AN ABSTRACT OF THE THESIS OF

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Title: Development of Microparticulate Feeds and Methods to Improve Acceptability of Artificial Diets by Blue Spotted Goby Larvae (*Asterropteryx semipunctata* L.)

Abstract approved:

Chris Langdon

An acceptable microparticulate diet for marine fish larvae may be defined as an artificial diet that contains, retains, and delivers the required nutrients to support survival and growth. Factors affecting ingestion rate of prey items by fish larvae include environmental factors such as light intensity, prey/background contrast, possible chemical cues, and co-feeding with live foods. The efficient addition of FAA (free amino acids) in microparticles, at concentrations similar to those found in live foods may reduce or eliminate the need for exogenous proteases and FAA from live feeds. Complex particles (CP), when coupled with optimization of environmental parameters, may result in ingestion of artificial diets at rates sufficient to support high survival and growth rates.

Lipid spray beads (LSB) have shown promise in their ability to deliver low molecular weight water-soluble (LMWS) nutrients to marine fish larvae. Furthermore, ingestion and digestion of zein-bound complex particles (CP), with incorporated LSB, by fish larvae have been reported. Here we describe improvements in LSB technology as well as feeding conditions for the effective delivery of FAA to blue spotted goby larvae (*Asterropteryx semipunctata*).

The FAA glycine was used as a model LMWS nutrient due to its high solubility in water. LSB performance was found to be optimized with an aqueous core concentration of 400 g l^{-1} glycine and was shown to have significantly higher DE over a 60 min period in aqueous suspension compared to that of beads with lower core glycine concentrations (two-way ANOVA; $p<0.0001$). Evaluation of core to lipid ratios (v/v) showed that retention and delivery efficiencies of LSB containing cores of 400 g glycine l^{-1} at a core to lipid ratio of 1:2 v/v were greater than those of LSB with other core to lipid ratios. The use of optimized LSB in zein-bound CP resulted in an encapsulation efficiency of 4% w/w glycine for the CP, which was within the range of FAA concentrations reported for rotifers, *Artemia* and copepods.

Blue spotted goby larvae showed peak feeding incidence at a light intensity of 5.44 $\mu E\ s^{-1}\ m^{-2}$ when fed on CP against a black background. Higher or lower light intensities and a white background, were shown to independently reduce feeding incidence. In a 5 day feeding trial, larvae fed CP containing LSB with core material consisting of casein, hydrolyzed casein, or a mix of FAA (based on the average of the amino acids found in casein and hydrolyzed casein) showed 6, 8, and 6% survival respectively, which was significantly lower than 37% survival for larvae fed the marine phytoplankton, Tahitian *Isochrysis galbana* and *Rhodomonas sp.* ($p>0.05$). At 0% survival, the starved control had significantly lower survival than that of all other treatments ($p<0.05$).

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Development of Microparticulate feeds and Methods to Improve Acceptability of Artificial Diets by Blue Spotted Goby Larvae (*Asterropteryx semipunctata* L.)

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature authorizes release of my thesis to any reader upon request.

Brendan W. Clack, Author

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CONTRIBUTION OF AUTHORS

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DEDICATION

This thesis is dedicated to my parents, Jillian and William Clack, who nurtured my curiosity from a young age and offered their encouragement and support throughout all of my adventures and misadventures.

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MICROPARTICULATE FOODS AND ENVIRONMENTAL CONDITIONS EFFECTING ACCEPTABILITY OF ARTIFICIAL DIETS BY FISH LARVAE

CHAPTER 1

INTRODUCTION

Currently marine larval fish culture relies almost solely on the use of live prey items, such as rotifers and *Artemia* for food. Production of live food is expensive, labor intensive, and often requires a significant portion of an aquaculture facility. Other disadvantages of live feeds include inconsistency in both production and nutritional value (Kanazawa et al. 1989). For these reasons, there is considerable interest in the development of artificial diets, but formulation and delivery of acceptable artificial diets are not simple. An acceptable microparticulate diet must incorporate sufficient nutrients to promote growth and survival, but due to high surface area-to-volume ratios of microparticles, low molecular weight, water-soluble (LMWS) nutrients tend to rapidly leach from particles. Leaching of LMWS nutrients is one of the greatest problems in production of satisfactory microparticulate diets when suspended in an aqueous environment (Yúfera et al. 2002, Langdon 2003, Önal and Langdon 2004b). Furthermore, fish larvae must consume and efficiently digest the food particles. This chapter will describe and categorize some microparticle types that have been developed for the aquaculture industry and identify environmental factors that influence the acceptability of food items by fish larvae.

Texture, taste, color, formulation and size all contribute to acceptability and digestibility, as well as leaching of nutrients from the food particle (Önal and Langdon 2000). A food item must be palatable to larvae and presented under appropriate conditions or it will simply not be eaten, rendering the diet ineffective. In general, early marine fish larvae are very small and the diet particles must be small enough to be ingested by the larvae; for example, early sea bass larvae require particles as small as 50 μm (Cahu and Infante 2001). Likewise the diet must be digestible by the larvae or the nutrients will simply pass through the digestive tract. Furthermore, the diet is rendered less nutritious if the nutrients leach out of the food particle prior to being consumed.

Buoyancy is another concern. In order for a particle to be available to larvae it must be suspended in the water column. If the particle sinks too fast it becomes unavailable and will simply decompose on the bottom of the culture vessel and degrade water quality. Likewise, if the particle floats at the surface it is less available to larvae. Ideally microparticulate food particles will be neutrally buoyant, or at least sink slowly to maximize availability to larvae.

There are several types of microparticulate feeds, each of which have strengths and weaknesses. Microbound particles are comprised of nutrients bound within a gelled or hydrocolloid binder (Southgate and Partridge 1998). The dietary components of these particles are randomly distributed throughout the matrix. A variety of binders have been used in the production of these particles, among these are alginate, carageenan, zein, and gelatin (Langdon 2003). The type of binder used affects particle characteristics, such as water stability, ingestion, and digestibility (Southgate and Partridge 1998). Permeability

to water is of concern in microbound particles. Pathways through the particle will allow water movement and, therefore, result in high leaching rates. Most particle types are very permeable to water and LMWS materials (Langdon 2003).

Leakage of LMWS nutrients can be reduced through encapsulation within a lipid shell. Lipid-walled microcapsules consist of a core of an aqueous solution of nutrients within a lipid wall (Langdon 2000). These capsules have been shown to retain 81% of encapsulated glycine after being suspended in seawater for a 24-hour period (Langdon et al. 1985). While lipid-walled capsules have been shown to decrease leaching losses of LMWS nutrients, they are not capable of providing high concentrations of LMWS nutrients to larvae because low solubility of the core material in water can severely limit the amount of material that can be encapsulated (Langdon and Sigfried 1984; Langdon and Buchal 1998).

One of the key concerns with any diet is digestibility. Digestibility of lipid-walled capsules depends largely on the lipid source used to form the walls as well as the digestive capacities of the intended recipient of the diet. Crustacean larvae have mouth structures to crush the walls of capsules made of harder lipid materials, such as tripalmatin, but marine fish larvae and bivalves lack mechanical means of breaking down food particles and therefore require that softer lipids be used to form capsule walls (Langdon 2000).

Lipid spray beads (LSB) consist of nutrient particles embedded throughout a bead of lipid. LSB are produced by spraying a lipid-nutrient mixture into a cooled chamber where the beads solidify prior to contact with the chamber walls, resulting in a spherical

bead of lipid with incorporated nutrients (Langdon 2000, Önal and Langdon 2004b). LSB may, in turn, be embedded into a microcapsule or microbound particle with other nutrients to form a complex particle, resulting in a complete diet. LSB have several advantages compared with lipid-walled capsules. Firstly, LSB can be prepared in the absence of water, avoiding leakage of LMWS nutrients during the preparation process. Secondly, nutrient water-solubility is not a limiting factor in the amount of nutrient that can be added to LSB and lastly, LSB are easier to produce than lipid walled capsules (Langdon and Buchal 1998, Langdon 2000).

A problem with LSB containing dry particulate core material is that because nutrients are not included as aqueous droplets surrounded by lipid, they are not confined to the core of the particle but rather are distributed throughout the beads and may protrude from their surfaces. This may create leaching problems due to dissolution of nutrients at the surface, creating channels through which water enters and permeates the bead. Önal and Langdon (2004b) showed that LSB containing aqueous solutions of glycine had higher retention efficiencies than LSB containing particulate glycine, perhaps as a result of channel formation in the beads. Preparation of LSB using wet slurries of particulate glycine might solve this problem of channel formation. The presence of water may result in aqueous pockets of core materials within the lipid, similar to those produced with aqueous solutions of core materials. This may create a shell of lipid surrounding the suspended nutrient particles, preventing them from protruding from the surface of the beads. The advantages of this approach would be both higher encapsulation efficiencies compared with those of LSB with aqueous cores as well as

higher retention and delivery efficiencies compared with those of LSB with particulate cores.

LSB may be positively buoyant and float to the surface, becoming unavailable to cultured fish and invertebrates. The hydrophobic nature of lipids may also cause clumping, producing clusters of beads that are too large to be consumed. Binding or encapsulating LSB in another particle, using a binder such as zein or gelatin, can decrease buoyancy and hydrophobicity, creating a neutrally buoyant, slow-sinking microparticle (Önal and Langdon 2004b). This combination of particle types is referred to as a complex particle (CP; Langdon 2000) and the component LSB are referred to as inclusion particles (Önal pers. comm. 2002). Depending on the type of lipid used, LSB may be soft and easily crushed during handling. The use of zein to coat and bind LSB helps to prevent premature crushing and breakdown of LSB during storage and handling.

Many factors may affect the acceptability of a diet by larvae, including environmental conditions such as lighting, rearing container color, prey contrast, and the presence of chemical feeding stimulants. Little information is available on the effect of lighting and contrast on the consumption of inanimate food particles by fish larvae, with most available data applying to live foods.

Based on the prominence of the eyes, marine fish larvae are thought to be primarily visual feeders (Blaxter 1968b; Huse 1994); therefore, adequate light levels have been shown to be necessary at the onset of exogenous feeding (Blaxter 1968a; Huse 1999). The eyes of many early fish larvae have been described as simple, with only cone cells present in the retinal tissue, thus limiting their ability to adapt to changes in

illumination (Blaxter 1968a,b; Neave 1984; Huse 1994). Downing and Litvak (2001) showed that feeding in larval haddock peaks at 1.7-18 μE s^{-1} m^{-2}, with feeding incidence and intensity declining both below and above the optimum lighting intensity. While light intensity has been shown to have a substantial effect on food consumption, survival, and growth of fish larvae, the optimal light intensity varies for different species (Batty 1987; Huse 1994; Downing et al. 1999). Quality of light may also play a significant role in larval feeding. In a study investigating the presence of visual pigments in fish larvae, Britt et al. (2001) showed that out of 22 species of fish studied, green sensitive single cone cells were the most common, being present in the eye tissue of 20 species, and 18 had ultraviolet or violet absorbing cones in their eye tissue. This implies that while the visible light spectrum is important in larval vision, many larvae also have the ability to detect light in the ultraviolet portion of the spectrum and that a full spectrum light source with an ultraviolet component may be beneficial for culture of marine fish larvae.

Tank color has also been shown to have a significant effect on the survival and incidence of feeding in the larvae of many fish species. Ostrowsky (1989) demonstrated that survival rates were improved by 130% in dolphin (*Coryphaena hippurus*) larvae reared in black tanks compared with tan tanks; furthermore, Denson and Smith (1996) reported that larval white bass (*Morone crysops*) showed a marked increase in survival rates at 24 days when reared in black tanks compared to 100% mortality at 6 days when reared in clear tanks and that dark tank walls were essential for prey capture under high illumination. Improvement in survival has been attributed to improved contrast between prey and background (Denson and Smith 1996; Downing and Litvak 1999). In contrast

Marietta et al. (1996) reported that larvae of orange spotted grouper (*Epinephelus suillus*) reared in tan tanks consumed rotifers at higher rates compared with those reared in black tanks. Furthermore, Downing and Litvak (1999) showed that larval haddock (*Melanogrammus aegelfinus*) had higher survival when reared in white tanks and that larvae displayed impaired growth when reared in black tanks at lower light levels. Naas et al. (1996) concluded that black rearing containers have less light reflection, better simulating natural light conditions in the pelagic ocean, whereas white tanks reflect and scatter a higher degree of light. Duray et al. (1996) reported larvae of the grouper, *Epinephelus suillus* showing significantly increased growth when reared in tan tanks compared with those reared in black tanks over a 14-day period, however there was no significant difference in survival. There is likely an interaction between light intensity and rearing tank color whereby at lower light intensities, white tanks may increase the overall intensity of light within the culture vessel through reflection, bringing the light to a suitable level for larvae.

Prey color and contrast between prey and background are likely to play a role in the ability of larvae to see and capture prey (Dendrinos 1984; Ostrowsky 1989; Duray 1996). Dendrinos et al. (1984) reported that Dover Sole (*Solea solea*) larvae showed a significant improvement in feeding efficiency when fed *Artemia* stained black against a clear background compared to larvae fed *Artemia* stained red, pink, blue, and yellow or naturally colored *Artemia*. Survival of larval dolphin (*Coryphaena hippurus*) was improved by 130% when reared in black tanks over those reared in tan colored tanks and larvae were noted to display side-to-side head motions at a higher frequency in tan tanks,

which was attributed to increased search effort due to decrease prey contrast (Ostrowski 1989).

The larvae of some fish species have been reported to display increased activity in the presence of certain dissolved metabolites. Newly hatched cod (*Gadus morhua*) displayed reduced swimming activity when exposed to increased concentrations of arginine and scanning micrographs of newly hatched cod larvae showing evidence of olfactory organs positioned in front of the eyes and above the mouth (Døving et al. 1994). Dempsey (1978) found that newly hatched herring (*Clupea harengus*) larvae displayed increased activity when exposed to extracts of *Balanus balanoides* nauplii, glycine and proline. Older herring larvae that had been exposed to *Artemia* showed increased activity when exposed to *Artemia* extracts, and the six amino acids glutamic acid, aspartic acid, glycine, methionine, alanine and proline. Kolkovski et al. (1997a, 1997b) and Koven et al. (2001) described live foods as having both a visual and a chemical stimulatory effect. Ingestion of microdiets by 20 day-old sea bream (*Sparus aurata*) larvae increased in the presence of the amino acids glycine, arginine, and alanine that were identified in culture media of *Artemia.* In contrast, Davis and Olla (1995) reported that walleye pollock (*Theragra chalcogramma*) larvae aggregation was not stimulated by prey scent, but rather by prey and light gradients, indicating that chemical stimulants have negligible effect on the behavior of walleye pollock larvae. It is apparent that the level of chemokinesis in fish larvae may depend on species, stage of development, prey experience, and mode of feeding. It is also important to acknowledge that larvae of

different species are at different stages of development at hatch and at first feeding, probably affecting the stage at which chemical detection of prey becomes important.

The presence of FAA in larval diets may also be important as a nutritional contribution and in the stimulation of digestive enzyme production. Larval sea bass (*Dicentrrarchus labrax*) displayed increased trypsin secretion when fed artificial diets containing 10% FAA (Cahu et al. 1995). Kolkovski (2001) suggested that live food organisms may contribute to digestion through autolysis or zymogens that may stimulate the production of endogenous enzymes by larvae. The digestive tract of many fish larvae have been described as poorly developed, having low enzymatic activity (Rønnestad et al 1999; Kolkovski 2001; Rønnestad et al 2003). Absorption of FAA has been shown to be higher than either peptide or protein bound amino acids by the larval gut, with an absorption rate of as much as 3.5 times faster than amino acids supplied in the form of soluble proteins (Rønnestad et al. 1999, 2000). Based on these results and the abundance of FAA found in live food organisms, it is likely that FAA play a major role in larval nutrition. A major goal of the production of microparticulate diets is to deliver FAA in quantities similar to those available from live food organisms. *Artemia* have been reported to have a FAA content of between 1.6% w/w and 2.8% w/w, and rotifers (*Brachionus plicatilis*) a FAA content of 1.4% w/w to 6.7% w/w, with the highest values being for animals fed on phytoplankton (Aragão et al. 2004). Srivastava et al. (2005) reported rotifers to have FAA contents of between 2.2 and 3.5% d/w.

In summary, LSB incorporated into complex particles result in a promising microparticle for the delivery of both LMWS and non-soluble nutrients to marine fish

larvae. While microparticle technology is rapidly advancing, an understanding of the environmental factors influencing consumption of inanimate particles by fish larvae is essential to the successful implementation of artificial diets in fish culture. The environmental requirements differ from species to species and thus optimization is required for individual species for accurate evaluation of particle performance as larval feeds. Furthermore the abundance of FAA in live food organisms may play a large role in the improved survival and growth of larvae fed on live foods versus currently available artificial microparticulate diets.

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DEVELOPMENT OF MICROPARTICULATE FEEDS AND METHODS TO IMPROVE ACCEPTABILITY OF ARTIFICIAL DIETS BY BLUE SPOTTED GOBY LARVAE (*Asterropteryx semipunctata* L.)

CHAPTER 2

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ABSTRACT

The rapid loss of low molecular weight water-soluble (LMWS) nutrients from microparticulate diets, together with low ingestion and digestion, are important problems in the development of artificial diets for marine fish larvae. Amino acids are important as both building blocks for protein and as a source of energy in developing larvae; however, first feeding larvae may lack the ability to effectively digest whole proteins. The delivery of free amino acids (FAA) to larvae presents amino acids in a form that is readily accessible and absorbed through the digestive tract. Lipid spray beads (LSB) have shown promise in their ability to deliver LMWS nutrients to marine fish larvae. Furthermore, ingestion and digestion of zein-bound, complex particles (CP) with incorporated LSB have been reported for fish larvae. Here we describe improvements in LSB technology, as well as feeding conditions, for the effective delivery of free amino acids (FAA) to blue spotted goby larvae (*Asterropteryx semipunctata*).

