Advances in Larval Development and Feed Regime Optimisation for Giant Kōkopu (*Galaxias argenteus*)

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ABSTRACT

Giant kōkopu, *Galaxias argenteus*, is an amphidromous fish species that is endemic to New Zealand. In its larval form, giant kōkopu are one of five species that are harvested in the whitebait fishery which holds significant social, cultural and economic value in New Zealand. Due to the high price per kilo ($100-160 kg^{-1}) and limited supply of whitebait from the wild fishery there is interest in developing whitebait species, including the giant kōkopu, for aquaculture production. However, there is little published literature on the giant kōkopu on which to base their development for aquaculture. Therefore, the research presented in this thesis aims to describe aspects of the morphological development and larval feeding requirements of giant kōkopu to assist in advancing their aquaculture.

Morphometric characteristics of larval giant kōkopu that are critical to the success of larviculture were measured over the 77 day production period, from hatch to harvest. The initial slow growth of the larvae appears to be a result of rapid depletion of endogenous energy reserves prior to initiation of first feeding, while the extent of eye pigmentation and the presence of an open mouth upon hatching indicate that feed provision should be brought forward from 3 DAH. Mouth gape width at hatch was 348 µm and increased rapidly in the first four weeks to 651 µm suggesting that larger live feed items could be used as the initial feed, such as instar-II *Artemia*. At harvest, the larvae varied enormously in size and condition, with larger fish showing early signs of metamorphosis, such as increasing opacity of flesh and colouration as organs. The results confirm that an improved understanding of the developmental biology of the larvae can assist in providing a more effective feeding regime, particularly for determining the timing of first feed provision and the feed particle size requirements throughout larval development. Consequently, heterogenous growth performance is an aspect of production that needs further attention for developing the efficient aquaculture production of this species.

A feeding experiment with early stage larval giant kōkopu compared the growth performance and survival of the larvae over a four week period when fed different proportions of instar-I and enriched instar-II *Artemia* for different durations. Larvae in the treatment group which received the greatest proportion of instar-I *Artemia* for the longest duration had the lowest mean wet weight and survival, leading to the lower total production at harvest when compared to other feeding treatments. The feed treatment that received only instar-II *Artemia* (i.e., no instar-I), achieved equal best total productivity. While the treatment fed a short initial duration of mixed instar-I

and instar-II followed by only instar-II *Artemia* was the best performing, producing larvae with the greatest mass, length, survival and total production. This feed treatment appears to offer the best balance between availability of feed particles in the optimum size ranges due to the initial combination of *Artemia* instars, with a higher level of digestibility.

Observations of larval ability to consume artificial diets across four ages (18, 21, 25 and 28 days after hatching, DAH) at each of the three daily feeds (Morning, Midday, Afternoon), demonstrated a potential to shorten the expensive live feeding period. At 18 DAH little to no artificial feed was consumed at either of the three feeding events throughout the day. At 21 DAH mean gut fullness increased and the number of fish with empty stomachs reduced by the end of the Morning and Afternoon feeding events. Both variables improved further for larvae at 25 and 28 DAH. Formulated feed consumption did not differ between larvae aged 25 and 28 DAH indicating that three days of feeding live feed could be removed from the feed regime. This change would save 13% on the cost of live feed, however, further research is required to determine the potential impacts on growth and survival.

Three artificial dry feed treatments (Otohime, Artemac and O.range) were experimentally tested with their effects on larval giant kōkopu growth performance compared. Larvae fed with Otohime outperformed both Artemac and O.range treatments by achieving the highest wet weight after 67 days, greater by at least 47 % on average than both Artemac and O.range. These differences in larval performance are likely to be due to the higher protein:energy ratio and EPA content of Otohime. High DHA and ARA in the diets in absolute terms or in relation to EPA did not result in added benefit for growth performance. This study provides an important first step in identifying the nutritional needs of larval giant kōkopu which can assist in improving their commercial aquaculture production

Collectively the outcomes of this research provide valuable information to assist in improving the development of giant kōkopu aquaculture. The results confirm that an improved understanding of the developmental biology of the larvae can assist in providing a more effective feeding regime, particularly for determining the timing of first feed provision and the feed particle size requirements throughout larval development. Furthermore, significant productivity gains and cost reductions (i.e., up to 27 % live feed cost) are likely to be achieved through the adoption of early shifts to instar-II *Artemia* and weaning from live food three days earlier than is current

practised in commercial production of giant kōkopu. Overall, the results from this study confirm the benefits of the close evaluation of larval morphology and feeding in improving the performance of the larval culture of fish.

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1 Chapter 1: General Introduction

1.1 Aquaculture and the Larval Bottleneck

Aquaculture is the production of aquatic organisms in fresh and salt water environments, including in land-based tanks. For the last thirty years aquaculture has been the fastest growing food production sector in the world but has slowed in recent years (FAO, 2014, 2016, 2018, 2020). The total volume of global aquaculture production doubled between the years of 2000 and 2012 and now contributes 46 % of total global fish production (FAO, 2014, 2016, 2018, 2020). In 2018 fin fish made up 47% of aquaculture production at 54.3 million tonnes, with a value of US$ 139.7 billion (FAO, 2020). The rising global demand for aquatic food products will be met by growing supplies from aquaculture production, with the global aquaculture production of fin fish by weight expected to exceed that of wild fisheries by 2021 (FAO, 2016).

Annual global aquaculture production grew 5.8 % between 2000 and 2016, being no longer able to sustain the double digit annual growth rate of the previous decades (FAO, 2018). The annual growth of the industry is expected to fall further below 2.3 % in the decade leading up until 2030 (FAO, 2020). This will come as a result of aquaculture increasingly competing with other industries for water and space, as well as being hindered by inadequate capital investment in regions with good aquaculture potential, as well as inhibition through poor governance and regulatory frameworks (FAO, 2016). However, the supplies of fish feed and fish seed are anticipated to be the two greatest limiting factors that will inhibit the growth of fin fish aquaculture (FAO, 2016). The large knowledge gaps in the nutritional requirements of larval fish and the high prices of fish oil and fish meal make it exceedingly difficult to produce enough viable young and deliver large quantities of high quality on growing feeds required to continue increasing the production of adult fish (Conceição et al., 2010; FAO, 2016; Glencross, 2009; Hamre et al., 2013; Qin, 2008; Rønnestad et al., 2013; Takeuchi, 2014).

A major advantage for aquaculture in overcoming production challenges is that it is a relatively young industry with significant scope for delivering solutions through research and development. For example, research directed at increasing the understanding of larval nutritional requirements, offers a substantial opportunity for significant improvements to fin fish aquaculture by reducing feed costs while increasing the production of high quality larval fish (Trushenski et al., 2006).

1.2 Galaxiidae

Galaxiidae are a family of freshwater fishes found in cool to cool-temperate regions of the southern hemisphere including New Zealand, New Caledonia, Australia, South Africa and South America (McDowall, 2010; McDowall & Frankenberg, 1981; Nelson, 2007). These fish are scaleless, and while some are elongate others are stocky and blunt in form, but all are relatively small, and usually less than 230 mm in length (McDowall, 1990, 2010; McDowall & Frankenberg, 1981). An exception is the giant kōkopu, *Galaxias argenteus*, which can grow to larger sizes (McDowall, 1990; McDowall & Frankenberg, 1981).

While most galaxiid species maintain an entirely freshwater life, amphidromy is a relatively common life history amongst this family (McDowall, 1988). Amphidromy is a form of diadromy and in freshwater fish it is where somatic growth and reproduction occurs in freshwater habitats after a short larval or juvenile marine period, after which the fish then migrate back into freshwater (McDowell 2009). Galaxiidae, with this life history, spawn their gametes on the banks of the lower reaches of tidally affected streams during periods of high water (Benzie, 1968; Franklin et. al., 2015). With fluctuating water levels in rivers, the incubation of the eggs often takes place terrestrially, where the adhesive and robust nature of the eggs acts to fix them in place and ensures they remain moist (Benzie, 1968; Mitchell, 1989). Hatching is triggered by re-inundation of eggs by the rise in water level from a rainfall event or spring tides at 15-40 days post fertilisation (McDowall et. al., 1994). Upon hatching the larvae are washed downstream and out to sea where a marine developmental phase of 3-6 months takes place. The larvae and early juveniles then migrate back into freshwater habitats occupied by the adults (McDowall, 1990; McDowall et al., 1994).

These larval migrations are the key driver behind the widespread distribution of species within this family (McDowall, 2010), however, this migration behaviour has also enabled their exploitation by fishing (McDowall, 1984). Larvae frequently migrate up fresh waterways in large shoals that are often of mixed species composition, and are channelled from the expanse of the ocean environment into narrow and shallow estuaries and rivers where they are harvested en masse. This is the basis of the vast majority of recreational, cultural and commercial galaxiid harvests in New Zealand and Australia (referred to as whitebait) and Chile (referred to as puye) (Mardones et al., 2008; McDowall, 2010).

1.3 New Zealand's Whitebait Fishery

The term whitebait is used around the world to refer to juvenile fish of various species. However, in New Zealand it is a generic name used for the larvae of five amphidromous native galaxiid species (McDowall, 1996). The most common in wild harvests of migrating whitebait is īnanga (*Galaxias maculatus*), which along with kōaro (*G. brevipinnis*) are not endemic, while short-jaw kōkopu (*G. postvectus*), banded kōkopu (*G. fasciatus*) and the giant kōkopu (*G. argenteus)* are all endemic to New Zealand (McDowall, 1996).

Whitebait species have been harvested as both migrating larvae and adult fish since the first human arrival in New Zealand. Whitebait were described as "among the most important fishes in our fresh waters… much eaten" by Māori (Phillips, 1940). Today, whitebait are a local delicacy that is in high demand, with limited and inconsistent supply which drives prices to NZ$100-160 kg^{-1} (Environment Southland, 2013; NZ Whitebait, n.d.). However, whitebait were once so abundant that they were used to cover acres of gardens, inches deep, presumably as a form of fertiliser at the turn of the 19th century and were still of sufficient abundance to be harvested to provide poultry feed in the 1950's (Clarke, 1899; Graham, 1956; McDowall, 2010).

Whitebait are easily exploited due to their seasonal concentrations in estuaries and river mouths as larvae migrate from the ocean into fresh waterways (McDowall, 1984). However, fishing pressure is only one of many challenges to the sustainability of this wild fishery. Perhaps the greatest threat to the sustainability of whitebait species is the extensive modification of native forests and wetlands for agriculture, forestry and urbanisation in New Zealand (Hanchet, 1990; McDowall, 1990; Minns, 1990). The most damaging is the reduction or total loss of riparian vegetation, which provides vital spawning grounds and is the source of important food, terrestrial invertebrates (Hanchet, 1990; McDowall, 1990). Furthermore, predation and competition by introduced invasive species, such as trout (Crowl et al., 1992; McDowall, 1968) and predation on terrestrially incubating eggs by introduced rodents, also add further pressures to wild galaxiid populations (Baker, 2006).

Historic whitebait catch records show annual harvests vary dramatically within and among regions, but with an overarching trend of decline (McDowall, 1984, 1990, 1991; Rowe et. al., 1992). Despite the social, cultural and economic value of this fishery little is known about the fishing effort, catch per unit effort or the total size of the whitebait harvest. Aquaculture production of whitebait has been identified as a means to overcome the unreliable

and diminishing supply of whitebait from the fishery whilst meeting consumer demand for this high value product (McDowall, 1995).

1.4 Current State of Whitebait Aquaculture

The unreliable and diminishing wild harvests of whitebait combined with their high value has led to the recognition of the potential for the aquaculture of galaxiid species in Chile, Argentina and New Zealand (Mardones et al., 2008; McDowall, 1995; Vega et al., 2013). However, the commercial aquaculture of galaxiid species that has occurred to date is only on a very limited scale.

Inanga (*G. maculatus*) are the galaxiid species of focus for aquaculture activity in Chile, where efforts have been centred on developing the technology for their production at the commercial scale since the 1990's (Mardones et al., 2008). However, farming of this species in Chile is yet to advance from only pilot scale production (Vega et al., 2013).

Early culturing experiments in New Zealand in the 1980's identified īnanga as being suitable for aquaculture development due to their adaptability to confinement, tolerance of poor water quality, and a willingness to accept commercially available fish feeds (Mitchell, 1989). In recent years, there has been success in breeding giant, short-jaw, banded kōkopu, as well as kōaro and īnanga at the research facility run by the Mahurangi Institute of Technology in Warkworth (O'Brien & Cooper, 2013). More recently giant kōkopu have gained greater aquaculture research attention which has led to increased understanding of their reproductive biology, and facilitated the closing of their life cycle enabling the production of large numbers of larvae (O'Brien & Cooper, 2013; Wylie et al., 2016; Wylie, 2011).

The greatest progress toward the development of commercial whitebait aquaculture has been achieved in recent years by New Zealand Premium Whitebait Limited (NZPWL) based in Warkworth, New Zealand. Optimisation of egg fertilisation, incubation and hatching processes of giant kōkopu by the company has enabled the first commercially cultured whitebait in New Zealand to be produced and sold in 2016.

The further development of the aquaculture of galaxiid fish is hampered by large knowledge gaps which greatly limit the security of production and potential profitability. Aspects requiring further research include; developing

a method for the selection of wild broodstock suitable for aquaculture, identification and management of ectoparasitic diseases, and the need for species-specific larval diets and feeding regimes (Vega et al., 2013). This latter area of research interest forms one of the main research objectives for the research that is presented in this thesis.

1.5 Galaxias argenteus

Galaxias argenteus (giant kōkopu) is the largest of the galaxiid fish species which can reach up to 580 mm in length and 2.7 kg in weight (Clarke, 1899; McDowall, 1990). Sightings of individuals greater than 400 mm are rare and according to the New Zealand Threat Classification, giant kōkopu are "At Risk – Declining" (David, 2002; Goodman, 2014; McDowall, 2010). Giant kōkopu were the first galaxiid species described by Europeans in New Zealand after samples were collected by Captain James Cook in 1773 (McDowall, 2010). Despite their early identification, very little is known about the biology and ecology of the giant kōkopu.

About 50 % of New Zealand's freshwater fish species are diadromous, the giant kōkopu and the four other whitebait species being further defined as amphidromous (David et al., 2004; McDowall, 1988; McDowall, 1996). However, giant kōkopu have also been found living in localised non-diadromous populations in land-locked lakes (David et al., 2004).

Giant kōkopu are found along the west coast of New Zealand from Auckland in the North Island through to Southland, and Stewart Island, however, they are largely absent from the east coast of New Zealand (Bonnett & Sykes, 2002). They inhabit a variety of habitats including estuaries, swamps, drains and the gravel beds of rivers in low lying coastal areas (Bonnett & Sykes, 2002; David et al., 2004; McDowall, 1990).

Giant kōkopu, like the other whitebait species in New Zealand, deposit their eggs on riparian margins during periods of elevated water levels during autumn and this results in subsequent terrestrial incubation of the eggs which are around 2 mm in diameter (Franklin et al., 2015, Charteris et al., 2003; Mitchell & Penlington, 1982). If another high flow event occurs in the following weeks, the developing eggs are inundated, hatch and the larvae are carried out to sea where they undergo a period of rapid larval growth (McDowall, 1984, 1988). Like the four other New Zealand whitebait species, larval giant kōkopu are 45-50 mm in length and are completely translucent

on their return from the sea as they migrate up freshwater ways (McDowall, 1990, 2010). Within a few days of re-entering freshwater, these fish begin to develop pigmentation, initially with dark spots and later dark body colouration with prominent gold markings (McDowall, 1990). Observations of the growth rings on otoliths of a 400 mm long giant kōkopu were used to estimate the age of the fish as 21 to 27 years of age (Jellyman, 1979; McDowall, 1990). Sexual maturity in this species is estimated to be reached at 2-3 years of age. The relative fecundity of giant kōkopu females is lower than other whitebait species and varies in relation to body length, with a female of 336 mm found to produce around 25,000 eggs (Jellyman, 1979; McDowall, 1990; McDowall et al., 1994). Giant kōkopu are a more suitable species for aquaculture because of higher absolute fecundity, ability to repeatedly spawn over many years, and the increased robustness of their larger eggs and larvae (Closs et al., 2013; McDowall, 1995).

Very little is known about the larval biology of giant kōkopu, as it is for all whitebait species, especially their feeding biology. Giant kōkopu larvae have been raised through to juveniles on combinations of rotifer, *Artemia* nauplii and copepod species (Mitchell, 1989; O'Brien & Cooper, 2013) while field and experimental studies on riverine juvenile īnanga have shown this species consumes a range of plankton prey (Catlin, 2015; Cervellini et al., 1993; Modenutti et al., 1993). However, further research is needed to understand the feeding biology of the larvae of giant kōkopu, so that the efficiency of feeding under aquaculture conditions can be improved.

1.6 Larval Feeding Parameters

Predation by fish is comprised of several key behavioural components including, encounter, detection, attack and capture (Eggers, 1977; Gerritsen & Strickler, 1977; O'Brien, 1979; Sullivan et al. 2016). Characteristics of the fish predator, the prey and the environment play important roles in determining the effectiveness of predation behaviour. These characteristics include prey abundance, predator and prey swimming ability, behaviour and size, as well as the predator's sensory abilities (Buskey, 2005; Buskey et al., 1993; Gerritsen & Strickler, 1977; Holling, 1966). Knowledge of these characteristics can be useful for helping to optimize feeding efficiency in aquaculture settings.

Larval and juvenile fish are frequently mouth gape-limited predators for which the size of their prey is limited by what they are able to capture in their mouths and ingest (Arts & Evans, 1987; Bremigan & Stein, 1994; Cunha &

Planas, 1999; Krebs & Turingan, 2003; Makrakis et al., 2008; Zaret, 1980). Therefore, it is important to understand how mouth gape changes during larval development due to its influence on the maximum size of ingestible food items (Qin & Fast, 1997). From an aquaculture perspective if a food item is too large it cannot be consumed by the larvae, resulting in unnecessary cost and reduction in fish productivity. In contrast, if food items are too small for the fish then they may be more difficult for the fish to detect, and the energy required to capture sufficient food particles rises dramatically culminating in reduced productivity (Schwartz, 2008). Furthermore, food particle volume tends to increase exponentially with diameter so feeding efficiency and feed delivery can be increased by ensuring larvae are feeding on particles toward their maximum ingestible size.

The mouth gape development of larval giant kōkopu has not been measured and therefore doing so, may help lead to improvements in the efficiency of their feeding in an aquaculture setting.

1.7 Larval Fish Nutrition and Digestion

Feeding is usually the single largest cost in fin fish aquaculture, regularly accounting for 30-70 % of production costs (Webster & Lim, 2001). Feeding is also a key limiting factor to commercial success in animal production industries where the profit margins are often small (Trushenski et al., 2006). Therefore, reducing feed costs is a high priority for emerging aquaculture businesses, particularly those commercialising species new to aquaculture production.

In general terms, the nutritional requirements of most larval fish are poorly understood (Hamre et al., 2013; Holt, 2011; Qin, 2008). Consequently, larval nutrition is frequently an area of concern in fin fish aquaculture due to the dominant effect that nutrition has on growth, health and survival of larvae (Conceição et al., 2010; Glencross, 2009).

For giant kōkopu, the nutritional requirements of the larvae are unknown and current commercial larviculture practice involves the transfer of simple techniques which have worked for the larviculture of other fin fish species. Subsequently, there is a need for research aimed at increasing the knowledge about the feeding behaviour and feed requirements of larval giant kōkopu that is likely to lead to improvements in the efficiency of feeding, growth,

survival, and health of larvae, whilst also minimising feeding costs during this important stage of aquaculture production.

As with the majority of fin fish aquaculture species, the current NZPWL giant kōkopu larval rearing technique involves the provision of live feeds to facilitate the transition from endogenous yolk sac and oil globule reserves to exogenous feeding in early stage larvae (Hamre et al., 2013; Schwartz, 2008). For culturing early larval giant kōkopu, feeding live *Artemia* nauplii is vital for ensuring growth and survival because they are readily captured and consumed (Léger et al., 1987). Currently the NZPWL feeding regime for giant kōkopu larvae begins two days after hatching with live *Artemia* nauplii and continues for at least 28 days, during which a slow weaning process takes place to a commercial artificial feed, Otohime A (Marubeni Nisshin Feed Co. Ltd). The artificial food ration that is supplied to the developing larvae is increased along with the particle size of the feed from 75 to 1410 µm (i.e., transitioning from Otohime grade A to grade C2) from weaning until the larval fish are harvested at approximately 11 weeks after hatching.

Digestion in larval fish is relatively poorly understood, especially for galaxiid species. Understanding the digestive capabilities of larvae is important because it is the source of all nutrients used to build and maintain tissues and consequently determines the growth, health and survival (Rønnestad et al., 2013). Larval fish generally have simple digestive systems made up of a straight undifferentiated gut with limited capacity for digestion and absorption of nutrients, which is often constrained by the low concentrations and activity of digestive enzymes in the rudimentary gut (Cara et al., 2003; Chen et al., 2006; Dabrowski, 1984; Schwartz, 2008). Consequently, live food items are often vitally important in the hatchery production of larval fish because of their increased digestibility when compared to artificial feeds (Schwartz, 2008). The release of digestive enzymes from the live food, such as *Artemia*, can also assist in the breakdown of food in the larval gut (Abatzopoulos et al., 2002; Dabrowski & Glogowski, 1977; Kolkovski et al., 1993; Lavens & Sorgeloos, 1996). The disadvantages of the use of live food in larval fish culture is their high cost and their labour intensive preparation (Southgate & Partridge, 1998). As a result, it is often of primary concern for the development of new species in aquaculture to determine the age at which the larval fish can be effectively weaned onto artificial dry food which is usually less costly to provide (Southgate & Partridge, 1998). The visual stimuli generated by the movement of live feeds is important for helping to facilitate the initiation of exogenous feeding in larval fish (Hubbs & Blaxter, 1986; Jones & Closs,

2016; Miller et al., 1988). The transition from live to inert feeds, which lack similar visual stimuli, is a critical phase in larval fin fish production. Understanding the behaviour of fish in response to artificial dry foods is vital to understanding whether they will consume them. This is pivotal information that must be attained before successful studies on the comparative growth performance of larval fish fed different artificial diets can take place.

The development of the capacity and efficiency of the digestive system is also important for designing more effective species-specific larval feeding regimes (Rønnestad et al., 2013). Optimising larval feed regimes can be achieved by managing the quantities of food, and the timing of their delivery to cultured larvae, that best match the limitations in larval feed intake and digestive processing. Consequently, research into the feeding behaviour and digestive capabilities of the larvae of giant kōkopu are important for maximising production whilst minimising feeding costs.

1.8 Aims of this Study

The overarching purpose of the research presented in this thesis is to help to develop a more efficacious commercial feeding regime for the aquaculture production of larval giant kōkopu, a species for which there is very little known about their feeding biology, especially through the larval phase. The thesis is divided into six chapters, with introductory and concluding chapters bookending four research chapters each presenting separate elements of research with specific aims. Each of these research chapters has been written as a self-contained chapter to facilitate publication, which has occurred for three of the four chapters, whilst the fourth is undergoing editorial processing.

The specific aims of the material presented in each chapter of the thesis are outlined below:

Chapter 1 provides an overview of the development of fin fish aquaculture globally, and provides a background of the limited knowledge of the biology of galaxiid species and their associated human exploitation, including the status of galaxiid aquaculture. The overall aims of the research presented in the thesis and the structure of the thesis are outlined.

Chapter 2 documents the growth and development of giant kōkopu larvae from hatch to harvest in the current NZPWL production system in terms of larval body length, depth, mass and mouth gape, as well as the moisture,

protein and lipid content. A particular focus of this research is to determine changes in larval mouth gape throughout the culture period because it is a key determinant of changes in the feeding abilities of the larvae. This research has been published as: McKay, W. J. G., & Jeffs, A. G. (2021). Morphometric and energetic development of artificially reared giant kōkopu (*Galaxias argenteus*). *Aquaculture*, 544, 737123. https://doi.org/10.1016/j.aquaculture.2021.737123

Chapter 3 presents the results of two experiments designed to improve the underlying knowledge about the nutritional accumulation of larval giant kōkopu provided with different food sources. The first experiment aims to compare the growth (length, depth and dry weight) and survival of larval giant kōkopu with different live feed treatments providing varying proportions and timing of delivery of instar-I and instar-II live *Artemia*. The second experiment compares the nutritional composition (protein, lipid and fatty acid profile) of instar-I and instar-II *Artemia* prepared under a variety of storage and enrichment techniques used routinely in the larviculture of giant kōkopu. The overall aim of the research presented in this chapter is to better understand the nutritional needs of larval giant kōkopu. This research is under review as: McKay, W. J. G., & Jeffs, A. G. (2022*). Optimization of Artemia feed regimes for larval giant kōkopu* (*Galaxias argenteus)*. [Manuscript submitted for publication]. Institute of Marine Science, University of Auckland.

Chapter 4 presents the results of research on the feeding of larval giant kōkopu over a development range from first feeding to 30 days after hatching. The aim of this experiment was to determine when ingestion of artificial feeds commences in larvae in an effort to identify the earliest possible age to wean the larvae off the more expensive live food diet. This research has been published as: McKay, W. J. G., & Jeffs, A. G. (2022). Improving the weaning of larval giant kōkopu*, Galaxias argenteus*: An emerging aquaculture species. *Journal of the World Aquaculture Society*, 12926. https://doi.org/10.1111/jwas.12926

Chapter 5 presents the results of an experiment comparing the growth (length, depth and mass), and nutritional composition (protein and lipid content and fatty acid profile) of larval giant kōkopu provided with three different commercially available artificial feeds. The aim of this research was to identify which of the three commercial artificial feeds is the most efficient for the production of giant kōkopu. The three artificial feeds selected for this experiment were chosen for their differences in protein, lipid and carbohydrate composition, as well as their presentation characteristics and cost. This research has been published as: McKay, W. J. G., & Jeffs, A. G. (2022).

Comparison of three artificial diets for the larviculture of giant kōkopu (*Galaxias argenteus*). *Fishes, 7*(6), 310; https://doi.org/10.3390/fishes7060310

Chapter 6 provides a general discussion of the results provided by the collective body of research presented in the preceding chapters. The overall aim of this general discussion is to provide some informed recommendations on the optimum approach to feeding in the rearing of larval giant kōkopu. Some of the limitations of the study are identified and recommendations for future research on whitebait aquaculture are provided.

2 Chapter 2: Morphometric and energetic development of artificially reared giant kōkopu (*Galaxias argenteus*)

Published as:

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2.1 Introduction

Whitebait is a generic term used worldwide for transparent juvenile or larval fish of a range of species that are often highly-prized as human food. In New Zealand the term whitebait refers to the transparent larval and early juvenile stages of five native species of the genus *Galaxias* (McDowall, 1996). These are īnanga (*G. maculatus*), kōaro (*G. brevipinnis*), short-jaw kōkopu (*G. postvectis*), banded kōkopu (*G. fasciatus*) and the giant kōkopu (*G. argenteus)* (McDowall, 1996). In local food markets, New Zealand whitebait fetch US$70-115 kg^{-1} (Environment Southland, 2013; *NZ Whitebait*, n.d.) and this, along with conservation concerns for wild populations of these fish species, offer an incentive for their development for production in aquaculture systems.

New Zealand Premium Whitebait Ltd (NZPWL) has developed the technology for raising two whitebait species (giant kōkopu and īnanga) in land-based aquaculture systems. Giant kōkopu has been chosen by the company as the species for commercialization as it can be reared for 77 days after hatching (DAH) before being harvested as market-ready whitebait. The larvae of most fish species require live prey as their first feed because prey movement triggers predatory behaviour (Qin, 2008). This is true of larval giant kōkopu, which are fed on live *Artemia* nauplii at the end of 3 DAH when they are thought to commence feeding. Live feeding continues for another ~28 days before a prolonged weaning period results in artificial diet provision until harvest at 77 DAH. In 2016 the first sales of whitebait to the local market were made of culture giant kōkopu.

Presently there are no reports detailing growth and morphological development during any period of the larval phase of giant kōkopu. An improved understanding of larval development has the potential to assist in advancing

commercial giant kōkopu aquaculture. For example, knowledge of changes in mouth gape may improve the efficiency of larval feed delivery, while understanding of changes in the biomass of larvae throughout the production cycle is critical for understanding the performance of larvae and the suitability of feeds and their timing.

A lack of understanding of larval biology and the high costs associated with providing feeds in larval culture are major limiting factors inhibiting the expansion of existing species, as well as the development of new species for aquaculture. For example, feed costs alone can account for 30-70 % of fin fish production costs (Verdal et al., 2018; Webster & Lim, 2001). The reduction of feed costs represents a large opportunity for research to greatly improve the efficiency of production. However, to achieve advances in the efficient feeding of fish larvae in culture it is essential to understand the developmental biology of the species.

In the early feeding of fish larvae, the mouth gape (i.e., the dorsoventral width between the upper and lower jaw) is a key determinant in the capture and consumption of food particles by the mouth (Bremigan & Stein, 1994; Cunha & Planas, 1999; Fernández-Diaz et al., 1994; Østergaard et al., 2005; Shirota, 1970). As predators larval fish are severely limited by the extent of their mouth gape as they consume prey items whole (Arts & Evans, 1987; Bremigan & Stein, 1994; China & Holzman, 2014; Cunha & Planas, 1999; Krebs & Turingan, 2003; Lavens & Sorgeloos, 1996; Makrakis et al., 2008; Zaret, 1980). In fin fish larviculture food particles that are too large to be consumed by larval fish are wasted. Likewise, the provision of food particles that are small relative to larval fish mouth gape can also result in suboptimal larval fish performance. This is due to the volume of food particles increasing at a cubic function of their diameter, and therefore smaller food particles result in a lower nutritional return for the same foraging effort (Dhont & Sorgeloos, 2002; Lavens & Sorgeloos, 1996). This lower return on effort reduces the nutritional surplus required for maintenance, growth and development in larval fish, resulting in reduced growth performance and health (Qin, 2008). Therefore, the feed particle size relative to the gape of the larval species in culture is a major consideration for achieving optimum growth performance. The ideal size range of food particles is normally in the range of 25 to 60 % of the mouth gape of larval fish, however, some species have achieved improved survival at ratios of up to 84 % (Bremigan & Stein, 1994; Fernández-Diaz et al., 1994; Hoestenberghe et al., 2015; Østergaard et al., 2005; Shirota, 1970).

The commencement of feeding is a particularly critical transitional period in larviculture. The timing of initiation of exogenous feeding varies enormously among species, being highly dependent on the functional development of a combination of sensory (eye diameter), locomotory, feeding and digestive structures, as well as the level of absorption of endogenous energy reserves (yolk sac and oil globule volumes) (Huang et al., 2005; Shan et al., 2009; Yin & Blaxter, 1989). In larviculture the timing of initiation of feeding is a critical point where delays in providing appropriate larval food frequently leads to morphological deformities, health problems and ultimately drives elevated larval mortality (Gisbert et al., 2004; Kjørsvik et al., 1991; McGurk, 1984; Shan et al., 2009).

Therefore, the aim of this research is to provide the first description of changes in larval morphology, during the larval development of giant kōkopu.

2.2 Materials and Methods

2.2.1 Larval Rearing

On the 19 August 2016 approximately 2.4 million giant kōkopu eggs were hatched in a conical 2500 l larval rearing tank from gametes provided by 160 female and 20 male broodstock. For the duration of the culture period larvae were kept under a 12:12 h light:dark lighting regime, in a recirculating aquaculture system with complete seawater exchange every 20 minutes. Seawater was maintained at 35 PSU and 16 °C and UV sterilized before return to larval culture tanks.

Live feeding began in the evening 3 DAH (days after hatching) and continued until 31 DAH. Larvae were fed at 0830, 1130 and 1630 h. The initial live feed consisted of instar-I *Artemia* nauplii until 14 DAH, after which instar-II was introduced and the proportion of instar-II *Artemia* nauplii was increased over a period of 7 days. This was followed by 7 days of instar-II only. Artificial feed (Otohime, Marubeni Nisshin Feed Co., Ltd, Japan) was introduced at 7 DAH, but initially only as a minute proportion of the total feed (<1 % by weight). Larvae were fully weaned from live feed onto Otohime at 31 DAH. From this point until the completion of the culture period (77 DAH), larvae were fed only this artificial food product, however, the particle size and total quantity of feed increased with fish age in accordance with the NZPWL larval rearing protocol.

