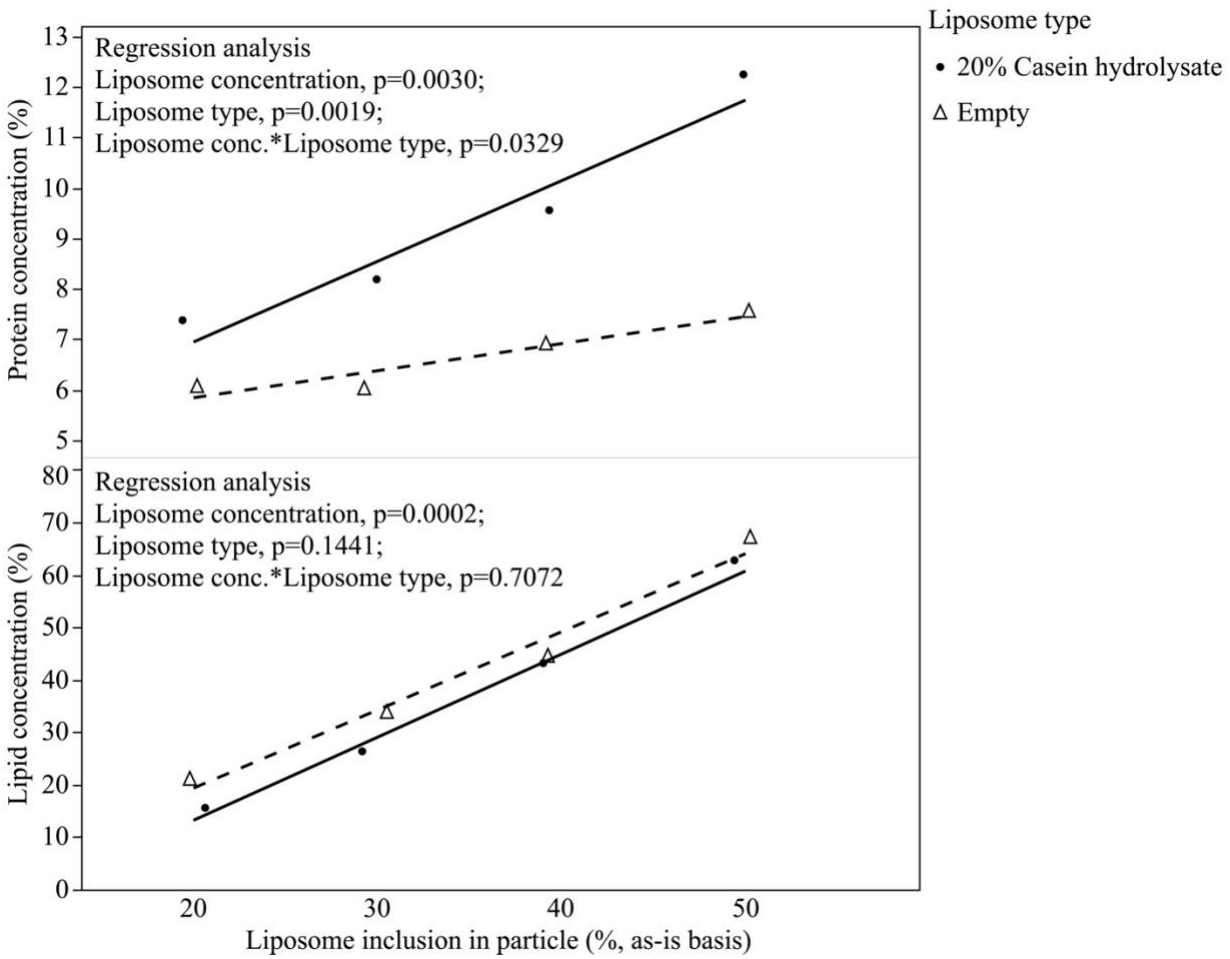

Figure 2.6 Protein (% w/w DM) and lipid concentrations (% w/w DM) measured in rice flour-based LCP as a result of increasing concentrations of liposomes (% w/v, as-is basis) that contained either 1) casein hydrolysates or 2) distilled water (empty) and were sprayed into 0.5% $CaCl_2$. Results of the multiple regression analysis are show in the top left corner.

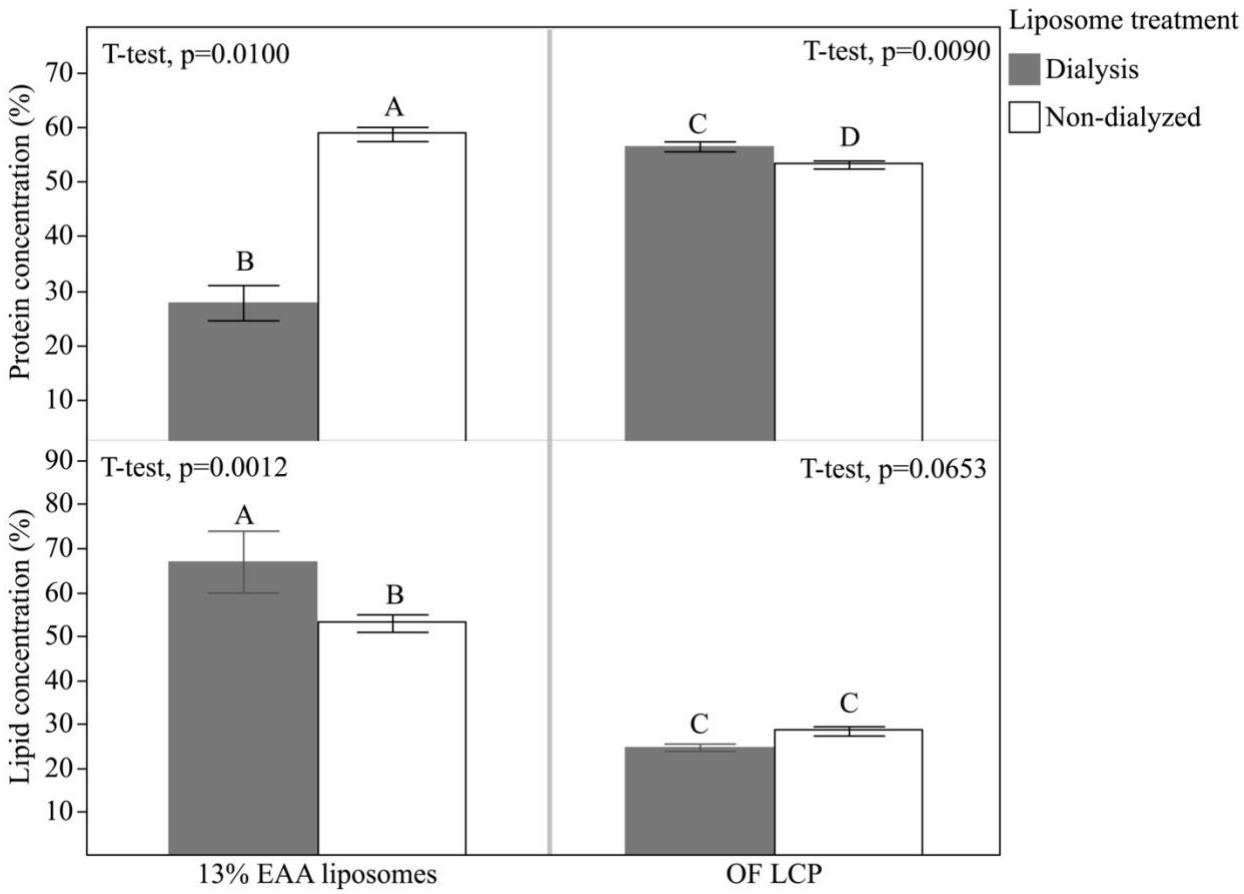

Figure 2.7 Protein and lipid concentrations (% w/w DM; mean ± 1 SD) of 13% EAA liposomes that were either dialyzed ("Dialysis") or underwent no treatment ("Non-dialyzed"; left) and open formula LCP (OF LCP) made from those respective liposomes (right). Results from one-way ANOVA analyses are depicted above. Different letters denotes significant differences between dialyzed and non-dialyzed treatments (see p-values for each pairwise T-test above). Letter codes "A,B" represents analyses done with respect to 13% EAA liposomes and codes "C, D" represents analyses done using OF LCP.

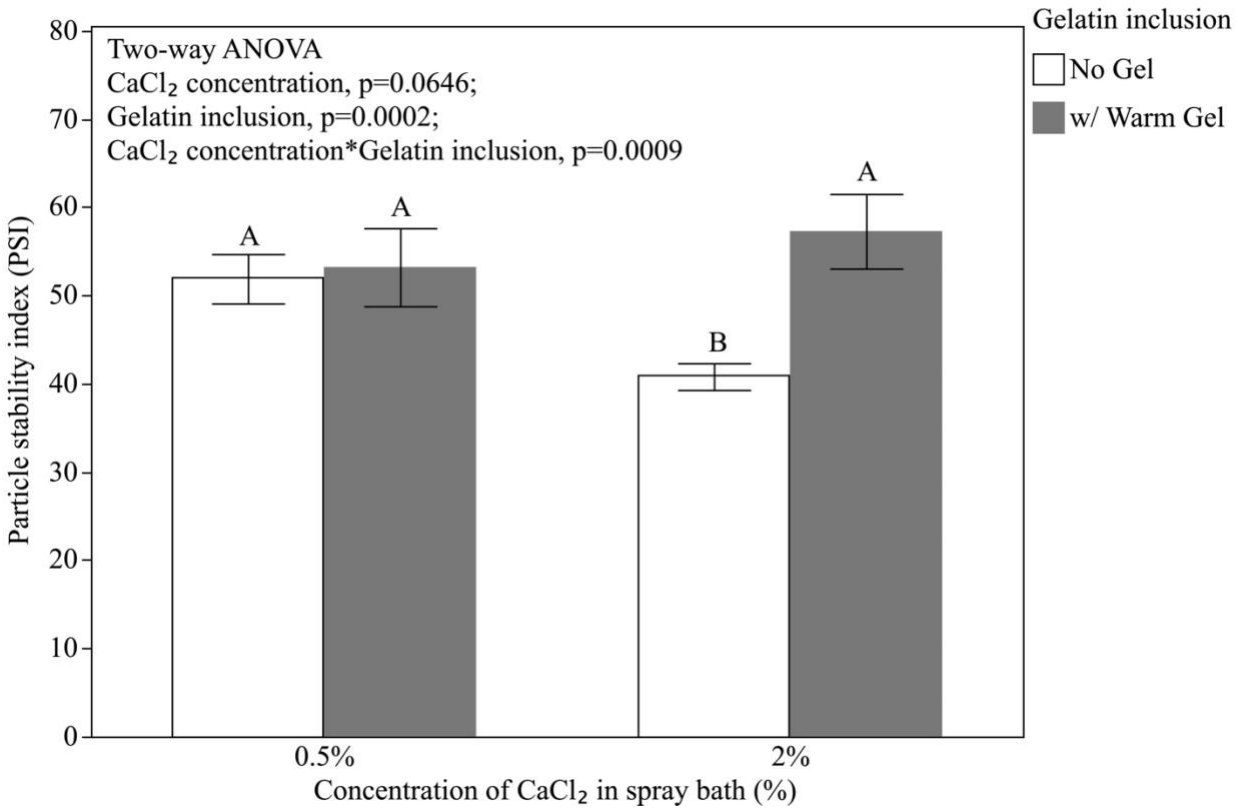

Figure 2.8 Particle stability index (PSI; mean ± 1 SD) of open-formula LCP determined by comparing the change in particle size at the 50th percentile before and after mechanical agitation (stirring at 1600 rpm). The results of the two-way ANOVA, with interaction, are shown in the top left.

