

**Effect of dietary vitamin A in live feeds on performance and skeletal malformations of Striped Trumpeter *Latris lineata* larvae and post-larvae**

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M. Sc. Experimental Biology

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of the requirements for the degree of  
Doctor of Philosophy

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## Abstract

Reducing malformation is a constant challenge in the aquaculture production of marine finfish throughout the world. Several nutritional studies have previously linked vitamin A (VA) to abnormalities in skeletogenesis during marine fish larval development. Striped Trumpeter, *Latris lineata*, is a marine fish native to south-eastern Australia and New Zealand that has been successfully cultured at a semi-commercial scale in the hatchery and sea cages. My project aim was to examine if dietary VA during the live feed period affects larval performance and skeletal development, especially the jaws and vertebral column, and determine the nutritional VA requirement. The first part of the project was to accurately measure and quantify VA in live feeds and fish larvae using two extraction methods. The first method measured total VA in terms of retinyl palmitate, retinol and retinoic acid and the second method measured total retinol. The second part of the project was to determine the optimal conditions to enrich rotifers and *Artemia* with VA (retinyl palmitate) through examining the effects of time and light. This was followed by examining the effect of water type (clear and greenwater) on VA content of enriched rotifers over time. The optimal enrichment time for VA enrichment was 2 h for rotifers in dark conditions and 12 to 24 h for *Artemia*. The enrichment of rotifers and *Artemia* with VA was not uniform, and rotifers displayed a higher retinoid inclusion pattern than *Artemia*. Water type, time spent in larval rearing water after enrichment and the previous VA enrichment levels affected the concentration of retinoids in the VA-enriched rotifers. In clearwater, the concentration of retinoids in VA-enriched rotifers decreased over time, while in greenwater, the retinoid concentration decreased in the high VA-enriched rotifers and increased in the low VA-enriched rotifers. The significant difference in VA content of prey from graded VA enrichments was maintained for at least 4 h in clearwater and 4 to 8 h in greenwater.

The third part of the project was to feed *L. lineata* larvae with VA-enriched rotifers or *Artemia* using dose-response experimental designs. The first experiment tested the effect of feeding VA-enriched

rotifers (eight doses of VA, 0.5 to 92.5 ng retinol mg<sup>-1</sup> DW rotifers) to *L. lineata* larvae in 24 replicate 300 l tanks and reared in greenwater from 6 to 18 days post hatch (dph), followed by a four week period on *Artemia* (from 17 to 43 dph) to allow post-larvae to fully develop the jaw and skeletal elements. The second experiment investigated the potential impact of retinoids derived from microalgae by testing the combined effect of green or clearwater and enriched rotifers (three doses of VA, 0.6 to 99.6 ng retinol mg<sup>-1</sup> DW rotifers). The last experiment tested the effect of feeding enriched *Artemia* (six doses of VA, 0.4 to 44.1 ng retinol mg<sup>-1</sup> DW *Artemia*) for four weeks (from 17 to 44 dph).

The results showed that VA incorporation into *L. lineata* larvae varied with different doses of VA in the enriched rotifers and *Artemia*, while the water type did not affect the VA content in the larvae. The performance of *L. lineata* larvae and post-larvae was not affected by dietary VA during either the rotifer or *Artemia* feeding periods, while greenwater affected growth (9.2% and 28.0% increase in length and weight, respectively, over clearwater, by 43 dph). Dietary VA did not affect the onset or types of jaw malformations in post-larvae during the rotifer or *Artemia* feeding period experiments (81 and 50% prevalence of commercially relevant malformations on 43 and 44 dph, respectively). However, jaw malformation severity was reduced when larvae were reared in greenwater (2%) compared with those reared in clearwater (14%). Dietary VA during the rotifer feeding period affected the prevalence of vertebral column malformations which were correlated with the larval VA content, where daily inclusion of  $\leq 123$  ng total VA mg<sup>-1</sup> DW rotifer, equivalent to  $\leq 35$  ng total retinol mg<sup>-1</sup> DW rotifer, can reduce the skeletal malformations to approximately 30%.

To my knowledge, this research is the first to examine the effects of increasing doses of VA through enrichment of live feeds, during two different larval developmental stages, on the performance and skeletal development of the same marine fish. Also, it is the second study to explore the effect of graded VA doses in enriched rotifers through dose-response experimental design on skeletal malformations in a marine finfish. The overall conclusion was that dietary VA has no effect on the

growth or survival of *L. lineata* larvae and also has little or no effect on the jaw malformation in *L. lineata* larvae, where physical factors, including the size of rearing tanks, tank colour, and water type, have a greater, and potentially masking, effect on jaw malformation. However, dietary VA was found to significantly affect vertebral column malformations only during the rotifer feeding period. The study highlights the species-specific nature of the effect of VA on malformations, and that marine larvae are more sensitive to the VA treatment when they are younger, during the formative stage of skeletal development.

# **Chapter 1**

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## **General introduction**

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## 1.1 Worldwide Aquaculture

As the world's human population continues to expand at an exponential rate, aquaculture is fast becoming an important source of food (FAO, 2010; Olsen, 2011). The natural stocks of fish in the oceans can only supply a finite amount of food (FAO, 2010). The average total quantity of captured fish was  $90 \pm 1.3$  million tonnes p.a. from 2004 to 2009 (FAO, 2010). Global warming, overfishing, pollution, and habitat destruction have severely limited seafood populations worldwide and prevent them from increasing (Combes et al., 2005). A total of almost 85% of the world's fisheries are fully to over-exploited, depleted, or in a state of collapse (Fig.1) (FAO, 2010). Faced with a growing population and shrinking food sources, the production of edible fish from aquaculture offers a promising answer to provide a reliable food source (ABARE, 2003; FAO, 2006, 2010). The world production from cultured fisheries increased from 42 to 55 million tonnes from 2004 to 2009 (FAO, 2010).

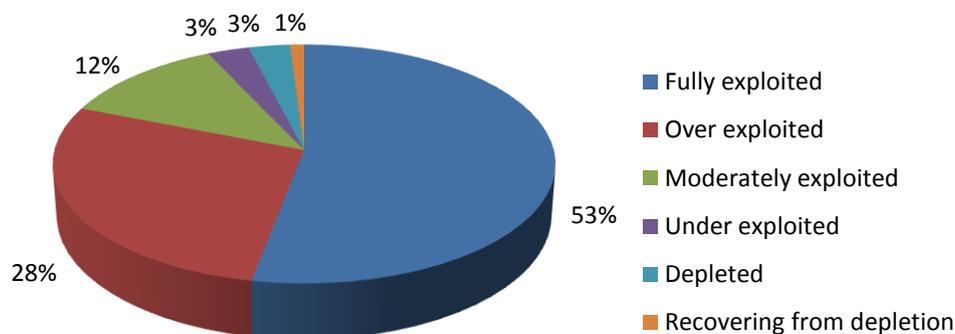


Figure 1: Status of the world fishery resources as reported in the state of world fisheries and aquaculture 2010 (modified from FAO, 2010).

In addition to the importance of cultured fish as a source of food, fish culture also contributes to improving the world economy. Aquaculture production was estimated to be less than 1 million

tonnes annually in the early of 1950s and increased to 53 million tonnes in 2008 with a value of US\$98 billion (FAO, 2010). Around 45 million people worldwide are working either directly or indirectly within the fishing industry, with employment opportunities decreasing in capture fisheries and increasing in aquaculture (FAO, 2010).

## 1.2 Aquaculture in Australia

Aquaculture is a diverse and rapidly expanding industry that makes a significant contribution to regional development in all Australian states and territories. It adds diversity to a region's economic base and creates demand for educational and training services, extension services, infrastructure and locally produced goods (ABARE, 2003; FAO, 2006). Australian production from aquaculture has grown over the years by 34% from 2001 to 2005 reaching AUD\$870 million in 2009-2010 (ABARE, 2004; ABARES, 2011a), although the gross total increase was relatively stable between 2008-09 and 2009-10 (ABARES, 2011b). Salmonids are the largest contributors to the Australian aquaculture production; between 2004/5 to 2009/10 production increased by 117% to AUD\$369 million, where the increase in the production is mostly from cage culture of Atlantic salmon, *Salmo salar* in Tasmania (ABARES, 2011a). In addition to salmonids, three other marine fin-fish are cultured in significant quantities, Southern Bluefin Tuna (SBT), *Thunnus maccoyii*, Barramundi, *Lates calcarifer* and Yellowtail Kingfish, *Seriola lalandii* (Battaglione et al., 2008).

Different species are grown in response to a wide range of climatic conditions in Australia (Table 1), for example tropical conditions in northern Australia are suited to pearl oysters, *Pinctada maxima*, prawns (shrimp), *Penaeus monodon* and *P. japonicas* and Barramundi farming (ABARES, 2012; Battaglione et al., 2008). Cooler conditions in southern Australia are suitable for on-growing wild captured juvenile SBT in South Australia and hatchery produced Atlantic salmon in Tasmania. Edible oysters, *Saccostrea glomerata* and mussel, *Mytilus edulis* aquaculture is mainly in New South Wales, South Australia and Tasmania where the temperature is temperate/cold (ABARES, 2012).

Table 1: Australian aquaculture production in 2009-10 by state. a: includes Silver Perch, *Bidyanus bidyanus*, eels, *Anguilla reinhardtii* and *A. australis*, aquarium fish and other native fish. b: includes Yabbies, *Cherax destructor*, Marron, *Cherax tenuimanus* and Redclaw, *Cherax quadricarinatus*. c: includes Scallops, *Pecten fumatus*, giant clams, *Tridacna gigas* and Abalone, *Haliotis laevis*. na, not available and \$'000, thousands Australian dollars. Modified from ABARES, 2011b.

	NSW	VIC	QLD	WA	SA	TAS	NT
<b>Production (tonnes)</b>							
<b>Fish</b>							
Salmon	150	815	0	na	na	30950	0
Tuna	0	0	0	na	7284	0	0
Barramundi	86	0	2405	na	na	0	699
Gross total production <sup>a</sup>	430	1030	2522	508	11456	30950	699
<b>Crustaceans</b>							
Prawn	165	0	5216	na	0	0	0
Gross total production <sup>b</sup>	175	0	5272	94	23	0	0
<b>Molluscs</b>							
Edible oysters	4960	0	na	0	6123	3721	0
Pearl oysters	0	0	0	na	na	0	na
Mussels	66	566	0	505	1343	982	0
Gross total production <sup>c</sup>	5026	566	na	505	7752	4874	na
<b>Value (\$'000)</b>							
<b>Fish</b>							
Salmon	1602	5102	0	na	na	362422	0
Tuna	0	0	0	0	102175	0	0
Barramundi	1046	0	20684	na	na	0	5770
Gross total production <sup>a</sup>	4984	8327	22755	5564	134205	362422	5770
<b>Crustaceans</b>							
Prawn	2400	0	57090	0	na	0	0
Gross total production <sup>b</sup>	2575	0	76023	2200	645	0	0
<b>Molluscs</b>							
Edible oysters	43000	0	520	0	35027	21212	0
Pearl oysters	0	0	0	85500	0	0	18980
Mussels	284	1981	0	1861	2530	3438	0
Gross total production <sup>c</sup>	43284	1981	520	87361	47898	29749	18980

NSW, New South Wales; VIC, Victoria; QLD, Queensland; WA, Western Australia; SA, South Australia; TAS, Tasmania; NT, Northern Territory.

Aquaculture production in Australia is projected to grow, driven by the increase in human population, decreased supply from wild fisheries and increased need for high quality protein sources (FAO, 2006). Aquaculture will likely benefit from the market development and the increased application of science and technology undertaken by various universities, state and federal government institutions, and industry, with additional funding from agencies such as the Australian SeaFood Cooperative Research Centre (CRC), Fisheries Research and Development Corporation (FRDC) and the Australian Research Council (ARC). Currently, one of the biggest impediments facing new aquaculture development is the supply of high quality seed stock (Battaglione et al., 2008; Battaglione and Fielder, 1997). Without the reliable production of high quality seed stock, the development of future marine fish culture is potentially limited (Battaglione et al., 2008). For example, the SBT industry is currently reliant upon wild-caught juveniles that are subject to a fishery quota. In 2009, the quota was reduced by 25% ([www.ccsbt.org](http://www.ccsbt.org)), which immediately reduced the volume of SBT that could be on-grown in South Australia. In this environment, there is some urgency in the research program to develop methods for the reliable production of quality SBT juveniles from eggs produced by captive SBT broodstock to underpin a sustainable industry (Hutchinson, 2009). In addition, reliable hatchery supplies of *L. calcarifer* and *S. lalandii* quality seed stock are also required (Cobcroft and Battaglione, unpublished data).

### **1.3 Why is high quality seed stock important?**

Aquaculture depends on the availability of high quality fish seed for stocking. Aquaculture involves growing a selected species, in a controlled environment, where the purpose is harvesting them and selling them commercially (ABARE, 2004). In this situation, healthy larvae are crucial to ensure a high quality end product for sale to the market. Any deviation from the normal structure or function of an animal is referred in the broad sense by the term “disease”, and morpho-anatomical abnormalities (deformities) are considered non-infectious diseases, to clearly distinguish them from morphological defects caused by contaminants and/or pathogens (Sindermann, 1978). In other literature, several

definitions are used: abnormality and anomaly are considered as synonyms, indicating a difference or deviation from the average or norm (The American Heritage Medical Dictionary, 2007); deformation indicates an alteration in shape and/or in structure of a previously normally formed part (The American Heritage Medical Dictionary, 2007); malformation is a morphological defect of an organ or larger region of the body, resulting from an intrinsically abnormal developmental process (Anderson, 2007). In this context, I have chosen to use malformation in the text, as the abnormalities in cartilage and bone structure identified in Striped Trumpeter, *Latris lineata* (Bloch and Schneider, 1801) occurred during development of larvae and post-larvae. Malformed fish appear in variable and unpredictable numbers in farmed stocks in both warm and cold waters, and cause severe losses to the production sector (Koumoundouros, 2010; www.finefish.info). In the face of increasing market competition, quality is crucial since malformations significantly decrease the product desirability. Malformations are a serious economical problem because they also add to the cost of farming by reducing survival and performance of cultured fish, require additional infrastructure and labour costs due to manual sorting, and reduce marketability and decreased final fish-flesh quality (Cahu et al., 2003a; Gavaia et al., 2002; Koumoundouros et al., 2002, 1997b). Malformations can have a negative effect on growth, viability and feed conversion rates as well as increased vulnerability of malformed fish to disease infection (Boglione et al., 2001; Koumoundouros et al., 1997a).

Without the production of healthy seed stock, the development of the sea cage culture, intensive shore-based recirculation grow-out systems and expanding saline water culture are limited (Battaglione and Fielder, 1997; Battaglione et al., 2008; Conceição et al., 2010a; Marte, 2003; Shields, 2001). Malformations have been reported in most commercially important aquaculture fish species both within Australia and overseas (Table 2). Skeletal malformations have caused severe economic losses in the aquaculture industry due to the reduced market value of the malformed fish (Boglione et al., 2001; Hattori et al., 2003; Koumoundouros et al., 1997b). The estimated loss due to malformed fish in European countries was > 50 million Euro in 2003-04 (Hough, 2009). Helland et al. (2009) found in a survey of Atlantic Cod, *Gadus morhua*, hatcheries that 25 to 80% of fish produced

had malformations (including minor deviations) and Sweetman (2004) reported that on average 17% of all juvenile fish produced in the European aquaculture industry were malformed. In Australia, a survey of 18 marine fish hatcheries revealed that 44% of these hatcheries indicated that skeletal malformations were a significant issue, with up to 70% malformation in some batches of cultured *S. lalandi*, and up to 95% in *L. lineata* (Cobcroft and Battaglione, in review).

## 1.4 Causes of malformations in cultured marine fish

Marine fish larvae hatch very early in their development compared with other vertebrates (Blaxter, 1986; Haga et al., 2002a; Villeneuve et al., 2005). Major changes occur in the body structure and shape as they develop and transform into juveniles (Cahu et al., 2003a; Giménez et al., 2007; Villeneuve et al., 2005). Most of the skeletal malformations that appear in cultured marine finfish develop during the larval and juvenile stages. They often occur due to a lack of optimal rearing requirements during early larval stages of fish development (Daoulas et al., 1991; Gavaia et al., 2002; Koumoundouros et al., 1997a). The development of skeletal disorders in larvae and juvenile fish can be due to environmental, genetic and nutritional factors (Cahu et al., 2003a; Divanach et al., 1997; Lall and Lewis-McCrea, 2007; Sadler et al., 2001). Unfavourable environmental conditions that can cause skeletal malformations may be abiotic conditions such as temperature (Pavlov and Moksness, 1997; Sfakianakis et al., 2004), light and photoperiod (Barahona-Fernandes, 1979; Villamizar et al., 2011), rearing conditions such as the density of the larvae in the rearing system (Bogliione et al., 2009; Izquierdo et al., 2010), water currents and quality (Divanach et al., 1997; Haaparanta et al., 1997) or salinity (Okamoto et al., 2009). They can be caused by biotic factors such as bacterial infections (Morrison and Macdonald, 1995; Norton et al., 1969) or parasites (Lom et al., 1991). Other causes of skeletal malformations are pollution (Bengtsson et al., 1988) and genetic variation, sometimes in association with triploid induction (McKay and Gjerde, 1986; Sadler et al., 2001).

Vertebrate skeletal tissues are commonly grouped into four major categories: 1) cartilage, 2) bone, 3) dentine, and 4) enamel (Witten et al., 2010). In contrast, the skeletal elements of teleost fishes

Table 2: Selected examples from the literature reporting malformed body regions in cultured marine fish.

Common name	Scientific name	Type of malformation	References
Barramundi	<i>Lates calcarifer</i>	Vertebral column (vertebrae), jaw, opercula	Fraser and de Nys, 2011, 2005; Fraser et al., 2004
Yellowtail Kingfish	<i>Seriola lalandi</i>	Jaw	Cobcroft et al., 2004
Striped Trumpeter	<i>Latris lineata</i>	Jaw and vertebral column	Cobcroft and Battaglione, 2009; Cobcroft et al., 2001a; Trotter et al., 2001
Dusky Grouper	<i>Epinephelus marginatus</i>	Vertebral column, opercula and jaw	Boglione et al., 2009; Glamuzina et al., 1998
Sea Bass	<i>Dicentrarchus labrax</i>	Vertebral column, swim bladder	Barahona-Fernandes, 1982; Cahu et al., 2003b; Chatain, 1994; Daoulas et al., 1991; Mazurais et al., 2008
Red Sea Bream	<i>Pagrus major</i>	Vertebral column and fins	Foscarini, 1988; Hattori et al., 2003; Matsuoka, 1987
Gilthead Sea Bream	<i>Sparus aurata</i>	Vertebral column, opercula, swim bladder, lateral line and eyes	Boglione et al., 2001; Carrillo et al., 1999, 2001; Chatain, 1994; Koumoundouros et al., 1997a; Paperna, 1978
Senegalese Sole	<i>Solea senegalensis</i>	Vertebral column and cranial	Gavaia et al., 2002, 2009
Red Porgy	<i>Pagrus pagrus</i>	Vertebral column and cranial	Izquierdo et al., 2010; Roo et al., 2009, 2010
Atlantic Halibut	<i>Hippoglossus hippoglossus</i>	Vertebral column	Lewis-McCrea and Lall, 2007; 2010; Lewis and Lall, 2006; Lewis et al., 2004
White Sea Bream	<i>Diplodus sargus</i>	Vertebral column	Saavedra et al., 2010a, 2010b
Japanese Flounder	<i>Paralichthys olivaceus</i>	Vertebral column	Hosoya and Kawmura, 1991; Tomoda et al., 2006
Common Dentex	<i>Dentex dentex</i>	Caudal fin	Koumoundouros et al., 2001
Common Pandora	<i>Pagellus erythrinus</i>	Caudal fin	Sfakianakis et al., 2004
Rainbow Trout	<i>Oncorhynchus mykiss</i>	Vertebral column	Deschamps et al., 2008
Atlantic Salmon	<i>Salmo salar</i>	Vertebral column	Fjellidal et al., 2007a, 2007b; Gjerde et al., 2005; Witten et al., 2009

are unique and display a full range of skeletal tissues that are intermediate between two or more of the above categories including several types of bone, different types of cartilage and tissues types that are intermediate between connective tissue and bone, and between bone and cartilage (Hall and Witten, 2007; Huysseune, 2000; Witten et al., 2010). Teleosts are the lowest vertebrates that have a mineralized skeleton (Haga et al., 2002b; Witten et al., 2010). In higher vertebrates the axial skeleton is formed by endochondral ossification, where the bones are formed as a cartilage first and then ossified into mineralized bone (Haga et al., 2002b). In teleosts, the axial skeleton is formed by intramembranous ossification in which the bone is formed as bone matrix and directly ossified and other bones by endochondral ossification (Ekanayake and Hall, 1988; Matsuoka, 1987). In this sense, skeletal structures are formed by two different ossification processes, by endochondral ossification, where a previously formed cartilage template is replaced by bone, and dermal or intramembranous ossification, where the bone is formed without a cartilage (mainly in teleosts fishes) (Fernandez and Gisbert, 2010). As mentioned above, several nutritional and/or environmental factors may affect the skeletogenesis process in teleosts causing skeletal disorders to different fish bone tissue types.

Jaws bones including the premaxilla and maxilla are formed by intramembranous ossification (mesenchymal cells differentiate into osteoblasts forming bone without a cartilaginous template) (Hall, 2005 ; Huysseune and Sire, 1992; Sire and Huysseune, 2003). Also the dentary (part of the lower jaw) is formed by intramembranous ossification while Meckel's cartilage (the internal support structure of the lower jaw) is formed by endochondral processes where condensed mesenchymal cells are differentiated into chondrocytes to form cartilage (Haga et al., 2002b; Hall, 2005 ; Huysseune and Sire, 1992; Sire and Huysseune, 2003). Regarding the vertebral column development, the vertebral centra are formed by intramembranous ossification while the vertebral arches are formed by endochondral ossification (Witten and Huysseune, 2009). Although the jaw bones (premaxilla, maxilla and dentary) and vertebral centra are formed by intramembranous ossification, they have different embryonic origin. The jaw bones originate from the neural crest while the

vertebral centra originate from the sclerotome-derived mesenchyme (Haga et al., 2002b; Smith and Hall, 1990). Because of the variation in bone tissue type and embryonic origin, nutritional and environmental factors may have variable effects on different skeletal elements.

Nutrition is one of the most common factors affecting skeleton formation, often in association with abiotic factors (Cahu et al., 2003a; Lall and Lewis-McCrea, 2007). In aquaculture, the knowledge of larval fish nutrition and feeding is still limited for most species (Teles et al., 2011). This is due to the large number of aquatic species cultivated and differences in their physiology and behaviour (Teles et al., 2011). Amino acids (Saavedra et al., 2010b), oxidized lipids (Lewis-McCrea and Lall, 2010), phospholipids (Geurden et al., 1997), polyunsaturated fatty acids (Roo et al., 2009), minerals (Ogino et al., 1978) and vitamins such as vitamin A (Fernández et al., 2008), C (Demartinez, 1990), D (Haga et al., 2004) and K (Roy and Lall, 2007) have previously been linked with malformations that appear both during the larval and juvenile stages (Cahu et al., 2003a; Lall and Lewis-McCrea, 2007).

## 1.5 Vitamin A

Vitamins are among the most common nutritional factors that can induce skeletal abnormality. They are organic catalytic compounds, which are required for an organism but cannot be synthesised by the body (Guillaume, 2001). They are different from essential amino acids or essential fatty acids in that they are required in minuscule amounts (Guillaume, 2001).

Vitamin A (VA) is essential throughout the lifecycle of vertebrates where its deficiency and excess affects the normal development of the vertebrate embryo (Ross et al., 2000). For example in: amphibians (Degitz et al., 2003; Eagleson and Theisen, 2008), reptiles (Comitato et al., 2006; Esposito et al., 2007), birds (Pawlowska et al., 2008; Wood et al., 2008), and mammals (Bowles and Koopman, 2007; See et al., 2008). Vitamin A's importance in fish is now well established, although the effects differ according to the species and developmental stage from broodstock (Alsop et al., 2008; Fontagne-Dicharry et al., 2010; Furuita et al., 2001, 2003; Santiago and Gonzal, 2000),

embryonic (Ellies et al., 1997; Herrmann, 1995; Suzuki et al., 1999, 2000) and larvae (Haga et al., 2002b; Miki et al., 1990; Takeuchi et al., 1998).

Vitamin A or retinoids are a group of nutrients with compounds that are structurally similar or have the biological activity of retinol, where they can bind or activate a specific receptor or a group of receptors. Many retinoid forms are available in the market for dietary incorporation, retinol (parent compound), VA alcohol, retinal (the aldehyde form), retinoic acid (the acid form of VA), retinyl acetate and retinyl palmitate (ester forms of VA, Fig. 2) (Wolf, 1984). Due to the presence of different VA compounds, VA activity is expressed in international units (IU), where 1 IU of VA is equivalent to 0.3  $\mu\text{g}$  retinol (Wolf, 1984). Vitamin A activity can also be expressed in terms of retinol equivalent (RE), where 1  $\mu\text{g}$  RE = 1  $\mu\text{g}$  retinol = 3.3 IU. Carotenoids are another source of VA, and some can be converted to VA but to different degrees according to other dietary factors such as availability of protein or, lipids and physiological factors associated with their cleavage into VA in the intestine (Olson, 1989; Ross and Ternus, 1993). Unlike VA, no toxicity occurs due to high doses of carotenoids (Takeuchi et al., 1995). Of the carotenoids,  $\beta$  carotene has the highest activity and it is generally accepted that 6  $\mu\text{g}$   $\beta$  carotene = 1  $\mu\text{g}$  retinol (Ross and Ternus, 1993).

Retinoid metabolism is a complex process that involves a number of proteins and enzymes that at the same time control the absorption, accumulation, transport and transformation into active forms (Ross, 1993a, 1993b). Dietary vitamin A sources are either of vegetable origin (carotenoids) or animal origin (retinyl esters) (Fernandez and Gisbert, 2011). They are absorbed from the intestine through chylomicrons (large lipoprotein particles), transported via the circulatory system and stored in the liver (Fig. 3) (Ross and Ternus, 1993). They are transferred from the liver when needed to the target cells via lipoproteins in the plasma (Combs, 2008). In the target cells retinyl esters are hydrolyzed to give retinol which is the vitamin A alcohol form (Giménez et al., 2007). Retinol is then oxidized once to give retinal 'vitamin A aldehyde form' and twice to give retinoic acid (Giménez et al., 2007). Importantly, retinol can give rise to all the different vitamin A compounds except  $\beta$

carotene, and is considered the parent compound. In addition, retinoic acid is the end product, once formed it cannot be converted back to retinol or retinal, and is therefore considered the active form of vitamin A (Ross and Ternus, 1993). Retinoic acid supports some biological functions (listed below) while retinol is required for reproduction and retinal for vision (Ross, 1993b; Ross and Ternus, 1993).

The proteins that are associated with retinol and other forms of VA are structurally similar but have a unique distribution in the tissues (Ross, 1993b; Ross and Ternus, 1993). This distribution differs according to the developmental stage. Among these proteins is the extracellular retinol-binding protein which transfers retinol in the plasma to the tissues and cells. The intercellular binding proteins are referred to as cellular retinol-binding protein (CRBP) and cellular retinoic acid binding protein (CRABP) (Ross and Ternus, 1993). The amount of CRABP in the cell is much less than CRBP, consequently excess retinoic acid may be more toxic than any other form of VA due to the high level of unbound retinoic acid in the cell (Takeuchi et al., 1998).

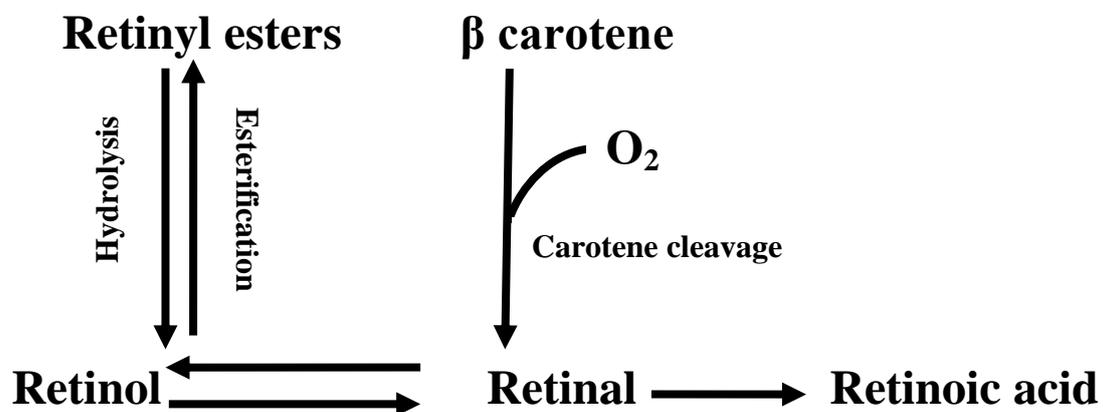


Figure 2: Biochemical relationships among dietary different vitamin A compounds. Modified from Ross, 1993b.

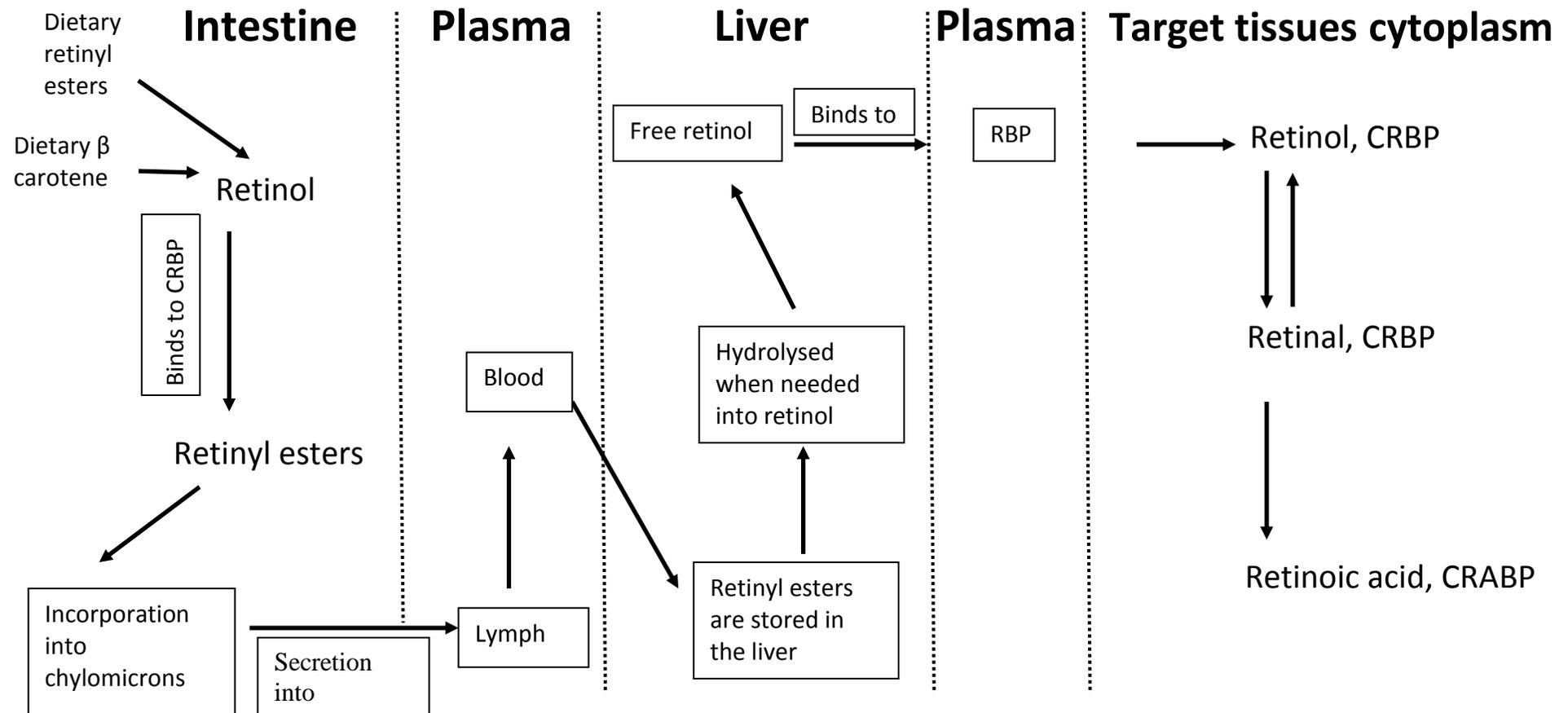


Figure 3: General Vitamin A metabolic reactions, illustrating vitamin compounds, extracellular and intracellular binding proteins and their tissue localization. Abbreviations: RBP, retinol binding protein; CRBP, cellular retinol binding protein; CRABP, cellular retinoic acid binding protein. Modified from Fernandez and Gisbert, 2011 and Ross, 1993b.

Retinoic acid, exists in two forms: 9-cis and all-trans retinoic acid, via the retinoid (nuclear) receptors and is involved in many pathways regulating skeleton formation, development, growth, morphogenesis, cellular differentiation, tissue homeostasis, bone metabolism, immunity, glycoprotein and glycosamioglycan synthesis (Combs, 2008; Mark et al., 2006; Srinivas and Chethankumar, 2007). The main effects of retinoic acid on development are mediated through its effect on gene expression (Mark et al., 2006; Means and Gudas, 1995; Ross et al., 2000). Retinoic acid functions in a hormone-like manner to mediate its effect on gene expression (Ross and Ternus, 1993). The mechanisms through which VA affects developmental gene expression involves two families of nuclear receptors, retinoic acid receptors (RARs) and retinoid "X" receptors (RXRs) (Liden and Eriksson, 2006; Mark et al., 2006; Villeneuve et al., 2006). Both RAR and RXR have three subfamilies (RAR $\alpha$ , RAR $\beta$  and RAR $\gamma$ , and RXR $\alpha$ , RXR $\beta$  and RXR $\gamma$ ). RARs and RXRs have been found in all the examined vertebrates, and have considerable structure similarity and a similar pattern of gene expression during development (Sucov and Evans, 1995). The retinoid pathway can act on more than 532 genes through direct or indirect control. The direct control involves the activation of the retinoic acid receptors while the indirect control is through activation of other protein receptors (Balmer and Blomhoff, 2002).