2.2.2 Sampling Design

Larval giant kōkopu were randomly sampled from the commercial larval rearing tank using a 1 l flask dipped into the tank every seven days beginning on 0 DAH until the tank was harvested at 77 DAH. Fifty fish from each sample were measured for total length (TL), measured from the tip of the snout to the tip of the caudal-fin membrane; and body depth at anus (BD_a), i.e., measured from the dorsal to ventral margin of the tail musculature at the anus (Figure 2-1). Fifteen randomly selected individuals were also measured for eye diameter (ED) and mouth gape diameter (GD). ED is the mean calculated from the maximum (ED_{max}) and minimum (ED_{min}) axes between the margins of the eye (Figure 2-1). GD was calculated using Equation 2-1, from Guma'a (1978) using upper jaw length (UJL), measured from the tip of the upper jaw to the axis of the mouth gape and the lower jaw length (LJL), measured from the tip of the dentary to the axis of the mouth gape (Figure 2-1). This method for calculating mouth gape was selected from a number of methods available because of the simplicity in dealing with multiple small, fragile larvae that required measurement immediately after being euthanized. Other methods for calculating mouth gape in larval fish can suffer from difficulties in precisely measuring mouth width, which may not be the most effective measure for estimating optimal food particle size (Cunha & Planas, 1999); while the equation used by Shirota (1970) does not consider the lower jaw length which had the potential of having a significant impact on gape measure in this study due to the lack of previous data on these morphological characters of giant kōkopu.

Equation 2-1

$$GD = \sqrt{(UJL^2 + LJL^2)}$$

Prey size as a percentage of gape compares the live feed prey item width to the mouth gape width of the giant kōkopu larvae. The width of instar-I *Artemia* (i.e., 195 µm) was compared with the calculated mean mouth gape of larvae at 0, 7 and 14 DAH. At 21 DAH instar-I and instar-II *Artemia* were fed to larvae so the larger prey item width, (i.e., 270 µm for instar-II) was used to calculate prey size as a percentage of gape at 21 DAH and again at 28 DAH when only instar-II was provided to the fish. Means of individual prey size as a percentage of gape were calculated and compared for each larval fish age (i.e., 0, 7, 14, 21 and 28 DAH).

Measures of yolk sac and oil globule diameter from 30 randomly selected fish took place at 0, 2, 3, 4, 5 and 7 DAH. Mean diameter was determined for the yolk sac (YSD) from the average of the maximum (YSD_{max}) and (YSD_{min}) axes between the margins of the yolk sac (Figure 2-1). Mean diameter of the oil globule (OGD) was calculated as the average of the maximum (OGD_{max}) and minimum (OGD_{min}) axes between the margins of the oil globule (Figure 2-1). The volume of each yolk sac and oil globule volume was estimated using the (Equation 2-2) from (Leitón et al., 2012):

Equation 2-2 $$Volume = \pi \div 6 \times L \times H^2$$

Where L is the length of the major axis ($_{max}$) (YSD_{max} or OGD_{max}) and H the length of the minor axis ($_{min}$) (YSD_{min} or OGD_{min}) (Leitón et al., 2012).

All measurements were taken by photographing fish on a transparent reference grid of 460 µm under a microscope fitted with an Olympus TG-4 digital camera. These images were then analysed using ImageJ64 software.

Mean yolk sac and oil globule absorption was determined by calculating using the difference between the measures of the yolk sac or oil globule volume at successive days of larval development and the mean volume at 0 DAH, then dividing by the mean volume at 0 DAH and multiplying by 100 to convert to a percentage (Equation 2-3).

Equation 2-3 $$Absorption = 100 - \left(\left(V^x - V^0\right) \div V^0\right) \times 100\right)$$

Where V^x is the volume at the time point at which absorption is being calculated, V^0 is volume at 0 DAH.

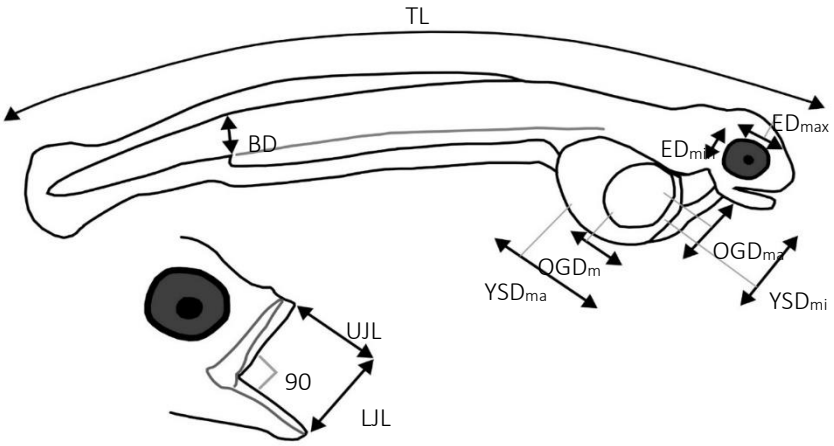

Figure 2-1. Diagram of morphometric measurements of a newly-hatched galaxiid larva showing measures as described above (Adapted from Jones and Closs 2016).

A mean wet weight (WW) of fish at each of the 7 day sampling intervals was measured by batch weighing (±0.001 g) three subsamples of 20 randomly selected fish dabbed dry with laboratory tissue, and then weighing to the nearest 0.001 g and dividing by 20.

Mean dry weight (DW) was measured after freeze drying pooled subsamples of varying sample size. Random samples of 100 (0, 7, 14, 21, 28 DAH), 50 (35, 42, 49 DAH), 40 (56 DAH) and 10 (63, 70, 77 DAH) fish were weighed to the nearest 0.0001 g and divided by the number of fish to get the average individual dry weight (DW) for each subsample. Three samples were taken at each sampling event for each of which mean DW was obtained.

Samples for lipid and protein content were taken from the same samples used to establish DW. Total lipid was determined using the Bligh and Dyer (1959) solvent extraction method which was modified to suit the sample size used in this experiment. Freeze dried samples were vortexed for 30 s with a 1.9 ml aliquot of chloroform, methanol and deionized water (2:1:0.8) and left to stand for 16 h. An aliquot of 0.5 ml of 0.7 % sodium chloride and 0.5 ml of chloroform were added prior to 30 s vortex then centrifugation at 1000 rpm for 10 min. Using a glass pipette the chloroform-lipid layer was removed and placed in a pre-weighed glass vial. The remaining residue was washed with a further 1 ml of chloroform, vortexed for 30 s, centrifuged at 1000 rpm for 10 min and removed

and placed in the same glass vial with the previous extract. This step was repeated again with 0.5 ml of chloroform to ensure all lipid was removed. The pre-weighed glass vials were placed in a thermal block at 39 °C under flowing nitrogen gas until chloroform was evaporated. The glass vials were then re-weighed to determine the total lipid mass. Mean total lipid per larva was calculated by dividing total subsample lipid by the number of fish in the subsample, then taking an average of the triplicate sub-samples of lipid content assayed for each fish age.

The total protein content of larvae was measured using a bicinchoninic acid (BCA) assay (Thermo Scientific Pierce BCA Protein Assay Kit). After removal of the lipid content as outlined above, samples were freeze dried and sodium hydroxide was added to each subsample before water bath incubation at 50 °C for 16 h. The resulting solution was homogenized and a subsample taken and centrifuged at 4000 rpm for 10 mins at 4 °C, then the BCA reagents were added before a 2.5 h incubation at 37 °C. The well plate was allowed to cool and absorbance was read at 562 nm. A series of bovine serum albumin standards, as well as the samples from the larvae were placed into a 96 well-plate and reagent added to each. Protein concentration was calculated using a standard curve produced from bovine serum albumin standards and presented as a percentage of dry weight (%DW). The mean total protein (per larvae) was determined by multiplying the protein concentration (%DW) by the mean DW of fish in the respective sample. Mean total protein was calculated for each larval age by taking an average of the triplicate sub-samples assayed for protein content.

2.2.3 Data Analyses

To characterize the association between total length and depth; gape and age; and eye diameter and age, linear regression was used. For the weight to length relationship a power curve was fitted to model weight increase relative to length. For covariates where the normality assumption did not hold (eye diameter) a log transformation was used on the response variable, subsequent to this transformation the residuals followed a normal curve and therefore estimates were multiplicative.

One-way ANOVA were used to assess if there were differences in lipid and protein between larval age groups. Normality and homogeneity of variances for all data were confirmed by testing prior to analyses. Where overall ANOVA results were significant, pairwise comparisons using the Tukey method were used to compare sequential

age groups. Statistical analyses were performed using R (RStudio, ver. 1.2.1335). All measures of variability of means are reported as standard error of the mean.

2.2.4 Use of animals in research

This research was conducted in compliance with New Zealand's Animal Welfare Act 1999, which strictly regulates the use of animals in research.

2.3 Results

2.3.1 Length and Depth

At hatching the giant kōkopu larvae averaged 9.17 mm (SE± 0.06) in length ranging from 7.60 to 9.87 mm. Larval depth at hatching ranged from 0.44 to 0.65 mm, with an overall mean 0.56 mm (SE± 0.01) in depth. At 77 DAH larvae reach 31.87 mm (SE± 0.90) in length and 3.05 mm (SE± 0.11) in depth (Figure 2-2). Body depth increased proportionately to TL, with a 1 mm increase in TL corresponding to a 0.11 mm increase in body depth ($P<0.01$, $R^2=0.98$).

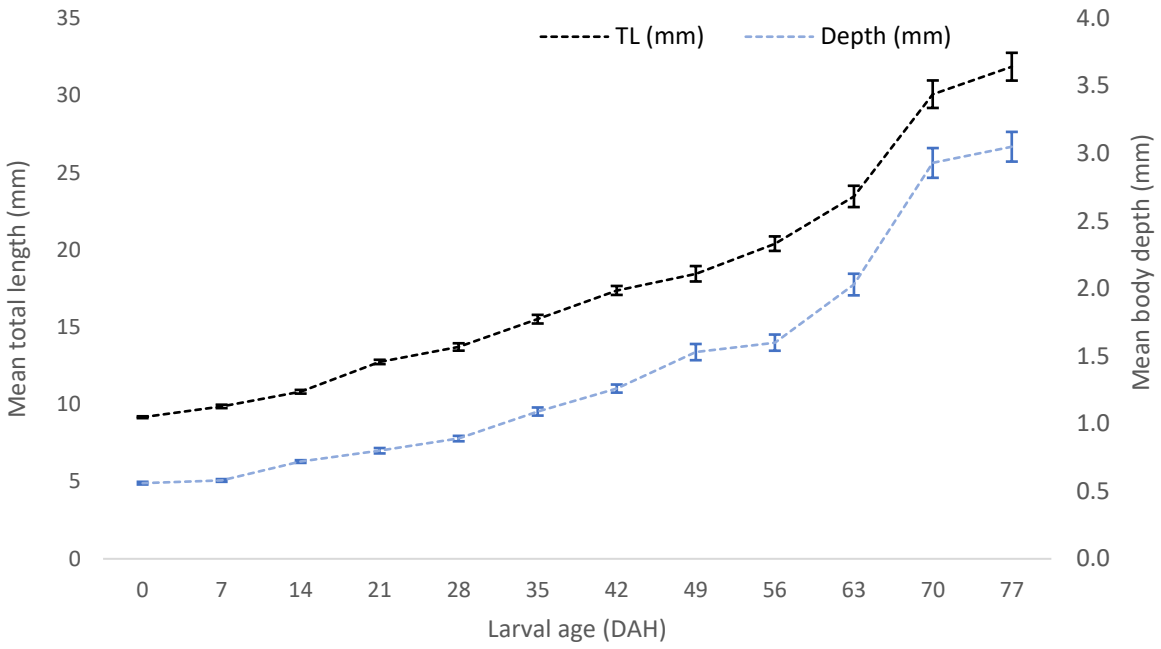

Figure 2-2. Mean total length (±SE) of larval giant kōkopu from 0 to 77 DAH on the primary-axis and mean body depth for larval giant kōkopu from 0 to 77 DAH on the secondary-axis.

2.3.2 Weight

Upon hatching larval giant kōkopu have a mean WW of 2.12 mg (SE± 0.01) and mean DW of 0.45 mg (SE± 0.00) (Figure 2-3). At harvest larvae (i.e., 77 DAH) had a mean WW of 177.05 mg (SE± 1.34) and a mean DW of 29.23 mg (SE± 1.26) (Figure 2-3), representing an overall increase from hatch of 8265 % and 6333 %, respectively.

For each millimetre increase in TL the WW increases by the equation WW = 0.0000004 × TL^3.8228 ($P<0.01$, $R^2=0.98$) (Figure 2-4).

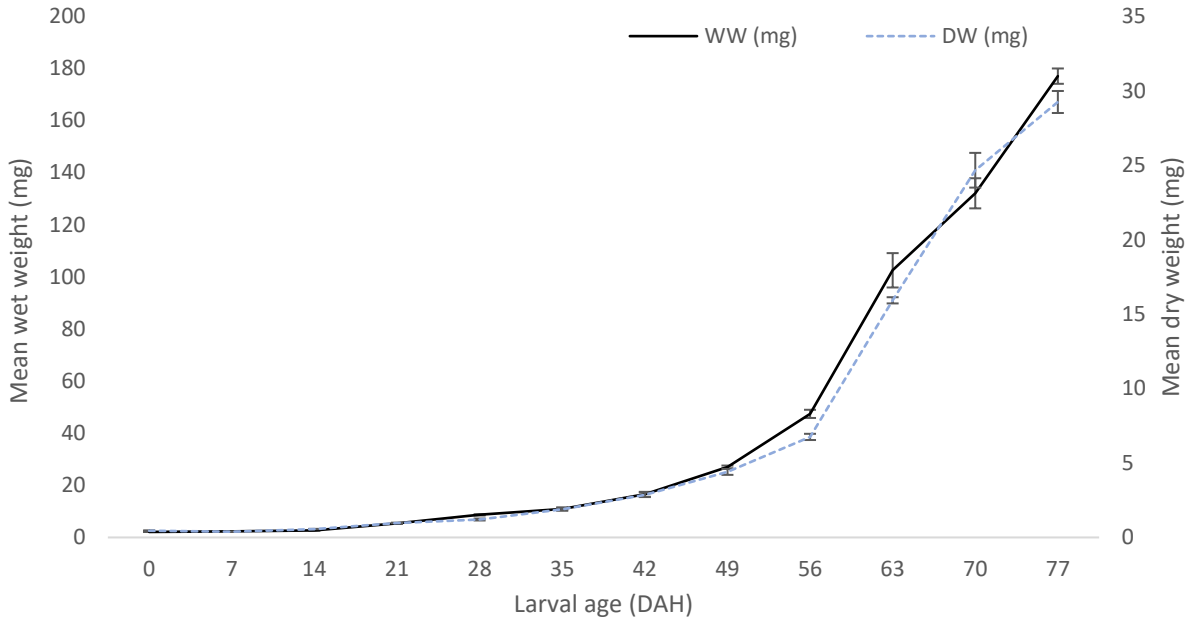

Figure 2-3. Mean wet weight (±SE) of larval giant kōkopu from 0 to 77 DAH on the primary-axis and mean dry weight for larval giant kōkopu from 0 to 77 DAH on the secondary-axis.

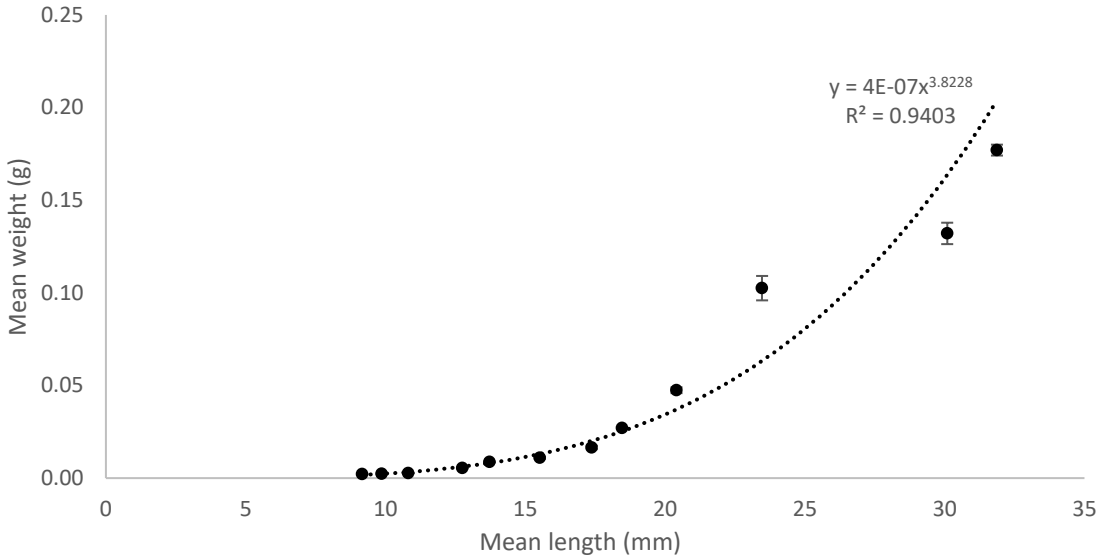

Figure 2-4. Mean total length (±SE) versus mean wet weight for larval giant kōkopu from 0 DAH to 77 DAH with black dotted line the line of best fit.

2.3.3 Mouth Gape

Larval giant kōkopu hatch with a mean mouth gape of 348 µm (SE± 15), which at 77 DAH reaches 1752 µm (SE± 98) (Figure 2-5). For each additional DAH the mouth gape increased by 1.8 % ($P<0.01$, $R^2=0.88$). For every millimetre increase in TL, giant kōkopu mouth gape increased by 58.81 µm ($P<0.01$, $R^2=0.96$) (Figure 2-6). At first feeding (3 DAH), the prey size (i.e., *Artemia* instar-I) is 48 % of mouth gape and decreases to 34.4 % (SE± 0.9) by 28 DAH (instar-II) (Figure 2-7).

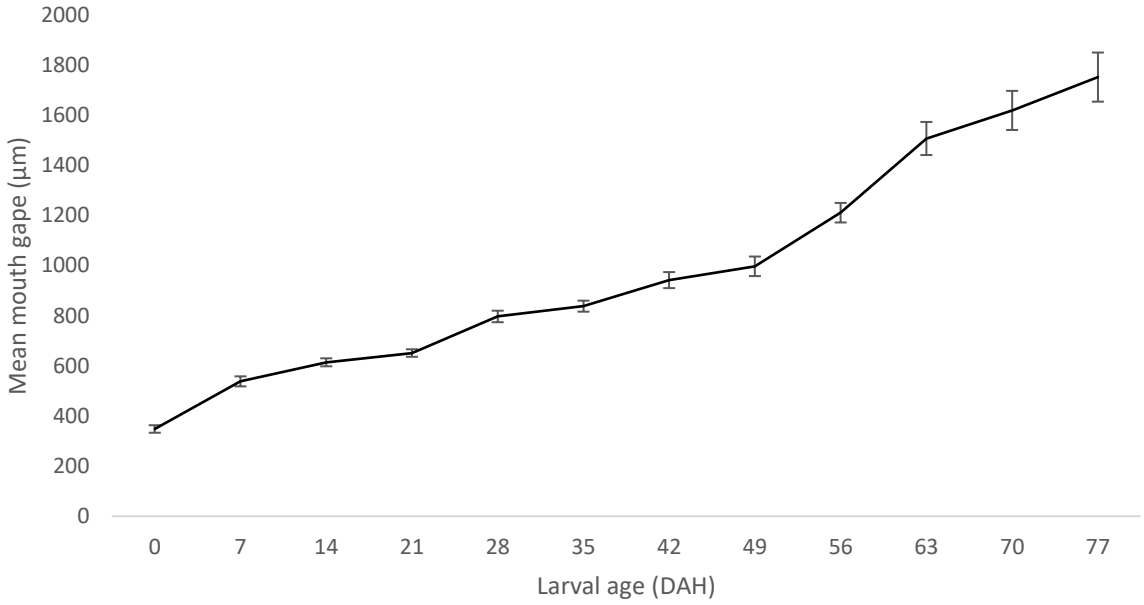

Figure 2-5. Mean mouth gape (±SE) of larval giant kōkopu over the 77 day rearing period.

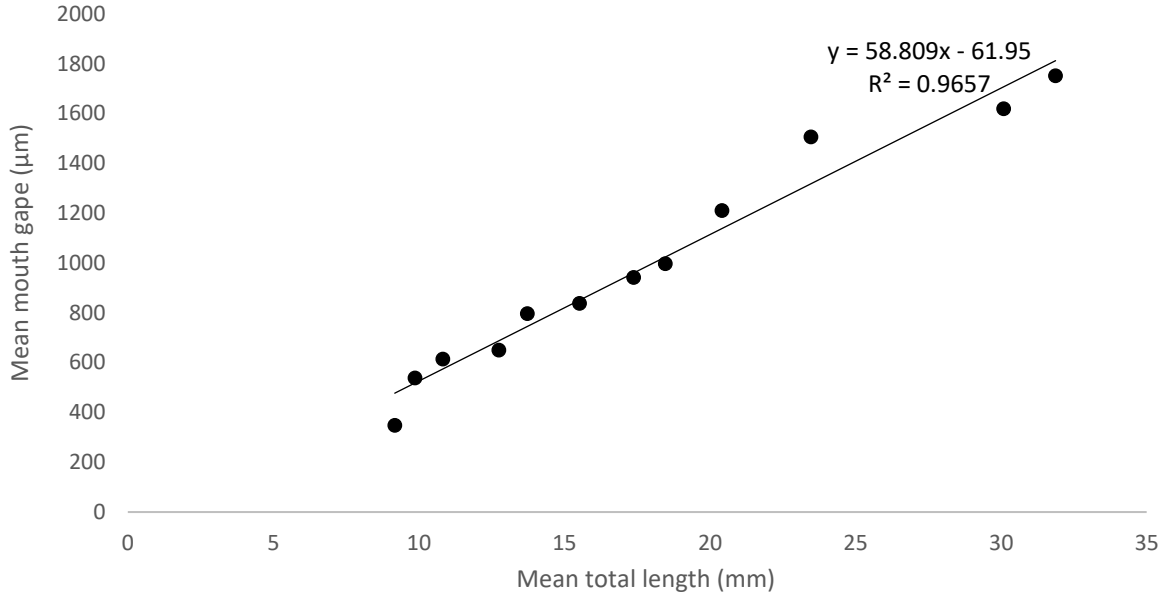

Figure 2-6. Comparison of mean total length and mean mouth gape of larval giant kōkopu during the 77 day rearing period.

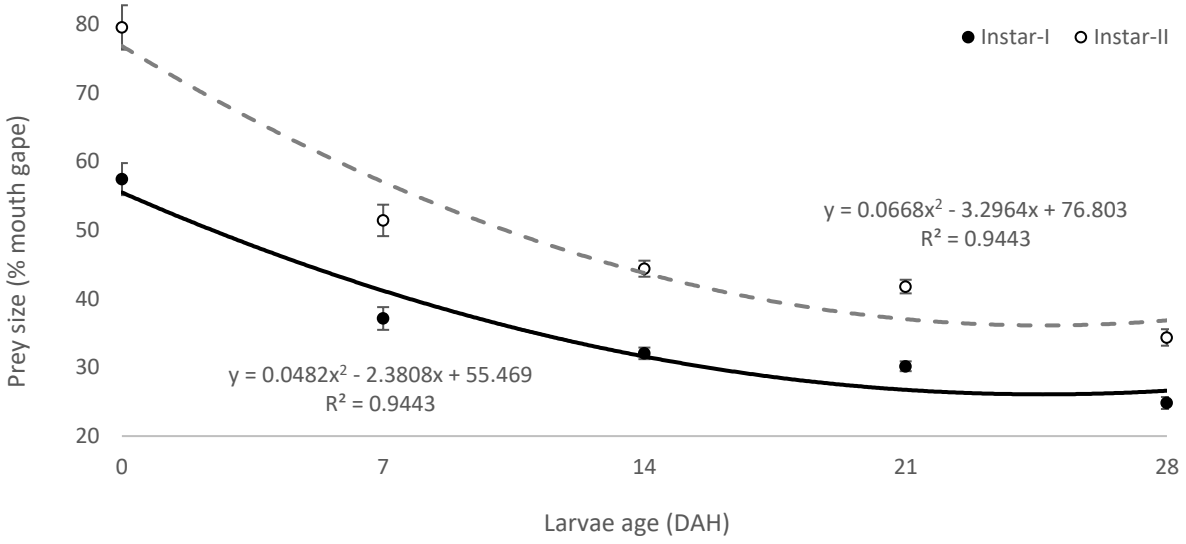

Figure 2-7. Mean prey size as a percentage of mean mouth gape for larval giant kōkopu during the period of live food provision up to 28 DAH for both instar-I (black) and instar-II (white). Black trend line with equation bottom left is for the prey size ratio of instar-I and grey trend line with associated equation middle right is for instar-II.

2.3.4 Eye Diameter

At hatch, larval giant kōkopu have a mean eye diameter of 431 µm (SE± 7) which increases steadily throughout the 77 day production period to 1315 µm (SE± 62) (Figure 2-8). With each day of larval development, the eye diameter increased by 1.5 % ($P< 0.01$, $R^2= 0.91$).

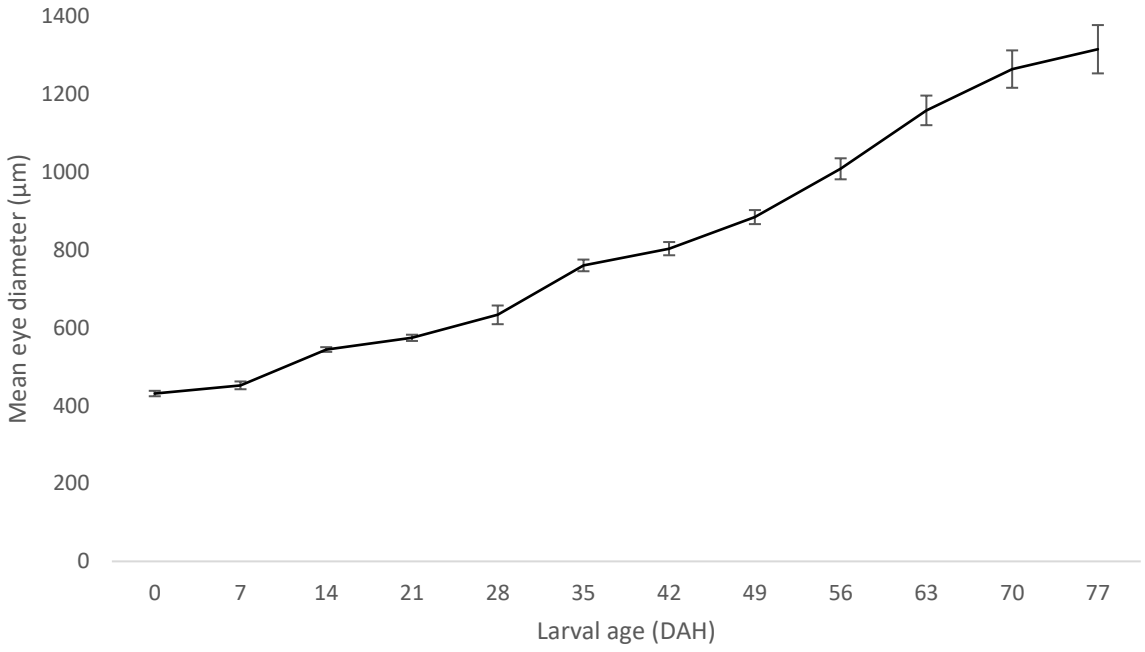

Figure 2-8. Mean eye diameter (±SE) for giant kōkopu larvae throughout the 77 day larval rearing period.

2.3.5 Yolk Sac and Oil Globule Size

The yolk sac of newly-hatched larval giant kōkopu diminished rapidly from a mean volume of 161 nl (SE± 6) to 2 nl (SE± 2) within the first 7 DAH. The rate of depletion was quite variable with 37 % of larvae having completely consumed (i.e., 0 nl) their yolk sac by 3 DAH and 63 % by 4 DAH (Figure 2-9).

At hatching, the oil globule reserve is smaller than the yolk sac at 26 nl (SE± 1). After 7 days the oil globule is depleted to 90 % of its original volume, with only one larva observed having completely consumed the oil globule by this time (Figure 2-9).

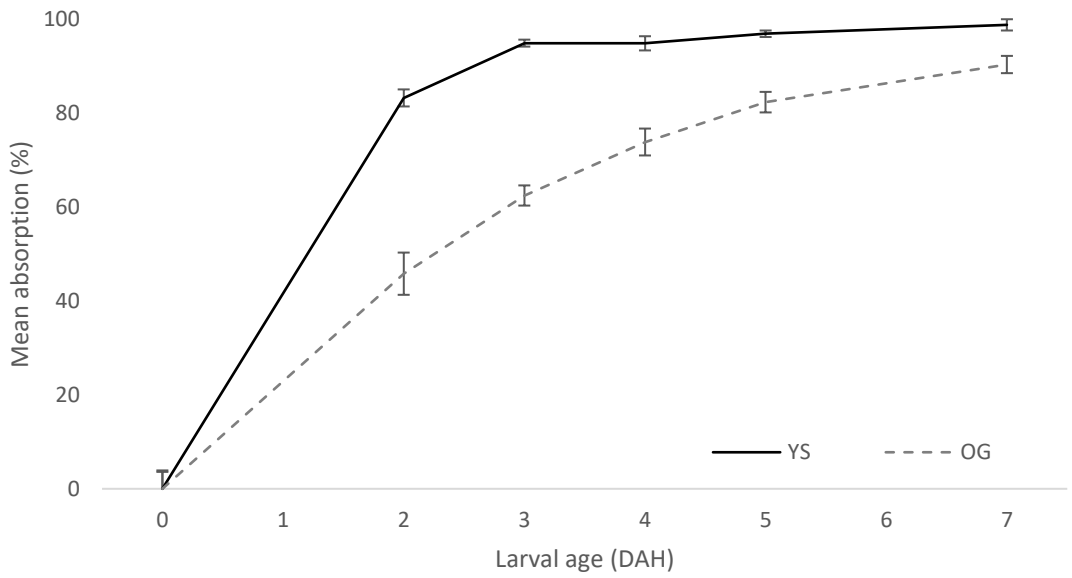

Figure 2-9. Mean absorption as a percentage of the mean starting volume of larval giant kōkopu yolk sac and oil globule over the first 7 days after hatching (mean ±SE). YS, yolk sac; OG, oil globule.

2.3.6 Lipid and Protein Composition

Mean total lipid of giant kōkopu larvae was not consistent through the 77 DAH rearing period ($F_{(11, 24)}$= 588.37, P< 0.01). Mean total lipid is greater immediately after hatching (0 DAH) than at 7 DAH (P< 0.1) (Figure 2-10). All but two of the remaining consecutive weekly comparisons resulted in a significant increase in total lipid in the larvae. There was no significant difference in total lipid from 21 DAH to 28 DAH (P= 0.09), nor was there from 70 to 77 DAH (P= 0.06) (Figure 2-10).

Mean total protein changed among larval ages ($F_{(11, 24)}$ = 624.4, P< 0.01). Total protein in giant kōkopu larvae did not differ between hatch (0 DAH) and 7 DAH (P=0.06) (Fig. 10). Thereafter, mean total protein of larvae increased in weekly samples until 63 DAH, after which it did not increase at 70 DAH (P=0.82) nor is 77 DAH greater than 70 DAH (P=0.06) (Figure 2-10).