CHAPTER III

EVALUATION OF LIPOSOME-CONTAINING COMPLEX PARTICLES AND MICROEXTRUDED MARUMERIZED DIETS AS POTENTIAL EARLY-WEANING MICRODIETS FOR CALIFORNIA YELLOWTAIL (*SERIOLA DORSALIS*) LARVAE

3.1 Abstract

The production of high-quality larvae and juveniles in marine finfish hatcheries is currently constrained by inadequacies in existing microdiet technology. New and improved microparticle types used for feeding marine fish larvae are needed to address inadequacies related to nutrient delivery, sink rate, and acceptability by fish larvae that compromise production outcomes. Unfortunately, ameliorating these issues has been very difficult since most existing commercial microdiets are closed formula, begging the need for an open formula platform to facilitate comparisons across species, systems, and institutions. This study aimed to evaluate the performance of two open formula microdiet types, liposome-containing complex particles (LCP) and microextruded marumerized (MEM) particles, in feeding trials with California yellowtail (*Seriola dorsalis*) larvae. Experimental microparticulate diets were evaluated against a commercial diet by measuring: 1) physical parameters of the diet (sink rate), 2) larval growth (standard lengths, larval dry weights and condition factor), and survival due to a controlled feeding experiment, and 3) diet acceptability (feeding incidence) following feed offerings. In the growth trial, larvae fed the MEM diets performed similarly to those fed the commercial diet Otohime in terms of final growth metrics (larval length, dry weight, and condition factor) and showed similar rates of consumption by the larvae as indicated by feeding incidence. However, larvae fed LCP showed reduced growth and lower feed incidence throughout the trial when compared to those fed commercial-type microdiets (MEM and Otohime). In a follow-up trial, low feeding incidence by CYT larvae was observed in all LCP-

fed treatments, regardless of LCP particle formulation. Though growth and feeding outcomes showed promise for MEM as a potential open formula reference diet for fish larvae, more research should be done to investigate the causes of poor growth outcomes and low feeding incidence associated with LCP diets.

3.2 Introduction

In 2022, global aquaculture production surpassed production from wild capture fisheries[5]. This increase was primarily due to an increase in finfish aquaculture worldwide, particularly in Asia. Unfortunately, the United States is only a minor contributor to this global output and is not even within the top 16 global producers[6] despite being the largest global importer. It is predicted that the United States imports more than 85% of its seafood, with more than half of this quantity being produced through foreign aquaculture (Rexroad et al., 2021). Most domestic production of finfish is comprised of species such as tilapia, catfish, and Atlantic salmon, with very little contribution from marine finfish. Some barriers to the expansion of marine finfish aquaculture in the United States comes from regulatory and policy constraints, while others come from constraints in production technology and research. In sum, output from marine finfish aquaculture in the United States currently relatively small, and an argument can be made for investment in the expansion and advancement to increase output to meet local demands for sustainable marine protein.

[5] See the State of World Fisheries and Aquaculture report (FAO, 2024) for a more detailed report.
[6] Top world marine and coastal aquaculture producers of finfish include China, Norway, and Chile (by thousand tons). See State of World Fisheries and Aquaculture report (FAO, 2022).

Shortcomings in production output for can be partially attributed to poor nutrition at the larval stage, which can lead to health outcomes for marine finfish in commercial hatcheries. Most currently available microparticulate diets (here forward 'microdiets') for marine finfish larvae are not effective at retaining low-molecular weight, water-soluble nutrients (Langdon, 2003; Langdon et al., 2007; Hamre et al., 2013; Langdon and Barrows, 2011; Stuart et al., 2018; Hawkyard et al., 2019). Losses of water-soluble nutrients from uneaten feeds have the potential to increase ammonia levels within the culture system and may cause detrimental health effects to fish if left untreated (Zhang et al., 2018). Additionally, they are prone to rapid sink rates, which limits the length of time a diet remains in suspension and is available for consumption by the slow-swimming larval predators (Baskerville-Bridges and Kling, 2000). Ultimately, low feeding incidence may result in poor growth outcomes, so it is advantageous for microdiets to be slow-sinking or neutrally buoyant. Another key limitation in the use of artificial microdiets is their poor acceptability, often in comparison to live feeds (Person-Le Ruyet et al., 1993; Hawkyard et al., 2019). This phenomenon may be partially due to the fact that quickly sinking microdiets do not provide ample time for slow-swimming larvae to capture them, as previously mentioned. Neutrally buoyant particles may mimic natural prey items like live feeds and can enhance the feeding response of fish larvae by mimicking natural predator-prey interactions (Langdon, 2003). Moreover, live feeds naturally contain a balanced array of essential nutrients including fatty acids, vitamins, and minerals that are easily digestible to larvae and difficult to replicate in artificial diets (Ohs et al., 2010). Because the sensory and digestive systems of fish larvae may not be fully developed, it is important to maximize the conditions that would make microdiets more palatable to fish larvae.

Another factor that makes larval nutrition research difficult is the ability to replicate and modify existing formulations and production methods. Commercial microparticulate diets are typically 'closed formula' wherein ingredients are listed, but the quantitative ingredient formulation is not publicly available (Barnard et al., 2009). Because microdiet structure, sinking rate, and nutrient leaching are influenced by both formulation and manufacture method and it is difficult to make advancements in microdiet technology if production information is unknown (Rust et al., 2015). Thus, there is the need for widespread use of an open formula, wherein ingredient source and formulation are publicly available, that can be modified to meet specific research needs and facilitate comparisons across institutions and experiments (Barnard et al., 2009). There is an additional need for a reproducible control diet with tested ingredient composition to allow for comparisons across species and institutions, otherwise known as a 'standard reference diet' (Watts and D'abramo, 2021). One example, Otohime (Marubeni Nissin Feed Co., Ltd., Tokyo, Japan) is a marine-based microdiet widely used in marine finfish hatcheries worldwide and can be considered a reference diet for many species. However, commercial reference diets currently on the market Otohime may be expensive, not always obtainable, and have inconsistent ingredients. Thus, it would be of great benefit to industry to create an open-formula reference diet that has comparable performance to commercial reference diets on the market, is made with widely accessible ingredients, and has a published formulation.

In this study, we evaluate an open formulation developed for marine fish larvae as the basis for two microparticulate diet types used to feed California yellowtail (*Seriola dorsalis*) larvae. The first was a microextruded marumerized (MEM) diet, which is a low-moisture, shaped microbound particle type that is similar to several commercial microdiets commonly used in marine finfish hatcheries. MEM particles can be formulated with a wide range of practical

ingredients and have been shown to be both palatable to larvae and sufficiently water-stable (Barrows and Lellis, 2006). These particles are formed via agglomeration that occurs during extrusion and subsequent marumerization, otherwise known as 'spheronization'. As a result of this production method, MEM particles are typically dense, fast-sinking particle (Langdon and Barrows, 2011). When characteristics like formulation, moisture levels, and extrusion and marumerization speeds are optimized, the MEM process can be very efficient and produce high yields of on-size particles. Made using open formula ingredients, MEM diets may act as a potential commercial reference diet for marine fish larvae providing a direct and consistent comparison for new and emergent feed technologies and formulations.

The second microdiet type evaluated in this research is a form of 'complex particles', which describes a microdiet whereby at least two complementary particle types are combined to capitalize on the attributes of each constituent particle type (Langdon, 2003; Langdon et al., 2007; Villamar and Langdon, 1993). Previous studies have shown that liposomes are an effective means to encapsulate water-soluble nutrients and deliver them to marine fish larvae via live feeds intermediates (Monroig et al., 2003, 2007; Barr and Helland, 2008; Hawkyard et al., 2015, 2016). Liposomes can be incorporated into larger carrier particles, hereafter referred to as 'liposome-containing complex particles' (LCP), which use alginate or similar binding agents to form larger particles that can be directly ingested by marine fish larvae, removing the need for bioencapsulation within live feeds. Previous research investigated the use of LCP to deliver compounds such as glycine and alanine to evaluate their effects on feed uptake by marine fish larvae (Hawkyard et al., 2019). In Chapter 2, we showed that additional feedstuffs, such as squid meal, krill meal, and fish oil can be included in these particles so that they may deliver complete nutrition to marine fish larvae. We also developed methods that attempted to optimize the

delivery of water-soluble compounds while maintaining a balanced nutrition profile in the LCP. One of the primary objectives of this study was to evaluate the use of LCP-type diets to deliver complete nutrition to marine fish larvae.

The goal of this research was to evaluate the performance of these two microdiet types using a variety of methods including a growth and acceptability trials with fish larvae. We selected California yellowtail (CYT; *Seriola dorsalis*) as the experimental species. Renowned for its rapid growth and desirable flavor, CYT is a promising candidate for marine aquaculture in the United States and a successful commercial species worldwide (Oladipupo et al., 2024; Rotman et al., 2021).

3.3 Methods

3.3.1 Microdiet production

3.3.1.1 Formulation

Liposome-based complex particles and MEM particles used in the growth trial were formulated to be equivalent on the basis of crude protein, crude lipid, and energy content. Moreover, all diets were formulated using the same ingredients and inclusion rates on a dry weight basis even when the means of incorporation differed. For example, the EAA mixture that was encapsulated in liposomes and included in LCP was directly added to the mash of MEM diets to ensure that they had both similar concentrations of protein and essential amino acids, regardless of particle type (by formula). Similarly, the same phospholipid type (PL-90H) that was used for producing liposomes, were included in MEM diets in same amounts on a dry-weight basis so they remained nutritionally equivalent (Figure 3.1 and Figure 3.2). Alginate powder was added to MEM diets so that both particle types would have the same alginate

concentrations (w/w DM), given that it is a necessary binder in LCP. Full ingredient list and nutrient comparisons are given in Tables 3.1 and 3.2. Formulation was accomplished using Excel (Microsoft Excel 2024, Version 16.85).

3.3.1.2 Production of liposome-containing complex particles (LCP)

Liposomes: Liposomes were produced based on methodology originally presented in Barr and Helland (2008) and later modified by Hawkyard et al., (2015, 2016, 2019). Liposomes either contained core solutions of: 1) 13% essential amino acid mix (refer to Table 2.2 for full ingredient list; "13% EAA liposomes"); 2) 10% taurine (CAS #107-35-7, Sigma-Aldrich, St. Louis, MO, "taurine liposomes"); 3) 20% casein hydrolysate (Peptone from casein, enzymatic digest, CAS #91079-40-2, Sigma-Aldrich, St. Louis, MO; 4) 3% stimulant mix [i.e. 1% w/v each of alanine (L-alanine, ≥98% TLC, CAS #56-41-7, Sigma-Aldrich, St. Louis, MO), betaine (≥98% perchloric acid titration, CAS #107-43-7, Sigma-Aldrich, St. Louis, MO), and glycine (CAS #56-40-6, Sigma-Aldrich, St. Louis, MO); or 5) distilled water only ("empty liposomes"). All liposomes were produced using PL-90H (Phospholipon 90H, CAS #97281-48-6, Lipoid GmbH, Ludwigshafen, Germany) as the primary phospholipid source. 13% EAA liposomes were dialyzed for five days in a membrane bag (Spectra/Por 5 RC Dialysis Membrane Tubing, 12 to 14 kDa, Spectrum Chemical Manufacturing Corp., New Brunswick, NJ) immersed in artificial seawater (35 ppt) with daily water changes before being added to LCP formulations.