The role of VA during larval skeletal development has been studied and documented in detail for relatively few species of fish (Table 3). An imbalance in VA, either a decrease or increase, can affect the normal skeletal development of the jaws, operculum, fins including the caudal complex, vertebra and body shape. Vitamin A is also among the factors that affects the quality of the bone matrix during bone formation (Binkley and Krueger, 2000; Ørnsrud et al., 2002). Excess of VA accelerates the maturation of chondrocytes and stimulates the osteoclasts, with a resultant delay in the production of bone matrix (Vasan and Lash, 1975). This leads to acceleration in the development of the vertebral column through precocious mineralisation, which can cause skeletal malformation including spinal malformations (Dedi et al., 1995, 1997; Estevez and Kanazawa, 1995), vertebral

compression (Dedi et al., 1995, 1997; Takeuchi et al., 1998), and vertebral fusion (Dedi et al., 1995, 1998; Takeuchi et al., 1995). Although VA affects normal skeletal development, the requirements for VA vary greatly among fish species. According to the United States National Research Council, VA requirements for different fish species such as, Rainbow Trout, *Oncorhynchus mykiss*, Channel Catfish, *Ictalurus punctatus*, Common Carp, *Cyprinus carpio* and Pacific Salmon, *Oncorhynchus* species range between 1,000 and 4,000 IU VA kg<sup>-1</sup> (National Research Council, 1993). Dedi et al. (1995) reported that the optimum level of VA in live feeds like *Artemia* to prevent skeletal malformations in Japanese Flounder, *Paralichthys olivaceus* is less than 50,000 IU VA kg<sup>-1</sup> DW while in Atlantic Halibut, *Hippoglossus hippoglossus* and Tilapia, *Oreochromis niloticus*, incorporation of 8,333 and 5,000 IU VA kg<sup>-1</sup> DW, respectively, into formulated diets is the optimum for normal growth (Moren et al., 2004b; Saleh et al., 1995). In contrast *Artemia* enriched with emulsion containing 4,333 IU VA kg<sup>-1</sup> results in a high incidence of skeletal malformations in Senegalese Sole, *Solea senegalensis* (Fernández et al., 2009).

As the above summary suggests, studying the requirements for VA on marine fish larval development is relatively complicated. Not only because VA metabolism is complex but because requirements differ greatly according to the species under study and the developmental stage tested. In addition, the requirement for VA may differ within the same species according to the skeletal part under study. The VA requirement to minimize jaw and hyoid malformations in European Sea Bass, *Dicentrarchus labrax* was < 9,402 IU VA kg<sup>-1</sup> DW diet while the requirement to minimize the vertebral and fin malformations was ≥ 9,402 IU VA kg<sup>-1</sup> diet (Mazurais et al., 2009). Factors to be considered include the levels of VA in the enrichment used, feeding route, vitamin form, experimental design (statistical power and range tested) and duration of exposure.

Table 3: Selected examples from the literature showing the effect of vitamin A on the skeletal development of different fish species exposed to or fed varying amounts of vitamin A in formulated diets and live feeds (rotifers and *Artemia*). Duration of exposure is expressed in days post hatch (dph) or fish size average total length (TL) or weight (WT) when applicable.

Fish species	Dietary route	VA form	Levels of enrichment (Range)	Duration of exposure	Main effects	Reference
<i>Danio rerio</i>	Injection into eggs, 1-4 cell stage embryo	Different retinoid compounds	0.25 nl from concentrations ranged from 0.04 to 1.25 M for retinal, 0.06 to 0.1 M for retinol and 0.01 M for $\beta$ -carotene	Injection into 1-4 cell stage embryos	At 72 h post fertilization, malformations in brain, jaw, heart induced by retinal, retinoic acid and retinol. No malformation for $\beta$ -carotene.	Haga et al., 2008
<i>Paralichthys dentatus</i>	Larvae were reared in water containing all-trans-retinoic acid (bath exposure)	all-trans-retinoic acid	0 to 20 nM (nanomolar) 20 l <sup>-1</sup>	10 days of exposure (TL 8.5 mm)	Malformations in the jaws, fins, hypurals, and vertebrae.	Martinez et al., 2007
<i>Paralichthys olivaceus</i>	Larvae were reared in water containing retinoic acid	Retinoic acid isomers (all-trans, 9-cis and 13-cis retinoic acid)	25 nM of different retinoic acid isomers	6 to 9 dph	Malformations in lower jaw, caudal fin and vertebrae.	Haga et al., 2002a
<i>Dicentrarchus labrax</i>	Formulated diet	Retinol	3,152 to 155,200 IU VA kg <sup>-1</sup> DW diet	9 to 45 dph	The development of malformations in all body parts dependant on the skeletal element under study.	Mazurais et al., 2009
<i>Dicentrarchus labrax</i>	Formulated diet	Retinyl acetate	39,600 to 646,800 IU VA kg <sup>-1</sup> DW diet	7 to 42 dph	Malformation in the skull and operculum and low incidence in the vertebral column.	Villeneuve et al., 2005

Fish species	Dietary route	VA form	Levels of enrichment (Range)	Duration of exposure	Main effects	Reference
<i>Chrysophrys major</i>	Formulated diet	Retinyl palmitate	95,700 to 5,685,900 IU VA kg <sup>-1</sup> DW diet	5 to 20 dph	Malformations in the mouth (low) and vertebra.	Hernandez-H et al., 2006
<i>Salmo gairdneri</i>	Formulated diet	Retinyl palmitate	4,000 to 8,104,000 IU VA kg <sup>-1</sup> DW diet	16 week (WT 0.6 g)	Malformations in the anal, caudal, pectoral and pelvic fins, and spine.	Hilton, 1983
<i>Oreochromis niloticus</i>	Formulated diet	NA	0 to 40,000 IU VA kg <sup>-1</sup> diet	18 week (WT 11.4 g)	Vertebral malformations.	Saleh et al., 1995
<i>Paralichthys olivaceus</i>	<i>Artemia</i>	Retinyl palmitate	1,300 to 1,282,900 IU VA kg <sup>-1</sup> <i>Artemia</i>	10 to 40 dph	Malformations in the vertebrae and body shape.	Dedi et al., 1995, 1998
<i>Solea senegalensis</i>	<i>Artemia</i>	Retinyl palmitate	1,320 to 12,900 ng total VA mg <sup>-1</sup> DW experimental emulsion	6 to 27 dph	Dietary VA accelerated the intramembranous ossification of vertebral centrums where the endochondral bones were more sensitive than intramembraneous bones resulting in vertebral and caudal fin malformations.	Fernández et al., 2009
<i>Sparus aurata</i>	Rotifers	Retinyl palmitate	1,698 to 16,947 ng total VA mg <sup>-1</sup> DW experimental emulsion	4 to 20 dph	Malformations in the cranial skeleton, opercular complex, vertebral centrums, body shape and caudal complex.	Fernández et al., 2008

There are generally two approaches that have been used to study the effect of VA on marine fish larval development. The first is rearing the larvae in water containing the VA treatment. The benefit of this approach is that it can be applied during the yolk sac stage before larvae are feeding and the mouth is open. This approach highlights the importance of VA during very early stages of development where organogenesis and morphogenesis processes occur (Fernandez and Gisbert, 2011). The other approach is the dietary dose–response, where the larvae are fed diets containing different graded levels of the VA. The earliest diets often need to be live prey, either rotifers or *Artemia*, enriched with VA or less frequently a compound diet. The bath exposure technique has been less studied (Suzuki et al., 1999, 2000), while examples of the dose-response approach in rotifers are also rare (Fernández et al., 2008) with a few more using *Artemia* (Fernández et al., 2009; Takeuchi et al., 1998; Tarui et al., 2006) and in the diet (Moren et al., 2004b; Villeneuve et al., 2005). Enrichment of rotifers or *Artemia* with VA is through bioencapsulation. Live feeds are cultured in a medium rich in VA. The non-selective feeding behaviour of the live prey enables them to feed on the correctly sized diffused particles which are incorporated into the digestive tract and assimilated into their bodies at different rates depending on enrichment periods, exposure to light and temperature (Conceição et al., 2010b; Sorgeloos et al., 2001; Sweetman, 2004). Retinoids are unstable compounds that can be oxidized and/or isomerised to other compounds especially in the presence of oxidants such as air, light or heat (Barua and Furr, 1998). For this reason, enrichment of the live prey, especially rotifers, is recommended under complete dark conditions or reduced light (Haga et al., 2006). Rotifers and *Artemia* are able to metabolize different VA compounds and accumulate them in their body (Haga et al., 2006). Due to species-specific differences between the two live preys, enrichment is not uniform and rotifers display a higher retinoid inclusion pattern than *Artemia* (Giménez et al., 2007). It is technically difficult to maintain the same VA levels during the whole live prey-feeding period of a larva. For that reason optimum VA levels are usually determined separately for the rotifer and *Artemia* feeding periods.

## 1.5.1 Enrichment of live feed with vitamin A

For the purpose of this study enrichment emulsions containing increasing levels of retinyl palmitate (VA) were prepared by Nutrakol, WA. The emulsions had an oil base ingredient (tuna oil) which contains 700 IU VA kg<sup>-1</sup> (NuMega certificate of analyses). The control treatment in this study was nominally designed without the addition of retinyl palmitate. Although retinoids exists in several forms (Fig. 2), only retinyl palmitate and acetate are recommended to fulfil the requirements of fish for retinoids (Fernandez and Gisbert, 2011). This is because they are less toxic than the other forms, and fish larvae at early stages have the required enzymes in their livers to metabolize them into the other VA forms (Fernandez and Gisbert, 2011; Takeuchi et al., 1998).

### 1.5.1.1 Enrichment of rotifers

For the purpose of our study, rotifers, *Brachionus plicatilis*, were enriched with different retinyl palmitate levels for 2 h under dark conditions according to Giménez et al. (2007) and Haga et al. (2006) studies and our preliminary results and methodology presented in Chapter 2. The levels of enrichment selected in the study were based on the enrichment curve of enriched rotifers with different levels of retinyl palmitate presented in Chapter 2, to ensure different significant incorporation from VA into the rotifers. After the enrichment, rotifers were rinsed, counted and volumetrically fed to larvae once per day.

### 1.5.1.2 Enrichment of *Artemia*

*Artemia* were enriched with different retinyl palmitate levels for 24 h under dark conditions according to Giménez et al. (2007) and experimentation presented in Chapter 2. The levels of enrichment used in the study were based on the enrichment curve of enriched *Artemia* with different levels of retinyl palmitate presented in Chapter 2, to ensure different significant incorporation from VA into the *Artemia*. After the enrichment, *Artemia* were rinsed, counted and volumetrically fed to larvae four times per day.

### 1.5.2 Quantifying vitamin A

All the biochemical analyses were carried at the Commonwealth Scientific and Industrial Research Organisation (CSIRO), Marine Laboratories, Hobart. Retinoids were measured as total VA (retinyl palmitate, retinol and retinoic acid) (Fig. 4) and total retinol (retinyl palmitate and retinol) (Fig. 5) using two different techniques modified from Moren et al. (2002, 2004a, 2005) and Takeuchi et al. (1998). In brief, samples were extracted, the retinoids were separated using high performance liquid chromatography (HPLC), and identified and quantified using photodiode array (PDA) and fluorescence detectors. Identification of the individual retinoid peaks was done by comparing their retention time to those of VA external standards. The concentration of different retinoids in the extracted samples was calculated by comparing their peak areas to those of the internal standard retinyl acetate (that was added to the samples during the extraction process) and the external standards. For further accuracy, identification and calculation of the peaks areas were done using the photodiode array detector and then the results were compared with those obtained from the fluorescence detector. Verifications of the peaks were further done by comparing their absorption spectrum to that of external standards (Fig. 6), since different retinoids have different spectrum absorption (Krinsky, 2004). In the samples that were low in their retinol and retinoic acid content, the fluorescence detector was more accurate in quantifying the concentration of retinoids than the photodiode array detector. Consequently, for the purpose of this thesis, the concentration of retinol and retinoic acid in all the extracted samples was always based on the reading of the fluorescence detector. The second technique, used for quantifying VA, makes use of the saponification process where the ester bonds in the retinyl palmitate are hydrolysed and total retinol is measured. Total VA and total retinol were measured in enrichment emulsions and live prey, rotifers and *Artemia*, while only total retinol was measured in larvae due to the small size of the larvae and the limited number of larvae available for the samples.

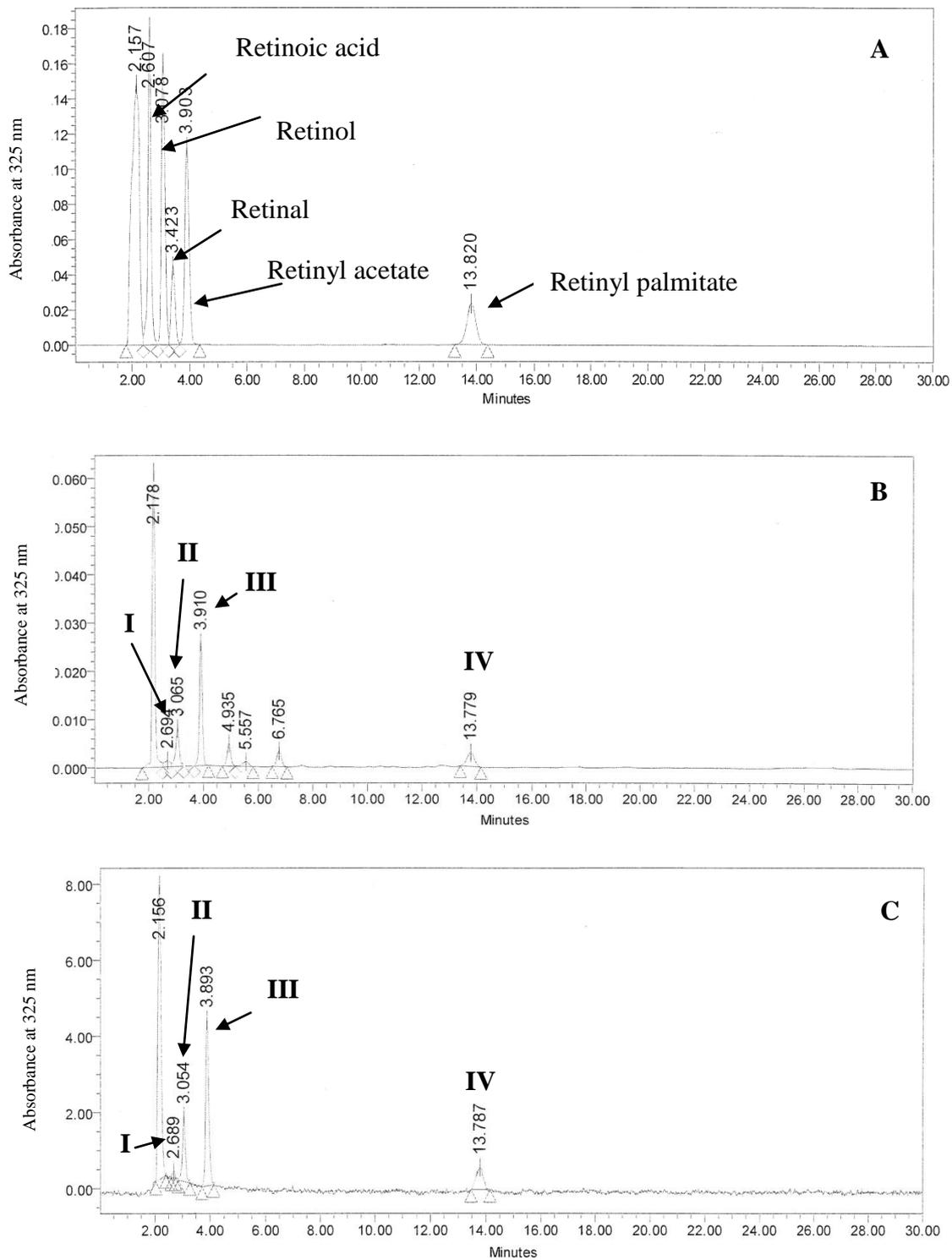


Figure 4: HPLC chromatograms illustrating the retention time of different VA compounds using a method modified from Takeuchi et al. (1998). (A) Vitamin A standards with PDA detector, (B) Extracted sample with PDA detector, (C) Extracted sample using scanning fluorescence detector. I = retinoic acid, II = retinol, III = internal standard retinyl acetate, IV = retinyl palmitate.

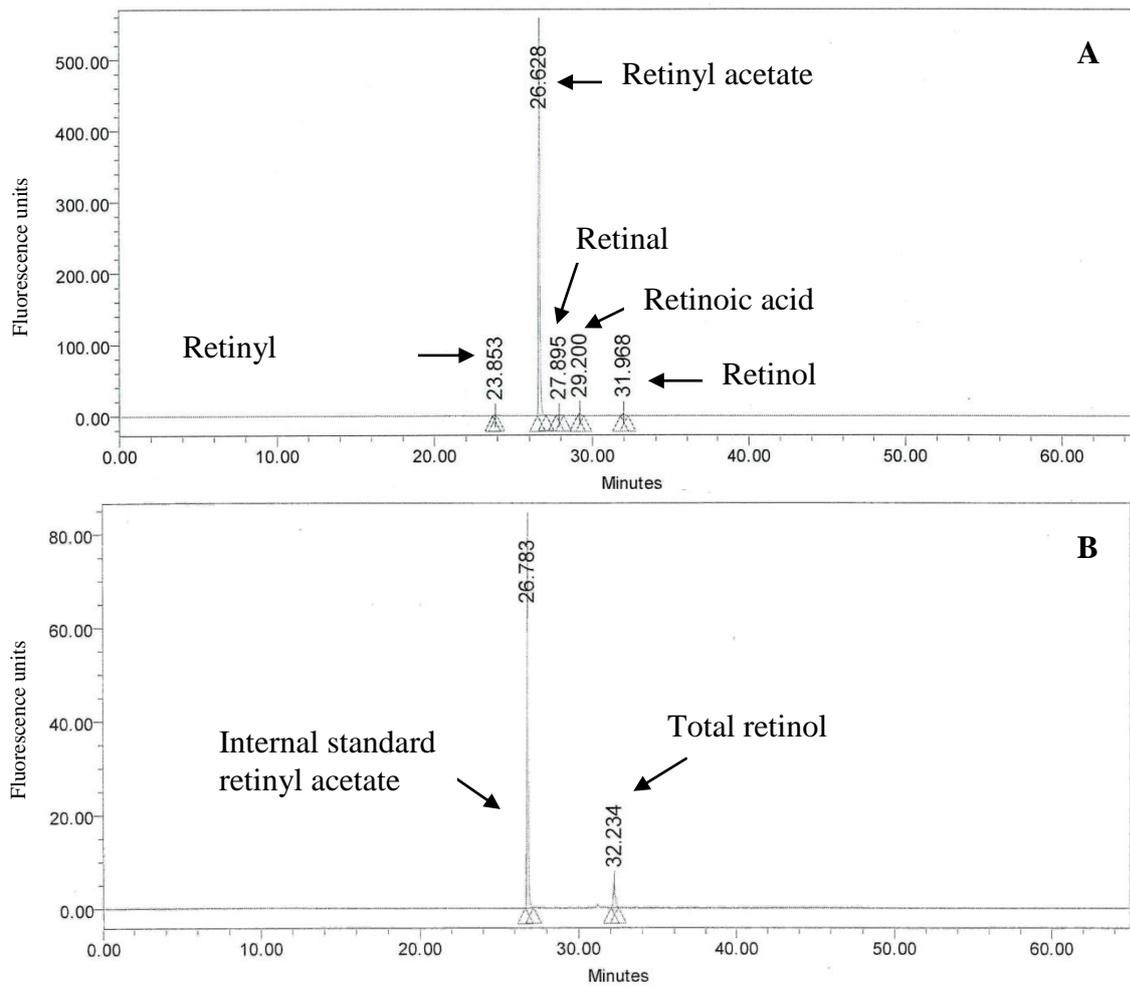


Figure 5: HPLC chromatograms illustrating the retention time of different VA compounds using a method modified from Moren et al. (2002, 2004a, 2005). (A) Vitamin A standards, (B) Extracted sample using scanning fluorescence detector.

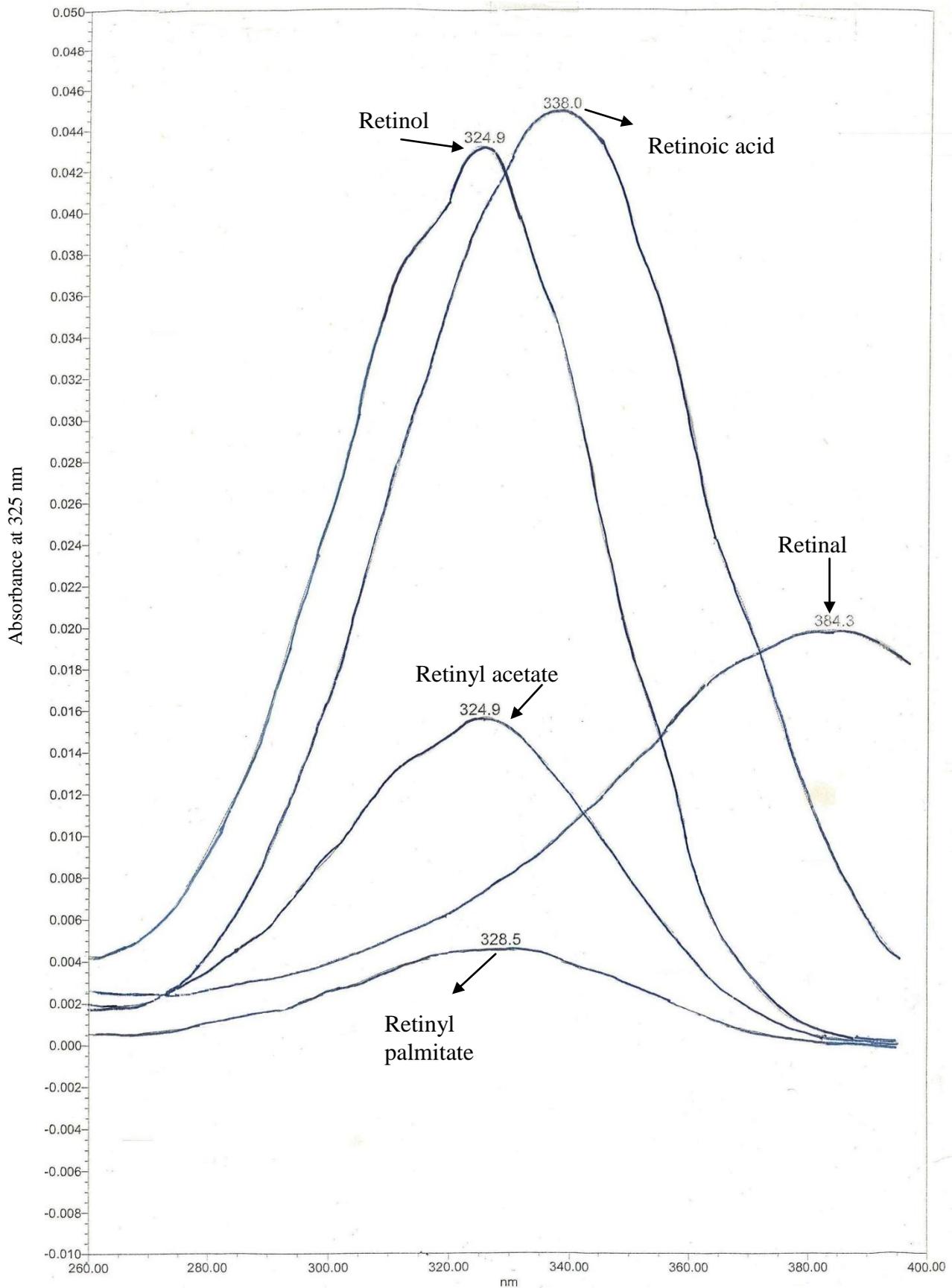


Figure 6: Absorption spectra measured by the photodiode array detector for different retinoid standards.

## 1.6 *Latris lineata* biology

*Latris lineata* has been selected as a new candidate for sea cage aquaculture in the temperate regions of Australia to complement salmonid farming which may be under threat from climate change (Battaglione et al., 2008; Battaglione and Cobcroft, 2007). In Australia, *L. lineata* distribution extends from the mid-coast of New South Wales to Kangaroo Island in South Australia, as well as being an endemic species in Tasmanian waters. *Latris lineata* is also widely distributed around the temperate regions of the southern hemisphere, where it has been identified in the Gough and Tristan Da Cunha Island groups in the southern Atlantic Ocean, the Amsterdam and St Paul Island groups in the southern Indian Ocean and in the Foundation Seamount in the southern Pacific Ocean (Andrew et al., 1995; Duhamel, 1989; Roberts, 2003). *Latris lineata* also occurs in New Zealand including the sub-Antarctic Auckland Island (Kingsford et al., 1989; Last et al., 1983). *Latris lineata* distribution is therefore limited to a latitudinal belt spanning from 35°S to 51°S (Tracey et al., 2007a).

*Latris lineata* is found on the continental shelf over rocky bottoms to depths of 300 m with the juveniles associated with shallow inshore reefs. The juveniles remain in the shallow reefs and do not move into deeper offshore reefs until they are 45 cm in size (Ziegler et al., 2007). *Latris lineata* maximum age is estimated to be 43 years (Tracey and Lyle, 2005), and they can grow up to 1.2 m in length and 25 kg in weight (Ziegler et al., 2007). Sexual maturity in the wild is reached at 6.8 years for females and 6.2 years for males (Tracey et al., 2007b). Although early *L. lineata* larval development is similar to other broadcast spawning marine fish, it has a complex and extended 9 month post-larval phase (paperfish) (Battaglione and Cobcroft, 2007; Furlani and Ruwald, 1999). According to (Leis and Trnski, 1989), the term “larvae” is the developmental stage between hatching and the completion of full external characters (fins and scales). The term “larvae” includes the yolk-sac, preflexion, flexion and postflexion stages (Leis and Trnski, 1989). In this context, for the purpose of this thesis the term “larvae” will be referring to *L. lineata* until 37 dph and the term “post-larvae” will be referring to older larvae that have completed flexion (44 dph in the thesis).

*Latris lineata* is commercially and recreationally exploited in Tasmania, for its high quality large white fillets. The fillets contain one of the highest concentrations of omega-3 oils among all of the commercially cultured available fish in the market not, only in Australia, but worldwide (Nichols et al., 2005). *Latris lineata* has positive characteristics for aquaculture, not only with high market value and tasty appeal, but also by the behaviour and response of the fish: its quiet nature, lack of cannibalism, ability to take formulated feeds and be held in captivity at high densities (Battaglione et al., 2008).

## **1.7 *Latris lineata* research**

Intensive research has been undertaken on *L. lineata* over the last two decades. Broodstock collected from the wild were held in captivity and routinely spawned all the year round through water temperature and photoperiod control (Morehead et al., 2000). Optimum temperature for egg incubation has been identified, and combined with the use of ozone as a disinfectant, resulted in high hatching rates (Battaglione and Morehead, 2006; Bermudes and Ritar, 1999; Morehead and Hart, 2003). Research has also included examination of larval sensory organ development and swim bladder inflation (Cobcroft and Pankhurst, 2003; Trotter et al., 2001). Larva feeding behaviour has been enhanced by examining the effect of turbidity and light intensity (Cobcroft et al., 2001b; Shaw, 2006). Early larva rearing requirements for the essential polyunsaturated fatty acids (PUFAs) and vitamins C and E were examined and research resulted in higher larval survival and increased growth (Bransden et al., 2004, 2005a, 2005b; Brown et al., 2005). The effect of temperature on *L. lineata* juveniles and weaning strategies has been studied and recommendations made regarding commercial grow-out (Choa et al., 2010a, 2010b). An important discovery was the identification and control of the myxozoan, *Kudoa neurophilia* that infects the nervous system of the juveniles after 30 dph (Grossel et al., 2003) and copepod parasites which infect *L. lineata* during culture (Andrews et al., 2009, 2011; Tang et al., 2007). Research has also examined the development of the immune

system (Covello et al., 2009). Cultured *L. lineata* were transferred to be commercial on-grown for the first time in sea cages in December 2006 (Battaglione et al., 2008; Battaglione and Cobcroft, 2010).

One of the impediments to the expansion of commercial grow-out of *L. lineata* has been a high level of malformations in cultured fish. All the hatcheries growing *L. lineata* have had problems with malformations in the post-larvae and juveniles with up to 95% malformation (Cobcroft et al., 2001a; Cobcroft and Battaglione, unpublished data). Intensive culture of *L. lineata* larvae and post-larvae has resulted in a particularly high incidence of jaw malformation in juveniles (Battaglione and Cobcroft, 2010; Cobcroft and Battaglione, 2009; Cobcroft et al., 2001a). Spinal malformations in *L. lineata* larvae or post-larvae are also reasonably common under intensive culture conditions. However, kyphosis associated with swim bladder malformation, where the viscera is misplaced and pushes upwards on the vertebral column, has been greatly reduced through a better understanding and optimising of initial swim bladder inflation (Trotter et al., 2001, 2005). Jaw malformation in *L. lineata* remains a major problem and has been linked with walling behaviour that is modified by tank colour, greenwater and the availability of live feed in the water column (Battaglione and Cobcroft, 2007; Cobcroft and Battaglione, 2009; Cobcroft et al., 2012). While few nutritional studies have specifically addressed malformation, jaw malformation does not appear to be affected by larval lipid nutrition or vitamin C and E incorporation in the live feed diets (Battaglione and Brown, 2006; Bransden et al., 2004; Brown et al., 2005).

## **1.8 Aim of study**

The aim of this study was to examine if VA is linked to jaw malformation and/or skeletal (vertebral) malformations in *L. lineata*. Specifically it was to determine the effect VA has on the larvae during two key feeding periods: first, feeding on rotifers, and then on *Artemia*. My study explores the question - Is there is a critical window during the course of the larval development where the effect of VA effect is most pronounced?

## 1.9 Thesis structure

The thesis is presented as a series of 6 Chapters, the research Chapters (Chapters 2-5) are written in manuscript format of the journal Aquaculture. This format has necessitated some duplication of introduction, methods and references.

### 1.9.1 Chapter 1

#### General introduction

This Chapter provides background and the general context for the thesis.

### 1.9.2 Chapter 2

#### Enrichment of rotifers and *Artemia* with retinyl palmitate

This Chapter presents the development of techniques for the enrichment of live feeds, rotifers and *Artemia*.

The aims were to 1) Determine the optimum enrichment time and effect of light on the enrichment process of live feeds (rotifers and *Artemia*), 2) Determine the uptake of retinyl palmitate by live feeds at different enrichment doses, 3) Determine the rotifer lipid levels after enrichment with different VA experimental emulsions, and 4) Determine VA levels in enriched rotifers held in green and clearwater over time.

### 1.9.3 Chapter 3

#### Effect of dietary vitamin A level in rotifers on performance and skeletal abnormality of Striped

#### Trumpeter *Latris lineata* larvae and post-larvae

The aims were to 1) Examine the effect of graded levels of VA on the performance of larvae and post-larvae during the rotifer feeding stage, and 2) Determine the requirement for VA during this early stage of development, and 3) Examine the effect of VA on selected skeletal malformations.

#### **1.9.4 Chapter 4**

Effect of dietary vitamin A level in rotifers and rearing in the presence and absence of greenwater on the performance and skeletal abnormality of Striped Trumpeter *Latris lineata* larvae and post-larvae

The aims of this Chapter were to 1) Examine the effect of rearing water type and VA-enriched rotifers on the performance of larvae and post-larvae, and 2) Examine the effect of rearing water type and enriched rotifers with VA on selected skeletal malformations.

#### **1.9.5 Chapter 5**

Effect of dietary vitamin A during *Artemia* feeding on performance and skeletal abnormality of Striped Trumpeter *Latris lineata* larvae and post-larvae

The aims of this Chapter were to 1) Examine the effect of graded levels of VA on the performance of larvae and post-larvae during the *Artemia* feeding stage, 2) Determine the requirement for VA during the *Artemia* feeding phase, and 3) Examine the effect of VA on selected skeletal malformations.

#### **1.9.6 Chapter 6**

General discussion

This Chapter discusses the implications of the research Chapters in this thesis in context with other published studies and future directions of the research.

## **1.10 Animal ethics approval**

All experimentation was conducted with the approval of the University of Tasmania Animal Ethics Committee, approval number A0009752, in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes - 7<sup>th</sup> edition, 2004.

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# Chapter 2

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## Enrichment of rotifers and *Artemia* with retinyl palmitate

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## 2.1 Abstract

The nutritional profiles of cultured live feed including rotifers, *Brachionus plicatilis*, and *Artemia* do not meet the requirements of many finfish larvae for optimal growth, survival and development. Enrichment of live feeds with different essential nutrients is therefore important to improve the nutritional profile required by the target larvae. Vitamin A (VA) plays a key role in vision, immunity and skeletogenesis in finfish larvae. In this study, the effects of light and time on the dynamics of different VA compounds were examined during the enrichment process of live feeds with retinyl palmitate. Enrichment time significantly affected the concentration of retinoids in both live feeds but light only affected the enrichment process of rotifers and the concentration of retinol in *Artemia*. Enrichment of rotifers and *Artemia* for 2 and 24 h, respectively, under dark conditions with levels of retinyl palmitate ranging from 0 to 10,000 mg l<sup>-1</sup> emulsions, resulted in a positive relationship between the concentration of retinyl palmitate in the enrichment emulsions and the concentration of retinoids in the enriched live feeds. The lipid level in rotifers enriched with VA emulsions for 2 h was found to be significantly lower than those enriched with Algamac-3050 for 8 h, and previous studies have shown the latter is necessary to meet the requirements of Striped Trumpeter, *Latris lineata*, larvae. The lipid profile of rotifers enriched for 6 h with Algamac-3050 followed by 2 h with VA emulsions resulted in a lipid profile sufficient to meet *L. lineata* larval lipid requirements, although the rotifers incorporated significantly lower retinoids than those enriched for only 2 h with VA emulsions. Therefore, when feeding VA-enriched rotifers to *L. lineata* larvae, this should be balanced with an appropriate lipid diet (Algamac-3050) to meet the larvae requirements for essential fatty acids. The greenwater larval culture method is optimal for *L. lineata*, although microalgae contain carotenoids that impact the VA content of live feeds. The concentration of retinoids in rotifers enriched with VA emulsions or Algamac-3050 and held in clear and greenwater over time revealed that the dynamics of VA compounds in the enriched rotifers were significantly affected by the initial VA enrichment levels, water type and time. The general trend, in

clearwater, was for the concentration of retinoids in VA-enriched rotifers to decrease over time, while in greenwater, the retinoid concentration decreases in high VA-enriched rotifers and increases in low VA-enriched rotifers. According to the post-enrichment rotifer holding conditions in this study, which were the same as those routinely used for culturing *L. lineata*, rotifers enriched with 0 and 500 mg retinyl palmitate l<sup>-1</sup> were significantly different for approximately 4 h in clear water and for 4 to 8 h in greenwater. Rotifers enriched with the highest VA treatment, i.e. 5,000 mg retinyl palmitate l<sup>-1</sup>, were significantly different from the other vitamin treatments in both clear and greenwater over 14 h. It is recommended that a wide range of initial emulsions are used in dose-response studies examining the effects of VA, especially when rearing larvae in clearwater to ensure that larvae receive graded VA doses.