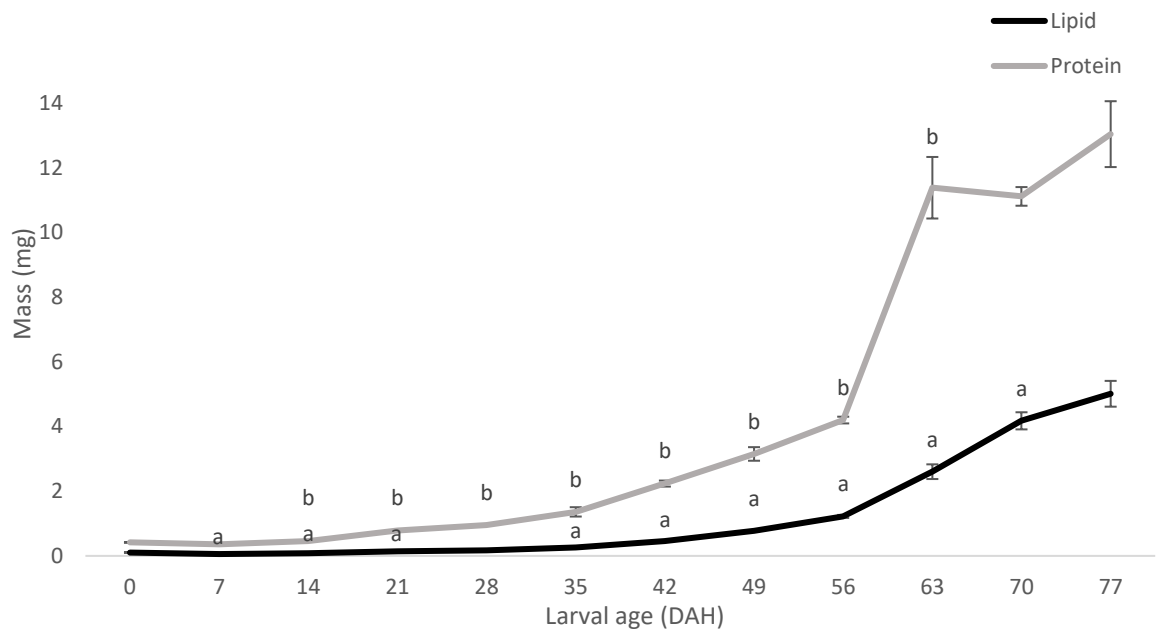

Figure 2-10. Mean total lipid (±SE) and total protein composition of giant kōkopu larvae throughout the 77 day larval rearing period (mean ±SE). 'a' Indicates a significant difference in total lipid between observed age and the previous age, 'b' indicates a significant difference in total protein between observed age and previous age.

2.4 Discussion

Very little is known about the morphological development of the larvae of the giant kōkopu larvae or any of New Zealand's five galaxiid species (Benzie, 1968c; McDowall et al., 1994; McDowall & Kelly, 1999). However, a greater understanding of their development is an important starting point for advancing their commercial aquaculture to produce whitebait.

Larval giant kōkopu total length and body depth increase considerably over the 77 DAH production period with both measures showing accelerated growth in the final 14 day period between 56 DAH and 70 DAH. The average 31.87 mm total length achieved in the larval rearing period (77 DAH) is greater than that recorded for the conspecific īnanga in culture, which are only <20 mm in total length at the equivalent age and requiring more than 140 days to reach the same total length as giant kōkopu larvae at harvest (Mitchell, 1989). This is a significant advantage in favour of the commercial culturing of giant kōkopu over the īnanga. In contrast to the larval size

achieved over 77 days of larval rearing in culture conditions (i.e., 31.87 mm total length), giant kōkopu have been caught in the wild upon their return migration to riverine systems at standard length of 34 to 36 mm (Woods, 1968) West Coast, 40.8 mm Waikato and 55.4 mm Buller (Yungnickel, 2017), the later with an estimated mean age of 130.9 days (McClintock, 2018). This indicates the potential of extending the 77 day rearing period to maximize productivity through growing larvae to these larger sizes or for potentially improving the culture regime to increase the growth rate.

The change in mass of the larval giant kōkopu through the 77 day culture period shows an exponential growth curve for WW and DW. The initial lag phase in WW and DW mass gain, from 0 to 7 DAH, reflects the significant decrease in total lipid as the maternally provisioned yolk sac and oil globules are consumed as endogenous energy sources. Over the same period, the protein content does not differ. This indicates the critical role of lipid in first feeds as an energy source to prevent the redirection of protein from somatic growth to supplying energetic demands. It also highlights the importance of an adequate maternal broodstock nutrition and conditioning regime to ensure a high degree of nutritional provisioning of the eggs.

Live feed provision did not begin until the end of 3 DAH when on average 95 % of the yolk sac and between 62-74 % of the oil globule had been absorbed. By this age, a large proportion of larvae (37 %) had completely consumed their yolk sac. Therefore, the reduced growth performance of giant kōkopu larvae in the first 7 DAH is likely to have been caused by providing initial live feeds too late. It is possible that diminished growth performance was caused by poor ability to consume live prey, however, observations during this study identified *Artemia* in the stomach of a high proportion of larvae within 30 minutes of their first exposure to live feeds. Timing of first feed provision is critical to achieving optimal growth performance and survival, where similar delays lead to detrimental outcomes for productivity in the culture of many other species (Blaxter & Hempel, 1963; McGurk, 1984; Peña & Dumas, 2005; Shan et al., 2009; Sulaeman & Fotedar, 2017; Zhang et al., 2009).

Timing of first feed initiation in other species of fin fish larvae usually coincides with the appearance of eye pigmentation, such as for the spotted bass which receives feed at 2 DAH upon eye pigmentation despite the yolk sac not being completely depleted until 3 DAH (Peña et al., 2003; Peña et al., 2004). The result is a period where the larvae are relying on endogenous and exogenous sources of nutrition, which is a common feature in the culture of the larvae of other fin fish species (Huang et al., 2005; Shan et al., 2009; Yu et al., 2003). In īnanga the

eye pigmentation develops during late egg stage development (Benzie, 1968b), while in giant kōkopu larval eye pigmentation was observed at hatch during this study so it is likely these larvae are able to detect food items upon hatching. Furthermore, in larval giant kōkopu the mouth is open at hatching, suggesting that prey size and foraging ability are likely the only barriers to successfully commencing feeding at hatch. Given the initial lag in growth performance and the likely ability to consume live prey from hatching, the introduction of live feed to larval giant kōkopu should commence earlier to investigate growth performance and survival outcomes, i.e., provision of live food between hatch and 2 DAH rather than the current regime of 3 DAH.

The mean eye diameter of giant kōkopu larvae at hatching is 431 µm, much larger than the ~280 µm for īnanga at the same stage (Mitchell, 1989). This is the only time in larval giant kōkopu production where eye diameter was greater than gape diameter and by 77 DAH, eye diameter reached 1315 µm, whereas for īnanga at the same age it is approximately 1163 µm (Mitchell, 1989). Eye diameter is a determinant of visual acuity and reactive distance, thereby significantly affecting the feeding abilities of fish larvae (Hubbs & Blaxter, 1986; Jones & Closs, 2016; Miller et al., 1988). Larger eye diameter and mouth gape allow for improved foraging in low light conditions and the capture of smaller prey (Mitchell, 1989) and may provide giant kōkopu larvae with greater initial foraging ability compared to īnanga.

Weaning from live feed is often a difficult period in fin fish larviculture and can cause severe mortalities if incorrectly managed (Barron et al., 2016; Hamre et al., 2013; Hoestenberghe et al., 2015; Lucas & Southgate, 2012; Rao, 2003). However, following the lag phase in the growth curve for giant kōkopu, subsequent rapid growth was apparent by 21 DAH with no obvious reduction in growth at the point of weaning from live food at 28 DAH, nor at 35 DAH (Figure 2-3). Furthermore, total protein of the larvae increased significantly over this period (i.e., 7 – 14 DAH). Giant kōkopu larvae appear to be well prepared for weaning at 28 DAH, so shortening the live feeding period should be investigated given the apparent ease with which larval giant kōkopu accepted the artificial diet, and the potential for significant cost savings in reducing live feed requirements in production.

Larval giant kōkopu reached an average of 177.05 mg in WW at harvest (77 DAH) increasing rapidly in the final weeks of production. However, total length and depth do not continue to increase at a similar rate from 70 – 77 DAH. The increasing opacity of the muscle mass from 63 DAH are comparable to wild specimens in the early stages of development i.e., “lightly pigmented” (Yungnickel, 2017). The presence

of increasing red coloration of internal organs from 70 DAH onward are indicators of the early stages of metamorphosis (Benzie, 1968b). These internal developments require significant energetic input and as a result energy could be expected to be diverted from growth, hence the likely cause for the reduction in the increases observed in TL, body depth and lipid deposition during this period (Gagnat et al., 2016; Osse & van den Boogaart, 2004). The progression of metamorphosis poses a problem for marketing of whitebait because premium "A Grade" whitebait are transparent and generally longer in length. Therefore, any increase in productivity resulting from culturing giant kōkopu for longer periods appears likely to result in a higher proportion of fish of lower quality as they develop further towards metamorphosis.

The mouth gape of giant kōkopu larvae at hatching is very similar to that of īnanga, (348 µm versus 343 µm respectively), despite īnanga being shorter in total length (Mitchell, 1989). Mouth gape is a major limiting factor in feeding for larval fish as it determines the maximum size and therefore the overall size range of food particles that can be consumed (Bremigan & Stein, 1994; Jones & Closs, 2016; Makrakis et al., 2008). However, from the observations in this study the mouth gape of giant kōkopu larvae increases at a greater rate than for īnanga whereby at the conclusion of the 77 DAH production period mouth gape is 1752 µm whereas for īnanga of an equivalent total length mouth gape is only 930 µm (Mitchell, 1989). A larger mouth gape is known to confer an ability to consume a wider size range of food particles in galaxiid fish (Bremigan & Stein, 1994; Jones & Closs, 2016) and is an important characteristic in the choice of giant kōkopu as the preferred whitebait species for aquaculture.

At first feeding (3 DAH) giant kōkopu larvae have a prey-gape ratio of 48.8 % when fed instar-I *Artemia*, this drops to 37.1 and 32.0 % at 7 and 14 DAH respectively. These values are at the lower end of the optimum theoretical prey size-gape ratio (Cunha & Planas, 1999; Fernández-Diaz et al., 1994; Hoestenberghe et al., 2015; Østergaard et al., 2005; Shirota, 1970). Therefore, it is possible that the relative lack of growth performance during the initial 14 DAH is due to larvae needing to capture more of the small live prey provided to reach satiation and in doing so, expending more energy foraging and reducing the resources available for growth (Cunha & Planas, 1999; Qin, 2008). Regardless, these baseline data on gape development can help to guide the feed selection throughout larval development in order to ensure larvae receive the optimum size food particle and therefore minimize wasted food and maximize productivity. Specifically, it is apparent that based on mouth gape measurements the

larval giant kōkopu could potentially consume a larger initial live feed particle. While there are a variety of methods for measuring mouth gape in relation to potential prey size, they all typically provide a generalized guide to the optimum size ratio of feed particle to gape. Further examination of the growth performance and survival outcomes of larvae fed live diets varying in size is critical for the specific species in culture to better understand their actual food capture and utilization capabilities as a basis for determining the optimum feed regime.

2.5 Conclusion

The measurement of the morphological characters and their development in larval giant kōkopu have resulted in the identification of priority areas for research attention that are aimed at improving efficiency of larviculture. The current initiation of first feeding at 3 DAH may be too late and consequently could have a detrimental impact on the growth and survival of larvae. The potential to improve larviculture through the earlier provision of live feeds sometime between hatch and 2 DAH needs to be tested as a priority. Giant kōkopu appear to be capable of consuming larger prey sizes than are presently delivered at first feeding suggesting that larger prey, such as instar-II *Artemia*, could be provided at first feed. The timing of weaning from live to artificial feed also needs further investigation as the current feeding regime shows little impact on growth performance when live food is eliminated. This indicates that larval giant kōkopu are well prepared for the transition, perhaps receiving expensive live feeds for longer than necessary, so that by making an earlier shift to artificial feed would result in reduced live feed costs. Finally, the current rearing protocol produces a smaller whitebait product than is common in the market from wild caught whitebait. A longer rearing period should be attempted to improve production volume whilst making closely monitoring the visual quality of the resulting whitebait flesh.

3 Chapter 3: Optimization of *Artemia* feed regimes for larval giant kōkopu (*Galaxias argenteus*)

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3.1 Introduction

Feed and feed preparation is often the largest single cost for fin fish aquaculture businesses, typically accounting for 30 – 70 % of the total cost of production (Webster & Lim, 2001). Furthermore, the relative lack of understanding of the specific nutritional requirements of larval fish (Hamre et al., 2013; Holt, 2011) means that research focusing on optimizing the use of feeds often provides the greatest improvements in cost efficiencies, especially for start-up aquaculture businesses (Trushenski et al., 2006).

Live feeds are one of the most expensive components of rearing larval fish due to the cost of supplying raw materials, extensive requirements for labour and capital equipment for live feed preparation, and the need to supply high quality water and production facilities (Southgate & Partridge, 1998). However, it is the use of live feeds, in particular *Artemia* spp. nauplii, which have unlocked the commercial aquaculture potential of many fin fish species (Dhert, 1996; Van Stappen, 1996). The high level of reliance on live feeds in fin fish larviculture is frequently due to the biological requirements for the initiation of exogenous feeding, which in the larvae of many fin fish species is triggered by prey movement (Qin, 2008). These first days of feeding are vital for achieving the optimum growth and survival of larvae under culture conditions and so the identification of the ideal first prey items and subsequent weaning to artificial inert diets is critical to commercial success in aquaculture production (Hoestenberghe et al., 2015). Therefore, the reliance on costly live feeds in commercial fin fish larviculture means that research to optimize production and minimize costs should be a priority.

Whitebait, a generic name for the larval form of galaxiid species, have been identified as a potential aquaculture species for their high market price, which is currently US$85 kg^{-1} (Taunton, 2020). However, as is the case with many fin fish aquaculture species, whitebait species have largely remained in the pilot stage of development due to challenges in larval production (Vega et al., 2013).

New Zealand Premium Whitebait Ltd (NZPWL), is a start-up company which has developed the fledgling technology to farm whitebait, specifically giant kōkopu, *Galaxias argenteus* on a commercial basis. As is the case for several other diadromous whitebait species, egg incubation was the former most significant step preventing scaling of whitebait production, attributed to the asynchronous spawning of broodstock and natural incubation

of eggs in terrestrial habitats (Benzie, 1968a; Mardones et al., 2008; Mitchell, 1989; O'Brien & Cooper, 2013; Vega et al., 2013). Using proprietary technologies and methods NZPWL has developed a scalable, cost and labour efficient incubation protocol which enables reliable mass production of high quality giant kōkopu larvae. Now, the critical challenge in larviculture of whitebait remains identifying nutritional requirements and suitable diets (P. Decker, personal communication, 2 July 2016; Mitchell, 1989; Vega et al., 2013).

Early efforts to raise larvae on the closely related īnanga (*Galaxias maculatus*), also known as "puye" in South America, were fed only *Artemia*, reaching 40 mm length in 160 days (Mitchell, 1989). More recent work on this species describes the feeding regime of the same species requiring 20 days feeding rotifers, before 20 days on a mix of rotifers and *Artemia*, followed by 140 days of artificial feed, reaching 40-60 mm (Mardones et al., 2008).

The current NZPWL larval rearing protocol used for commercial scale production of giant kōkopu larvae has been developed incrementally, largely through trial and error rather than any systematic experimental research. Under the NZPWL protocol, *Artemia* nauplii are used as live feed for approximately 29 days commencing two days after hatching (DAH), and being fed out three times a day at 0830, 1230 and 1630 h. For the first 14 days of feeding, only instar-I *Artemia* are provided, followed by a week in which the proportion of enriched instar-II *Artemia* and later stages (i.e., referred to as Artemia-II+) fed to the larvae increases from 0 to 100 %. Therefore, in the final week of live food provision, only instar-II+ *Artemia* are provided. For the remaining approximately 47 days only artificial feed is provided with larvae reaching a length of 32 mm and wet weight (WW) of 177.05 mg (McKay & Jeffs, 2021). This feeding protocol has been implemented by NZPWL because it has been shown to provide a slow and conservative transition in feed particle size and is based on NZPWL's experience with closely related species (īnanga), however, to date no directed experimentation on larval feeding has taken place for giant kōkopu. There are marked morphometric differences between the larvae of īnanga and giant kōkopu at comparable ages, most notably mouth gape width and total length (McKay & Jeffs, 2021), which would suggest their feeding abilities are also likely to differ. The high cost of providing feed for the production of giant kōkopu provides a strong incentive for research that may lead to reduction in feed costs.

Therefore, the purpose of this study is to better understand the growth and survival of larval giant kōkopu that are fed *Artemia* diets which differ by the proportions and timing of the transition from instar-I and instar-II+ *Artemia*. This information, together with knowledge of the relative costs of preparation of instar-II+ versus instar-I *Artemia*, will enable the opportunity to maximize fish production and minimize feed costs through optimizing the use of *Artemia* cysts. These data are useful for comparison with larval rearing protocols for many other fin fish species for which provisioning instar-I and instar-II+ *Artemia* is a significant cost of larval production.

3.2 Methods

3.2.1 Experimental Animals

Approximately 2.4 million giant kōkopu fertilized eggs from 160 female and 20 male captive broodstock were hatched on 9 September 2016 into commercial larval rearing tanks consisting of conical 2500 l tanks. Three days later 18,000 larval fish were randomly sampled from the commercial larval rearing tank and divided evenly into nine 20 l experimental tanks, i.e., around 2000 larvae per tank. This was achieved by estimating the total number of fish per litre in the transfer vessel by careful mixing and taking random 200 ml samples and then counting the number of fish in each sample to produce a mean estimate of the total number of fish.

"Instar-I" *Artemia* refer to the first naupliar stage, as emerged from the cyst. The term "instar-II+" is used out of convenience due to the continual development and moulting of *Artemia*-I to *Artemia*-II and subsequently advancing to later metanaupliar stages throughout the enrichment process.

3.2.2 Tank Design and Recirculation System

Nine identical experimental tanks were manufactured from round 20 l plastic (HDPE) pails, blue in colour, 270 mm in diameter and 380 mm high. Tanks were designed to hold 18 l of water by situating an outflow pipe 80 mm below the rim of the pail. The outflow pipe was fitted with a banjo filter using 600 µm filter mesh to prevent the escape of larvae while allowing the passage of suspended particles. Black PVC adhesive tape was used to line the wall of the tank from 25 mm below to 25 mm above water level to inhibit the climbing ability of the fish larvae.

All nine experimental tanks were operating on the same recirculation system. Outflow from each tank was directed to a filter basket through which seawater passed through a 5 µm filter mat into the sump, with the mat changed daily. The sump was filled to 300 l and operated as the biological filter containing 40 l of plastic Kaldnes-K3 media (Krüger Kaldnes AS, Norway). Each day 100 l of seawater was removed from the sump and replaced with natural seawater of 35 ppt filtered to 5 µm and UV sterilized. Manual skimming of protein from the sump was undertaken daily or as necessary.

Seawater was pumped from the sump via a UV filter and entered each tank via 4 mm tubing delivering water to both the surface and the bottom of the tank. For the first seven days of the experiment water flow rate was 0.28 l min^{-1} to each tank, with water entering perpendicular to the tank wall. From day seven to day 14 the flow was angled to be parallel to the tank wall to produce circular flow in the tank. From day 14 onwards flow rate was increased to 0.37 l min^{-1} with flow remaining parallel to tank wall.

Tanks were aerated by an air-stone at the bottom of the tank producing two medium sized (0.5 mm diameter) bubbles per second.

The tanks were illuminated by three 58 W fluorescent tubes, suspended 100 cm above the top edge of the tanks. Lights were operated between the 0745 and 1800 h with shade cloth being used to dim the light intensity for 30 min after switching on and before turning off.

Seawater temperature was not controlled but was measured every 6 h during the experimental period and found to vary between 14 and 18 °C over the course of the experiment but was consistent among the nine experimental tanks.

Nitrate, nitrite, ammonia, carbonate hardness and pH were measured every second day using API© test kits and acceptable levels were maintained throughout the experiment, while temperature was measured with a glass thermometer (Aqua One).

3.2.3 Experimental Design

Three experimental live food treatments for feeding larval giant kōkopu were tested over a period of 28 days starting on the first day of feeding (3 DAH). Each experimental tank received 3 g of feed three times a day at 0830, 1230 and 1630 h throughout the experimental period (i.e., 3 – 31 DAH). Each feed treatment was conducted in three randomly selected replicate tanks.

The first treatment, "NZPWL", conformed to the existing commercial feeding regime in terms of the timing and transition of instar-I to instar-II+ *Artemia* as used for the commercial production of larval giant kōkopu by NZPWL. For the initial 14 days of feeding, fish were fed only instar-I *Artemia* followed by a transitional phase with two days of a 2:1 ratio by wet weight of instar-I to instar-II+ *Artemia* at each feed, followed by two days of 1:1 ratio, and finally two days 1:2 ratio. For the remainder of the experimental period (8 days) the larvae were fed only instar-II+ *Artemia* (Table 3-1).

The two other experimental feeding treatments were "Instar-II+" and "5050." For the Instar-II+ treatment, only instar-II+ *Artemia* were provided to the fish in this treatment for the 28 day duration of the experiment. This treatment was selected as earlier work (McKay & Jeffs, 2021) identified the potential for larger feed items to be consumed as first feeds. These later stage *Artemia* moults enable the testing of this theory as they are larger than instar-I and importantly are readily produced in commercial scale culture. For the 5050 treatment the larvae were fed with a 1:1 ratio of instar-I to instar-II+ *Artemia* for the first 7 days by wet weight, and for the remaining 21 days the fish were provided with only instar-II+ *Artemia* (Table 3-1). The 50:50 treatment acts as a middle ground between the other two treatments where the average size of feed particle is concerned.

Artemia cysts used throughout this experiment *A. franciscana* from GSL Sep-Art (INVE Aquaculture Inc, USA) from the same batch. Feeds were administered by total wet weight, by pouring harvested *Artemia* through a 100 µm sieve and placing the sieve on a towel to drip dry for 1 min before measuring with tared electronic scales to the nearest 0.1 g. This method was used to standardise feed volumes and had no impact on the performance or

quality of *Artemia.* The number of instar-I *Artemia* per gram were in the order of 110,000 g^{-1}, while for instar-II+ there were 85,000 g^{-1}. At the initiation of live feeding, in NZPW Lt's commercial larviculture tanks, feed particle density is 8800 l^{-1}. By the end of live feeding (30 DAH), in commercial scale tanks, particle density nears 15,300 l^{-1}.

Table 3-1. Feed particle density in each experimental tank by treatment throughout the experimental period given as the number of live fed particles per litre (l^{-1}). Three grams of live feeds were added to each tank containing 2000 larvae for each feeding event in the respective ratios of instar-I and instar-II+ *Artemia*. The range presented for the NZPWL treatment at 17-23 DAH reflects the change in feed particle density from the start to the finish of the week as the ratio of instar-I to instar-II+ *Artemia* changed during this period.

Treatment	3-9 DAH	10-16 DAH	17-23 DAH	24-30 DAH
Instar-II+	14200	14200	14200	14200
5050	16300	14200	14200	14200
NZPWL	18300	18300	16900-14200	14200

Upon hatching of giant kōkopu eggs, the commercial tanks at NZPWL are stocked with fish larvae at densities in the region of 500 l^{-1}, and the number of feed particles per larva is estimated to begin at 18 per feeding event. At the conclusion of live feeding, this increases to at least 32 food particles per larva per feeding event. In contrast to commercial conditions, the experimental tanks were stocked at 111 larvae l^{-1} as a result of the smaller diameter of the experimental tanks. Due to the five-fold difference in larval fish stocking densities between commercial rearing and experimental tanks, feed particles per larva are greater for the duration of this experiment than would be used under typical commercial production conditions. This is a function of the practical limitations of conducting small scale experiments on fish larvae, as a result of an inability to run replicated commercial scale experiments owing to the logistic and infrastructure constraints of running such large-scale experimental studies.

Instar-I *Artemia* production involved a 17 h incubation of cysts in 250 l *Artemia* cones at 29 °C at 35 ppt with constant, vigorous bubbling while exposed to light. Live *Artemia* were then separated from cysts via magnet and rinsed in a 100 µm sieve with clean 35 ppt salt water. Instar-I *Artemia* that were fed to larvae at 0830 h had been cold stored at a density of 1.67 g ml^{-1} at 4 °C for 16 h after their harvest late the previous day. Instar-I *Artemia* fed to giant kōkopu larvae at 1230 h were cold stored for 2 h, while those used for the 1630 h feed had undergone 6 h of cold storage.

To produce enriched instar-II+ *Artemia* for all experimental treatments, the cysts were incubated for 27 h at 29 °C at 35 ppt with constant, vigorous aeration while exposed to a 58 W tube light 100 cm above the water surface.

Live animals were then separated from cysts via magnet and rinsed in a 100 µm sieve with 35 ppt clean seawater prior to the 21 – 29 h enrichment period. The enrichment was specially formulated by NZPWL using a proprietary combination of commercially available concentrated instant algae products (Rotigrow Plus, Nanno 3600, *Tetraselmis* 3600, Reed Mariculture Inc., USA). An aliquot of 60 ml of enrichment formula was provided upon the initial transfer of instar-II+ *Artemia* to the enrichment tank, with another 60 ml added 18 h later. Instar-II+ *Artemia* fed to larvae at 0830 h had been enriched for 21 h, while those fed out at 1230 h were enriched for 25 h, and those fed at 1630 h for a period of 29 h.

3.2.4 Sampling of Larvae

Experimental giant kōkopu larvae were sampled twice; on the first day (Day 0, age 3 DAH) and the last day (Day 28, 31 DAH) of the experiment. The first sampling event was taken from the temporary transfer vessel used to move fish from the commercial larval rearing tank to the experimental tanks. Three samples containing 50 fish were taken at random for subsequent determination of mean wet weight and mean dry weight (DW) which was measured by freeze drying, re-weighing and dividing by the total number of fish. The total length (i.e., snout to tip of tail) and body depth (i.e., centre of body at the anus across to the lateral surface) were measured in a further 50 randomly sampled fish. The length:depth ratio (LDR) was calculated by dividing the total length of each fish by its depth. Total length and depth of larvae were measured by taking a digital image of each fish placed on a 460 µm grid using an Olympus TG-4 camera and processing images with the computer software ImageJ64 to derive the measures.

At the final sampling event three samples of 20 fish each were taken at random from each of the replicate experimental tanks. Also, for each of the nine tanks the total length and depth of 20 fish were measured in the same manner as described previously.

Mortality of larvae was estimated at 2 and 3 day intervals by carefully siphoning the floor of each tank and the number of dead fish that were removed were counted. Mortality in the first seven days was too great to count and appeared to be consistent among tanks and was most likely related to the stress of handling the larvae for the transfer to the small tanks. As a consequence, the first count of mortality occurred at 9 DAH. Mortality in the first seven days of the experiment was estimated by back calculation using the knowledge of how many fish were alive at the end of the experiment, how many were in each tank at the start, and how many died at each siphoning event throughout the experiment. Cumulative mortality, calculated as a percentage of the initial tank population, was determined for each tank by adding the number of dead fish at each siphoning event. Mean cumulative mortality for each treatment was calculated from the three replicate tanks within each treatment.

Total production was calculated by multiplying the total number of fish alive by the mean wet weight of fish for each tank at the end of the experiment. Fish mean wet weight was determined for each tank by taking the average of three samples of randomly selected 10 fish that were weighed to the nearest 0.001 g.

3.2.5 Sampling of *Artemia*

Triplicate samples of 10 g wet weight *Artemia* were taken at time points 0 h, (i.e., at harvest after the 17 h incubation) then after 2, 6 and 16 h cold storage for instar-I and sampling was repeated for a total of three batches of instar-I production/feed. The same sampling program was also used for instar-II+ *Artemia,* however, 0 h sample was taken after 27 h incubation, then 21, 25 and 29 h enrichment.

The lipid proportion of the *Artemia* samples was determined with a modified Bligh and Dyer (1959) solvent extraction methodology as described in Wang et al. (2015). In brief, after lyophilizing the samples of larvae, the lipids were extracted from the tissues into a mix of chloroform and methanol solvents, and the lipid recovered by fractionating with the addition of deionized water. The lipid fraction was recovered, and solvent removed through evaporation and the remaining lipid was weighed and lipid mass which was then divided by the total dry weight of the *Artemia* sample and multiplied by 100 and presented as (%DW).

A modified bicinchoninic acid (BCA) assay (Micro BCA™ Protein Assay Kit, ThermoFisher Scientific) was used to determine the protein proportion of the *Artemia* sample as described by Smith et al. (1985). In brief, the *Artemia* samples were hydrolysed with heated sodium hydroxide and then cuprous cations generated from the reduction of Cu^{2+} to Cu^{1+} by protein were measured via a colorimetric reaction with BCA against a set of bovine serum albumin standards. Protein content was then calculated as a percentage of dry weight (%DW).

Fatty acid (FA) analyses were undertaken on the previously extracted lipid using a derivatization and preparation process based on Lepage & Roy (1986) and modified as described in Spreitzenbarth & Jeffs (2022). In brief, The derivatized samples were analysed using an Agilent 7890B gas chromatograph coupled to a 5977C mass spectrometer (GC-MS) with a split/splitless inlet (Jeffs et al., 2002) With instrument parameters based on Kramer et al. (2008). The measured FA was calculated as a proportion of *Artemia* dry weight.

3.2.6 Statistical Analyses

The final mean weight, total length and depth of larvae, as well as total tank production were compared among treatments using one-way ANOVA. Normality and equality of variances were confirmed using the Shapiro-Wilk's and Levene's tests before analyses took place. For each of these response variables a linear mixed model was fitted to control for the random effects of the tanks. When overall experimental treatment effects were identified by ANOVA, pairwise Tukey's comparisons of treatment means, with adjustment for false discovery, were made to identify differences between the individual treatments. The same methods were used to compare differences in lipid and protein proportion as % DW, however, these response variables were first arc-sine transformed to correct for any data distribution bias associated with percentage data (Zar, 1999). The difference between instar-I and instar-II+ *Artemia* fatty acid profiles was done using the non-parametric Kruskal-Wallis test due to the difference in number of samples in each group and non-normality. Estimated mean difference and 95 % confidence intervals were calculated and are presented.

The response variable, mortality, is a binary measure so a generalized mixed effects model with binomial distribution (logistic regression) was used to compare mortality among treatments over the course of the experiment, whilst also controlling for the random effects of the tanks. Where ANOVA identified effects of experimental differences at age-time points a Z-score test was implemented to establish whether the difference was significant.

All statistical analyses were performed using R (RStudio, ver. 1.2.1335). All measures of variability of means are reported as standard error of the mean.

3.3 Results

3.3.1 Weight

At the start of the experiment the mean WW for the larvae that were distributed at random among all the treatment tanks was 2.30 ± 0.02 mg.

Mean WW was significantly different among treatment groups at the conclusion of the experiment ($F_{(2,6)}$ = 26.49, P < 0.01). The 5050 treatment mean WW was 10.48 ± 0.35 mg, which was between 1.32 and 1.62 times greater than the NZPWL mean WW of 7.19 ± 0.26 mg (P< 0.01) (Figure 3-1). The mean WW in the 5050 treatment was also between 1.16 and 1.42 times greater than for the Instar-II+ treatment mean WW of 8.18 ± 0.17 mg (P < 0.01) (Figure 3-1). Mean WW for the Instar-II+ treatment was also between 1.03 and 1.27 times greater than in the NZPWL treatment (P < 0.05).

The mean DW of fish at the start of the experiment was 0.380 ± 0.001 mg. However, the mean DW of larval giant kōkopu by the end of the experiment was different among treatments ($F_{(2,6)}$ = 10.67, P = 0.01). The mean DW of fish in the 5050 treatment was 2.00 ± 0.15 mg, which was between 1.24 and 1.77 times larger than NZPWL treatment with a mean DW of 1.35 ± 0.09 mg (P = 0.01) (Figure 3-1). The mean DW of the larvae in the 5050 treatment at the end of the experiment was also 1.13 to 1.61 times larger than Instar-II+ treatment mean DW of 1.47 ± 0.04 mg (P = 0.02) (Figure 3-1). The mean DW of the larvae in the Instar-II+ and NZPWL treatments were not significantly different (P = 0.31).

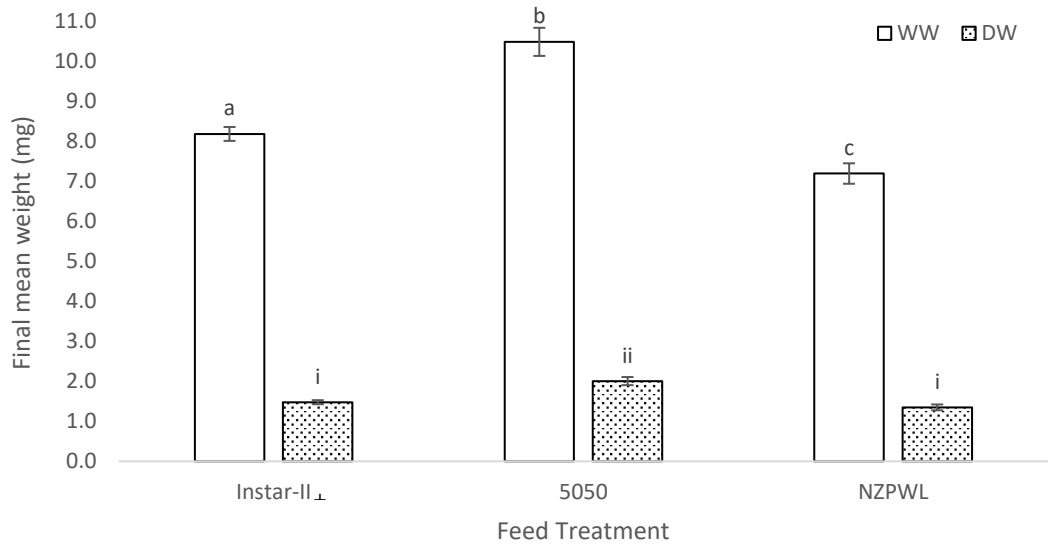

Figure 3-1. Final mean wet weight (WW) and mean dry weight (DW) for larval giant kōkopu from three different feed treatments; Instar-II+, 5050 and NZPWL at the end of a 28 day experimental period (mean ±SE). Means with different superscripts are significantly different for WW or DW($P<0.05$).