Complex particles: Complex particles were made using a modified apparatus described in Hawkyard et al. (2019) with the addition of a 4-L pressure pot (Figure 2.1). The basal open formula dry ingredients were first weighed in and finely ground (Ultra Centrifugal Mill ZM 300, Retsch GmbH, Haan, Germany). Liposomes were added to LCP formulations at a 30% w/v (as-

is) inclusion rate. A 10% alginate (Alginic acid sodium salt from brown algae, low-viscosity A-2158, CAS#9005-38-3, Sigma-Aldrich, St. Louis, MO) solution was prepared approximately 24 h in advance and included as an organic binder to achieve a final alginate concentration of 1% w/v (as-is; approximately 3.27% DM) of the LCP (by formula). A concentration of 0.5% hollow silica microspheres were included in all LCP formulations based on previously derived experimental data. Hollow silica microspheres were not included in MEM diets because they would be destroyed during extrusion and spheronization. LCP mash was pushed by means of low-pressure nitrogen gas through the spray apparatus (Air Atomizer, ¼ JBCJ SS fitted with 60100 brass fluid cap for air atomizing spray, Spraying Systems Co., Pomona, CA) and droplet formation was facilitated using 10 to 15 psi atomizing air. The resultant particles were collected in a bath containing an 0.5% $CaCl_2$ (≤7.0 mm, ≥93.0%, CAS #10043-52-4, Sigma-Aldrich, St. Louis, MO) solution. LCP were filtered over stacked 12-inch sieves to isolate the desired particle size range for California yellowtail larvae at this stage (600 μm to 1.2 mm). The particle mass was stored in 250 mL plastic sample containers with no more than 100 g (as-is) of particles in each container. Five milliliters of 10% acetic acid (glacial ≥99%, CAS #64-19-7, Sigma-Aldrich, St. Louis, MO) was added each container to serve as a preservative during storage. Diets were then shipped to Hubbs-SeaWorld Research Institute (San Diego, CA) to be fed to California yellowtail larvae.

3.3.1.3 Production of microextruded marumerized (MEM) diets

Microextruded marumerized particles were produced as follows: All pre-milled dry ingredients were combined and mixed using a commercial stand mixer (KitchenAid, Inc., Benton, Harbor, MI) for 10 minutes, prior to the addition of fish oil. MEM diets were produced using an open formula like that of LCP, but with an initial moisture content in the mash of

approximately 30% w/v to facilitate the formation of noodles during extrusion. The mash was extruded through a LCI MG55 extruder (LCI Corporation, Charlotte, NC) using a 700 µm die rotating at 55 rpm to form noodles. Approximately 200 g of noodles at a time were placed in a marumerizer (QJ 230 T-2, LCI Corporation, Charlotte, NC) with a rotational speed of 1000 rpm for two minutes. The resultant particles were then dried with a fluidized bed dryer (Model 501, Sherwood Scientific, Cambridge, UK) for two hours to achieve a moisture content of between 7 and 10.5% (HE73 Moisture Analyzer, Mettler Toledo, Greifensee, Switzerland). The dried MEM particles were sifted through a series of sieves until the desired particle size range (700 µm to 1.2 mm) was isolated. Particles were stored in sealed containers and shipped to Hubbs-SeaWorld Research Institute for the growth trial.

3.3.2 Growth trial using California yellowtail (*Seriola dorsalis*) larvae

3.3.2.1. Sinking rate trial

Sinking rates (cm sec^{-1})were compared between the LCP, MEM, and Otohime diets used in the growth trial with CYT larvae. Sinking trials were conducted in triplicate using a 17-inch tall 2000-mL graduated cylinder filled with 35 ppt seawater at Hubbs-SeaWorld Research Institute. One gram of diet was suspended in 10 mL of seawater and suspended in 5 mL of seawater before being added to the full cylinder. A timer began at the point in which suspended particles touched the surface of the cylinder and stopped at the point in which the first particle touched the base of the cylinder. These time values were turned into sink rates (cm sec^{-1}) and were statistically compared using an ANOVA across diet treatments.

3.3.2.2 Larval rearing

California yellowtail (*Seriola dorsalis*) larvae were obtained from F1 broodstock housed at the Hubbs-SeaWorld Research Institute (HSWRI). Larvae were initially reared through 15

days post-hatch (dph) in a single 1,600-L black conical bottom tank. From 2 to 8 dph, larvae were fed Ori-Green (Skretting, Tooele, UT) enriched rotifers (*Brachionus plicatilis*). On 6 dph, larvae were co-fed with second-instar *Artemia* (*Artemia franciscana*) enriched with S.Presso (Inve Aquaculture, Salt Lake City, UT) in addition to the rotifers. Larvae were weaned onto commercial diet Otohime (Marubeni Nisshin Feed Co., Ltd., Tokyo, Japan) until approximately 25 dph when they were transferred to their respective experimental tanks.

3.3.2.3 Experimental design

On 25 dph, larvae were stocked into a recirculating system containing twenty 350-L tanks. They were stocked at 800 larvae $tank^{-1}$ with four replicate tanks dedicated to each of the three diet types (LCP, MEM, and Otohime). Seawater was originally obtained from the Mission Bay (San Diego, CA) and maintained at 21 ºC. The flow provided to each tank was maintained at a rate of 5 L min^{-1}. A 700-micron screen was placed over the standpipe of each tank to prevent larval escape, and two air stones were placed in each tank to help circulate and aerate water.

3.3.2.4 Feed regime and sampling

Larvae were reared in the experimental system from 25 dph to 53 dph and fed either 1) open formula LCP containing 13% EAA liposomes (“LCP"), 2) MEM containing free EAA and produced with an open formulation (“MEM”) or 3) a commercial microparticulate diet containing approximately 50.0% protein, ≥ 10% crude fat, and 17.0% crude ash (“Otohime”). MEM and Otohime diets were fed once per day so feeding response could be observed, then continuously throughout the rest of the 24 h period using an automatic feeder. Experimental LCP diets were fed to larvae three times per day, where rations were pre-suspended in 30 mL (35 ppt) seawater before being delivered to each tank to reduce clumping and create a more even dispersion. Starting at 25

dph, larvae in each treatment were initially offered a total of 10 grams of feed (as-is) tank^{-1} day^{-1}. On 33 dph the daily ration was increased to 20 g of feed tank^{-1} day^{-1}, and then 30 g tank^{-1} day^{-1} from 46 dph until the end of the trial. Larvae were co-fed with *Artemia* until 34 dph after which they were completely reliant on their respective microdiets. LCP were delivered within a size range of 600 µm to 1.2 mm for the entire duration of the trial. Larvae initially received MEM particles around 700 µm[7] until 36 dph before being transitioned onto a 1 mm particle for the duration of the trial. Larvae fed Otohime were given C1 (600 to 850 µm) initially and were weaned onto C2 (850 µm to 1.2 mm) at 36 dph.

Ten larvae from each tank were sampled at six different time points to assess feeding incidence. One hour post-feed, ten larvae were sampled from each tank and euthanized in buffered MS-222 (Syncaine Fish Anesthetics [MS-222], Syndel, Ferndale, WA) in accordance with institution animal care and use committee approved protocols. Larvae were observed under a microscope (MZ1, Leica Microsystems, Inc., Bannockburn, IL) and gut contents were individually dissected to identify the presence or absence of feed. Feeding incidence for each tank was determined by dividing the number of larvae observed with feed in the gut by total number of larvae sampled (N=10). Photographs of larval gut dissections were also taken to coarsely assess the early digestion of the different microdiets. Standard lengths were measured once weekly by subsampling 20 larvae tank^{-1} over the 28-day trial. Standard length was measured manually using a ruler to the nearest 0.1 mm. Larval samples from each tank were then pooled, dried in an oven

[7] MEM particles were made using an extruder die with 700 µm holes and passed through 850 µm and 600 µm sieves to isolate the correct size fraction. We use 700 µm as a general title for this size range. 1.0 mm particles were made using an extruder die with 1.0 mm holes and were passed through 1.0 mm and 1.2 mm sieves post-production.

at 65C for 48 h, and then weighed on a pre-dried and pre-weighed GF/C filter. Condition factor (K), was calculated as follows:

$$K = (DW \div SL^3) \times 100$$

Where: K is Fulton's condition factor, DW is the average individual larval wet weights measured for each tank at the end of the trial and SL is the average individual standard length measured for each tank at the end of the trial. The final proportion of surviving larvae in each tank was calculated upon termination of the trial as:

Survival for each treatment was also calculated based on numbers of surviving larvae at the end of the trial. Survival numbers were measured on an individual tank basis and average survival for each treatment was calculated over four replicate tanks. 800 larvae per tank was used as the number of larvae originally stocked for each calculation.

$$Survival\ (\%) = \frac{\#\ of\ surviving\ larvae\ upon\ termination\ of\ the\ trail\ per\ tank}{\#\ of\ larvae\ originally\ stocked\ per\ tank} \times 100$$

3.3.2 Feeding incidence trials with California yellowtail (*Seriola dorsalis)* larvae

Two subsequent acceptability trials were conducted to attempt to how various particle formulations affect the uptake rates of LCP by CYT larvae. The following experimental treatments and design are as follows.

Trial 1 All LCP diets were made with an open formula base using methodology outlined in 3.3.1.2 LCP and contained either 13% EAA liposomes, empty liposomes, or 20% casein hydrolysate liposomes added at an inclusion rate of 30% w/v of the overall particle (as-is). LCP

were sprayed into collection baths containing 0.5% w/v CaCl2 and either 0.1% v/v sodium benzoate (ReagentPlus, 99%, CAS #532-21-1, Sigma-Aldrich, St. Louis MO) or 2% v/v acetic acid (glacial, ACS reagent, ≥99.7%, CAS #64-19-7, Sigma-Aldrich, St. Louis, MO) to assess the effect of preservative agents on feed ingestion rates. Both acetic acid and sodium benzoate (C_6H_5COONa) have been widely used in the food industry to potentially prevent or delay food spoilage (Shahmohammadi et al., 2016). LCP diet treatments were as follows: 1) LCP with empty liposomes ("Empty lipo LCP"); 2) LCP with 3% Stim mix (1% glycine, 1% alanine, 1% betaine w/v) liposomes sprayed into $CaCl_2$ and sodium benzoate ("3% Stim mix LCP in $CaCl_2$/NaBenz"); 3) LCP with 13% EAA liposomes sprayed into $CaCl_2$ and acetic acid ("13% EAA LCP in $CaCl_2$/AcetA"); 4) LCP with 13% EAA liposomes sprayed into $CaCl_2$ and sodium benzoate; and 5) LCP containing 20% casein hydrolysate liposomes sprayed into $CaCl_2$ and sodium benzoate ("20% Cas LCP in $CaCl_2$/NaBenz"). LCP treatments were compared to Otohime (C1) as well as MEM particles produced with the same open-formula ingredients (by formula on a dry weight basis) as those used for the LCP containing empty liposomes. The MEM diet also included alginate and PL90H at similar concentrations when compared to LCP so that these could be directly compared.

At 35 dph, CYT larvae reared from captive broodstock at HSWRI were stocked into an experimental recirculating system containing twenty-four 350-L tanks. Due to space constraints within the experimental system, trial 1 took place over a two-day period wherein two replicate tanks from each treatment were fed and sampled each day, for a total of four replicate tanks per treatment. "Day" was initially included in the statistical model; however, it was found to not have a statistically significant effect on feed uptake rates and was removed (i.e. all treatments were statistically compared to each other, regardless of what day the feeding took place). The

same experimental protocol took place on both days and was as follows: two randomly selected replicate tanks were assigned to each experimental diet and were each stocked at 20 larvae per tank. Larvae were acclimated to the system for 24 h, after which 1 g of respective LCP diet was delivered to each tank. After one hour, the larvae in each tank were euthanized using MS-222. Manual gut checks were conducted to assess feeding incidence, as described in Section 3.3.2.3 Feeding incidence was calculated as follows:

$$FI = (F \div T) \times 100$$

Where FI is the feeding incidence (%), F is the number of larvae in each tank with food observed in their gut and T is the total number of larvae sampled per tank.