## 2.2 Introduction

Feeding most finfish larvae for aquaculture still depends on live feeds during the earliest phases of development (Conceição et al., 2010a), despite the fact that progress has been achieved in the production of inert diets for some fish larvae (Cahu and Infante, 2001; Koven et al., 2001; Lazo et al., 2000). The reason is that when newly hatched marine larvae start to feed they are characterized by a small and simple digestive system (Ronnestad et al., 1999; Watanabe and Kiron, 1994), where there is no stomach and much of the protein digestion takes place in hindgut epithelial cells (Govoni et al., 1986). The larval digestive system is incapable of processing formulated diet as efficiently as live feeds, and consequently limits larval growth and survival when compared to larvae fed on live feeds (Conceição et al., 2010a). The two main live foods offered to marine fish larvae in culture are rotifers, *Brachionus plicatilis*, and *Artemia* species. Average body size is 50-200 µm for rotifers and 200-500 µm for *Artemia*, depending on the strain and age (Conceição et al., 2010a; Watanabe and Kiron, 1994). The advantages of using live feeds in aquaculture, in addition to their high digestibility for larvae (water content > 80%), are their small size, which makes it easier for the larvae to prey upon them, and their swimming ability, which allows larvae to detect and capture the prey.

Despite the simple characteristics of the larval gut, larval fish require sufficient balanced nutrients to support normal development. The nutritional profile of cultured rotifers and *Artemia* do not meet these requirements for many fish species. However, dietary manipulation to improve the nutritional profile of the cultured live feed is accomplished by a post-culture enrichment process (Conceição et al., 2010a; Lubzens and Zmora, 2007). Enrichment is through bioencapsulation where live feeds are cultured in a medium rich in fatty acids, amino acids, vitamins or other substances such as hormones or vaccines (Conceição et al., 2010a; Coutteau and Sorgeloos, 1997; Dhert et al., 2001). The non-selective feeding behaviour of the live prey enables them to feed on the correctly sized diffused particles and incorporate them into the digestive tract and assimilate them into their bodies depending on enrichment periods and temperature (Conceição et al., 2010a; Sorgeloos et al., 2001; Sweetman, 2004). The enrichment of rotifers and *Artemia* to deliver important nutrients including lipids, amino acids, vitamins and minerals to the marine larvae to support normal development is widely used in aquaculture (Aragao et al., 2004; Coutteau and Sorgeloos, 1997; Sargent et al., 1999).

Vitamins such as A, C, D, E and K play an important role to support the optimal growth and development of fish larvae (Brown et al., 2005; Demartinez, 1990; Haga et al., 2004b; Hamre et al., 2010; Lall and Lewis-McCrea, 2007; Lock et al., 2010; Mazurais et al., 2008; Roy and Lall, 2007; Villanueva et al., 2009; Waagbø, 2010). Vitamin A (VA), also known as retinoid, in fish larvae plays a key role in vision, immunity, differentiation of epithelial tissue, morphogenesis, tissue homeostasis and skeletogenesis (Blomhoff and Blomhoff, 2006; Combs, 2008; Fernández et al., 2009; Ross et al., 2000; Srinivas and Chethankumar, 2007; Thompson et al., 1995). Retinoids also have a role in establishing body and organ axes in conjunction with other nutrients such as vitamin D and fatty acids (Balmer and Blomhoff, 2002; Hamre et al., 2010; Villeneuve et al., 2006). Vitamin A deficiency in fish leads to retarded growth, blindness, restlessness, abnormal movement, exophthalmia and haemorrhages of the eye, fins or skin, decreased immunity and harm to the intestinal epithelia (Goswami and Basumatari, 1988; Goswami and Dutta, 1991; Moren et al., 2004; Saleh et al., 1995; Yang et al., 2008). On the other hand, excess VA induces developmental abnormalities including

patterning defects, skin haemorrhage, abnormal pigmentation and abnormal bone formation in the fish (Dedi et al., 1997; Fernández et al., 2008; Haga et al., 2002; Hernandez-H et al., 2006; Hilton, 1983; Martinez et al., 2007; Suzuki et al., 1999; Takeuchi et al., 1998; Tarui et al., 2006).

The aim of this research Chapter was to examine the enrichment process of live feeds, rotifers and *Artemia*, with VA in the retinyl palmitate form, and to determine the concentration of retinoids over time in both live feeds. The research reported here forms the basis for the enrichment protocols used to enrich live feeds with VA. For later Chapters, VA-enriched live feeds were fed to Striped Trumpeter, *Latris lineata*, larvae in dose-response experimental designs (Chapters 3, 4 and 5). My thesis was designed to assess the effect of dietary VA delivered in live feeds on skeletal malformations and determine the VA requirement for optimal development of *L. lineata*. Since retinoids are unstable compounds and can be oxidized and/or isomerised to other compounds, especially in the presence of oxidants including air, light or heat (Barua and Furr, 1998), the effect of the absence or presence of light on the enrichment process was examined. The effect of light on the enrichment process of live feeds with VA has been examined by Haga et al. (2006). He found that light affected the enrichment process of rotifers, with the concentration of retinoids being higher in dark conditions, but not the *Artemia*. I also examined the lipid profile of the VA-enriched rotifers to determine if they met *L. lineata* requirements for fatty acids, based on previous research (Bransden et al., 2004, 2005b).

*Latris lineata* culture is optimal with the addition of the microalga *Nannochloropsis oculata* into the larval rearing tanks during the rotifer feeding period (Battaglione and Cobcroft, 2007; Cobcroft et al., 2001; Shaw, 2006), known as the “greenwater technique”. This microalga contains small amounts of retinol ( $< 0.25\text{ng mg}^{-1}$ ) and  $290 \pm 40 \text{ ng mg}^{-1}$   $\beta$ -carotene (Brown et al., 1999), where  $\beta$ -carotene is a major dietary precursor of VA (Ross and Ternus, 1993). I also assessed the effect of adding rotifers enriched with VA into the larval rearing tanks with the presence or absence of microalgae, in conditions to mimic the culture environment of the larvae. The change in the retinoid content of

rotifers was examined with respect to the water type, and the time after transfer from the enrichment.

## 2.3 Materials and methods

### 2.3.1 Source culture of rotifers and *Artemia*

*Brachionus plicatilis* (Austria strain) were cultured on algal paste (*Nannochloropsis* sp, Reed Mariculture Inc. USA) at a density up to 1,000 rotifers ml<sup>-1</sup> at 25 °C and 33 ppt in a recirculation system with ozone disinfected sea water (> 700 mV ORP for 10 min). Harvested rotifers were rinsed with sea water and counted before distribution into 20 l enrichment containers at 500 rotifers ml<sup>-1</sup> and 25 °C.

*Artemia* cysts (AAA, INVE Aquaculture Nutrition USA) were decapsulated and stored in brine at 4 °C (Sorgeloos et al., 1977) and hatched when needed at 26 °C under strong illumination and aeration. Rotifers and *Artemia* were enriched with the experimental emulsions at 0.11 and 0.6 g l<sup>-1</sup> enrichment respectively, at 26 °C and 33 ppt. The required weight of the experimental emulsions was blended for 3 min in fresh tap water at 20 °C, then poured through a 63 µm screen before adding to the rotifers and *Artemia*. Following the enrichment periods, rotifers and *Artemia* were gently siphoned, collected into 63 and 150 µm bag screens respectively, and rinsed for 10 min in clean water before counting and distribution to experimental tanks if required, or transferred immediately to -80 °C prior to biochemical analyses.

### 2.3.2 Experiments with rotifers and *Artemia*

#### 2.3.2.1 Determining the enrichment time and the effect of light

In preparation for experiments with fish larvae, this research investigated the optimal enrichment times and lighting conditions (light or dark) for VA incorporation in live feeds. Rotifers were enriched with 3,000 mg retinyl palmitate l<sup>-1</sup> emulsion (Nutrakol Nutrition, WA Australia) while *Artemia* were

enriched with 5,000 mg retinyl palmitate l<sup>-1</sup> emulsion. Basic emulsion constituents were tuna oil 57%, vitamin E 4% and vitamin C 4% (Nutrakol, WA). Enrichments were added to the rotifers and harvested after 2, 4, 6, 8, 10 and 12 h, while *Artemia* were harvested after 3, 6, 12, 18, 22 and 24 h in both dark and light conditions. The light intensity in the 'light' treatment was  $18.45 \pm 2.34 \mu\text{mol s}^{-1} \text{m}^{-2}$ , and the enrichment vessels were covered with black plastic curtains (sheets) for the 'dark' treatment.

### 2.3.2.2 Enrichment curve

In order to set dietary levels of VA, it was necessary to determine the uptake of retinyl palmitate by live feeds at different VA enrichment doses. Rotifers were enriched with 13 increasing levels of retinyl palmitate (0, 50, 100, 200, 300, 400, 500, 750, 1,000, 1,500, 3,000, 5,000 and 10,000 mg retinyl palmitate l<sup>-1</sup> emulsion) for 2 h in the dark. *Artemia* were enriched with 9 increasing levels of retinyl palmitate (0, 250, 500, 750, 1,000, 1,500, 3,000, 5,000 and 10,000 mg retinyl palmitate l<sup>-1</sup>) for 24 h in the dark. Triplicate samples were collected from each VA level at the end of the enrichment time, for VA and lipid biochemical analyses.

### 2.3.2.3 Determining the lipid content

This experiment was designed to allow the comparison of the lipid profile of the rotifers enriched with Algamac-3050 (Aquafauna Biomarine, USA), which is optimal for *L. lineata* growth and survival (Battaglione and Cobcroft, 2007; Battaglione et al., 2006), with the lipid profile of the rotifers enriched with the VA experimental emulsions. Rotifers were enriched in 20 l vessels with Algamac-3050 for 8 h in constant light at 0.2 g million<sup>-1</sup> rotifer according the manufacturer's instructions. The required weight of Algamac-3050 was hydrated in 1 l fresh tap water at 20 °C for 10 min, before blending for 3 min, then screened and added to the rotifers in the enrichment vessels. Rotifers were also enriched with 10,000 mg retinyl palmitate l<sup>-1</sup> experimental emulsion for 2 h in complete darkness at 0.11 g l<sup>-1</sup> enrichment. After enrichment, enriched rotifers were collected into 63  $\mu\text{m}$  bag screens and

rinsed for about 10 min in clean seawater and transferred immediately to  $-80^{\circ}\text{C}$  prior to biochemical lipid analyses.

Initial results indicated that the experimental emulsions did not provide sufficient dietary lipids for *L. lineata* requirements in the 2 h enrichment time, which was optimal for VA enrichment. To determine whether rotifer lipid levels could be increased by initial enrichment with Algamac-3050, followed by a top-up of VA enrichment with the experimental emulsions, rotifers were first enriched with Algamac-3050 for 6 h under constant light at  $0.2 \text{ g million}^{-1}$  rotifer. Rotifers were then further enriched with experimental emulsions containing 0, 500 or  $5,000 \text{ mg l}^{-1}$  retinyl palmitate for 2 h under dark conditions. Enriched rotifers were rinsed and collected as mentioned above and transferred immediately to  $-80^{\circ}\text{C}$  prior to lipid biochemical analyses. These rotifers were also extracted to determine their retinoid content, in order to test whether the retinoid content in the rotifers was affected by the 6 h Algamac-3050 enrichment before the 2 h enrichment with the VA emulsions.

#### **2.3.2.4 Determining vitamin A levels in enriched rotifers held in clear and greenwater over time**

Larval rearing of *L. lineata* is optimal using greenwater. This experiment was designed to examine the effect of green or clearwater on the VA level in rotifers. Rotifers enriched for 2 h with 0, 500 or  $5,000 \text{ mg l}^{-1}$  retinyl palmitate, and rotifers enriched with Algamac-3050 alone for 8 h, were transferred to green and clear sea water in  $24 \times 300 \text{ l}$  larval rearing tanks at a density of  $5 \text{ ml}^{-1}$ , simulating larval experiment conditions (Chapters 3 and 4). For greenwater treatments, live algae *Nannochloropsis oculata* were added to achieve a turbidity of 3 NTU ( $\sim 400,000 \text{ cells ml}^{-1}$ ). Live algae were acclimated to the experimental temperature in an aerated reservoir tub the day before the experiment started and transferred to the tanks just before adding the enriched rotifers. Rotifers were sampled from the three tested vitamin levels and Algamac-3050 at different times over 14 h (0, 4, 8, and 14 h), then analysed to determine the content of retinoids.

For the 0 h sample, rotifers were taken immediately following rinsing from the enrichment vessels. At 4, 8 and 14 h, one experimental tank from each treatment was harvested using a 63 µm bag screen, and rotifers were rinsed with 0.5 M ammonium formate to remove salt and transferred immediately to -80 °C until biochemical analyses. There was no replication of tanks for any of the treatments due to logistical constraints associated with the available number of experimental tanks, and sample size required, although three replicate samples from each tank were analysed for each treatment and time combination.

### 2.3.3 Biochemical analysis

Retinoids were measured as total VA (the sum of retinyl palmitate, retinol and retinoic acid) using a technique modified from Takeuchi et al. (1998). To determine total VA, 1 g of live feed was homogenised within a 20 ml culture tube containing 10 ml chloroform: methanol (2:1 v/v), 0.01% butylated hydroxytoluene and 50 ng retinyl acetate as an internal standard. After adding 2.5 ml 0.88% KCl and shaking for 30 s, each sample was left on ice for 1 h. Sample tubes were centrifuged for 10 min and then the lower chloroform phase was transferred to 10 ml vials and diluted to 10 ml with additional chloroform. Subsamples of 4 ml were transferred to 5 ml vials and evaporated to dryness by blowing with nitrogen. The dry fraction derived from each of the live feed samples was redissolved in 2 ml acetone: methanol (1:1 v/v) and filtered through 0.45 µm syringe filter. Samples (20 µl) were analysed using a Waters Model 600E liquid chromatograph system (Waters Corporation, Milford, MA, USA) supplied with a Waters Model 996 photodiode array (PDA) detector and 475 scanning fluorescence detector set for an excitation maximum at 325 nm and emission maximum at 470 nm. The retinoids eluted within 30 min, using an Alltima C18 Column (250 mm x 4.6 mm 5µm; Grace Davison Discovery Sciences, Rowville, Vic., Australia) set to 30 °C and using as isocratic mobile phase of 98% methanol with 0.5% ammonium acetate and chloroform (85:15 v/v) at a flow rate of 1.5 ml min<sup>-1</sup>. Peak areas were quantified using Waters Millennium software.

The concentration of retinoids in samples was calculated by comparing peak area to those of internal and external standards. Live feed samples were not freeze-dried and appropriate calculations were made to convert wet weight into dry weight (DW). Rotifer and *Artemia* DW were based on measurements for different freeze-dried samples of live feed during this experiment. Data are presented as  $\text{ng mg}^{-1}$  DW.

Total lipids and fatty acid methyl esters in live prey were determined using a modified version of Bligh and Dyer (1959). Freeze-dried samples (50 mg) were extracted overnight in chloroform: methanol: water (1:2:0.8 v/v/v) in 100 ml separatory funnels. Separation was initiated by adding chloroform and water to give a final ratio of (1:1:0.9 v/v/v). The chloroform was removed, then concentrated by rotary evaporation and the total lipids were determined gravimetrically after blowing with nitrogen until constant weight. The extracts were then redissolved in 1.5 ml chloroform, transferred to vials and stored under nitrogen at  $-20\text{ }^{\circ}\text{C}$ .

Fatty acid methyl esters (FAME) were determined by transmethylation of 100  $\mu\text{l}$  of the extract. The aliquot was first blown with nitrogen until the solvent evaporated, then 3 ml of methanol: hydrochloric acid: chloroform (10:1:1 v/v/v) were added and the sample was heated at  $80\text{ }^{\circ}\text{C}$  for 2 h. FAME were extracted three times with 3 ml hexane: chloroform (4:1 v/v), where each time the upper layer was transferred to a round bottom flask (RBF). The combined extracts within the RBF were rotary evaporated and then rinsed 3 times with 0.5 ml dichloromethane to recover all the FAME. The recovered FAME were transferred into 2 ml gas chromatography (GC) vials and blown with nitrogen until dryness. Then 1.5 ml chloroform containing the internal standard methyl tricosanoate ( $\text{C}_{23:0}$ ) was added. FAME were analysed by GC (Agilent Technologies 7890A) equipped with methyl-silicone, fused capillary column (15 m x 0.1 mm internal diameter, 0.1  $\mu\text{m}$  film thickness). Samples were injected in splitless mode at  $120\text{ }^{\circ}\text{C}$  using an Agilent Technologies 7683B injector with helium as carrier gas. Acquired peaks were quantified with Agilent Technologies Chemstation software (Palo Alto, CA, USA). Preliminary peak identifications were made by

comparing retention time data with authentic and laboratory standards. GC-mass spectrometric (GC-MS) analyses were performed on a Finnigan GCQ plus GC-MS (Finnigan corp., San Jose, CA, USA) ion trap fitted with a capillary column similar to that described above.

### **2.3.4 Statistical analysis**

One-way ANOVA was used to test the effect of different enrichment levels on VA incorporation and lipid content of enriched live feeds. Two-way ANOVA was used to test the effect of the fixed factors time and light on VA incorporation into live feeds. Two-way ANOVA was also used to test the effect of the fixed factors VA enrichment with or without Algamac-3050 enrichment on the retinoid concentration in enriched rotifers. Three-way ANOVA was used to test the effects of water type, enrichment levels and time on the VA content of live feeds. Dry weight data were  $\log_{10}$  transformed to achieve homogeneity of variance and normal distribution. Levene's test was used to assess data for homogeneity of variance. Where significant treatment effects were found, a post hoc Tukey test was used to determine differences among means. A polynomial regression was used to examine the relationship between dietary (emulsion) VA and the concentration of retinoids in live feeds. Significant differences were accepted at  $P < 0.05$ . Statistical analyses were performed using SPSS 13.0 (SPSS Inc.).

## **2.4 Results**

### **2.4.1 Enrichment time and the effect of light**

#### **2.4.1.1 Rotifers**

Concentration of retinoids (retinyl palmitate, retinol, retinoic acid and total VA) in rotifers enriched with a nominal concentration 3,000 mg retinyl palmitate  $l^{-1}$ , were significantly affected by the enrichment time and the illumination conditions (Table 1). The highest concentration of total VA was in the rotifers enriched in dark conditions for 2 h ( $205.53 \pm 4.83$  ng total VA  $mg^{-1}$  DW rotifers, all VA values are expressed as ng  $mg^{-1}$  DW live prey, here and throughout) while the lowest concentration

was in the rotifers enriched in the light for 12 h ( $75.31 \pm 3.85$  ng total VA  $\text{mg}^{-1}$ ) (Fig. 1a). There were no significant differences in the concentration of total VA for 2, 4 and 6 h of enrichment under light, with an average of  $139.15 \pm 6.69$  ng total VA  $\text{mg}^{-1}$ .

Table 1: Results of two-way ANOVAs to test the effects of time and illumination conditions on the retinyl palmitate, retinol, retinoic acid and total VA content of rotifers enriched with 3,000 mg retinyl palmitate  $\text{l}^{-1}$ . Time has 6 levels (2, 4, 6, 8, 10, 12 h). Illumination conditions has two levels, light and dark. \* indicates significant effect  $P < 0.05$ .

	Source of variation	F value	P value
<b>Retinyl palmitate</b>	Time	302.540	< 0.001*
	Illumination Condition	93.252	< 0.001*
	Time x Illumination Condition	47.653	< 0.001*
<b>Retinol</b>	Time	403.706	< 0.001*
	Illumination Condition	32.684	< 0.001*
	Time x Illumination Condition	5.758	< 0.001*
<b>Retinoic acid</b>	Time	6.774	< 0.001*
	Illumination Condition	12.482	0.002*
	Time x Illumination Condition	4.283	0.006*
<b>Total VA</b>	Time	195.026	< 0.001*
	Illumination Condition	144.961	< 0.001*
	Time x Illumination Condition	46.601	< 0.001*

Under dark enrichment conditions, retinyl palmitate concentration reached a maximum peak after 2 h from the onset of enrichment ( $198.18 \pm 5.06$  ng retinyl palmitate  $\text{mg}^{-1}$ ) and then gradually decreased where the lowest concentration was at 8 and 12 h from the onset of enrichment ( $82.54 \pm 1.45$  ng retinyl palmitate  $\text{mg}^{-1}$ ). In contrast, under dark conditions retinol and retinoic acid were lowest at 2 h and increased with the increasing enrichment period (Fig. 1b). Retinol concentration increased from  $5.10 \pm 0.06$  ng  $\text{mg}^{-1}$  after 2 h from the onset of enrichment to an average of  $25.47 \pm 0.44$  for 6, 8, 10 and 12 h of enrichment. The lowest retinoic acid concentration was 2 h from the onset of enrichment ( $2.26 \pm 0.31$  ng retinoic  $\text{mg}^{-1}$ ) and the average was  $5.16 \pm 0.72$  ng retinoic  $\text{mg}^{-1}$  from 4 to 12 h of enrichment.

Enriched rotifers in the light showed a similar trend, although the concentration of retinoids was either significantly lower or the same as the corresponding times from the dark enrichment conditions. The only exception was the concentration of retinyl palmitate at 8 h which was significantly lower under dark conditions ( $78.89 \pm 2.81$  ng retinyl palmitate  $\text{mg}^{-1}$ ) compared with light conditions ( $93.12 \pm 10.25$  ng retinyl palmitate  $\text{mg}^{-1}$ ).

Under light conditions, the retinyl palmitate concentration peaked at 2 h ( $141.55 \pm 6.57$  ng retinyl palmitate  $\text{mg}^{-1}$ ) and then gradually decreased until 12 h, where the concentration was  $52.00 \pm 4.35$  ng retinyl palmitate  $\text{mg}^{-1}$ . Retinol showed two peaks at 6 and 8 h, average of  $25.26 \pm 1.13$  ng retinol  $\text{mg}^{-1}$ , and then decreased. Retinoic acid showed a peak at 6 h of enrichment ( $6.85 \pm 1.67$  ng retinoic  $\text{mg}^{-1}$ ) and an average of  $2.82 \pm 0.21$  ng retinoic  $\text{mg}^{-1}$  for 4, 8, 10 and 12 h of enrichment.

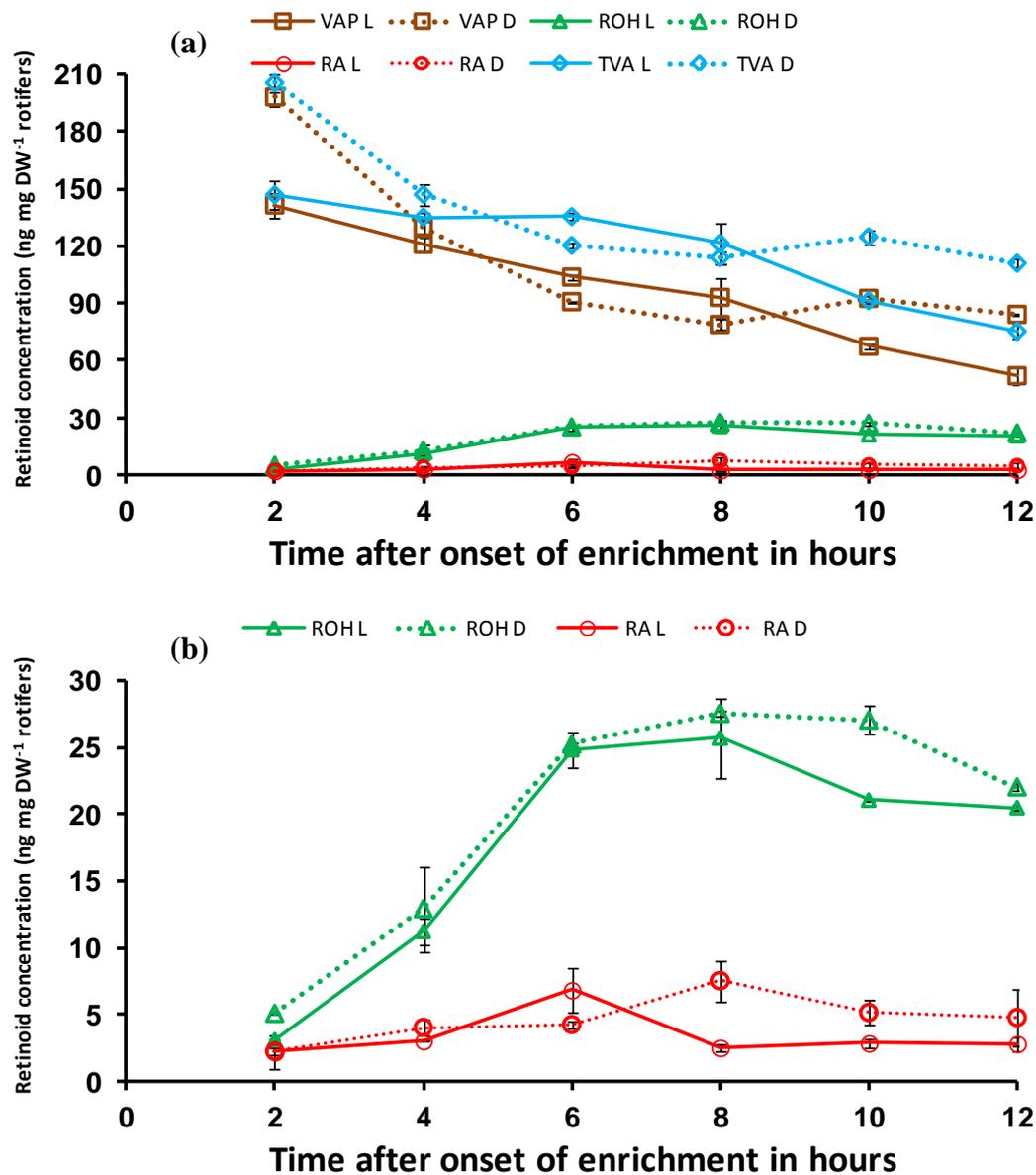


Figure 1: (a) Concentration of retinoids in rotifers enriched with nominal concentration emulsion 3,000 mg retinyl palmitate l<sup>-1</sup> over time under dark and light conditions, (b) Showing only retinol and retinoic acid. Values are mean  $\pm$  SD (n = 3). Abbreviations: D, dark; L, light; VAP, retinyl palmitate; ROH, retinol; RA, retinoic acid; TVA, total vitamin A.

#### 2.4.1.2 *Artemia*

The concentration of retinoids in *Artemia* enriched with 5,000 mg retinyl palmitate l<sup>-1</sup> was significantly affected by the enrichment time but not by illumination, except for retinol (Table 2). Total VA in the enriched *Artemia* was significantly higher after 3 h of enrichment ( $36.35 \pm 2.01$  ng total VA mg<sup>-1</sup>) and significantly lower after 6 h of enrichment ( $25.07 \pm 4.24$  ng total VA mg<sup>-1</sup>) while

there was no significant difference in the total VA concentration between the *Artemia* enriched for 12, 18, 22 and 24 h, with an average of  $30.37 \pm 4.50$  ng total VA  $\text{mg}^{-1}$  (Fig. 2a).

Table 2: Results of two-way ANOVAs to test the effects of time and illumination conditions on retinyl palmitate, retinol, retinoic acid and total VA content of *Artemia* enriched with 5,000 mg retinyl palmitate  $\text{l}^{-1}$ . Time has 6 levels (3, 6, 12, 18, 22, 24 h). Illumination condition has two levels, light and dark. \* indicates significant effect  $P < 0.05$ .

	Source of variation	F value	P value
<b>Retinyl palmitate</b>	Time	7.388	< 0.001*
	Illumination Condition	0.001	0.973
	Time x Illumination Condition	3.385	0.019*
<b>Retinol</b>	Time	21.786	< 0.001*
	Illumination Condition	7.342	0.012*
	Time x Illumination Condition	12.381	< 0.001*
<b>Retinoic acid</b>	Time	33.505	< 0.001*
	Illumination Condition	1.512	0.231
	Time x Illumination Condition	6.040	< 0.001*
<b>Total VA</b>	Time	5.498	0.002*
	Illumination Condition	0.060	0.809
	Time x Illumination Condition	2.422	0.065

There was no significant difference in the concentration of retinyl palmitate at 3 h dark and light conditions, 6 to 18 h light conditions and 18 to 24 h dark conditions with an average of  $18.62 \pm 1.72$  ng  $\text{mg}^{-1}$ . The minimum concentration was from 6 to 12 h dark conditions and 22 to 24 light conditions with an average of  $15.43 \pm 1.85$  ng  $\text{mg}^{-1}$ . Minimum concentration of retinoic acid was recorded after 3 h from the onset of enrichment for both dark and light conditions with an average of  $3.31 \pm 0.04$  ng  $\text{mg}^{-1}$  (Fig. 2b). There was no significant difference in the retinoic acid content between *Artemia* enriched in light for 12 to 24 h and those enriched in dark for 12 and 24 h with an average of  $7.66 \pm 0.89$  ng  $\text{mg}^{-1}$ . Retinol concentration in enriched *Artemia* was affected by the light conditions and reached a peak for both dark and light enrichments at 12 h, and was not significantly different at 24 h for the dark enrichment only, with an average of  $10.55 \pm 0.83$  ng retinol  $\text{mg}^{-1}$ .

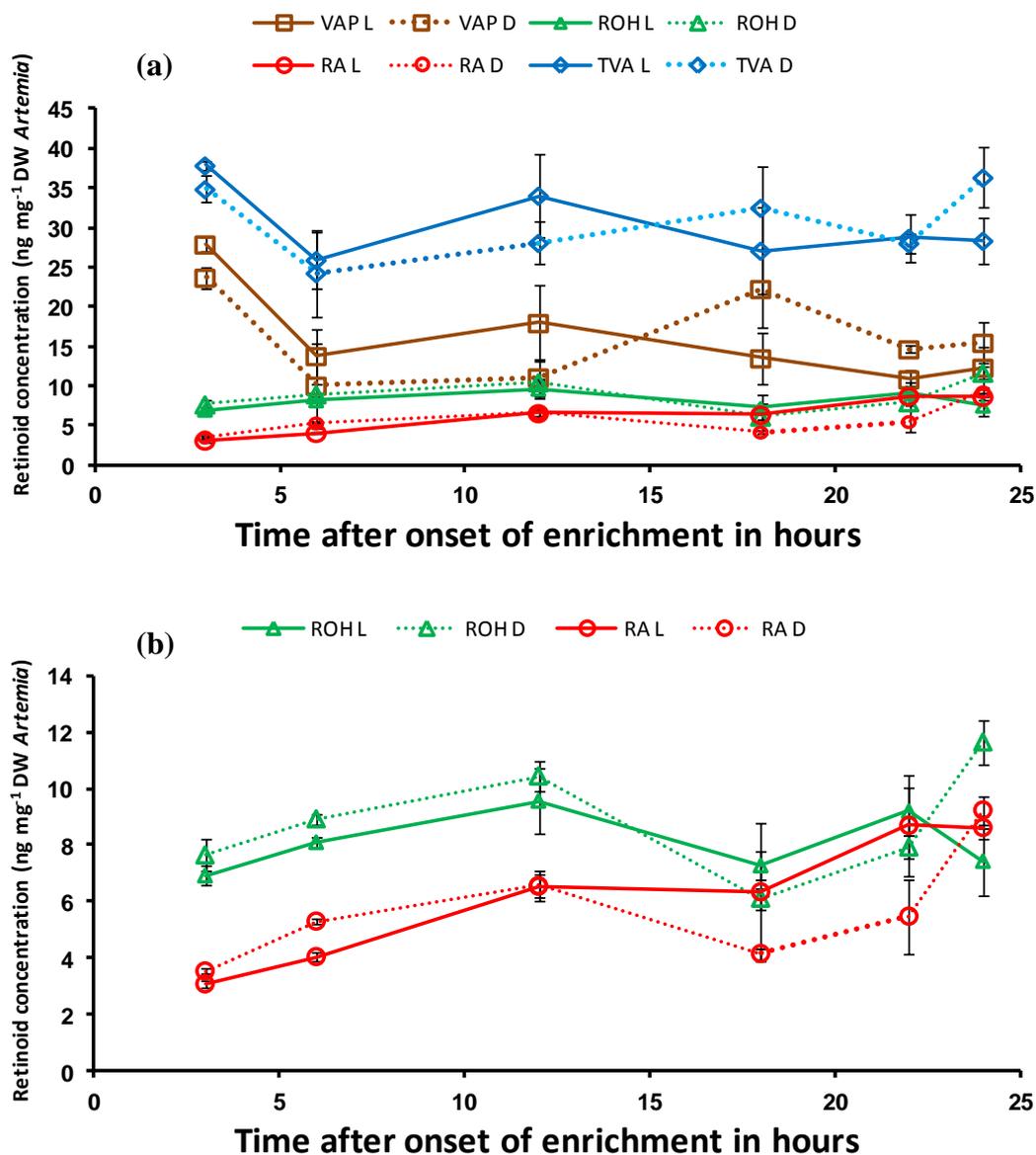


Figure 2: (a) The concentration of retinoids in *Artemia* enriched with nominal concentration emulsion 5,000 mg retinyl palmitate l<sup>-1</sup> over time under dark and light conditions, (b) Showing only retinol and retinoic acid. Values are mean  $\pm$  SD (n = 3). Abbreviations: D, dark; L, light; VAP, retinyl palmitate; ROH, retinol; RA, retinoic acid; TVA, total vitamin A.

## 2.4.2 Enrichment curve

### 2.4.2.1 Rotifers

There was a significant positive relationship between the concentration of retinyl palmitate in the enrichment emulsions and the concentration of retinoids in the rotifers enriched with graded levels of retinyl palmitate for 2 h in dark conditions (Fig. 3) ( $P < 0.001$  for all). The retinoid content in the

rotifers was continuing to increase at the highest enrichment level, indicating that the maximum VA enrichment may not have been achieved using 10,000 mg retinyl palmitate l<sup>-1</sup> emulsion.