3.3.2 Length and Depth

The mean total length of fish at the start of the experiment was 10.20 ± 0.10 mm. However, the mean total length of giant kōkopu larvae at the end of the experiment differed significantly among the treatment groups ($F_{(2,6)}$ = 21.44, $P < 0.01$). The mean total length of fish in the 5050 treatment was 15.55 ± 0.25 mm, which was between 0.16 and 0.30 mm longer than those in the NZPWL treatment with a mean total length of 13.28 ± 0.26 mm ($P < 0.01$) (Figure 3-2). The mean final total length of the larvae in the 5050 treatment was between 0.08 and 0.22 mm longer than the Instar-II+ treatment with a mean of 14.03 ± 0.25 mm ($P < 0.01$) (Figure 3-2). There was no difference between the mean total length of the larvae in the Instar-II+ and NZPWL treatments ($P = 0.08$).

There was no significant difference in mean LDR ($F_{(2,6)}$ = 0.277, P= 0.77), Instar-II+ 14.46 ± 0.32, 5050 14.59 ± 0.38 and NZPWL 14.87 ± 0.49 (Figure 3-2).

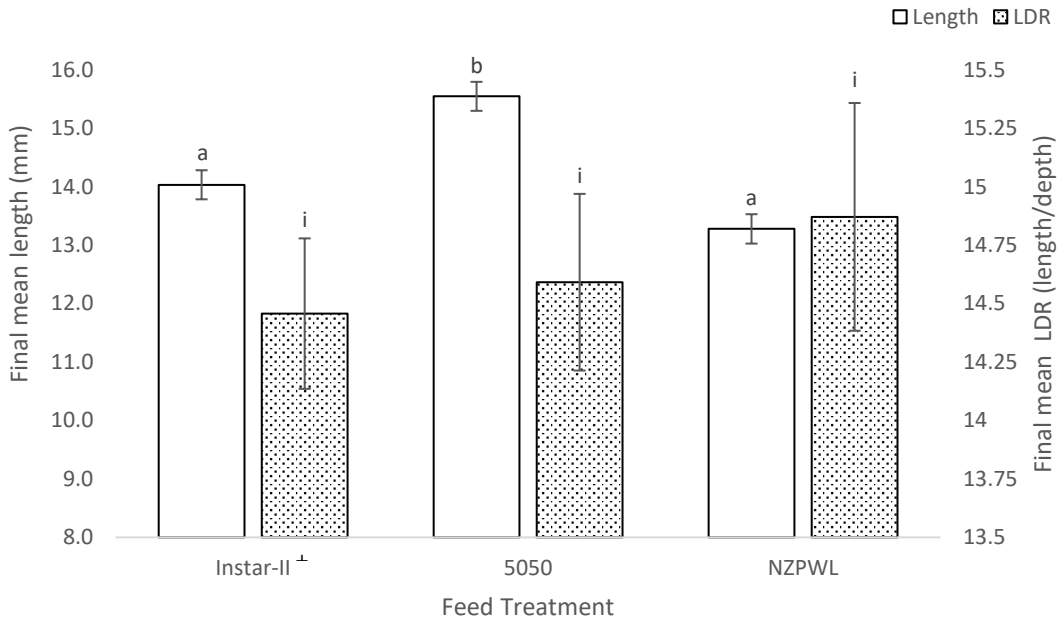

Figure 3-2. Final mean total length (primary axis) and mean length:depth ratio (secondary axis) of larval giant kōkopu for three different feed treatments; Instar-II+, 5050 and NZPWL at the end of the 28 day experimental period (mean ±SE). Means with different superscripts are significantly different among either final mean total length or mean length:depth ratio (P <0.05).

3.3.3 Mortality

Initial mortality was very high for all treatment groups in the first four days of the experiment. Mortality rates then slowed in all groups for the next seven day period and plateaued with little additional mortality from age 14 DAH until the completion of the experiment at age 31 DAH. Significant differences in cumulative mortality were found among the treatments at several different times during this experiment (χ^2 = 426.0, P < 0.001). The mortality at 7 DAH, did not differ significantly among treatment groups, i.e., Instar-II+ - 63.3 ± 2.13 %, 5050 - 66.65 ± 2.72 % and NZPWL - 67.72 ± 4.30 % (P > 0.05).

At 14 DAH the mean cumulative mortality of larvae in the NZPWL treatment (i.e., 90.3 ± 2.2 % SE) was greater than for the Instar-II+ treatment (i.e., 81.9 ± 1.3 %) (P < 0.01) and the 5050 treatment, which had a mean mortality of 83.2 ± 3.0 % (P < 0.01) (Figure 3-3).

The mean cumulative mortality of the NZPWL treatment remained significantly higher than both of the other treatment groups for the remainder of the experiment (Figure 3-3). At no point in the experiment did the cumulative mean mortalities of the 5050 and Instar-II+ treatments differ.

At the conclusion of this experiment the mean cumulative mortality of the larvae in NZPWL (i.e., 95.1 ± 1.6 %) was greater than that of Instar-II+ by between 1.7 and 4.1 % (P <0.01) with a cumulative mean mortality of 88.2 ± 1.5

% (Figure 3-3). The NZPWL cumulative mortality was also greater than that of the 5050 treatment which had a mean mortality of 90.2 ± 2.4 % which was between 1.4 and 3.3 % lower than for the NZPWL treatment (P < 0.01).

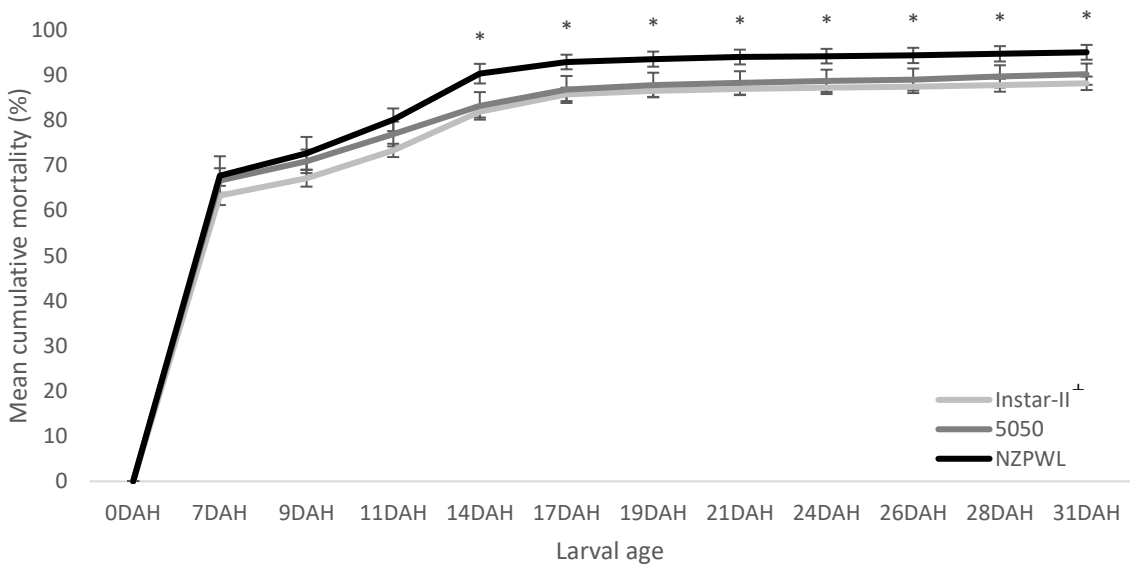

Figure 3-3. Mean cumulative mortality of larval giant kōkopu in three different feed treatments; Instar-II+, 5050 and NZPWL over the 28 day experimental period (mean ± SE). Sets of three treatment means marked with asterisk are significantly different for that DAH sampling event (P < 0.05).

3.3.4 Artemia Lipid and Protein Composition

Lipid proportion was very consistent among instar-I samples, showing no significant difference even after 16 h (in-1:16h-f) cold storage when compared to immediately post-harvest (in-1:0h) (t= -0.24, P = 0.91) (Figure 3-4). It is also notable that there was no difference in total lipid proportion between instar-I and instar-II+ immediately after harvest (in-1:0h and in-2:0h) (t = -0.02, P = 0.99) (Figure 3-4). However, all of the enriched instar-II+ *Artemia* treatments (in-2:21h-e, in-2:25h-e and in-2:29h-e) had significantly lower lipid relative to their dry weight compared with those immediately after harvest (in-2:0h) (t = 5.72, P < 0.01; t = 6.90, P < 0.01; t = 6.91, P < 0.01, respectively) (Figure 3-4).

A similar trend was present in the protein proportion in *Artemia* where there was no difference among any of the instar-I samples from the time of their harvest and subsequent cold storage (i.e., in-1:0h and in-1:2h t = 0.21, P = 0.87; in-1:0h and in-1:6h t = 0.53, P = 0.73; or in-1:0h and in-1:16h t =-2.10, P = 0.11) (Figure 3-4). All instar-II+ *Artemia* all had lower protein proportion than instar-I after 16 h cold store, (in-2:0h t = 3.39, P = 0.02; in-2:21h-e t = 4.47, P = 0.01; in-2:25h-e t = 4.19, P = 0.01; in-2:29h-e t = 4.08, P = 0.01) while 21 h enriched instar-II+ had a

lower protein proportion than freshly hatched instar-I (t = 1.39, P = 0.03). There were no other differences in protein composition among instar-I and instar-II+ *Artemia*.

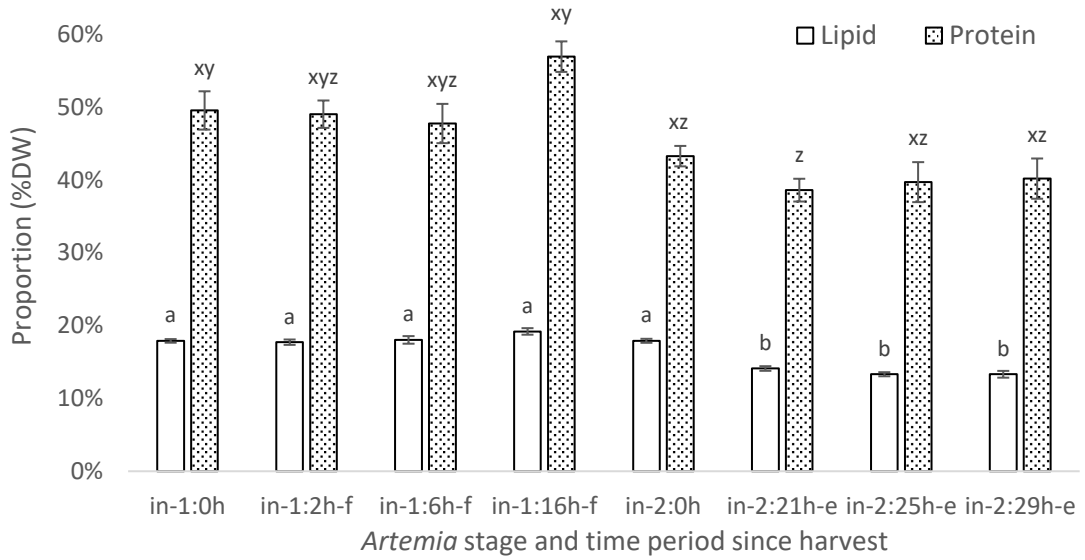

Figure 3-4. Mean lipid (no fill) and protein (shaded) proportions of *Artemia* as % dry weight. Annotations: in-1 refers to instar-I *Artemia* and 0 h denotes immediately after harvest; 2, 6 and 16 h-f denote 2, 6 and 16 h in cold store, respectively. In-2 refers to instar-II+ *Artemia* and 0 h immediate sampling on harvest; 21, 25 and 29h-e signify 21, 25 and 29 h enrichment, respectively. Means with different superscripts are significantly different among means for lipid and protein (P < 0.05).

3.3.5 *Artemia* Fatty Acid Profile

The fatty acid profiles of instar-I and instar-II+ *Artemia* were markedly different with instar II+ Artemia containing higher proportional amounts of saturated fatty acids on a dry weight basis (Table 3-2). For example, Instar-II+ were higher than instar-I in C18:0 2.162 ± 0.043 % versus 1.853 ± 0.026 % ($\chi\chi^2$ = 29.82, df = 1, P < 0.01), C20:0 0.049 ± 0.001 % versus 0.049 ± 0.001 % (χ^2 = 30.21, df = 1, P < 0.01) and C22:0 0.095 ± 0.003 % versus 0.051 ± 0.001 % (χ^2 = 49.60, df = 1, P < 0.01).

No differences were found between instar-I and Instar-II+ *Artemia* for C18:1n-9t (χ^2 = 2.34, df = 1, P = 0.13) and C20:4n-6c (ARA) (χ^2 = 0.15, df = 1, P = 0.70), however, Instar-II+ had higher C18:1n-7c 0.935 ± 0.017 % versus 0.671 ± 0.010 % (χ^2 = 48.95, df = 1, P < 0.01).

All other fatty acids were in higher concentration in instar-I than instar-II+ *Artemia*. For example, C20:5n-3 0.245 ± 0.005 % versus 0.205 ± 0.014 % (χ^2 = 4.76, df = 1, P < 0.05). Total PUFA and total HUFA were both higher in instar-I *Artemia* comprising PUFA 6.841 ± 0.079 % versus 4.164 ± 0.206 % (χ^2 = 48.30, df =

1, P < 0.01), and HUFA 0.518 ± 0.011 % versus 0.401 ± 0.024 % (χ^2 = 17.10, df = 1, P < 0.01). DHA was not detected at any level in either form of *Artemia.*

Table 3-2. Mean percent fatty acid composition (±SE) of total dry weight of instar-I and instar-II+ Artemia. Superscript characters indicate significant differences along the row. ARA: arachidonic acid; EPA: eicosapentaenoic acid; PUFA: polyunsaturated FA; HUFA: highly unsaturated FA; ND: not detected.

Fatty Acid		**Instar-I**	**Instar-II+**
C14:0		0.286 ± 0.006 [a]	0.146 ± 0.004 [b]
C15:0		0.420 ± 0.009 [a]	0.210 ± 0.007 [b]
C16:0		4.529 ± 0.062 [a]	3.333 ± 0.072 [b]
C16:1n-7c		0.417 ± 0.006 [a]	0.272 ± 0.007 [b]
C16:1n-7t		0.099 ± 0.002 [a]	0.054 ± 0.002 [b]
C17:0		0.186 ± 0.003 [a]	0.163 ± 0.003 [b]
C17:1n-7c		0.106 ± 0.002 [a]	0.063 ± 0.002 [b]
C18:0		1.853 ± 0.026 [b]	2.162 ± 0.043 [a]
C18:1n-7c		0.671 ± 0.010 [b]	0.935 ± 0.017 [a]
C18:1n-9c		2.461 ± 0.032 [a]	2.392 ± 0.048 [b]
C18:1n-9t		0.030 ± 0.001 [a]	0.029 ± 0.001 [a]
C18:2n-6c		0.892 ± 0.011 [a]	0.518 ± 0.020 [b]
C18:2n-6t		0.080 ± 0.001 [a]	0.038 ± 0.001 [b]
C18:3n-3c		5.207 ± 0.060 [a]	3.125 ± 0.160 [b]
C18:3n-6c		0.102 ± 0.002 [a]	0.044 ± 0.002 [b]
C20:0		0.049 ± 0.001 [b]	0.049 ± 0.001 [a]
C20:1n-9c		0.082 ± 0.002 [a]	0.074 ± 0.003 [b]
C20:2n-6c		0.042 ± 0.001 [a]	0.039 ± 0.001 [b]
C20:3n-3c		0.180 ± 0.004 [a]	0.106 ± 0.006 [b]
C20:3n-6c		0.014 ± 0.000 [a]	0.011 ± 0.000 [b]
C20:4n-6c	ARA	0.080 ± 0.002 [a]	0.079 ± 0.004 [a]
C20:5n-3	EPA	0.245 ± 0.005 [a]	0.205 ± 0.014 [b]
C22:0		0.051 ± 0.001 [b]	0.095 ± 0.003 [a]
C22:6n-3	DHA	ND	ND
Total PUFA		6.841 ± 0.079 [a]	4.164 ± 0.206 [b]
Total HUFA		0.518 ± 0.011 [a]	0.401 ± 0.024 [b]

3.3.6 Total Production

The mean total production of larvae by weight differed among treatments ($F_{(2,6)}$ = 7.21, P = 0.03). The mean total production of larvae in the 5050 treatment was 1.82 ± 0.31 g and was between 0.52 and 1.94 g greater than the NZPWL treatment with a mean of 0.59 ± 0.23 g (P = 0.03) (Figure 3-5). The Instar-II+ treatment mean total production 1.74 ± 0.22 g was between 0.43 and 1.85 g greater than that of the NZWPL treatment (P = 0.03) (Figure 3-5). There was no difference in the mean total production for the Instar-II+ and 5050 treatments (P = 0.82).

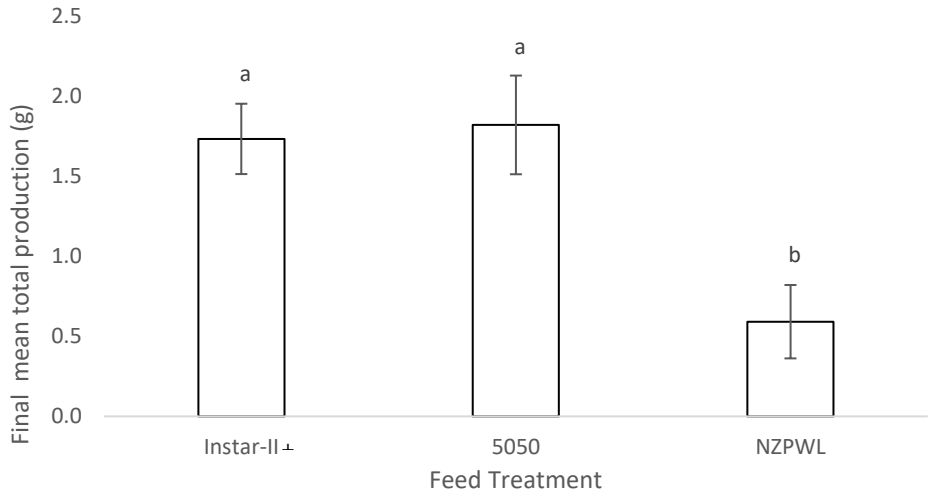

Figure 3-5. Mean total production of larval giant kōkopu resulting from three different feed treatments; Instar-II+, 5050 and NZPWL over the 28 day experimental period (mean ± SE). Means with different superscripts are significantly different (P < 0.05).

3.4 Discussion

The performance of cultured larval giant kōkopu was influenced by three different *Artemia* live feed treatments tested in this research. The 5050 treatment achieved the greatest final mean WW, DW and total length of larvae when compared with the NZPWL and the Instar-II+ treatments. The final mean WW of the Instar-II+ treatment was greater than that of the NZPWL treatment, however, there were no other significant differences between these two treatment groups for the remaining performance measures.

Mortality was extensive in the first seven days of this experiment and consistent across all three treatments. This could be attributed to the physical and physiological stress involved in the transfer of the fish from commercial to experimental tanks. However, the subsequent cumulative mortality was not consistent among treatments from day 14 when larval mortality in the NZPWL treatment first became higher than for the other two treatments. This difference increased further over the remainder of the experimental period. In contrast, there was no difference in cumulative mortality between the Instar-II+ and 5050 treatments at any time during the experiment.

Growth and survival in fish larvae rely to a large extent on the ability to capture and digest food with an appropriate nutritional composition. The nutritional value of the two stages of *Artemia* nauplii are not equivalent with instar-I being richer in lipid with a higher proportion of unsaturated fatty acids and marginally higher in protein than instar-II+. Interestingly, the feed treatments containing lower proportions of these two vital energetic and growth nutrients (i.e., Instar-II+ and 5050) achieved greater growth performance and survival than the NZPWL treatment, which received only instar-I for the first two weeks. The lower EPA, PUFA and HUFA composition of instar-II+ *Artemia* compared to instar-I used in these feed treatments did not appear to constrain growth in these feed treatments despite higher dietary supply of these essential fatty acids generally being considered to be critical for greater growth performance and survival in larval fish (Bell et al., 1995; Bengtson et al., 1991; Boglino et al., 2012; Izquierdo & Koven, 2011; Izquierdo, 1996; Koven et al., 2012, 2018; Sargent et al., 2003) that is not the case in the present study. This suggests that despite the lower overall EPA, PUFA and HUFA supplied by the instar-II+ *Artemia*, it is sufficient to meet the demands for somatic growth of the larvae. The closely related *Galaxias maculatus* has shown that when cultured under high salinity conditions, higher levels of EPA and PUFA are not critical while DHA is important in larval development of eye and neural networks, which can lead to detrimental impacts on feeding ability, growth performance and survival (Dantagnan et al., 2010, 2013; Izquierdo & Koven, 2011; Koven et al., 2018; Mourente et al., 1991; Mourente & Tocher, 1992; Navarro et al., 1997). In the present study, the DHA fatty acid profile was absent in both *Artemia* feeds. This is unsurprising for instar-I as they are naturally deficient in DHA (Koven et al., 2012; Peykaran Mana et al., 2014), however, the key role of enrichment of instar-II+ *Artemia* is to provide these essential fatty acids (Navarro et al., 1999; Viciano et al., 2015). This is a direct result of using enrichment products that are very low in DHA and the delay between enrichment and feeding out. This low DHA content is likely to have had a detrimental impact on early larval performance, however, given the lack of DHA in all treatments, the nutritional profile of feed items cannot be the sole determinant of the greater success seen in the treatments with earlier, and higher provisions of instar-II+ *Artemia.*

Provision of live prey of suitable size, type, and stocked at sufficient density to make them available to larvae for consumption is paramount to achieving optimum productivity (van der Meeren & Næss, 1993). Despite the importance of first feeding and subsequent weaning from live feed for larval fin fish growth and survival (Hoestenberghe et al., 2015), few studies have directly compared the impacts of timing and proportion of instar-I and instar-II+ *Artemia* on the growth performance and survival of cultured larvae.

A primary limitation on the size of prey that can be consumed by larval fin fish is the size of the mouth opening or mouth gape (Arts & Evans, 1987; Bremigan & Stein, 1994; Cunha & Planas, 1999; Krebs & Turingan, 2003; Makrakis et al., 2008; Zaret, 1980). Consequently, it is vital that feed items meet the species-specific capture capabilities of larval fish in culture. This is of particular importance at the transitional stage from endogenous to exogenous resource utilization when larval fish are highly vulnerable (Black & Pickering, 1998). Any delay in the availability of suitable food items can have immediate and ongoing effects on growth performance and survival for larval fish.

Larval giant kōkopu have a mouth gape of 348 ± 15 µm upon hatching and by 7 DAH gape has increased to 538 ± 20 µm (McKay & Jeffs, 2021). The provision of food for larval giant kōkopu in this current experiment began at 3 DAH, which is consistent with the commercial rearing protocol of NZPWL. The ratio of prey size:mouth gape has been a key determining factor in food item choice in aquaculture with 25-60 % considered to be optimum (Bremigan & Stein, 1994; Fernández-Diaz et al., 1994; Østergaard et al., 2005; Shirota, 1970). The prey size:mouth gape ratio for giant kōkopu larvae at the initiation of exogenous feeding (at 3 DAH) if provisioned with instar-I *Artemia* (minimum width 195 µm) is 48 %, and 70 % for instar-II+ (width minimum 270 µm) (Hoestenberghe et al., 2015; McKay & Jeffs, 2021). At this stage of development of the larval giant kōkopu their mouth gape could be expected to limit capture success of the larger instar-II+ *Artemia* (520 µm length) in comparison to instar-I *Artemia* (450 length). However, the Instar-II+ group still outperformed the NZPWL group for several measured variables, most importantly the total biomass production. This would suggest any potential size limitation has been overcome to some degree by other characteristics of the instar-II+ *Artemia* nauplii.

Feed particle density plays a vital role in the initiation of exogenous feeding in larviculture with foraging success shown to increase with increasing feed particle density until an asymptote is reached when feeding in excess (Houde & Schekter, 1980; Puvanendran & Brown, 1999; Temple et al., 2004; Wyatt, 1972). Low feed densities result in lower growth performance and survival due to increased energy partitioning to foraging effort (Kiørboe & Munk, 1986; Munk & Kiørboe, 1985; Puvanendran & Brown, 1999). Unfortunately, as a new species being developed for aquaculture there is no existing information on larval feed density requirements for giant kōkopu.

The NZPWL treatment received greater feed particle density over the first 14 days of this experiment. However, it was the worst performing treatment in terms of growth performance and achieved the greatest mortality. This suggests that feed particle density in this treatment could have exceeded the requirements of larval giant kōkopu. Reduced growth performance and survival can occur despite increased encounters between predator and prey when the predator's assimilation capacity is exceeded (Werner & Blaxter, 1980). For example, the growth performance of Atlantic cod (*Gadus morhua*) larvae did not increase with a corresponding increase in prey density from 4000 l^{-1} to 16,000 l^{-1} (Puvanendran & Brown 1999), a response that is thought to be due to larval fish continuing to consume prey despite having a full gut, resulting from a lack of satiety feedback (Rønnestad et al., 2013). As a consequence of this continued larval feeding, the gut throughput increases, reducing residence times and decreasing the efficiency of digestion. Ultimately this results in an overall reduction in the net energy budget for the larvae. Additionally, high densities of live food items frequently cause reduced growth and survival through degrading water quality (Houde, 1975; Puvanendran & Brown, 1999). This may also help to explain the increased mortality observed in the NZPWL treatment from 14 DAH.

Successful diets are not only dependent on the ability of cultured species to capture the feed item or the nutritional quality but also the digestive capabilities of the larval fish which is restricted by their simple gut with little physical processing capacity combined with the relative lack of the quantity, activity and diversity of digestive enzymes (Cara et al., 2003; Chen et al., 2006; Dabrowski, 1984; Schwartz, 2008). This restricts the ability of larval

fish to hydrolyse the digesta to breakdown fractions which can then be more effectively absorbed and assimilated. Likewise, an inability to physically breakdown ingested food, such as the tough exoskeleton of crustaceans, can severely inhibit access to the nutrient rich tissues contained within (Camus, 2012; Léger et al., 1987; Luizi et al., 1999; Schipp, Bosmans, & Marshall, 1999). Instar-II+ *Artemia* are more readily digestible than instar-I as their mouth is open, providing a second point of entry for the digestive enzymes of fish larvae (Léger et al., 1987; Sorgeloos et al., 2001; Van Stappen, 1996). Furthermore, instar-II+ have greater diversity of endogenous digestive enzymes which once released in the gut of the fish can also help to break down the digesta (Abatzopoulos et al., 2002; Dabrowski & Glogowski, 1977; Kolkovski et al., 1993; Lavens & Sorgeloos, 1996; Léger et al., 1987; Naz, 2008). This increased digestibility of instar-II+ *Artemia* may have played an important role in the improved growth performance of giant kōkopu larvae by improving their ability to hydrolyse what was a slightly lesser amount of available protein than fish which received a higher proportion of instar-I *Artemia.*

The current study suggests instar-I *Artemia,* could be substituted with instar-II+ for greater effect in the larviculture of giant kōkopu, a substitution that may be useful for larviculture of other species. For example, the Senegalese sole, *Solea senegalensis*, showed no negative consequences when the instar-I *Artemia* was replaced with instar-II+ in larval rearing (Ferreira de Sá, 2016). However, the larvae of the mandarin fish, *Synchiropus splendidus*, achieved greater survival when given instar-II+ *Artemia* at 25 DAH than 28 DAH, however, at 22 DAH survival was reduced, suggesting any substitution between the two *Artemia* stages may also be highly sensitive to the state of larval development (Shao, 2016). Earlier provision of, and increasing the proportion of instar-II+ *Artemia*, in the diet of larval giant kōkopu has the potential to markedly improve the commercial production of this species. Comparison of the total productivity of each of the treatments by final biomass demonstrates the advantage gained by the Instar-II+ and 5050 feed regimes. The 5050 treatment produced between 88 and 328 % more larval giant kōkopu biomass than the NZPWL treatment, while the Instar-II+ treatment produced between 73 and 314 % more, which would translate to a significant increase in productivity at the commercial scale. There is potential for further significant gains through the development of a more suitable enrichment formula and protocol for the instar-II *Artemia* to ensure EPA, DHA and ARA are in suitable proportions.

The most significant savings through switching to one of the proposed feed regimes is the reduced *Artemia* costs. The *Artemia* product currently used by NZPWL costs $282NZ per kg of cysts with a total of 11.1 kg required for the live feeding stage for approximately 2.4 million larval giant kōkopu. Estimating from the 2016 harvests, this number of fish results in approximately 75 kg of whitebait product at harvest. In adopting the timing and proportion of instar-II+ provision in the 5050 regime the total *Artemia* cost for production would be reduced by 21 %, while the Instar-II+ regime would offer a 25 % saving. Further savings will be made as a result of the lower labour associated with instar-II+ *Artemia* production.

Furthermore, better condition (i.e., larger size and better health) have been shown to reduce the age at which larval fish will accept artificial dry foods (Kubitza & Lovshin, 1997; Luz et al., 2015; Moura et al., 2000). The increased size of larvae at younger age and improved condition achieved when using the 5050 and Instar-II+ diets

has the potential to allow weaning from live to artificial feeds at an earlier age than currently practiced. This outcome would result in greater productivity and further savings on food associated costs.

3.5 Conclusion

The results of this study demonstrate the major potential benefits that earlier provision of, as well as increasing the proportion of, instar-II+ *Artemia* has on the larviculture of giant kōkopu. By improving the growth performance and survival of larvae productivity increased substantially, an obvious commercial benefit. These changes to the diet directly reduce the cost of live feed costs and bolster the commercial production benefits.

To further optimize the live feeding regime of larval giant kōkopu future research should focus on understanding growth performance and survival of larvae fed a lower range of feed particle densities. Additionally, the impacts of nutrient composition of instar-II+ *Artemia* with differing enrichment formulas should be assessed on growth performance and survival of this species. It is evident from this study that attempts to improve the nutritional quality of instar-II+ *Artemia* may not currently be achieved given the lower levels of EPA, PUFA and HUFA and the lack of solid evidence in the form of increased ARA or DHA levels compared to instar-I. As such the current enrichment protocols used in the current commercial production of giant kōkopu should be assessed with it being likely that composition, quantity and timing of enrichment is inadequate to maximise essential fatty acid composition of the enriched *Artemia*.

4 Chapter 4: Improving the weaning of larval giant kōkopu (*Galaxias argenteus*), An emerging aquaculture species

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4.1 Introduction

Larval galaxiid fisheries (whitebait) are over-exploited but their products remain highly valued in the Southern Hemisphere and in New Zealand, four of the five species which make up this harvest are native or endemic and threatened or endangered (David, 2002; Goodman, 2014; McDowall, 2010; Vega et al., 2013). The aquaculture of these species in land-based facilities has taken on renewed interest for its potential to alleviate pressure from wild harvesting, while taking advantage of the high market price of US$70-115 kg^{-1} (Environment Southland, 2013; *NZ Whitebait*, n.d.).