Glycine was used as a model LMWS nutrient due to its high water solubility. LSB performance was found to be optimized with an aqueous core concentration of 400 g l^{-1} glycine and were shown to have significantly higher DE over a 60 min period in aqueous suspension compared to that of beads with lower core glycine concentrations (two-way ANOVA; $p<0.0001$). Evaluation of core to lipid ratios (v/v) showed that retention and delivery efficiencies of LSB containing cores of 400 g glycine l^{-1} at a core to lipid ratio of 1:2 v/v were greater than those of LSB with other core to lipid ratios. The use of optimized LSB in zein-bound complex particles (CP) resulted in an encapsulation

efficiency of 4% w/w glycine CP, which was within the range of FAA contents reported for rotifers, *Artemia* and copepods.

Blue spotted goby larvae showed peak feeding incidence at a light intensity of 5.44 μE s^{-1} m^{-2} when fed on CP against a black background. Higher or lower light intensities and white background color, were shown to independently reduce feeding incidence.

Larvae fed CP containing LSB with casein, hydrolyzed casein, or FAA (in ratios averaged between those present in casein and hydrolyzed casein) showed lower survival rates than larvae fed a combination of Tahitian *Isochrysis galbana* and *Rhodomonas sp*. in 5 day feeding trials, but were significantly improved compared with that of the starved control, which showed 100% mortality on day 4.

INTRODUCTION

The use of microparticulate diets in marine fish larval culture is limited due to lower growth and survival of larvae fed on these diets compared with live foods. The high surface area-to-volume ratios of microparticulate diets results in rapid leakage losses of low molecular weight water-soluble (LMWS) nutrients, such as free amino acids (FAA), when diet particles are placed in an aqueous environment. Diets must retain their nutritional integrity until consumed by larvae, but must be digestible once consumed. Furthermore, food particles must be presented in a manner that stimulates a feeding response in fish larvae to ensure adequate ingestion rates.

Many microparticle types developed to date have displayed high levels of leaching of LMWS nutrients within the first few minutes of suspension in an aqueous environment, limiting their potential effectiveness as a larval food. While microbound particles utilizing zein, alginate or carageenan have been shown to lose >80% of FAA within the first 2 min of aqueous suspension (Lopez-Alvarado et al., 1994), lipid-based particle types, such as lipid-walled capsules (LWC) and lipid spray beads (LSB), have shown greater retention of LMWS nutrients. LWC consist of LMWS nutrients encapsulated within a lipid capsule. LWC are produced by first forming an emulsion of an aqueous solution of LMWS nutrients in lipid. This emulsion is then re-emulsified in an aqueous solution to form a double emulsion consisting of spheres of lipid encapsulating the LMWS nutrients (Langdon 2003). In contrast, LSB are created by

spraying a lipid-nutrient mixture into a cooled chamber where the beads solidify prior to contact with chamber walls (Langdon 2000, Önal and Langdon 2004b).

Microparticle performance is often described in terms of inclusion efficiency (IE), encapsulation efficiency (EE), retention efficiency (RE), and delivery efficiency (DE), where IE is the percentage of core material in the original dietary mixture present in the particles post production, EE is the percent of the total particle composed of core material, RE is the percentage of core material remaining in the particle after a period of time in aqueous suspension, and DE is the percentage of the particle composed of core material after a period of time in aqueous suspension.

LWC have been shown to reduce the rate of loss of water-soluble nutrients, but due to the limited solubility of some water-soluble nutrients in the aqueous core, LWC are unlikely to be capable of delivering enough LMWS nutrients to meet the nutritional requirements of larvae (Langdon and Sigfried 1984), making them of limited use for larval fish culture. Langdon and Buchal (1998) developed a method of forming LSB that contained finely ground nutrients, which allowed higher concentrations of water-soluble nutrients to be incorporated in the beads. Önal and Langdon (2004a and 2004b) showed that spraying a mixture of LMWS nutrients and molten menhaden stearine (MS) into a cooled chamber to create LSB improved retention of LMWS nutrients during production, allowing higher quantities to be encapsulated.

Önal and Langdon (2000a) showed that LSB formulated using MS could be incorporated into complex particles (CP) using zein as a binder. In addition to LSB, other dietary components could be incorporated in CP to form a more complete diet.

Furthermore, coating LSB with zein had the added advantage of improving dispersion of LSB in water.

Acceptability of artificial diets depends not only on their biochemical composition, but also on environmental factors. Based on the size of eyes in relation to body size, it is likely that blue spotted goby larvae and most other marine fish larvae depend largely on sight to detect and target prey (Blaxter 1968b; Huse 1994). Light intensity and contrast of prey against background may be directly linked to the ability of larval fish to identify and catch prey items (Blaxter 1968a; Puvanendran and Brown 1998).

Light intensity has been shown to significantly effect the incidence of feeding for cod, *Gadus morhua*; plaice, *Pleuronectes platessa*; and turbot, *Scophthalmus maximus* (Huse 1994), haddock, *Melanogrammus aeglefinus* (Downing and Litvak 2001) and spotted sand bass, *Paralabrax maculatofasciatus* (Pena et al 2004). The optimum light intensity has been shown to differ among species. A narrow range in light intensity, required by many species of fish larvae, is due to the undeveloped nature of larval fish eyes, which lack rod cells in the retinal tissues during early development, thus limiting the ability of larvae to adapt to changes in illumination (Blaxter 1968a;Neave 1984; Huse 1994).

Light quality may also play an important role in larval feeding. Britt et al. (2001) reported that cone cells in the eyes of larvae were sensitive to specific wavelengths of light with green-sensitive cones being the most common. Of 22 species of fish investigated, 18 showed sensitivity to ultraviolet light. This indicates that the quality of

the light source may be as important as light intensity in larval feeding. The use of full spectrum lighting that includes ultraviolet wavelengths may be necessary for efficient captive rearing of some species of fish larvae.

Tank wall color has been shown to have a significant effect on the incidence of feeding in larvae of many fish species. Ostrowsky (1989) showed that survival rates were improved by 130% in dolphin (*Coryphaena* hippurus) larvae reared in black compared with tan tanks. Denson and Smith (1996) reported that larval white bass (*Morone crysops*) showed a marked increase in survival rate at 24 days when reared in black tanks compared to 100% mortality at 6 days when reared in clear tanks. This was thought to be partly due to improved contrast between prey and background. In contrast Marietta et al. (1996) reported that larvae of orange spotted grouper (*Epinephelus suillus*) reared in tan tanks displayed a higher consumption of rotifers compared with those reared in black tanks and Downing and Litvak (1999) showed that larval haddock (*Melanogrammus aegelfinus*) had higher survival when reared in white tanks.

Studies have indicated that some free amino acids stimulate a feeding response in larval fish. Kolkovski et al. (1997a, 1997b) and Koven et al. (2001) described live foods as having both a visual and a chemical stimulatory effect on the ingestion of microdiets by 20 day-old sea bream larvae, with the FAA's glycine, arginine, and alanine acting as chemical stimulants of feeding activity.

FAA and short chain peptides may play an essential role in survival and growth in larval fish (Conceiçã et al. 2003). The digestive tract of many fish larvae is poorly developed, having low enzymatic activity (Rønnestad et al 1999; Kolkovski 2001;

Rønnestad et al 2003) and absorption of FAA has been shown to be higher and up to 3.5 times faster than either peptide-bound or protein-bound amino acids (Rønnestad et al. 1999, 2000). Furthermore it has been suggested that live food organisms may contribute to digestion through autolysis or zymogens that may stimulate the production of endogenous enzymes by larvae (Kolkovski 2001). Larval sea bass (*Dicentrrarchus labrax*) have displayed increased trypsin secretion when fed artificial diets containing 10% FAA (Cahu et al. 1995). This indicates that the presentation of FAA to fish larvae through the use of CP may improve early survival and growth.

The blue spotted goby is ideal for feeding trials for a number of reasons. The compact size of mature adults (19 mm – 40 mm) limits the space required to house broodstock. The high fecundity relative to body size and short time periods between spawns also reduces the number of broodstock required. Privitera (2001, 2002) reported a minimal 6-day, inter-clutch period for spawning, with clutch sizes ranging from 296 – 1552 eggs per female and eggs hatching within 4-5 days of spawning. At the time of hatch blue spotted goby larvae are elongated and transparent with a notochord length of approximately 1.8 mm. The transparent body walls of larvae allow direct observation of ingestion and breakdown of food particles within the digestive tract. The small size of blue spotted goby larvae indicates the need for a small food item. It is likely that wild, first feeding, blue spotted goby larvae feed on a combination of prey items such as phytoplankton by filter feeding and ciliates, and copepod nauplii by selective predation.

The purpose of this investigation was to improve both the nutritional quality and physical acceptability of microparticulate diets by blue spotted goby larvae.

Improvement of nutritional quality of LSB characteristics was through manipulation of core glycine concentrations and core-to-lipid ratios to increase the concentration of LMWS nutrients in LSB, with the goal of creating CP with FAA levels near those found in live food. The second goal was to improve CP acceptability by blue spotted goby larvae by manipulating light intensity and tank color of larval cultures, as well as chemical environmental properties by the addition of possible feeding stimulants of FAA and phytoplankton to cultures of larvae fed on CP.

METHODS AND MATERIALS

General LSB Preparation

LSB were prepared using a modification of the method described by Önal (2004). MS was warmed in a water bath at 65^{o}C until molten. Glycine was chosen as a model LMWS nutrient because it is one of the most soluble amino acids (water solubility at 200 g l^{-1} at 25^{o}C) and has been shown to result in high leakage rates from LSB in past research (Önal 2004b).

Finely powdered, glycine was prepared by spray-drying (Mini Spray Drier B-191, Büchi, Switzerland) under the following parameters: nutrient solution flow rate: 5 ml min^{-1}, inlet temperature: 100^{o}C, outlet temperature: 65^{o}C, atomizing air pressure 40 psi, atomizing airflow rate: 600 cc sec^{-1}. The spray-dried glycine was then added at the desired concentration to distilled water and the resulting suspension or solution was added to molten MS (Omega Protein), contained in a 55 ml conical centrifuge tube, at the

desired ratio (v/v) of core to lipid. A water-in-oil emulsion was produced using a Braun Labsonic sonicator probe (L. B. Braun Biotech Inc.) on high power for 30 sec. The resulting emulsion was shaken constantly until added to the spray apparatus. The time period from emulsification to LSB spraying was no greater than 3 min.

The spray apparatus (Fig. 2.1) was modified from the equipment described by Önal (2004a, 2004b). Modifications to the apparatus included a direct feed line from the hopper containing the lipid-core emulsion to the spray nozzle. Furthermore, the lipid emulsion was not sonicated during the spray procedure as it was found that further sonication resulted in aggregation and uneven distribution of suspended core materials. The temperature of the spray apparatus was maintained using a heating coil and thermocouple in conjunction with a temperature controller (model CN9000A, Omega Engineering Inc.).

After filling the hopper with the lipid-core emulsion, dry nitrogen gas was used to pressurize the hopper and the emulsion was immediately sprayed through a commercial spray nozzle (1/4 JBCJ, Spraying Systems Co.) using pressurized dry nitrogen gas to force the mixture through the nozzle. The mixture was sprayed into a conical stainless steel chamber chilled to $-20^{o}C$ with nitrogen vapor delivered from a liquid nitrogen tank. Atomized droplets of the spray mixture solidified in the conical cooling chamber and collected on the walls of the chamber. LSB were then air-brushed into a container positioned at the bottom of the cone, using a stream of dry nitrogen forced through a 5 ml pipette tip. LSB were stored under dry nitrogen at $-80^{o}C$.

Optimizing LSB

Core concentration

The effect of core concentration on LSB characteristics was investigated using LSB containing core glycine concentrations of 100, 200, 400 and 600 g l^{-1} with a core to lipid ratio of 1:1.5 (v/v). Finely powdered glycine was prepared by spray-drying, as described above. Aliquots of 1, 2, 4, and 6 g of the glycine powder were weighed out and water (65^{o}C) was added to the glycine aliquots to bring the total volume 10 ml. The aqueous glycine preparations were mixed vigorously for 20 sec using a Vortex-Genie (Fisher Scientific), then 15 ml of molten menhaden stearine (65^{o}C) were added and LSB were prepared following the procedures described. EE, IE, RE, and DE were determined for the resulting LSB, as described below.

Core to lipid ratio

The effect of core to lipid ratio (v/v) on LSB was tested using LSB with a core glycine concentration of 400 g l^{-1} at core to lipid ratios (v/v) of 1:1, 1:1.25, 1:1.5, 1:2, and 1:2.5. Ten ml of glycine suspension were prepared by mixing (Vortex–Genie, Fisher Scientific) 4 g of spray-dried glycine with distilled water at 65^{o}C for 20 sec. The core glycine suspension was mixed with 10, 12.5, 15, 20, or 25 ml of molten MS (65^{o}C) and LSB were prepared as described above. EE, IE, RE, and DE of the resulting LSB were determined as described below.

CP preparation

CP were prepared by dissolving 1.5 g of zein in 20 ml of 90% aqueous solution of ethanol. Two grams of dry nutrients were thoroughly mixed with the zein/alcohol solution and the resulting mixture was chilled to 5^{o} C; then 1.5 g of LSB were added to the chilled mixture and mixed vigorously (Vortex-Genie, Fisher Scientific) and added to the hopper of the spray apparatus (Fig. 2.2). The mixture was immediately sprayed through a commercial spray nozzle (1/4 JBCJ, Spraying Systems Co.) using pressurized dry nitrogen gas to force the mixture through the nozzle. The particles were sprayed at room temperature (22^{o}C) into a polyethylene cylinder (60 cm diameter by 100 cm) with a conical bottom. Alcohol was allowed to evaporate off for an additional 10 minutes and CP were collected using a soft brush to gently sweep them into a collection cup at the bottom of the cone.

Loss of core glycine by LSB during CP Production

Retention of glycine by LSB during production of CP was investigated by suspending triplicate samples of LSB containing a core concentration of 400 g l^{-1} glycine at a core to lipid ratio of 1:2 v/v in 90% ethanol. RE was measured at 2 and 10 min. LSB were not exposed to the zein/alcohol solution for more than 3 min during production of CP; therefore it was not necessary to test leakage past 10 min.

Comparison of LSB vs. CP containing LSB

CP containing (by weight) 30% zein, 30% LSB (with a core of 400 g l^{-1} glycine at a 1:2 (v/v) core to lipid ratio) and 40% rice starch were produced using the method described above. RE and DE of the resulting CP were compared against those for freely suspended LSB containing glycine at 400 g l^{-1} and a core to lipid ratio of 1:2 (v/v).

Measures of microparticle performance

Amino Acid Analysis

Glycine concentrations were determined using the ninhydrin method described by Doi et al. (1981). One ml samples containing glycine were acidified by the addition of 10 μl of 1% acetic acid. One ml of ninhydrin solution (Sigma Chemicals, Inc) was added to each sample and the samples were placed in an aluminum heat block at 100^{o}C for 10 min to cause color change. At 10 min, the samples were transferred to an ice bath and 5 ml of 95% ethanol were added to each sample to stabilize the color. Absorbance of samples was spectrophotometrically determined at 570nm and regression equations derived from standard curves were utilized to determine glycine concentration of experimental solutions.

Inclusion efficiency (IE)

Inclusion efficiency was expressed as the percentage of glycine originally present in the lipid mixture, prior to spraying that was successfully encapsulated. IE was determined by dissolving triplicate samples of 25 mg of each microparticle type in 5 ml of chloroform in a tared 25 ml test tube. Glycine was removed from the chloroform by

washing with 5 ml of distilled water five times. The aqueous supernatant was removed after each washing and all five washings were combined. A sample from each replicate was treated with ninhydrin and absorbance was compared spectrophotometrically against distilled water at the absorbance peak for ninhydrin (570 μm) using a Beckman DU 530 UV/Vis spectrophotometer (Beckman inc.). Solution concentrations were calculated using a regression equation derived from standard curves for glycine, and total amounts of glycine in the aqueous extracts were calculated. The chloroform remaining in the test tubes was removed by evaporation using a stream of dry nitrogen and the weight of wall material was determined from the remaining product.

To determine the quantity of glycine on the outside of the particles, triplicate LSB samples on a 0.65 μm membrane filter (Durapore; Millipore) were rinsed with 10 ml of distilled water and the resulting rinsate collected in 15 ml centrifuge tubes. Concentrations of glycine were determined in the rinsate as described above. The amount of glycine in the rinsate was subtracted from the total glycine in the aqueous fraction of the extractions described previously, to determine the true value of the glycine from within the particles.

IE = [(ratio of AA to lipid in LSB) / (ratio of AA to lipid in formula)] X 100

Encapsulation efficiency (EE)

Encapsulation efficiency was described as the percentage wet weight of particles (lipid matrix and suspended aqueous glycine) that was composed of glycine (dry weight).

Encapsulation efficiency was calculated from the adjusted glycine content, as determined above, and the total weight of LSB extracted.

EE = (dry weight of glycine / wet weight of particles) X 100

Retention efficiency (RE)

Retention efficiencies were expressed as the percentage of initial core material retained after particle suspension in distilled water at 22^{o}C for a specified period of time. Triplicate samples of particles (10 mg for LSB, 33 mg for CP) were added to 25 ml test tubes for each sample time and mixed using a Tissue Culture Rotator (Glas-Col). At time intervals of 2, 10, 30, and 60 min the contents of triplicate test tubes for each treatment were filtered onto a 0.65 μm membrane filter (Durapore; Millipore). The filtrate was collected in a 15 ml centrifuge tube and the concentration of glycine was determined using the ninhydrin method as previously described.

RE = {[(initial glycine)-glycine in filtrate)] / (initial glycine)} X 100

Delivery efficiency (DE)

Delivery efficiency was calculated as the percent wet weight of particle (lipid matrix and suspended aqueous core glycine) composed of glycine (d/w) after a given time period of suspension in water.