Trial 2: We investigated the effects of liposome type (i.e. liposomes made with different core solutions) and particle base on feed ingestion rates by larvae. LCP diets were all prepared with 2% w/v alginate solution and then basal ingredient mixture that was either 1) based on the open formula developed for marine fish larvae, 2) derived from a milled commercial microdiet (Otohime C1), or 3) produced without basal ingredients and formulated similar to those originally reported by Hawkyard et al. (2019). Each of these were produced with liposomes containing either a composite mixture of 1% betaine, 1% alanine, and 1% glycine ("3% Stim mix liposomes") or distilled water ("empty liposomes"). All LCP diets were produced using methodology presented in Section 3.3.1.2. LCP diets made for both trials were sieved to isolate particles between 700 μm to 1 mm and were produced at the University of Maine, Orono campus before being shipped to Hubbs-SeaWorld Research Institute (San Diego, CA) for the uptake trial.

Experimental design, tanks, and sampling protocol was similar to that of Trial 1. However, larvae were aged 31 dph during trial 2 and the experiment was completed on a single day. CYT larvae were stocked at 20 larvae $tank^{-1}$ into a twenty-four tank experimental system. Each tank received 1 g of experimental feed. Larvae were euthanized one hour post-feed and manual gut checks were conducted to assess feeding incidence for each treatment.

3.3.3 Statistical analysis

Statistical analyses were performed using JMP software (JMP Pro Version 17.1.0). ANOVA assessments were used to evaluate significant differences in growth, condition factor, and feed ingestion rates of larvae between treatments. Pairwise T-tests were used to make comparisons between treatments at sampling points of interest (i.e. endpoint sampling between MEM and Otohime treatments). Assumptions of equal variance were verified where necessary using Levene's test and normality was checked graphically. In the case of non-normality, data were transformed using an arcsine square root transformation before analyses were run. Tukey's HSD was used as a post-hoc test after ANOVA and regression analyses. Connecting letter reports were used to identify significant differences between treatments and are delineated where necessary.

3.4 Results

3.4.1 Sinking rate trial

A statistical comparison of sinking rates (cm sec^{-1}) of the three microdiets used in the growth study showed significantly different sinking rates between the three microdiet treatments (ANOVA, $p<0.0001$; Figure 3.1). LCP sinking rates were lower than those of Otohime and MEM diets (Tukey's HSD, threshold $p<0.05$).

3.4.2 Growth trial using California yellowtail (*Seriola dorsalis*) larvae

3.4.2.1 Larval growth and survival

Results from a one-way ANOVA reveals significant differences in standard lengths (mm) between treatments (ANOVA, $p<0.0001$; Figure 3.2). Standard lengths of larvae at 39 dph were significantly different between the three microdiet treatments (ANOVA, $p<0.0001$). An endpoint comparison of larval standard lengths on 53 dph revealed no significant differences between MEM and Otohime treatments (T-test, $p=0.5008$). Data was not collected on larvae fed LCP after 39 dph due to a large mortality event, which caused the termination of that treatment at 42 dph. Thus, LCP treatments are not represented in endpoint statistics in all subsequent analyses.

Average individual dry weights (mg) of larvae fed LCP were significantly different from those fed MEM and Otohime (ANOVA, $p=0.0027$; Figure 3.3). At 39 dph, individual larval dry weights were significantly different between larvae fed LCP when compared to those of fed Otohime or MEM (ANOVA, $p=0.0107$, Tukey's HSD). In a comparison of endpoint dry weights of larvae fed Otohime or MEM at 53 dph, no significant differences were found between treatments (T-test, $p=0.6727$).

The growth and survival metrics assessed during this study are presented in Table 3.3. Condition factors of larvae were not statistically different between treatments (ANOVA, $p=0.7637$). However, at the 39 dph sampling point statistical differences in condition factor were observed between Otohime, MEM, and LCP treatments (ANOVA, $p<0.0001$). Upon conclusion of the trial on 53 dph, larvae fed MEM and Otohime showed no statistical differences in condition factor between the two treatments (T-test, $p=0.5580$).

Endpoint survival, measured on 53 dph, was significantly different between the Otohime treatment (92.8 ± 4.3%) when compared to the MEM treatment (79.6 ± 2.22%; T-test, p=0.0036). Endpoint survival was not assessed for LCP because those treatments were terminated on 42 dph.

3.4.2.2 Feeding incidence during the growth trial

Lower feeding incidences were observed in LCP treatments when compared to those observed in Otohime and MEM at every sampling point until 35 dph sampling point (ANOVA, $p<0.05$; Figure 3.4). However, at the 40 dph sampling point there was no difference in feeding incidence between treatments (ANOVA, p=0.0737). Otohime and MEM treatments did not display significant differences at this time point (Tukey's HSD). At 50 dph, the last sampling point for feeding incidence, larvae in both MEM and Otohime treatments displayed a feeding incidence rate of 100%.

3.4.3 Feeding incidence trials with California yellowtail (*Seriola dorsalis*) larvae

Trial 1: Feeding incidence was significantly different between treatments (ANOVA, $p<0.0001$; Figure 3.5). Since this trial took place over two days, a statistical analysis was run on the impact of 'Day' on feeding incidence. The impact of 'Day' was not found to be significant (T-test, p=0.7068) which allowed all results to be compared regardless of what day feeding took place. Feeding incidence rates of MEM and Otohime were statistically different from each other and all LCP treatments (ANOVA, $p<0.0001$). All LCP treatments displayed no statistical differences between each other (Tukey's HSD, significance threshold $p<0.05$). Mean feeding incidence (Mean ± SD%) was the higher in fish offered Otohime (85.7 ± 8.3%) when compared to those offered MEM particles (60.7 ± 3.9%; Tukey's HSD).

Trial 2: The main effect 'Liposome type' (i.e. 3% Stim mix liposomes or empty liposomes) was not significant (Two-way ANOVA, p=0.9091). Main effect 'Particle base' (i.e. Alginate, MCD, or OF) was not significant (p=0.4049). The interaction effect 'Liposome type*Particle base' was significant (p=0.0087). Statistics were done using Arcsine square root-transformed data, but raw data was used for graphing purposes.

3.5 Discussion

In this study, there was no significant difference in the final endpoint of growth metrics including standard length, individual dry weight, and condition factor between larvae fed MEM and Otohime. Feed ingestion rates remained comparable between the two treatments through the duration of the growth trial. These two diets performed similarly in terms of larval growth over time. In a similar study by Orihuela et al. (2018), MEM particles were compared to Otohime in a growth trial using fine flounder *(Paralichthys adspersus*) larvae. The results from this trial showed that the dry weight and total length were significantly higher ($p<0.05$) for larva fed Otohime than those fed MEM and no significant differences in survival (%) were found between larvae fed each diet. In comparison, our experiment with CYT larvae showed no statistical differences in length and dry weight between treatments; however, there was a significant difference between survival rates. Unfortunately, survival was lower when larvae were fed MEM when compared to those fed Otohime. While the lower survival in MEM was not ideal, the benefits of having an open formula reference diet for future research purposes likely outweighs the drawback of somewhat reduced survival. Overall, these are very promising results given the early stages of this particle type and formulation. Likewise, this microdiet type appears to be a viable open-formula reference diet for use in early-stage CYT nutritional studies. There are only few instances where an open formula has been used, and even fewer when used in the MEM

form. Stuart et al. (2018) applied this same open formula to the PARA (particle-assisted rotational agglomeration) process to make microdiets which were used to deliver taurine to CYT larvae. In their abstract advocating for the need for an open-formula reference diet, Rust et al. (2015) mentions the nutrient composition of open-formula MEM and PARA diets and compares these with the nutrient composition of *Artemia* and rotifers. However, they did not mention details of the open-formula MEM's performance when fed to fish larvae, evidence of a gap in research that we addressed in this study. Future research is needed to optimize MEM so that it surpasses Otohime in terms of growth metrics like length, dry weight, and condition factor as well as long-term survival. Other research paths may work to adjust formulation and characteristics of MEM to make optimize their use for other marine finfish species.

California yellowtail larvae offered LCP diet showed poorer growth metrics when compared to those provided with MEM or Otohime diets. This was evidenced in statistically different standard length, individual dry weight, and condition factor of larvae fed LCP in comparison to the other two diets at 39 dph. The poor growth in the LCP treatment could be related to low feeding incidence in LCP treatment observed throughout the trial and compared statistically at 39 dph across all treatments. The low feed uptakes rates observed in the LCP treatment were not likely caused by the sink rates of the particles. In chapter 2, we found that both gas forming agents and silica microspheres can be used to adjust the sink rate of LCP. This resulted in a significantly slower sink rate for LCP diets than MEM and Otohime (Figure 3.1). However, this did not seem to increase ingestion rates in LCP-fed larvae. Even though these methods of buoyancy manipulation did not result in high ingestion rates in the present studies, these methods may have utility in existing microparticulate diets and provide tools for future LCP applications.

Additionally, it should be noted that LCP diet had a different nutrient composition on an as-is basis in comparison to MEM and Otohime. LCP were formulated to have a moisture content of approximately 70%, while MEM and Otohime diets had an average moisture content of 9.88% (as measured by HE73 Moisture Analyzer, Mettler Toledo, Columbus, OH) and 6.5%[8], respectively. Equal amounts of each diet were offered to larvae on an as-is basis. However, feed consumption rates are important and would ultimately dictate the quantity of nutrients ingested per unit of feed. If feed ingestion rates were equal across treatments, larvae fed LCP would have received approximately 1/3rd the amount of nutrients on a dry-weight basis for the same amount of MEM or Otohime ingested. In theory, this may be a plausible explanation for why larvae fed LCP grew more slowly, assuming that LCP, MEM, and Otohime were consumed at similar rates on an as-is basis.

Another potential theory for low ingestion rates observed in LCP treatments during the growth trial may be partially due to the use of acetic acid as a post-production preserving agent during storage. However, upon investigation with a subsequent acceptability trial (Trial 1), we found no difference in uptake rates between larvae fed LCP containing acetic acid and sodium benzoate, an alternative preserving agent. Feeding incidence remained low regardless of the used and while these compounds do not appear to be the primary reason for the low feeding incidences observed, they can neither be ruled out until more palatable formulations of LCP can be tested.

Low ingestion rates of LCP were also observed in the second acceptability trial which compared LCP produced with different nutrient bases (i.e. OF, MCD, or Alginate) with and

[8] Moisture content (%) for Otohime (C2) was derived from product information sheet for provided by distributor https://reedmariculture.com/collections/otohime-pellet-feeds.

without liposomes containing a stimulant mix. The low feeding incidence observed was consistent with those of the first acceptability trial, where LCP treatments containing 13% EAA, empty, and 20% casein hydrolysate liposomes did not produce uptake rates that were significantly different from one another. These results run counter to those observed by Hawkyard et al (2019), whereby Alginate-LCP containing 3% Stim mix liposomes resulted in higher feeding incidence by CYT larvae than those containing empty liposomes. This is particularly troubling, in that the conditions of the trials, including the tanks, fish age, and formulation (of Alginate-LCP with and without stimulants) were nearly identical to those of the previous study. Additionally, amino acids such as betaine, alanine, and glycine, among others, are known chemoattractants for many fish species and have been previously found to elicit a greater feed response when included in feed (Carr et al., 1996; Polat and Beklevik et al., 1999; Hawkyard et al., 2019). However, this phenomenon was not observed by larvae in this study.