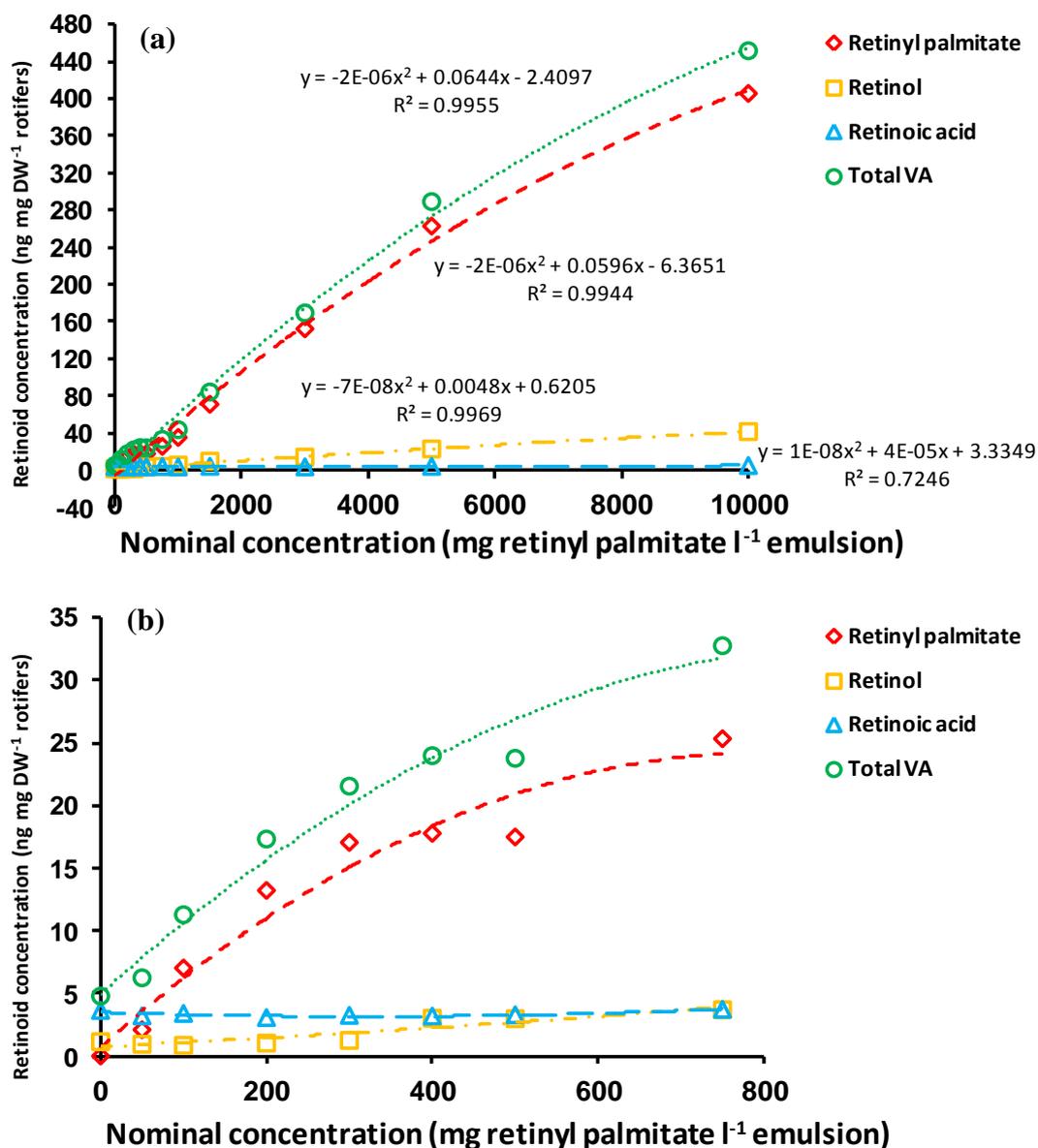


Figure 3: (a) Concentration of retinoids in rotifers enriched with graded levels of retinyl palmitate for 2 h in dark conditions, (b) Showing the concentration of retinoids in rotifers enriched with ≤ 750 mg retinyl palmitate l<sup>-1</sup>. Values are mean, n = 3. Line indicates polynomial regression.

#### 2.4.2.2 Artemia

There was a significant positive relationship between the concentration of retinyl palmitate in the enrichment emulsions and the concentration of retinoids in the *Artemia* enriched with graded levels of retinyl palmitate for 24 h in dark conditions (Fig. 4) ( $P < 0.001$  for all). A similar pattern was

observed to rotifers, where the retinoid content continued to increase at the highest enrichment level, indicating the maximum VA enrichment may not have been achieved by the 10,000 mg retinyl palmitate l<sup>-1</sup> emulsion.

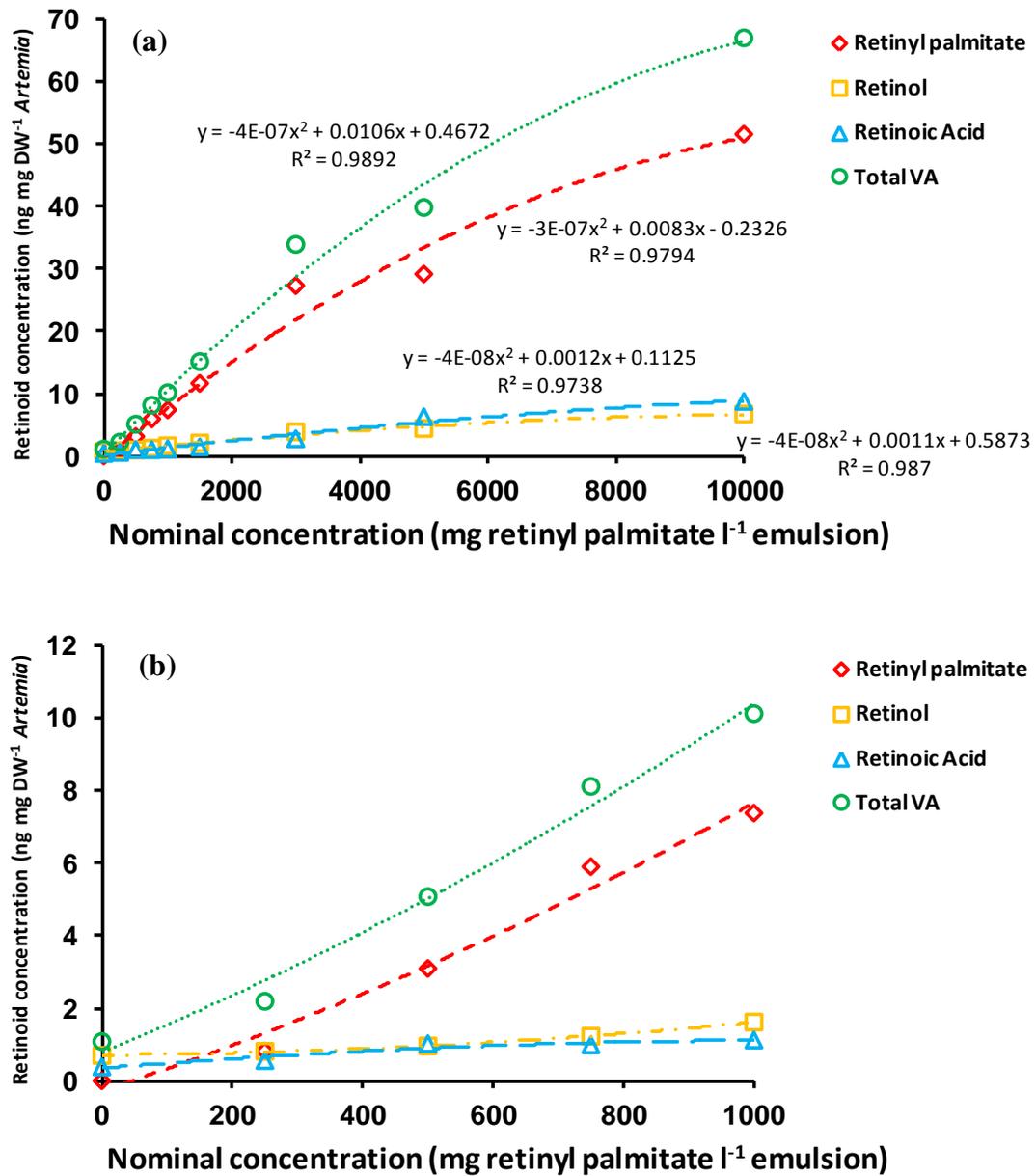


Figure 4: (a) Concentration of retinoids in *Artemia* enriched with graded levels of retinyl palmitate for 24 h in dark conditions, (b) Showing the concentration of retinoids in *Artemia* enriched with  $\leq$  1,000 mg retinyl palmitate l<sup>-1</sup>. Values are mean, n = 3. Line indicates polynomial regression.

### 2.4.3 Lipid content of live prey

Concentrations of saturated fatty acids (SFA), polyunsaturated fatty acids (PUFA), arachidonic acid (ARA, 20:4 $\omega$ 6), docosahexaenoic acid (DHA, 22:6 $\omega$ 3) and total fatty acids (total FA) were significantly lower in the rotifers enriched with 10,000 mg retinyl palmitate l<sup>-1</sup> for 2 h than those rotifers enriched for 8 h, with either Algamac-3050 (6 h) followed by enrichment emulsions (2 h), or with Algamac-3050 only (8 h) (Table 3) (ANOVAs,  $F_{4,10} = 17.54, 159.75, 45.09, 792.05$  and  $21.70$ , respectively,  $P < 0.001$  for all). Monounsaturated fatty acid (MUFA) and eicosapentaenoic acid (EPA, 20:5 $\omega$ 3) were significantly higher in the rotifers enriched with 10,000 mg retinyl palmitate l<sup>-1</sup> than the other treatments (ANOVAs,  $F_{4,10} = 91.82$  and  $10.39$ , and  $P < 0.001$  and  $P = 0.001$ , respectively).

Table 3: Selected fatty acid groups and polyunsaturated fatty acids in rotifers (mg g<sup>-1</sup> DW) enriched with 0, 500 and 5,000 mg retinyl palmitate l<sup>-1</sup> for a total of 8 h (6 h Algamac-3050 + 2 h enrichment emulsions), 10,000 mg retinyl palmitate l<sup>-1</sup> for 2 h and Algamac-3050 for 8 h. Values are mean of 3 replicates  $\pm$  SD. SFA = saturated fatty acid, MUFA = monounsaturated fatty acid, PUFA = polyunsaturated fatty acid and FA = fatty acid. Different letters within the same row show significant differences among dietary groups (ANOVA,  $P < 0.05$ ).

Enrichment type	mg retinyl palmitate l <sup>-1</sup> emulsion				Algamac-3050
	0	500	5000	10000	
Enrichment period	6 h Algamac-3050 + 2 h VA emulsion			2 h	8 h
Total SFA	28.23 $\pm$ 0.81 <sup>b</sup>	24.81 $\pm$ 0.45 <sup>b</sup>	25.33 $\pm$ 2.70 <sup>b</sup>	19.44 $\pm$ 0.18 <sup>a</sup>	25.37 $\pm$ 1.76 <sup>b</sup>
Total MUFA	16.49 $\pm$ 0.51 <sup>b</sup>	15.04 $\pm$ 0.82 <sup>b</sup>	15.82 $\pm$ 1.48 <sup>b</sup>	24.45 $\pm$ 0.41 <sup>c</sup>	13.34 $\pm$ 0.87 <sup>a</sup>
Total n: $\omega$ 6	15.28 $\pm$ 0.45 <sup>b</sup>	14.29 $\pm$ 0.72 <sup>b</sup>	14.06 $\pm$ 1.17 <sup>b</sup>	8.30 $\pm$ 0.17 <sup>a</sup>	14.38 $\pm$ 1.15 <sup>b</sup>
Total n: $\omega$ 3	50.31 $\pm$ 1.58 <sup>c</sup>	46.21 $\pm$ 1.89 <sup>bc</sup>	45.06 $\pm$ 2.08 <sup>b</sup>	22.85 $\pm$ 0.87 <sup>a</sup>	45.00 $\pm$ 2.44 <sup>b</sup>
Total PUFA	65.59 $\pm$ 2.03 <sup>c</sup>	60.50 $\pm$ 2.61 <sup>bc</sup>	59.12 $\pm$ 3.25 <sup>b</sup>	31.18 $\pm$ 1.03 <sup>a</sup>	59.37 $\pm$ 3.59 <sup>b</sup>
Fatty acid (mg g <sup>-1</sup> DW)					
20:4 $\omega$ 6	2.98 $\pm$ 0.08 <sup>b</sup>	2.72 $\pm$ 0.05 <sup>b</sup>	2.76 $\pm$ 0.14 <sup>b</sup>	2.01 $\pm$ 0.07 <sup>a</sup>	2.72 $\pm$ 0.15 <sup>b</sup>
20:5 $\omega$ 3	7.89 $\pm$ 0.19 <sup>a</sup>	7.32 $\pm$ 0.28 <sup>a</sup>	7.94 $\pm$ 0.42 <sup>a</sup>	9.18 $\pm$ 0.35 <sup>b</sup>	7.23 $\pm$ 0.61 <sup>a</sup>
22:6 $\omega$ 3	31.53 $\pm$ 0.97 <sup>c</sup>	28.97 $\pm$ 0.63 <sup>bc</sup>	27.16 $\pm$ 0.77 <sup>b</sup>	8.78 $\pm$ 0.38 <sup>a</sup>	28.28 $\pm$ 1.03 <sup>b</sup>
Total FA (mg g <sup>-1</sup> DW)	104.72 $\pm$ 2.66 <sup>c</sup>	95.22 $\pm$ 1.54 <sup>bc</sup>	95.26 $\pm$ 5.77 <sup>bc</sup>	77.98 $\pm$ 1.79 <sup>a</sup>	93.08 $\pm$ 4.85 <sup>b</sup>
22:6 $\omega$ 3/ 20:5 $\omega$ 3	3.99 $\pm$ 5.10 <sup>c</sup>	3.96 $\pm$ 2.30 <sup>c</sup>	3.42 $\pm$ 1.86 <sup>b</sup>	0.96 $\pm$ 0.03 <sup>a</sup>	3.91 $\pm$ 1.69 <sup>c</sup>

There was no significant difference in the SFA, ARA and EPA between the rotifers enriched with Algamac-3050 for 8 h and those enriched with Algamac-3050 (6 h) followed by enrichment emulsions (2 h). Monounsaturated fatty acids were significantly higher in the rotifers from the combined enrichment of Algamac-3050 followed by enrichment emulsions than those enriched with the Algamac-3050 only.

The total VA concentration in rotifers enriched for 6 h with Algamac-3050, followed by 2 h enrichment with VA enrichment emulsions (0, 500 and 5,000 mg retinyl palmitate l<sup>-1</sup> emulsion), was significantly lower (22, 27 and 47% lower, respectively) than the rotifers enriched with VA enrichment emulsions only for 2 h without the Algamac-3050 enrichment (Table 4 and 5 and Fig. 3).

Table 4: Results of two-way ANOVAs to test the effect of enriching rotifers with different levels of vitamin A for 2 h in dark conditions with or without an earlier enrichment with Algamac-3050 for 6 h in light conditions on the concentration of retinyl palmitate, retinol, retinoic acid and total VA content in the enriched rotifers. Vitamin treatment has 3 levels of increasing retinyl palmitate (0, 500 and 5,000 mg retinyl palmitate l<sup>-1</sup> emulsion). Algamac-3050 has 2 levels, absence or presence of an earlier enrichment. \* indicates significant effect  $P < 0.05$ .

	Source of variation	F value	P value
Retinyl palmitate	Vitamin treatment	6024.992	< 0.001*
	Algamac	54.979	< 0.001*
	Vitamin treatment x Algamac	19.181	< 0.001*
Retinol	Vitamin treatment	108.509	< 0.001*
	Algamac	43.230	< 0.001*
	Vitamin treatment x Algamac	22.097	< 0.001*
Retinoic acid	Vitamin treatment	9.056	0.004*
	Algamac	45.982	< 0.001*
	Vitamin treatment x Algamac	3.092	0.083
Total VA	Vitamin treatment	2321.417	< 0.001*
	Algamac	87.093	< 0.001*
	Vitamin treatment x Algamac	4.964	0.027*

Table 5: Retinoid concentrations (retinyl palmitate, retinol, retinoic acid and total VA)  $\text{ng mg}^{-1}$  in DW rotifers enriched with Algamac-3050 for 6 h followed by 2 h enrichment with 0, 500 and 5,000 mg retinyl palmitate  $\text{l}^{-1}$ . Values are mean  $\pm$  SD and  $n = 3$ . Different letters within the same column show significant differences among dietary groups (ANOVA,  $P < 0.05$ ).

Nominal concentration (mg retinyl palmitate $\text{l}^{-1}$ emulsion)	Retinyl palmitate	Retinol	Retinoic acid	Total VA
0	0.00 $\pm$ 0.00 <sup>a</sup>	1.42 $\pm$ 0.48 <sup>a</sup>	2.29 $\pm$ 0.58 <sup>a</sup>	3.71 $\pm$ 0.36 <sup>a</sup>
500	13.29 $\pm$ 1.44 <sup>b</sup>	2.42 $\pm$ 0.23 <sup>ab</sup>	1.74 $\pm$ 0.25 <sup>ab</sup>	17.45 $\pm$ 1.24 <sup>b</sup>
5000	146.22 $\pm$ 25.03 <sup>c</sup>	4.17 $\pm$ 0.83 <sup>b</sup>	3.01 $\pm$ 0.30 <sup>b</sup>	153.41 $\pm$ 24.32 <sup>c</sup>

#### 2.4.4 Vitamin A levels in enriched rotifers held in clear and greenwater over time

Retinoid concentrations in the rotifers enriched with different retinyl palmitate levels or Algamac3050 were influenced by the water type in which they were held, and the time they spent in the water (Fig.5). After the enrichment process and before distribution to the experimental tanks (0 h), the concentration of retinyl palmitate, retinol and total VA content was highest in rotifers enriched with 5,000 mg retinyl palmitate  $\text{l}^{-1}$  (313.76  $\pm$  12.65, 7.80  $\pm$  1.24 and 324.84  $\pm$  14.08  $\text{ng mg}^{-1}$ , respectively), followed by 500 mg retinyl palmitate  $\text{l}^{-1}$  (14.56  $\pm$  0.96, 3.20  $\pm$  0.51 and 21.00  $\pm$  1.32  $\text{ng mg}^{-1}$ , respectively), and significantly lower in rotifers enriched with 0 mg retinyl palmitate  $\text{l}^{-1}$  and Algamac-3050 (not detected, 1.16  $\pm$  0.16 and 5.01  $\pm$  0.73  $\text{ng mg}^{-1}$ , respectively) (ANOVAs,  $F_{3,8} = 14977.93, 89.32$  and  $931.82$ , respectively,  $P < 0.001$  for all). Retinoic acid content in the enriched rotifers was not significantly different among the four treatments after the completion of the enrichment process (3.56  $\pm$  0.51  $\text{ng retinoic acid mg}^{-1}$ ) (ANOVA,  $F_{3,8} = 1.48$  and  $P = 0.292$ ).

In clear water, retinyl palmitate was not detected in the rotifers enriched with 500 mg retinyl palmitate after 4 h. In the rotifers enriched with the 5,000 mg retinyl palmitate  $\text{l}^{-1}$ , retinyl palmitate content decreased by 87% to 40.18  $\pm$  1.47 at 4 h and to 18.69  $\pm$  1.50  $\text{ng mg}^{-1}$  at 8 h and was not detected at 14 h. Retinol and retinoic acid also decreased over time in the clear water. Retinol was not detected in rotifers enriched with 0 mg retinyl palmitate  $\text{l}^{-1}$  and Algamac-3050 from 4 h, nor in

rotifers enriched with 500 mg retinyl palmitate l<sup>-1</sup> from 8 h. In rotifers enriched with 5,000 mg retinyl palmitate l<sup>-1</sup>, retinol continued to decrease over time to reach 1.84 ± 0.13 ng mg at 14 h. The pattern of change in retinoic acid concentration in the enriched rotifers held in clear water over time was similar to retinol.

In greenwater, retinyl palmitate in the enriched rotifers significantly increased at 4 h for rotifers enriched with either 0 mg retinyl palmitate l<sup>-1</sup> or Algamac-3050 compared with the concentrations at 0 h. In comparison, the concentration of retinyl palmitate decreased for the rotifers enriched with 500 and 5,000 mg retinyl palmitate l<sup>-1</sup> compared to the initial concentrations. The concentration of retinyl palmitate increased at 8 h and 14 h for rotifers enriched with 500 mg retinyl palmitate l<sup>-1</sup>, while it continued to decrease in rotifers enriched with 5,000 mg retinyl palmitate l<sup>-1</sup>. At 14 h, there was no significant difference in the concentration of retinyl palmitate in rotifers enriched with 0 and 500 mg retinyl palmitate l<sup>-1</sup> and Algamac-3050 with an average of 9.10 ± 0.78 ng mg<sup>-1</sup>, and it was significantly higher in rotifers enriched with 5,000 mg retinyl palmitate l<sup>-1</sup> with an average of 13.98 ± 1.63 ng mg<sup>-1</sup> (ANOVA, F<sub>23,48</sub> = 513.12 and P < 0.001).

In greenwater, retinol content at 4 h in the rotifers enriched with 0 and 5,000 mg retinyl palmitate l<sup>-1</sup> (average 9.29 ± 0.71 ng mg<sup>-1</sup>) was significantly higher than the retinol content of rotifers enriched with 500 mg retinyl palmitate l<sup>-1</sup> (5.04 ± 0.92 ng mg<sup>-1</sup>). At 8 h, there was no significant difference in the concentration of retinol for the rotifers enriched the VA treatments, with an average of 13.69 ± 0.46 ng mg<sup>-1</sup>. There was no significant change in the retinol concentration between 4 and 8 h in rotifers enriched with Algamac-3050, with an average of 9.69 ± 0.94 ng mg<sup>-1</sup>. By 14 h, the concentrations of retinol in rotifers enriched with 0 or 500 mg retinyl palmitate l<sup>-1</sup>, or Algamac-3050 were significantly lower (4.94 ± 0.63 ng mg<sup>-1</sup>) than the concentration of retinol in rotifers enriched with 5,000 mg retinyl palmitate l<sup>-1</sup> (6.65 ± 0.13 ng mg<sup>-1</sup>) (ANOVA, F<sub>23,48</sub> = 372.74 and P < 0.001). In greenwater, the concentration of retinoic acid increased over time in all of the rotifer enrichments and reached a peak at 14 h with an average of 11.72 ± 1.69 ng mg<sup>-1</sup>.

The highest concentration of total VA after the transfer to the experimental tanks was at 4 h for rotifers held in both clear and greenwater and initially enriched with 5,000 mg retinyl palmitate l<sup>-1</sup> with an average of 50.71 ± 1.28 ng mg<sup>-1</sup>. Likewise, at 8 h in rotifers held in greenwater, total VA was significantly higher in the rotifers previously enriched with 5,000 mg retinyl palmitate l<sup>-1</sup> (38.15 ± 3.22 ng total VA mg<sup>-1</sup>) than those previously enriched with 0 or 500 mg retinyl palmitate l<sup>-1</sup> (29.50 ± 1.00 ng total VA mg<sup>-1</sup>), while Algamac-3050 enriched rotifers had a significantly lower level (22.04 ± 0.99 ng total VA mg<sup>-1</sup>). By 14 h, the total VA in rotifers previously enriched with 0 or 500 mg retinyl palmitate l<sup>-1</sup> or Algamac-3050 were significantly lower (26.01 ± 1.71 ng total VA mg<sup>-1</sup>) than in the rotifers previously enriched with 5,000 mg retinyl palmitate l<sup>-1</sup> (31.61 ± 2.50 ng total VA mg<sup>-1</sup>) (ANOVA, F<sub>23, 48</sub> = 2710.52 and P < 0.001).

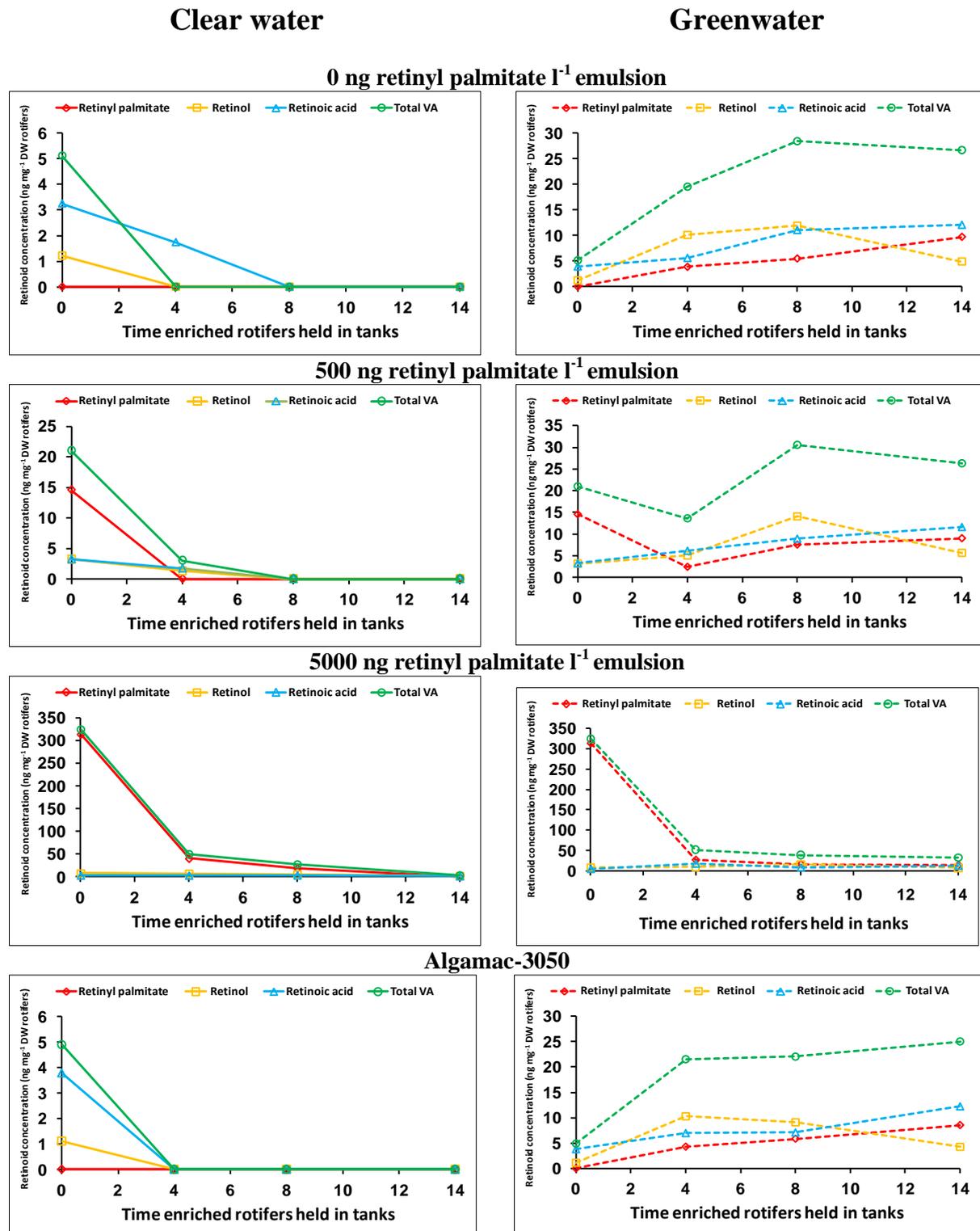


Figure 1: Retinoid concentrations (retinyl palmitate, retinol, retinoic acid and total VA) in rotifers with time held in clear and greenwater after enrichment with 0, 500 and 5,000 mg retinyl palmitate l<sup>-1</sup> for 2 h or Algamac-3050 for 8 h. Values are mean and n = 3. SD not shown for clarity. Note the different y-axis scales in each figure part.

## 2.5 Discussion

The retinoid content of live prey was affected by enrichment conditions of time and light, with different effects in rotifers and *Artemia*. Rotifer retinoid content was significantly affected by both the enrichment time and the illumination conditions, with the highest concentration after 2 h enrichment and in dark conditions. This result is in agreement with those reported by Haga et al. (2006), who found that the retinoid content in rotifers enriched with VA in darkness was always higher than that enriched under light conditions. Haga et al. (2006) suggested that the denaturation of VA in light occurred after VA had been incorporated into the rotifer body. This is supported by the observation that feeding VA-enriched rotifers decreased the incidence of pseudoalbinism in the Japanese flounder, *Paralichthys olivaceus* but the rate of pseudoalbinism occurrence increased in fish exposed to longer light cycles during feeding (Nakamoto, 1991). This implies that the light caused VA denaturation in the rotifers, to the extent that it decreased its efficiency in reducing the pseudoalbinism. After ingestion of VA by vertebrates, it is converted into retinol in the intestine where it binds to retinol binding proteins (RBP) (Napoli, 1996). Retinol binding proteins transport retinol as a complex and stabilize it from light (Blaner and Olson, 1994). Haga et al. (2006) hypothesised that rotifers do not have RBP that bind and transport retinol as a complex and therefore it is not stabilized and protected from light.

In contrast, total VA, retinyl palmitate and retinoic acid in *Artemia* were affected by the enrichment time but not by light. This result agrees with Haga et al. (2006), who found light did not affect VA enrichment in *Artemia*, although, higher mortality of VA-enriched *Artemia* observed in dark conditions, was not apparent in my study. It is possible that physiological differences in the *Artemia* strains may have contributed to the higher mortality observed by Haga et al. (2006). In addition, salinity, temperature, aeration, hydrodynamics in the enrichment media, *Artemia* density, size of the particles in the emulsions and the stage of development of the *Artemia*, are all possible factors that affect the enrichment process (Conceição et al., 2010a). Haga et al. (2006) suggested *Artemia* have a

mechanism to stabilize VA in the body, such as RBP, to protect denaturation by light. Another characteristic of the RBP is that they are highly regulated and not influenced by the dietary VA status of the body in vertebrates (Blaner et al., 1986). Haga et al. (2004a) concluded that the metabolic pathway of retinoids in *Artemia* is similar to that of vertebrates. In my study, there was no significant difference in the total VA content for *Artemia* enriched over 12 h (from 12 to 24 h of enrichment), which implies that *Artemia* were able to control the accumulation and metabolism of VA. There is also the possibility that the body pigment of *Artemia* (red or orange) compared with almost transparent rotifers might shade the VA in the body and protect it from light. The results of the current study suggest the optimal incorporation of retinoids into live feeds is 2 h in dark conditions for rotifers and from 12 to 24 h of enrichment for *Artemia*.

Using the optimal enrichment time determined in the first enrichment trial, the concentration of total VA in the live feeds increased significantly with increasing levels of dietary retinyl palmitate. However, this increase was not proportional to the rise in total VA in the enrichment emulsions. The rotifer retinoid concentration was 12.5% of the nominal emulsion content at the lowest VA level, decreasing to 4.5% at the highest level, and *Artemia* retinoid content was 0.9% at the lowest VA level decreasing to 0.7% at the highest VA level (excluding the 0 retinyl palmitate l<sup>-1</sup> emulsions). These results agree with Giménez et al. (2007), who found total VA content in the enriched live preys were significantly correlated with the concentration of VA in the enrichment emulsions. In contrast, Srivastava et al. (2011) found no relationship between dietary VA and rotifer content, where rotifers enriched with retinyl palmitate ranging from 0 to 200 ng mg<sup>-1</sup> incorporated retinol ranging from 4.9 to 6.98 ng mg<sup>-1</sup> rotifers. One potential reason for the difference in results may be the extraction method and VA analysis used by Srivastava et al. (2011) who combined retinyl palmitate with retinol, while retinol and retinyl palmitate were detected separately by Giménez et al. (2007).

Rotifers accumulated 6.7 times more total VA than *Artemia* following enrichment with 10,000 mg retinyl palmitate l<sup>-1</sup> emulsion and in a shorter enrichment time, 2h compared with 24 h. Giménez et

al. (2007) indicated the differential pattern in VA accumulation between rotifers and *Artemia*, and attributed that difference between the two species to their developmental stage, function of the digestive system and filtration rate. In the current study, rotifers and *Artemia* were able to absorb, digest and metabolize the retinyl palmitate in the enrichment emulsion, indicated by their VA content which was not maintained in the form of retinyl palmate only, but retinoic acid and retinol were detected. The result also agrees with Fernández et al. (2009), Takeuchi et al. (1998) and Tarui et al. (2006) for *Artemia* and Fernández et al. (2008) and Giménez et al. (2007) for rotifers. Overall, the results highlight the difficulties in utilising live prey for VA delivery, because the VA form and content are not stable over time.

Rotifers and *Artemia* also showed different levels of total VA incorporation following enrichment with different VA compounds (Table 6). Giménez et al. (2007) used retinyl acetate to enrich the live feeds and the enrichment levels were low compared with the current study, yet resulted in higher incorporation of total VA in the live preys. In both studies, the enrichment time was 2 and 24 h in rotifers and *Artemia*, respectively, although the enrichment was in dark conditions in the current study. The enrichment volume, temperature and density of live preys were different between the two studies and this might have also affected the enrichment processes and the incorporation of retinoids into the live feed (Conceição et al., 2010b). Retinyl acetate and palmitate were recommended to fulfil the larvae requirements for retinoids because they are less toxic than other VA forms (Fernandez and Gisbert, 2011), but my results suggest the different levels of incorporation into live feeds must be considered when designing nutritional studies.

The short enrichment time necessary to optimise the VA enrichment of rotifers (2 h), had a negative impact on the lipid profile of the live feed in the context of meeting requirements for some marine fish larvae. Live feeds do not have the EFA needed by most marine larvae for optimal growth, and fish larvae have no, or very limited ability to synthesize them; they must be sourced in their diet (Grote et al., 2011; Sargent et al., 1999; Tocher, 2010). Enrichment of live feed with n-3 PUFAs is

commonly used to enrich the live feeds especially during the early stages of larval development

(Conceição et al., 2010b; Olivotto et al., 2006, 2011; Vagelli, 2004).

Table 6: Comparison between total vitamin A incorporation into rotifers and *Artemia* after enrichment with two different vitamin A compounds. \* mg l<sup>-1</sup> enrichment media and \*\* ng mg<sup>-1</sup> DW. Data from Giménez et al. (2007) and the current study.