DE = (dry weight glycine at t_n/ initial particle wet weight) X 100

Where t_n is the length of the time particles were immersed in distilled water.

Short term feeding of blue spotted goby larvae (Asterropteryx semipunctata) on zein-bound complex particles

Three-day-old, first-feeding larvae were stocked in one-liter beakers at a density of 100 larvae l^{-1} and allowed to recover for two hours prior to commencement of feeding. The experimental containers were maintained at 26^{o}C under full spectrum fluorescent lighting (Aquasun 10,000K, Ultraviolet Resources International). Light intensity was dependent on the experiment and treatment. Larvae were fed CP at a rate of 100 mg l^{-1} added each fifteen min for a period of one hour. CP were composed of 30% zein, 30% LSB, and 40% starch.

At the end of the experimental period, larvae were sieved onto an 80 μm Nytex screen and stored in 5% buffered (pH 8.3) formalin at 5^{o}C. Sub-samples of 30 larvae from each replicate were observed microscopically and the presence of food particles within the gut noted as evidence of feeding incidence. Digital images were taken of all larvae containing food within the digestive tract using a Spot Insight QE digital system (Diagnostic Instruments Inc.), and gut fullness, defined as the total area of food within the gut, was calculated using an image analysis system (Image Pro v. 4.5.1; Media Cybernetics). The percentage of larvae observed to have consumed CP was calculated using all 30 larvae in the sample to determine feeding incidence and a mean gut fullness value was calculated for those larvae observed to have fed.

Effect of light intensity on larval feeding on zein-bound complex particles

The influence of light intensity on the proportion of larvae feeding was investigated by stocking first-feeding larvae into 1 l beakers at a density of 100 larvae l^{-1}. Containers were placed under light intensities of 0, 0.15, 1.04, 2.90, 5.50, and 11.52 μE s^{-1} m^2. Different light intensities were achieved using layers of mesh screen to reduce the light emitted by a 24-watt full spectrum fluorescent bulb (Aquasun 10,000K, Ultraviolet Resources International). Light intensity was measured using a LiCor LI-1000 Data Logger (Li-Cor inc.) fitted with a SPH Quantum light probe (Li-Core Inc). Larvae were fed CP containing LSB with a core of distilled water. Larvae were collected at the end of the 1 h experimental period, preserved, and feeding incidence and gut fullness values were determined as previously described.

Effect of prey contrast on larval feeding on zein-bound complex particles

The effect of prey contrast against background on larval feeding was investigated using experimental vessels consisting of 1 l beakers with an internal coating of either black or white epoxy paint. First-feeding larvae were added to the beakers at a density of 100 larvae l^{-1} and fed CP colored either black or white with incorporated finely ground epoxy paint (<10 μm; McCrone micronizing mill, McCrone Scientific Ltd.). CP were composed of 30% zein, 30% fresh water core LSB, 20% starch, and 20% finely ground epoxy paint. Treatments consisted of larvae fed black particles against white background, black particles against black background, white particles against white background and white particles against black background. Experimental vessels were

maintained at a light intensity of 5.44 μE s^{-1} m^2 using a 24 watt full spectrum fluorescent bulb (Aquasun 10,000K, Ultraviolet Resources Inc.). Larvae were collected at the end of the 1 h experimental period, preserved, and feeding incidence and food consumption values were determined as previously described.

Effect of addition of free amino acids to culture media on larval feeding

Koven et al. (2001) indicated that the free amino acids glycine, alanine and arginine had a stimulatory effect on feeding for gilthead seabream larvae presented with microparticulate diets. The potential of these amino acids as feeding stimulants for first-feeding blue spotted goby larvae was investigated following similar methods to those employed by Koven et al. (2001). Artificial seawater was used to ensure the absence of potential feeding stimulants being tested in the base culture media. Artificial seawater was prepared at 31 ppt using Instant Ocean Salt (Aquarium Systems Inc) and heated to 26^{o}C. Larvae were stocked into 1 l beakers coated with black epoxy paint at a density of 100 larvae l^{-1} under a 24 watt full spectrum fluorescent bulb (Aquasun 10,000K, Ultraviolet Resources International) muted to light intensity of 5.44 μE s^{-1} m^{-2}. Treatments (Table 2.2) consisted of artificial seawater, artificial seawater with 9.1 ng ml^{-1} glycine, artificial seawater with 5.9 ng ml^{-1} alanine, and artificial seawater with 0.2 ng ml^{-1} arginine (Kolkovski et al. 1997). CP were composed of 30% (w/w) zein, 30% (w/w) fresh water core LSB, and 40% (w/w) starch. At the time of the first addition of CP, potential stimulants were added to beakers to obtain the previously described amino acid concentrations per treatment. Larvae were collected at the end of the 1 h experimental

period, preserved, and feeding incidence and gut fullness values were determined as previously described.

Effect of addition of microalgae on larval feeding on zein-bound complex particles

First-feeding larvae were stocked into 1-l beakers coated with black epoxy paint at a density of 100 larvae l^{-1} under a light intensity of 5.44 $\mu Es^{-1}m^2$. Larvae were larvae fed CP with or without additions of the marine phytoplankton *T. Isochrysis galbana* (T. *Iso*.) and *Rhodomonas sp.* (3C) as described in Table 2.3. Larvae were collected at the end of the 1 h experimental period, preserved, and feeding intensity and food consumption was determined as previously described.

Digestibility of CP containing LSB by blue spotted goby larvae

Digestibility of CP was investigated using qualitative observations. First-feeding blue spotted goby larvae were stocked into a 2 l mesh-bottomed rearing container with a flow rate of 1 ml sec^{-1} filtered seawater. Larvae were fed *ad libitum* on CP containing LSB with an aqueous riboflavin core (400 g l^{-1}). At the end of 1 h, a sample of larvae was taken to observe gut contents using an epifluorescent microscope (Leica DM1000, Leica inc) fitted with a UV light source. Fluorescence of free riboflavin under UV lighting indicated release of riboflavin from ingested CP.

Survival of blue spotted goby larvae (Asterropteryx semipunctata) fed CP vs. micro algae

First-feeding larvae were stocked into a flow-through larval rearing system at 150 larvae per 1^{-1}. Culture vessels consisted of black, 2-l, polypropylene containers with 250 μm Nytex screening across the bottom. Water was added at the top of the water column at a rate of 1 ml sec^{-1}. Treatments consisted of a starved control, a control fed the phytoplankton Tahitian *Isochrysis galbana* (T. *Iso.*) and *Rhodomonas sp*. (3C) at a concentration of 50,000 cells ml^{-1} each, and three treatments fed CP containing LSB with incorporated casein, hydrolyzed casein or FAA. Amino acid profiles of casein and hydrolyzed casein were determined using HPLC (AAA Laboratories; Boring, Oregon; Table 2.4). Amino acid weight ratios of casein and hydrolyzed casein were averaged to determine the ratios of free amino acids incorporated into LSB added to CP. Diet compositions were as described in Table 2.5. Water temperature was maintained at 26°C, lighting at 5.44 μE s^{-1} m^2, and salinity at approximately 32 ppt. Algae were added to the algal-based treatments once every 6 h and the artificial diet treatments were fed at rations of 50 mg of CP every hour over a 20 h period each day. At the end of the five-day experiment, surviving larvae were harvested and stored in 5% buffered formalin (pH 8.3) at 5°C. Larvae were counted to determine total survival. Growth was measured as notochord length by image analysis (Image Pro v. 4.5.1; Media Cybernetics). Notochord length was defined as the length from the tip of the upper jaw to the tip of the notochord.

Statistical Analysis

Data were checked for normality and homogeneity of variance using normal probability plots and Bartlett's test, at a 5% level of significance, to ensure that assumptions of ANOVA were met. Where necessary, data were transformed to conform to the assumptions of ANOVA. Two-way ANOVA was used to analyze RE and DE data using time and treatment as variables and one-way ANOVA was used to analyze all other data. Tukey HSD test was used to test the significance, at a level of 5%, of differences among treatments at a 5% level of significance.

RESULTS

Optimizing LSB

Core Concentration

Core concentration of glycine did not have a significant effect on RE of glycine by LSB (two-way ANOVA; $p=0.066$); in contrast, suspension time had a significant effect (two-way ANOVA; $p<0.0001$) (Figure 2.3). A significant time x core concentration interaction effect was observed (two-way ANOVA; $p=0.034$), but there was no obvious pattern to explain this interaction.

There was a significant difference in DE among treatments (two-way ANOVA; $p<0.0001$), and LSB with a core concentration of 400 g l^{-1} glycine had a significantly higher DE than with either 100 or 200 g l^{-1} core glycine concentrations (Tukey HSD, $p<0.05$; Figure 2.4). Time (two-way ANOVA; $p<0.0001$), and time x core concentration interaction effects (two-way ANOVA; $p<0.009$) had significant effects on DE of glycine.

While DE of all treatments decreased over time, differences among treatments also decreased over time (Figure 2.4). Significant differences were noted among all treatments at each time interval with 400 g l^{-1} showing the highest DE at each interval. LSB containing 400 g l^{-1} glycine cores had a DE of 14% w/w at time 0 which decreased to 8% w/w at 60 min.

There was a significant difference (Tukey HSD, $p<0.0001$; Table 2.1) in IE between LSB containing 600 g l^{-1} core and IE of all other treatments (Table 2.1), with a core concentration of 600 g l^{-1} having the lowest IE at only 55.95% w/w. LSB containing glycine cores at 100, 200, and 400 g l^{-1} showed no significant differences with IE of 88.44, 97.37, and 86.35% w/w respectively (Tukey HSD; $p>0.0001$). Furthermore, it was found that it was difficult to form an emulsion during production of LSB with a core concentration of 600 g l^{-1} resulting in greater difficulty in spraying.

Core concentration had a significant effect on EE (two-way ANOVA, $p<0.0001$; Table 2.1). All tested core concentrations of LSB showed significant differences in EE, except at 400 and 600 g l^{-1}, which displayed the highest EE at 19.62 and 19.17% w/w, respectively.

Core to lipid ratio

There were significant differences (two-way ANOVA; $p<0.0001$; Figure 2.5) in RE among all core-to-lipid treatments. LSB prepared with a core to lipid ratio of 1:2 had a significantly higher 60 min RE than all LSB prepared with lower ratios (Tukey HSD; $p<0.05$). LSB prepared with a core to lipid ratio of 1:2 and 1:2.5 did not have a

significantly different RE (Tukey HSD; $p>0.05$) with 60 min RE values of 64% and 54% w/w, respectively. Time also had a significant effect on the RE of glycine by LSB (two-way ANOVA; $p<0.0001$). No significant time x treatment interaction was indicated (two-way ANOVA; $p=0.1275$). All treatments showed a general trend towards lower RE over time.

Core to lipid ratio had a significant effect on DE (two-way ANOVA; $p<0.0001$; Figure 2.6). DE of LSB with a core to lipid ratio of 1:1.25 v/v did not differ significantly from those with ratios of either 1:1.5 or 1:2.5 (Tukey HSD; $p>0.05$). Significant differences were seen among all other core to lipid ratios. Time had significant effects on DE (two-way ANOVA; $p<0.0001$) with DE decreasing over time. No time-treatment interaction was noted (two-way ANOVA; $p=0.1122$). All treatments appeared to follow the same trend over time. LSB with a core to lipid ratio of 1:2 displayed a significantly higher average DE across the test period than all other treatments with an initial DE of 11.3% w/w decreasing to 8.6 w/w at 60 min. LSB with a core to lipid ratio of 1:1 had the lowest DE over the test period with an initial DE of 5.8% w/w decreasing to 3.0% w/w at 60 min.

LSB containing different core to lipid ratios showed significant differences in IE (ANOVA; $p<0.0001$; Table 2.1). IE increased with an increase in core to lipid ratio. IE for LSB with a core to lipid ratio of 1:1 was significantly lower than that for all other treatments. LSB with ratios of 1:2 and 1:2.5 were not significantly different from each other, but were significantly higher than for all other treatments.

Different core to lipid ratios had a significant effect on EE (ANOVA; $p<0.0001$), with LSB prepared with a core to lipid ratio of 1:1.25 having the highest EE of 17.13%. EE of LSB prepared with ratios of 1:1, 1:2, and 1:2.5 were not significantly different. EE increased as the ratio of core to lipid increased up to a ratio of 1:1.25, above which EE decreased.

Loss of core glycine by LSB during CP production

LSB containing a core glycine concentration of 400 g l^{-1} at a 1:2 ratio v/v had an RE of 99% w/w after an initial rinse with 90% alcohol, followed by an RE of 97% w/w after 2 min, and 96% w/w after 10 min of suspension (Figure 2.7). There was a significant difference in RE among periods of 0, 2, and 10 min suspension (Tukey HSD; $p<0.05$). While the differences among RE at the different time intervals were statistically different, the overall loss of glycine was well within acceptable limits for the production of CP.

Comparison of RE and DE of free LSB versus CP containing LSB

Zein-bound CP containing LSB with a 400 g l^{-1} glycine core and a ratio of 1:2 core to MS had a significantly lower RE than freely suspended, similarly composed LSB (Tukey HSD; $p<0.05$; Figure 2.8), with a 60 min RE of 53% for LSB and 21% for CP. Time had a significant effect on RE and there was a significant time x particle type interaction effect (two-way ANOVA; $p<0.05$).

CP had a significantly lower DE compared to LSB (Tukey HSD; $p<0.05$; Figure 2.9). After an initial rinse, LSB had a DE of 11.9 % w/w while CP had a DE of 3.9%

w/w. After 60 min in aqueous suspension, LSB had a DE of 7.3% w/w while CP had a DE of 1.2% w/w.

Effect of light intensity on larvae feeding on zein-bound complex particles

Light intensity had a significant effect on the feeding incidence of larvae fed on CP (ANOVA HSD; $p>0.0001$). The average percentage of larvae that ingested CP at different light intensities ranged from 2 to 24% w/w (Figure 2.10). Percent feeding significantly increased with light intensity, peaking at 5.44 $\mu E\ s^{-1}\ m^{-2}$ (Tukey HSD; $p<0.0001$).

Effect of prey contrast on larval feeding on zein-bound complex particles

The average percentage of goby larvae feeding on CP with different contrast combinations ranged from 6 to 35% w/w (Figure 2.11). Particle color (two-way ANOVA; $p=0.0332$) and background color (two-way ANOVA; $p<0.001$) both had a significant effect on the percentage of larvae ingesting CP, with larvae fed white particles against a black background having the highest feeding incidence. White CP were shown to have significantly higher feeding incidences against both white and black backgrounds when compared to feeding incidence larvae fed black CP against like backgrounds (Tukey HSD; $p<0.05$). Larvae fed white CP against a black background displayed the highest feeding incidence (Tukey HSD; $p<0.05$). No significant difference was observed in gut fullness of larvae that had fed on the different CP (two-way ANOVA; $p=0.7154$).

Effect of addition of free amino acids to culture media on larval feeding

The percentage goby larvae that fed on FAA free CP in a culture media consisting of artificial seawater, or artificial seawater mixed with individual amino acids glycine, alanine, and arginine ranged from 32% to 36% over a 1 h feeding period (Fig. 2.12). These additions of amino acids had no significant effect on either the feeding incidence of larvae fed CP (ANOVA; $p = 0.6206$), nor on the quantity of CP ingested (ANOVA; $p=0.2342$).

Effect of addition of microalgae on larval feeding on zein-bound complex particles

The percentage goby larvae that fed on CP, phytoplankton, or a combination of the two ranged from 39% to 59%. While there was no statistical difference among these frequencies (Tukey HSD; $p<0.05$), larvae fed on CP showed the lowest incidence of feeding at 39% and co-fed larvae had the highest incidence of feeding at 59%. There was no significant difference in the percentage of larvae feeding among treatments (ANOVA; $p=0.1610$; Fig. 2.13a), or in gut fullness measurements (ANOVA; $p=0.1191$; Fig. 2.13b). While no statistical difference was found among treatments, there was a trend indicating that larvae fed on CP had the highest gull fullness values, followed by those of co-fed and algae-fed larvae (Fig. 2.13b).

Digestibility of CP containing LSB by blue spotted goby larvae

First-feeding goby larvae ingested CP containing LSB with riboflavin cores, as observed by epifluorescent microscopy. Riboflavin was released from CP into the fore-

and hindgut (Fig. 2.14a). Furthermore physical breakdown of CP were noted as particles reached the hindgut (Fig. 2.14b).

Survival of blue spotted goby larvae (Asterropteryx semipunctata) fed CP vs. micro algae

The percent survival of five-day post feeding larvae fed on different types of CP or algae ranged from 6% to 37% (Figure 2.15). Starved larvae showed 100% mortality on day four of the experiment. While there were no significant differences in survival of larvae fed on the different CP types, their survival was significantly less than that of larvae fed on algae (Tukey HSD; $p=0.0002$). Average survival of larvae fed on algae was 37% compared to 6%, 8%, and 6% of larvae fed on casein CP, hydrolysed casein CP, and FAA CP respectively. While larvae fed CP did not have higher survival than those fed phytoplankton they did display significantly higher survival than that of the starved control, which showed 0% survival after 4 days. At day 5 there was no significant difference in the notochord length of blue spotted goby larvae.

Exp #	Core Glycine Conc. (g l^{-1})	Core:lipid (v/v)	Wall Material (%) w/w	IE (%) w/w	EE (%) w/w
1					
	100	1:1.5	100% MS	88.44[a]	5.88[a]
	200	1:1.5	100% MS	97.37[a]	12.10[b]
	400	1:1.5	100% MS	86.35[a]	19.62[c]
	600	1:1.5	100% MS	55.95[b]	19.17[c]
2					
	400	1:1	100% MS	34.85[a]	12.88[a]
	400	1:1.25	100% MS	60.92[b]	17.13[b]
	400	1:1.5	100% MS	61.82[b]	14.88[c]
	400	1:2	100% MS	80.78[c]	14.62[a,c]
	400	1:2.5	100% MS	86.78[c]	12.82[d]

Table 2.1: Inclusion (IE) and encapsulation efficiencies (EE) of LSB containing glycine at different core concentrations and different core to lipid ratios. Letters denote significant differences (Tukey HSD; $p<0.05$).