Aside from low ingestion rates, the digestibility of LCP may have also inhibited the utilization of this diet by the larvae. Though digestibility was not quantitatively evaluated, visual observations during feeding incidence checks showed that LCP diets were not well-digested. This was evidenced by observing large, in-tact particles in the gut of CYT larvae fed LCP (Figure 3.7). In contrast, MEM and Otohime particles appeared to be more well-digested, evidenced by homogenous rather than granular gut contents. This observation was peculiar considering that both LCP and MEM were produced with the same ingredients. While both diets contained alginate, the alginate was not cross-linked in the MEM diets, via Ca^{2+} bridging, as they were in LCP diets. One possible explanation for these results is that the simple inclusion of alginate in the formulation may not be an issue, but cross-linked alginate via the Ca^{2+} in the collection bath may inhibit digestion by CYT larvae. Another potential explanation and notable

caveat to these observations is that these individual gut checks were conducted only 1 h post-feeding event. There is the potential that ingested diets could have been in early stages of digestion, or that LCP are slower to digest than MEM and Otohime and slower digestion was not observed at the point of sampling.

In sum, the largest hindrance to successful growth of CYT larvae fed LCP seemed to be its low acceptability. Unfortunately, it is still unclear why acceptability of LCP is so low among CYT larvae, despite having tested multiple formulations. Further investigation of ingredients, formulation, and production methods is necessary to improve performance with marine finfish larvae. It is known that larval feeding behavior is influenced both by the ontogeny and developmental stage of the larvae as well as diet characteristics including the organic properties, dimensions, movement characteristics (Rønnestad et al., 2013). Microdiet shape my influence food capture, whereas taste and texture influence ingestion (Stradmeyer, 1989). This suggests there may be some attribute, whether it may be mouth feel or texture, taste, or some unknown characteristic, about LCP production or formulation that is influencing ingestion rates by larvae. Further studies are needed to better understand this issue and its applicability to a broader range of fish species.

3.6 Conclusion

The goal of this study was to evaluate the performance of two open formula microdiet types, liposome-containing complex particles (LCP) and microextruded marumerized (MEM) particles, in trials with California yellowtail (*Seriola dorsalis*) larvae and compare them with a commercial microdiet (Otohime) commonly used for marine fish larvae. The results of this research showed promise for the use of MEM particles as an open-formula reference diet due to its comparable performance to Otohime based on length, weight, feed uptake, and condition

factor of CYT larvae observed in the growth trial. This result will benefit industry as the open formula ingredient mixture that can be adopted by commercial feed companies and is a very useful research tool in marine larvae fish nutrition studies. Future research may work to adjust the open formula MEM formulation to fit the nutritional needs of other commercial finfish species and assess uptake and growth on these diets using similar trials. In addition, the open formula ingredient mixture may be used as the basis for additional particle types in the future.

With respect to LCP diets, further investigation is needed to understand the limitations of this particle and why it is ingested at such low rates by CYT larvae, even when compared to diets formulated with similar ingredients. Future trials should also investigate the application of LCP with other commercial finfish and shellfish species.

Overall, this study resulted in a better understanding of the benefits and drawbacks of these particle types and led to many advancements with respect to production, formulation, and scaling of both MEM and LCP diets. With respect to MEM diets, these are the first results suggesting that open-formula MEM particles provide a suitable alternative for the commercial diet Otohime, currently one of the top performing commercial diets. Future research with MEM diets should focus on optimizing buoyancy, nutrient composition and nutrient delivery as well as application and adaptation for other finfish species.

3.7 Acknowledgements

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purpose of providing specific information and does not imply recommendation or endorsement by the United States Department of Agriculture (USDA). We are grateful for the help and assistance from researchers and technicians at Hubbs-SeaWorld Research Institute for the use of their labor, facilities, expertise, and resources.

TABLES

Table 3.1 Formulation of ingredients for MEM and LCP diets (grams as-is by formula) for growth trial with Seriola dorsalis larvae.

	MEM w/ EAA mix	LCP w/ 13% EAA liposomes
Ingredient	*Ingredient inclusion rate (g as is, by formula)*	*Ingredient inclusion rate (g as-is by formula)*
Squid meal	23.93	11.05
Krill meal	15.54	7.20
Fish oil	7.75	3.65
Wheat gluten meal	5.41	2.53
Vitamin premix (ARS mix)	1.48	0.52
Dicalcium phosphate	1.48	0.52
Taurine	0.53	0.00
Stay-C	0.15	0.05
Astazanthin	0.07	0.07
EAA mix (as-is)	3.16	0.00
Alginate (10% solution)	0.00	10.00
13% EAA liposomes	0.00	29.56
PL-90H	6.96	0.00
D.I. water	31.35	29.40
10% Taurine liposomes	0.00	4.93
Silica beads	0.00	0.50
Alginate (powder)	2.11	0.00
Yttrium Oxide	0.074	0.029
Total added (g)	**100.00**	**100.00**

Table 3.2 Nutritional profile of MEM with EAA mix and LCP with 13% EAA liposomes on as-is (g) and dry matter (%) basis, by formula.

	MEM w/ EAA mix		LCP w/ 13% EAA liposomes	
Diet nutrient summary	***As-is (g)***	***DM (%)***	***As-is (g)***	***DM (%)***
Moisture (%)	34.93	0.00	69.56	0.00
Crude protein (%)	35.04	53.85	16.21	53.24
Crude lipid (%)	19.38	29.78	9.05	29.71
Carbohydrate (%)	2.11	3.24	1.00	3.29
Ash (%)	4.46	6.85	2.40	7.89
Total EAA (%)	8.18	12.57	3.73	12.26
Phospholipid (%)	8.65	13.29	4.01	13.17
Taurine (%)	1.04	1.59	0.50	1.64
Energy (kcal)	2428.93	3732.94	1124.41	3693.69
Fat/Lipid, total (%)	12.54	19.28	5.86	19.27
EAA supplement (%)	2.50	3.84	1.10	3.61
Alginate (%)	2.11	3.24	1.00	3.29
Dicalcium phosphate (%)	1.48	2.27	0.52	1.70
Vitamin premix (%)	1.48	2.27	0.52	1.70
Astaxanthin	0.07	0.11	0.07	0.23
Fish oil (%)	7.60	11.67	3.57	11.74
PL 90 supplement (%)	6.82	10.48	3.16	10.39
Yttrium oxide (%)	0.07	0.11	0.03	0.10
Silica beads (%)	0.00	0.00	0.50	1.64

Table 3.3 Summary table of metrics used to evaluate and compare performance of Otohime, MEM, and LCP diets in growth trial with *Seriola dorsalis* larvae.

	Microdiet treatments					
	Otohime		**MEM**		**LCP**	
Growth metric	*39 dph*	*Endpoint*	*39 dph*	*Endpoint*	*39-40 dph*	*Endpoint*
Avg. standard length (mm)	31.34±3.79 mm	42.86±4.67 mm	30.45±3.42 mm	43.43±5.81 mm	22.34±2.15 mm	x
Avg. individual dry weight (mg)	190.24±127.33 mg	285.65±18.65 mg	189.56±103.29 mg	294.95±37.53 mg	44.41±73.50 mg	x
Feeding incidence (%)	100±0.00%	100±0.00%	97.5±5.00%	100±0.00%	77.5±22.2%	x
Condition factor	2.11	1.71	1.977	1.76	1.70	x
Survival (% of initial stocked)	x	92.8±4.3%*	x	79.6±2.2%*	x	1.7±0.9%*

FIGURES

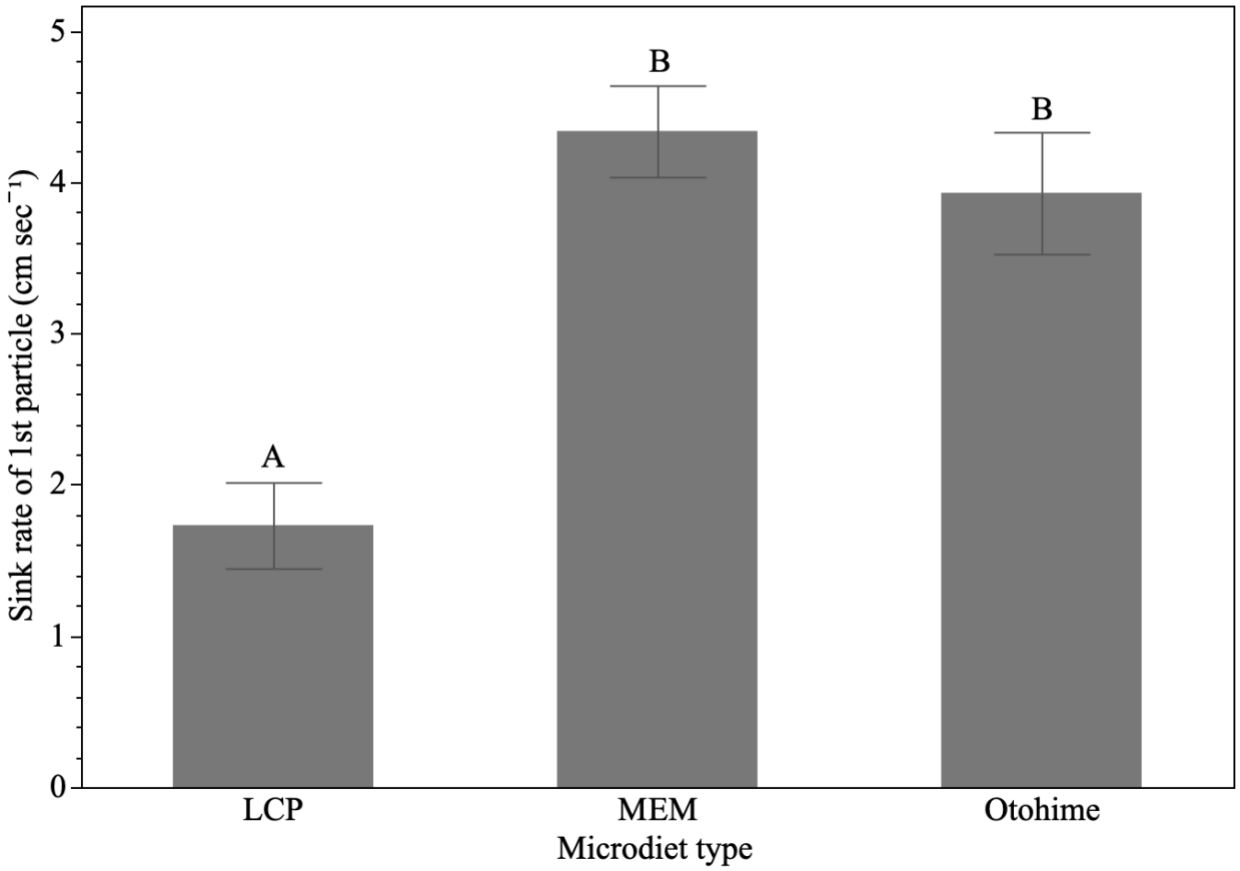

Figure 3.1 Bar chart depicts sink rate (cm sec^{-1}; mean ± 1 SD) of first particle for three different microdiet types used in growth trial with CYT larvae. Connecting letters report depicts significant differences between treatments (Tukey's HSD, significance threshold $p<0.05$).