The giant kōkopu, *Galaxias argenteus*, is the whitebait species of choice at New Zealand Premium Whitebait Ltd (NZPWL) a start-up whitebait aquaculture venture, because of their high fecundity, annual spawning, robustness in culture and potential to restock wild populations (P. Decker, personal communication, 2 July 2016). As an emerging aquaculture species, for which there is limited biological information, the development of commercial aquaculture of giant kōkopu has many challenges. However, the greatest current challenge in the aquaculture of giant kōkopu is the high cost of the hatchery use of live feeds. Larval fish require a live feed as a first diet because the movement of live feed triggers predatory behaviour and the initiation of exogenous feeding (Conceição et al., 2010; Holt, 2011; Pousão-Ferreira et al., 2003; Qin, 2008). The high cost of raw materials, labour, capital investment and water quality requirements of live food production, as well as their variable nutritional quality, means that early weaning of the larvae from live feed to formulated food is highly desirable (Southgate & Partridge, 1998; Lucas & Southgate, 2012). *Artemia* are used as the first feed for larval giant kōkopu (Mardones

& De los Ríos-Escalante, 2012; Mitchell, 1989) and is it therefore desirable to wean the larvae onto formulated feeds as quickly as possible (Barron et al., 2016). However, weaning larval fish too early can have deleterious effects on larval fish growth performance and survival (Barron et al., 2016; Hamre et al., 2013; Hoestenberghe et al., 2015; Lucas & Southgate, 2012; Rao, 2003). Lack of visual stimulation, unsuitable olfactory cues, unpalatability, incompatible particle size, rapid particle settlement rate and poor larval predatory skills can all play a role in limiting the consumption of formulated diets by early stage fish larvae (Lucas & Southgate, 2012). Furthermore, the weaning regime itself (i.e., the speed and duration of transition, as well as the proportions of diet type) has a large part to play in successful weaning (Lucas & Southgate, 2012).

Very little is known about the ability of larval galaxiids to wean onto formulated diets. Inanga, *Galaxias maculatus*, (or "puye" in South America) are described as weaned at 40 days after hatching (DAH) (Mardones et al., 2008; Mardones & De los Ríos-Escalante, 2012) while the current hatchery techniques for giant kōkopu at NZPWL has larvae weaned at 28 DAH. However, with the need to further reduce cost of production, it is vital that this stage of feeding is better understood. Therefore, the present study describes the feeding capabilities of giant kōkopu larvae on live and formulated diets across a 10 day period within a commercial hatchery run using their standard rearing protocol. The aim of the research is to identify the appropriate age at which larvae are able to effectively and consistently consume formulated feeds.

4.2 Materials and Methods

4.2.1 Broodstock Rearing and Larval Production

Gametes from 160 female and 20 male giant kōkopu were stripped and fertilized on 15 August 2016. Broodstock ranged between 100-500 g, and were not fed for seven days before being sedated in an AQUI-S (AQUI-S New Zealand Ltd) solution for 5-10 mins prior to stripping. Eggs were fertilized and incubated for a 4 week period in UV treated freshwater filtered to 1 µm at 4 °C. On 9 September 2016, approximately 2.4 million giant kōkopu were hatched directly into one 2500 L conical commercial rearing tank. The tank was supplied with natural seawater, of 35 ppt, fully exchanged at least hourly, filtered to 5 µm, UV treated. Water temperature was not controlled, with ambient conditions maintaining < 18 °C.

The large scale of the larval rearing tanks (i.e., 2500 L) is the standard size for commercial rearing of these larvae. Sampling from this tank environment serves to represent their actual feeding in a commercial production setting, versus establishing small scale tanks for sampling. The latter would likely influence the results because of the unique hydrodynamic conditions and the much higher tank wall area to water volume ratio. Replication of tanks for sampling larvae was not possible at this large scale, however, the production from this experimental run was consistent with previous commercial batches in the same larval rearing tanks. Consequently, the results from sampling for this study were likely representative of commercial production. For the duration of the experimental period fish larvae were kept under a 12:12 hrs light:dark illumination regime and seawater quality was monitored twice daily throughout the experiment and remained well within the acceptable parameters for rearing marine larval fish (Brownell 1980a, 1980b).

The feeding of live feed began in the Afternoon 3 DAH and continued until 28 DAH. Larvae were fed live food three times a day at 0830, 1230 and 1630 hrs. The first 10 days of live feed consisted only of instar-I *Artemia* nauplii, after which the proportion of instar-II *Artemia* nauplii increased over a period of 7 days with the live food provision for the following 8 days containing only instar-II *Artemia* (Table 4-1). GSL Sep-Art (INVE Aquaculture Inc, USA) *A. franciscana* cysts were used throughout this experiment. *Artemia* production involved incubation of cysts in 250 L cones at 29 °C at 35 ppt with constant, vigorous bubbling while exposed to a 58W tube light. After 17 hrs incubating instar-I *Artemia* were then separated from cysts via magnet and rinsed in a 100 µm sieve with clean 35 ppt salt water. They were then fed immediately to the larvae or cold stored at a density of 1.67 g ml^{-1} at 4 °C until required (max 16 hrs). Instar-II *Artemia* were produced in separate batches using the same set up but were incubated for 27 hrs. These were separated from cysts and cleaned in the same manner, before a 21 – 29 hrs enrichment period. A proprietary enrichment, combining commercially available algae products (Rotigrow Plus, Nanno 3600, Tetraselmis 3600, Reed Mariculture Inc., USA) was formulated by NZPWL. Enrichment formula (60 ml) was added as instar-II were initially transferred to the enrichment tank and the same volume added after 18 hrs.

Formulated feed (A1 75-150 µm Otohime; Marubeni Nisshin Feed Co., Ltd, Japan) (75-150 µm, brick red colour, minimum 58% crude protein, minimum 8% crude fat, maximum 3% crude fibre, maximum crude ash 16 %, 8% maximum crude moisture) began at 7 DAH and was administered 1 hr prior to live food (i.e., 0730, 1130 and 1530

hrs, referred to as Morning, Midday and Afternoon, feeding events respectively). Initially, formulated feed was provided at the Morning feed event only, then each feed event from 11 DAH onwards (Table 4-1). Due to the 1 hour delay between the provision of formulated food and the subsequent introduction of live food, all uneaten formulated food settled to the bottom of the tank where it was unable to be consumed by the larvae, which only consume suspended particles (pers. obs.). Residual uneaten feed, which settled to the bottom of the culture tank, was removed by siphon three times a day, 2 hrs after each formulated feed provision. Between feeding events (i.e., the provision of live food after the Morning feed event at 0830 hrs and the formulated food at the Midday event at 1130 hours), there was a three hour period. Tank water was replaced completely each hour ensuring that after three hours *Artemia* were either consumed, flushed out of the tank or siphoned out during tank cleaning and to ensure high water was maintained. The removal of *Artemia* was confirmed by visual sampling of the water prior to commencing the next feeding event. Consequently, there was no food present at any feeding event remaining from the previous event, i.e., at no time were the two feeds (formulated Otohime and live *Artemia*) simultaneously present in the tank.

The Otohime formulated weaning feed was used for this study because it has been proven to be effective for weaning larval giant kōkopu, as well as being widely used in the aquaculture industry as an effective weaning diet for a wide range of larvae of fin fish species (Orihuela et al., 2018). The wide use of this weaning diet has resulted in it commonly being used as reference diet for comparative assessment of experimental weaning diets (Orihuela et al., 2018; Rust et al., 2015).

Table 4-1 Feeding regime for larval giant kōkopu from 3 DAH to 28 DAH at New Zealand Premium Whitebait Ltd showing quantities of each type of feed provided at each feeding event. *Artemia* density ranged from 8.8 ml^{-1} at first feeding to 15.3 ml^{-1} at the last live feeding.

DAH	Morning			Midday			Afternoon		
	Dry Feed (g)	Instar-I (g)	Instar-II (g)	Dry Feed (g)	Instar-I (g)	Instar-II (g)	Dry Feed (g)	Instar-I (g)	Instar-II (g)
0									
1									
2									
3								200	
4		200			200			200	
5		200			200			200	
6		200			200			200	
7	40	200			200			200	
8	40	250			250			250	
9	40	250			250			250	
10	40	250			250			250	
11	40	250		40	250		40	250	
12	40	300		40	300		40	300	
13	40	350		40	350		40	350	
14	65	300	100	65	300	100	65	300	100
15	65	300	100	65	300	100	65	300	100
16	65	250	100	65	250	100	65	250	100
17	65	175	175	65	175	175	65	175	175
18	65	175	175	65	175	175	65	175	175
19	65	100	250	65	100	250	65	100	250
20	65	100	250	65	100	250	65	100	250
21	165		350	165		350	165		350
22	165		400	165		400	165		400
23	165		450	165		450	165		450
24	165		450	165		450	165		450
25	165		450	165		450	165		450
26	165		450	165		450	165		450
27	165		450	165			165		450
28	165			165			165		450

Notes.

Morning feeding event started at 0730 hrs, Midday feeding event at 1130 hrs, and Afternoon feeding event at 1530 hrs. Each feeding event followed the same routine with formulated feed introduced at the start of the feeding event, and *Artemia* 1 hr later. Sampling days on 18 (light grey), 21 (grey), 25 (dark grey) and 28 DAH (black) marked with outlined rows. For reference larvae wet weight and length at age 14 DAH is 2.70 mg ± 0.03 and 10.82 ± 0.12 mm, 5.38 ± 0.17 mg and 12.75 ± 0.14 mm at 21 DAH and 8.67 ± 0.24 mg and 13.72 ± 0.24 mm at 28 DAH (McKay & Jeffs, 2021). The operation of the larval culture system meant that unconsumed *Artemia* were flushed from the system prior to dry feed (Otohime) being introduced at the next feeding event, and the dry feed settled out prior to the following feeding of *Artemia*.

4.2.2 Sampling of Larvae

Larvae were sampled to make observations for gut fullness at 0, 15 and 30 mins after the provision of formulated food at each feeding event at 18, 21, 25 and 28 DAH. Observations of gut fullness were also made after the provision of live feeds at 0, 15, 30, 60, 120 and 180 mins where live feeds were provided.

Larvae were sampled by randomly scooping approximately 20 individuals from the surface of the tank and pouring them into a 100 µm sieve. The underside of the sieve was quickly dried with a towel, and a digital image taken of the larvae using an Olympus TG-4 camera. This was repeated at least three times to ensure at least 50 individuals of appropriate orientation were photographed.

Gut fullness was determined by comparing the number of pixels along the total length of the gut occupied by food particles relative to that of the total length of the gut (Canino & Bailey, 1995; Dunbrack et al., 2009) (Equation 4-1) (Figure 4-1). Because larvae were not often perfectly horizontal, segmented lines were used to more accurately make these measurements. Digital images of the larvae were assessed using ImageJ64 (version 1.52q) software.

Equation 4-1 $$\text{Gut fullness} = (GL^T - GL^F)/GL^T \times 100$$

Where GL^T is the total gut length, GL^F is the gut length occupied by food.

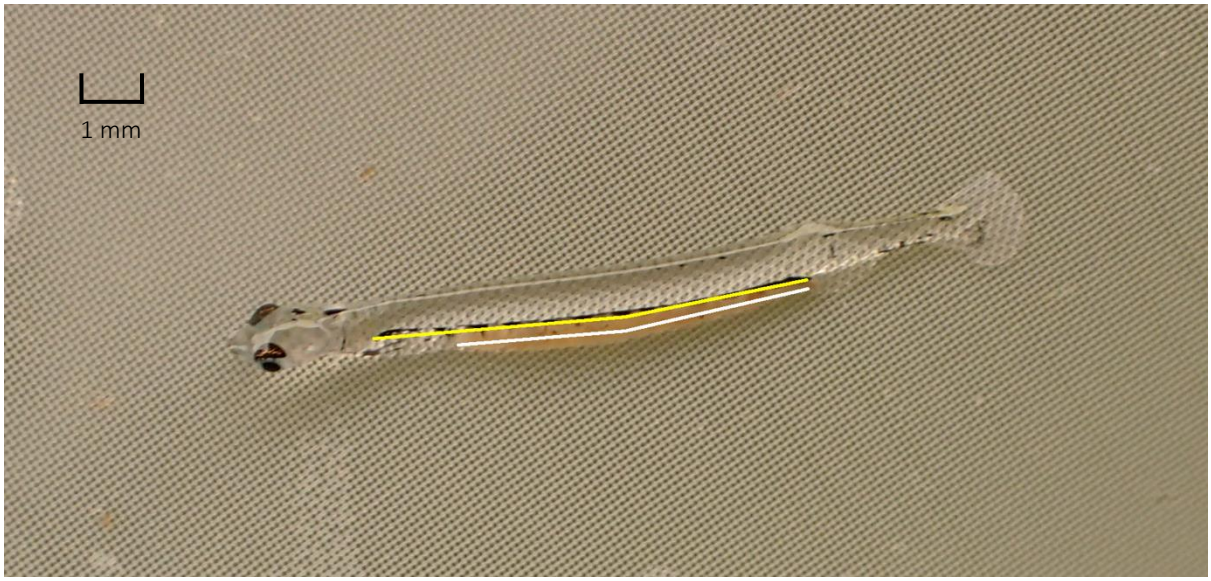

Figure 4-1 Screenshot taken from ImageJ64 analyses of a 25 DAH larva 60 minutes post-afternoon feeding. The yellow line was used to count the number of pixels along the total length of gut and white line the total number of pixels along the length of the gut occupied by food particles. Larva total length 13.5 mm, scale bar shows 1 mm

The change in gut fullness was calculated by subtracting the mean gut fullness at the start of the feeding event (0 mins) from each measure of larval gut fullness 30 mins after formulated feed was provided (for the same feeding event) and taking a mean from all 50 sampled fish. Change in gut fullness was calculated for each of the three feeding events.

4.2.3 Statistical Analyses

The mean gut fullness between larval ages (i.e., DAH) at 30 mins after the initiation of feeding was compared using an ANOVA where feeding event was nested within age. Pairwise comparisons were calculated between ages using Tukey's method where ANOVA identified an overall significant difference among means. Prior to analysis the proportional data were arc-sine transformed to correct for any bias in the distribution of the data (Zar, 1998). Normality and equality of variances were confirmed using the Shapiro-Wilk's and Levene's tests, respectively.

The change in individual gut fullness at 30 mins after feeding was compared to the mean at time zero using the non-parametric Kruskal-Wallis test due to non-normality. Dunn's test was subsequently used to compare the results between larval ages and p-values were corrected for multiple comparisons using the Benjamini-Hochberg method.

A logistic regression model was used to predict the proportion of larvae with an empty gut by using age, feeding event and time point, as well as the interactions among these covariates. To find the most parsimonious model an analysis of deviance table was used to determine whether each parameter resulted in a significant change in deviance. Pairwise comparisons were calculated for each combination of age, feeding event and time point, and adjusted for multiple comparisons using the Benjamini-Hochberg method.

All statistical analyses were performed using R (RStudio, ver. 1.2.1335). All measures of variability of means are reported as standard error of the mean.

4.2.4 Animal Welfare

This research was fully compliant with the Animal Welfare Act 1999 of New Zealand, which regulates the use of animals in scientific research in New Zealand.

4.3 Results

The mean gut fullness of larval giant kōkopu was different among ages at 30 mins after the initial feeding event ($F_{(3,588)} = 77.16$, $P < 0.01$).

At 25 DAH and 28 DAH mean gut fullness was greater than at both 18 DAH and 21 DAH for each of the three feeding events ($P < 0.01$ in each case) (Figure 4-2). There was no difference in mean gut fullness of larval giant kōkopu at 25 DAH and 28 DAH at 30 mins after any of the formulated feeding events (Morning $P = 1.00$, Midday $P = 1.00$, Afternoon $P = 1.00$) (Figure 4-2). Similarly, there was no difference in mean gut fullness between ages 18 and 21 DAH at 30 mins after the provision of formulated feeds for any of the feeding events (Morning $P = 0.51$, Midday $P = 0.92$, Afternoon $P = 0.98$) (Figure 4-2).

Larval giant kōkopu gut fullness was different between event and time based on age ($F_{(6, 1175)}$ = 3.45, P < 0.01). At 18 DAH gut fullness was not different between 0730 and 0800 hrs (P = 1.00); 1130 and 1200 hrs (P = 0.32); nor between 1530 and 1600 hrs (P = 1.00) (Figure 4-2). Gut fullness of larvae aged 21, 25 and 28 DAH was always greater 30 mins post feeding (i.e., 0800, 1200 and 1600 hrs), compared to prior (i.e., 0730, 1130 and 1530 hrs, respectively) for each of the three feeding events (P < 0.01 in each case (Figure 4-2)).

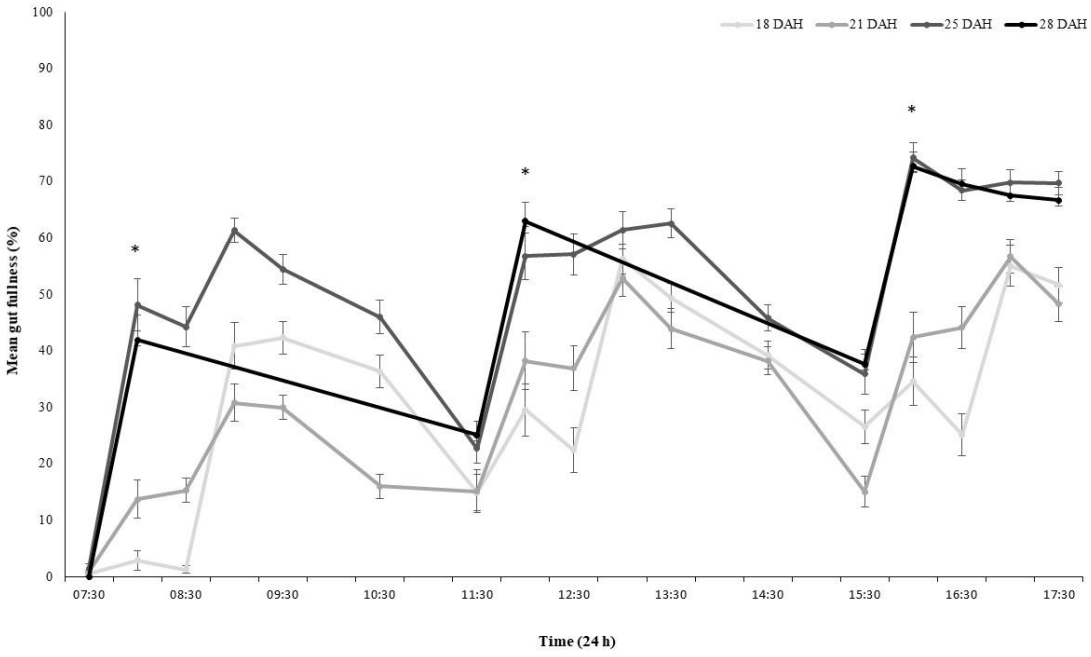

Figure 4-2 Mean gut fullness of larval giant kōkopu for four days (i.e., 18, 21, 25, and 28 DAH) during the weaning from live to formulated feeds. Larvae were fed as per Table 4-1. Asterisk indicates the presence of a significant difference among treatment means at the corresponding time point (P<0.05). Comparison was only made at 30 mins post formulated feed event and not every time point on the graph. n = 50 larvae for each sampling point.

The change in mean gut fullness for each of the three formulated feeding events per day differed among the ages of larval giant kōkopu (i.e., for Morning X^2 = 69.08, df = 3, P < 0.01; Midday X^2 = 14.04, df = 3, P < 0.01; Afternoon X^2 = 29.55, df= 3, P < 0.01). At the Morning feeding event the change in larvae gut fullness at 25 DAH and 28 DAH was not different (Z = 1.06, P = 0.17) (Figure 4-3). However, change in gut fullness at both 18 and 21 DAH was

lower than that observed for 25 and 28 DAH for the Morning feed (18 DAH, Z = -4.59, P < 0.01 and Z = -5.89, P < 0.01; 21 DAH, Z = -5.74, P< 0.01 and Z = -6.68 P < 0.01) (Figure 4-3). There was no difference in the change in gut fullness across the Morning feed between 18 and 21 DAH (Z = 0.79, P = 0.21) (Figure 4-3).

The changes in gut fullness of larvae showed the same pattern for both the Midday and Afternoon feed events. Change in gut fullness at 28 DAH was not different to 25 or 21 DAH (Midday, Z = -0.55, P = 0.35 and Z = -0.83, P = 0.31 respectively; Afternoon, Z = 1.76, P = 0.17 and Z = -0.28, P = 0.39, respectively) (Figure 4-3). There was also no difference in gut fullness between 25 and 21 DAH for the Midday and Afternoon feeds (Z = -0.28, P = 0.39 and Z = -1.34, P = 0.13, respectively) (Figure 4-3). Change in gut fullness was smaller for 18 DAH when compared to 21, 25 and 28 DAH for the Midday feed (Z = -2.62, P < 0.01; Z = -2.89, P < 0.01 and Z = -3.44, P < 0.01, respectively) and the Afternoon feed (Z = -3.74, P < 0.01; Z = -5.09, P < 0.01 and Z = -4.01, P < 0.01, respectively) (Figure 4-3).

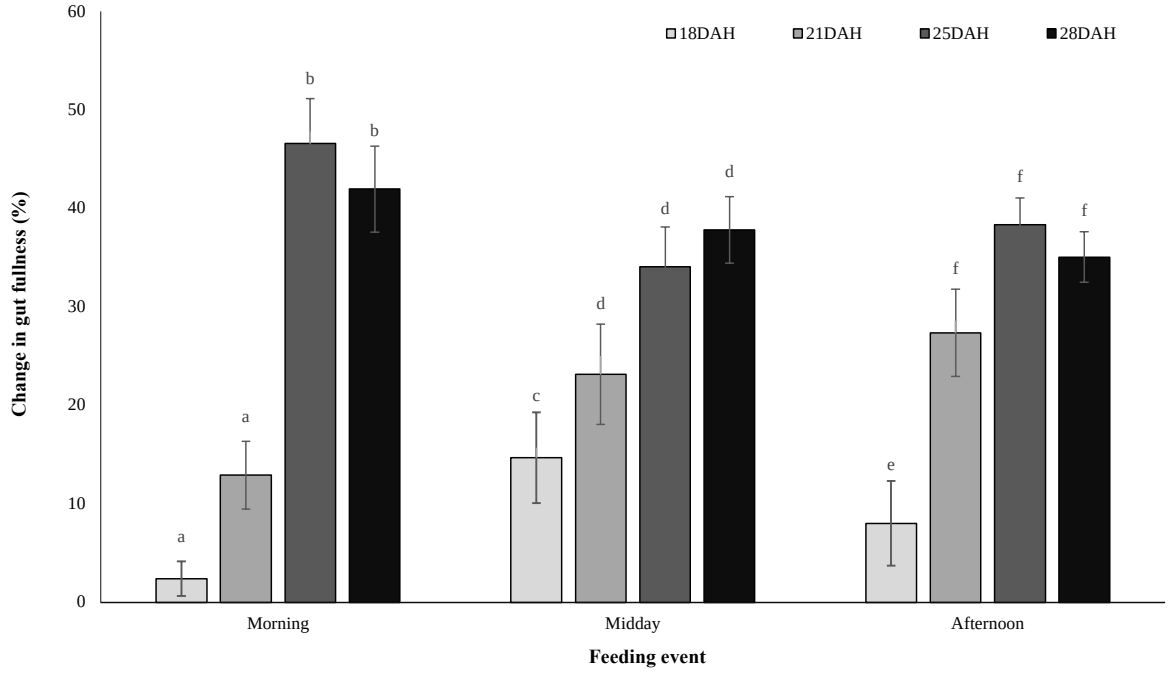

Figure 4-3 Mean change in giant kōkopu larvae gut fullness across each of the three daily feeding events for larvae at 18, 21, 25 and 28 DAH. Letters indicate differences in change of gut fullness within each feeding event where P<0.05 and n = 50 larvae for each sampling point.

Overall, there were differences in the mean proportions of larvae with an empty gut among larval age and between the start and finish of the feeding events (i.e., 0 mins and 30 mins) (deviance = 550.13, df = 23, $P < 0.01$) (Figure 4-4).

At 18 DAH there was no difference in proportion of larvae with an empty gut at the start or finish of each feeding event (Morning $Z = -0.39$, $P = 0.73$; Midday $Z = -1.61$, $P = 0.14$; Afternoon $Z = 0.42$, $P = 0.71$) (Figure 4-4). The proportion of larvae with an empty gut was higher at the finish of each feeding event for larvae aged 18 DAH compared to 21 DAH for the Morning feed event only ($Z = 2.80$, $P < 0.01$), and for 25 and 28 DAH for all three feeding events (Morning $Z = 6.20$, $P < 0.01$ and $Z = 6.07$ and $P < 0.01$; Midday $Z = 3.84$, $P < 0.01$ and $Z = 4.10$, $P < 0.01$; and Afternoon $Z = 3.15$, $P < 0.01$ and $Z = 3.15$, $P < 0.01$, respectively) (Figure 4-4).

There was a decrease in the proportion of 21 DAH larvae with empty guts between the start and finish for the Morning ($Z = -2.58$, $P = 0.02$) and Afternoon feed events ($Z = -2.21$, $P = 0.04$) (Figure 4-4). For each of the three feed events, the proportion of 21 DAH larvae with empty guts was higher at the finish than for both 25 and 28 DAH (Morning $Z = 4.76$, $P < 0.01$ and $Z = 4.60$ and $P < 0.01$; Midday $Z = 3.20$, $P < 0.01$ and $Z = 3.68$, $P < 0.01$; and Afternoon $Z = 2.88$, $P < 0.01$ and $Z = 2.88$, $P < 0.01$, respectively) (Figure 4-4).

Comparing 25 DAH and 28 DAH for the finish of each of the three feeding events there was no difference in the proportion of fish with an empty gut (Morning $Z = -0.25$, $P = 0.83$; Midday $Z = 1.41$, $P = 0.20$; and Afternoon $Z = 0.00$, $P = 1.00$) (Figure 4-4).

The proportion of 25 DAH larvae with an empty gut was lower at the finish versus the start of each of the three feeding events (Morning $Z = -6.21$, $P < 0.01$; Midday $Z = -2.34$, $P = 0.03$; and Afternoon $Z = -2.58$, $P = 0.02$) (Figure 4-4). The proportion of 28 DAH larvae with empty guts was lower at the finish versus the start for the Morning and Midday feed events at 28 DAH but not the Afternoon, although there was trend toward a decrease ($Z = -4.93$, $P < 0.01$; $Z = -2.39$, $P = 0.03$; and $Z = -1.52$, $P = 0.16$, respectively) (Figure 4-4).

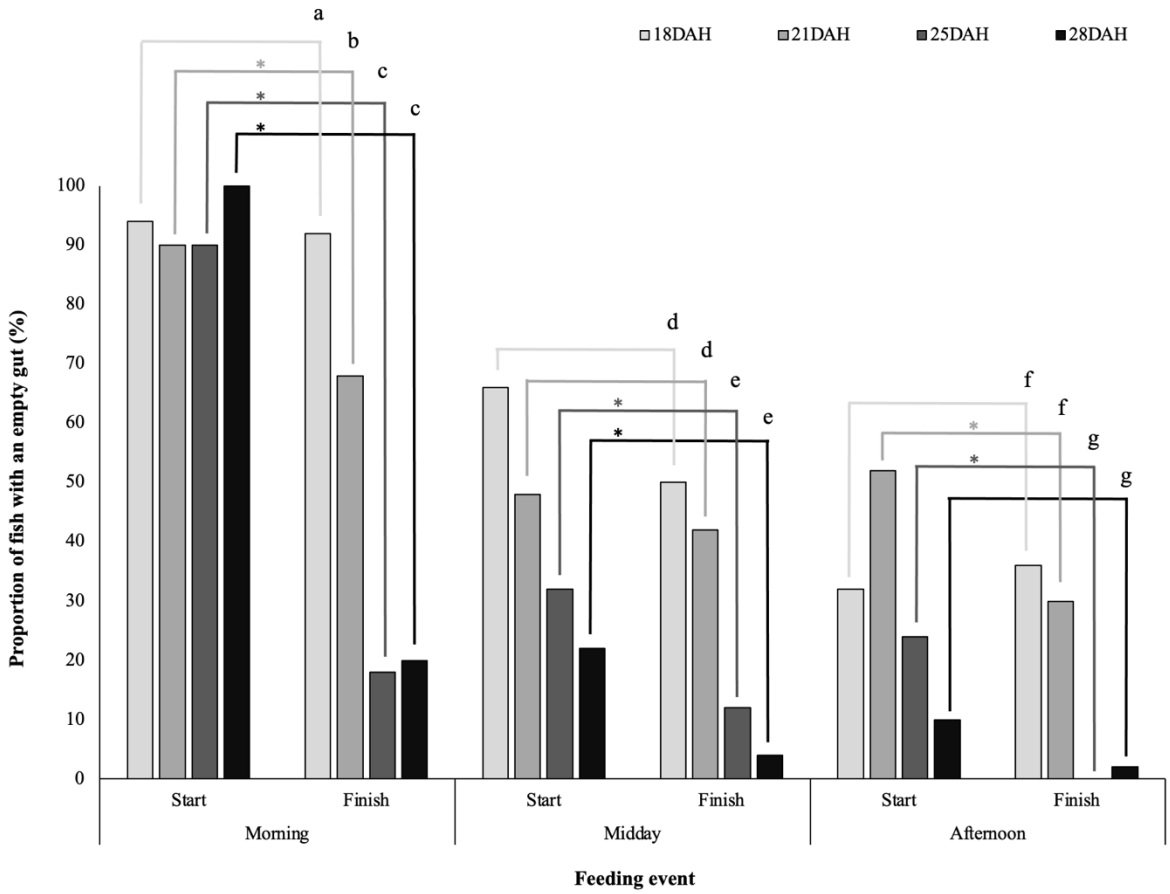

Figure 4-4 Proportion of giant kōkopu larvae with an empty gut at the start and finish of each feeding event at ages 18, 21, 25 and 28 DAH. An asterisk indicates a significant difference in the proportion of empty guts between the start and finish time points for larvae of one age and for one feeding event, while different letters indicate differences in the proportion of empty guts at the finish time point within each feeding event. In both cases significance refers to $P<0.05$, n = 50 larvae for each sampling point

4.4 Discussion

This study provides the first report of the ability of larval galaxiid fishes to wean onto formulated diets. Giant kōkopu absorb their yolk sac by 3 DAH with exogenous feeding regimes providing live *Artemia* from 3 DAH to 28 DAH (McKay & Jeffs, 2021). Because of the higher cost of live feeds over artificial feeds, reducing the duration of live feeding is critical to the reduction of cost of production and therefore the success of the commercialization this species. Therefore, the aim of this research was to identify a more optimal weaning age and in so doing

provide some initial insights into the potential reasons for the changing ability of giant kōkopu of different ages to consume artificial feeds. The results show that the consumption of formulated diets by larval giant kōkopu increases significantly over the 10 day period from 18 DAH to 28 DAH. Of particular importance is the ability of larvae aged 25 DAH to achieve the same level of gut fullness when fed a formulated diet as larvae at 28 DAH. This is an indication that the larvae could be weaned onto formulated diet at least three days earlier, which if confirmed by further research to be the case, would provide a significant cost saving for commercial production. It is critical to note that the two feeds (Otohime and *Artemia*) were never in the water column at the same time due to settlement rate, time between each provision of feed and the water turnover rate within the tank. As such, changes in gut fullness or proportion of empty guts across the 30 minute period post-formulated feed provision were due to consumption of Otohime only and cannot be attributed to preferential selection of *Artemia.*

Giant kōkopu larvae at 18 DAH were largely incapable of consuming formulated diet. The change in mean gut fullness of 18 DAH larvae across each feeding event was lower than for the older larvae and the proportion of 18 DAH larvae with an empty gut did not decrease over the period of the three separate feed events during the day. Despite their poor ability to consume artificial feeds, 18 DAH larvae demonstrate very good ability to consume live feeds when they are present. This is a strong indication that weaning giant kōkopu larvae at this age is too early and would likely result in significant losses in productivity due to insufficient feed intake leading to decreased growth and survival. At 21 DAH larvae appear to be able to consume formulated feed as confirmed by the overall increase in gut fullness across feeding events, and the decrease in the proportion of empty guts observed for Morning and Afternoon feed events. However, the change in gut fullness achieved 30 mins after the delivery of formulated feed for larvae at 21 DAH consistently tended to be smaller than for 25 DAH and 28 DAH for all three feeding events, suggesting that weaning of the larvae from live feed was not complete at 21 DAH. Measures of changes in gut fullness and the proportion of larvae with empty guts were not different for larvae of 25 DAH versus 28 DAH for each of the three feeding events, which indicated that feed ingestion had not increased further over this period of larval development. This suggests the larvae had been weaned and consequently the delivery of live feed could potentially be shortened by three days, i.e., live feed eliminated at 25 DAH versus 28 DAH.