Treatment	Culture Media	Feeding Stim Conc. (ng ml^{-1})
1	SW	-
2	SW, glycine	9.1
3	SW, alanine	5.9
4	SW, Arginine	0.2

Table 2.2: Potential feeding stimulant concentrations added to artificial seawater for feeding experiments with blue spotted goby larvae (*Asterropteryx semipunctata*). FAA added at concentrations described by Kolkovski et al. (1997)

Treatment	CP (mg)	T. ISO (cells ml^{-1})	3C (cells ml^{-1})
Starved Control	-	-	-
Live food	-	50,000	50,000
Co-feed	100	50,000	50,000
ZBP	100	-	-

Table 2.3: Dietary treatments to determine the effects of co-feeding complex particles (CP) with microalgae (*Isochrysis galbana*, Tahitian strain, T-ISO, and *Rhodomonas sp.,* 3C) on first-feeding blue spotted goby (*Asterropteryx semipunctata*) larvae.

		FAA contents		
AMINO ACID		Casein (mg g^{-1} casein)	Hydrolyzed Casein (mg g^{-1} hydro. Casein)	Mean FAA Content (mg g^{-1} mix)
ASP	(D)	58.97	62.04	60.50
THR	(T)	35.08	36.49	35.79
SER	(S)	45.40	43.47	44.44
GLU	(E)	191.44	192.84	192.14
PRO	(P)	92.86	77.52	85.19
GLY	(G)	13.64	14.05	13.84
ALA	(A)	22.48	24.20	23.34
VAL	(V)	58.10	59.76	58.93
MET	(M)	25.76	23.50	24.63
ILE	(I)	41.13	42.11	41.62
LEU	(L)	81.18	72.13	76.65
TYR	(Y)	50.94	26.14	38.54
PHE	(F)	49.72	41.26	45.49
HIS	(H)	24.08	20.42	22.25
LYS	(K)	66.76	67.37	67.06
ARG	(R)	29.67	27.74	28.70
Total		887.20	831.03	859.11

Table 2.4: Amino acid compositions (w/w) of casein and hydrolysed casein with average percent compositions calculated to provide an FAA mixture for incorporation into LSB for use in feeding trials. FAA compositions were determined by HPLC (AAA Laboratories, Boring, Oregon).

Ingredient		Casein CP	Hydro. Casein CP	FAA CP
		(g)	(g)	(g)
Rice Starch		0.75	0.75	0.75
Cod Roe Meal		3	3	3
Cod liver oil		0.25	0.25	0.25
LSB Component		3	3	3
MS		2	2	2
Water		0.5028	0.5028	0.5028
Amino Acids/ Casein/ Hydro Casein		0.4	0.4	0.4
	ASP			0.028
	THR			0.017
	SER			0.021
	GLU			0.089
	PRO			0.040
	GLY			0.006
	ALA			0.011
	VAL			0.027
	MET			0.011
	ILE			0.019
	LEU			0.036
	TYR			0.018
	PHE			0.021
	HIS			0.010
	LYS			0.031
	ARG			0.013
Lipid Sol. Vit.		0.0096	0.0096	0.0096
	Retinol	3.79E-05	3.79E-05	3.79E-05
	Cholcalciferol	1.14E-05	1.14E-05	1.14E-05
	Tocopherol	9.41E-03	9.41E-03	9.41E-03
	Menadione	1.51E-04	1.51E-04	1.51E-04
Water Sol. Vit.		0.088	0.088	0.088
	Thiamine	0.00036	0.00036	0.00036
	Riboflavin	0.00053	0.00053	0.00053
	Pyrodoxin	0.00036	0.00036	0.00036
	Pantothenic	0.00267	0.00267	0.00267
	Niacin	0.00267	0.00267	0.00267
	Biotin	0.00002	0.00002	0.00002
	Folic acid	0.00018	0.00018	0.00018
	Cyanocobalmin	0.000003	0.000003	0.000003
	Choline	0.05330	0.05330	0.05330
	Inositol	0.00799	0.00799	0.00799
	Ascorbic acid	0.01954	0.01954	0.01954

Table 2.5: Approximate nutritional composition of CP containing LSB with casein, hydrolysed casein, or FAA cores (g/10g particle).

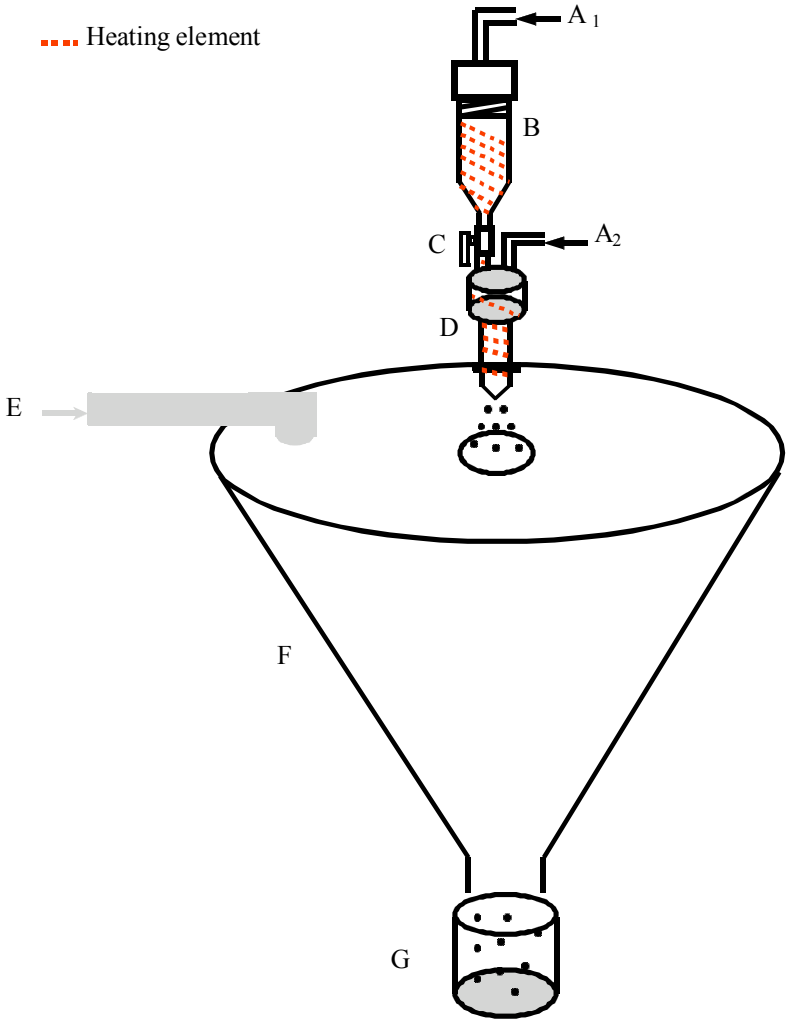


Figure 2.1: Spray system used for the preparation of lipid spray beads (LSB). An emulsion of core material and molten menhaden stearine (MS) at 65°C was added to the metal hopper (B) heated to 65°C using a heating coil (shown as a red dotted line). The emulsion was then atomized into the cooled stainless steel cone using pressurized nitrogen (N_2). See materials and methods for a detailed description of the procedure. (A_1) pressurized N_2 inlet to pressurize the hopper and push out the spray material, (A_2) pressurized N_2 for atomization, (B) metal hopper, (C) fluid control valve, (D) spray nozzle, (E) inlet for liquid nitrogen vapour, (F) stainless steel cone, (G) collection cup. Black arrows indicate direction of pressurized N_2. Grey arrow indicates direction of liquid N_2 vapour. Cone Dimensions: Top diameter = 60 cm, bottom diameter = 10 cm, Cone height = 60 cm.

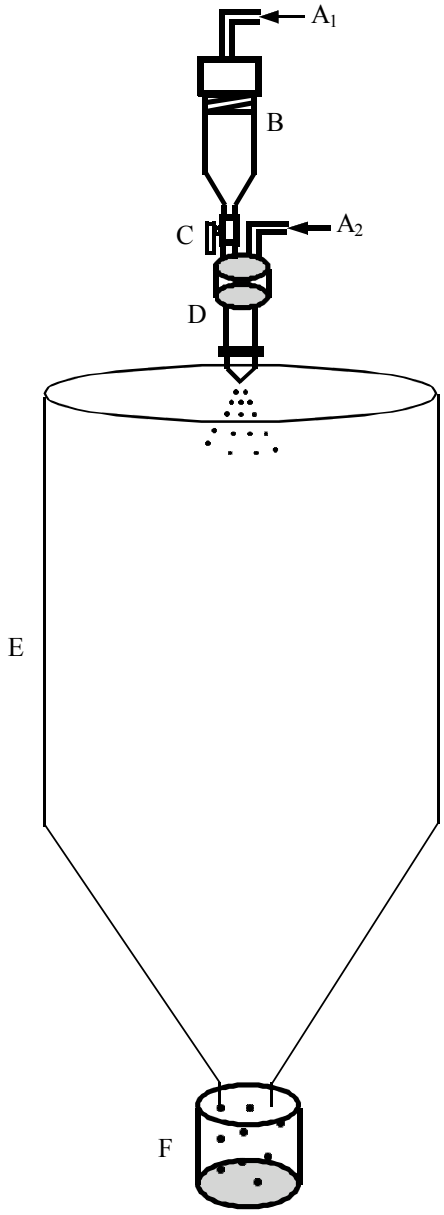


Figure 2.2: Spray system used for the preparation of complex particles (CP) bound with zein. LSB were suspended in a zein/dietary mixture and added to the hopper. The resulting mixture was then atomized into a polyethylene cylindro-conical drum using pressurized nitrogen (N_2) using the methods described in methods and materials. (A_1) pressurized N_2 inlet to pressurize the hopper and push the dietary mixture, (A_2) pressurized N_2 for atomization, (B) metal hopper, (C) fluid control valve, (D) spray nozzle, (E) polyethylene cylindrical-conical drum, (f) collection cup. Black arrows indicate direction of pressurized N_2. Cylinder dimensions: Top diameter = 60 cm, bottom diameter 10 cm, cylinder height 100 cm.

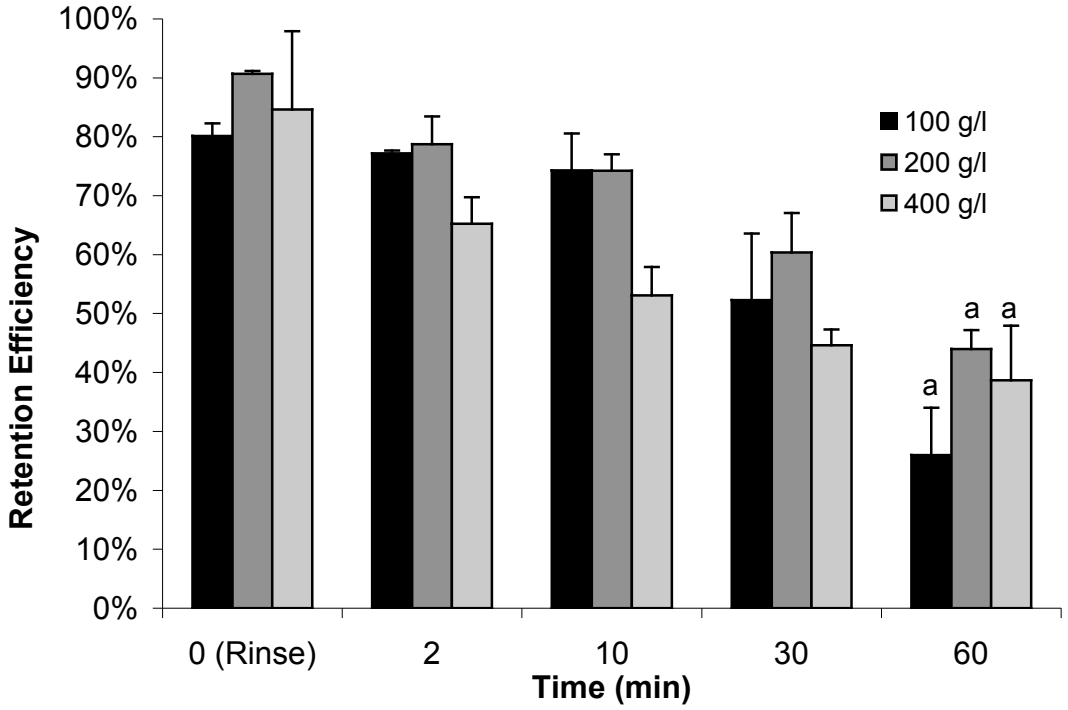


Figure 2.3: Retention efficiencies (RE) of LSB containing glycine cores at a concentration of 100, 200, and 400 g l^{-1} at a core to lipid ratio of 1:1.5 v/v over a 1 h period of aqueous suspension. Error bars represent one standard deviation. Rinse indicates the quantity of external glycine rinsed from particles by a quick rinse with distilled water. Similar letters denote no significant differences in RE at 60 min (Tukey HSD; $p>0.05$).

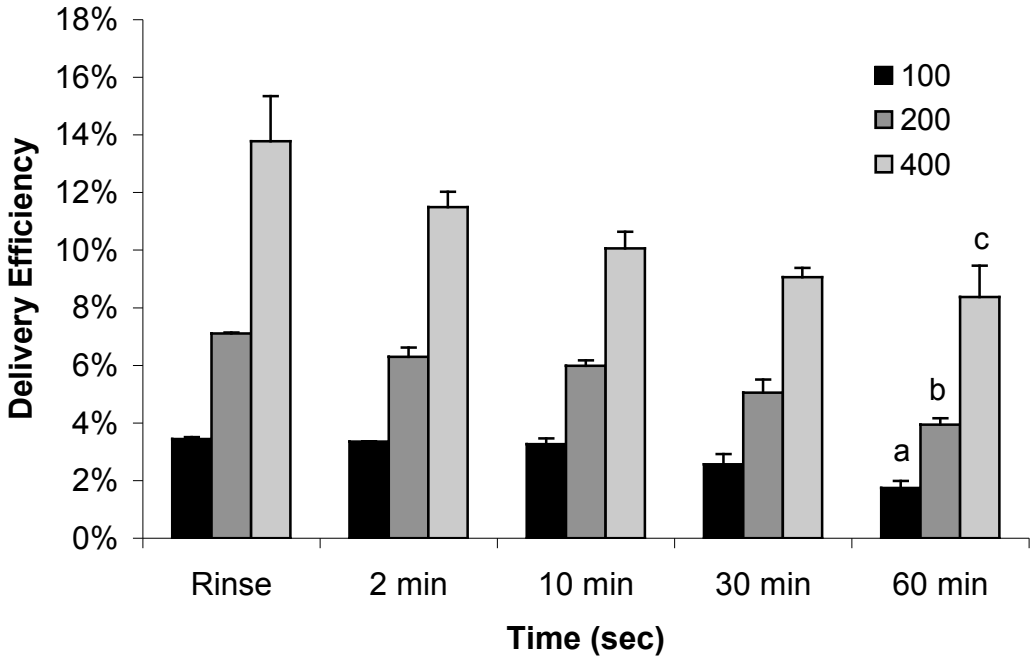


Figure 2.4: Delivery efficiencies (DE) of LSB containing glycine cores at a concentration of 100, 200, and 400 g l^{-1} with a core to lipid ratio of 1:1.5 v/v over a 1 h period in aqueous suspension. Error bars represent one standard deviation. Rinse indicates the delivery efficiency after external glycine was rinsed from particles using a quick rinse of distilled water. Letters denote significant differences in DE at 60 min (Tukey HSD; $p<0.05$).

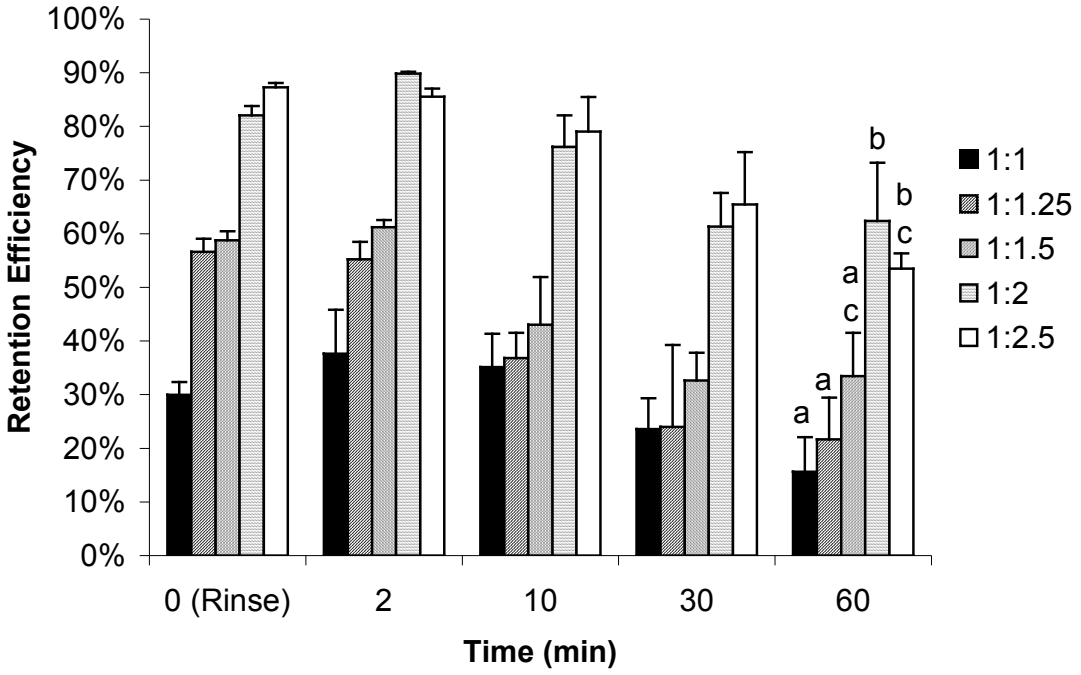


Figure 2.5: Retention efficiencies (RE) of LSB containing glycine at a core concentration of 400 g l^{-1} at core to lipid ratios of 1:1, 1:1.25, 1:1.5, 1:2, 1:2.5 v/v over a 1 h period of aqueous suspension. Error bars represent one standard deviation. Rinse indicates the quantity of external glycine rinsed from particles using a quick rinse of distilled water. Letters denote significant differences in RE at 60 min (Tukey HSD; $p<0.05$).