Giménez et al. (2007)					Current study				
Temp. (°C)	Volume (l)	Density (ml <sup>-1</sup> )	Retinyl acetate*	Total VA**	Temp. (°C)	Volume (l)	Density (ml <sup>-1</sup> )	Retinyl palmitate*	Total VA**
<b>Rotifers</b>									
20	30	230	0.124	190	26	20	500	0.000	4.78
			0.138	180				0.006	6.24
			0.151	190				0.011	11.28
			0.165	185				0.022	17.32
			0.178	170				0.033	21.54
			0.192	200				0.044	23.97
			0.226	300				0.055	23.76
			0.259	190				0.083	32.75
			0.327	806				0.110	43.29
								0.165	83.86
								0.330	168.90
								0.550	288.84
								1.100	451.35
<b>Artemia</b>									
20	10	200	0.494	4	26	20	50	0.00	1.08
			0.548	9				0.15	2.19
			0.602	15				0.30	5.07
			0.629	17				0.45	8.11
			0.710	14				0.60	10.12
			0.764	28				0.90	15.10
			0.899	29				1.80	33.88
			1.034	24				3.00	39.80
			1.334	52				6.00	66.98

The essential fatty acids DHA and ARA in the VA-enriched rotifers were significantly lower than the recommended standard requirements for *L. lineata* larvae during the rotifer feeding period. In the culture of *L. lineata* larvae, Algamac-3050 is used routinely to enrich live feeds (Battaglione and

Cobcroft, 2007; Battaglione et al., 2006; Bransden et al., 2005a, 2005b). Algamac-3050 could not be used as the base of the VA enrichment emulsions in my study, due to the physical properties of Algamac-3050 which is a freeze-dried algal product, in a dry flake form. It would have been technically very difficult for VA to be combined into the emulsified product for consistent and effective delivery to the live feeds, requiring manual weighing out VA for addition to the hydrating Algamac-3050 at each enrichment event. In order to obtain consistent graded levels of VA, tuna oil (liquid form) was used as the lipid base for the VA enrichment emulsions and allowed for even blending of the VA into the 'stock' emulsion. The control emulsion contained no retinyl palmitate, although 4.78 and 1.08 ng total VA mg<sup>-1</sup> was detected in rotifers and *Artemia*, respectively. This was due to the tuna oil lipid base that contained 700 IU VA kg<sup>-1</sup> (NuMega certificate of analyses). Other studies have used commercial emulsions e.g., Easy Selco as the lipid base and added VA (Fernández et al., 2008, 2009). We did not specifically test Easy Selco but other similar products do not meet *L. lineata* larval lipid requirements (Battaglione et al., 2006), unlike other fish species such as Gilthead Sea Bream, *Sparus aurata* which is less sensitive to lower lipids (Fernández et al., 2008). Given the need to provide high lipid profile, rotifers were enriched with Algamac-3050 for 6 h followed by VA graded emulsions for another 2 h. This resulted in an appropriate lipid profile for *L. lineata* larvae, although VA incorporation was not sufficient to create increasing VA levels in enriched rotifers. Insufficient lipids during the rotifer feeding period of *L. lineata* has been demonstrated to affect the tissue content of the larvae, resulting in erratic swimming behaviour, abnormal lipid assimilation, transport and subsequent deposition, highly vacuolated hepatocytes, and altered prostanoid production (Bransden et al., 2004, 2005b). These results highlight the necessity of supplying *L. lineata* larvae with an adequate source of lipids.

Transferring VA and Algamac-3050 enriched rotifers to clear water or greenwater had different effects on the VA content, which was influenced by initial VA enrichment levels and the time after transfer. In clearwater VA decreased rapidly, and was not detected after 4 h in rotifers with low initial VA enrichment (0 mg retinyl palmitate l<sup>-1</sup> emulsion and Algamac-3050), and after 8 h with

intermediate initial VA enrichment (500 mg retinyl palmitate l<sup>-1</sup> emulsion). Total VA concentration in the rotifers enriched with 5,000 mg retinyl palmitate l<sup>-1</sup> decreased by 85, 91 and 99% by 4, 8 and 14 h, respectively. The decline in the VA content could be due to the metabolism and excretion of VA by the rotifers or degradation by the light. These results highlight the rapid decline of VA in the enriched rotifers when held in clearwater. Depending on the feeding time and ‘flushing’ of uneaten rotifers, larvae may not receive differentially VA-enriched rotifers under these conditions. Therefore, it is important to use a high and wide range of initial emulsions.

In greenwater the total VA content in rotifers enriched with higher levels of VA initially (500 and 5,000 mg retinyl palmitate l<sup>-1</sup>) decreased rapidly by 4 h (35 and 84%, respectively). In contrast, the VA content of the rotifers with low initial VA enrichment (0 mg retinyl palmitate l<sup>-1</sup> and Algamc3050) accumulated 3.8 and 4.4 times more total VA than at 0 h. Despite the effect of greenwater on VA content of previously enriched rotifers, total VA content at 4 h was significantly different among different VA treatments. Over time there was no significant difference between treatments, indicating that the rotifers lost or accumulated VA to a stable level, sourced from the retinol and  $\beta$ -carotene present in the microalgae in the greenwater. Only at the highest VA enrichment level (5,000 mg retinyl palmitate l<sup>-1</sup>), where the total VA content dropped by 84, 88 and 90% at 4, 8 and 14 h respectively, was the total VA content higher than the other treatments, which was attributed to the initially high content (324.8 ng total VA mg<sup>-1</sup>). In addition to microalgae being a direct source of nutritional VA, the turbidity produced from the addition of the microalga, *N. oculata*, into the water, would have decreased the light penetration into the water which may have contributed to protecting, or at least decreasing, the VA denaturation by light. The study highlights the importance of understanding the potential masking effects of greenwater when extrapolating experimental results derived in clearwater to recommended levels of VA in commercial production using greenwater.

## 2.6 Conclusion

The optimal conditions determined for VA enrichment were 2 h in dark conditions for rotifers and 24 h for *Artemia*. Enrichment of rotifers and *Artemia* with increasing levels of retinyl palmitate from 0 to 10,000 mg l<sup>-1</sup> did not reach a plateau and showed a polynomial relationship between the nominal concentration of retinyl palmitate in the emulsion and different VA compounds accumulating in the both live feeds. The lipid profile in rotifers enriched for 2 h with VA emulsions did not meet *L. lineata* requirements for EFA. Rotifer VA content in rotifers enriched with retinyl palmitate and reared in clear and greenwater was affected by the initial levels of enrichment, water type and time post transfer, and must be considered when designing dose response experiments with fish larvae. The results presented in this Chapter are utilized in the following experimental research reported in Chapters 3, 4 and 5, where *L. lineata* larvae were fed increasing levels of dietary retinyl palmitate through live feeds to examine the effects on growth parameters, survival and skeletal development.

## 2.7 References

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# Chapter 3

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**Effect of dietary vitamin A level in rotifers on performance and skeletal abnormality of Striped Trumpeter *Latris lineata* larvae and post-larvae**

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### 3.1 Abstract

Several nutritional studies have linked dietary vitamin A (VA) to skeletogenesis in marine fish larvae. In my study, rotifers were enriched with eight levels of retinyl palmitate. Striped trumpeter (*Latris lineata*) larvae were fed rotifers twice daily, VA-enriched rotifers (morning feed) and Algamac-3050 enriched rotifers (afternoon feed), in greenwater systems from 6 to 18 days post-hatch (dph). The VA-enriched rotifers had incorporated 0, 6, 14, 26, 57, 109, 215 and 388 ng retinyl palmitate mg<sup>-1</sup> dry weight (DW) following 2 h enrichment with emulsions containing 0 (control), 68, 187, 532, 1402, 2670, 4808 and 9523 ng retinyl palmitate mg<sup>-1</sup> emulsion. After the rotifer feeding phase (6 to 18 dph), the larvae were fed Algamac-3050 enriched *Artemia* until 43 dph. The pattern of increasing VA in the enriched rotifers was not reflected in the larvae. Larvae incorporated  $11.08 \pm 0.27$  ng total retinol mg<sup>-1</sup> DW (mean  $\pm$  SD) when fed rotifers containing 0.93 to 2.32 ng total retinol mg<sup>-1</sup> DW (6 to 14 ng retinyl palmitate mg<sup>-1</sup> DW) and incorporated a lower level of  $5.57 \pm 0.30$  ng total retinol mg<sup>-1</sup> DW when fed rotifers with 0.52 (control) or  $\geq 16.31$  ng total retinol mg<sup>-1</sup> DW (0 and  $\geq 57$  ng retinyl palmitate mg<sup>-1</sup> DW). Possibly reflecting the masking effect of using two sources of rotifers, whereby there was reduced feeding on rotifers enriched with higher amounts of VA, although there was no evidence of reduced feeding in morning feeds or reduced growth. By 43 dph, neither larval growth in length ( $16.0 \pm 0.1$  mm) or dry weight ( $4.57 \pm 0.20$  mg), nor survival ( $34.8 \pm 10.6\%$ ), were significantly affected by increasing dietary levels of retinyl palmitate. The prevalence of vertebral column malformations in 43 dph post-larvae were positively correlated with total retinol content of larvae at 16 dph ( $R^2 = 0.55$ ,  $P < 0.001$ ). Unlike other studies on a range of marine fish species, retinyl palmitate enrichment in rotifers did not affect the type or severity of jaw malformations. By 43 dph,  $81 \pm 9\%$  of post-larvae displayed severe jaw malformations. Vitamin A daily inclusion of more than 123 ng total VA mg<sup>-1</sup> DW rotifer, equivalent to more than 35 ng total retinol mg<sup>-1</sup> DW rotifer (109 ng retinyl palmitate mg<sup>-1</sup> DW rotifers), is recommended to reduce vertebral column malformations when *L. lineata* larvae are reared in greenwater. Carotenoids in the greenwater consumed by the rotifers and

metabolized either in the rotifer or larvae are likely to have influenced results and merit further investigation.

### **3.2 Introduction**

Reducing malformation is a continuous challenge in the production of marine finfish throughout the world (Boglione et al., 2001; Chatain, 1994; Izquierdo et al., 2010; Koumoundouros et al., 1997, 2002; Nagano et al., 2007; Paperna, 1978; Saavedra et al., 2010). The development of skeletal disorders in larvae and juvenile fish can be due to nutritional, environmental and genetic factors (Cahu et al., 2003; Divanach et al., 1997; Lall and Lewis-McCrea, 2007; Sadler et al., 2001). Skeletal disorders are early indicators of low quality fish for farming (Roo et al., 2010). Although there is now a growing understanding of the factors that contribute to malformations, they remain an important issue for the international finfish hatchery industry and animal welfare (Chandoo et al., 2004; Gavaia et al., 2009; Sfakianakis et al., 2006).

One of the key parameters that affect skeletogenesis in larval fish is nutrition, especially at first feeding. Optimum forms and sufficient levels of lipids, amino acids and vitamins are essential for normal development (Cahu et al., 2003). Among the vitamins, A, D, E, K and C are all important (Lall and Lewis-McCrea, 2007). Vitamin A (VA) or retinoids are the group of nutrients with compounds that are structurally similar or have the biological activity of retinol, where they can bind or activate a specific receptor or a group of receptors. Many retinoid forms are available in the market for dietary incorporation, retinol (parent compound), VA alcohol, retinal (the aldehyde form), retinoic acid (the acid form of VA), retinyl acetate and retinyl palmitate (esters forms of VA) (Wolf, 1984). Only retinyl palmitate and acetate are recommended to fulfil the requirements of fish for retinoids. This is because they are less toxic than the other forms, and fish larvae at early stages have the required liver enzymes to metabolize them into the other VA forms (Fernandez and Gisbert, 2011; Takeuchi et al., 1998). Due to presence of different VA compounds, VA activity is expressed in international units (IU), where 1 IU of VA is equivalent to 0.3 µg retinol (Wolf, 1984).

It is now generally recommended that retinoids be added to the broodstock diet and to the diet of all developmental larval fish stages (Alsop et al., 2008; Dedi et al., 1995; Zile, 2001). Determining optimal amounts and forms of VA for different developmental stages needs to be assessed for each species (Fernández et al., 2009; Fontagne-Dicharry et al., 2010; Fontagné et al., 2006; Moren et al., 2004b; Villeneuve et al., 2005). This is because excess or diminished VA affects normal growth and development (Dedi et al., 1995, 1998; Furuita et al., 2001; Tarui et al., 2006; Villeneuve et al., 2005). The effects differ according to the species and there are often symptoms of hypervitaminosis with larger doses of VA where there is growth retardation and increased mortalities, malformation to different elements such as the brain, otoliths and otic placodes, skull, vertebral segments, caudal region, jaws and fins and abnormal pigmentation (Fernández et al., 2008, 2009; Haga et al., 2002a, 2002b; Takeuchi et al., 1998).

A dietary dose–response approach where the larvae are fed diets containing graded levels of VA is one of the best ways to study the effect of VA (or any other nutrient) on larval development.

Determining the optimal amount of VA is complicated by the use of live prey. Rotifers and *Artemia* are able to metabolize different VA compounds and accumulate them in their body (Haga et al., 2006). In addition, due to species-specific differences between the two live preys, enrichment is not uniform and rotifers display a higher retinoid inclusion pattern than *Artemia* (Giménez et al., 2007; Chapter 2). Thus, it is technically difficult to maintain the same VA levels during the whole live prey-feeding period of a larva. For that reason optimum VA levels are usually determined separately for the rotifer and *Artemia* feeding periods. To the best of my knowledge only one study has examined in detail the VA requirements of marine finfish using dose-response experiments in early larval stages (i.e., during rotifer feeding) (Fernández et al., 2008).

Striped trumpeter, *Latris lineata* (Bloch and Schneider, 1801), is native to south-eastern Australia and New Zealand and has been a candidate for sea cage aquaculture in the temperate regions of Australia to complement salmonid farming which is under threat from climate change (Battaglione

and Cobcroft, 2007). Intensive culture of *L. lineata* larvae and post-larvae has resulted in a high incidence of either jaw and/or spinal malformation in juveniles (Cobcroft and Battaglione, 2009; Cobcroft et al., 2001a; Trotter et al., 2001). Studies have examined the early requirements for the essential polyunsaturated fatty acids (PUFAs) and vitamins C and E, often in dose-response experiments (Battaglione et al., 2006; Bransden et al., 2005a, 2005b; Brown et al., 2005). *Latris lineata* larvae are usually reared in greenwater, by adding the microalga *Nannochloropsis oculata*, which has a number of benefits in relation to improved prey intake, digestion, microbial management and larval performance (Cobcroft et al., 2001b; Makridis et al., 2010; Shaw, 2006). This microalga contains very small amounts of retinol ( $< 0.25 \text{ ng mg}^{-1}$ ) and  $290 \pm 40 \text{ ng mg}^{-1}$   $\beta$ -carotene (Brown et al., 1999).  $\beta$ -carotene is a major dietary precursor of VA, with the nutritional equivalency accepted at  $6 \mu\text{g } \beta\text{-carotene equal to } 1 \mu\text{g retinol}$  (Ross and Ternus, 1993).

The only published accounts of spinal malformation in *L. lineata* larvae or post-larvae under intensive culture conditions describe kyphosis associated with swim bladder malformation, where the viscera is misplaced and pushes upwards on the vertebral column (Trotter et al., 2001, 2005). Jaw malformation in *L. lineata* is a significant problem and has been linked with a walling behaviour that is modified by tank colour, greenwater and the availability of live feed in the water column (Battaglione and Cobcroft, 2007; Cobcroft and Battaglione, 2009).

The aim of the current study was to determine the effect of VA on early larval development of *L. lineata*, particularly on jaw and spinal malformations. Larvae were reared using standard practises in greenwater and fed from 6 to 18 days post hatch (dph) with rotifers enriched with eight graduated concentrations of retinyl palmitate. The larvae were then fed on *Artemia* until 43 dph to allow them to fully develop jaw and skeletal elements. Growth, survival and development were assessed in relation to rotifer enrichment with VA.

### 3.3 Materials and methods

#### 3.3.1 Eggs and stocking of larvae

Gametes were obtained from wild-caught acclimated broodstock held at the Institute for Marine and Antarctic Studies, Fisheries, Aquaculture and Coasts facility (IMAS-FACC), Hobart under controlled light and ambient water temperature. Eggs stripped from one female were fertilised by mixing with sperm from three males. Immediately after fertilization the embryos were disinfected in ozonated seawater 1.05 ppm for 57 s, in order to prevent the transmission of pathogens (Battaglione and Morehead, 2006). The seawater used in the egg incubation and larval rearing systems was filtered to 1  $\mu\text{m}$  and ozonated to 700 mV ORP for  $\geq 10$  min, treated with UV at 254 nm and filtered with carbon before distribution to the tanks at 300-350 mV ORP.

The embryos were incubated in 250 l conical tanks at  $14.2 \pm 0.2$  °C (mean  $\pm$  SD, here and throughout). Incubators were kept on flow-through at approximately  $150 \text{ l h}^{-1}$ , photoperiod 14 h L: 10 h D, salinity ranged from 33.6 to 34.5 ppt, pH 8.13 to 8.26 and DO 95.8 to 106.3%. The central screens were 250  $\mu\text{m}$ . Paper towels were applied to the surface for the removal of any oil or debris. The fertilized eggs hatched after 5 days. From 1 dph the temperature was slowly increased at  $0.1$  °C  $\text{h}^{-1}$  to  $16.2 \pm 0.2$  °C. Yolk-sac larvae were stocked to larval rearing tanks on 1 dph at  $10 \text{ larvae l}^{-1}$ .

#### 3.3.2 Experiment design

The effect of dietary VA on the performance, growth, survival and skeleton malformation was determined in early *L. lineata* larvae using emulsions containing graded levels of retinyl palmitate (1,600,000 IU  $\text{g}^{-1}$ , MP Biomedicals, Australia) prepared by Nutrakol, WA ; 0 (control), 100, 300, 750, 1,500, 3,000, 5,000 and 10,000 mg retinyl palmitate  $\text{l}^{-1}$  WW (wet weight) emulsion. The emulsion basic constituents were lipids 57%, vitamin E 4% and vitamin C 4% (Nutrakol, WA). Rotifers enriched with these graded levels of retinyl palmitate were fed to larvae from 6 to 18 dph in three replicate tanks per treatment.

### 3.3.3 Larval rearing conditions

Larvae were reared in black 300 l hemispherical tanks, from 1 dph until 43 dph. The temperature ranged from 15.5 to 16.5 °C ( $16.1 \pm 0.2$ ), salinity from 32.1 to 33.5 ppt ( $32.9 \pm 0.3$ ), pH from 8.04 to 8.63 ( $8.18 \pm 0.07$ ) and dissolved oxygen  $> 84.7\%$  ( $101.6 \pm 5.5$ ). Photoperiod was 16 h L: 8 h D (lights on at 09:00 and off at 01:00) from stocking until 18 dph. The light period increased gradually to 18 h L: 6 h D on 19 dph, 22 h L: 2 h D on 20 dph and 24 h L from 21 dph onward to reduce downward nocturnal migration and associated mortality (Bransden et al., 2005a). The light intensity was  $10.56 \pm 1.71 \mu\text{mol s}^{-1}$  at the water surface, provided by a 50W halogen globe.

Tanks were static from 1 dph (stocking) until 5 dph. On 6 dph (first feeding) the incoming flow of seawater exchange was  $103 \pm 4 \text{ l h}^{-1}$  for 9 h from 23:00 to 08:00. Additional recirculation flow started on 19 dph and duration of incoming flow was increased to 24 h, and both continued until the end of the experiment, increasing the total flow to  $200 \pm 33 \text{ l h}^{-1}$  for 24 h. Each tank had a surface skimmer to remove the oily film from the water surface in order to facilitate initial swim bladder inflation (Trotter et al., 2005). The skimmers were applied from 8 dph until 13 dph, and occasionally thereafter until 19 dph ( $< 2 \text{ h day}^{-1}$ ) to ensure a clean water surface.

Larvae were reared in greenwater, from 6 dph till 20 dph; live *Nannochloropsis oculata* was added to each tank to achieve a turbidity of 3 NTU ( $\sim 350,000 \text{ cell} \times 10^6 \text{ tank}^{-1}$ ). Live algae was acclimated to the larval rearing temperature by pumping to an aerated reservoir tub the day before its addition to the tanks. During the *Artemia* feeding period, to ensure larvae had not been feeding prior to sampling for biochemical composition, bag screens of 63  $\mu\text{m}$  were fitted into the recirculated water reservoirs  $\sim 12 \text{ h}$  before sampling to collect the live prey in the tanks. Water quality parameters were measured daily and tanks were siphoned daily to remove any detritus or dead larvae. Mortalities were counted and observations of fish behaviour and tank conditions were recorded daily.

### 3.3.4 Feeding rates

The larvae were fed enriched rotifers *Brachionus plicatilis* (Austria strain) at 5 ml<sup>-1</sup> twice daily at 09:00 and 17:00, starting from 6 dph until 16 dph. On 17 and 18 dph the 17:00 rotifer feed was replaced with enriched *Artemia* at 0.25 ml<sup>-1</sup>, which were also fed at 21:00. Enriched *Artemia* were fed four times each day at 09:00, 13:00, 17:00 and 21:00 from 19 dph at 0.25 ml<sup>-1</sup> feed<sup>-1</sup> until the end of the experiment (43 dph). *Artemia* feeding rates were increased during the experiment to account for increasing prey consumption due to the high survival in most of the tanks. *Artemia* were fed at 0.25 ml<sup>-1</sup> feed<sup>-1</sup> until 22 dph when the 21:00 feed was increased to 0.50 ml<sup>-1</sup> until the end of the experiment. From 36 dph, the 09:00, 13:00 and 17:00 were increased to 0.30 ml<sup>-1</sup>, except for four low survival tanks that were kept constant at 0.25 ml<sup>-1</sup>.

### 3.3.5 Enrichment

Rotifers in culture were fed with commercial concentrated algae paste (*Nannochloropsis* sp, Reed Mariculture Inc. USA) and harvested every day. They were rinsed in seawater before being distributing into eight 20 l containers at 500 rotifers ml<sup>-1</sup> and 25 °C. Rotifers for the 09:00 feed were enriched with the experimental emulsions in complete darkness for 2 h using 0.11 g emulsion l<sup>-1</sup>. The required weight for each emulsion was blended for 3 min in tap water at 20 °C then screened through 63 µm mesh before adding it to the rotifers.

Rotifers for the 17:00 feed were enriched with Algamac-3050 (Aquafauna Biomarine, USA) for 8 h in constant light at 2 g million<sup>-1</sup> rotifer to ensure adequate lipid nutrition (Brandsen et al., 2005b). The Algamac-3050 was hydrated in 1 l tap water at 20 °C for 10 min, before blending for 3 min, then screened as above and added to the rotifers. After enrichment of rotifers with the emulsions or Algamac-3050, they were gently harvested by siphoning, collected into 63 µm bag screens and rinsed for about 10 min in clean seawater. The rotifers were counted and volumetrically fed to larvae.

*Artemia* cysts (AAA, INVE Aquaculture Nutrition USA) were decapsulated and stored in brine at 4 °C until required (Sorgeloos et al., 1977). *Artemia* were hatched daily from 16 dph, enriched with Algamac-3050 the day after hatching at 26 °C under strong illumination and aeration. The 09:00 and 17:00 feeds were enriched for 16 h, while the 13:00 and 21:00 feeds were enriched for 20 h. After enrichment, *Artemia* were siphoned and collected into 150 µm screen bags, rinsed with freshwater and then seawater. The *Artemia* were counted and volumetrically fed to larvae.

### 3.3.6 Sampling

Before stocking, 20 yolk sac larvae from the incubator were anaesthetised in 0.06% 2-phenoxyethanol. Their standard length was measured using an eyepiece graticule fitted to an Olympus SZ stereomicroscope. Samples of 10 larvae were taken out of each tank on 5 dph, and larval standard length was measured and the general condition of the larvae was assessed before starting the diet treatments.

On 9, 25 and 36 dph, 20 larvae were siphoned from each tank prior to feed addition. These larvae were anaesthetised and examined with a stereomicroscope. They were scored for absence or presence of the swim bladder, feeding (retained food in the gut, an indication for poor digestion), general condition, grey gut (an indication of lipid deposition) (Bransden et al., 2005b), and the standard (SL) or total length (TL). On 16 and 43 dph, 50 larvae from each tank were sampled, only 20 on 16 dph were measured for the SL, and the entire 50 larvae from both sampling days were scored as above. Immediately after scoring, these larvae were placed on pre-weighed filter paper, rinsed with 15 ml 0.5 M ammonium formate, excess moisture was removed and the filters were re-weighed to determine larval wet weight. The filter papers were folded, placed in aluminium foil pockets, frozen in liquid nitrogen, and transferred within 4 h to storage in a -80 °C freezer. Afterwards, the samples were freeze-dried and re-weighed to determine the dry weight of the larvae.

To quantify the retinoid content in the larvae, 200 larvae on 16 dph were siphoned out of each tank, rinsed with 0.5 M ammonium formate, transferred to 5 ml vials and immediately stored in liquid

nitrogen before transfer to a -80 °C freezer. These larvae were extracted and analysed by high performance liquid chromatography (HPLC) for total retinol.

To quantify the retinoid content in the live feed, samples were taken from the enriched rotifers fed to larvae on 11 and 15 dph and *Artemia* on 23 and 33 dph, rinsed and stored as mentioned above. Enriched rotifers were also extracted for their lipid content.

To identify jaw malformations, 20 larvae on 25 dph and 50 on 43 dph were randomly sampled from each tank and rated on a scale from 0 to 3 (Cobcroft and Battaglione, 2009). The score for a normal jaw was 0 and 0.5 was used for a jaw showing very minor deviation from normal that will not affect its market value. Scores 1, 2 and 3 were malformed jaws where 1 had minor deviation from normal structure, 2 was a moderate deviation in jaw element(s) in shape and/or position, and 3 indicated severe malformations where jaw elements had abnormal shape and position. The severity of malformations was indicated in each treatment by the proportions of fish with each score, and then the overall percentage of malformed fish with scores of 1, 2, or 3. The prevalence of jaw malformation type for each tank was calculated as the proportion of fish with the particular type out of all fish assessed, and then averaged for the treatment prevalence. This means that individual fish may have been classified with more than one malformation type.

To identify other skeletal malformations, 50 post-larvae per tank were sampled on 43 dph and fixed in 10% neutral buffered formalin. These post-larvae were stained with alizarin red and alcian blue for bone and cartilage on whole mounts using the method described by Taylor and VanDyke (1985).

These post-larvae were examined for vertebral column malformations. The vertebral column was divided into four distinct regions based on morphological features according to Bogleione et al. (2001). The first two cephalic vertebrae were characterized by only neural processes. Pre-haemal vertebrae (3 to 16) were characterized by centra showing pleural and epipleural ribs followed by the haemal vertebrae (17 to 34) that were characterized by neural and haemal processes (spines). The

last three caudal vertebrae (35 to 37) were characterized by longer neural and haemal processes.

Malformations in the caudal region (Chapters 3, 4 and 5) include malformations in the hypural, epural and parahypural (caudal fin complex).

Final larval survival was determined by draining each tank onto a mesh screen to collect the post-larvae, anaesthetising the larvae and then counting. Final survival was calculated by adjusting for the sampled larvae using the following formula:

Survival (%) = total alive post-larvae on 43 dph / (yolk sac larvae stocked on 1 dph – (total sampled 9 to 36 dph + live larvae removed in mortality siphoning)) \*100.

### 3.3.7 Biochemical analysis

Retinoids were measured as total VA (the sum of retinyl palmitate, retinol and retinoic acid) and total retinol using two different techniques modified from Moren et al. (2002 and 2004a) and Takeuchi et al. (1998). Total VA and retinol were measured in enrichment emulsions and live prey while only total retinol was measured in 16 dph larvae.

Determination of total VA in live feed was as described in Chapter 2. While for the enrichment emulsions, 0.1 g was homogenised within a 20 ml culture tube containing 10 ml chloroform: methanol (2:1 v/v), 0.01% butylated hydroxytoluene and 50 ng retinyl acetate as an internal standard. Each sample was left on ice for 1 h after adding 2.5 ml 0.88% KCl and shaken for 30 s. Sample tubes were centrifuged for 10 min and then the lower chloroform phase was transferred to 10 ml Maxi-vials and diluted to 10 ml with additional chloroform. Subsamples of 4 ml were transferred to 5 ml Mini-vials and evaporated to dryness by blowing with nitrogen. The dry fraction derived from each of emulsion samples was redissolved in 2 ml chloroform: methanol (2:1 v/v) and filtered through 0.45 µm syringe filter. Samples from 5 to 100 µl were analysed using a Waters Model 600E liquid chromatograph system (Waters Corporation, Milford, MA, USA) supplied with a Waters Model 996 photodiode array (PDA) detector and 475 scanning fluorescence detector set for

an excitation maximum at 325 nm and emission maximum at 470 nm. The retinoids eluted within 30 min, using an Alltima C18 Column (250 mm x 4.6 mm 5 $\mu$ m; Grace Davison Discovery Sciences, Rowville, Vic., Australia) set to 30 °C and using as isocratic mobile phase of 98% methanol with 0.5% ammonium acetate and chloroform (85:15 v/v) at a flow rate of 1.5 ml min<sup>-1</sup>. Peak areas were quantified using Waters Millennium software. Retinyl palmitate results were obtained using the PDA detector while retinol and retinoic acid were determined from the fluorescence detector.

The HPLC method for total retinol extraction makes use of the saponification process where the ester bonds in the retinyl palmitate are hydrolysed and total retinol is measured. In this process, 0.5 g of the live feed or emulsions or 0.1 g larvae was mixed vigorously with 4.5 ml of 2% KOH in ethanol in 15 ml culture tubes. Ascorbic acid and pyrogallol (20 mg of each) and 0.5 ml saturated ethylenediaminetetraacetic acid were added before the containers were capped and heated for 20 min at 100 °C in a waterbath. After each sample was cooled, 1 ml milli-Q water was added and the mixture was extracted three times with 3 ml hexane. The combined hexane extracts were transferred to a 10 ml extraction vial and 50 ng of retinyl acetate was added as an internal standard. The entire 9 ml was blown dry with nitrogen. The dried fraction was redissolved in 2 ml hexane and stored in the fridge at 4 °C. Samples (50  $\mu$ l from live feed, 1 to 25  $\mu$ l from emulsions and 100  $\mu$ l from larvae) were analysed using the HPLC system as previously described. The column used was an Alltima<sup>™</sup> silica column-W (250 x 4.6 mm 5 $\mu$ m; Grace Davison Discovery Sciences) set at 30 °C. The mobile phase consisted of gradient of n-heptane and 1,4-dioxane and the elution time was 65 min (Moren et al., 2004a). Peaks were detected and quantified with the PDA detector (325 nm). The concentration of retinoids in samples was calculated by comparing peak area to those of internal and external standards. Live feed samples and larvae were not freeze-dried and appropriate calculations were made to change wet weight into dry weight (DW). Larvae, rotifer and *Artemia* DW were based on measurements for 16 dph larvae and different freeze-dried samples of live feed. Data are presented as ng mg<sup>-1</sup> DW.

Total lipids and fatty acid methyl esters in live prey were determined using a modified version of Bligh and Dyer (1959), as described in Chapter 2.

### **3.3.8 Statistical analysis**

One way ANOVA was used to test the effect of dietary vitamin levels on the larval performance parameters; length, dry weight, swim bladder inflation, survival, and jaw and vertebral column malformations. Mean percentage data were transformed by  $\arcsin \sqrt{x}$ , where  $x$  is the proportion, and the dry weight data was  $\log_{10}$  transformed to achieve homogeneity of variance and normal distribution. Levene's test was used to assess data for homogeneity of variance. Where significant treatment effects were found, a Tukey's test was used to determine differences among means. Correlation was used to test the association between larvae retinoid content and skeletal malformations. Significant differences were accepted at  $P < 0.05$ . Statistical analyses were performed using SPSS 13.0 (SPSS Inc.).

## **3.4 Results**

### **3.4.1 Biochemical analyses**

#### **3.4.1.1 Retinoid content in experimental emulsions, live prey and larvae**

Retinyl palmitate was the predominant form of VA in the emulsions but a small retinol peak was observed that was difficult to quantify as it was masked by other, higher concentration peaks. This retinol peak was due to the base emulsion ingredients (lipids base contained tuna oil 700 IU VA  $\text{kg}^{-1}$ , NuMega certificate of analyses) prior to retinyl palmitate inclusion. Retinol was quantified using the saponification process and expressed as total retinol, which is the retinyl palmitate hydrolysed into retinol plus the retinol from the emulsion ingredients.

Emulsions in the current study were designed nominally to contain 0 to 10,000  $\text{mg l}^{-1}$  retinyl palmitate. Retinyl palmitate and total retinol content in the emulsions increased significantly with

increasing addition of retinyl palmitate (Table 1). The concentration of retinyl palmitate was 62 to 70% of that theoretically added in the lower range of 100 to 750 mg retinyl palmitate l<sup>-1</sup> emulsion, and 89 to 96% of the added quantity in the 1,500 to 10,000 mg retinyl palmitate l<sup>-1</sup> range. The corresponding values of total retinol for the designed emulsions ranged from 30 to 1406 ng total retinol mg<sup>-1</sup> emulsion, and there was a small detectable quantity of total retinol (0.29 ng mg<sup>-1</sup>) in the 0 emulsion.

Table 1: Retinyl palmitate and total retinol (saponification method) in experimental live prey enrichment emulsions. Values are mean  $\pm$  SD and n = 3. Different letters within the same column show significant differences among emulsions (ANOVA, P < 0.05).

Emulsion (mg retinyl palmitate l <sup>-1</sup> )	Retinyl palmitate ng mg <sup>-1</sup>		Total Retinol ng mg <sup>-1</sup>	
0	0 $\pm$	0.00 <sup>a</sup>	0.29 $\pm$	0.02 <sup>a</sup>
100	68.20 $\pm$	1.14 <sup>b</sup>	30.36 $\pm$	2.81 <sup>b</sup>
300	187.28 $\pm$	1.24 <sup>c</sup>	73.15 $\pm$	13.25 <sup>c</sup>
750	532.15 $\pm$	46.99 <sup>d</sup>	141.21 $\pm$	14.35 <sup>d</sup>
1500	1,402.32 $\pm$	78.64 <sup>e</sup>	310.30 $\pm$	12.71 <sup>e</sup>
3000	2,670.08 $\pm$	14.31 <sup>f</sup>	428.26 $\pm$	73.25 <sup>e</sup>
5000	4,808.35 $\pm$	628.82 <sup>g</sup>	778.96 $\pm$	94.89 <sup>f</sup>
10000	9,522.70 $\pm$	182.38 <sup>h</sup>	1,406.08 $\pm$	190.98 <sup>g</sup>

In the enriched rotifers, average total VA and total retinol were significantly different with increasing doses of retinyl palmitate (Table 2). Retinyl palmitate was the predominant VA in the enriched rotifers with detectable amounts of retinol and retinoic acid. The different concentrations of retinyl palmitate in the emulsions were reflected in the amount of retinyl palmitate detected in the rotifers with an approximate doubling of retinyl palmitate in rotifers with each increase in enrichment emulsions.