The improved feeding performance of the 21 DAH over 18 DAH larvae is also likely to have been affected by the increased ration of formulated feed of 165 g at 21 DAH per feeding event versus 65 g at 18 DAH. Fish larvae

require formulated feeds in excess, due to the particles needing to be captured before settling onto the tank floor and to increase rates of encounter with feed particles (Cahu & Zambonino Infante, 2001). This is a limited time window and so the delivery of excess feed allows for greater opportunity to feed on formulated diets, especially for early stage fish larvae (Cahu & Zambonino Infante, 2001). Equal volumes of feed were provided for the 21, 25 and 28 DAH larvae, however, feeding ability was lower at 21 DAH, indicating other factors must be contributing to their lower feeding success.

The size limit for the ingestion of feed particles for fin fish larvae is usually set by the extent of their mouth gape (Arts & Evans, 1987; Bremigan & Stein, 1994; Cunha & Planas, 1999; Krebs & Turingan, 2003; Makrakis et al., 2008; Zaret, 1980). At 28 DAH larval giant kōkopu gape is 22% and 30% greater than at 21 and 14 DAH, respectively (McKay & Jeffs, 2021). Maximum feed particle size (150 µm) relative to gape width was 24%, 23%, and 19% at 14, 21 and 28 DAH, respectively (McKay & Jeffs, 2021). Therefore, the larger gape of the older larvae in this experiment makes successful capture more likely, which may also help to explain the greater gut fullness and fewer fish with an empty gut at 28 and 25 DAH versus 21 and 18 DAH. However, these relative feed particle sizes are at the lower end of what is generally considered the optimum size range of 25-60% of mouth gape width (Bremigan & Stein, 1994; Fernández-Diaz et al., 1994; Østergaard et al., 2005; Shirota, 1970). Furthermore, giant kōkopu have been shown to achieve greater growth performance when fed live diets with much larger particle size to mouth gape ratios up to at least 70% (McKay & Jeffs, 2022b). Larval fish rely on visual and olfactory cues to locate and capture prey (Hubbs & Blaxter, 1986; Jones & Closs, 2016; Miller et al., 1988; Southgate & Partridge, 1998). It is during this period (i.e., 14 to 28 DAH) that eye diameter (ED) of larval giant kōkopu increases significantly. At 28 DAH the ED (633 µm) is 9% larger and 14% larger than 21 (574 µm) and 14 (544 µm) DAH, respectively (McKay & Jeffs, 2021). Larger ED results in greater visual acuity, which is likely to result in 25 and 28 DAH larvae having greater food capture abilities. Therefore, it is probable that the poor uptake of artificial feed at the earlier stages, i.e., 18 and 21 DAH, is caused by the small size of the feed particles (i.e., 75-150 µm) and/or limited visual ability of the larvae to detect them, in contrast to the larger size of the *Artemia* nauplii (400-500 µm). Prior research has already shown Otohime to produce greater growth performance and survival over other formulated larval feeds of similar particle sizes (McKay & Jeffs, 2022a). However, from the present study it would be advantageous to experiment with larger initial feed particle sizes with variable sinking rates to determine whether visual acuity at these very early stages of larval development are preventing earlier uptake of artificial

feeds. For example, further research could assess the use of larger Otohime weaning feeds, such as Otohime B1 (360 µm) or B2 (360-650 µm).

There may be further potential to reduce the age at weaning through alteration of weaning regime for giant kōkopu larvae. For example, future research could take into account feeding capabilities throughout the day. Further assessment of the behavioural feeding biology of the giant kōkopu may enable better understanding of how and when this species naturally accept food. By designing the feeding regime around the natural species-specific behaviours then improvements in weaning success, growth performance and survival are more likely to be achieved (Rønnestad et al., 2013). From this study it appears that larvae are better able or more likely to consume formulated feeds at the Midday and Afternoon feeding events as evidenced by the greater change in gut fullness of 25 DAH larvae at the corresponding feeding events relative to the Morning feeding event. Larval fish differ in their diel feeding behaviours by species due to their biological and environmental circumstances (Rønnestad et al., 2013). The diel feeding pattern of wild, marine stage larval giant kōkopu is not known, however, studies indicate a high level of feeding flexibility by larval whitebait species including both opportunistic and selective feeding approaches depending on prey size and density and are to the point of being able to undertake completely freshwater life cycles if required (Catlin et al., 2019; David et al., 2019, 2022; Manosalva et al., 2021). Similarly, studies on adult giant kōkopu indicate flexibility between feeding levels between day and night, depending on season (David & Closs, 2003; Hansen et al., 2004), however, given the relatively poor visual acuity at early stages of development, larvae in this study may in fact benefit from the increased level of natural light intensity at the Midday and Afternoon feeding events, leading to greater capture success and therefore gut fullness (Fiksen & Jørgensen, 2011). Were this found to be the case, backed up by feeding behaviour analyses, feeding live food only in the Morning with higher proportions of formulated feeds for the Afternoon feeds may lead to greater productivity outcomes. Additional modifications to the pattern of feeding could be made, for example, co-feeding of live and artificial diets, adjusting the light intensity for morning feeds or using different sizes of weaning diet particles. The current feeding pattern is in use simply because it has been shown to work for this species and so was employed in this study to form a baseline from which further experimental regimes can be compared against.

It is important to recognize that the same consumption levels of artificial feeds at different larval ages do not necessarily equate to the same long term larval performance. The digestive and assimilation capabilities of larvae at different ages ultimately determines the success of reduced weaning age and so further research is required before achieving full confidence in implementation of altered weaning regimes at the commercial scale (Cahu & Zambonino Infante, 2001). Live feeds are important in larval fish for the development of their sensory systems through the stimulation of predatory behaviour, and for the development of the gastrointestinal tract and digestive enzymes. Consequently, weaning larval fish too early can compromise their development, ultimately negatively impacting productivity and becoming more costly in the long run (Abatzopoulos et al., 2002; Barron et al., 2016; Cara et al., 2003; Chen et al., 2006; Dabrowski & Glogowski, 1977; Hamre et al., 2013; Hoestenberghe et al., 2015; Kolkovski et al., 1993; Van Stappen, 1996). The findings of this study only provide a preliminary understanding of weaning ability of this species and further study is required. A better understanding of the implications and potential for success of earlier weaning is only possible with longer term studies tracking the subsequent growth rate and survival impacts on larvae, weaned at earlier ages. These studies should include further levels of replication, additional observations variables such as weight, length and mortality, as well as seek to determine the influence ambient temperature and lighting levels have on weaning success.

The financial implications of this initial research on larval giant kōkopu weaning show that by simply reducing the period of feeding live feed to 25 DAH the direct cost of *Artemia* would be reduced by 13% due to the reduced volume of cysts required. Additional savings would be made in the reduced labour effort from reduced *Artemia* production. This change is easily and rapidly implementable with no set up costs and one that would be advised immediately. Furthermore, this study shows the formulated feed from 7 DAH is being wasted. Although at these very early ages this is a small volume (and cost) of feed, left uneaten it contributes to the rapid deterioration of the water quality and provides an opportunity for bacterial growth and disease to proliferate (Kong et al., 2020; Luz et al., 2015; D. M. Smith et al., 2002). Combined these issues will lead to reduced productivity and increased costs which should be avoided.

4.5 Conclusion

This study provides important preliminary findings in the understanding of larval giant kōkopu feeding abilities, which may lead to significant cost savings in their aquaculture by reducing the period for feeding live prey. However, before establishing new commercial protocols, further research is required to understand, the impacts of earlier weaning on multiple response variables including growth performance, survival, gastrointestinal tract development, and enzyme secretion.

5 Chapter 5: Comparison of three artificial diets for the larviculture of giant kōkopu (*Galaxias argenteus*)

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5.1 Introduction

Reliable larviculture to produce high quality juvenile fin fish in large quantities is one of the greatest challenges facing the aquaculture industry currently (Crooks et al., 2013; Nhu et al., 2010). Low survival rates and poor growth of fin fish larvae are frequently major contributing factors to this production bottleneck (Conceição et al., 2010; Shields, 2001). Poor outcomes from larviculture is often the result of a combination of factors, such as a lack of knowledge of larval nutritional requirements, difficulties in supplying feeds that can be processed by rudimentary larval digestive systems, providing husbandry of small and fragile larval fish, as well as the appropriate management of the rapid and complex changes that fish undergo during this early phase of development (Conceição et al., 2010; Crooks et al., 2013; Hamre et al., 2013; Holt, 2011; Lavens & Sorgeloos, 1996; Nhu et al., 2010). The preparation and provision of appropriate feed is particularly critical for successful fish larviculture and typically accounts for 30 – 70 % of total production costs (Webster & Lim, 2001), so that the optimization of larval nutrition and feeding regimes are priority targets for research in early stage aquaculture businesses (Trushenski et al., 2006).

Generally, feeding regimes for the larviculture of fin fish begin with several weeks of live food provision, followed by a period of co-feeding with live and artificial diets before larvae can be fully weaned to artificial diets (Conceição et al., 2010; Holt, 2011; Pousão-Ferreira et al., 2003). Rotifers and *Artemia* are the most commonly used live feeds, but while these organisms have unlocked the potential for aquaculture of a multitude of fin fish species in the last few decades, they still impose limitations to successful commercial larviculture (Lavens & Sorgeloos, 1996). These limitations include their high costs, from both their purchase and the infrastructure and resources

required to culture or prepare them (Pousão-Ferreira et al., 2003). Furthermore, the nutritional condition of live feeds can vary widely, with poor nutritional condition resulting in inferior survival and growth performance in cultured larvae (Kolkovski et al., 1993; Pousão-Ferreira et al., 2003). As a result, significant effort is focus on identifying optimal artificial diets to replace live feeds.

Artificial diets provide several key advantages over live prey for larviculture. Most importantly, they can be customized to the species and age-specific nutritional needs of the animal in culture and are reliably uniform in composition (Langdon & Barrows, 2011; Pousão-Ferreira et al., 2003). This allows the reliability that is required for consistently delivering optimal survival and growth in commercial larviculture. Artificial diets are also far more practical, in that they are easily stored, require far fewer resources for preparation than live feeds, and are immediately dispensable when required (Langdon & Barrows, 2011).

Issues remain in preparing artificial larval feeds which provide for nutrient stability, as well as perceptibility, palatability, digestibility and nutritional value (Hamre et al., 2013; Jones et al., 2015; Langdon & Barrows, 2011). Of critical importance is identifying the specific needs of the species in culture, such that the optimal feed can be provided and economical production be achieved (Hauville et al., 2014). The provision of essential fatty acids are especially critical for larval marine fin fish (Izquierdo, 1996; Sargent, Bell, et al., 1999; Watanabe & Kiron, 1994). The absolute and relative requirements of essential fatty acids, particularly EPA (eicosapentaenoic acid), ARA (arachidonic acid) and DHA (docosahexaenoic acid), are highly species-specific due to differences in their metabolic capabilities and nutritional requirements for development (Bell et al., 1995; Benítez-Santana et al., 2007; Hauville et al., 2014; Koven, 2003; Rodríguez et al., 1997; Sargent, Bell, et al., 1999; Tocher, 2003).

Galaxiid fishes are a family of freshwater fishes, found in cool-temperate southern hemisphere (McDowall, 2010). Most galaxiids maintain an entirely freshwater life cycle, however, amphidromy is common, such as in the case of the giant kōkopu (*Galaxias argenteus*) which is endemic to New Zealand (McDowall, 1988, 2009). The eggs of this species are deposited on river-banks, incubating terrestrially, until flooding rain events stimulate hatching, washing the larvae down rivers and out to sea (Benzie, 1968a; Franklin et al., 2015; McDowall et al., 1994; Mitchell, 1989). After 3-6 months, giant kōkopu larvae, along with larvae from four other galaxiid species, undertake a mass migration back into the freshwater habitats occupied by adults (McDowall, 1990; McDowall et al., 1994). These mass migrations occurring in confined fresh waterways provide the opportunity for targeting

their harvesting to provide highly prized "whitebait" (McDowall, 1984, 1991). Increasingly, studies show that the abundance of the species which make up this fishery are in decline due to habitat loss and predation by introduced species, with the giant kōkopu now considered threatened (Main, 1988; McDowall, 1991; Swales & West, 1991).

Protection of these galaxiid species largely involves increasing regulatory controls on wild fisheries activities, however, larviculture of giant kōkopu is in the early stages of commercialisation and is proposed to reduce the reliance on harvesting threatened wild populations (Environment Southland, 2013; Mitchell, 1989; O'Brien & Cooper, 2013). Optimum diet selection for larvae is a significant issue impeding further commercialisation of giant kōkopu aquaculture. Recent work on this species has developed knowledge on their morphometry and energetic demands, indicating the benefits of earlier provision of larger feed particles (McKay & Jeffs, 2021, 2022c). Still, a significant portion of the larval production cycle (approximately 30 of 77 days) expensive live feeds are required, in large part due to the unknown nutritional of requirements of the larvae. Some early research that has been undertaken on the closely related īnanga, *Galaxias maculatus*, identified that its larval diet requires high levels of alpha-linolenic fatty acid under certain salinity culture conditions (Dantagnan et al., 2013). However, these studies were undertaken at much lower salinities (0 and 15 ppt) than those in which giant kōkopu are reared.

This study aims to improve knowledge of the nutritional requirements of larval giant kōkopu through the comparison of the growth performance of larvae fed on three different commercially available artificial dry feeds. The results have the potential to be useful for improving the efficiency of larviculture, which is important for securing the future commercial success of the giant kōkopu aquaculture industry.

5.2 Materials and Methods

5.2.1 Experimental Animals

Gametes from 80 female and 20 male giant kōkopu were stripped and fertilized before being subjected to a 4 week incubation period in UV treated freshwater filtered to 1 µm at 4 °C. On 4 July 2016, approximately 1.2 million giant kōkopu were hatched directly into one 2500 l conical commercial larval rearing tank containing UV treated water, 35 ppt and ambient temperature < 18 °C. At 2 DAH (days after hatching) around 9000 fish were randomly selected from the commercial tank and split evenly among nine 20 l experimental tanks. This was achieved by estimating the total number of fish per litre in the transfer vessel by careful mixing and taking random 200 ml

samples and then counting the number of fish in each sample to produce a mean estimate of the total number of fish.

5.2.2 Tank Design and Recirculation System

Experimental tanks were made from 20 l plastic (HDPE), round, blue pails, 270 mm in diameter and 380 mm in height. The water outflow pipe was set 80 mm below the rim of the pail so that each tank held 18 l. For the first 4 weeks the outflow pipe was fitted with a banjo filter using 600 µm filter mesh to prevent the escape of giant kōkopu larvae while allowing the passage of suspended particles. For the remainder of the experiment, banjo filters with 1 mm mesh were used. Surfboard wax was applied in a thick, 40 mm width strip around the inside of the tank at the water level to inhibit the climbing ability of the fish larvae.

The experimental tanks were connected to a recirculation system with the outflow from each tank being directed to a filter basket for removal of insoluble particles by a 5 µm filter mat. After passing through the filter mat, the water entered a 300 l sump containing 40 l of plastic Kaldnes-K3 media (Krüger Kaldnes AS, Norway) for biological filtration which had been preconditioned in a commercial giant kōkopu RAS system for at least six months immediately prior to experimental use. Protein was skimmed from the sump manually, as required. Each day the filter mat was changed, and 100 l of seawater was removed from the sump and replaced with natural seawater, 35 ppt, filtered to 5 µm and UV sterilized.

From the sump seawater was pumped through a UV filter and then distributed into each experimental tank using 4 mm tubing connected at the surface and bottom of the tank. Water flow to each tank was 0.28 l min^{-1} over the first 14 days of the experiment. However, inflow was suspended for 30 minutes during feeding events for the first 7 days. Flow rate was increased to 0.37 l min^{-1} from 15-28 days before increasing to 0.49 l min^{-1} for the following 14 days, and finally to 0.62 l min^{-1} for the remainder of the experiment.

Tanks were aerated by an air-stone at the bottom of the tank producing two medium sized (0.5 mm diameter) bubbles per second for the first 28 days of the experiment. The air-stone was then changed to provide a high number of very fine bubbles for the remaining experimental period.

Illumination of experimental tanks was provided by three 58 W fluorescent tubes, suspended 100 cm above the top edge of the tanks. Light reaching the tanks was dimmed by hanging shade cloth over the tanks for 30 minutes either side of the lights coming on at 0745 h and off at 1800 h.

Seawater temperature was not controlled, but was measured every 6 h with a glass thermometer (Aqua One) during the experimental period and found to vary between 14 and 18 °C, and was consistent among all tanks. Nitrate (<5 mg/L), nitrite (<0.25 mg/L), ammonia (NH3/NH4+) (<0.25 mg/L), carbonate hardness and pH (7-8) were measured every second day using API® test kits to ensure water treatment was maintaining suitable conditions for the larvae and was within the acceptable ranges reported for the rearing of larvae (Brownell, 1980a, 1980b; Richardson, 1997; West et al., 1997). Water quality was never found to be outside these acceptable ranges for rearing larvae from set up through to the conclusion of the experiment and was consistent among experimental tanks due to the recirculation system.

5.2.3 Experimental Design

Three commercially available artificial dry feeds for larval giant kōkopu were tested. Larvae were provided solely live food for the first 14 days before a prolonged weaning period after which (from 45 DAH) only artificial food was provided (Table 5-1).

The first feeding treatment “OTO” used the Otohime products A (75 – 250 µm), B1 (250 – 360 µm) and B2 (360 – 650 µm) (Marubeni Nisshin Feed Co., Ltd, Japan) and are referred to as Small Particle, Medium Particle and Large Particle or “SP,” “MP,” and “LP” respectively (Table 5-2).

The second treatment “ART” made use of the Artemac products 2 (100 – 200 µm), 3 (200 – 300 µm) and 4 (300 – 500 µm) (Aquafauna Bio-Marine, Inc., USA), again referred to as “SP,” “MP,” and “LP” respectively (Table 5-2).

The final feed treatment “ORA” used O.range products START-S (100 – 200 µm), WEAN-S (200 – 400 µm) and WEAN-L (300 – 500 µm) (INVE Aquaculture Inc., USA) also referred to as “SP,” “MP,” and “LP” respectively (Table 5-2).

Table 5-1. Experimental feeding regime for giant kōkopu larvae showing the feed provision of each feeding event and number of feeding events per day by larvae age. Instar-I Artemia (in-I), instar-II Artemia (in-II), small particle (SP), medium particle (MP), large particle (LP). *Indicates that for the period 36 – 44 DAH a ration of 2 g of instar-II *Artemia* were also administered with the fourth feed of the day only.

DAH	Feed	Number of Events
0 - 2	NIL	-
3 - 14	3 g in-I	4
15-21	0.1 g SP + 3 g in-II	4
22 - 28	0.1 g SP + 2 g in-II	4
29 - 35	0.25 g SP + 0.1 g MP + 2 g in-II	4
36 - 44 *	0.6 g MP	4
45 - 49	1.2 g MP	4
50 - 56	0.6 g MP	5
57 - 64	0.6 g MP + 0.3 g LP	4
64 - 66	0.3 g MP + 0.6 g LP	4

Table 5-2. Percent dry matter feed composition, energy content, and fatty acid profiles of the three larval diets tested in the present study.

	Colour	Protein (% DW)	Digestible Energy (kcal/g)	Gross Energy (kcal/g)	Est. Protein:Energy (mg/kcal)	Lipid (%)	EPA (mg/g)	DHA (mg/g)	ARA (mg/g)	Carbohydrate (%DW)	Fiber (%DW)	Ash (%DW)	Moisture (%DW)
OTO	Brick Red	53	4.07	4.66	123	9	22.9	20.3	1.1	14.5	3.5	15	6.5
ART	Mustard	57	5.01	5.50	113	9	11.2	12.7	0.9	12.0	2.0	5	7.0
ORA	Orange	56	4.39	5.01	124	13	10.0	20.0	1.2	15.1	1.0	10	5.9

i. *Data on percent dry matter was obtained from product manufacturer's specifications except for the moisture content of O.range which came from* (Bonaldo et al., 2011).

ii. *Digestible energy calculated with the equation: crude protein × 5.64 kcal/g + crude lipid × 9.44 kcal/g* (Csargo et al., 2013; Jobling, 2012).

iii. *Gross energy calculated with the equation: crude protein × 5.64 kcal/g, crude lipid × 9.44 kcal/g* (Portz & Cyrino, 2004) *and carbohydrate (nitrogen free extract) × 4.11 kcal/g* (Csargo et al., 2013; Gallagher, 1997; Jobling, 2012).

iv. *Protein energy ratio calculated with the equation: crude protein × 5.64 kcal/g × 0.877, crude lipid × 9.44 kcal/g × 0.982* (Portz & Cyrino, 2004)*, and carbohydrates (nitrogen free extract) × 4.11 kcal/g × 0.90* (Csargo et al., 2013; Gallagher, 1997; Jobling, 2012).

v. *EPA, DHA data obtained from manufacturers for Artemac and O.range, and for Otohime as well as ARA for each product from* (Bonaldo et al., 2011; Hauville et al., 2014; Thériault & Pernet, 2007).

5.2.4 Live Food Production

Artemia cysts used to produce live feed throughout this experiment were GSL Sep-Art (INVE Aquaculture Inc., USA) from the same batch.

Live feeds were administered by total wet weight, with *Artemia* being harvested and poured through a 100 µm sieve that was allowed to drip dry on a towel for 1 min and measured with electronic scales to the nearest 0.1 g.

Instar-I *Artemia* were produced by incubating cysts in natural seawater 35 ppt for 17 h at 29 °C with constant, vigorous aeration while exposed to light in 250 l *Artemia* cones. Instar-I *Artemia* were separated from unhatched cysts and husks with a magnet before rinsing in a 100 µm sieve with clean 35 ppt water. Instar-I *Artemia* were fed out immediately after harvesting from cysts.

Instar-II *Artemia* were produced and prepared for feeding under the same conditions, however, received a 27 h incubation with enrichment. After separation live animals were enriched in a 400 l tank for between 23 – 31 h using a proprietary enrichment formula that combines the commercially available instant algae products – Rotigrow Plus, Nanno 3600 and Tetraselmis 3600 (Reed Mariculture Inc., USA). At the beginning of enrichment and 23 h later an aliquot of 60 ml of enrichment formula was added to the enrichment tank.

5.2.5 Sampling of larvae

Two sampling events of larval giant kōkopu took place in this experiment. The initial sampling took place on 27 July 2016 when larvae were 23 DAH, once larvae had been exposed to SP weaning diets for the first week to establish if initial weaning performance may set a foundation for subsequent outcomes. The second sampling was at the conclusion of the experiment on 9 September 2016 when larvae were 67 DAH to determine the overall outcome of the comparative weaning treatments.

At both sampling events, randomly sampled larvae were euthanized by placing in ice water (0 °C) for 20 mins, measured for total length (i.e., snout to tip of tail) and body depth (i.e., centre of body at the anus across to the dorsal surface) from each tank. Measurements were conducted by placing fish on 46 µm grid plastic sheets and photographing fish under a microscope using an Olympus TG-4 camera. Images were later processed using ImageJ (ver. 1.53, National Institutes of Health) to derive measurements.

For the initial sampling event from each tank three samples of 50 fish were taken at random by gently swirling the tank, collecting fish with a small jar and pouring through 300 µm mesh. Mean wet weight (WW) of fish was determined by weighing followed by mean dry weight (DW) after freeze drying, re-weighing and dividing by the total number of fish. These lyophilized samples were then used to determine total lipid and total protein content. A further 20 fish were randomly sampled from each tank to measure total length and body depth.

For the final sampling event 20 fish were randomly sampled to total length and body depth measurements. Due to reduced numbers of fish from mortality amongst all treatments early in the experiment resulting from stress

of transfer of larvae into the experimental tanks, three samples of 20 fish per sample were taken from each tank to undertake WW and DW measurements. These same sampled fish were then also lyophilized and used for protein and lipid analyses. The ORA treatment was an exception where one tank had sufficient numbers for only 20, 20 and 19 fish per replicate sample and another tank with only 15 fish per replicate sample. Unfortunately, it was not possible to accurately recover and record respective mortalities of larvae throughout the experiment.

5.2.6 Specific Growth Rate

The mean specific growth rate (SGR) for WW was determined for each treatment tank across the duration of the 44 day experimental period between initial and final sampling events using Equation 5-1 (Ricker, 1958).

Equation 5-1 $SGR = 100\ (e^{g} - 1)$

$g = (\ln m_2 - \ln m_1) / (t_2 - t_1)$

m2 = final wet weight

m1 = initial wet weight

t2 = number of days after hatching at final sampling

t1 = number of days after hatching at initial sampling

5.2.7 Lipid and Protein Composition

Lipid was extracted from larval fish samples using a modified Bligh and Dyer (Bligh & Dyer, 1959) solvent extraction method (Wang et al., 2013). A 1.9 ml aliquot of chloroform, methanol and deionized water mixture (ratios 2:1:0.4) was added to the lyophilized samples before being vortexed for 30 s and then left to stand for 16 h. An aliquot of 0.5 ml of 0.7 % sodium chloride and 0.5 ml of chloroform were added, followed by 30 s of vortexing, then centrifuging for 10 min at 1000 rpm. The chloroform-lipid layer was removed and placed in a pre-weighed glass vial. The residual layer was washed with 1 ml of chloroform, followed by 30 s vortexing, centrifuging for 10 min at 1000 rpm and the chloroform-lipid removed and added to pre-weighed glass vial. This step was repeated again using only 0.5 ml of chloroform. The glass vials were then placed in a thermal evaporator held at 39 °C under flowing nitrogen gas to remove the chloroform. The glass vials were then re-weighed to determine lipid mass which was then divided by the total larval sample dry mass and multiplied by 100 to provide lipid content as a percentage of dry weight (%DW). The total lipid (per larva) was determined by multiplying the lipid proportion (%DW) by the mean DW of individual larvae in the respective sample. For both the three replicate lipid proportion measures and the three replicate total lipid measurements per replicate tank were then used to determine the tank average, with the results from the three tanks per treatment averaged to give the treatment mean.

The protein content of larvae was measured using a bicinchoninic acid (BCA) assay (Micro BCA™ Protein Assay Kit, ThermoFisher Scientific). After removing the lipid content the residual larval tissues were freeze dried and ground before the addition of sodium hydroxide and incubation in a water bath at 50 °C for 16 h. Samples were diluted and then centrifuged at 4000 rpm for 10 mins at 4 °C. The resulting samples and a set of bovine serum albumin standards were placed into a 96 well-plate and reagents added to each well followed by reading absorbance at 562 nm. The protein content of the larvae was calculated using the standard curve as a percentage of dry weight (%DW). The mean total protein (per larva) was determined by multiplying the protein proportion (%DW) by the mean DW of larvae in the respective sample.

5.2.8 Fatty Acid Profiles

Fatty acid analyses were conducted on an aliquot of the total lipid previously extracted gravimetrically. The derivatization process was based on Lepage & Roy (Lepage & Roy, 1986). Laboratory controls were included during the derivatization process. This comprised a positive control containing 52 reference standards of FAs all with different concentrations and a negative control containing C19 and C23 FAs in the same concentration range as the samples. An extraction solution of 2 ml of methanol:toluene (4:1 v/v, Analytical Grade, Merck) containing internal standards (C19: nonadecanoic acid 0.083 mg ml^{-1} and C23: tridecanoic acid 0.082 mg ml^{-1}, Nu-Chek Prep., USA) was added to each sample and transferred to borosilicate tubes with Teflon-lined screw caps. Magnetic stirring bars were added to each tube. Acetyl chloride (200 ml, ECP) was added slowly, dropwise to each sample over a period of 1 min. The tubes were placed in a heating and stirring dry block at 100 °C for 1 h. After 1 h, the tubes were cooled in water and 5 ml of an aqueous solution of 6% potassium carbonate were added to each tube. The tubes were vortexed, then centrifuged at 3,500 g (5 min at room temperature). The upper toluene phase was recovered and transferred to a gas chromatography (GC) vial with an insert, and a further 25% dilution was done using toluene, for analysis by GC-mass spectrometry (GC–MS) at the Auckland Science Analytical Services, at the University of Auckland. GC–MS instrument parameters were based on Kramer et al. (Kramer et al., 2008). The instrument used was an Agilent 7890B gas chromatograph coupled to a 5977C mass spectrometer with a split/splitless inlet (Jeffs et al., 2002). A sample of 1 µl was injected using a CTC PAL autosampler into a glass 4 mm ID straight inlet liner packed with deactivated glass wool (Restek Sky®). The inlet temperature was 250 °C, in splitless mode, and the column flow was set at 1 ml min^{-1}, with a column head pressure of 62 kPa, giving an average linear velocity of 19 cm s^{-1}. Purge flow was set to 50 ml min^{-1} at 1 min after injection. Column selection was based on the recommendations from the official methods for the determination of trans fat (American Oil Chemists Society - (Mossoba & Kramer, 2009)). The column was a fused silica Rtx-2330, which was 100 m long, 0.25 mm internal diameter, 0.2 µm highly polar stationary phase (90% biscyanopropyl and 10% cyanopropylphenyl polysiloxane, Shimadzu). Carrier gas was instrument grade helium (99.99%, BOC). The GC oven temperature programming started isothermally at 45 °C for 2 min, increased by 10 °C min^{-1} to 215 °C, held for 35 min and then increased by 40 °C min^{-1} to 250 °C and held for 10 min. The transfer line to the mass spectrometric detector (MSD) was maintained at 250 °C, the MSD source at 230 °C and the MSD quadropole at 150°C. The detector was turned on 14.5 min into the run. The detector was run in positive-ion, electron-impact ionization

mode, at 70 eV electron energy, with electron multiplier set with no additional voltage relative to the autotune value. Data were acquired at 1,463 amu s^{-1} in scan mode from 41 to 420 amu, with a detection threshold of 100 ion counts. Resulting GC-MS peaks were identified on fatty acid methyl ester mass spectral library and each FA peak was quantified using an inhouse R package (RStudio, ver. 1.2.1335). The data were screened for chromatographic retention time drift, and manual correction/integration was carried out where necessary. The data set was normalized by the response of the internal standard (nonadecanoic acid), and a blank treatment was applied to correct the baseline response. The resulting normalized peak area values were used to quantify the total of each FA using linear calibration information obtained from seven calibration curve standards. The total amount of FA measured in the lipid aliquot was then adjusted for the total lipid extract to calculate the proportional contribution of each FA to the total FAMEs.

5.2.9 Statistical analyses

The initial and final mean DW, WW, total length, body depth, total lipid and protein, percent lipid and protein, fatty acids, and SGR were compared among treatments using ANOVA where parametric data assumptions were satisfied. Initial and final lipid and protein concentrations as well as fatty acid profiles and SGR were arc-sine transformed prior to analysis to correct for any data distribution bias associated with percentage data (Zar, 1999). Normality and equality of variance of data were tested and confirmed using the Shapiro-Wilk's and Levene's tests prior to analyses. Where data conformed to parametric assumptions a linear mixed model ANOVA was fitted to control the random effects of the tanks in each analysis. When ANOVA identified overall experimental treatment effects the differences between pairs of individual means were identified with a Tukey's test with adjustment for false discovery. Estimated mean difference and 95 % confidence intervals were calculated and are presented.

Data requirements for performing parametric tests were not met for the mean final total length and depth variables for giant kōkopu larvae. Consequently, Kruskal-Wallis tests were used to compare these data and where significant differences were found, then Mann-Whitney-Wilcox post-hoc comparison tests were used to compare means between treatment groups.

All statistical analyses were performed using R (RStudio, ver. 1.2.1335). All measures of variability of sampled means is reported as standard error of the mean.

5.3 Results

5.3.1 Weight

There was no difference among treatments for mean initial WW of larval fish ($F_{(2,6)}$= 0.38, P= 0.70); i.e., OTO 7.45 ± 0.13 mg, ART 7.52 ± 0.24 mg and ORA 7.21 ± 0.10 mg (Figure 5-1). However, at the final sampling the mean WW of the fish was different among the treatments ($F_{(2,6)}$= 15.99, P< 0.01). Fish in the OTO treatment had a greater mean final WW (73.23 ± 3.14 mg), between 12.70 and 34.60 mg greater than ART (49.58 ± 2.15 mg, P< 0.01), and

between 19.10 and 41.00 mg greater than ORA (43.16 ± 1.74 mg, P< 0.01) (Figure 5-1). The mean final WW of the ART and ORA treatment groups were not different (P= 0.30).