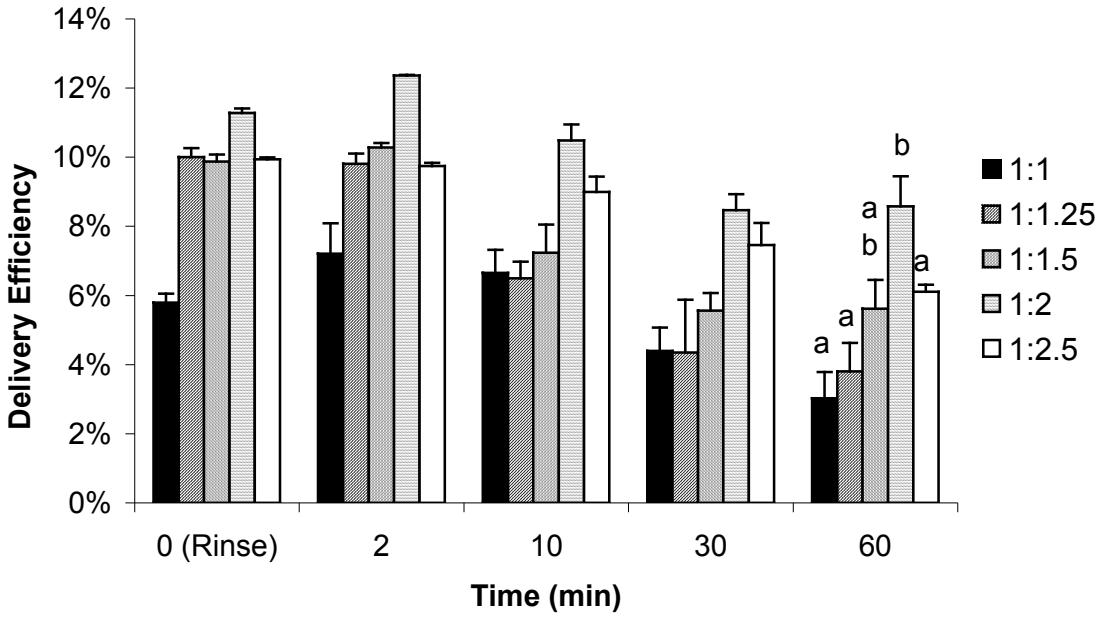


Figure 2.6: Delivery efficiencies (DE) of LSB containing glycine at a core concentration of 400 g l^{-1} at core to lipid ratios of 1:1, 1:1.25, 1:1.5, 1:2, 1:2.5 v/v over a 1 h period of aqueous suspension. Error bars represent one standard deviation. Rinse indicates the delivery efficiency after external glycine was rinsed from particles using a quick rinse of distilled water. Letters denote significant differences in DE at 60 min (Tukey HSD; $p<0.05$).

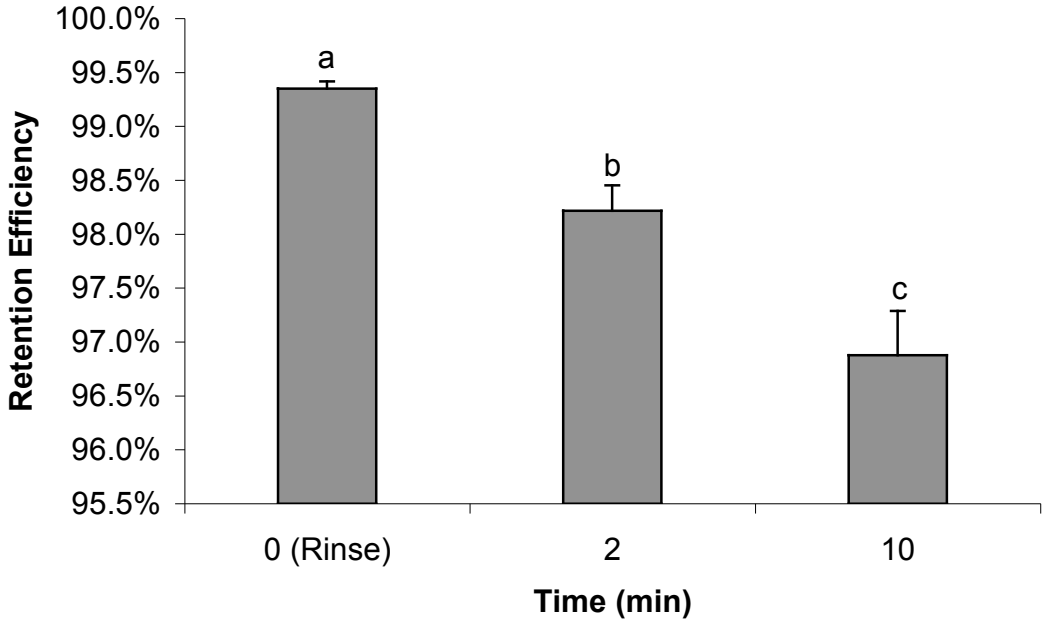


Figure 2.7: Retention efficiencies (RE) of LSB containing glycine at a core concentration of 400 g l^{-1} at core-to-lipid ratio of 1:2 v/v when suspended in 90% ethanol over a 10 min period as an indicator of the quantity of glycine lost during the incorporation of LSB into CP bound with zein. Error bars represent one standard deviation. Rinse indicates the quantity of external glycine rinsed from particles using a quick rinse of 90% ethanol. Different letters denote significant differences in RE. (Tukey HSD, $p<0.05$)

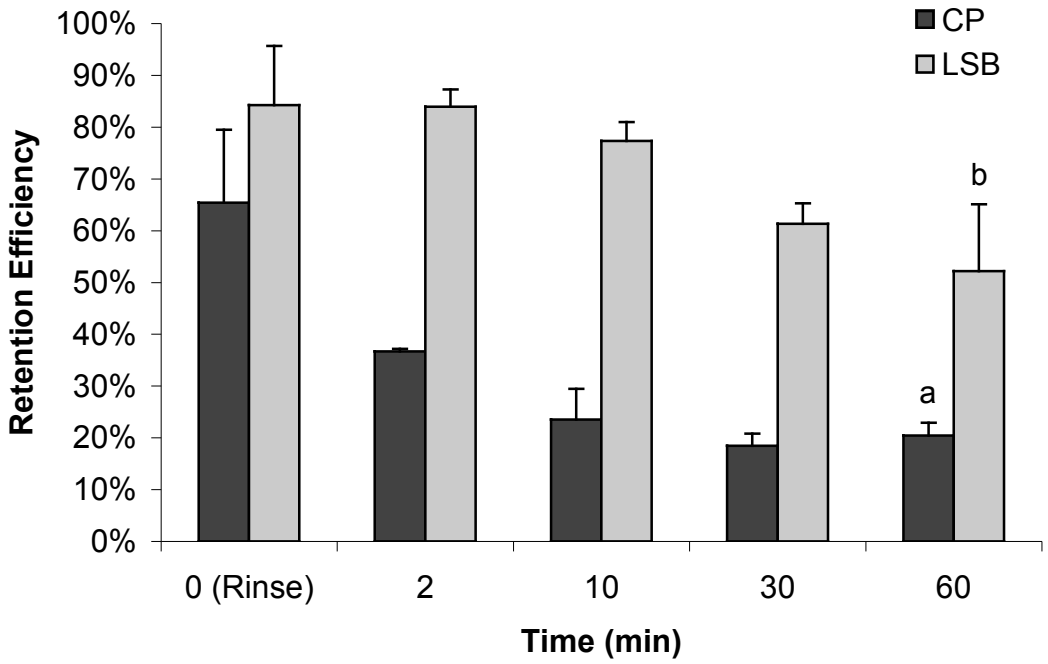


Figure 2.8: Retention efficiencies (RE) of LSB containing core glycine at 400 g l^{-1} and a core to lipid ratio of 1:2 v/v compared to the same LSB when bound in CP composed of 30% w/w zein, 40% w/w rice starch, and 30% w/w LSB over a 1 h period in aqueous suspension. Error bars represent one standard deviation. Letters denote significant differences in RE at 60 min (Tukey HSD; $p<0.05$).

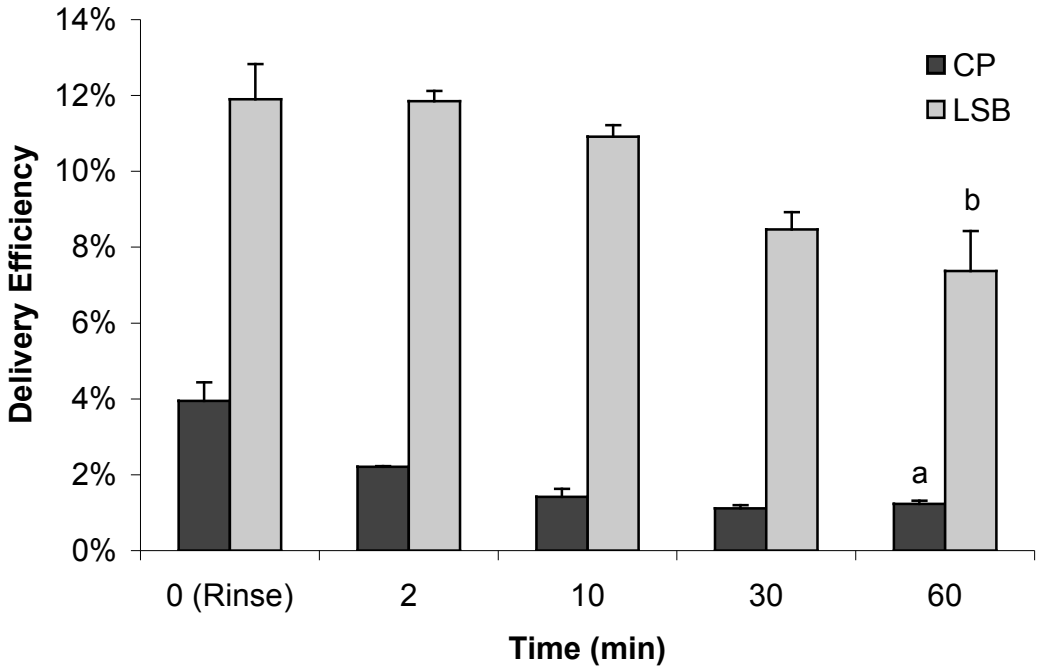


Figure 2.9: Delivery efficiencies (DE) of LSB containing core glycine at 400 g l^{-1} and a core to lipid ratio of 1:2 v/v compared to the same LSB when bound in a CP composed of 30% w/w zein, 40% w/w rice starch, and 30% w/w LSB over a 1 h period in aqueous suspension. Error bars represent one standard deviation. Letters denote significant differences in DE at 60 min (Tukey HSD; $p<0.05$).

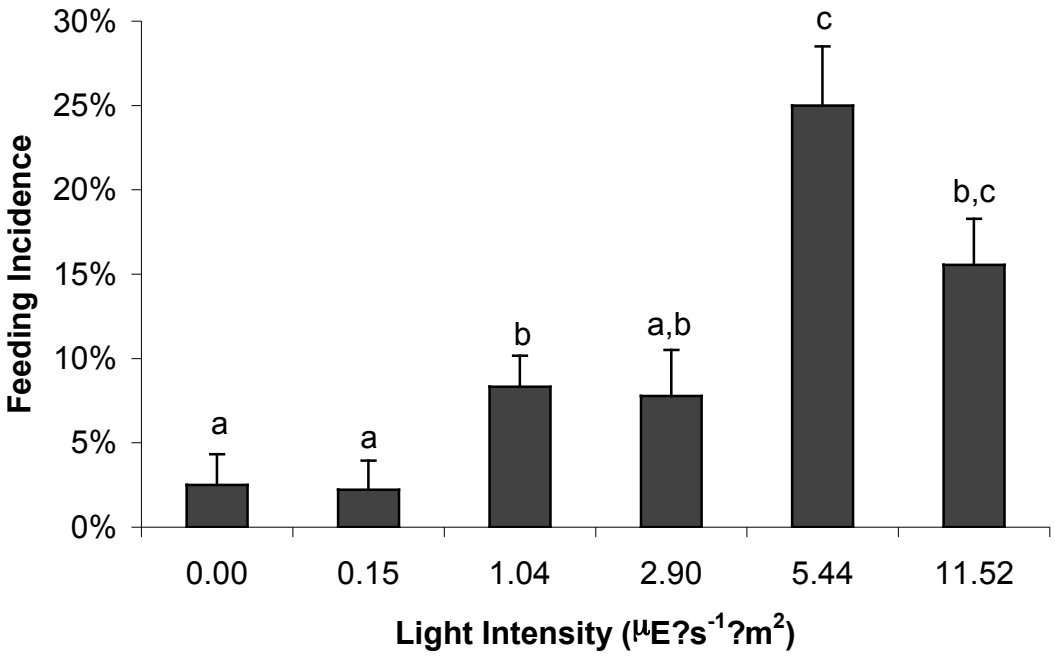


Figure 2.10: Effect of light intensity on the feeding incidence of blue spotted goby larvae (*Asterropteryx semipunctata*) feeding on CP. Error bars represent one standard deviation. Letters denote significant differences (Tukey HSD; $p<0.05$).

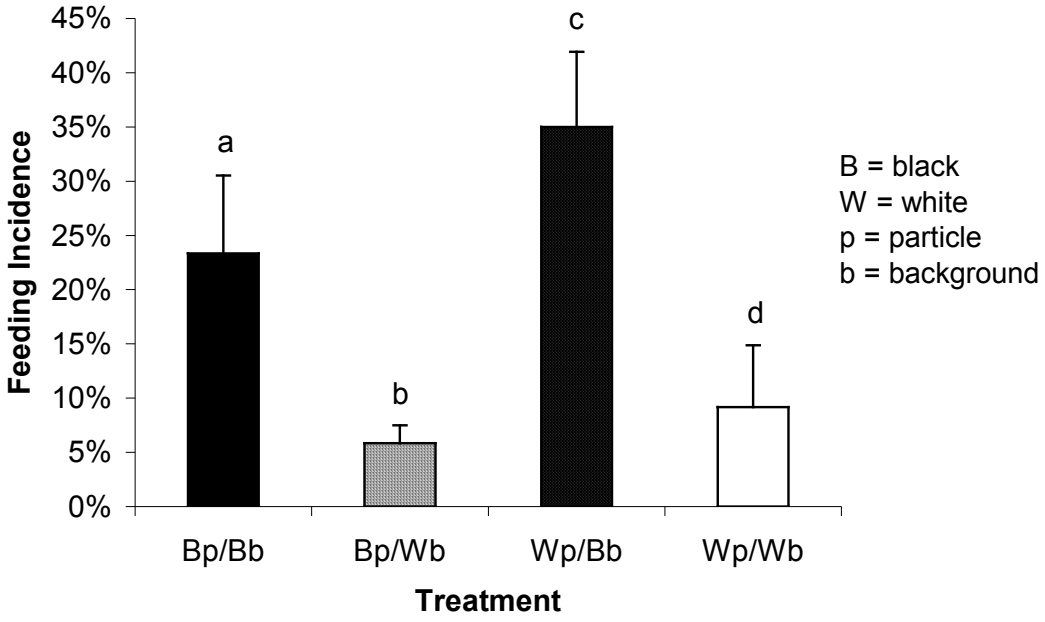
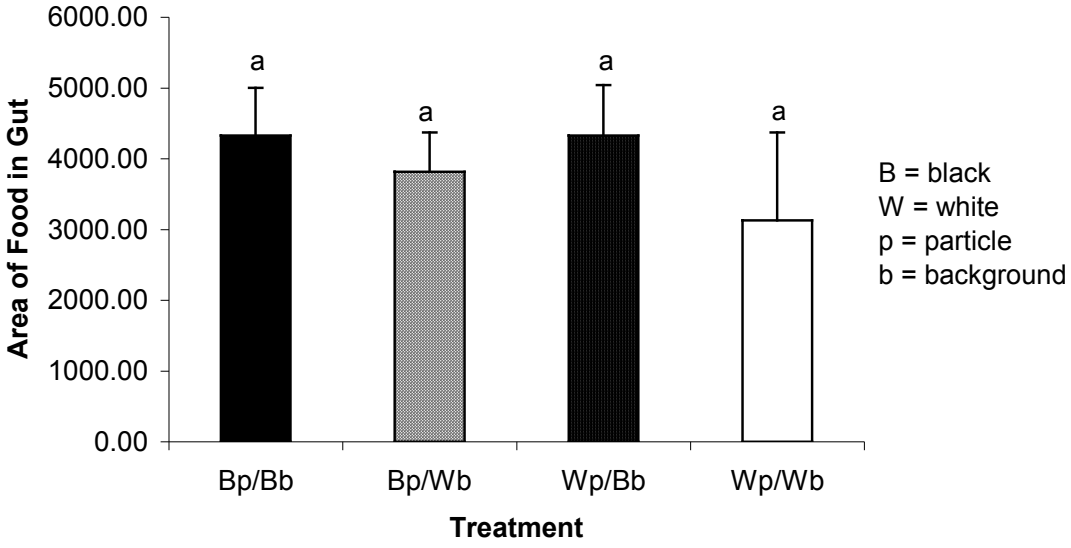


Figure 2.11a: Effect of prey/background contrast on feeding incidence of blue spotted goby larvae (*Asterropteryx semipunctata*) fed on CP. Error bars represent one standard deviation. Letters denote significant differences (Tukey HSD; $p<0.05$)

Figure 2.11b: Effect of prey/background contrast on gut fullness of blue spotted goby



larvae (*Asterropteryx semipunctata*) defined in terms of the two-dimensional area (μm^2) of food within the gut. Error bars represent one standard deviation. Letters denote significant differences (Tukey HSD; $p>0.05$).