Retinyl palmitate was not detected in rotifers and *Artemia* enriched with Algamac-3050 but after the samples were saponified, total retinol was 0.53  $\pm$  0.01 ng mg<sup>-1</sup> DW in rotifers enriched for 8 h and 0.76  $\pm$  0.00 and 0.69  $\pm$  0.03 ng mg<sup>-1</sup> DW for *Artemia* enriched for 16 and 20 h, respectively.

Table 2: Total VA content (retinyl palmitate, retinol and retinoic acid) and total retinol (saponification method) ng mg<sup>-1</sup> in DW rotifers enriched with graded levels of retinyl palmitate. Values are mean ± SD and n = 3. Different letters within the same column show significant differences among dietary groups (ANOVA, P < 0.05).

Retinyl palmitate in emulsion	Retinyl palmitate	Retinol	Retinoic acid	Total VA	Total Retinol (retinyl palmitate + retinol)
<b>0</b>	0 ± 0.00 <sup>a</sup>	2.41 ± 0.53 <sup>b</sup>	4.53 ± 0.81	6.94 ± 1.34 <sup>a</sup>	0.52 ± 0.09 <sup>a</sup>
<b>68</b>	6.45 ± 0.76 <sup>b</sup>	0.93 ± 0.75 <sup>a</sup>	4.00 ± 0.36	11.38 ± 1.87 <sup>b</sup>	0.93 ± 0.02 <sup>b</sup>
<b>187</b>	13.60 ± 4.13 <sup>c</sup>	1.08 ± 0.42 <sup>ab</sup>	4.36 ± 0.63	19.04 ± 5.18 <sup>c</sup>	2.32 ± 0.10 <sup>c</sup>
<b>532</b>	26.33 ± 2.26 <sup>d</sup>	2.88 ± 0.34 <sup>bc</sup>	4.54 ± 0.40	33.75 ± 2.99 <sup>d</sup>	6.67 ± 0.14 <sup>d</sup>
<b>1402</b>	57.15 ± 6.75 <sup>e</sup>	7.08 ± 0.96 <sup>cd</sup>	4.71 ± 0.63	68.95 ± 8.33 <sup>e</sup>	16.31 ± 0.72 <sup>e</sup>
<b>2670</b>	108.56 ± 12.38 <sup>f</sup>	10.80 ± 3.67 <sup>de</sup>	3.87 ± 0.49	123.23 ± 16.54 <sup>f</sup>	34.76 ± 0.87 <sup>f</sup>
<b>4808</b>	215.49 ± 22.49 <sup>g</sup>	21.67 ± 5.32 <sup>ef</sup>	3.15 ± 0.76	240.31 ± 28.57 <sup>g</sup>	52.73 ± 1.70 <sup>g</sup>
<b>9523</b>	387.95 ± 31.79 <sup>h</sup>	38.28 ± 3.76 <sup>f</sup>	3.01 ± 0.45	429.24 ± 35.99 <sup>h</sup>	92.54 ± 2.12 <sup>h</sup>

Due to small larvae size on 16 dph, larvae retinoid content was determined using the saponification technique only. There was a significant effect of dietary retinyl palmitate on total retinol content of larvae at 16 dph (ANOVA,  $F_{7,16} = 12.55$ ,  $P < 0.001$ ) (Fig. 1). Average total retinol in larvae 16 dph was significantly higher ( $11.08 \pm 0.27$  ng mg<sup>-1</sup> DW larvae) for those fed on rotifers with total retinol levels between 0.93 to 2.32 ng mg<sup>-1</sup> DW (low treatments) compared with those ( $5.57 \pm 0.30$  ng mg<sup>-1</sup> DW larvae) fed on rotifers with total retinol levels between 16.31 to 92.54 ng mg<sup>-1</sup> DW (high treatments). Retinol content was intermediate in larvae fed 6.67 ng mg<sup>-1</sup> DW total retinol. There was no significant difference in the larvae total retinol content between those fed on the control and high treatment rotifers (Fig. 1).

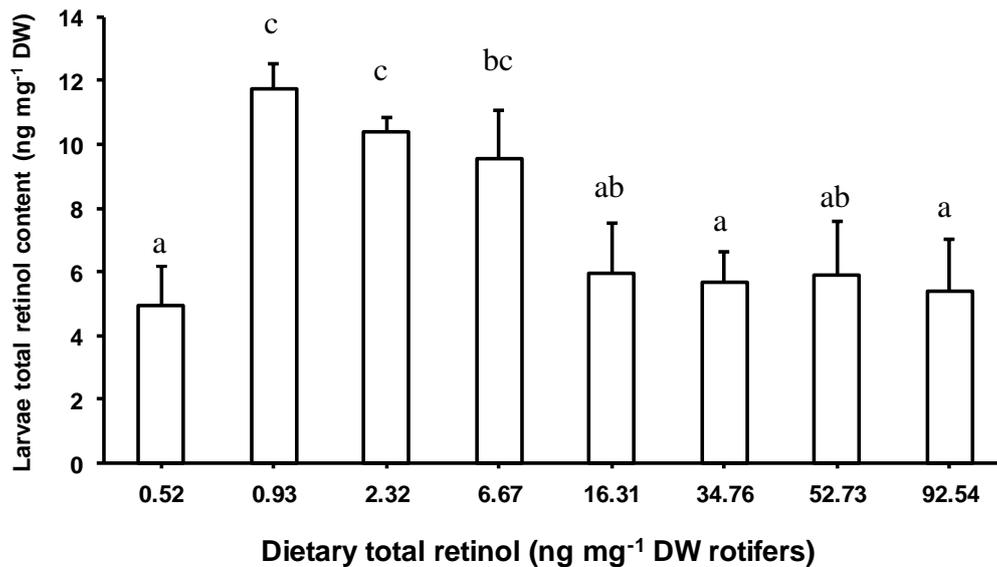


Figure 1: Total retinol in *Latris lineata* larvae at 16 dph fed rotifers enriched with graded levels of retinyl palmitate. Retinoid content is expressed as ng mg<sup>-1</sup> DW larvae. Values are mean + SD and n = 3 replicate tanks. Histograms not sharing a common letter indicate significant differences among means (ANOVA, P < 0.05).

#### 3.4.1.2 Lipid content of live prey

Total fatty acids in rotifers enriched with Algamac-3050 for 8 h were significantly higher than in rotifers enriched with the graded VA emulsions for 2 h (Table 3) (ANOVA,  $F_{3,8} = 214.45$ ,  $P < 0.001$ ).

There was no significant difference in the total fatty acids between rotifers enriched with graded VA emulsions (Table 3). Docosahexaenoic acid (DHA, 22:6 $\omega$ 3), eicosapentaenoic acid (EPA, 20:5 $\omega$ 3) and arachidonic acid (ARA, 20:4 $\omega$ 6) were significantly higher in rotifers enriched with Algamac-3050 than the rotifers enriched with the VA emulsions (ANOVAs,  $F_{3,8} = 151.81$ , 2130.48, and 21.15, respectively,  $P < 0.001$ ).

Table 3: Selected fatty acid groups and polyunsaturated fatty acids in rotifers ( $\text{mg g}^{-1}$  DW) enriched with 750, 1,500 and 3,000  $\text{mg l}^{-1}$  retinyl palmitate and Algamac-3050 for 2 and 8 h respectively. Values are mean of 3 replicates  $\pm$  SD. SFA = saturated fatty acid, MUFA = monounsaturated fatty acid, PUFA = polyunsaturated fatty acid and FA = fatty acid. Different letters within the same row show significant differences among dietary groups (ANOVA,  $P < 0.05$ ).

Enrichment ( $\text{mg l}^{-1}$ retinyl palmitate)	750	1500	3000	Algamac-3050
<b>Total SFA</b>	$17.45 \pm 0.78^b$	$16.38 \pm 0.81^a$	$18.68 \pm 0.80^c$	$20.12 \pm 0.37^d$
<b>Total MUFA</b>	$21.92 \pm 1.71^b$	$21.26 \pm 1.75^b$	$22.10 \pm 1.15^c$	$16.41 \pm 0.25^a$
<b>Total n:<math>\omega</math>6</b>	$8.09 \pm 0.26^{ab}$	$8.20 \pm 0.74^b$	$7.95 \pm 0.30^a$	$16.61 \pm 0.05^c$
<b>Total n:<math>\omega</math>3</b>	$19.13 \pm 0.29^a$	$18.98 \pm 1.52^a$	$18.84 \pm 0.77^a$	$43.18 \pm 0.38^b$
<b>Total PUFA</b>	$27.22 \pm 0.54^a$	$27.18 \pm 2.25^a$	$26.79 \pm 1.07^a$	$59.79 \pm 0.43^b$
<b>Fatty acid (<math>\text{mg g}^{-1}</math> DW)</b>				
<b>20:4<math>\omega</math>6</b>	$1.84 \pm 0.06^a$	$1.90 \pm 0.18^a$	$1.74 \pm 0.06^a$	$3.09 \pm 0.01^b$
<b>20:5<math>\omega</math>3</b>	$8.23 \pm 0.14^b$	$8.35 \pm 0.52^b$	$7.64 \pm 0.25^a$	$9.02 \pm 0.02^c$
<b>22:6<math>\omega</math>3</b>	$6.64 \pm 0.01^a$	$6.42 \pm 0.43^a$	$7.81 \pm 0.27^b$	$28.17 \pm 0.18^c$
<b>Total FA (<math>\text{mg g}^{-1}</math> DW)</b>	$69.25 \pm 2.09^a$	$67.05 \pm 2.44^a$	$66.77 \pm 2.73^a$	$98.52 \pm 0.81^b$
<b>22:6<math>\omega</math>3/ 20:5<math>\omega</math>3</b>	$0.81 \pm 0.01^b$	$0.77 \pm 0.00^a$	$1.02 \pm 0.00^c$	$3.12 \pm 0.01^d$

### 3.4.2 Larval growth and survival

#### 3.4.2.1 Larval development

The amount of VA in rotifers had no significant effect on the mean TL (ANOVA,  $F_{7,16} = 0.19$ ,  $P = 0.99$ ) (Table 4) or weight of the larvae (ANOVA,  $F_{7,16} = 0.24$ ,  $P = 0.97$ ) (Fig. 2). Average larval SL was  $4.35 \pm 0.01$  mm ( $n = 20$  larvae) on 1 dph and  $5.23 \pm 0.13$  mm ( $n = 240$ ) on 5 dph. On 43 dph, average TL was  $15.96 \pm 0.13$  mm ( $n = 1200$  larvae) and average DW was  $4.57 \pm 0.20$  mg ( $n = 480$  larvae) across treatments.

Table 4: Larval growth in standard length (SL) for 9, 16 and 25 dph, total length (TL) for 36 and 43 dph of *Latris lineata* larvae and post larvae fed rotifers enriched with graded levels of retinyl palmitate. Values are mean  $\pm$  SD (n = 60 for 9, 16, 25 and 36 dph and n = 150 for 43 dph). No statistical differences among treatments within days (ANOVA, P > 0.05).

Retinyl palmitate ng mg <sup>-1</sup> DW rotifers	Larval length (mm) within age (dph)				
	9 dph (SL)	16 dph (SL)	25 dph (SL)	36 dph (TL)	43 dph (TL)
<b>0.00</b>	5.97 $\pm$ 0.03	7.51 $\pm$ 0.15	9.23 $\pm$ 0.19	13.28 $\pm$ 0.62	15.82 $\pm$ 0.65
<b>6.45</b>	6.00 $\pm$ 0.04	7.73 $\pm$ 0.10	9.30 $\pm$ 0.26	13.55 $\pm$ 0.11	16.02 $\pm$ 0.39
<b>13.60</b>	5.98 $\pm$ 0.01	7.52 $\pm$ 0.18	9.29 $\pm$ 0.25	13.65 $\pm$ 0.35	15.89 $\pm$ 0.83
<b>26.33</b>	5.92 $\pm$ 0.08	7.69 $\pm$ 0.17	9.43 $\pm$ 0.13	13.33 $\pm$ 0.26	16.10 $\pm$ 0.44
<b>57.15</b>	5.96 $\pm$ 0.05	7.67 $\pm$ 0.05	9.40 $\pm$ 0.21	13.52 $\pm$ 0.27	15.78 $\pm$ 0.02
<b>108.56</b>	5.99 $\pm$ 0.02	7.72 $\pm$ 0.14	9.38 $\pm$ 0.36	13.41 $\pm$ 0.82	16.14 $\pm$ 0.60
<b>215.49</b>	5.93 $\pm$ 0.04	7.76 $\pm$ 0.22	9.41 $\pm$ 0.22	13.49 $\pm$ 0.39	16.05 $\pm$ 0.47
<b>387.95</b>	5.97 $\pm$ 0.01	7.65 $\pm$ 0.10	9.22 $\pm$ 0.06	13.12 $\pm$ 0.14	15.91 $\pm$ 0.49

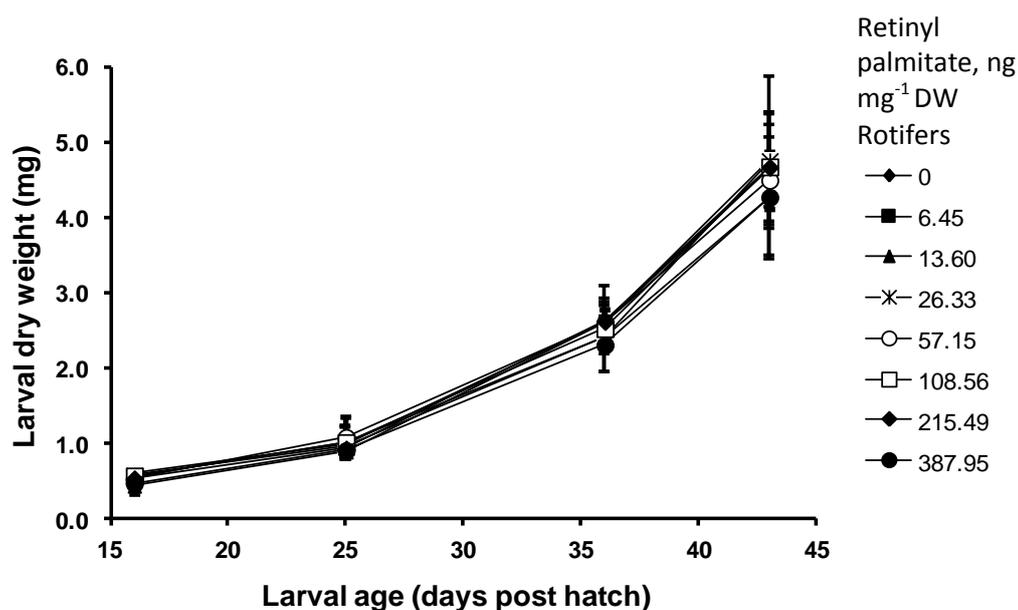


Figure 2: Increase in dry weight of *Latris lineata* larvae and post-larvae fed rotifers enriched with graded levels of retinyl palmitate. Values are mean  $\pm$  SD (n = 3 replicate tanks). No statistical differences among treatments within days (ANOVA, P > 0.05).

There was no significant difference in survival among treatments (ANOVA,  $F_{7,16} = 0.80$ , P = 0.60) (Fig. 3). Average survival was  $34.8 \pm 10.6\%$  among treatments except for the 108.56 retinyl palmitate ng mg<sup>-1</sup> DW rotifers treatment which had an average of  $25.1 \pm 20.7\%$  where two tanks had low survival.

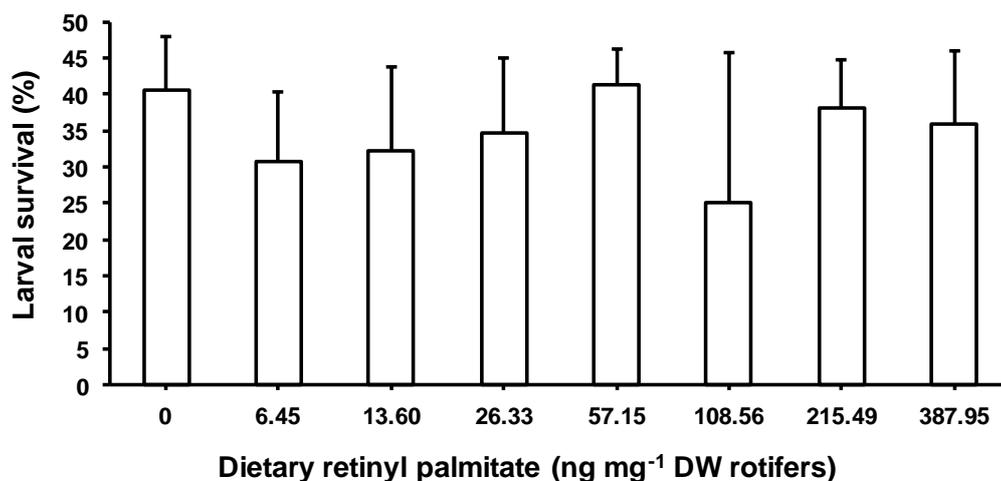


Figure 3: Survival of *Latrix lineata* post-larvae at 43 dph fed rotifers enriched with graded levels of retinyl palmitate. Values are mean + SD (n = 3 replicate tanks). No statistical differences among treatments (ANOVA,  $P > 0.05$ ).

### 3.4.2.2 Jaw malformation

Graded retinyl palmitate treatments did not significantly affect incidence, severity or types of jaw malformation (Table 5 and Fig. 4). Some larvae showed moderate and severe malformations with multiple jaw elements involved; larvae scoring 2 and 3 (Fig. 5 B, C, E and F). The most severe jaw malformations (larvae scoring 3) increased from  $1.04 \pm 0.86\%$  on 25 dph to  $12.7 \pm 2.89\%$  on 43 dph (Fig. 4). Commercially important malformations (scores 1 + 2 + 3) were not affected by dietary VA, and were  $81.4 \pm 9.3\%$  across treatments at 43dph (ANOVA,  $F_{7,16} = 1.40$ ,  $P = 0.27$ ) (Fig. 5). Only  $5.0 \pm 4\%$  of the post-larvae by 43 dph had normal jaws (score zero).

Table 5: Mean percentage of different types and time of occurrence (dph) of jaw malformations in *Latrix lineata* larvae fed rotifers enriched with graded levels of retinyl palmitate. Three replicate tanks per treatment, n = 20 on 25 and 36 dph and n = 50 on 43 dph. Values are mean  $\pm$  SD.

Larval age	Incidence of different jaw malformations (%)			
	Lowered hyoid arch	Open jaw	Lower jaw	Upper jaw
<b>25 dph</b>	$8 \pm 7$	$13 \pm 12$	$42 \pm 20$	$0 \pm 0$
<b>43 dph</b>	$8 \pm 4$	$55 \pm 11$	$63 \pm 12$	$10 \pm 7$

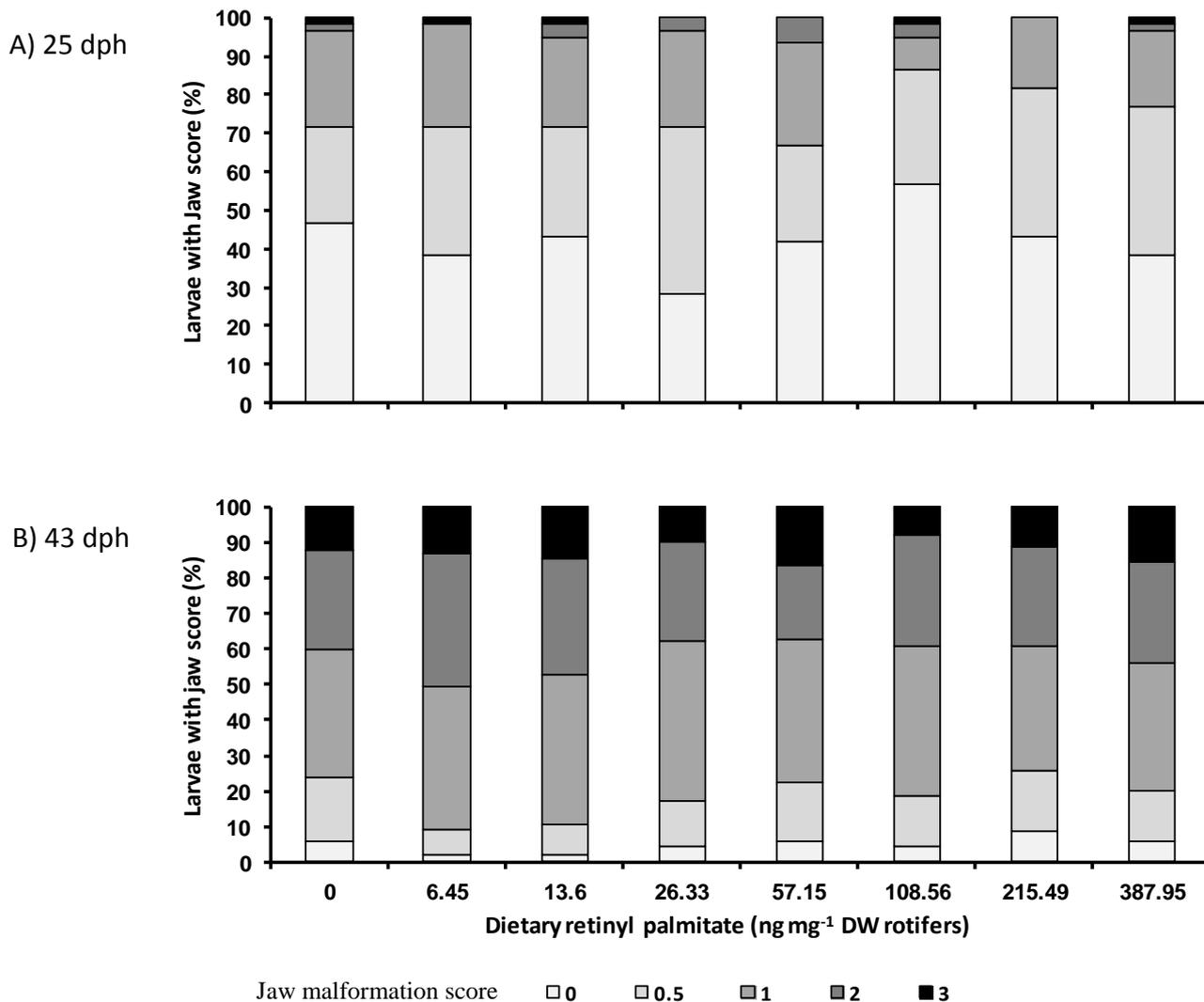


Figure 4: Incidence, severity and development of jaw malformation in *Latris lineata* larvae and post-larvae fed rotifers enriched with graded levels of retinyl palmitate. Bar shading represent jaw malformation index as shown in legend. Descriptions of the jaw scores are as mentioned in text. Three replicate tanks per treatment, n = 20 fish per tank on 25 dph and n = 50 on 43 dph.

Lower jaw malformations included short, thickened or twisted jaw on 25 dph, in addition to long or bent jaw on 36 dph and abraded jaw on 43 dph (Fig. 5). Upper jaw malformations included short jaw on 25 dph in addition to thickened, short or thin maxilla or short premaxilla on 36 dph, in addition to curled or abraded maxilla and premaxilla, or maxilla aligned forward on 43 dph (Fig. 5). No significant correlation was found between different jaw malformations types and dietary VA.

By 43 dph, lowered hyoid arch and upper jaw malformations were the least commonly observed malformations among treatments. Opened jaws (Fig. 5B and C), where the maxilla and pre-maxilla have an abnormal shape or position and there was limited or no movement to open and close the mouth, and malformations of the lower jaw were the most common.

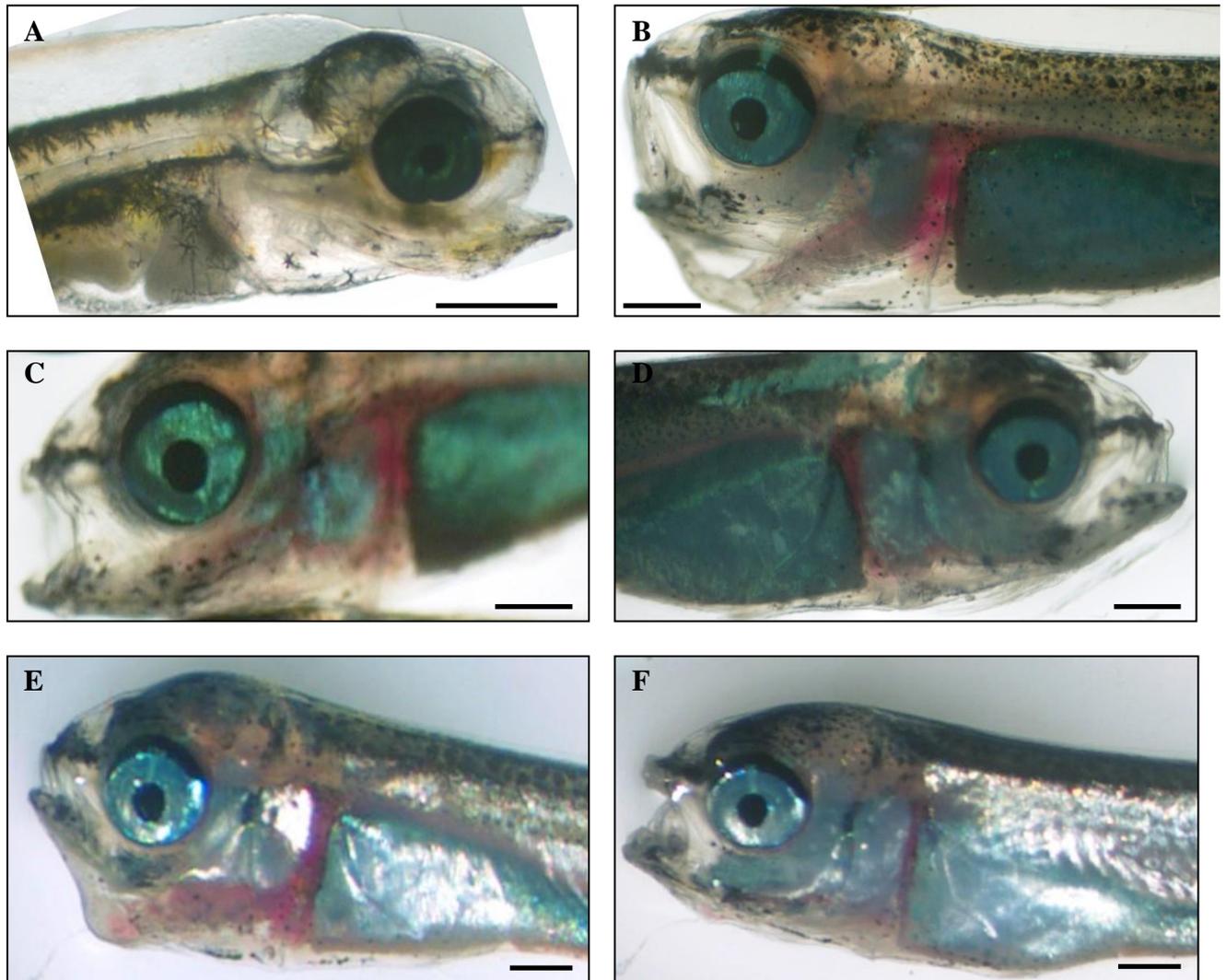


Figure 5: Different jaw malformations in *Latris lineata* larvae and post-larvae fed rotifers enriched with graded levels of retinyl palmitate. A) 16 dph larvae showing short upper jaw (score 1). B) 36 dph larvae showing opened jaw and lowered hyoid arch (score 3). C) 36 dph post-larvae showing bent maxilla (upper jaw), open and thickened lower jaw (score 3). D) 36 dph post-larvae showing thickened upper and lower jaw (score 1). E) 43 dph post-larvae showing short lower jaw and lowered hyoid arch (score 2). F) 43 dph post-larvae showing missing premaxilla, twisted and thin maxilla, and thickened and abraded lower jaw (score 3). Scale bars are 1 mm.

### 3.4.2.3 Vertebral column malformation

Graded VA levels significantly affected overall vertebral column malformations (Fig. 6E) (ANOVA,  $F_{7,16} = 15.28$ ,  $P < 0.001$ ) but did not significantly affect the malformations in specific vertebral regions (Fig. 6A, B, C and D). The percentage of post-larvae with vertebral malformation was  $51 \pm 5\%$  when fed on rotifers incorporating 6.45 to 26.33 ng retinyl palmitate  $\text{mg}^{-1}$  DW and  $31 \pm 5\%$  when fed on rotifers incorporating 108.56 to 387.95 ng retinyl palmitate  $\text{mg}^{-1}$  DW. Significant correlation was found between total retinol content in 16 dph larvae and the percentage of post-larvae showing vertebral malformation at 43 dph ( $R^2 = 0.55$ ,  $P < 0.001$ ) (Fig. 7). By the end of the experimental period, there was no significant difference in the mean number of vertebrae ( $37 \pm 0$ ) among the larvae fed different levels of VA-enriched rotifers (ANOVA,  $F_{7,16} = 1.228$ ,  $P = 0.344$ ) (Fig. 8).

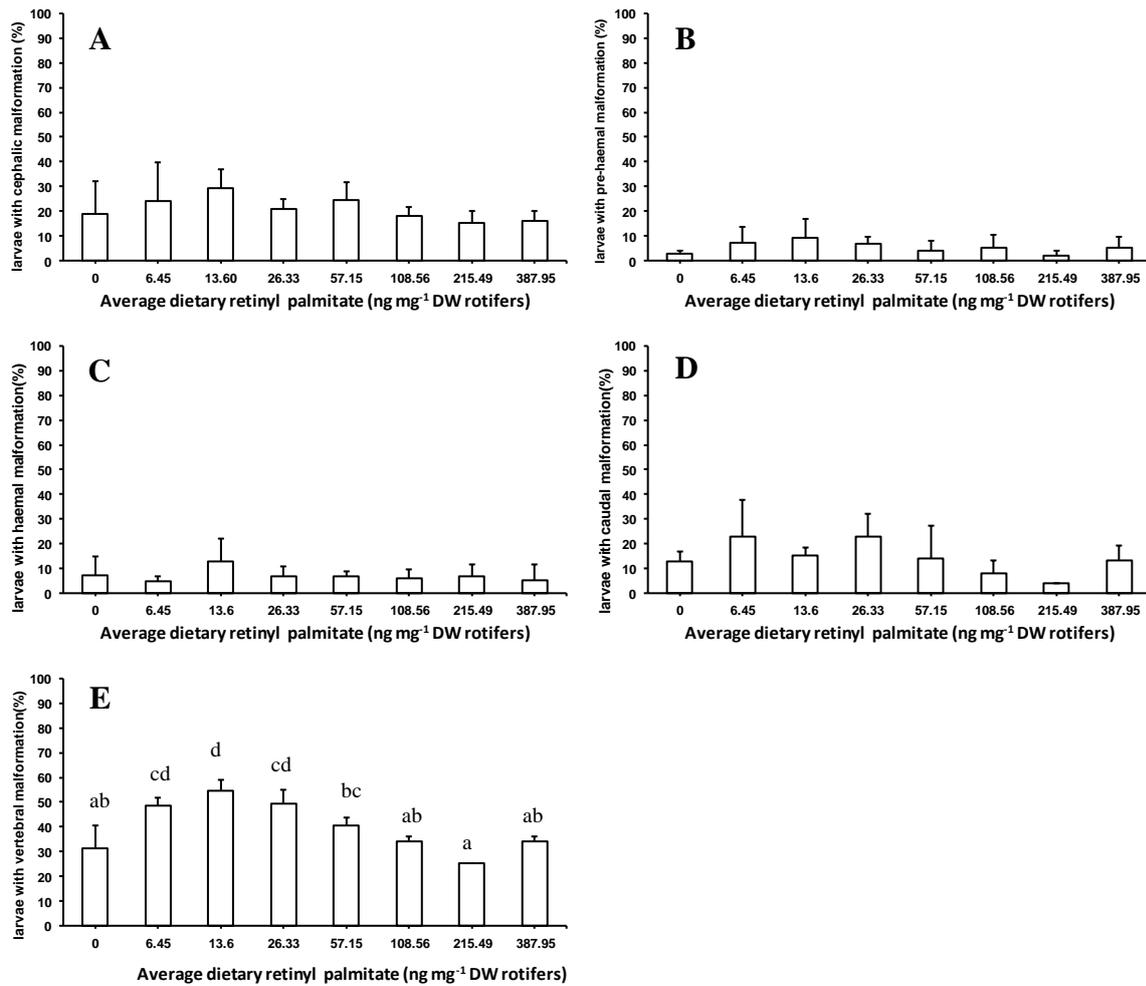


Figure 6: Prevalence of skeletal malformations in *Latris lineata* post-larvae at 43 dph fed rotifers enriched with graded levels of retinyl palmitate, in A) cephalic (first two vertebrae) B) pre-haemal C) haemal D) caudal fin complex regions of vertebrae and E) vertebral malformations in any region. Values are mean + SD. Three replicate tanks, n = 50 fish per tank. Different letters indicate statistically significant differences in E ( $P < 0.05$ ).