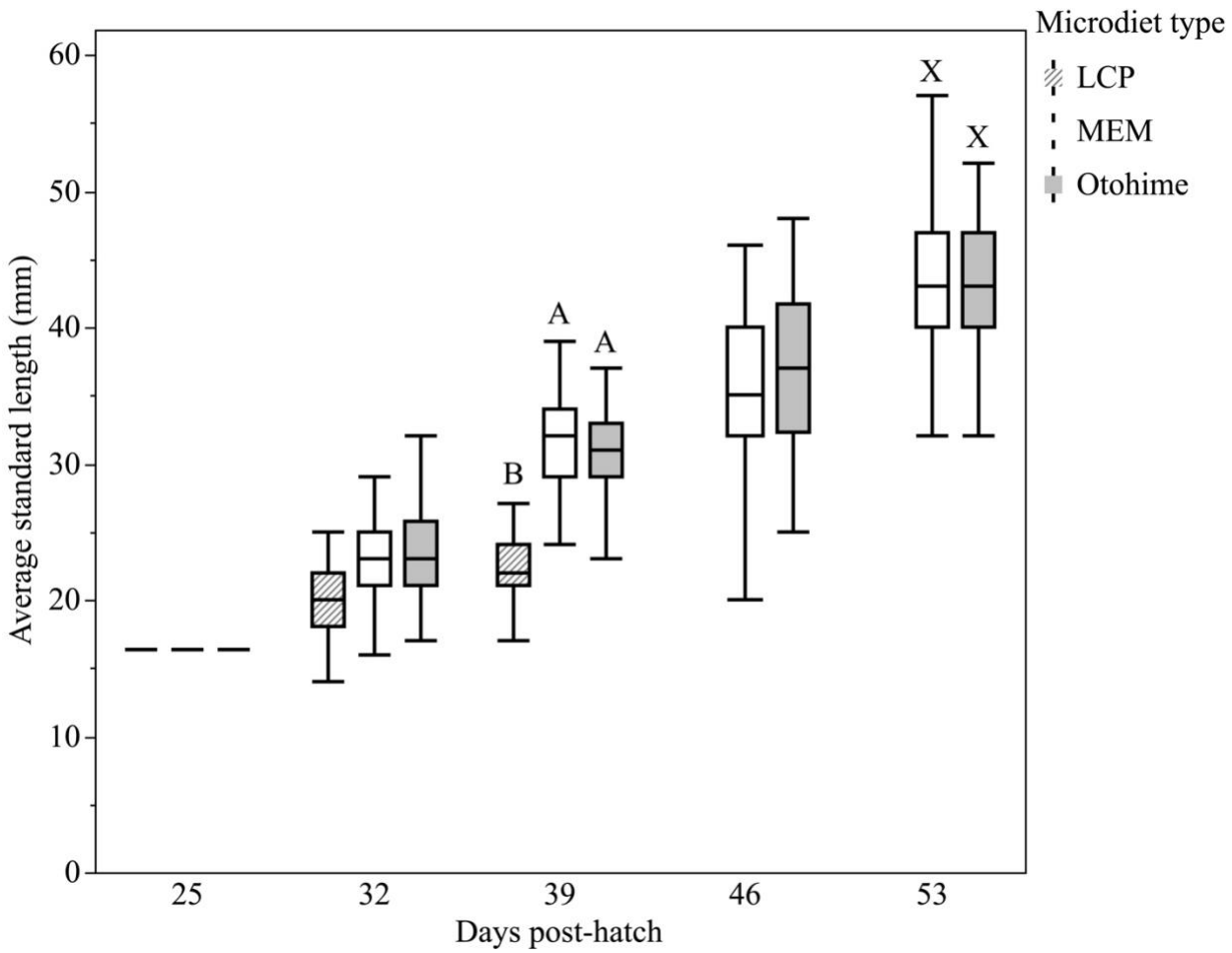

Figure 3.2 Standard length (mm; mean ± 1 SD) of CYT larvae measured throughout the 28-day feeding trial. Graph depicts median standard length, upper and lower quartiles (box), and maximums and minimums (whiskers) for each treatment at each dph. Significant differences between treatments are denoted graphically using connecting letters (Tukey's HSD). Different letter codes denote statistical comparisons at individual sampling points (i.e. 39 dph= A, B, C; 53 dph= X, Y, Z).

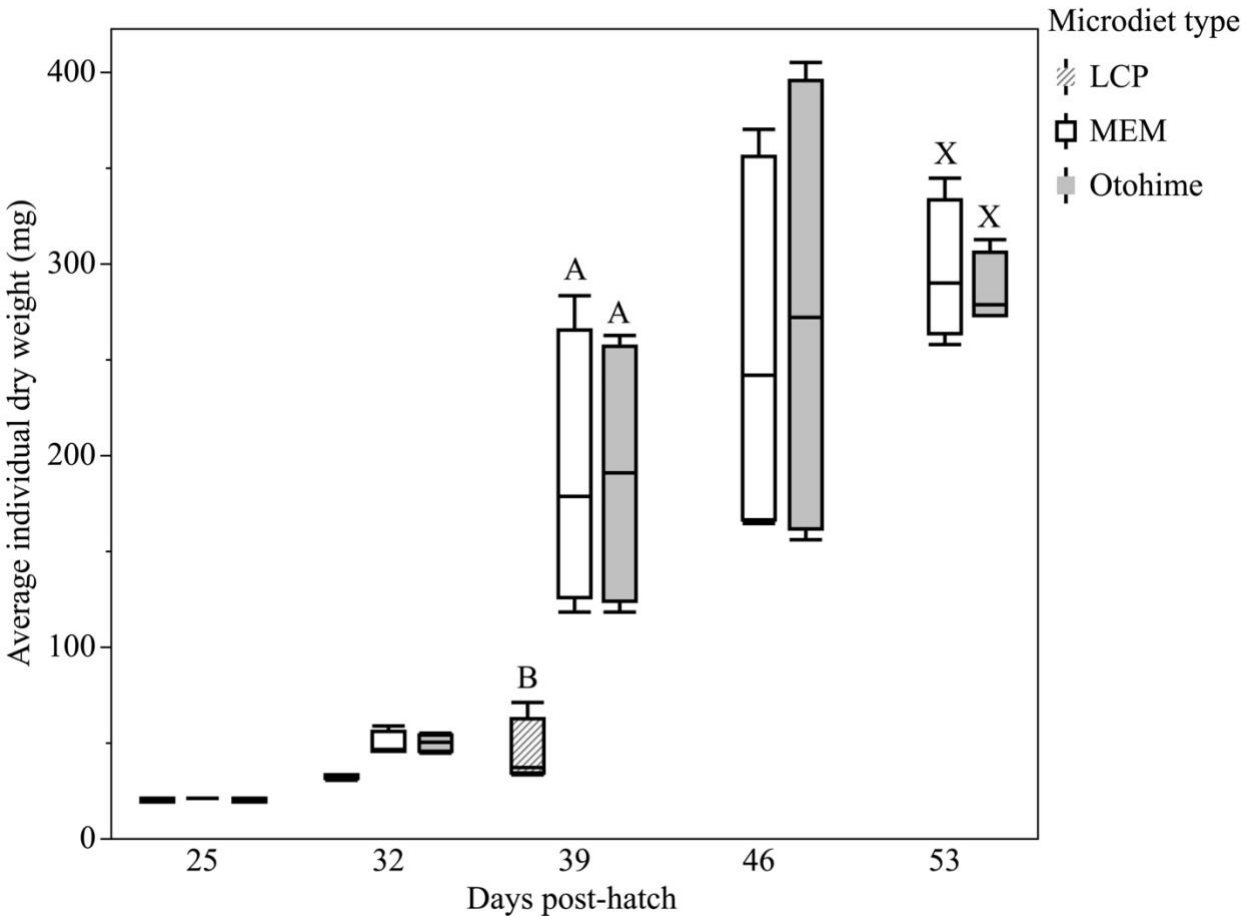

Figure 3.3 Individual dry weight (mg fish^{-1}) of CYT larvae measured throughout the 28-day feeding trial. Graph depicts median dry weight, upper and lower quartiles (box), and maximums and minimums (whiskers) for each treatment at each dph. Significant differences between treatments are denoted graphically using connecting letters (Tukey's HSD). Different letter codes denote statistical comparisons at individual sampling points (i.e. 39 dph= A, B, C; 53 dph= X, Y, Z).

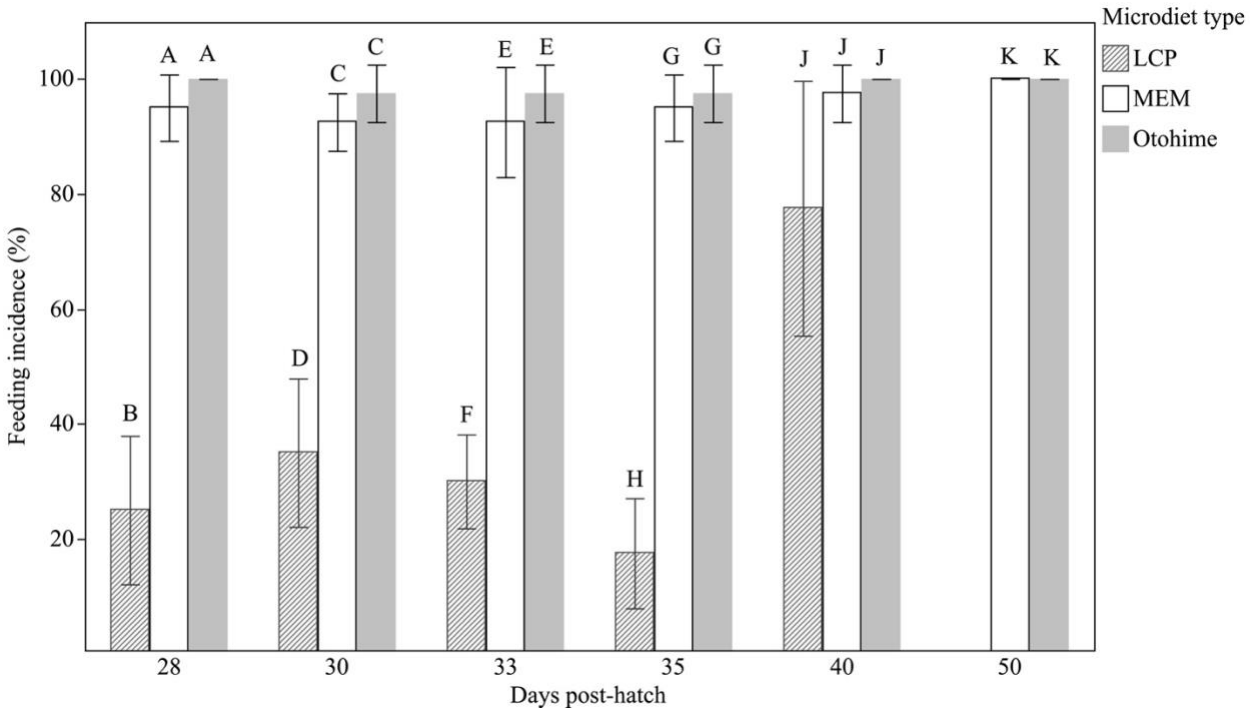

Figure 3.4 Average percentage of CYT larvae observed with feed in gut (feeding incidence, %) during feeding sampling (10 fish tank^{-1} subsample^{-1}) over 53 day feeding trial. Bars depict feeding incidence per treatment diet with standard deviation shown (zig-zag pattern, LCP; dots, MEM; and solid, Otohime). Significant differences between treatments are denoted graphically using connecting letters (Tukey's HSD). Different letter codes denote individual statistical comparisons at marked sampling points throughout the trial (i.e. 28 dph =A, B; 30 dph= C, D; 33 dph= E, F; 35 dph=G, H; 40 dph= J; 50 dph= K).