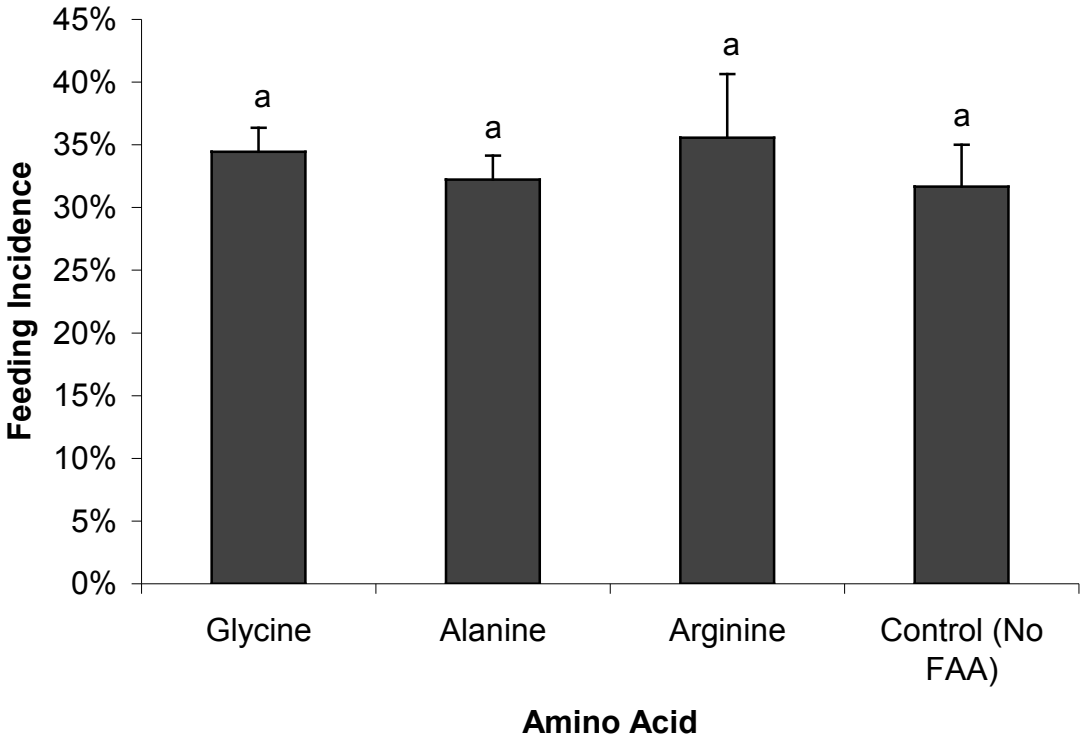


Figure 2.12a: Effect of separate additions of the free amino acids at concentrations of 9.1 ng ml^{-1} glycine, 5.9 ng ml^{-1} alanine, and 0.2 ng ml^{-1} arginine to larval culture media on the feeding incidence of blue spotted goby larvae (*Asterropteryx semipunctata*) fed on CP over a 1 h feeding period. Error bars represent one standard deviation. Letters denote significant differences (Tukey HSD; $p>0.05$).

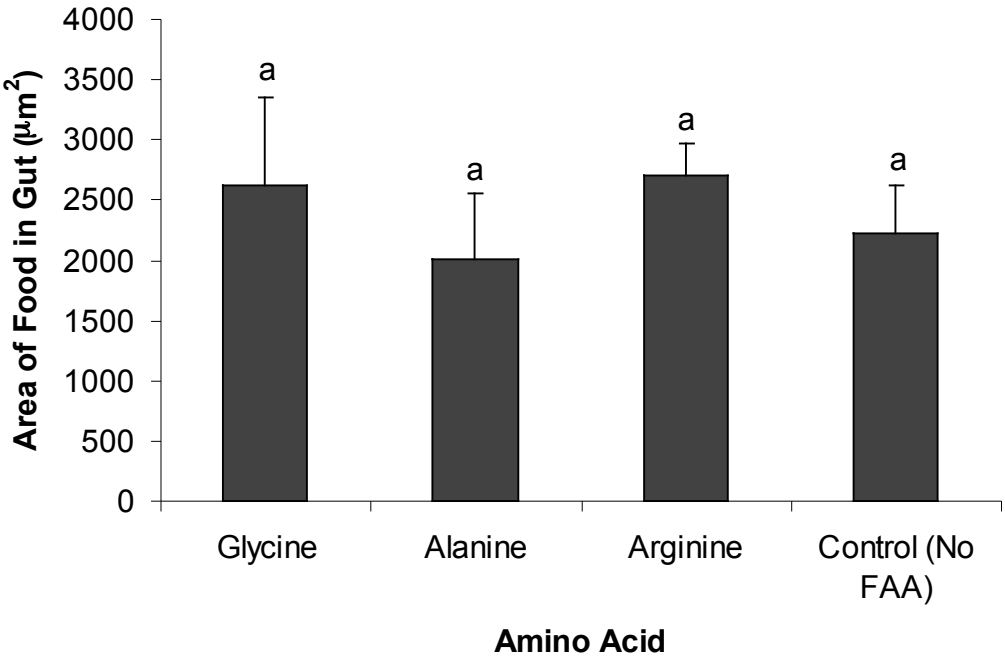


Figure 2.12b: Effect of additions of the free amino acids at concentrations of 9.1 ng ml^{-1} glycine, 5.9 ng ml^{-1} alanine, and 0.2 ng ml^{-1} arginine to larval culture media on gut fullness, defined in terms of two-dimensional area (μm^2) of food within the gut of blue spotted goby larvae (*Asterropteryx semipunctata*). Error bars represent one standard deviation. Letters denote significant differences (Tukey HSD; $p>0.05$).

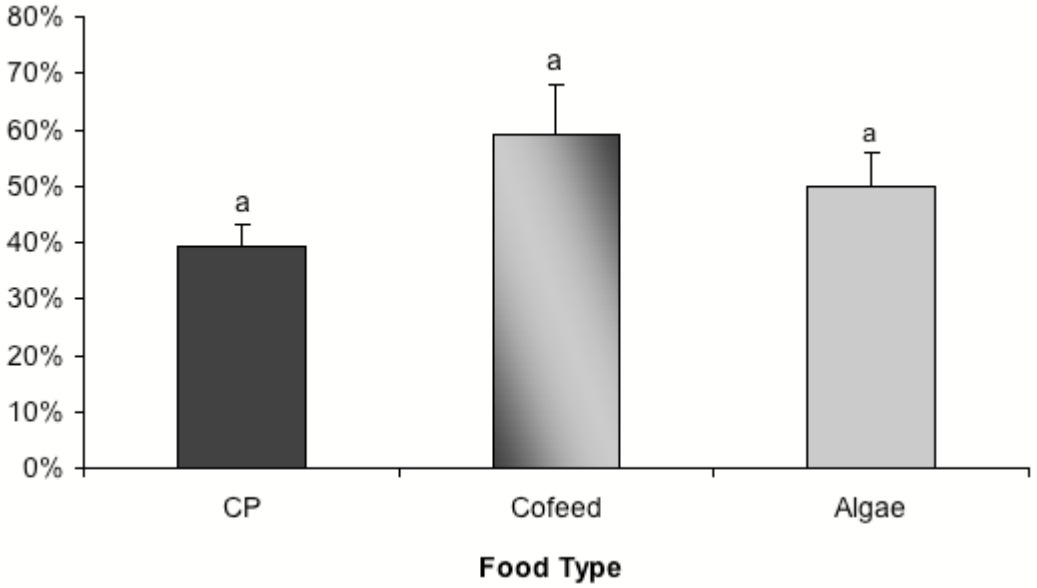


Figure 2.13a: Effect of co-feeding a mixture of algae and zein-bound complex particles (CP) on feeding incidence of blue spotted goby larvae (*Asterropteryx semipunctata*). Error bars represent one standard deviation. Letters denote significant differences (Tukey HSD, p>0.05)

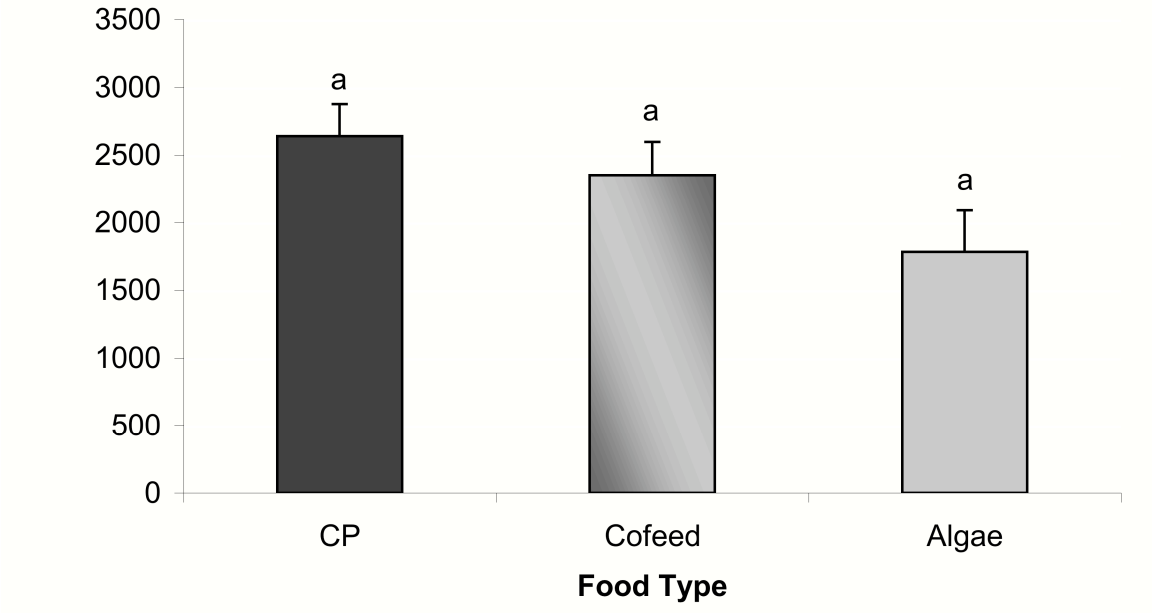


Figure 2.13b: Effect of co-feeding a mixture of algae and zein-bound complex particles (CP) to blue spotted goby larvae (*Asterropteryx semipunctata*) on gut fullness, defined as the two-dimensional area (μm^2) of food within the gut. Error bars represent one standard deviation. Letters denote significant differences (Tukey HSD; p>0.05).

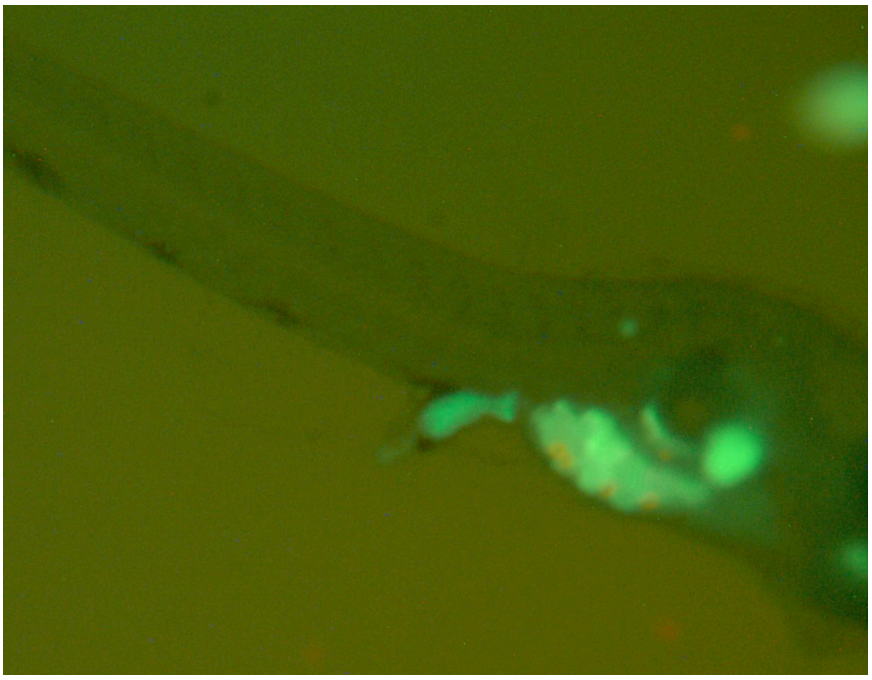


Figure 2.14a: Blue spotted goby (*Asterropteryx semipunctata*) fed CP containing LSB with a riboflavin core. CP are visible in the foregut and free *riboflavin* is visible in the hindgut and exiting the anus.

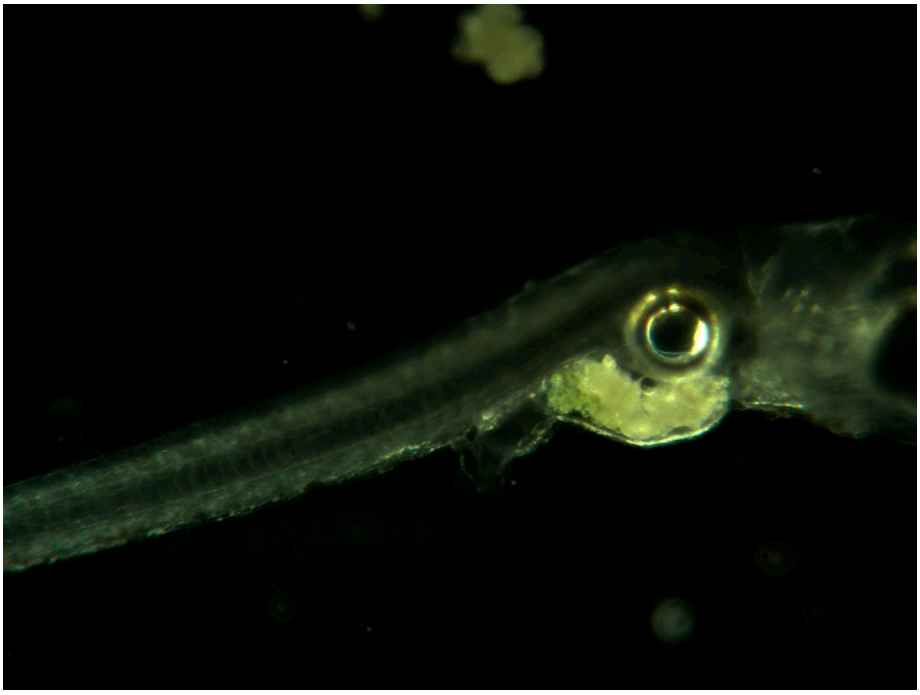


Figure 2.14b: Blue spotted goby (*Asterropteryx semipunctata*) fed CP containing LSB. Mechanical breakdown of CP is evident towards the hindgut.

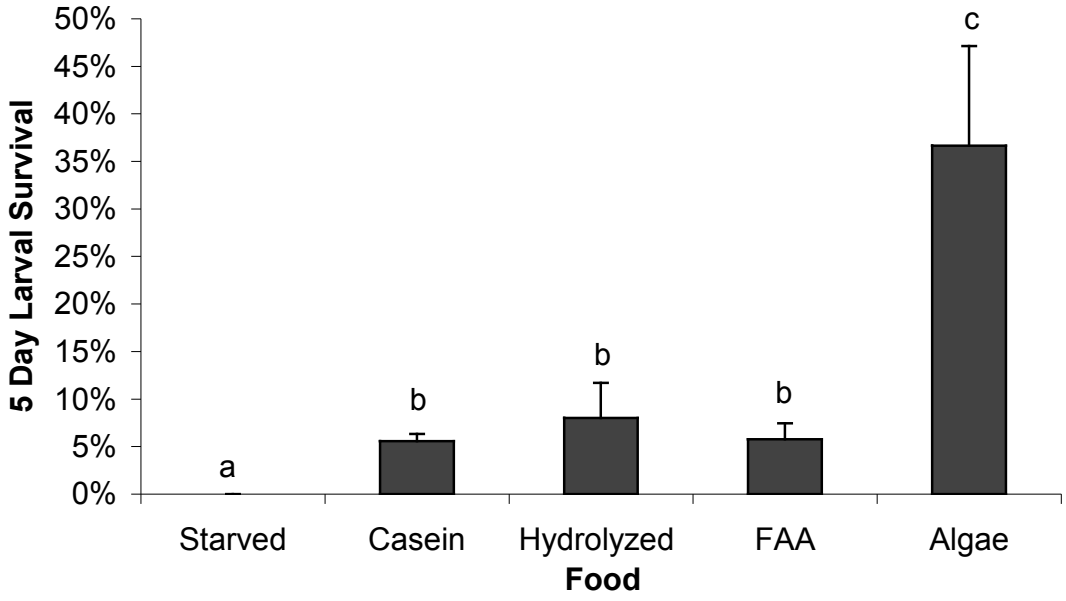


Figure 12.15a: Survival of blue spotted goby (*Asterropteryx semipunctata*) larvae fed CP containing LSB with core contents of casein, hydrolysed casein, or free amino acids compared to that of larvae fed phytoplankton over a five-day period. CP were composed of approximately 4% w/w casein, hydrolysed casein, or free amino acids added in the ratios (w/w) averaged between those of casein and hydrolysed casein. Error bars represent one standard deviation. Letters denote significant differences (Tukey HSD; $p<0.05$).