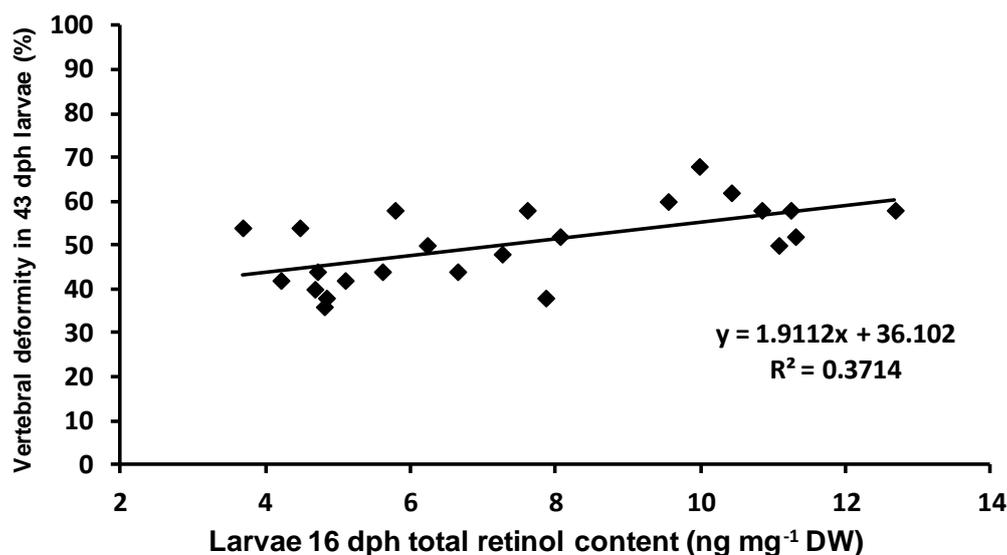


Figure 7: Linear correlation between the percentage of vertebral malformations in *Latris lineata* post-larvae at 43 dph from each tank and total retinol content in 16 dph larvae ( $R^2 = 0.55$ ,  $P = 0.00$ ).

A range of skeletal malformations were observed in 43 dph post-larvae fed different VA levels. The malformations included paired, short and branched haemal spines, thickened or short neural spines (Fig. 9A, B, C and D). The vertebrae malformations included fused (Fig. 9A) and compressed vertebrae (Fig. 9F). Scoliosis, kyphosis and sometimes combinations of both were observed (Fig. 9E).

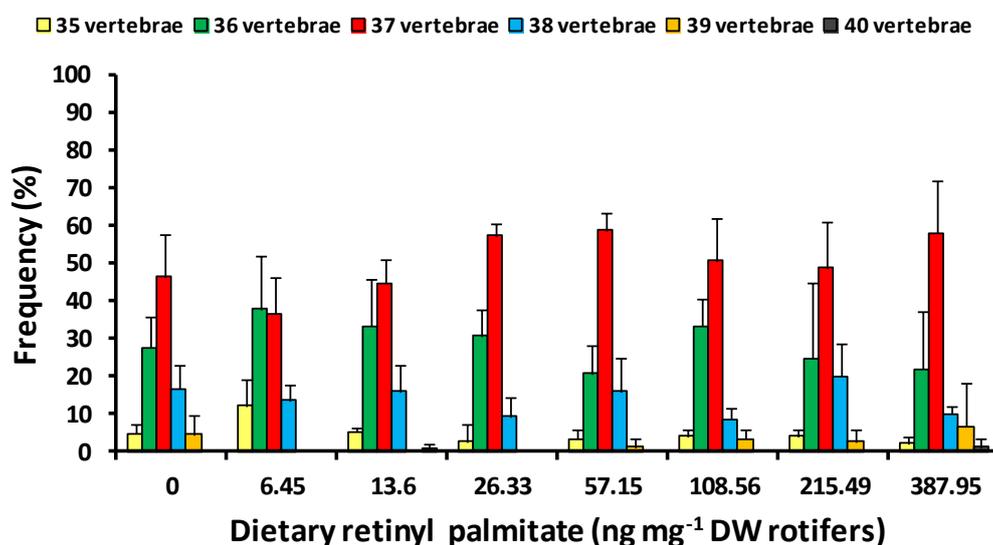


Figure 8: Frequency (%) of number of vertebra in *Latris lineata* at 43 dph post-larvae fed rotifers enriched with graded levels of retinyl palmitate. Values are mean + SD. Three replicate tanks,  $n = 50$  fish per tank. No statistical differences among treatments (ANOVA,  $P > 0.05$ ).

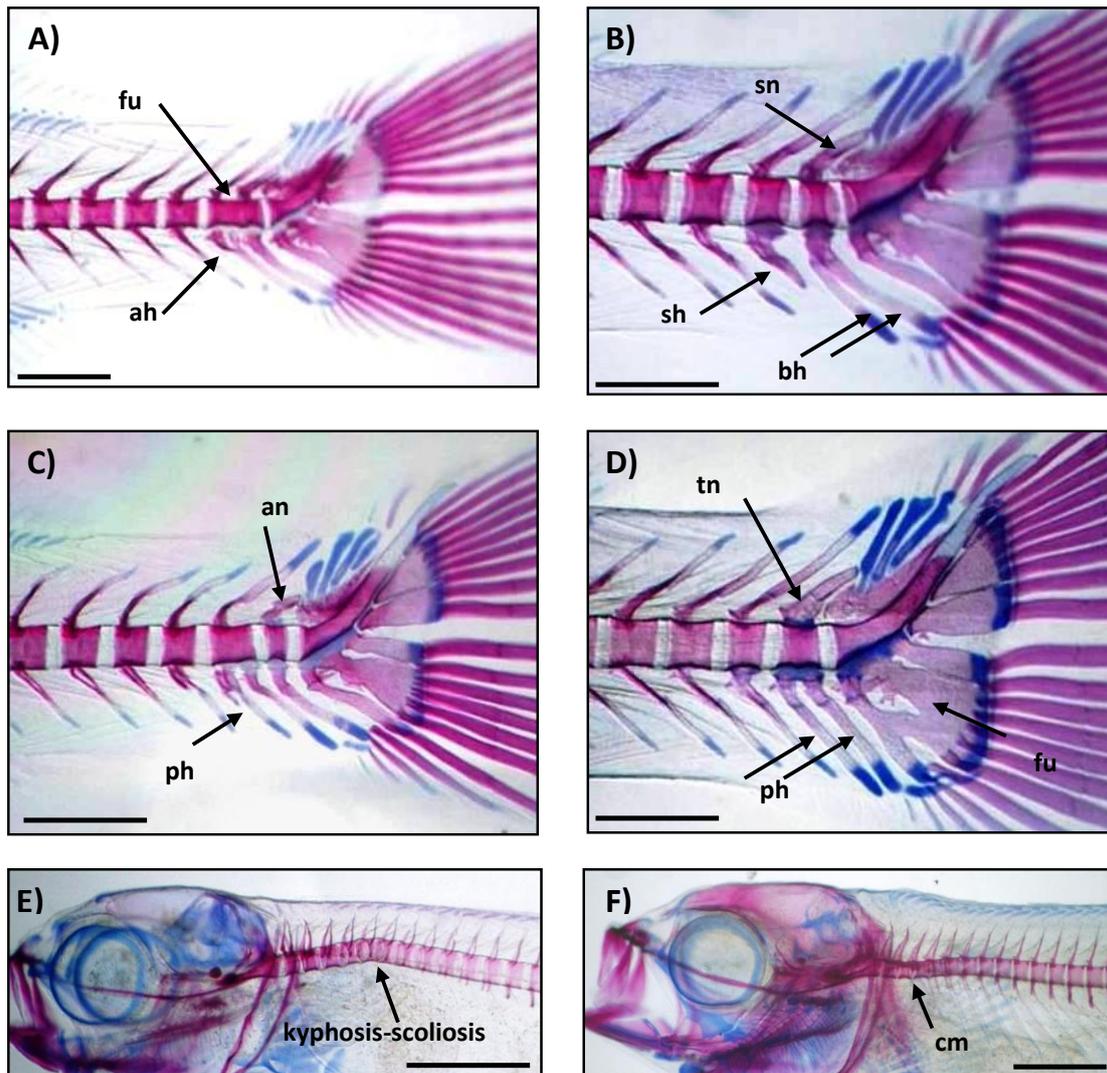


Figure 9: Different skeletal malformations in *Latris lineata* post-larvae at 43 dph fed rotifers enriched with graded levels of retinyl palmitate. Abbreviations: ah, abnormal haemal; an, abnormal neural; bh, branched haemal; cm, compressed vertebra; fu, fusion; ph, paired haemal; sh, short haemal; sn, short neural; tn, thickened neural. Scale bars are 1 mm.

### 3.5 Discussion

The effects of increasing dietary VA (retinyl palmitate) levels on *L. lineata* larval performance parameters and skeletal development were studied by means of a dose-response experiment through enrichment of rotifers. Rotifers were enriched with eight levels of retinyl palmitate and enriched for 2 h in complete darkness (Giménez et al., 2007; Haga et al., 2006). Total VA in the rotifers was higher than that reported by Fernández et al. (2008). Both studies used a comparable

range of retinyl palmitate concentrations, with the same enrichment duration (2 h). However, in the current study rotifers were enriched in complete darkness, as retinoid content in rotifers enriched under dark conditions has been shown to be higher than those enriched under light conditions (Haga et al., 2006). It is also possible that physiological differences in the rotifer strains may have contributed to the higher VA in the present study.

Due to the short enrichment period with VA (2 h), the concentration of dietary essential fatty acids (EFA), especially DHA was below the standard requirements for *L. lineata* larvae during the rotifer feeding period. Average DHA concentration in the enriched rotifers with the experimental VA emulsions was  $9.28 \pm 0.82 \text{ mg g}^{-1} \text{ DW}$ , below the minimum recommended dose of  $13 \text{ mg g}^{-1} \text{ DW}$  (Bransden et al., 2003, 2004, 2005b). In addition, ARA and the ratio between DHA to EPA were also below the recommended dose for *L. lineata* (Bransden et al., 2004, 2005b). Deficiency or imbalance in the EFA during the rotifers feeding period in *L. lineata* can result in erratic swimming behaviour, abnormal lipid assimilation, and highly vacuolated hepatocytes (Bransden et al., 2004, 2005b).

Commercial products with high levels of DHA and low EPA are normally used to enrich live feeds for marine fish larvae (Conceição et al., 2010; Koven et al., 2001). Algamac-3050 was chosen as the source of lipid enrichment following research which showed it was superior to other commercial and fish oil based enrichments for *L. lineata* including those used in previous VA studies with rotifers (Battaglione et al., 2006; Bransden et al., 2005b; Fernández et al., 2008, 2009; Giménez et al., 2007). I examined using Algamac-3050 enrichment for 6 h followed with enrichment with VA for a further 2 h in order to provide just one source of enriched rotifers. This resulted in a satisfactory lipid profile in the enriched rotifers but very low VA inclusion in the rotifers (Chapter 2). I settled on using two different enrichments one to provide a dosed VA diet the other adequate lipid nutrition. The morning feed when larvae were most hungry was the VA enriched rotifers and the afternoon feed the Algamac-3050 enriched rotifers. Both diets were available for the same time and at the same concentration 8 h at a 5 rotifer  $\text{ml}^{-1}$ . Thus, larval performance was not compromised by poor lipid

nutrition during the experiment and differences can be attributed to the VA treatments. I acknowledge the potential for differential feeding by the larvae, particularly that rotifers enriched with high doses of VA may have been less palatable. If this occurred it would potentially mask the results and may explain the bell shaped response. As a counter argument I note three factors in support of equal feeding on the two sources of enriched rotifers. First, there was no detectable difference in the feeding responses of larvae among treatments. Second, there was no significant difference in growth or survival of larvae among treatments. Third, the growth and survival was well above that reported in earlier studies with *L. lineata* and was achieved without addition of antibiotics (Battaglione et al., 2006; Bransden et al., 2004, 2005b; Cobcroft and Battaglione, 2009).

The pattern of increasing VA in the enriched rotifers was not reflected in the larvae fed those rotifers. Larvae incorporated  $11.08 \pm 0.27$  ng total retinol  $\text{mg}^{-1}$  DW when fed rotifers containing 0.93 to 2.32 ng total retinol  $\text{mg}^{-1}$  DW (6 to 14 ng retinyl palmitate  $\text{mg}^{-1}$  DW rotifers) and incorporated a lower level of  $5.57 \pm 0.30$  ng total retinol  $\text{mg}^{-1}$  DW when fed rotifers with 0.52 (control) or  $\geq 16.31$  ng total retinol  $\text{mg}^{-1}$  DW (0 and  $\geq 57$  ng retinyl palmitate  $\text{mg}^{-1}$  DW rotifers). Although this pattern of incorporation is unexpected, it is worth pointing out that the levels of retinyl palmitate used in the current study (6 to 14 ng retinyl palmitate  $\text{mg}^{-1}$  DW rotifers) and incorporation of the highest concentration of retinoids in the larval body, are the lowest levels examined on any marine larvae during the rotifer feeding period and early stages of development. Fernández et al. (2008) found that the lowest level of retinyl palmitate in the enriched rotifers was 67 ng retinyl palmitate  $\text{mg}^{-1}$  DW rotifers, which is 5 times higher than my lowest dose. It might be that with the very low doses of VA, the larvae metabolize and accumulate VA more efficiently than at the higher doses, where in the current study *L. Lineata* larvae incorporated double the concentration of retinoids when fed on rotifers incorporating  $\geq 26$  ng retinyl palmitate  $\text{mg}^{-1}$  DW than when fed on rotifers incorporating  $\geq 57$  ng retinyl palmitate  $\text{mg}^{-1}$  DW.

The apparent disconnection between VA concentrations in larval tissue and the rotifers on which they were fed remains unclear, further research will be needed to determine if this is a species specific issue, related to the method of rotifer enrichment or a masking effect of using two sources of enriched rotifers. One other potential reason may be regulation of proteins for metabolising VA. In a study on male rats, if there are sufficient retinoids provided and available to keep the animal in a non-deficient state, then the levels of CRPB (cellular retinol binding protein) are highly regulated and independent of the levels of retinol available in the tissue (Blaner et al., 1986). Recently, Darias et al. (2012) found that the amount of VA absorbed by Senegalese sole, *Solea senegalensis* larvae was controlled at the intestinal level to prevent excessive accumulation in the target tissues. Since VA metabolism is similar among vertebrate animals (Duester, 2000), this might explain why there is no significant difference (increase) in the amount of total retinol incorporated in the larval tissues with increasing VA levels in the higher treatments (rotifers > 53,823 IU VA kg DW) but does not explain why the incorporation levels were high in the larvae tissues with lower treatment.

A further possible explanation for the larvae incorporating higher retinoid levels in the low VA treatments than in the higher treatments may be the use of greenwater, which might have influenced the uptake of retinoids. *Nannochloropsis oculata* contains undetectable amounts of retinol (< 0.25 ng mg<sup>-1</sup>) but 290 ± 40 ng mg<sup>-1</sup> β-carotene (Brown et al., 1999). Atlantic halibut, *Hippoglossus hippoglossus*, Japanese parrotfish, *Oplegnathus fasciatus* and spotted parrotfish, *O. punctatus* were able to convert β-carotene in their diet into VA (Moren et al., 2002; Tachibana et al., 1997). *Latris lineata* larvae fed rotifers low in VA, potentially obtained additional VA from the carotenoids in the algae (either through direct ingestion, or via the rotifer), leading to an overall increase of the total VA. Larvae fed higher VA doses may not have metabolised and absorbed the alternative β-carotene substrate as efficiently, though the reason for this is not clear and this hypothesis would need to be proven by further investigation. Other studies investigating VA requirement in larval fish have not used greenwater, but it was used in the current study as it is the standard rearing regime for *L. lineata* (Battaglione and Cobcroft, 2007).

*Latris Lineata* larvae fed on the control treatment (0 ng retinyl palmitate mg<sup>-1</sup> DW rotifers), incorporated 4.92 ± 1.27 ng total retinol mg<sup>-1</sup> DW larvae, which was statistically equivalent to the total retinol incorporated by the larvae fed the highest four treatments (≥ 57 ng retinyl palmitate mg<sup>-1</sup> DW rotifers). Although, there was no retinyl palmitate added to the control emulsion, 0.29 ng mg<sup>-1</sup> DW total retinol was present (revealed by the biochemical analyses) due to the composition of other emulsion ingredients (tuna oil). This base level of total retinol in the control emulsion was reflected in the enriched rotifers, where they had no retinyl palmitate incorporated in their body, but 2.41 and 4.53 ng mg<sup>-1</sup> DW retinol and retinoic acid, respectively were detected. The amount of retinoic acid identified in the control treatment was not significantly different from all the other treatments. This suggests that its source was the base emulsion ingredients. Consequently, larvae fed on rotifers enriched with the control treatment obtained their VA as retinol and RA with no retinyl palmitate. In contrast, larvae from the other treatments were fed on rotifers incorporating mainly retinyl palmitate. The incorporation of total retinol in the larvae fed the control treatment was equivalent to larvae fed on the high retinyl palmitate treatments, likely because the VA form in the control was different and apparently efficiently metabolized and accumulated.

There was no sign of toxicity (hypervitaminosis) in terms of mass mortality of *L. lineata* during the experiment and neither growth in length nor weight or survival were significantly affected. Similar larval performance results to the current study, with no effect of dietary VA, were reported for, *S. senegalensis* and summer flounder, *Paralichthys dentatus* (Fernández et al., 2009; Martinez et al., 2007). In contrast, VA levels in the diet affected both growth and survival of gilthead sea bream, *Sparus aurata*, Tilapia, *Oreochromis niloticus* and red sea bream, *Chrysophrys major* (Fernández et al., 2008; Hernandez-H et al., 2006; Saleh et al., 1995). While in Japanese flounder, *Paralichthys olivaceus*, European sea bass larvae, *Dicentrarchus labrax* and Atlantic halibut, *Hippoglossus hippoglossus* survival was not affected by VA levels but growth in length was affected (Dedi et al., 1995; Mazurais et al., 2009; Moren et al., 2004b). The difference in the results among species highlights the species-specific nature of the effects of VA.

Jaw malformations are a major concern in the culture of *L. lineata* (Battaglione and Cobcroft, 2007; Cobcroft and Battaglione, 2009; Cobcroft et al., 2001a), and the current study found that early dietary intake of retinyl palmitate (VA) did not influence the severity of jaw malformation or alter the onset. Larvae were examined for jaw malformation throughout the experiment, and its severity increased with larval age, to 81% with commercially important jaw malformation by 43 dph. This result concurs with Martinez et al. (2007), where the overall frequency and severity of jaw defects (42% slight and 54% severe) increased in *P. dentatus* larvae when exposed to different VA doses (retinoic acid) for 10 days, but no dose response was observed. The result also agrees with Fernández et al. (2009), where increasing levels of retinyl palmitate fed to *Solea senegalensis* larvae from 6 to 27 dph did not cause skeletal malformations in the jaw apparatus or neurocranium. Although, the lack of any detectable VA effect on jaw malformations is in contrast to reports for other marine finfish species. Studies by Fernández et al. (2008); Haga et al. (2002b); Mazurais et al. (2009); Suzuki et al. (2000) and Villeneuve et al. (2005) on *P. olivaceus*, *D. labrax* and *S. aurata* larvae demonstrated that VA exerts strong effects on the formation of different skeletal elements including the jaw and different mouth parts. Despite the significant incorporation of VA in *L. lineata* larvae at 16 dph, jaw malformation was not affected at any stage through the experiment. This result supports the hypothesis that the jaw malformation in *L. lineata* is mainly due to physical factors.

Jaw malformation in *L. lineata* has previously been correlated with walling behaviour (Cobcroft and Battaglione, 2009), and this behaviour was recorded across all treatments in the current study, although, the number of larvae walling per tank was not assessed. Walling behaviour is affected by the larval rearing environment (e.g. tank colour and greenwater) (Cobcroft and Battaglione, 2009) and it is likely that the tank size and stocking density at first feeding in the current study increased walling behaviour (hard-surface interaction) and jaw malformation. Cobcroft and Battaglione (2009) found *L. lineata* post-larvae had an average of 60% normal jaws (score 0) on 44 dph compared with an average of 5% normal jaws (score 0) in the current study on 43 dph. The difference between the

two studies can be attributed to different stocking density and age of stocking into the small tanks. In the current study, yolk-sac larvae were stocked at 1 dph into 300 l tanks at a stocking density of 10 larvae l<sup>-1</sup>, while in Cobcroft and Battaglione (2009), 2 dph yolk-sac larvae were stocked into 3,000 l tanks at a density of 7.5 larvae l<sup>-1</sup> and transferred to the 300 l tanks on 16 dph at a density of 1.7 larvae l<sup>-1</sup>. In smaller tanks, the wall-surface to water volume ratio was much higher than in the large tanks, which increases the chance of hard surface interaction. The increased interaction between individual larvae at higher stocking density may also cause more escape responses that increase the chance of swimming into and staying associated with the wall. Consequently, stocking yolk-sac larvae in 300 l tanks on 1 dph in the current study, increased the number of days that larvae had an increased chance of hard-surface interaction compared with the Cobcroft and Battaglione (2009) study, and may be why I observed higher jaw malformation. The approach in the current study could not be avoided, due to the logistical requirement to run replicated diet treatments in small tanks.

Skeletal malformations in the vertebral column of *L. lineata* post-larvae were affected by increasing VA levels in the enriched rotifers and the subsequent incorporation of VA into the larval body. However, the mean number of vertebra in *L. lineata* post-larvae was not affected by dietary VA. Total retinol content of *L. lineata* at 16 dph was correlated with the prevalence of vertebral malformations in 43 dph post-larvae, suggesting an effect of VA accumulation on subsequent skeletal development. However, malformations in the vertebral column of *L. lineata* significantly decreased with increasing dietary VA levels but not for a specific vertebral region.

By the end of the study, an average of 69% of the larvae which were fed on rotifers incorporating retinyl palmitate  $\geq 108.56 \text{ ng mg}^{-1} \text{ DW}$  ( $\geq 173,696 \text{ IU VA kg}^{-1}$ ), had normal vertebral column development compared with 49% for those fed on rotifers incorporating retinyl palmitate between 6.45 and 26.33  $\text{ ng mg}^{-1}$  (between 10,320 and 42,128  $\text{ IU VA kg}^{-1}$ ). Fernández et al. (2008) found a similar pattern where the percentages of vertebral malformations were significantly lower in higher VA treatments. Vertebral compression, fusion and malformation in at least one vertebral centrum in

*S. aurata* was significantly lower in larvae fed on rotifers incorporating retinyl palmitate  $\geq 139 \text{ ng mg}^{-1} \text{ DW}$  ( $\geq 222,569 \text{ IU VA kg}^{-1}$ ) than those fed on rotifers incorporating  $100 \text{ ng retinyl palmitate mg}^{-1} \text{ DW}$  ( $160,480 \text{ IU VA kg}^{-1}$ ) (Fernández et al., 2008). Studies of other species have found different trends. For example, for *S. senegalensis*, *P. olivaceus* and, *C. major* larvae, high VA levels significantly increased the vertebral malformations (Fernández et al., 2009; Hernandez-H et al., 2006; Tarui et al 2006).

Fernández et al. (2009) and Villeneuve et al. (2005) found that increasing VA concentration in the diet or enriched live prey was correlated or reflected in the larval body content of *S. senegalensis* and *D. Labrax* larvae, effecting the subsequent skeletal development. Though VA concentration in the larvae was significantly different for the higher treatments only;  $646,800$  and  $13,333,000 \text{ IU VA kg}^{-1} \text{ DW}$  extracted diet and experimental emulsion, respectively. In the current study, the correlation between the total retinol content in 16 dph larvae and the prevalence of vertebral column malformations observed in 43 dph explains why the vertebral malformations were reduced in higher treatments, where the pattern of increasing VA in the enriched rotifers was not reflected in the larvae fed those rotifers. *Latris lineata* larvae at 16 dph incorporated higher amounts of total retinol ( $11.08 \text{ ng mg}^{-1} \text{ DW}$ ;  $36,564 \text{ IU VA kg}^{-1}$ ) when fed rotifers containing  $0.93$  to  $2.32 \text{ ng total retinol mg DW}$  ( $3,069$  to  $7,656 \text{ IU VA kg}^{-1} \text{ DW rotifers}$ ) and incorporated lesser amount of total retinol ( $5.57 \text{ ng mg}^{-1} \text{ DW}$ ;  $18,381 \text{ IU VA kg}^{-1}$ ) when fed rotifers  $> 16.31 \text{ ng total retinol mg}^{-1} \text{ DW}$  ( $> 53,823 \text{ IU VA kg}^{-1} \text{ DW rotifers}$ ). This suggests that the prevalence of skeletal malformations at 43 dph post-larvae were less in the higher treatments because the 16 dph larvae were incorporating less total retinol.

### 3.5 Conclusion

The pattern of increasing VA in the enriched rotifers was not reflected in the larvae, although skeletal malformations in post-larvae at 43 dph were correlated to larval retinoid content at 16 dph. Larvae were reared in greenwater and it is likely that the carotenoids in the live algae consumed by the rotifers were metabolized either in the rotifer and/or the larval body into further VA

compounds. Increasing dietary levels of retinyl palmitate during the rotifer feeding period did not affect *L. lineata* growth, survival or degree of jaw malformation. Further research is needed to determine the effect of greenwater on rotifer VA content and consequently on the larvae they are fed to (Chapter 4). Further research is also required to examine the effect of VA during later stages of *L. lineata* development (*Artemia* feeding period) (Chapter 5).

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## **Chapter 4**

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**Effect of dietary vitamin A level in rotifers and rearing in the presence and absence of greenwater on the performance and skeletal abnormality of Striped Trumpeter *Latris lineata* larvae and post-larvae**

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## 4.1 Abstract

This Chapter investigated both the effect of dietary vitamin A (VA), previously linked with the appearance of skeletal malformations in marine fish and the addition of greenwater microalgae, *Nannochloropsis oculata* to the culture tanks of Striped Trumpeter (*Latris lineata*) larvae. *Latris lineata* larvae were fed in the morning (09:00) with rotifers enriched with 3 doses of retinyl palmitate, 0 (control), 3,000 and 10,000 mg<sup>-1</sup> l retinyl palmitate, and in the afternoon (17:00) with rotifers enriched with Algamac-3050. Larvae were reared during the rotifer feeding period in the absence or presence of microalgae from 6 to 18 days post hatch (dph). After the rotifer feeding period, the larvae were fed Algamac-3050-enriched *Artemia* until 43 dph. By 18 dph, the retinoid content of *L. lineata* was positively correlated with the enriched rotifers they fed on, while larval uptake of retinoids was not influenced by the greenwater. Generally, larvae reared in greenwater grew longer, heavier and were more developed at the same age (15.84 ± 0.41 mm and 4.14 ± 0.27mg at 43 dph) compared with those reared in clearwater (14.39 ± 0.64 mm and 2.99 ± 0.49 mg at 43 dph), while survival was significantly higher in clearwater (64.9 ± 17.0 and 52.8 ± 5.6% in clear and greenwater, respectively). Dietary VA had no effect on growth, survival and the prevalence of skeletal malformations. The severity and prevalence of jaw malformations in 43 dph post-larvae was significantly lower in greenwater compared with clearwater, while the absence or presence of greenwater did not significantly affect the prevalence of vertebral column malformation in *L. lineata*.

## 4.2 Introduction

Skeletal malformations are frequently observed in hatchery reared commercially important species such as European Sea Bass, *Dicentrarchus labrax*, Red Sea Bream, *Pagrus major*, Gilthead Sea Bream, *Sparus aurata*, Japanese Flounder, *Paralichthys olivaceus*, Senegalese Sole, *Solea senegalensis*, Seven-Band Grouper, *Epinephelus septemfasciatus* and Dusky Grouper, *Epinephelus marginatus* (Barahona-Fernandes, 1982; Boglione et al., 2001; Foscarini, 1988; Gavaia et al., 2009; Glamuzina et

al., 1998; Hosoya and Kawamura, 1991; Nagano et al., 2007; Tomoda et al., 2006). The prevalence of skeletal malformations varies among these species and it is reported to peak at 50 to 100% of variable severity skeletal malformations (Barahona-Fernandes, 1982; Boglione et al., 2001, 2009; Gavaia et al., 2009; Hosoya and Kawamura, 1991; Matsuoka, 1987; Nagano et al., 2007). Skeletal malformation has caused severe economic losses in the aquaculture industry due to the reduced market value of the malformed fish (Boglione et al., 2001; Hattori et al., 2003; Koumoundouros et al., 1997). The estimated loss due to malformed fish in European countries was > 50 million Euro in 2003-04 (Hough, 2009).

Marine fish larvae are immature when they hatch compared with other vertebrates where organ differentiation starts during the yolk absorption period and further development and growth continue during the exotrophic period (Falk-Petersen, 2005; Haga et al., 2002, 2011). Skeletogenesis of marine fish larvae is affected by exogenous nutrients including vitamin A (VA) (Cahu et al., 2003; Lall and Lewis-McCrea, 2007). Imbalances in VA cause impaired bone and cartilage metabolism and growth retardation in fish (Lall and Lewis-McCrea, 2007; Weston et al., 2003; Wolbach, 1947).

Vitamin A, especially retinoic acid, induces the maturation of chondrocytes and activates the mineralization of the cartilage matrix in vertebrates including fish (Iwamoto et al., 1994; Vasan and Lash, 1975), and consequently it affects the replacement of cartilage with bones. Excess VA accelerates cartilage degeneration and accordingly, the rapid replacement with bone, leading to skeletal malformations in vertebrates (Dedi et al., 1995; Lall and Lewis-McCrea, 2007; Wolbach, 1947).

Striped Trumpeter, *Latris lineata* (Bloch and Schneider, 1801), has been selected as a new candidate for finfish sea cage aquaculture in the temperate regions of Australia to complement salmonid farming which is under threat from climate change (Battaglione et al., 2008; Battaglione and Cobcroft, 2007). One of the impediments to the expansion of commercial grow-out of *L. lineata* has been a high level of malformations in cultured fish. Intensive culture of *L. lineata* larvae and post-larvae has

resulted in a particularly high prevalence of jaw malformation in juveniles (up to >95%) (Battaglione and Cobcroft, 2007, 2010; Cobcroft and Battaglione, 2009; Cobcroft et al., 2001b). Spinal malformation in *L. lineata* larvae or post-larvae has also been observed under intensive culture conditions (Battaglione and Cobcroft, 2010; Trotter et al., 2001).

Apart from the requirement for microalgae for enriching live prey such as *Artemia* and rotifers, microalgae are often used directly in the seawater when rearing marine fish larvae. This greenwater technique is commonly applied for rearing larvae of marine finfish, including *S. aurata*, *D. labrax*, groupers such as *E. marginatus* and Humpback Grouper, *Cromileptis altivelis*, Milkfish, *Chanos chanos*, Atlantic Halibut, *Hippoglossus hippoglossus*, California Yellowtail, *Seriola lalandi*, and Atlantic Cod, *Gadus morhua* (Boglione et al., 2009; Dimitrios et al., 2010; Marte, 2003; Naas et al., 1992; Papandroulakis et al., 2000; Shields, 2001; Skiftesvik et al., 2003; Stuart and Drawbridge, 2011). The advantages of adding microalgae to newly hatched marine larvae include maintenance of the nutritional value of the live feeds, production of beneficial substances by the microalgae that enhance the digestive system and increase immunity of the larvae, availability of beneficial bacteria associated with the microalgae cultures, changes in the light intensity penetration and hence the distribution and feeding behaviour of the larvae, and a reduction in the concentrations of ammonia and other harmful substances in the water (Cobcroft et al., 2001a; Makridis et al., 2010; Shaw, 2006). In *L. lineata* culture, adding microalgae also decreases wall nosing behaviour which is linked to jaw malformation (Battaglione and Cobcroft, 2007; Cobcroft and Battaglione, 2009).

In Chapter 3, the effect of increasing dietary VA (retinyl palmitate) on *L. lineata* larval performance parameters and skeletal development was examined. The larvae were fed VA-enriched rotifers and reared in greenwater by adding the microalga *Nannochloropsis oculata* from 6 to 18 dph. In that study, growth, survival and the prevalence of jaw malformation were not affected by increasing doses of dietary retinyl palmitate, while vertebral column malformation was correlated with VA levels detected in the larvae. The safe level of VA inclusion during the rotifer feeding period when *L.*

*lineata* larvae reared in greenwater was estimated at  $\geq 123$  ng total VA  $\text{mg}^{-1}$  dry weight (DW) rotifers (Chapter 3). However, the influence of greenwater on the effect of dietary VA on larval performance was unknown.

*Nannochloropsis oculata* contains very small amounts of retinol ( $< 0.25$  ng  $\text{mg}^{-1}$ ) and  $290 \pm 40$  ng  $\text{mg}^{-1}$   $\beta$ -carotene (Brown et al., 1999).  $\beta$ -carotene is a major dietary precursor of VA, with the nutritional equivalency accepted at 6  $\mu\text{g}$   $\beta$ -carotene equal to 1  $\mu\text{g}$  retinol (Ross and Ternus, 1993). The VA levels in VA-enriched rotifers held in green and clearwater over time are presented in Chapter 2. Vitamin A levels in enriched rotifers were found to increase over time when held in greenwater and decrease when they were held in clearwater. Rotifers held in greenwater were able to digest and metabolize the retinol and  $\beta$ -carotene in *N. oculata* and increase their total VA content. Some larval fish, for example, *H. hippoglossus*, Japanese parrotfish, *Oplegnathus fasciatus* and spotted parrotfish, *O. punctatus*, convert  $\beta$ -carotene in their diet into VA (Moren et al., 2002; Tachibana et al., 1997). This means that larvae reared in greenwater and fed rotifers enriched with VA will potentially obtain additional VA from the carotenoids in the algae (either through direct ingestion, or via the rotifers feeding on algae), leading to an overall increase of the total VA in the larval body tissues.

The aim of this chapter was to determine the effect of increasing dietary VA (retinyl palmitate) doses in rotifers on *L. lineata* larval performance parameters and skeletal development in the presence or absence of greenwater (microalgae). Larvae were reared in either greenwater or clearwater and fed from 6 to 19 days post hatch (dph) with rotifers enriched with three graduated concentrations of retinyl palmitate (0, 3,000 and 10,000 mg retinyl palmitate  $\text{l}^{-1}$ ). The larvae were then fed on *Artemia* until 43 dph to allow them to fully develop jaw and skeletal elements. Growth, survival and development of skeletal malformations were assessed in larvae exposed to the different treatments.

## 4.3 Materials and methods

### 4.3.1 Eggs and stocking of larvae

*Latris lineata* gametes were obtained from naturally spawning broodstock held at the Institute for Marine and Antarctic Studies, Fisheries, Aquaculture and Coasts facility (IMAS-FAC), Hobart, under controlled light and ambient water temperature. Eggs stripped from one female were fertilised by mixing with sperm from three males. Immediately after fertilization, the embryos were disinfected in ozonated seawater, in order to prevent the transmission of pathogens (Battaglione and Morehead, 2006). The seawater used in the egg incubation and larval rearing systems was filtered at 20 µm and ozonated to  $\geq 700$  mV ORP for 10 min and then treated with UV at 254 nm. Water was then filtered with activated carbon before distribution to the tanks at 300-350 mV ORP.