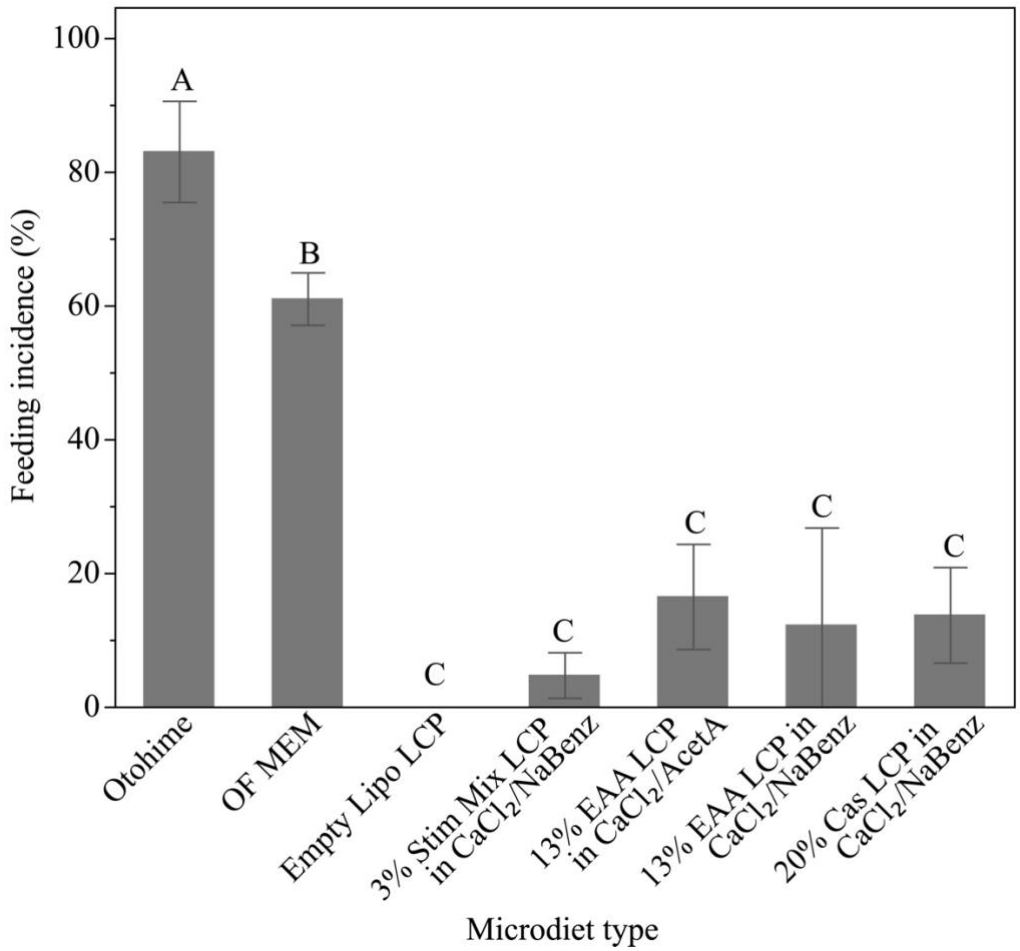

Figure 3.5 Percentage of fish observed with feed in gut (feeding incidence; %) 1 h post-feeding. Bar chart depicts uptake rates of sample larvae per microdiet treatment (Mean $\pm$ 1 SD). Connecting letters report depicts key differences in mean feed uptake rates between treatments (Tukey's HSD, threshold $p<0.05$). Key OF= open formula; Empty lipo = liposomes containing distilled water, 3% Stim mix = liposomes contained 1% glycine, 1% alanine and 1% betaine (w/v); EAA = essential amino acid [liposomes]; 20% Cas = 20% casein hydrolysate [liposomes], Alg-LCP = LCP produced with only alginate in the matrix (no gelatin or nutrient mix); AcetA = LCP were sprayed into acetic acid bath with $CaCl_2$; NaBenz = LCP were sprayed into a bath containing sodium benzoate and $CaCl_2$.

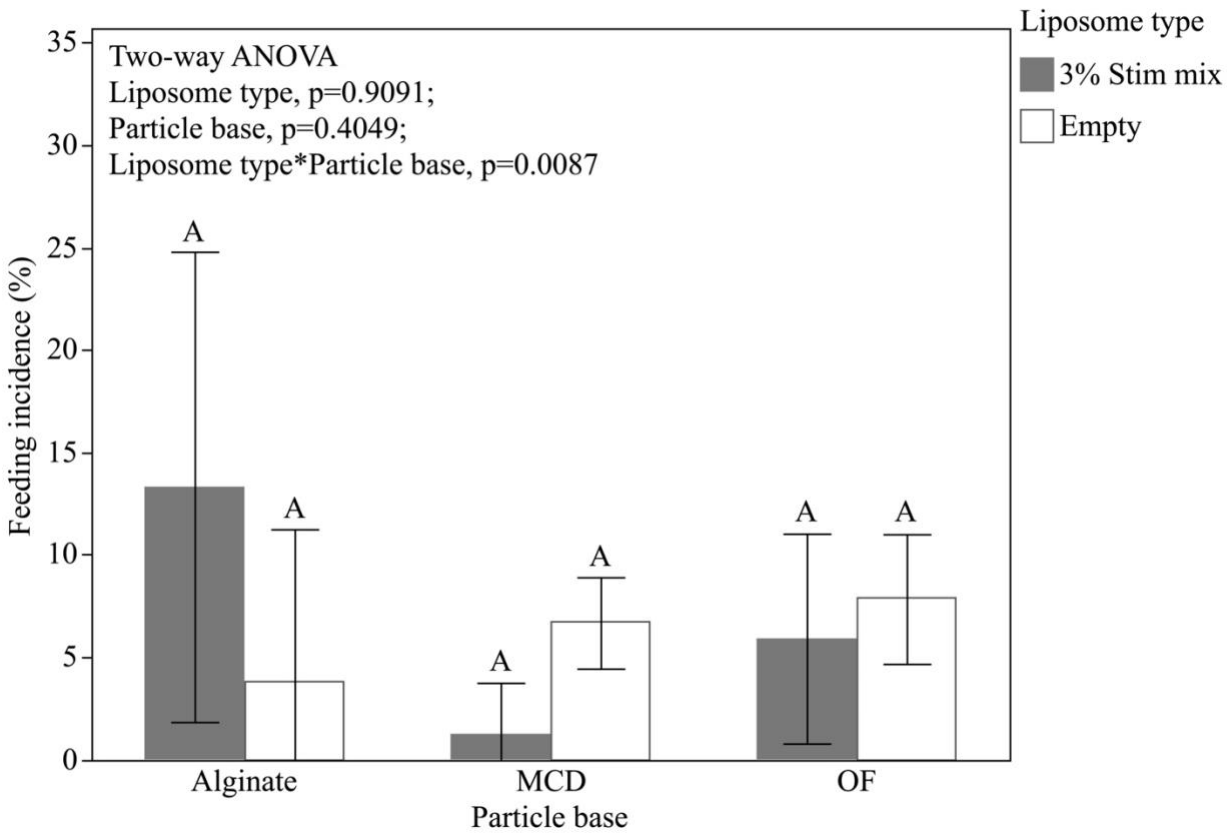

Figure 3.6 Percentage of fish observed with feed in gut (feeding incidence; %) 1 h post-feeding. Bar chart depicts uptake rates of sample larvae per microdiet treatment (Mean $\pm$ 1 SD). LCP were made with a particle base consisting of alginate, milled commercial diet (MCD), or open formula (OF) and either 3% Stim mix (i.e. 1% glycine, 1% alanine, 1% betaine) liposomes or empty liposomes. Bars depict mean uptake rates of sampled larvae per microdiet treatment, with grey bars representing LCP containing 3% Stim mix liposomes and white bars representing those containing empty liposomes. Connecting letters report depicts differences in mean uptake rates between treatments (Tukey's HSD, threshold $p<0.05$).

Figure 3.7 California yellowtail larvae photographed under microscope during manual gut checks performed 1 h post-feeding. Red arrows highlight undigested or poorly digested LCP found in larvae gut.

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CHAPTER IV

CONCLUSION

4.1 Overarching goals

The overarching goals of this research were to: 1) develop and evaluate the use of liposome-based complex particles (LCP) for the delivery of complete nutrition to marine fish larvae and 2) validate the use of microextrdued marumerized (MEM) particles produced with an open formula developed for early-stage marine fish as a potential open formula reference diet (OFRD) for *Seriola* larvae. Both LCP and MEM were formulated using an open formula developed for marine fish larvae (Objective 1). Chapter 2 describes the evaluation of LCP performance through a series benchtop trials aimed to optimize particle performance related to payload incorporation and delivery. Changes in protein were observed as a result of changes in collection bath concentration, liposome inclusion, and liposome dialysis (Objective 2). We found success in buoyancy manipulation of LCP diets using hollow silica microspheres and gas-forming agents the inclusion of which resulted in slower sink rates in LCP when compared to MEM and commercial Otohime diets (Objective 3). In chapter 3, LCP and MEM, both produced with the open formula, were evaluated using growth and acceptability trials using California yellowtail (*Seriola dorsalis*) larvae and compared with Otohime. California yellowtail larvae fed LCP showed poorer growth and survival compared to both Otohime and MEM diets, which appeared to be largely due to low acceptability and ingestion rates of LCP. However, larvae fed OF-MEM showed similar growth and feed ingestion rates but with minor reductions in survival when compared to those fed with Otohime (Objective 4). Overall, these results validate the use of the OF for use with Seriola larvae and suggest that OF-MEM could be used as an open-formula reference diet more broadly. The low ingestion rates of LCP, when compared to MEM and Otohime, observed in the growth trial were not due to buoyancy as this issue was corrected

though the addition of hollow-silica microspheres. In addition, alternative formulations, tested in follow-up acceptability trials, were not able to ameliorate this issue (Objective 5).

4.2 Statement of major findings

Major findings of this research include:

- Both gas-forming $NaHCO_3$ and hollow silica microspheres can be added to LCP formulation to modify particle buoyancy. However, hollow silica microspheres were more effective at doing this at lower inclusion rates. Addition of 0.5% w/w hollow silica microspheres was selected over $NaHCO_3$ as the selected buoyancy-altering agent for LCP because it did not require the addition of potentially unpalatable acetic acid to the collection bath. This inclusion was used in feed uptake and growth trials with CYT larvae.

- LCP produced with liposomes containing casein-hydrolysate showed decreasing protein concentrations as a function of increasing $CaCl_2$ concentrations in the collection bath. This trend was similar in LCP produced with both empty and 20% casein hydrolysate liposomes. These observations were not consistent with the previously-held hypothesis that osmolality may be influencing protein concentration of particles but rather suggest that LCP may absorb $CaCl_2$ during suspension thus diluting payload concentrations.

- Protein and lipid concentrations increased in LCP with increasing inclusion rates of casein-hydrolysate containing liposomes. A 30% inclusion rate for liposomes in LCP appeared to be the maximum level that can be used without providing total lipids in excess of those typically recommended for marine fish larvae. However, using this inclusion rate of casein-hydrolysate liposomes only resulted in a 3% increase in total protein of the particle that can be directly attributed to the casein hydrolysate.

- Washing via dialysis is an effective method to remove unencapsulated protein present in liposomes. Liposome protein content decreased after a 5-day dialysis; however, this had only minor impacts on final LCP protein contents.

- California yellowtail larvae fed OF-LCP showed reduced growth and survival when compared to those fed open-formula MEM and Otohime. These differences appeared to be largely due to poor feed uptake of LCP.

- California yellowtail larvae fed MEM particles produced with an open formula showed similar growth outcomes and only modest decreases in survival when compared to those

fed Otohime suggesting that OF-MEM may be used as an open-formula reference diet for *Seriola*, if not marine finfish, more broadly.