The mean initial DW of the fish larvae was not different among treatments ($F_{(2,6)}$= 1.03, P= 0.41), OTO 1.39 ± 0.03 mg, ART 1.32 ± 0.03 mg and ORA 1.34 ± 0.02 mg (Figure 5-1). However, at the final sampling there were significant differences among treatments for DW ($F_{(2,6)}$= 20.84, P< 0.01). OTO mean final DW (15.05 ± 0.62 mg), was between 0.26 and 0.59 times greater than ART (9.83 ± 0.42 mg, P< 0.01), and between 0.35 and 0.69 times greater than ORA (8.95 ± 0.37 mg, P< 0.01). There was no significant difference in the mean final DW between ART and ORA (P= 0.32) (Figure 5-1).

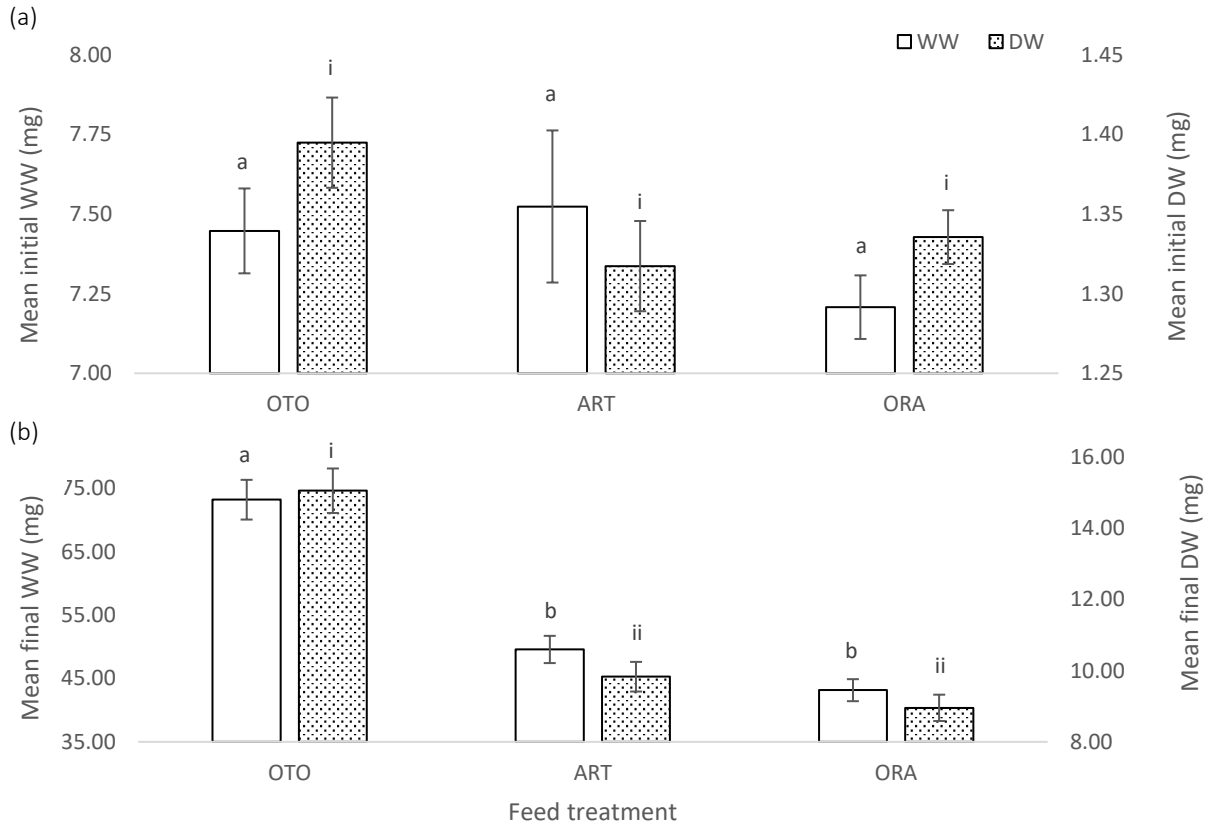

Figure 5-1. **(a)** Mean initial wet weight (WW) and dry weight (DW) for larval giant kōkopu from three different feed treatments; OTO (Otohime), Artemac (ART) and ORA (O.range) (mean ±SE). Means with different superscripts are significantly different among treatments for WW and among treatment for DW (P<0.05). **(b)** Mean final wet weight (WW) and dry weight (DW) for larval giant kōkopu from three different feed treatments; OTO (Otohime), Artemac (ART) and ORA (O.range) (mean ±SE). Means with different superscripts are significantly different among treatments for WW and among treatment for DW (P<0.05).

5.3.2 Length and Depth

At the initial sampling the total length of the giant kōkopu larvae was not significantly different among treatments ($F_{(2,6)}$= 0.11, P=0.90); i.e., OTO 15.05 ± 0.21 mm, ART 15.02 ± 0.21 mm and ORA 14.87 ± 0.18 mg (Figure 5-2). However, at final sampling the mean total length of larvae was different among treatments (X^2= 8.52, P= 0.01); i.e., OTO 27.97 ± 0.55 mm, ART 24.85 ± 0.44 mm and ORA 25.74 ± 0.58 mm (Figure 5-2). Mean final total length was greater in the OTO treatment than ART (P< 0.01), while there was no difference between OTO and ORA (P= 0.10), and between ART and ORA (P= 0.13).

The mean initial depth of the body of the larval fish was the same among treatments (i.e., 23 DAH) ($F_{(2,6)}$= 0.02, P= 0.98); i.e., OTO 1.09 ± 0.02 mm, ART 1.09 ± 0.02 mm and ORA 1.10 ± 0.02 mm (Figure 5-3). At the final sampling there was a significant difference in mean body depth among treatments (X^2= 22.08, P< 0.01); i.e., OTO 2.55 ± 0.06 mm, ART 2.11 ± 0.06 mm and ORA 2.23 ± 0.07 mm (Figure 5-3). Mean final total body depth was greater in the OTO treatment than both ART (P< 0.01) and ORA (P< 0.01). There was no difference in final total body depth between ART and ORA (P= 0.13).

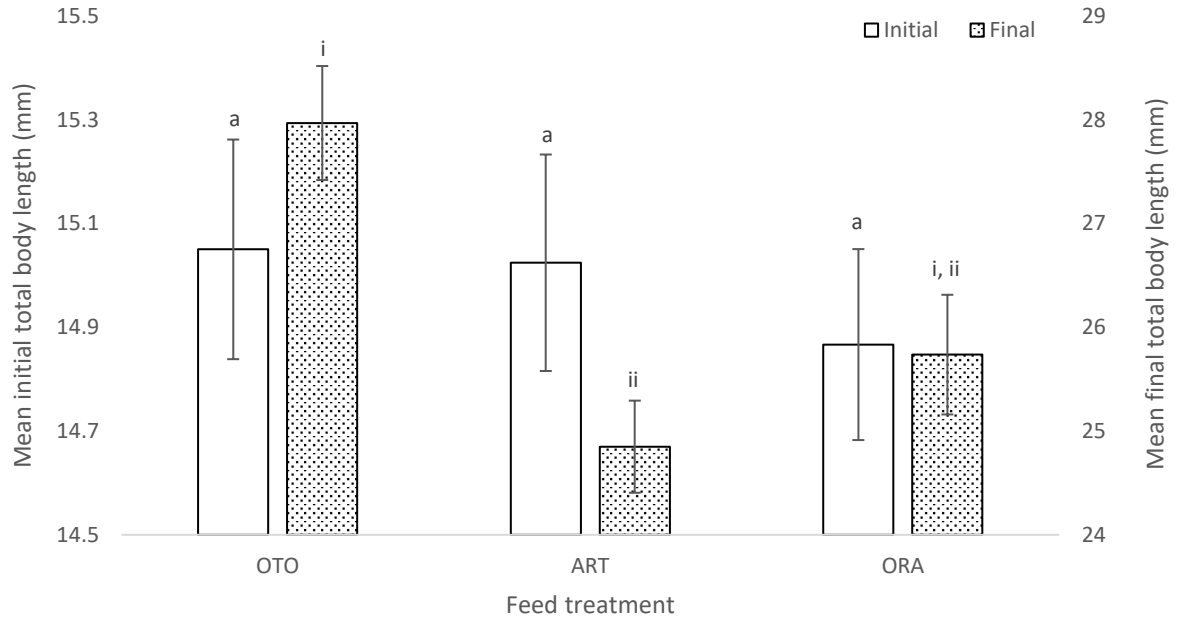

Figure 5-2. Mean initial and final total length of larval giant kōkopu from three different feed treatments; OTO (Otohime), Artemac (ART) and ORA (O.range) (mean ±SE). Means with different superscripts are significantly different among treatments for mean initial total length and among treatments for mean final total length (P<0.05).

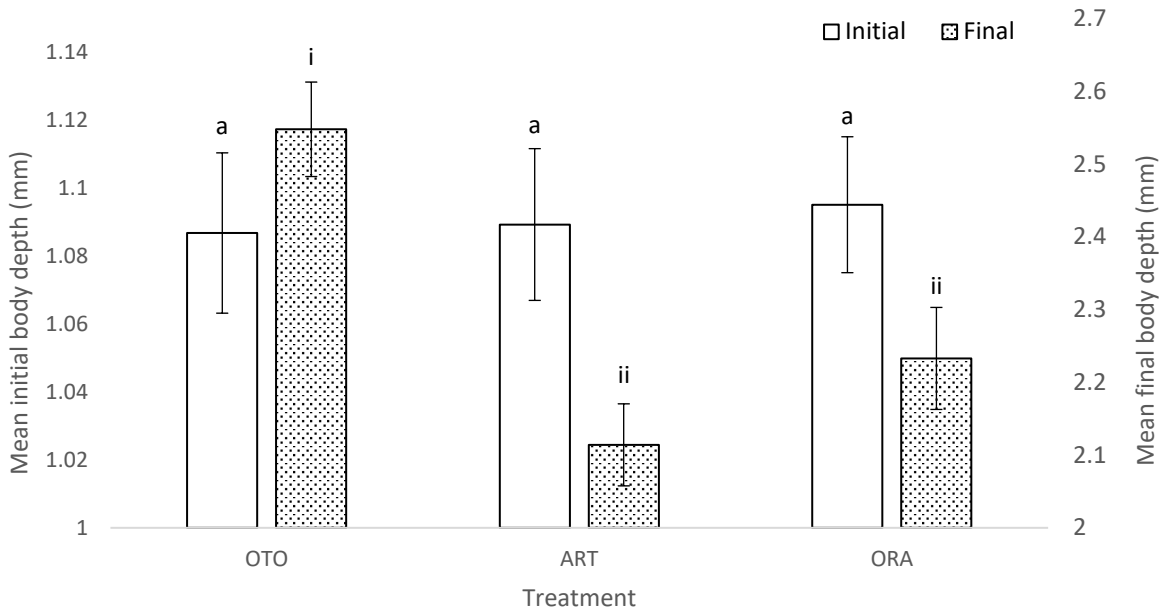

Figure 5-3. Mean initial and final body depth for larval giant kōkopu in three different feed treatments; OTO (Otohime), Artemac (ART) and ORA (O.range) (mean ±SE). Means with different superscripts are significantly different among treatments for mean initial body depth and among treatments for mean final body depth (P<0.05).

5.3.3 Specific Growth Rate

There was a significant difference in the SGR among the three feed treatments ($F_{(2,6)}$= 30.09, P< 0.01). The OTO treatment achieved a greater SGR, 5.18 ± 0.10 %, than both ART, 4.41 ± 0.06 % (P< 0.01) and ORA, 4.10 ± 0.09 % (P< 0.01) (Figure 5-4). No difference was found in the SGR between ORA and ART treatments (P= 0.20).

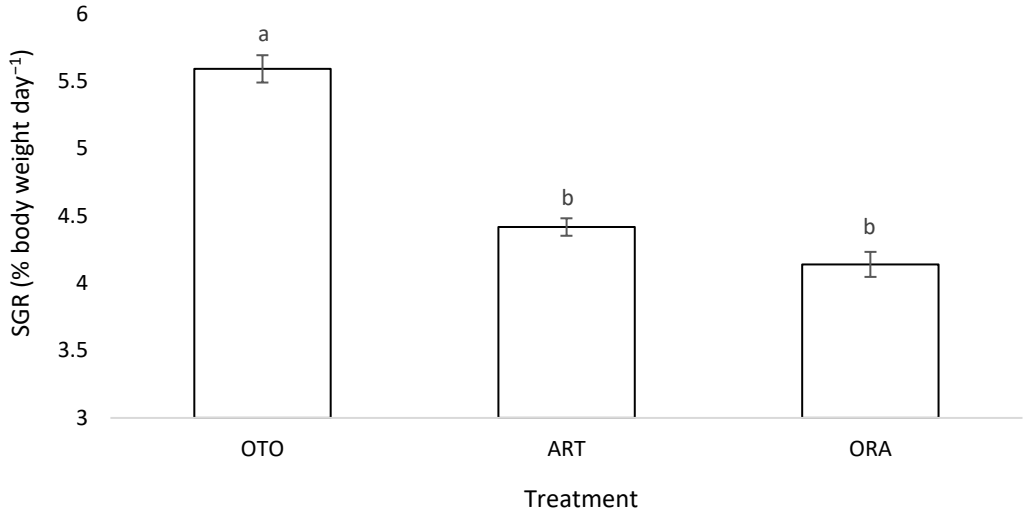

Figure 5-4. Mean specific growth rate (SGR) for larval giant kōkopu for three different feed treatments; OTO (Otohime), Artemac (ART) and ORA (O.range) (mean ±SE). Means with different superscripts are significantly different (P<0.05).

5.3.4 Lipid and Protein

At the initial sampling there was no difference in proportional lipid content of the larvae among the three feed treatments ($F_{(2,6)}$= 1.30, P= 0.34); i.e., OTO 16.1 ± 0.2 %DW, ART 15.8 ± 0.2 %DW and ORA 16.0 ± 0.4 %DW (Figure 5-5). There was no significant difference in proportional lipid content of the larvae among treatments at the final sampling ($F_{(2,6)}$= 0.21, P= 0.82); i.e., OTO 17.0 ± 0.6 %DW, ART 15.5 ± 0.8 %DW and ORA 16.7 ± 0.6 %DW (Figure 5-5).

There was also no difference in total lipid among treatments at the initial sampling ($F_{(2,6)}$= 3.08, P= 0.12); i.e., OTO 0.24 ± 0.00 mg, ART 0.20 ± 0.00 mg and ORA 0.22 ± 0.00 mg (Figure 5-6). However, there was a significant difference in mean total lipid among the three feed treatments at the final sampling event ($F_{(2,6)}$= 17.72, P< 0.01); i.e., OTO 2.43 ± 0.10 mg, ART 1.56 ± 0.05 mg and ORA 1.43 ± 0.01 mg (Figure 5-6). OTO accumulated between 0.51 and 1.23 mg more lipid than ART (P< 0.01) and between 0.64 and 1.35 mg more lipid than ORA (P< 0.01). There was no difference in the total lipid content of larvae between the ART and ORA treatments (P> 0.1).

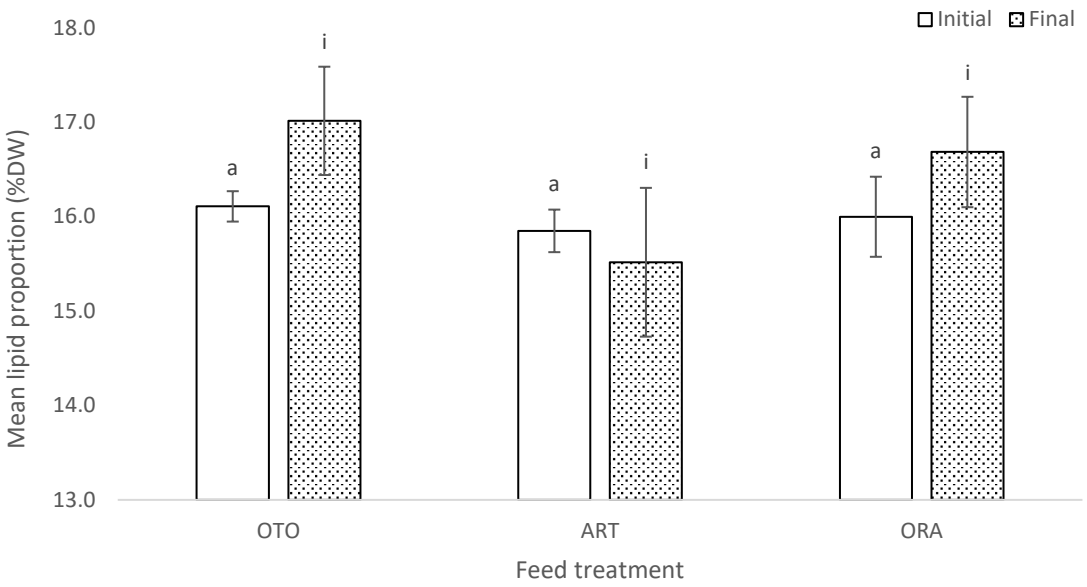

Figure 5-5. Mean proportional lipid content of larval giant kōkopu as a percentage of dry weight (DW) for at the initial and final sampling events for three different feed treatments; i.e., OTO (Otohime), Artemac (ART) and ORA. There were no significant differences among mean lipid proportions for the three different treatments, for either initial or final sampling.

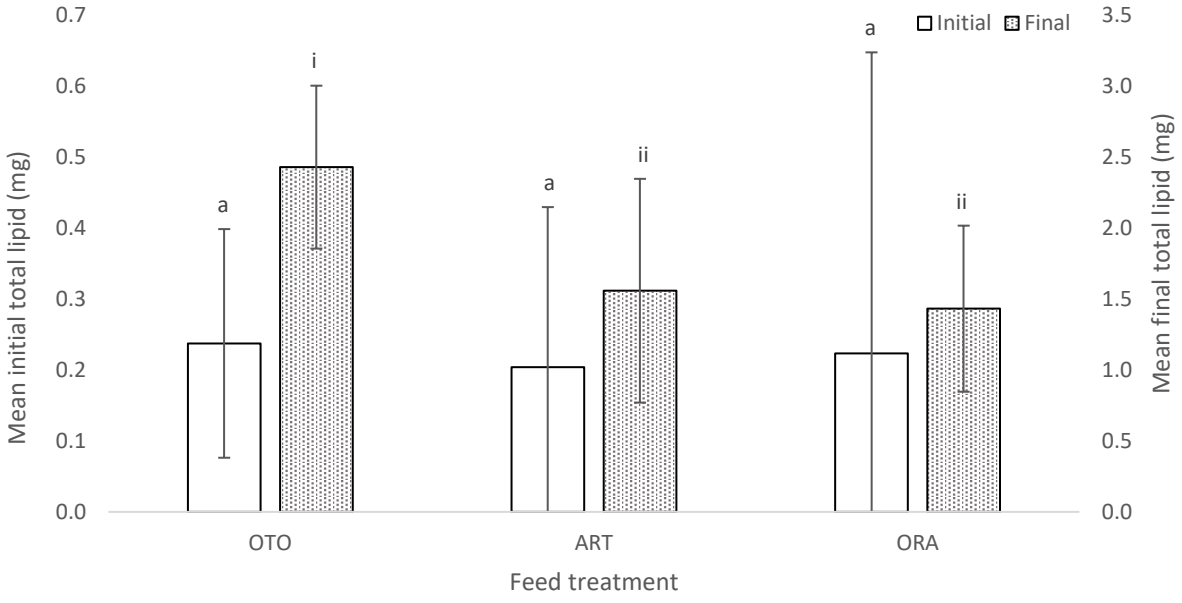

Figure 5-6. Mean initial and final total lipid for larval giant kōkopu in three different feed treatments; i.e., OTO (Otohime), Artemac (ART) and ORA (O.range) (mean ±SE). Means with different superscripts are significantly different within each of the set of three treatment means for each sampling event ($P<0.05$).

At the initial sampling there was no difference in the proportional protein content of the larval giant kōkopu among the three feed treatments ($F_{(2,6)}$= 2.29, P= 0.18), i.e., OTO 76.8 ± 2.3 %DW, ART 77.7 ± 1.5 %DW and ORA 72.0 ± 1.6 %DW (Figure 5-7). Likewise, there was no difference in the proportional protein content of larvae among the three feed treatment groups at the final sampling ($F_{(2,6)}$= 0.22, P= 0.81); i.e., OTO 73.5 ± 2.5 %DW, ART 77.6 ± 4.7 %DW and ORA 73.8 ± 1.9 %DW (Figure 5-7).

There were no differences among treatments in the mean initial total protein of the larvae ($F_{(2,6)}$= 1.17, P= 0.37); i.e., OTO 1.07 ± 0.04 mg, ART 1.03 ± 0.03 mg and ORA 0.96 ± 0.03 mg (Figure 5-8). However, there was a significant difference in the total protein of larval fish at the final sampling ($F_{(2,6)}$= 13.82, P< 0.01) (Figure 5-8). The mean final total protein for OTO was 10.65 ± 0.67 mg, between 1.92 and 5.18 mg more than for ART (7.42 ± 0.11 mg, P= 0.01), and between 2.45 and 5.98 mg more than for ORA (6.77 ± 0.17 mg, P= 0.01) (Figure 5-8). There was no difference in mean final total protein between ART and ORA (P= 0.50) (Figure 5-8).

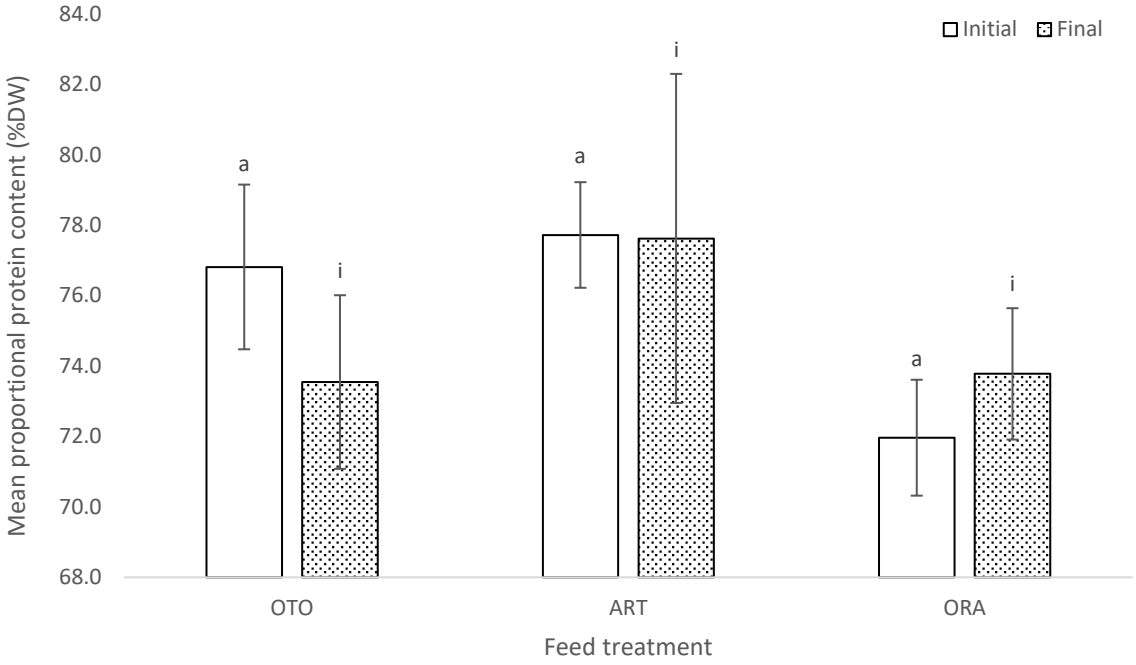

Figure 5-7. Mean proportional protein content measured as a percentage of dry weight (DW) for larval giant kōkopu at initial and final sampling for three feed treatments; i.e., OTO (Otohime), Artemac (ART) and ORA (O.range) (mean ±SE). There were no differences detected among treatment means for either initial or final sampling.

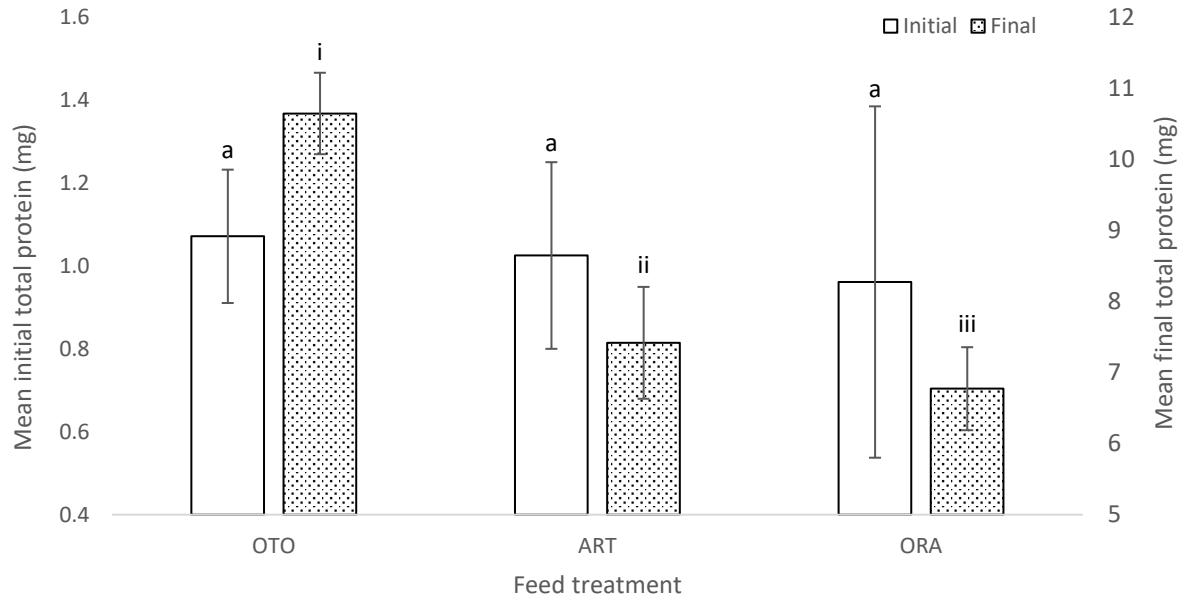

Figure 5-8. Mean initial and final total protein content of larval giant kōkopu for three feed treatments; i.e., OTO (Otohime), Artemac (ART) and ORA (O.range) (±SE). Means with different superscripts are significantly different within each of the set of three treatment means for each sampling event (P<0.05).

5.3.5 Fatty Acid Profiles

Thirty one fatty acids were identified across the three treatment groups with 18 fatty acids being present as >1% of total fatty acids (Table 5-3).

For the initial sampling of larvae at the outset of the experiment, there were no differences for the proportion of any individual fatty acid detected among treatments.

For the final sampling the proportions of some fatty acids differed among treatments. ARA ($F_{(2,6)}$= 6.15, P= 0.04) was higher in ORA (1.33 % ± 0.03) than OTO (1.11 % ± 0.03, P= 0.04) but not greater than in ART (1.27 % ± 0.05, P= 0.41), while ART did not have a higher proportion of ARA than OTO (P= 0.07). EPA ($F_{(2,6)}$= 56.21, P< 0.01) was higher in OTO (10.08 % ± 0.08) than ART (7.73 % ± 0.05, P< 0.01) and ORA (7.55 ± 0.06 %, P< 0.01) (Table 5-3). DHA ($F_{(2,6)}$= 21.37, P< 0.01) was proportionately higher in larvae from the ORA treatment (14.86 % ± 0.11) compared to ART (11.00 ± 0.15, P< 0.01) and OTO (11.07 % ± 0.08, P< 0.01) (Table 5-3).

Larvae in the ORA treatment had a higher proportion of PUFA ($F_{(2,6)}$= 58.55, P< 0.01) (48.19 % ± 0.12) than OTO (40.09 % ± 0.08, P< 0.01) and ART (37.8 % ± 0.08, P< 0.01) (Table 5-3). Likewise, HUFA ($F_{(2,6)}$= 19.13, P< 0.01) were proportionately more abundant in ORA (26.66 % ± 0.12), followed by OTO (24.76 % ± 0.08, P< 0.05) and ART (22.11 % ± 0.11, P< 0.01) (Table 5-3).

Ratios of EPA, DHA and ARA were different among treatments EPA:ARA ($F_{(2,6)}$= 66.21, P< 0.01) and DHA:APA ($F_{(2,6)}$= 62.97, P< 0.01), but not differ for DHA:ARA EPA ($F_{(2,6)}$= 4.14, P= 0.07) (Table 5-3). OTO had the highest EPA:ARA

ratio (9.15 % ± 0.11), greater than that of ART (6.13 % ± 0.07) (P< 0.01) and ORA (5.69 % ± 0.03) (Table 5-3). ORA DHA:EPA (1.97 % ± 0.03) was greater than OTO (1.10 % ± 0.04) (P< 0.01) and ART (1.43 ± 0.06) (P< 0.01) (Table 5-3).

Table 5-3. Mean initial and final percent fatty acid composition (±SE) of larval giant kōkopu for three feed treatments. Only fatty acids that are present at >1% are included, "Other" is the sum of all other fatty acids present at <1% (12:0, 15:0, 16:0, 16:1n-7t, 17:1-7c, 18:1n-9t, 18:3n-6c, 20:0, 20:2n-6c, 20:3n-6c, 21:0, 22:0, 24:0, 24:1n-9c). Different superscripts indicate significant differences along the row. There were no differences among initial fatty acids. ARA: arachidonic acid; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; PUFA: polyunsaturated FA; HUFA: highly- unsaturated FA.