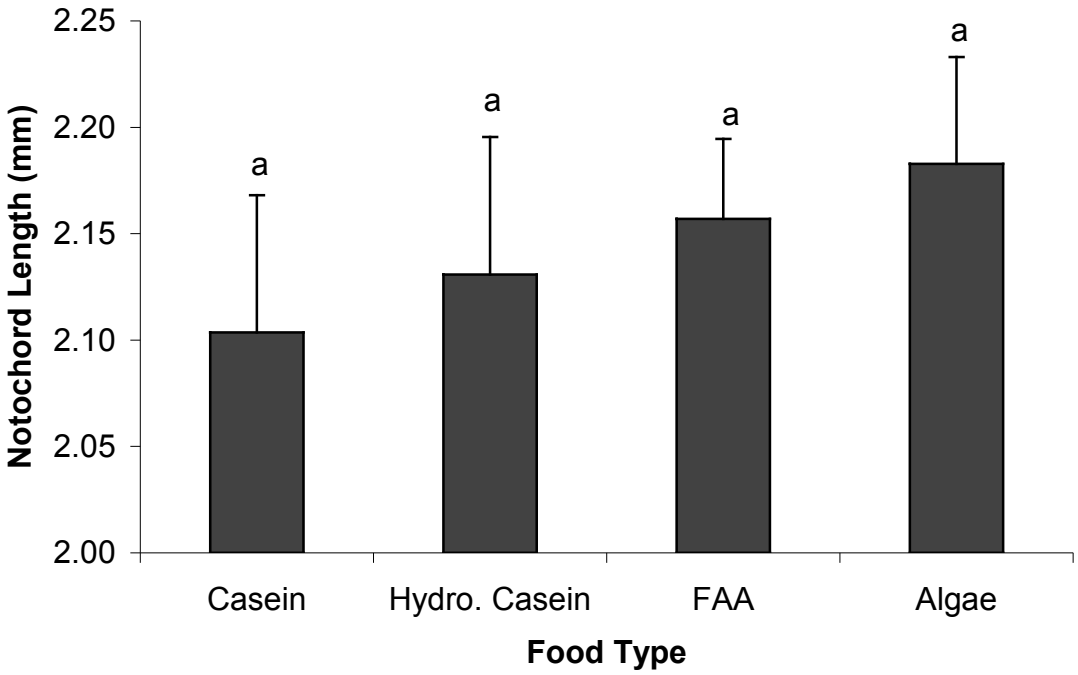


Figure 12.15b: Final notochord length (mm) of blue spotted goby (*Asterropteryx semipunctata*) larvae fed CP containing LSB with core contents of casein, hydrolyzed casein, or free amino acids compared to that of larvae fed phytoplankton over a five-day period. CP were composed of approximately 4% w/w casein, hydrolyzed casein, or free amino acids added in the ratios (w/w) averaged between casein and hydrolyzed casein. Starved control was not included due to 100% mortality on day 4. Error bars represent one standard deviation. Letters denote significant differences (Tukey HSD; $p>0.05$).

DISCUSSION

One of the greatest hurdles in the development of microparticulate diets is creating a diet that efficiently incorporates enough LMWS nutrients to support survival and growth, and remain in suspension long enough to be ingested by the target larvae. Once ingested, the larvae must be able to access and utilize the nutrients. Önal and Langdon (2004a, 2004b) reported the use of menhaden stearine (MS) in LSB improved RE and DE of LMWS nutrients when LSB were placed in aqueous suspension, compared with mixtures of MS, spermaceti and coconut oil. One of the goals of this project was to improve IE, EE, RE, and DE of LSB containing glycine, using MS as the wall material.

It was found that an EE of 19.62% and IE of 86.35% could be obtained for LSB containing an aqueous slurry core of glycine at 400 g l^{-1}, while a core of 600 g l^{-1} resulted in LSB with a similar EE at 19.17%, but lower IE at 55.95%. These results can be compared with those of Önal and Langdon (2004b) who reported LSB with an aqueous glycine core having a higher IE of 89.56%, but lower EE of 4.95%. The lower IE for LSB created with 600 g l^{-1} glycine, compared with that of LSB with lower core concentrations, was likely due to difficulty in creating an emulsion of core material in the molten lipid. While the RE was not significantly different from those of other LSB types, the DE of LSB with a core of 400 g l^{-1} glycine was 8% w/w after 60 min of aqueous suspension and was greatly improved compared with those of LSB with core concentrations of 100 g l^{-1} and 200 g l^{-1} that had DE of 2% and 4%, respectively. Önal and Langdon (2004b) reported that LSB containing an aqueous glycine core performed better than those containing particulate glycine, with aqueous or particulate core LSB

showing a 60 min DE of approximately 2.9% and 0.9%, respectively. Langdon and Buchal (1998) showed that LWC had a higher DE for OTC·HCl when encapsulated in an aqueous solution. This higher RE and DE for LMWS materials dissolved in aqueous solution is possibly due to the formation of hydrophobic lipid walls surrounding the core. In contrast, particulate glycine may protrude from the surface of the lipid beads, resulting in rapid leakage losses. The use of an aqueous slurry of glycine resulted in LSB displaying the advantages of dry core LSB by carrying a higher dose of glycine and of LSB with a completely aqueous core. Due to the presence of water resulted in a hydrophobic interaction between the core material and molten lipid, resulting in lipid-coated pockets of glycine.

Core to lipid ratio had a significant effect on the IE, EE, RE, and DE of glycine at a core concentration of 400 g l^{-1}. A core to lipid ratio of 1:2 resulted in the best overall performance, with IE, RE and DE decreasing significantly at ratios both above and below 1:2. The poor performance of LSB with ratios higher than 1:2 was likely due to reduced lipid available to coat the core material, resulting in thinner bead walls. In extreme cases, such as with a ratio of 1:1 core to lipid, the emulsion may have become reversed, resulting in the intended core material being on the outside of a lipid core. In support of this explanation, Jalil and Nixon (1990) showed that microcapsules containing high core to polymer wall ratios released significantly more of the core material within the first 10 minutes of aqueous suspension compared with those prepared with lower core to wall ratios. LSB with a core to lipid ratio of 1:2.5 had lower DE and IE due to less core material being added to the spray mixture.

LSB containing glycine displayed low leakage when immersed in 90% ethanol for 10 minutes, retaining 96% of the initial glycine. The typical time period between addition of LSB to the zein/alcohol solution and spraying CP is under three minutes. While this indicates that relatively little leaching of glycine occurs during the preparation of CP, the effect of CP preparation on losses of other water-soluble nutrients needs to be determined.

LSB alone cannot provide a complete and balanced diet for fish larvae because the ratio of lipid to dietary protein and carbohydrates would be greater than optimal. For this reason LSB must be incorporated in a CP to form a complete diet. When compared to CP, LSB showed much higher RE and DE for glycine. The difference in DE is of no surprise since CP initially contained only 30% LSB and, therefore, less than one third of the glycine content of LSB. The DE of CP was 4% w/w after an initial rinse and decreased to 1.2% w/w after 60 minutes aqueous suspension. These glycine concentrations are comparable to the FAA pools of commonly used live foods, such as rotifers and *Artemia*. Aragão et al. (2004) reported that *Artemia* had a FAA pool of between 1.6% w/w and 2.8% w/w and rotifers had a FAA pool of 1.4% w/w to 6.7% w/w, with the highest values being for animals fed on phytoplankton, while Srivastava et al. (2005) reported rotifers to have FAA reserves of between 2.2 and 3.5% d/w. The ability to deliver similar quantities of FAA using CP is important since it is believed that early fish larvae lack the digestive enzymes required to break down proteins and large peptide chains. The ability to deliver FAA and small peptides to larvae may bypass this physical limitation.

A diet must not only be of sufficient nutritional quality and palatability, but must be presented to the larvae in a manner that results in a feeding response. This may be achieved through environmental manipulation. Light intensity had a significant effect on the feeding incidence in blue spotted goby larvae, with an optimum light of intensity of 5.44 μEs^{-1} m^{-2}. Incidence of larval feeding was shown to decrease at both lower and higher light levels. This is likely due to the absence of light sensing rod cells in the eyes of fish larvae resulting in an inability to adjust to a range of light intensities, and is supported by previous studies that have reported light intensity to have a substantial effect on the percent of larvae feeding in many other species of marine fish larvae.

Light intensity appears to be closely linked to background color and prey contrast. Blue spotted goby larvae display a significant increase in feeding incidence when fed in tanks lit with the optimum lighting intensity of 5.44 $\mu mol\ s^{-1}$ m^{-2} in a black culture vessel versus a white culture vessel. Similarly, Denson and Smith (1996) showed that under high illumination, white bass, *Marone chrysops* larvae displayed a higher level of feeding on rotifers when reared in a black tank than reared in a transparent tank, indicating that prey contrast had an influence on feeding incidence. Nass et al. (1996) indicated that black colored tanks mimicked natural light conditions more accurately than white tanks or black tanks with white bottoms. The data showed that a much higher percentage of blue spotted goby larvae consumed CP when fed against a black background than a white background and larvae fed on white particles had a significantly higher feeding incidence than larvae fed on black particles against either white or black background. The combination of white particles on a black background ultimately resulted in the highest

percentage of larvae feeding. This is likely due to the relatively higher proportion of light reflected by white particles. In the natural environment very little light is reflected from the background, but small particles and organisms maybe highly reflective. This light reflectance may allow fish larvae to see, recognize and judge the distance to food items. White colored tanks reflect much more light than black tanks and this extra reflectance may overwhelm the ability of larvae to discern individual particles and judge distances. The black particles used in this study resulted in a lower percentage of larvae feeding, likely as a result of black absorbing rather than reflecting light, making black particles more difficult to detect than white.

The percentage of larvae consuming CP increased from the light intensity experiment to the contrast experiment. The results of these experiments suggest that light intensity and tank color may have a cumulative effect. The reflectance of white tank surfaces and light absorbance of black tank surfaces may have different effects on larval feeding at different light intensities, with each background color having a different optimum light level. Further investigation is needed to determine the cumulative effect using a matrix of light intensities and background color.

While the presence of the free amino acids glycine, alanine, and arginine have been reported to increase feeding incidence for 20 day old gilthead seabream, *Sparus aurata* larvae fed on microparticles (Kolkovski et al. 1997a, 1997b, 2001), first-feeding blue spotted goby larvae showed no improvement in feeding incidence when FAA were introduced to the culture water at the same concentrations described to be effective by Koven et al. (1997a). While this may be due to blue spotted goby larvae being poorly

developed at first feeding, the association between food and chemical cues may be a learned behavior and may be only displayed in older, more experienced larvae. Further studies should incorporate treatments with larvae of different ages to investigate the effect of age and experience. The effect of encapsulated amino acids on prey detection and feeding behavior and activity should be investigated.

Kolkovski et al. (1997a) described live foods as having both a visual and chemical stimulatory effect on ingestion of microdiets by 20 day old sea bream larvae when fed microdiets in the presence of *Artemia*. First-feeding blue spotted goby larvae did not display a significant difference in either percent feeding or gut fullness in the presence of phytoplankton, CP alone, or a mixture of CP and phytoplankton. The lack of significant differences may have been due to differences in the mode of consumption of the different food items. The cell size of the phytoplankton species used in the feeding experiment ranged from 8 to20 μm. It was likely that consumption of these items relied on filter feeding similar to the mode of filtering algae used by cod larvae (*Gadus morhua*) described by Van der Meeren (1991). In contrast, ingestion of larger CP was more likely to be the result of targeted feeding as observed through s-strike mode of feeding in tanks fed on CP. While rotifers or another common live food would make a better comparison than phytoplankton, a suitable sized live food has yet to be found for blue spotted goby larvae as both L- and SS- strains of rotifers are too large. Oyster or mussel trochophores may be a suitable size prior to the D stage of development, but developmental rate of trochophores at 26^{o}C may prove too rapid resulting in the food items out-growing the maximum gape of the fish larvae.

CP containing LSB with riboflavin cores displayed release of riboflavin in the fore- and hindgut of first-feeding blue spotted goby larvae, indicating the availability of ingested core material to the larvae. This is similar to the release of riboflavin from complex particles containing LSB with riboflavin cores in the foregut and hindgut of zebrafish larvae as reported by Önal and Langdon (2004a).

Blue spotted goby larvae fed CP displayed significantly lower survival 5 days post feeding than those fed the algae species Tahitian *Isochrysis sp.* and *Rhodomonas sp.*, though survival of larvae fed on CP exceeded that of starved larvae, which showed 100% mortality on day 4 of the trial. As blue spotted goby larvae have been shown to consume CP the probable cause for the low survival is inadequate nutrition. This may be due to an imbalance or absence of one or more essential nutrients. This result suggests that CP could be used as a food source by blue spotted goby larvae given further investigation into their nutritional requirements. Önal and Langdon (2000) showed that larval zebrafish survival was significantly improved when microparticulate diets were co-fed with *Artemia* nauplii, compared with microparticulate diets alone. Further investigation into the delivery methods and nutritional composition of artificial microdiets is required to fully evaluate the effectiveness of CP and LSB as vehicles for nutrient delivery to marine fish larvae. Due to the inability to raise blue spotted goby larvae past 12 days on live foods, they may not be suitable for initial survival and growth studies using microparticulate diets. Clownfish of the genus *Amphiprion* may prove a more suitable test model as the life cycle has been well documented, they have large and well

developed larvae, and they are readily reared on standard live foods. Furthermore, an efficient method of delivering CP to culture vessels at a constant rate is needed.

CONCLUSION

LSB incorporated in CP can efficiently deliver FAA to fish larvae at concentrations similar to those found in live foods. By delivering free amino acids and short peptide chains, bypassing the need for enzymatic protein digestion through exogenous enzymes typically received via prey, it may be possible to support survival and growth in early fish larvae. This may also circumvent some of the physical limitations of early larval digestive systems. Furthermore, the ability of CP to deliver a complete compliment of both water-soluble and non-water soluble nutrients make them ideal for use in investigations into larval nutrition studies involving the manipulation of specific nutrients.

With optimization of culture conditions, such as light intensity and tank color, feeding of larvae on CP can be improved. With co-feeding, CP may be used as an effective supplement for larval diets or perhaps, with further improvements in nutritional composition and particle delivery methods, as a primary food source and replacement of live foods.

Future investigations for the culture of blue spotted goby larvae and the use of complex particles should include the interaction of light intensity, tank color, and particle color, methods of delivery for CP particles to culture vessels, alternative live feeds for comparison with CP, and nutritional requirements of blue spotted goby larvae. However,

survival and growth studies utilizing a species for which the larval cycle and nutritional requirements are better understood such as *Amphiprion sp.* are a logical next step in the implementation of CP as live food alternatives and would provide the greatest opportunity for success.

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CHAPTER 3

CONCLUSION

This study investigated the improvement of lipid spray beads (LSB) and incorporation of LSB into complex particles (CP) for the delivery of free amino acids (FAA) to fish larvae using glycine as a model water-soluble nutrient and environmental factors that influence the consumption of CP by blue spotted goby (*Asterropteryx semipunctata*) larvae.

LSB containing glycine core material at concentrations of 400 gl^{-1} showed increased delivery efficiency (DE), encapsulation efficiency (EE) and similar retention efficiency (RE) and inclusion efficiency (IE) to LSB with aqueous cores, while displaying improved RE over previous works using dry particulate glycine as core material. Core concentrations higher than 400 g l^{-1} resulted in greatly reduced IE, but similar EE. Manipulation of core to lipid ratios yielded an optimum DE at a ratio of 1:2, whereby higher ratios displayed much lower RE, DE, and IE with minimal improvements to EE and lower ratios displayed similar RE, but decreased DE.

LSB did not lose substantial quantities of glycine when immersed in 90% alcohol for 10 minutes, indicating that minimal core material is lost during preparation of CP using zein as a binder. CP using zein as a binder and containing LSB displayed higher initial leaching than LSB alone. This indicates that the water/particle interaction between the less hydrophobic zein allows for greater mixing of the water allowing for a higher rate of leaching than LSB alone.

First feeding blue spotted goby larvae showed breakdown of CP as evidenced through the presence of free riboflavin observed throughout the foregut and hindgut under epifluorescent microscopy, indicating that CP particles consisting of the binder zein, LSB and other nutritional components are capable of delivering water soluble nutrients to first feeding fish larvae and that the larvae are able to access those nutrients.

The improved incidence of feeding by blue spotted goby larvae as light intensities increased from 2.9 $\mu Es^{-1}m^{-2}$ to 5.4 $\mu Es^{-1}m^{-2}$ and subsequent decrease as light intensity was increased to 11.5 $\mu Es^{-1}m^{-2}$ indicates that blue spotted goby larvae have a narrow threshold for visual acuity that is likely related to the undeveloped nature of larval fish eyes.

The improvement in feeding incidence against a black background over a light background indicates that background color has an effect on the ability of larvae to target prey. While contrast did not appear to have an effect on incidence of feeding, the minor, although not statistically different increase in feeding incidence on white particles over black particles may be due to an increased level of backscattered light improving the ability of larvae to target prey.

The null effect of the three amino acids glycine, alanine, and arginine as feeding stimulants may be due to the poorly developed nature of first feeding blue spotted goby larvae. The amino acids may have a stimulatory effect in later stages of the larval cycle when chemoreception is more fully developed.

While co-feeding CP with phytoplankton did not greatly improve the total food incidence of feeding, the use of co-feeding may improve long-term nutrition and health in

larvae. While CP did improve the survival of blue spotted goby larvae over the starved control over the 5-day feeding trial, Cp performed poorly when compared to the microalgae's Tahitian *Isochrysis* and *Rhodomonas sp*.

The use of LSB composed of MS to encapsulate wet amino acid paste's can efficiently deliver FAA to fish larvae when incorporated into a CP matrix. CP of this type can effectively deliver quantities of FAA similar to those found in some live foods as well as a full compliment of essential nutrients. With optimization of culture conditions such as light intensity and tank color incidence of feeding can be improved and with co-feeding CP may be used as an effective supplement to larval diets or perhaps as a primary food source.