- Follow-up feed acceptability trials using CYT larvae resulted in low feeding incidence for LCP treatments, despite modifications to bath type (preservative addition), liposome core type (13% EAA, empty, 3% stimulant mixture), and particle protein base (alginate, milled commercial diet, open formula). More research must be done with respect to why LCP is being ingested at such low rates before this diet can be considered a viable microdiet for fish larvae.

4.3 Future research

The results of this study highlight major advancements, limitations, and areas of future research and optimization with respect to the use of LCP as a microdiet for marine fish larvae. Further studies are needed to understand why LCP are not ingested at high rates by larvae. Several formulations and preserving agents were evaluated, but no improvements in ingestion rates were observed. Future research may aim to investigate inclusion rates and type of binders (i.e. alginate) in both LCP and MEM diets. Future efforts should also quantify the leaching of water-soluble materials from LCP. Understanding particle efficiencies (i.e. encapsulation, inclusion, and retention efficiency) would add to our understanding of the limitations on nutrient encapsulation and retention within the particle, as well as what proportion of our desired water-soluble payload is being ingested by fish larvae after time in suspension. As previously noted, liposomes deliver disproportionate amounts of lipid in comparison to protein. Without substantial improvements in this area, LCP would be better suited for delivering substances like taurine, conditionally essential amino acids, vitamins, and minerals that are required by marine larvae in smaller amounts. There may be more utility for LCP as a vehicle for delivering these trace materials rather than larger, macronutrient-oriented, payloads.

This research demonstrates support for the use of an open formula MEM diet as a potentially viable reference diet for marine fish larvae with performance comparable to

commercial standard Otohime. However, their performance has yet to surpass Otohime, leaving room for future optimization. MEM diets certainly suffer a whole suite of challenges typical of commercial-type microdiets, including losses of water-soluble nutrients when suspended in water. Thus, leaching trials should be conducted to assess losses of water-soluble nutrients when suspended in water over time.

In sum, if issues regarding acceptability and digestion by larvae are remedied and LCP-fed larvae are able to grow well with low mortality, LCP diets have the potential to act as an early-weaning diet for marine fish larvae. If LCP are produced to be small enough to reflect the size of live feeds (approximately 400 to 550 µm), retain their water-soluble nutrients, and have a buoyancy that reflects the motility of live feeds, LCP may be able to reduce reliance on live feeds by allowing larvae to transition onto microdiets earlier than they do currently. Additionally, LCP may be used to deliver other bioactive compounds to fish larvae in the future, including vaccines, drugs, and antibiotics. Moreover, the open formulation, particle size, and characteristics of the present LCP may be changed to suit the needs of other marine finfish species, and eventually other marine suspension feeders like shrimp. With respect to the open formulation in MEM diets, more refinement in formulation and production processes may validate and support this microdiet for use with marine fish larvae. If these diets leach their water-soluble nutrients at lower rates than commercial microdiets, they may provide more utility in hatcheries and be selected over commercial diets like Otohime as an OFRD.

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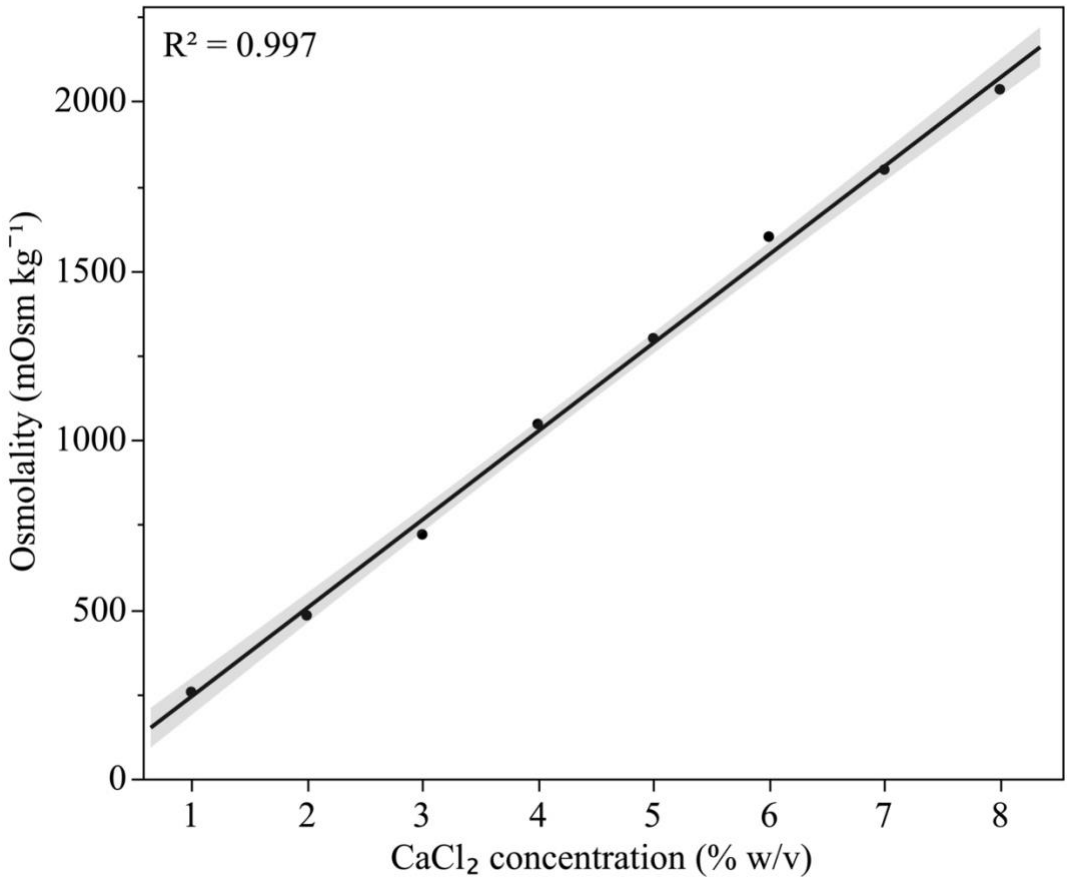
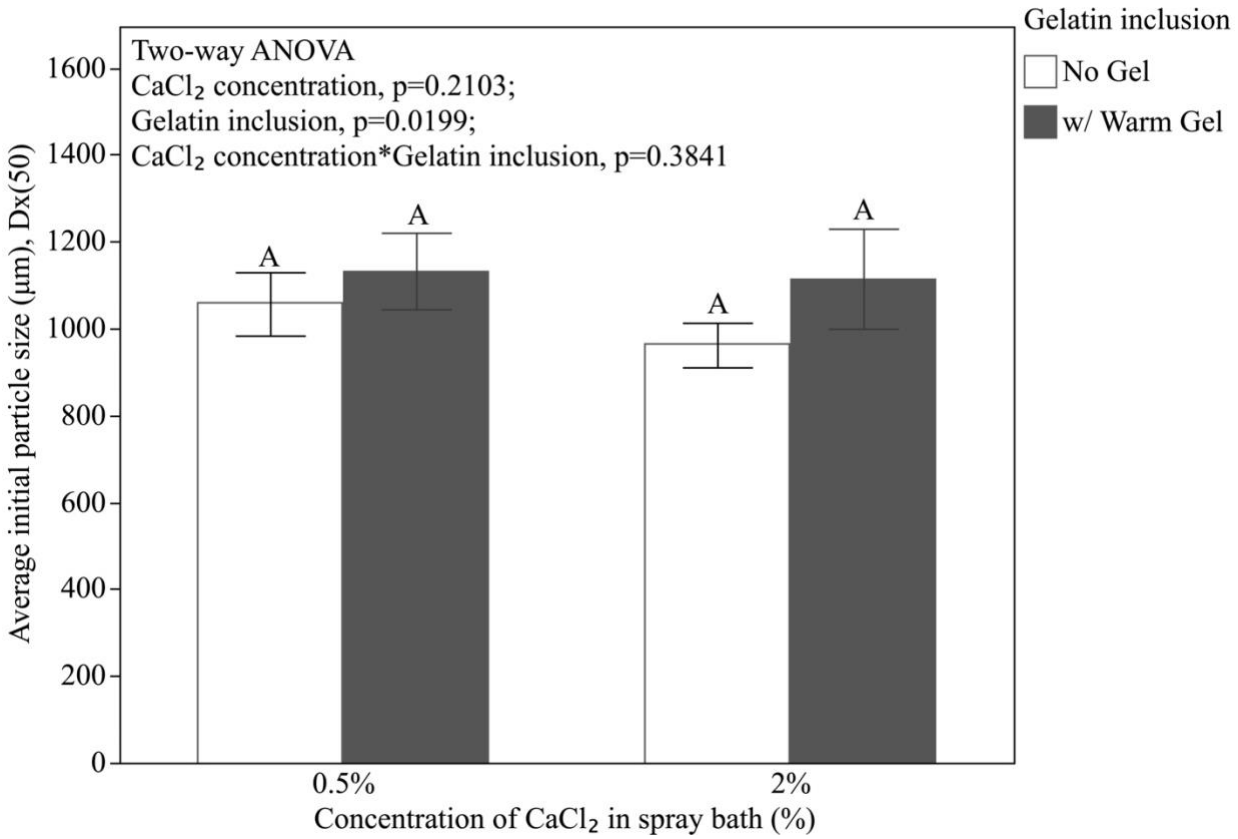
APPENDICES



Appendix A. Osmolality (mOsm kg^{-1}) of increasing concentrations (w/v) of $CaCl_2$ prepared in distilled water. For reference, seawater is predicted to be approximately 1000 mOsm kg^{-1}.



Appendix B. Initial particle size [Dx(50); µm] of OF-based LCP (%; mean±1 SD) analyzed at 500 rpm in quadruplicate for each diet treatment. No gel treatments had smaller average particle size regardless of the $CaCl_2$ concentration in collection bath. Two-way ANOVA results reveal that main effect '$CaCl_2$ concentration' was not significant (p=0.2103). Main effect 'Gelatin inclusion' was significant' (p=0.0199). The interaction between $CaCl_2$ concentration and Gelatin inclusion was ultimately not significant (p=0.3841).

BIOGRAPHY OF THE AUTHOR

Kara Chuang was born on February 12, 1998 in the suburbs of Los Angeles, California to Yeou-Chyi and Michelle Chuang. She was raised in the San Gabriel Valley and graduated from Mark Keppel High School in 2016. She went on to attend the University of California, Berkeley where she played collegiate rugby and achieved a B.A. in Integrative Biology with a degree focus in Ecology and Evolutionary Biology. The onset of COVID-19 and a few random twists and turns led her to the island of Hawai'i in 2020 where she worked for Sea Warden, Inc., an aquaculture technology start-up. Here, she fell in love with the aquaculture industry as she conducted global industry research, observed aquaculture farms using satellite data, and established direct working relationships with farmers from around the world. She soon put her hands to the water as a Hatchery Technician at Blue Ocean Mariculture, a commercial fish farm on Hawai'i's coast, where she raised Amberjack yellowtail (*Seriola rivoliana*) in production hatchery. In 2022, she left Hawai'i and began her longest move to Maine in pursuit of her M.S. in Marine Biology under Dr. Matt Hawkyard. Here, she focused her research on developing a novel microdiet type for the improved delivery of nutrients to marine fish larvae. Her research was done in collaboration with the Aquaculture Research Institute, USDA National Cold Water Marine Aquaculture Facility, Hubbs-SeaWorld Research Institute, and the University of Maine. In her free time, she finds joy in playing and coaching rugby, hosting local pub trivia, participating in community theater, and exploring Maine's hiking trails. Kara is a candidate for the Master of Science degree in Marine Biology from the University of Maine in August 2024.