		Initial			Finish		
FA		**Otohime**	**Artemac**	**O.Range**	**Otohime**	**Artemac**	**O.Range**
C14:0		1.35 ± 0.06	1.33 ± 0.07	1.48 ± 0.06	7.34 ± 0.12 ^{a}	5.47 ± 0.11 ^{b}	3.56 ± 0.08 ^{c}
C16:0		25.92 ± 0.11	24.7 ± 0.14	25.48 ± 0.1	26.82 ± 0.19 ^{a}	25.80 ± 0.16 ^{a}	25.22 ± 0.09 ^{a}
C16:1n-7c		1.56 ± 0.03	1.60 ± 0.04	1.72 ± 0.02	2.22 ± 0.08 ^{b}	3.48 ± 0.04 ^{a}	1.55 ± 0.01 ^{c}
C17:0		1.22 ± 0.04	1.15 ± 0.05	1.23 ± 0.04	2.18 ± 0.10 ^{a}	1.07 ± 0.10 ^{b}	0.90 ± 0.04 ^{b}
C18:0		12.95 ± 0.12	12.15 ± 0.13	12.85 ± 0.16	6.33 ± 0.11 ^{a}	7.24 ± 0.14 ^{a}	7.73 ± 0.11 ^{a}
C18:1n-7t		3.06 ± 0.07	2.93 ± 0.07	2.88 ± 0.04	1.14 ± 0.10 ^{a}	1.22 ± 0.16 ^{a}	0.90 ± 0.06 ^{a}
C18:1n-9c		11.93 ± 0.06	12.32 ± 0.07	12.06 ± 0.08	7.97 ± 0.08 ^{b}	9.64 ± 0.11 ^{a}	7.40 ± 0.04 ^{b}
C18:2n-6c		4.07 ± 0.03	4.17 ± 0.03	4.15 ± 0.02	3.47 ± 0.03 ^{c}	4.31 ± 0.03 ^{b}	7.27 ± 0.03 ^{a}
C18:2n-6t		4.19 ± 0.03	4.30 ± 0.03	4.29 ± 0.02	3.60 ± 0.03 ^{c}	4.46 ± 0.03 ^{b}	7.43 ± 0.03 ^{a}
C18:3n-3c		17.62 ± 0.15	17.93 ± 0.14	16.69 ± 0.15	2.11 ± 0.18 ^{a}	1.85 ± 0.03 ^{a}	2.10 ± 0.03 ^{a}
C20:1n-9c		0.41 ± 0.01	0.44 ± 0.03	0.45 ± 0.01	2.04 ± 0.13 ^{a}	2.32 ± 0.20 ^{a}	0.74 ± 0.05 ^{b}
C20:4n-6c	ARA	1.84 ± 0.03	2.00 ± 0.04	1.95 ± 0.04	1.11 ± 0.03 ^{b}	1.27 ± 0.05 ^{ab}	1.33 ± 0.03 ^{a}
C20:5n-3	EPA	4.28 ± 0.06	4.63 ± 0.07	4.36 ± 0.06	10.08 ± 0.08 ^{a}	7.73 ± 0.05 ^{b}	7.55 ± 0.06 ^{b}
C22:1n-9c		0.34 ± 0.03	0.33 ± 0.02	0.33 ± 0.02	0.96 ± 0.13 ^{b}	2.02 ± 0.21 ^{a}	0.73 ± 0.04 ^{b}
C22:2n-6c		2.82 ± 0.04	3.07 ± 0.05	2.99 ± 0.03	5.14 ± 0.08 ^{a}	4.36 ± 0.05 ^{b}	4.19 ± 0.03 ^{b}
C22:4n-6c		1.04 ± 0.02	1.13 ± 0.03	1.09 ± 0.03	0.62 ± 0.02 ^{a}	0.48 ± 0.02 ^{b}	0.47 ± 0.01 ^{b}
C22:5n-3c		0.48 ± 0.02	0.54 ± 0.03	0.54 ± 0.03	1.77 ± 0.04 ^{b}	1.51 ± 0.03 ^{c}	2.33 ± 0.03 ^{a}
C22:6n-3c	DHA	0.41 ± 0.05	0.71 ± 0.25	0.68 ± 0.16	11.07 ± 0.08 ^{b}	11.00 ± 0.15 ^{b}	14.86 ± 0.11 ^{a}
Other		4.54 ± 0.05	4.57 ± 0.05	4.78 ± 0.05	4.05 ± 0.12 ^{a}	4.77 ± 0.11 ^{a}	3.73 ± 0.05 ^{a}
Sum PUFA		37.98 ± 0.13	39.86 ± 0.18	38.18 ± 0.16	40.09 ± 0.08 ^{b}	37.80 ± 0.08 ^{b}	48.19 ± 0.12 ^{a}
Sum HUFA		8.27 ± 0.06	9.26 ± 0.15	8.88 ± 0.12	24.76 ± 0.08 ^{b}	22.11 ± 0.11 ^{c}	26.66 ± 0.12 ^{a}
EPA:ARA		2.32 ± 0.02	2.31 ± 0.01	2.23 ± 0.01	9.15 ± 0.11 ^{a}	6.13 ± 0.07 ^{b}	5.69 ± 0.03 ^{b}
DHA:EPA		0.1 ± 0.03	0.15 ± 0.10	0.15 ± 0.07	1.10 ± 0.04 ^{c}	1.43 ± 0.06 ^{b}	1.97 ± 0.03 ^{a}
DHA:ARA		0.23 ± 0.05	0.34 ± 0.16	0.34 ± 0.11	10.10 ± 0.16 ^{a}	8.79 ± 0.18 ^{a}	11.21 ± 0.10 ^{a}

5.4 Discussion

The results from this experiment demonstrate the effectiveness of the artificial dry food (Otohime) for the rearing of larval giant kōkopu compared to two other commercially available artificial dry foods, ART and ORA. The OTO treatment produced giant kōkopu larvae with the greatest WW, DW, SGR, total body length and depth when

compared to the ART and ORA treatments. The artificial dry food treatments did not have any influence on the proportions of either lipid or protein of the larvae at the end of the experiment. However, both the total lipid and protein content of the larvae were greater for the OTO treatment as a result of the larger overall size of the fish, than for fish provided with the ART and ORA feed treatments. At the end of the experiment there were no differences in any of the morphometric or biochemical parameters between the larvae provided with the ART and ORA treatments.

The intake of food particles is a crucial determining factor of the suitability of a feed products for larval fish and is affected by several characteristics. Perceptibility, capture/handling and palatability can impact the intake and the effectiveness of a feed item on the growth performance of larval fin fish (Holt, 2011). Despite similar size and colour of the different feed particles, perceptibility may have impacted on growth performances among the different dietary treatments in this experiment. The visual attractiveness of the feed, the speed at which feed particles sink through the water column of the tank and any chemical attractant can influence differences in the feeding response by the larvae (Langdon & Barrows, 2011). Larval eyes are pigmented at hatch in giant kōkopu and relative eye size throughout the first 77 DAH of development indicate have good visual acuity (McKay & Jeffs, 2021). However, weaning experiments show artificial feed intake is very low in the first 21 DAH indicating that these larvae are not able to recognize these feed particles (McKay & Jeffs, 2022c). Given that the current experiment included a weaning period, the non-nutritional characteristic of the artificial feed products used will have had an impact on the growth performance of larvae, potentially to the benefit of the darker coloured and wider size range of Otohime. Future experiments should seek to separate out the weaning period and the artificial only period so as to determine both the best feeds by non-nutritional and nutritional characteristics and include larger feed particle items which giant kōkopu larvae have proven well adept at capturing (McKay & Jeffs, 2021, 2022c). The incremental increases in feed particle sizes and rates of water flow during the experiment, although kept consistent among all treatments, may have influenced food availability through affecting the period that food particles remained in suspension and available for consumption. The density and sinking rate of feed particles and their suspension via water turbulence are collectively important factors that influence feeding intake but are difficult to measure in practice (Kato et al., 2012; Rønnestad et al., 2013).

Nutritional value is a key consideration in the selection of feed in fin fish aquaculture and is likely to have influenced the variation in growth performance amongst the feed treatments in this experiment. Formation of musculature accounts for the majority of mass increase in larval fish with dietary protein providing the amino acids required for muscle construction (Qin, 2008). Dietary lipid is thought to be the primary source of energy, which is in significant demand in rapidly developing larval fish (Qin, 2008). In order to realize maximal growth rates the optimum balance between the two macronutrients must be achieved (Lucas & Southgate, 2012). The protein to energy ratio (P:E) is species-specific, with diets providing P:E ratios either side of the optimum will typically result in reduced growth. A low P:E can incur inadequate protein intake because consumption is also regulated by energetic requirements (Csargo et al., 2013; Webster & Lim, 2001), while excessive dietary P:E lacks the energy required for catabolic and anabolic activity (Csargo et al., 2013; Goddard, 1996; Webster & Lim, 2001). Despite the OTO feed treatment containing the lowest total protein (i.e., 51-51 % versus 57 % for ORA and 56 %

for ART) it is possible that the 123 mg $kcal^{-1}$ P:E is most suitable or more readily available to meet the requirements of larval giant kōkopu as this treatment group achieved the greatest final mass and total protein. The ART diet has a lower P:E of 113 mg $kcal^{-1}$ and corresponding with lower growth in the larvae. However, the ORA diet has a similar P:E (i.e., 124 mg $kcal^{-1}$) to that of OTO, but resulted in significantly lower growth performance. This outcome exemplifies the difficulty of larval fin fish diet selection due to the complex and interacting factors characteristics of aquaculture feeds where proximate analyses and ratios alone cannot be used in isolation to determine the optimum larval diet.

Generally, the fatty acid composition of a fish is a reflection of its diet (Salhi & Bessonart, 2012; Turchini et al., 2010; Watanabe et al., 2016; Xu et al., 2020). However, digestive capability in larval fin fish is generally poor, limited by the lack of development of organs and physiological activities required to break down feed into usable nutrients (Cousin et al., 1987; Kvåle et al., 2007; Nordgreen et al., 2009). Digestibility is fundamental to the transformation of food into utilizable nutrients and subsequently biomass and it is likely that this has had a material impact on the growth performance of larvae in this trial.

The delivery of sufficient essential PUFAs through the diet is critical during larviculture because they are utilized in tissue construction, especially for vital nervous and optic tissues (Izquierdo, 1996; Sargent et al., 2003). The levels of EPA, DHA and ARA in each of the treatment diets did not always correlate directly with levels of accumulation in larval tissues. Otohime and O.range have equally high levels of DHA in the feed particles but larvae in the ORA treatment accumulated more DHA as a proportion of total fatty acids. Although Otohime has almost twice as much DHA as Artemac, larvae from the OTO and ART treatments accumulated similar relative levels of this fatty acid. Subsequently, DHA was likely not the limiting factor to growth in these feed treatment. However, for each treatment Final DHA levels were two orders of magnitude greater than respective Initials indicating the demand and possible lack of DHA in the early diet which largely consisted with *Artemia* nauplii. This is consistent with earlier studies where DHA appears to be absent from these live feeds and a limiting factor to growth (McKay & Jeffs, 2022c). Furthermore, it has been highlighted that, current weaning protocols in the commercial hatchery have little effect on growth performance, and that weaning could be undertaken earlier as a result may in fact also reflect the improved nutritional provision of the artificial over the live feeds (McKay & Jeffs, 2021, 2022c).

ARA content in the three larval diets is an order of magnitude lower in the diet than EPA and DHA, and was accumulated in larval tissues to a similarly low degree. ARA is highest in O.range, of the three diets, with larvae in the ORA treatment accumulating a higher relative proportion of ARA in tissue when compared against the other treatments. Despite the importance of ARA to early development of larvae the differences in accumulated ARA within the tissues of the larvae of each treatment indicates that higher levels of ARA are not advantageous and that the levels found in the OTO diet appear to be sufficient (Bell et al., 1997; Sargent, Bell, et al., 1999). This is contrary to previous findings for other species where higher levels of ARA and lower ratios of EPA:ARA were advantageous, further indicating the likely critical importance of EPA to larval giant kōkopu development (Hauville et al., 2014; Sargent, et al., 1999; Tocher, 2003). Otohime has the highest EPA proportion of the three diets tested

and larvae in the OTO treatment accumulated the highest levels of EPA relative to total fatty acid content. Both Artemac and O.range have similar, low levels of EPA (roughly half that of Otohime) and EPA accumulated in larvae was equal at the end of the experiment for the latter two treatments. Furthermore, the larvae fed the O.range diet which has an equal proportion of DHA to Otohime and slightly more ARA, performed as poorly as larvae fed Artemac which has much lower levels of each of these fatty acids. These observations are critical as they highlight the importance of high levels of EPA to giant kōkopu growth performance, as has been noted in many other species (Bell et al., 1995; Boglino et al., 2012; Izquierdo, 1996; Izquierdo & Koven, 2011; Koven et al., 2012; Sargent et al., 2003).

Although not measured in this study, the leaching of nutrients from feed particles (i.e., the loss of water soluble compounds out of feeds) can have negative implications for the performance of the feed for provisioning for fish growth. Upon introduction of feed particles to rearing tanks, any rapid leaching of water soluble compounds represents a subsequent loss of the nutritional value of subsequently consumed feed particles to the larvae. Due to high surface area to volume ratio of larval fish microdiets as much as 50 – 95 % of free amino acids and protein hydrolysates can be lost through leaching within minutes of being fed into water (Baskerville-Bridges & Kling, 2000; Hamre et al., 2013; Kvåle et al., 2006; Nicklason & Johnson, 2008; Nordgreen et al., 2009; Önal & Langdon, 2005; Yúfera et al., 2002). Any differences in nutrient leaching of the feeds among the three treatments may have resulted in differing biomass growth rates between feed treatments with the OTO treatment potentially being more stable and therefore better able to provide nutrients to larvae after their consumption. A further study should determine the differences in the nutrient leaching among these three weaning diets.

The experimental set up may have had an impact on the performance on the artificial feeds due to stress caused due to initially transferring larvae from the commercial tanks in which they hatched. The majority of mortality occurred in the first 48 h after larvae were transferred into the experimental tanks as has been previously observed in prior experiments with this species (McKay & Jeffs, 2022b). Furthermore, recent studies have shown that the predominance of instar-I Artemia is not optimal as a first feed for giant kōkopu larvae, potentially compounding the post-transfer stress (McKay & Jeffs, 2022b). As such, the larvae that survived and became the subjects of this study may not best represent the performance of larvae in the commercial scale setting where the transfer in particular does not occur.

Given the scale of the experimental systems water quality was easily managed, maintaining very low ammonia levels which may not be possible at commercial scale. Closely related species Galaxias maculatus and Galaxias fasciatus have demonstrated tolerance to ammonia toxicity 1.47 and 0.80 mg/L respectively, however, future experiments on this species should confirm the species-specific optimum ranges for giant kōkopu (Richardson, 1997). Furthermore, to ensure the most commercially relevant information is attained, this should focus on the early larval rearing stage (van der Meeren & Mangor-Jensen, 2020; Wang et al., 2015). A critical learning from this experiment is that the use of full commercial scale larviculture systems would be advantageous for future experimentation to avoid any unnecessary impact on larvae performance. As previously mentioned, larval performance may have been impacted in this study through initial handling for tank transfers, low quality live

feeds as well as the small experimental scale for the tank experiment. The latter of which can impact water flow dynamics, affecting larvae behaviour, as well as water quality as a result of RAS performance, all of which is likely to impact the performance of the larvae themselves (Davidson & Summerfelt, 2004; Espmark et al., 2017; Holan et al., 2020; Oppedal et al., 2011). These variations need to be removed in order to attain the most commercially relevant information possible.

This study has confirmed marked differences in performance of larval giant kōkopu fed with different commercially available feeds and that examining the differences among the nutritional status of the resulting fish and that of their diet can provide insights into basis of the observed differences. EPA appears to be more critical than DHA and ARA while a high P:E ratio is also advantageous. There are still several key aspects for future research which would lead to better understanding of the variation among these artificial diets and consequently their suitability as larval giant kōkopu diets. Nutrient leaching rate on rehydration of diets may impact whether key nutrients are still available to larvae when consumed. Furthermore, understanding differences in settling rates among diets may help to identify the characteristics of artificial diets which initiates a feeding response from larval giant kōkopu. Analyses should also be undertaken to better understand the digestive capabilities of these larvae. Furthermore, additional variables should be examined, particularly mortality rates, given the impact this has on total productivity of a larviculture system. However, the present study enables a baseline for a suitable diet from which future studies can build upon to further optimize growth performance and survival from dietary provisions. Given the paucity of information on galaxiid species in aquaculture more generally, these data should also act to inform the development of techniques for closely related species globally.

6 Chapter 6: General Discussion

Successful aquaculture ventures require sound knowledge of key biological and production metrics of the cultured species, such as nutritional requirements, growth morphometrics and survival. These data inform management decisions for optimising productivity and maximizing financial returns (Ernst et al., 2000; Khan, 2015). In the commercialisation of new aquaculture species this information is often lacking, and such is the case for giant kōkopu. Therefore, it was the purpose of the research presented in this thesis to generate knowledge for improving the production performance and financial viability of culturing giant kōkopu larvae.

The lack of knowledge of the morphological ontogeny of giant kōkopu larvae and the effectiveness of feeding regimes was the focus for the initial research presented in this thesis. In summary, the research describes the larval development of giant kōkopu during the production period from hatching through to 77 days after hatching when commercial harvesting normally occurs (Chapter Two). This research includes a description of the ontogenetic changes in the important morphometric characteristics of total length and depth, mass, mouth gape and eye size. This information provides a baseline for growth of larval giant kōkopu through the aquaculture production cycle and affords new insights into the larval feeding abilities. The research also compared the growth performance and survival of larvae fed different early live feed and artificial diets to assess the potential for changes in these feeding regimes to improve production efficiency (Chapters Three and Four). Finally, the research examined the feeding ability of giant kōkopu larvae on live and artificial diets during the weaning process (Chapter Five). Collectively, the research findings presented in these three chapters greatly increase our understanding of larval giant kōkopu feed intake and nutritional requirements, from a starting point of almost no information whatsoever.

6.1 Contribution of the Research to Knowledge on Larval Development

There are published descriptions of the morphological changes during the larval development of several New Zealand galaxiid species including īnanga (Benzie, 1968b; Mitchell, 1989), however, the larval development of giant kōkopu has remained undescribed. The current research indicates the larval development in this species differs markedly from other species in the genus. For example, the key morphometric character of mouth gape is similar for giant kōkopu and īnanga at hatching (i.e., 348 µm and 343 µm respectively), however, by 77 DAH giant kōkopu gape is almost three times greater (Chapter Two; Mitchell, 1989). The importance of this in an aquaculture context highlights the need for studies such as this one. Even closely related species cannot be relied on for the designing of larviculture protocols, instead, research at the species-specific level is required to optimise productivity.

6.2 Contribution of the Research to the Development of Larval Feeding Regimes

The timing of the provision of first feeding in larval fish culture is critical as any delays will detrimentally affect the subsequent growth performance and survival of the fish (Blaxter & Hempel, 1963; McGurk, 1984; Peña & Dumas,

2005; Shan et al., 2009; Sulaeman & Fotedar, 2017; Zhang et al., 2009). During the first 7 DAH the giant kōkopu larvae achieved relatively low increases in wet weight. The yolk sac and oil globule reserves are severely depleted prior to the introduction of first feeds to the larviculture system at the end of 3 DAH. This is an indication that the current feeding protocol is likely to be introducing the initial live feed too late. First feeds should be introduced prior to 3 DAH to provide an extended opportunity for larvae to transition, an approach which has been used to good effect for the larval culture other fish species (Huang et al., 2005; Shan et al., 2009; Yu et al., 2003). Furthermore, the larvae of closely related galaxiid species have been observed feeding within two days post hatch when half of the yolk sac was still present (Benzie, 1968d), while other galaxiid species seldom survive starvation of 48 h in captivity and therefore in the wild they are likely to be utilising exogenous feed prior to this time (Mitchell, 1989).

Future research should examine the potential benefits of providing giant kōkopu larvae with live feeds beginning at 1, 2 and 3 DAH and continuing for a period of at least two weeks to provide an effective comparison of growth performance and survival. This would identify the optimum productivity and most cost-efficient method for the initiation of feeding in giant kōkopu.

The significant majority of fin fish larvae require live first feeds because the movement of the live feed triggers a predatory response (Conceição et al., 2010; Holt, 2011; Pousão-Ferreira et al., 2003; Qin, 2008). The selection of an appropriately sized live feed item is critical as larvae must consume these organisms whole, therefore, mouth gape is a critical determining factor in whether larval fish are capable of consuming a feed item (Arts & Evans, 1987; Bremigan & Stein, 1994; Cunha & Planas, 1999; Krebs & Turingan, 2003; Makrakis et al., 2008; Zaret, 1980). Mouth gape has not previously been measured for giant kōkopu, with only one previous study observing gape for the closely related īnanga (Mitchell, 1989). The disparity in the mouth gape of these two species suggests the week long period of feeding rotifers and instar-I *Artemia* nauplii used for culturing larval īnanga, may not be required for giant kōkopu. The research in the present study supports this proposition because live feed containing greater quantities and earlier initiation of the larger instar-II *Artemia* outperformed larvae fed diets with greater proportions of instar-I *Artemia.*

Very few studies have compared the growth performance and survival of larval fin fish fed diets that vary the provisioning of instar-I and instar-II *Artemia* nauplii. The generalisation in the literature that the width of prey items for larvae should fall in the region of 25-60 % of the mouth gape width has tended to result in instar-II *Artemia* not commonly being considered for first feeding of fin fish larvae (Bremigan & Stein, 1994; Fernández-Diaz et al., 1994; Østergaard et al., 2005; Shirota, 1970). However, the present study in larval giant kōkopu demonstrates that providing a live feed with an initial prey size to mouth gape ratio of 78 % outperformed live feed that began at a ratio of 56 %. This contributes to evidence from the larval culture of other fin fish species that have achieved greater growth performance and survival when feed items with prey size to mouth gape ratio of 84 % (Hoestenberghe et al., 2015). Consequently, future research for many larval fin fish species should re-test the assumption that larger prey will result in reduced performance, particularly where instar-II *Artemia* are used as first feed.

A critical finding of this body of research is that the instar-II *Artemia* enrichment formula currently used by NZPWL is likely limiting potential growth performance and survival (Chapter 4). Profiling of the fatty acids present in the commercially produced instar-II *Artemia* identified that no DHA was present, caused by the selection of unsuitable enrichment products. Very low levels of DHA will have a considerable impact on larval marine fish, for its roles in eye and neural network development, lack of which directly leads to poor feeding ability, growth performance and survival (Dantagnan et al., 2010, 2013; Izquierdo & Koven, 2011; Koven et al., 2018; Mourente et al., 1991; Mourente & Tocher, 1992; Navarro et al., 1997). Increasing DHA in larval giant kōkopu feeds is very easily achieved simply by replacing some or all of the current commercially available frozen microalgae enrichment products.

Artificial feeds are frequently more cost effective, less labour intensive and more consistent in their nutritional quality than live feeds for the larval culture of aquatic organisms, such as fin fish (Kolkovski et al., 1993; Langdon & Barrows, 2011; Pousão-Ferreira et al., 2003; Southgate, 2013). Consequently, weaning fish larvae off live feeds and onto artificial feeds as soon as possible is usually favourable from an financial stand point, provided it can be achieved with minimal mortalities (Barron et al., 2016; Southgate & Partridge, 1998). However, transitioning to artificial feeds before fin fish larvae are capable of identifying, consuming and digesting artificial feeds can cause significant detrimental impacts to their growth and health, which will impact overall production.

The present study has determined the capabilities of giant kōkopu to consume artificial feeds for the first time at several stages during the first 31 DAH. This knowledge is critical for improving decision making on weaning age in this species. However, this is merely a starting point for further research to shorten the live feeding period as well as optimising larval performance and production.

One important issue to resolve in the culture of larval giant kōkopu is the disparity between the longest and shortest larva which increases in the seven days leading up to 28 DAH. This seven day period is the critical weaning stage and evidently some larvae are better able to consume and digest the artificial diet than others. Previous studies have shown that issues with weaning can cause latent effects, including reduced survival and increased heterogeneity in growth performance within the cohort (Barron et al., 2016; Qin, 2008). Feed provision that is better suited to the larger and more competent feeders will result in these fitter individuals outcompeting the less competent individuals for food and they subsequently will grow more quickly. This further widens the gap in consumptive ability and subsequently generates a greater divergence in size within the cohort. The results of the present study reflects this with the total length of larvae at 77 DAH varying between 23.17 to 40.86 mm with an average of 31.87 mm total length. This means a large proportion of larvae are harvested prematurely, and an opportunity cost exists as maximum potential harvest mass is not achieved.

It is possible that the culture period could be prolonged to allow the smaller larvae to catch up, achieving greater overall mass and bolstering production volumes. However, the presence of red coloration in the abdomen observed during in the later weeks of the production cycle indicates development of the mesonephros (Benzie, 1968d). This is indicative of the early phase of metamorphosis in galaxiids and preparation for life in fresh water. The appearance of organs and opaque colouration of flesh due to metamorphosis is detrimental to the market acceptability of the “whitebait” product on organoleptic quality assessment. Therefore, simply prolonging the

culture period would likely prove problematic for this reason. A more effective weaning programme that reduces the divergence in the transition of larval feeding, or a method of size sorting larvae would assist in overcoming this problem.

Another possible approach to avoiding the divergence in larval size during weaning would be to artificially manipulate metamorphosis. To achieve this, greater understanding is required on how and why giant kōkopu undertake their return migration to freshwater and initiate metamorphosis. While it is believed that olfactory cues from adult populations, as well as freshwater signals, lead galaxiids back to riverine habitats (Baker & Hicks, 2003; Ellien et al., 2020; Keith, 2003; Keith et al., 2008; McDowall, 1992; Miles et al., 2014; Taillebois et al., 2011), the species-specific physiological mechanisms which induce metamorphosis on this return migration are poorly understood for New Zealand galaxiids. General control of metamorphosis in fish larvae is by thyroid hormones or thyroid hormone-like compounds (Ellien et al., 2020; Paris & Laudet, 2008) and the use of thiourea has been effective in slowing or halting metamorphosis for flounder (Miwa & Inui, 1987) as well as at the marine to freshwater migration stage for gobies (Taillebois et al., 2011). Experimentation with this compound may provide initial insights into the triggers of metamorphosis in giant kōkopu larvae. Salinity has also been examined with regard to the metamorphic changes undertaken by *Sicyopterus lagocephalus,* a goby which has the same amphidromous life history as giant kōkopu (Ellien et al., 2020). However, given that giant kōkopu larvae were cultured entirely in 35 ppt water it is unlikely that low salinity is required to trigger metamorphosis, rather it might be that larval growth itself induces the hormonal release that stimulates metamorphosis (Ellien et al, 2020). Therefore, controlling metamorphosis may be a difficult potential solution for achieving more consistent and higher quality product from cultured whitebait. Consequently, the most effective solution to reducing the heterogeneity of larval size at harvest age will most likely involve improving the feeding regime, most critically starting with the weaning process. Further research is required in several areas to ensure the feeding regime is optimised and specifically suited to giant kōkopu larvae.

There are multiple further opportunities for future experiments in the search for satisfactory growth performance and survival with a reduced live feeding window. These could include varying the frequency of provision of both live and artificial feeds throughout the day. For example, providing only artificial foods for the first feed of the day or providing artificial feed constantly. Alternatively, the period over which weaning occurs could be tested by abruptly ceasing live feed provision rather than slowly changing the composition of live to artificial feed over a number of weeks. However, it is firstly important to better understand the natural feeding behaviour exhibited by the species in culture and develop the feeding protocol to suit (Rønnestad et al., 2013). For example, understanding natural feeding positions in the water column is critical for the determination of the most effective sinking rates for artificial feed pellets.

6.3 Financial Impacts of the Research on Laval Giant Kōkopu Feed Bill

The costs associated with feeding fin fish in aquaculture can be up to 70% of total operational costs (Webster & Lim, 2001). Therefore, even small improvements in reducing these costs or increasing productivity from the same

financial input can result in significant improvements to the economics of an aquaculture venture. If employed, the findings of this research would potentially lead to significant potential improvements to the financial viability of larval giant kōkopu culture (Figure 6-1). Based on the research results, providing three live feeds per day from 2 DAH will incur an initial greater production cost, however, the total feed requirement at this stage is very small and will likely be returned through improved larval growth performance and survival. Feeds should be a 1:1 mix of instar-I and II *Artemia* for the first seven days of feeding and instar-II only from the eighth day of feeding. Live feed provision would cease at 25 DAH. Including the increase in required artificial dry feeds, the food cost in the first 28 DAH under this recommended protocol would result in an estimated cost saving of around 26.6 %. It is also anticipated the recommended feeding protocol would also produce an increase in total fish production by at least 88 %. Obviously, this greater biomass will require an increase in feed volume and subsequently increase the total feed cost but it will be significantly lower relative to the increase in biomass output.

6.4 General Conclusions

Overall, this research presented in this thesis provides highly valuable, novel information, that has the potential to greatly improve the commercial scale culture of giant kōkopu larvae. New fundamental knowledge about the developmental changes in morphology, and feed requirements for the larval culture of this species also provide baselines for comparisons for potential future improvements. Future research is likely to provide further improvements to culture and productivity of this species that will bolster the financial viability of giant kōkopu culture.

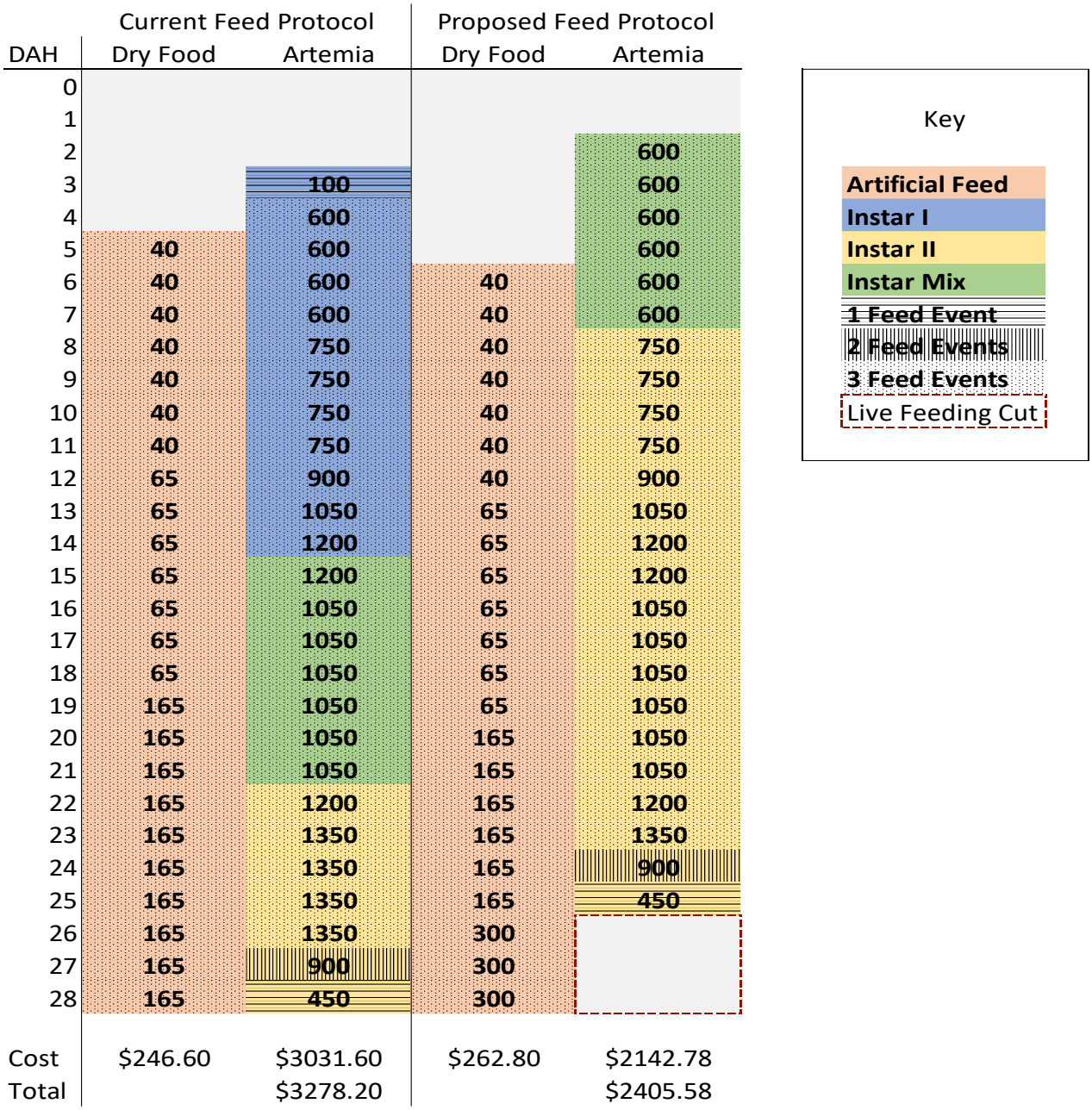

Figure 6-1. Schematic showing the current and proposed feed protocols by both feed type, mass (g) and number of feeding events per day for artificial and live feed items.

APPENDICES

Appendix 1 – Tabulated format of data captured in Chapter 2 by weekly sampling of Total Length (TL), Body Depth (BD), Wet Weight (WW), Dry Weight (DW), Mouth Gape (MG) and Eye Diameter (ED), from 0 -77 days after hatching (DAH) displayed as mean ± SE.

Age	TL (mm)	BD (mm)	WW (mg)	DW (mg)	GD (um)	ED (um)
0DAH	9.17 ± 0.06	0.56 ± 0.01	2.12 ± 0.02	0.45 ± 0.003	348 ± 15	431 ± 7
7DAH	9.87 ± 0.11	0.58 ± 0.01	2.30 ± 0.03	0.39 ± 0.004	538 ± 20	452 ± 10
14DAH	10.82 ± 0.12	0.72 ± 0.01	2.70 ± 0.03	0.57 ± 0.010	614 ± 16	544 ± 6
21DAH	12.75 ± 0.14	0.80 ± 0.02	5.38 ± 0.17	0.98 ± 0.017	651 ± 15	574 ± 8
28DAH	13.72 ± 0.24	0.89 ± 0.02	8.67 ± 0.24	1.20 ± 0.065	797 ± 23	633 ± 24
35DAH	15.52 ± 0.28	1.09 ± 0.03	10.93 ± 0.63	1.90 ± 0.080	838 ± 22	760 ± 15
42DAH	17.38 ± 0.29	1.26 ± 0.03	16.55 ± 0.95	2.87 ± 0.152	942 ± 32	803 ± 17
49DAH	18.46 ± 0.50	1.53 ± 0.06	27.07 ± 0.52	4.40 ± 0.198	997 ± 39	884 ± 18
56DAH	20.41 ± 0.47	1.60 ± 0.06	47.43 ± 1.57	6.75 ± 0.208	1211 ± 39	1008 ± 27
63DAH	23.47 ± 0.69	2.03 ± 0.08	102.53 ± 6.59	15.93 ± 1.166	1507 ± 66	1158 ± 38
70DAH	30.09 ± 0.89	2.93 ± 0.11	132.08 ± 5.78	24.66 ± 0.739	1619 ± 78	1264 ± 48
77DAH	31.87 ± 0.90	3.05 ± 0.11	177.05 ± 2.94	29.23 ± 1.261	1752 ± 98	1315 ± 62

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