Further investigations utilizing blue spotted goby larvae should include the interaction of light intensity, tank color, and particle color, methods of delivery for CP particles to culture vessels, alternative live feeds for comparison with CP, and nutritional requirements of blue spotted goby larvae. However, survival and growth studies utilizing a species for which the larval cycle and nutritional requirements are better understood such as *Amphiprion sp.* are recommended as the next step for the implementation of CP for the replacement of live foods.

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APPENDICES

Source of variation	SS	df	MS	F-Value	P-Value
Core Concentration	1084.235	3	361.412	68.366	<0.0001
Residual	42.291	8	5.286		

Comparison	Mean Diff.	Crit. Diff.	Significance
100, 200	-1.867	6.013	
100, 400	1.771	6.013	
100, 600	21.718	6.013	S
200, 400	3.638	6.013	
200, 600	23.585	6.013	S
400, 600	19.947	6.013	S

Table A.1: ANOVA and Tukey HSD results for incorporation efficiencies of LSB composed of menhaden stearine and glycine at concentrations of 100, 200, 400, and 600 g l^{-1} at a core to lipid ratio of 1:1.5 v/v. S denotes a significant difference at $p<0.05$.

Source of variation	SS	df	MS	F-Value	P-Value
Core Concentration	191.458	3	63.819	639.875	<0.0001
Residual	0.798	8	0.1		

Comparison	Mean Diff.	Crit. Diff.	Significance
100, 200	-4287	0.826	S
100, 400	-9.853	0.826	S
100, 600	-9.187	0.826	S
200, 400	-5.566	0.826	S
200, 600	-4.900	0.826	S
400, 600	0.666	0.826	

Table A.2: ANOVA and Tukey HSD results for encapsulation efficiencies of LSB composed of menhaden stearine and glycine at concentrations of 100, 200, 400, and 600 g l^{-1} at a core to lipid ratio of 1:1.5 v/v. S denotes a significant difference at $p<0.05$.

Source of variation	SS	df	MS	F-Value	P-Value
Time	3672.014	4	918.004	58.931	<0.0001
Core Concentration	92.921	2	46.461	2.983	0.0659
Time x Core Conc	309.093	8	38.637	2.480	0.0340
Residual	467.326	30	15.578		

Comparison	Mean Diff.	Crit. Diff.	Significance
100, 200	-1.993	3.557	
100, 400	1.517	3.557	
200, 400	3.509	3.557	

Table A.3: Two-way ANOVA and Tukey HSD results for retention efficiencies of LSB composed of menhaden stearine and glycine at concentrations of 100, 200, and 400 g l^{-1} at a core to lipid ratio of 1:1.5 v/v. S denotes a significant difference at $p<0.05$.

Source of variation	SS	df	MS	F-Value	P-Value
Time	97.819	4	21.955	64.912	<0.0001
Core Concentration	637.106	2	318.553	941.836	<0.0001
Time x Core Conc	8.708	8	1.088	3.218	0.0092
Residual	10.147	30	0.338		

Comparison	Mean Diff.	Crit. Diff.	Significance
100, 200	-4.031	0.524	S
100, 400	-9.194	0.524	S
200, 400	-5.162	0.524	S

Table A.4: Two-way ANOVA and Tukey HSD results for delivery efficiencies of LSB composed of menhaden stearine and glycine at concentrations of 100, 200, 400, and 600 g l^{-1} at a core to lipid ratio of 1:1.5 v/v. S denotes a significant difference at $p<0.05$.

Source of variation	SS	df	MS	F-Value	P-Value
Ratio	2012.009	4	503.002	31.631	<0.0001
Residual	159.024	10	15902		

Comparison	Mean Diff.	Crit. Diff.	Significance
1:1, 1:1.25	-15.126	10.706	S
1:1, 1:1.5	-15.660	10.706	S
1:1, 1:2	-27.991	10.706	S
1:1, 1:2.5	-33.296	10.706	S
1:1.25, 1:1.5	-0.534	10.706	
1:1.25, 1:2	-12.865	10.706	S
1:1.25, 1:2.5	-18.170	10.706	S
1:1.5, 1:2	-12.331	10.706	S
1:1.5, 1:2.5	-17.636	10.706	S
1:2, 1:2.5	-5.305	10.706	

Table A.5: ANOVA and Tukey HSD results for incorporation efficiencies of LSB composed of menhaden stearine and core glycine at a concentration of 400 g l^{-1} at core to lipid ratios of 1:1, 1:1.25, 1:1.5, 1:2, 1:2.5 v/v. S denotes a significant difference at $p<0.05$.

Source of variation	SS	df	MS	F-Value	P-Value
Ratio	24.490	4	6.123	20.148	<0.0001
Residual	3.039	10	0.304		

Comparison	Mean Diff.	Crit. Diff.	Significance
1:1, 1:1.25	-3.419	1.480	S
1:1, 1:1.5	-1.660	1.480	S
1:1, 1:2	-1.442	1.480	
1:1, 1:2.5	0.054	1.480	
1:1.25, 1:1.5	1.760	1.480	S
1:1.25, 1:2	1.977	1.480	S
1:1.25, 1:2.5	3.473	1.480	S
1:1.5, 1:2	0.217	1.480	
1:1.5, 1:2.5	1.714	1.480	S
1:2, 1:2.5	1.496	1.480	S

Table A.6: ANOVA and Tukey HSD results for encapsulation efficiencies of LSB composed of menhaden stearine and core glycine at a concentration of 400 g l^{-1} at core to lipid ratios of 1:1, 1:1.25, 1:1.5, 1:2, 1:2.5 v/v. S denotes a significant difference at $p<0.05$.

Source of variation	SS	df	MS	F-Value	P-Value
Time	3926.059	4	981.515	54.150	<0.0001
Ratio	10184.640	4	2546.161	140.471	<0.0001
Time x Ratio	442.743	16	27.671	1.527	0.1275
Residual	906.292	50	18.126		

Comparison	Mean Diff.	Crit. Diff.	Significance
1:1, 1:1.25	-6.130	4.408	S
1:1, 1:1.5	-10.668	4.408	S
1:1, 1:2	-28.389	4.408	S
1:1, 1:2.5	-28.330	4.408	S
1:1.25, 1:1.5	-4.538	4.408	S
1:1.25, 1:2	-22.259	4.408	S
1:1.25, 1:2.5	-22.201	4.408	S
1:1.5, 1:2	-17.721	4.408	S
1:1.5, 1:2.5	-17.663	4.408	S
1:2, 1:2.5	0.059	4.408	

Table A.7: Two-way ANOVA and Tukey HSD results for retention efficiencies of LSB composed of menhaden stearine and core glycine at a concentration of 400 g l^{-1} at core to lipid ratios of 1:1, 1:1.25, 1:1.5, 1:2, 1:2.5 v/v. S denotes a significant difference at $p<0.05$.

Source of variation	SS	df	MS	F-Value	P-Value
Time	301.448	4	75.362	37.889	<0.0001
Ratio	244.891	4	61.223	30.780	<0.0001
Time x Ratio	50.039	16	3.127	1.572	0.1122
Residual	99.452	50	1.989		

Comparison	Mean Diff.	Crit. Diff.	Significance
1:1, 1:1.25	-1.570	1.460	S
1:1, 1:1.5	-2.714	1.460	S
1:1, 1:2	-5.346	1.460	S
1:1, 1:2.5	-3.579	1.460	S
1:1.25, 1:1.5	-1.144	1.460	
1:1.25, 1:2	-3.776	1.460	S
1:1.25, 1:2.5	-2.009	1.460	S
1:1.5, 1:2	-2.632	1.460	S
1:1.5, 1:2.5	-0.865	1.460	
1:2, 1:2.5	1.767	1.460	S

Table A.8: Two-way ANOVA and Tukey HSD results for delivery efficiencies of LSB composed of menhaden stearine and core glycine at a concentration of 400 g l^{-1} at core to lipid ratios of 1:1, 1:1.25, 1:1.5, 1:2, 1:2.5 v/v. S denotes a significant difference at $p<0.05$.

Source of variation	SS	df	MS	F-Value	P-Value
Time	2385.636	4	596.409	23.644	<0.0001
Particle Type	4371.271	1	4371.271	173.296	<0.0001
Time x Particle Type	364.975	4	91.244	3.617	0.0225
Residual	504.486	20	25.224		

Comparison	Mean Diff.	Crit. Diff.	Significance
LSB,CP	24.142	3.825	S

Table A.9: Two-way ANOVA and Tukey HSD results for retention efficiencies of LSB composed of menhaden stearine, core glycine concentration of 400 g l^{-1} at core to lipid ratios of 1:2 v/v with LSB wall composed of 100% MS compared to CP containing 30% LSB w/w. S denotes a significant difference at $p<0.05$.

Source of variation	SS	df	MS	F-Value	P-Value
Time	94.034	4	23.508	23.985	<0.0001
Particle Type	841.619	1	841.619	858.665	<0.0001
Time x Particle Type	14.526	4	3.631	3.705	0.0205
Residual	19.603	20	0.980		

Comparison	Mean Diff.	Crit. Diff.	Significance
LSB,CP	10.593	0.754	S

Table A.10: Two-way ANOVA and Tukey HSD results for delivery efficiencies of LSB composed of menhaden stearine, core glycine concentration of 400 g l^{-1} at core to lipid ratios of 1:2 v/v with LSB wall composed of 100% MS compared to CP containing 30% LSB w/w. S denotes a significant difference at $p<0.05$.

Source of variation	SS	df	MS	F-Value	P-Value
Light Intensity	1451.792	5	290.358	19.093	<.0001
Residual	273.743	18	15.208		

Comparison	Mean Diff.	Crit. Diff.	Significance
0.00, 0.15	0	8.755	
0.00, 1.04	-8.809	8.755	S
0.00, 2.90	-6.830	8.755	
0.00, 5.44	-21.431	8.755	S
0.00, 11.52	-15.484	8.755	S
0.15, 1.04	-8.809	8.755	S
0.15, 2.90	-6.830	8.755	
0.1512, 5.44	-21.431	8.755	S
0.1512, 11.52	-15.484	8.755	S
1.04, 2.90	1.979	8.755	
1.04, 5.44	-12.621	8.755	S
1.04, 11.52	-6.674	8.755	
2.90, 5.44	-14.600	8.755	S
2.90, 11.52	-8.653	8.755	
5.44, 11.52	5.947	8.755	

Table A.11: ANOVA table and Tukey HSD table for feeding incidence of blue spotted goby larvae (*Asterropteryx semipunctata*) at a light intensity of 0.00, 0.15, 1.04, 2.90, 5.44, and 11.52 $\mu E\ s^{-1}\ m^{-2}$ when fed CP against a white background for a 1 h period. S denotes a significant difference at $p<0.05$.

Source of variation	SS	df	MS	F-Value	P-Value
Light Intensity	1291.249	3	430.416	21.975	<.0001
Residual	235.043	12	19.587		

Comparison	Mean Diff.	Crit. Diff	Significance
Bp on Bb, Bp on Wb	14.850	9.294	S
Bp on Bb, Wp on Bb	-7.496	9.294	
Bp on Bb, Wp on Wb	11.699	9.294	S
Bp on Wb, Wp on Bb	-22.345	9.294	S
Bp on Wb, Wp on Wb	-3.151	9.294	
Wp on Bb, Wp on Wb	19.195	9.294	S

Table A.12: ANOVA and Tukey HSD results for feeding incidence of blue spotted goby larvae (*Asterropteryx semipunctata*) at a light intensity of 5.44 $\mu E\ s^{-1}\ m^{-2}$ when fed on black or white colored CP against black or white colored backgrounds for a 1 h period. S denotes a significant difference at $p<0.05$.

Source of variation	SS	df	MS	F-Value	P-Value
Light Intensity	3872394	3	1290797.918	0.460	0.7154
Residual	33680453	12	2806704.421		

Comparison	Mean Diff.	Crit. Diff.	Significance
Bp on Bb, Bp on Wb	512.748	3518.177	
Bp on Bb, Wp on Bb	-0.862	3518.177	
Bp on Bb, Wp on Wb	1198.591	3518.177	
Bp on Wb, Wp on Bb	-513.609	3518.177	
Bp on Wb, Wp on Wb	685.843	3518.177	
Wp on Bb, Wp on Wb	1199.453	3518.177	

Table A.13: ANOVA and Tukey HSD results for area of food ingested (μm^2) by blue spotted goby larvae (*Asterropteryx semipunctata*) at a light intensity of 5.44 $\mu E\ s^{-1}\ m^{-2}$ when fed on black or white colored CP against black or white colored backgrounds for a 1 h period. S denotes a significant difference at $p<0.05$.

Source of variation	SS	df	MS	F-Value	P-Value
Light Intensity	2.110	2	1.060	0.129	0.8805
Residual	73.526	9	8.170		

Comparison	Mean Diff.	Crit. Diff	Significance
ala, arg	0.497	5.645	
ala, gly	-0.530	5.645	
arg, gly	-1.027	5.645	

Table A.14: ANOVA and Tukey HSD results for feeding incidence of blue spotted goby larvae (*Asterropteryx semipunctata*) at a light intensity of 5.44 μE s^{-1} m^{-2} when fed on CP against black colored backgrounds for a 1 h period in the presence of individual free amino acids at concentrations of 9.1 ng ml^{-1} glycine, 5.9 ng ml^{-1} alanine, and 0.2 ng ml^{-1} arginine as potential feeding stimulants. S denotes a significant difference at $p<0.05$.

Source of variation	SS	df	MS	F-Value	P-Value
Light Intensity	1129267	2	564633.415	1.912	0.2032
Residual	2657916	9	295323.968		

Comparison	Mean Diff.	Crit. Diff.	Significance
Ala, Arg	-684.559	1073.288	
Ala, Gly	-610.626	1073.288	
Arg, Gly	73.933	1073.288	

Table A.15: ANOVA and Tukey HSD results for area of food consumed by blue spotted goby larvae (*Asterropteryx semipunctata*) fed at a light intensity of 5.44 μE s^{-1} m^{-2} fed CP against black colored background for a 1 h period in the presence of individual free amino acids at concentrations of 9.1 ng ml^{-1} glycine, 5.9 ng ml^{-1} alanine, and 0.2 ng ml^{-1} arginine as potential feeding stimulants. S denotes a significant difference at $p<0.05$.

Source of variation	SS	df	MS	F-Value	P-Value
Feed	280.942	2	140.471	2.252	0.1610
Residual	561.327	9	62.370		

Comparison	Mean Diff.	Crit. Diff	Significance
Algae	-5.520	15.597	
Cofeed	6.323	15.597	
CP	11.843	15.597	

Table A.16: ANOVA and Tukey HSD results for feeding incidence of blue spotted goby larvae (*Asterropteryx semipunctata*) at a light intensity of 5.44 μE s^{-1} m^{-2} when fed on the phytoplankton *Rhodomonas sp.* and Tahitian *Isochrysis sp.*, CP or a combination of both phytoplankton and CP against black colored background for a 1 h period. S denotes a significant difference at $p<0.05$.

Source of variation	SS	df	MS	F-Value	P-Value
Light Intensity	1523268	2	761633.816	2.720	0.1191
Residual	2520028	9	280003.085		

Comparison	Mean Diff.	Crit. Diff.	Significance
Algae, Co-feed	-567.387	1045.078	
Algae, ZBP	-857.958	1045.078	
Co-feed, ZBP	-290.571	1045.078	

Table A.17: ANOVA and Tukey HSD results for area of food consumed by blue spotted goby larvae (*Asterropteryx semipunctata*) fed at a light intensity of 5.44, $\mu E\ s^{-1}\ m^{-2}$ fed the phytoplankton *Rhodomonas sp.* and Tahitian *Isochrysis sp.*, CP or a combination of both phytoplankton and CP against black colored background for a 1 h period. S denotes a significant difference at $p<0.05$.

Source of variation	SS	df	MS	F-Value	P-Value
Light Intensity	2136.712	4	534.178	41.935	<.0001
Residual	127.381	10	12.738		

Comparison	Mean Diff.	Crit. Diff.	Significance
Algae, Casein	23.503	9.582	S
Algae, Control	37.114	9.582	S
Algae, FAA	23.305	9.582	S
Algae, Hydrolyzed	21.004	9.582	S
Casein, Control	13.611	9.582	S
Casein, Hydrolyzed	-2.499	9.582	
Casein, FAA	-1.980	9.582	
Control, Hydrolyzed	-16.110	9.582	S
Control, FAA	-13.809	9.582	S
Hydrolyzed, FAA	-2.302	9.582	

Table A.18: ANOVA and Tukey HSD results for survival of blue spotted goby larvae (*Asterropteryx semipunctata*) at a light intensity of 5.44, μE s^{-1} m^{-2} when fed on the phytoplankton *Rhodomonas sp.* and Tahitian *Isochrysis sp.*, CP containing LSB with a casein core, CP containing LSB with a hydrolysed casein core or CP containing LSB with a FAA core, against a black colored background over a 5 day period with 1 ml s^{-1} flow rate. S denotes a significant difference at $p<0.05$.

Source of variation	SS	df	MS	F-Value	P-Value
Light Intensity	0.010	3	0.003	1.133	0.3921
Residual	0.025	8	0.003		

Comparison	Mean Diff.	Crit. Diff.	Significance
Algae, Casein	0.079	0.145	
Algae, Hydro	0.026	0.145	
Algae, FAA	0.052	0.145	
Casein, Hydro	-0.027	0.145	
Casein, FAA	-0.053	0.145	
Hydro, FAA	0.026	0.145	

Table A.19: ANOVA and Tukey HSD results for final notochord length of blue spotted goby larvae (*Asterropteryx semipunctata*) at a light intensity of 5.44, μE s^{-1} m^{-2} when fed on the phytoplankton *Rhodomonas sp.* and Tahitian *Isochrysis sp.*, CP containing LSB with a casein core, CP containing LSB with a hydrolysed casein core or CP containing LSB with a FAA core, against a black colored background over a 5 day period with 1 ml s^{-1} flow rate. S denotes a significant difference at $p<0.05$.