The embryos were incubated in 250 l conical tanks at  $14.1 \pm 0.1$  °C (mean  $\pm$  SD, here and throughout). Incubators were flow-through at approximately  $150 \text{ l h}^{-1}$ , photoperiod 14 h L: 10 h D, salinity, pH and DO ranged from 33.7 to 34.0 ppt, 8.00 to 8.02 and 96.8 to 102.9%, respectively. The central screens were 250 µm. Paper towels were applied to the surface when necessary for the removal of any oil or debris. From 1 dph, the temperature was slowly increased at  $0.1$  °C  $\text{h}^{-1}$  to  $15.3 \pm 0.4$  °C. Fertilized eggs hatched after 5 days. Yolk sac larvae were stocked on 1 dph into 24 x 300 l tanks at  $10 \text{ larvae l}^{-1}$ .

### 4.3.2 Experiment design

*Latris lineata* larvae were fed daily at 09:00 from 6 to 19 dph with rotifers enriched with three retinyl palmitate (VA) emulsions prepared by Nutrakol Nutrition, WA Australia; 0 (control), 3,000 and 10,000 mg retinyl palmitate  $\text{l}^{-1}$  ( $1,600,000 \text{ IU g}^{-1}$ , MP Biomedicals, Australia). The emulsion base constituents included tuna oil 57%, vitamin E 4% and vitamin C 4% (Nutrakol). To ensure adequate lipid nutrition, which was not possible to achieve by the VA enrichment emulsions due to the short

enrichment period, larvae were also fed at 17:00 with Algamac-3050 (Aquafauna Biomarine, USA) enriched rotifers (Chapters 2 and 3). The experiment investigated the effect of dietary VA during the rotifer feeding period, with rearing in either green or clearwater, on *L. lineata* larval performance, growth, survival and skeletal malformation, using four replicate tanks per treatment.

Larvae were reared in either green or clearwater from 6 to 20 dph; live microalgae, *Nannochloropsis oculata*, was added to the 12 greenwater tanks to achieve a turbidity of 3 NTU ( $\sim 400,000$  cells  $\text{ml}^{-1}$ ). The same amount of seawater was added to the other 12 clearwater tanks. Live algae or seawater were acclimated to the larval rearing temperature by pumping them to an aerated reservoir tub the day before addition to the tanks. The algae or seawater was added at 08:30 each morning, just before the addition of the enriched rotifers.

### 4.3.3 Larval rearing conditions

Larvae were reared in marble coloured 300 l hemispherical tanks, from 1 dph until 43 dph. The temperature ranged from 15.1 to 16.3 °C, salinity from 33.2 to 34.1 ppt, pH from 7.95 to 8.11 and dissolved oxygen was maintained > 90 %. Photoperiod was 16 h L: 8 h D (lights on at 09:00 and off at 01:00) from stocking until 18 dph. The light period increased gradually to 18 h L: 6 h D on 19 dph, 22 h L: 2 h D on 20 dph and 24 h L from 21 dph onward to reduce downward nocturnal migration (Bransden et al., 2005a). The light intensity was  $6.44 \pm 0.84 \mu\text{mol s}^{-1}$  at the water surface, provided by a single 50 W halogen globe above each tank.

Tanks were static with no water exchange or aeration from 1 dph (stocking) until 5 dph. From 6 dph (first feeding), the incoming flow of seawater exchange was  $112.6 \pm 2.84 \text{ l h}^{-1}$  in 9 h from 23:00 to 08:00 during the dark period to flush out any uneaten live prey. Additional recirculation flow started on 20 dph until the end of the experiment, increasing the total flow to  $217 \pm 3.83 \text{ L h}^{-1}$  for 24 h. Each tank had a surface skimmer to remove the oily films from the water surface in order to facilitate initial swim bladder inflation (Trotter et al., 2005). The skimmers were applied from 8 dph until 14

dph. Water quality parameters were measured daily and tanks were siphoned daily to remove any detritus or dead larvae.

#### 4.3.4 Feeding rates

The larvae were fed enriched rotifers *Brachionus plicatilis* (Austria strain) at 5 ml<sup>-1</sup> twice daily at 09:00 and 17:00, starting from 6 dph until 17 dph. On 18 and 19 dph the 17:00 rotifer feed was replaced with enriched *Artemia* at 0.25 ml<sup>-1</sup>, which were also fed at 21:00. Enriched *Artemia* was added four times each day at 09:00, 13:00, 17:00 and 21:00 from 20 dph until the end of the experiment (43 dph). *Artemia* feeding rates were increased during the experiment to account for increasing prey consumption due to the high survival in most of the tanks. *Artemia* were added at 0.25 ml<sup>-1</sup> feed<sup>-1</sup> until 25 dph when the 09:00 feed was increased to 0.5 ml<sup>-1</sup> until the end of the experiment. From 32 dph the 13:00, 17:00 and 21:00 feeds were increased to 0.3 ml<sup>-1</sup> until 36 dph where they were further increased to 0.5 ml<sup>-1</sup> until the end of the experiment.

#### 4.3.5 Enrichment

Rotifers in culture were fed with commercial concentrated algae (*Nannochloropsis* sp, Reed Mariculture Inc. USA) and harvested every day. They were rinsed in seawater before being distributed into three 40 L cylindroconical tanks at 500 rotifers ml<sup>-1</sup> and 25 °C. Rotifers for the 09:00 feed were enriched with the experimental emulsions in complete darkness for 2 h using 0.11 g emulsion l<sup>-1</sup>. The required weight of each emulsion was blended for 3 min in tap water at 20 °C then screened through 63 µm mesh before adding it to the rotifers.

Rotifers for the 17:00 feed were enriched with Algamac-3050 based on preliminary enrichment trials (Chapter 2), to ensure adequate lipid nutrition (Brandsen et al., 2005b). Enrichment with Algamac-3050 was for 8 h in constant light at 2 g million<sup>-1</sup> rotifer. The Algamac-3050 was hydrated in 1 l tap water at 20°C for 10 min, before blending for 3 min, then screened as above and added to the rotifers. After enrichment with the emulsions or Algamac-3050, the rotifers were gently harvested

by siphoning, collected into 63 µm bag screens and rinsed for about 10 min in clean seawater. The rotifers were counted and volumetrically fed to larvae.

*Artemia* cysts (AAA, INVE Aquaculture Nutrition USA) were decapsulated and stored in brine at 4 °C until required (Sorgeloos et al., 1977). *Artemia* were hatched daily from 16 dph, enriched with Algamac-3050 the day after hatching at 26 °C under strong illumination and aeration. The 09:00 and 17:00 feeds were enriched for 16 h, while the 13:00 and 21:00 feeds were enriched for 20 h. After enrichment, *Artemia* were siphoned and collected into 150 µm screen bags, rinsed with freshwater and then seawater. The *Artemia* were counted and volumetrically fed to larvae.

### **4.3.6 Sampling**

Yolk sac larvae from the incubator (n = 20) on 1 and 5 dph were anaesthetised in 0.06% 2-phenoxyethanol. Their standard length was measured using an eyepiece graticule fitted to an Olympus SZ stereomicroscope and general condition of the larvae was assessed.

On 11, 18, 25, 36 and 43 dph, 20 larvae were siphoned from each tank prior to feed addition. These larvae were anaesthetised and examined with a stereomicroscope. They were scored for absence or presence of the gas-inflated swim bladder, feeding (retained food in the gut, an indication for poor digestion), general condition (overall appearance and colour of the body), grey gut (an indication of lipid deposition) (Bransden et al., 2005b), and the standard (SL) or total length (TL). On 18 and 43 dph, 50 larvae from each tank were sampled; only 20 larvae from each sampling day were scored as above and the entire 50 larvae were scored for jaw malformation. Jaw malformation identification was also examined in 20 larvae from each tank on 25 and 36 dph. Immediately after scoring, these larvae were placed on pre-weighed filter paper, rinsed with 15 ml 0.5 M ammonium formate, excess moisture was removed and the filters were re-weighed to determine larval wet weight. The filter papers were folded, placed in aluminium foil pockets, immediately frozen in liquid nitrogen, and

transferred within 4 h to storage in a -80 °C freezer. Afterwards, the samples were freeze-dried and re-weighed to determine the dry weight of the larvae.

On 43 dph, 20 post-larvae from each tank were fixed in 10% formaldehyde for later analysis of skeletal malformation. Whole larvae were cleared and stained for bone and cartilage using the method described by Taylor and VanDyke (1985). Recognition of jaw and skeletal malformations was done using the scoring system and morphological region identification, respectively, as described in Chapter 3.

To quantify the retinoid content in the larvae on 18 dph, at the end of the rotifer feeding period, 200 larvae were siphoned out of each tank, rinsed with 0.5 M ammonium formate, transferred to 5 ml vials and immediately stored in liquid nitrogen before transfer to a -80 °C freezer. These larvae were subsequently extracted and analysed by high performance liquid chromatography (HPLC) for total retinol (details below).

To quantify the retinoid content in the live feed, samples were taken from the enriched rotifers fed to larvae on 6 and 11 dph and *Artemia* on 21 and 33 dph, rinsed and stored as mentioned above. Enriched rotifers were also extracted for their lipid content (details below).

Final larval survival was determined by draining each tank onto a mesh screen to collect the post-larvae, anaesthetising the post-larvae and then counting them on white trays. Final survival was calculated using the following formula:

$$\text{Survival (\%)} = \frac{\text{total alive post-larvae on 43 dph}}{(\text{yolk sac larvae stocked on 1 dph} - \text{total live larvae removed from 11 to 42 dph})} * 100$$

Where removed = sampled + alive removed in daily siphoning.

### 4.3.7 Biochemical analysis

Retinoids were measured as total VA (the sum of retinyl palmitate, retinol and retinoic acid) and total retinol using two different techniques modified from Moren et al. (2002, 2004) and Takeuchi et al. (1998). Total VA and total retinol were measured in enrichment emulsions and live prey, while only total retinol was measured in 18 dph larvae due to the small volume of sample available. Total lipids and fatty acid methyl esters in live prey were determined using a modified version of Bligh and Dyer (1959). The extraction methods for VA and lipids were as described in Chapter 2. Live feed samples and larvae were not freeze-dried and appropriate calculations were made to convert to concentration per dry weight ( $\text{ng mg}^{-1}$  DW (dry weight)). Larvae, rotifer and *Artemia* dry weights were based on dry weight measurements for 18 dph larvae and different freeze-dried samples of live feed during this experiment, respectively.

### 4.3.8 Statistical analysis

Two way ANOVA was used to test the effect of both water type and vitamin doses on the larval performance parameters (length, dry weight, survival, jaw and vertebral column malformations). A two way ANOVA was also used to compare larval retinoid content in greenwater from the current Chapter at 18 dph with Chapter 3 larval content at 16 dph (fixed factors were: VA enriched rotifers, 3 nominal doses, and experiments, 2 levels). Since there were only 3 replicate tanks per treatment in Chapter 3 compared with 4 in the current study, the average of the larval VA content of the three tanks was used as the fourth replicate value to enable a balanced ANOVA ( $n=4$ ) (Sokal and Rolf, 1981). Mean percentage data were transformed by  $\arcsin \sqrt{x}$ , where  $x$  is the proportion, and the dry weight data was  $\log_{10}$  transformed to achieve homogeneity of variance and normal distribution. Levene's test was used to assess data for homogeneity of variance. Where significant treatment effects were found, a post-hoc Tukey test was used to determine differences among means. Correlation was used to test the association between survival and the prevalence of vertebral, and jaw malformations.

Significant differences were accepted at  $P < 0.05$ . Statistical analyses were performed using SPSS 13.0 (SPSS Inc.).

## **4.4 Results**

### **4.4.1 Biochemical analysis**

#### **4.4.1.1 Retinoid content in experimental emulsions, live prey and larvae**

Retinyl palmitate was the predominant form of VA in the emulsions but a small retinol peak was observed that was difficult to quantify as it was masked by other, higher concentration peaks. This retinol peak was due to the base emulsion ingredients (lipids base contained tuna oil 700 IU VA  $\text{kg}^{-1}$ , NuMega certificate of analyses) prior to retinyl palmitate inclusion. Retinol was quantified using the saponification process and expressed as total retinol, which is the retinyl palmitate hydrolysed into retinol plus the retinol from the emulsion ingredients.

Emulsions in the current study were designed nominally to contain 0, 3,000 and 10,000  $\text{mg l}^{-1}$  retinyl palmitate. Retinyl palmitate and total retinol content in the emulsions increased significantly with increasing addition of retinyl palmitate (Table 1). Retinyl palmitate concentration was 104.7 and 105.8% of the nominal concentration in the 3,000 and 10,000  $\text{mg l}^{-1}$  retinyl palmitate, respectively. The corresponding values for total retinol were 439 and 1,455  $\text{ng total retinol mg}^{-1}$  emulsion, and there was a detectable quantity of total retinol (0.34  $\text{ng mg}^{-1}$ ) in the 0 (control) emulsion.

Table 1: Retinyl palmitate and total retinol (saponification method) in experimental live prey enrichment emulsions. Values are mean  $\pm$  SD and n = 3. Different letters within the same column show significant differences among emulsions (ANOVA, P < 0.05).

Emulsion Nominal (mg retinyl palmitate l <sup>-1</sup> )	Retinyl palmitate ng mg <sup>-1</sup>		Total Retinol ng mg <sup>-1</sup>	
0	0.00	$\pm$ 0.00 <sup>a</sup>	0.34	$\pm$ 0.12 <sup>a</sup>
3000	3140.06	$\pm$ 86.37 <sup>b</sup>	438.51	$\pm$ 150.33 <sup>b</sup>
10000	10580.28	$\pm$ 273.67 <sup>c</sup>	1454.95	$\pm$ 28.98 <sup>c</sup>

In the enriched rotifers, average total VA and total retinol increased and were significantly different among different concentrations of retinyl palmitate in the emulsions (Table 2). Retinyl palmitate was the predominant VA form in the enriched rotifers with detectable amounts of retinol and retinoic acid. Average retinol content in the enriched rotifers increased significantly with increasing retinyl palmitate in the enriching emulsions while there was no significant difference in the retinoic acid content.

Retinyl palmitate was not detected in rotifers and *Artemia* enriched with Algamac-3050. However, after saponification of the samples, total retinol was  $0.55 \pm 0.06$  ng mg<sup>-1</sup> DW for rotifers enriched for 8 h and  $0.71 \pm 0.05$  and  $0.63 \pm 0.03$  ng mg<sup>-1</sup> DW for *Artemia* enriched for 16 and 20 h, respectively.

Table 2: Total VA content (retinyl palmitate, retinol and retinoic acid) and total retinol (saponification method)  $\text{ng mg}^{-1}$  in DW rotifers enriched with graded doses of retinyl palmitate. Values are mean  $\pm$  SD and  $n = 3$ . Different letters within the same column show significant differences among dietary groups (ANOVA,  $P < 0.05$ ).

Retinyl palmitate in emulsion ( $\text{ng mg}^{-1}$ )	Rotifer VA content ( $\text{ng mg}^{-1}$ DW)				Total Retinol (retinyl palmitate + retinol)
	Retinyl palmitate	Retinol	Retinoic acid	Total VA	
<b>0</b>	0.00 $\pm$ 0.00 <sup>a</sup>	2.96 $\pm$ 0.62 <sup>a</sup>	4.20 $\pm$ 0.88	7.17 $\pm$ 1.49 <sup>a</sup>	0.57 $\pm$ 0.12 <sup>a</sup>
<b>3140</b>	120.16 $\pm$ 10.49 <sup>b</sup>	14.20 $\pm$ 4.31 <sup>b</sup>	4.67 $\pm$ 1.25	139.04 $\pm$ 13.45 <sup>b</sup>	38.39 $\pm$ 6.17 <sup>b</sup>
<b>10580</b>	442.53 $\pm$ 50.97 <sup>c</sup>	44.79 $\pm$ 6.35 <sup>c</sup>	5.19 $\pm$ 1.06	492.52 $\pm$ 44.46 <sup>c</sup>	99.56 $\pm$ 6.78 <sup>c</sup>

Due to the small size of the larvae on 18 dph, larvae retinoid content was determined using the saponification technique only. By 18 dph, larval uptake of retinoids was not influenced by the water type, where there was no significant difference in the larvae total retinol content between the fish reared in clear or greenwater ( $F_{5,18} = 1.176$ ,  $P = 0.292$ ) (Fig. 1A). Average larvae total retinol increased with increasing dietary VA (Fig. 1B). Total retinol content was 2.5 times higher in larvae fed the highest VA treatment compared with control larvae ( $F_{5,18} = 8.186$ ,  $P = 0.003$ ). By 18 dph, the retinoid content of *L. lineata* was positively correlated with the enriched rotifers they fed on (Fig 2). There was no significant interaction between water type and rotifer enrichment on the larvae uptake of retinoids ( $F_{5,18}=0.489$ ,  $P = 0.621$ ).

In a comparison of the VA content of larvae reared in greenwater in this study at 18 dph with Chapter 3 larval retinoid content at 16 dph, there was no significant difference in the larvae retinoid content among VA levels (ANOVA,  $F_{5,18} = 2.34$ ,  $P = 0.125$ ) and there was a trend in an effect of experiment (ANOVA,  $F_{5,18} = 3.19$ ,  $P = 0.091$ ), with values in this Chapter lower (except for the 10,000  $\text{mg l}^{-1}$  retinyl palmitate emulsion), but not significantly different than the values in Chapter 3 (Fig. 3).

There was no significant effect of the interaction between VA level and experiment (ANOVA,  $F_{5,18} = 1.64$ ,  $P = 0.221$ ).

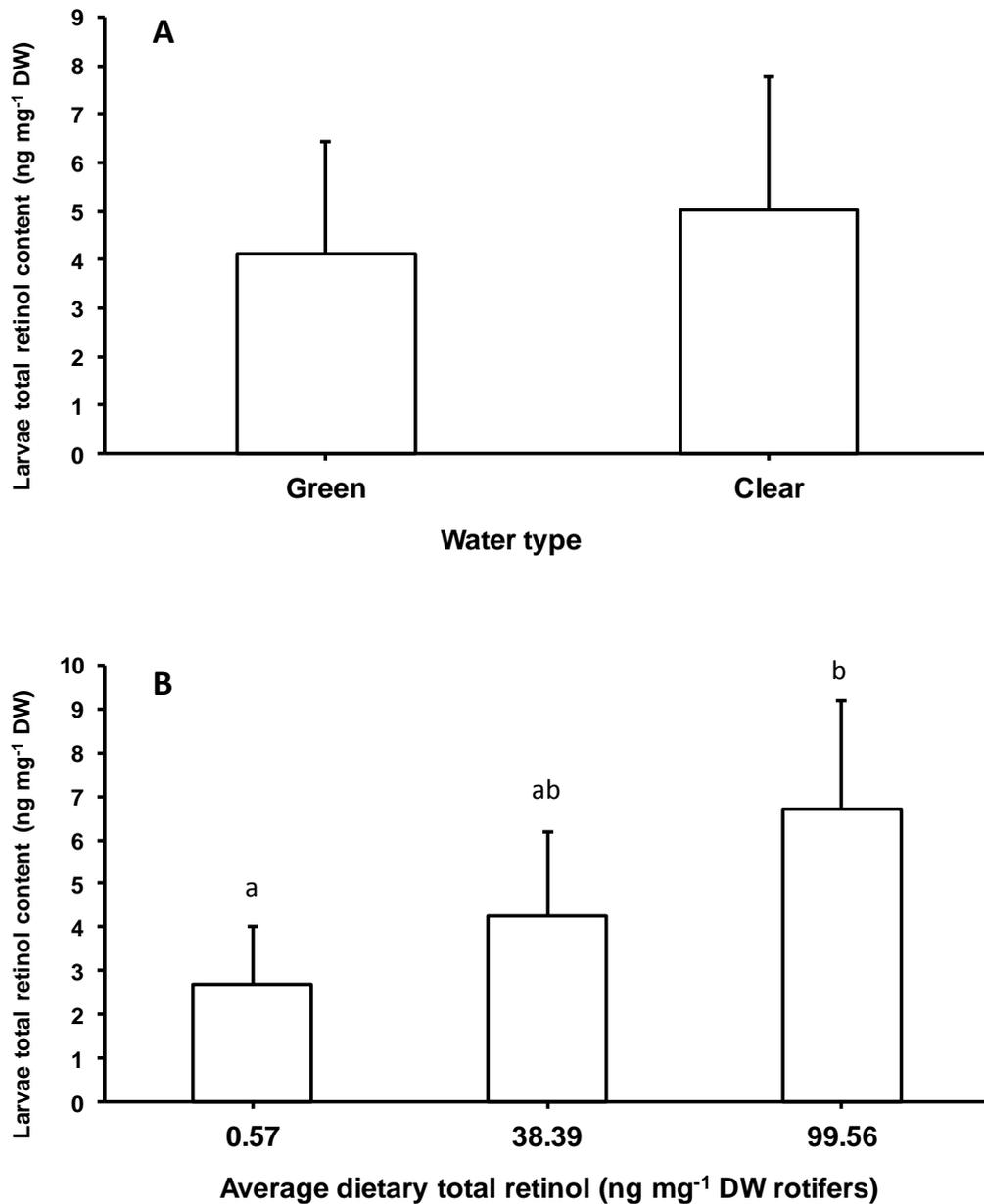


Figure 1: Total retinol in *Latris lineata* larvae at 18 dph fed rotifers enriched with graded doses of retinyl palmitate and reared in green or clearwater. A) according to water type,  $n = 12$  replicate tanks, and B) according to vitamin level,  $n = 8$  replicate tanks. Retinol content is expressed as ng mg<sup>-1</sup> DW larvae. Values are mean + SD. Histograms not sharing a common letter indicate significant differences among means (ANOVA,  $P < 0.05$ ).

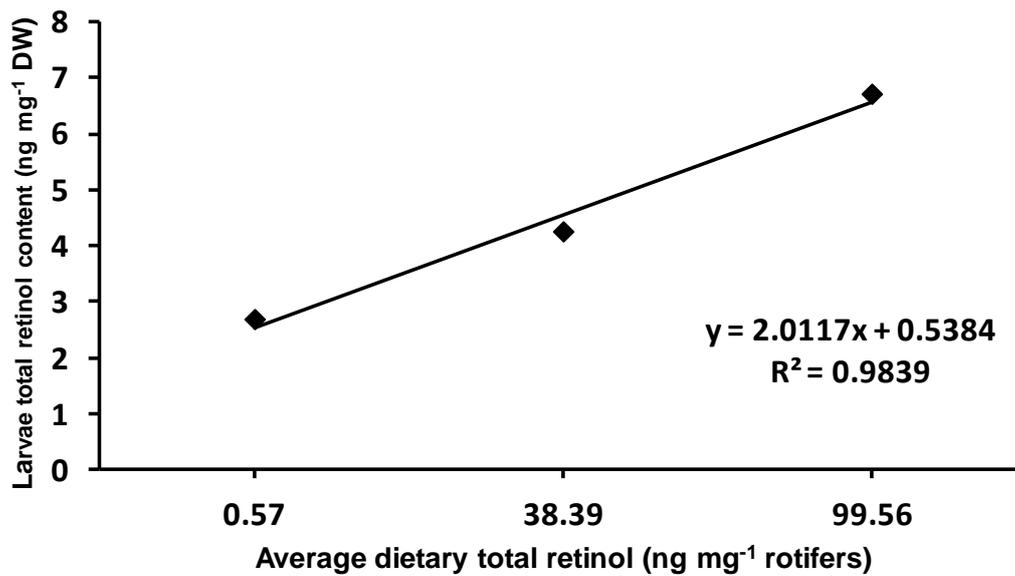


Figure 2: Linear correlation between larvae total retinol content (ng mg<sup>-1</sup> DW) at 18 dph and total retinol content of the rotifers they were fed on ( $R^2 = 0.98$ ,  $P = 0.005$ ).

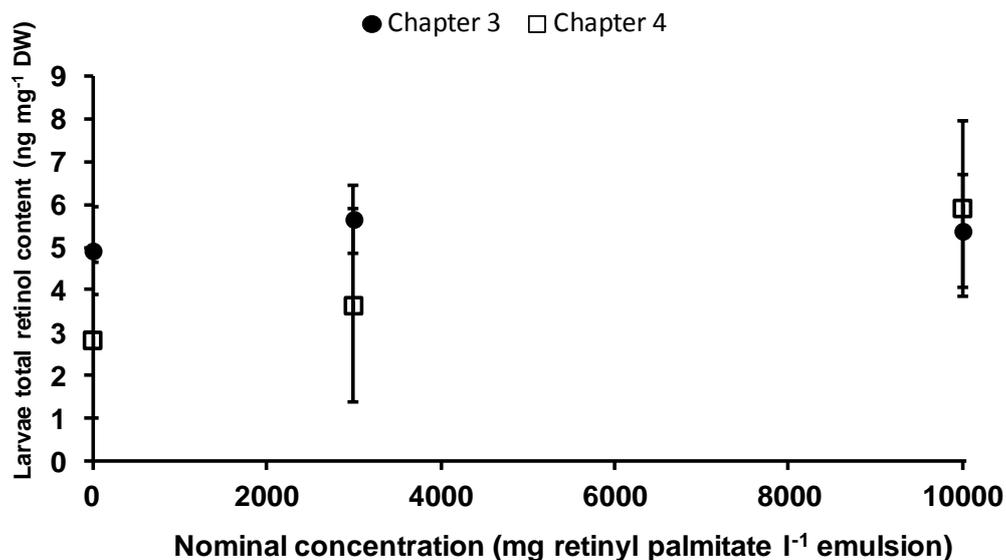


Figure 3: Comparison between larval retinoid content at 16 dph (Chapter 3) and 18 dph (Chapter 4) with dietary VA. Mean  $\pm$  SD,  $n = 4$ .

#### 4.4.1.2 Lipid content of live prey

Total fatty acids (FA) in rotifers enriched with Algamac-3050 were significantly higher than in rotifers enriched with 3,140 and 10,580 ng retinyl palmitate mg<sup>-1</sup> emulsions (Table 3) (ANOVA,  $F_{3,4} = 16.521$ ,

P = 0.010). While saturated fatty acids (SFA) were significantly higher in rotifers enriched with 0 ng retinyl palmitate mg<sup>-1</sup> emulsion than in rotifers enriched with 3,140 and 10,580 ng retinyl palmitate mg<sup>-1</sup> emulsion and Algamac-3050 (Table 3) (ANOVA,  $F_{3,4} = 14.549$ , P = 0.013). In addition, monounsaturated fatty acids (MUFA) were significantly higher in rotifers enriched with 0 ng retinyl palmitate mg<sup>-1</sup> emulsion than in rotifers enriched with 10,580 ng retinyl palmitate mg<sup>-1</sup> emulsion and Algamac-3050 (ANOVA,  $F_{3,4} = 166.65$ , P < 0.000). Docosahexaenoic acid (DHA, 22:6 $\omega$ 3) and arachidonic acid (ARA, 20:4 $\omega$ 6) were significantly higher in rotifers enriched with Algamac-3050 than the rotifers enriched with the VA emulsions (ANOVA,  $F_{3,4} = 281.39$  and 61.46 and P < 0.000 and 0.001, respectively), while there was no significant difference for the eicosapentaenoic acid (EPA, 20:5 $\omega$ 3).

Table 3: Selected fatty acid groups and polyunsaturated fatty acids in rotifers (mg g<sup>-1</sup> DW) enriched with 0, 3,140 and 10,580 ng mg<sup>-1</sup> retinyl palmitate for 2 h. Values are mean of 2 replicates  $\pm$  SD. SFA = saturated fatty acid, MUFA = monounsaturated fatty acid, PUFA = polyunsaturated fatty acid and FA = fatty acid. Different letters within the same row show significant differences among dietary groups (ANOVA, P < 0.05).

Enrichment (mg l <sup>-1</sup> retinyl palmitate)	0	3140	10580	Algamac-3050
<b>Total SFA</b>	22.07 $\pm$ 0.75 <sup>b</sup>	19.47 $\pm$ 0.25 <sup>a</sup>	19.43 $\pm$ 0.44 <sup>a</sup>	19.01 $\pm$ 0.66 <sup>a</sup>
<b>Total MUFA</b>	27.80 $\pm$ 1.02 <sup>c</sup>	25.05 $\pm$ 0.64 <sup>bc</sup>	24.44 $\pm$ 1.03 <sup>b</sup>	15.53 $\pm$ 0.54 <sup>a</sup>
<b>Total n:<math>\omega</math>6</b>	9.25 $\pm$ 0.35 <sup>a</sup>	8.41 $\pm$ 0.22 <sup>a</sup>	8.30 $\pm$ 0.32 <sup>a</sup>	18.09 $\pm$ 0.62 <sup>b</sup>
<b>Total n:<math>\omega</math>3</b>	26.18 $\pm$ 1.03 <sup>a</sup>	23.34 $\pm$ 0.91 <sup>a</sup>	22.92 $\pm$ 1.27 <sup>a</sup>	41.30 $\pm$ 1.42 <sup>b</sup>
<b>Total PUFA</b>	35.43 $\pm$ 1.38 <sup>a</sup>	31.75 $\pm$ 1.13 <sup>a</sup>	31.22 $\pm$ 1.59 <sup>a</sup>	59.39 $\pm$ 2.05 <sup>b</sup>
<b>Fatty acid (mg g<sup>-1</sup> DW)</b>				
<b>20:4<math>\omega</math>6</b>	2.31 $\pm$ 0.09 <sup>a</sup>	2.07 $\pm$ 0.08 <sup>a</sup>	2.02 $\pm$ 0.08 <sup>a</sup>	3.21 $\pm$ 0.11 <sup>b</sup>
<b>20:5<math>\omega</math>3</b>	10.42 $\pm$ 0.27 <sup>a</sup>	9.40 $\pm$ 0.38 <sup>a</sup>	9.26 $\pm$ 0.45 <sup>a</sup>	8.93 $\pm$ 0.31 <sup>a</sup>
<b>22:6<math>\omega</math>3</b>	10.22 $\pm$ 0.51 <sup>a</sup>	8.93 $\pm$ 0.37 <sup>a</sup>	8.70 $\pm$ 0.50 <sup>a</sup>	27.60 $\pm$ 0.95 <sup>b</sup>
<b>Total FA (mg g<sup>-1</sup> DW)</b>	88.74 $\pm$ 3.31 <sup>bc</sup>	79.60 $\pm$ 0.98 <sup>ab</sup>	78.26 $\pm$ 2.43 <sup>a</sup>	93.93 $\pm$ 3.24 <sup>c</sup>
<b>22:6<math>\omega</math>3/ 20:5<math>\omega</math>3</b>	0.98 $\pm$ 1.86 <sup>a</sup>	0.95 $\pm$ 0.97 <sup>a</sup>	0.94 $\pm$ 1.10 <sup>a</sup>	3.17 $\pm$ 0.11 <sup>b</sup>

## 4.4.2 Larval growth and survival

### 4.4.2.1 Larval development

There was no significant interaction between water type and rotifer enrichment with different retinyl palmitate doses on larval growth in length or weight (Table 4). Overall, greenwater reared larvae grew longer and heavier than larvae reared in clearwater (Figs. 4 and 5). Differences in growth were apparent at the end of rotifer feeding 18 dph, when larvae reared in greenwater averaged  $7.93 \pm 0.85$  mm and  $0.43 \pm 0.09$  mg, significantly longer and heavier than larvae reared in clearwater,  $7.38 \pm 0.18$  mm and  $0.33 \pm 0.04$  mg, respectively (Figs 4A and 5A). Differences in growth became more apparent after *Artemia* feeding when greenwater reared larvae were significantly longer  $15.84 \pm 0.41$  mm and heavier  $4.14 \pm 0.27$  mg than those reared in clearwater,  $14.39 \pm 0.64$  mm and  $2.99 \pm 0.49$  mg, respectively. Rotifer enrichment with different retinyl palmitate doses had no significant effect on growth of post-larvae, either in length or weight by 43 dph, where the average was  $15.11 \pm 0.91$  mm and  $3.56 \pm 0.70$  mg across treatments, respectively (Table 4, Figs 4B and 5B).

There was no significant interaction between water type and rotifer enrichment with different retinyl palmitate doses on larval survival by 43 dph (Table 4). By the end of the study, survival of the post-larvae reared in clearwater was significantly higher ( $64.9 \pm 17.0\%$ ) compared with the post-larvae reared in greenwater ( $52.8 \pm 5.6\%$ ) (Fig. 6A). Different retinyl palmitate treatments did not affect larval survival by 43 dph, average  $58.8 \pm 13.8\%$  across treatments (Table 4 and Fig 6B).

Table 4: Results of two way analysis of variance on larval growth in length and weight and survival during different days post hatch (dph). Water type, green or clear, and different enrichment doses of retinyl palmitate 0, 3,000 or 10,000 mg L<sup>-1</sup> are fixed factors. \* indicates significant effect (P < 0.05).

	dph	Source of variation	F value	P value
<b>Growth in length</b>	<b>11</b>	Vitamin treatment	1.398	0.273
		Water type	127.907	0.000*
		Vitamin treatment x water type	0.230	0.797
	<b>18</b>	Vitamin treatment	1.288	0.300
		Water type	89.040	0.000*
		Vitamin treatment x water type	0.228	0.798
	<b>25</b>	Vitamin treatment	1.359	0.282
		Water type	48.147	0.000*
		Vitamin treatment x water type	0.279	0.760
	<b>36</b>	Vitamin treatment	1.004	0.386
		Water type	30.206	0.000*
		Vitamin treatment x water type	0.650	0.534
	<b>43</b>	Vitamin treatment	1.120	0.348
		Water type	42.891	0.000*
		Vitamin treatment x water type	0.748	0.487
<b>Growth in weight</b>	<b>11</b>	Vitamin treatment	1.185	0.329
		Water type	24.736	0.000*
		Vitamin treatment x water type	2.992	0.076
	<b>18</b>	Vitamin treatment	2.421	0.117
		Water type	16.996	0.001*
		Vitamin treatment x water type	1.165	0.334
	<b>25</b>	Vitamin treatment	3.163	0.066
		Water type	25.048	0.000*
		Vitamin treatment x water type	1.035	0.375
	<b>36</b>	Vitamin treatment	2.381	0.121
		Water type	19.102	0.000*
		Vitamin treatment x water type	0.796	0.466
	<b>43</b>	Vitamin treatment	2.755	0.090
		Water type	58.927	0.000*
		Vitamin treatment x water type	0.948	0.406
<b>Survival</b>	<b>43</b>	Vitamin treatment	0.347	0.711
		Water type	5.296	0.034*
		Vitamin treatment x water type	0.861	0.439

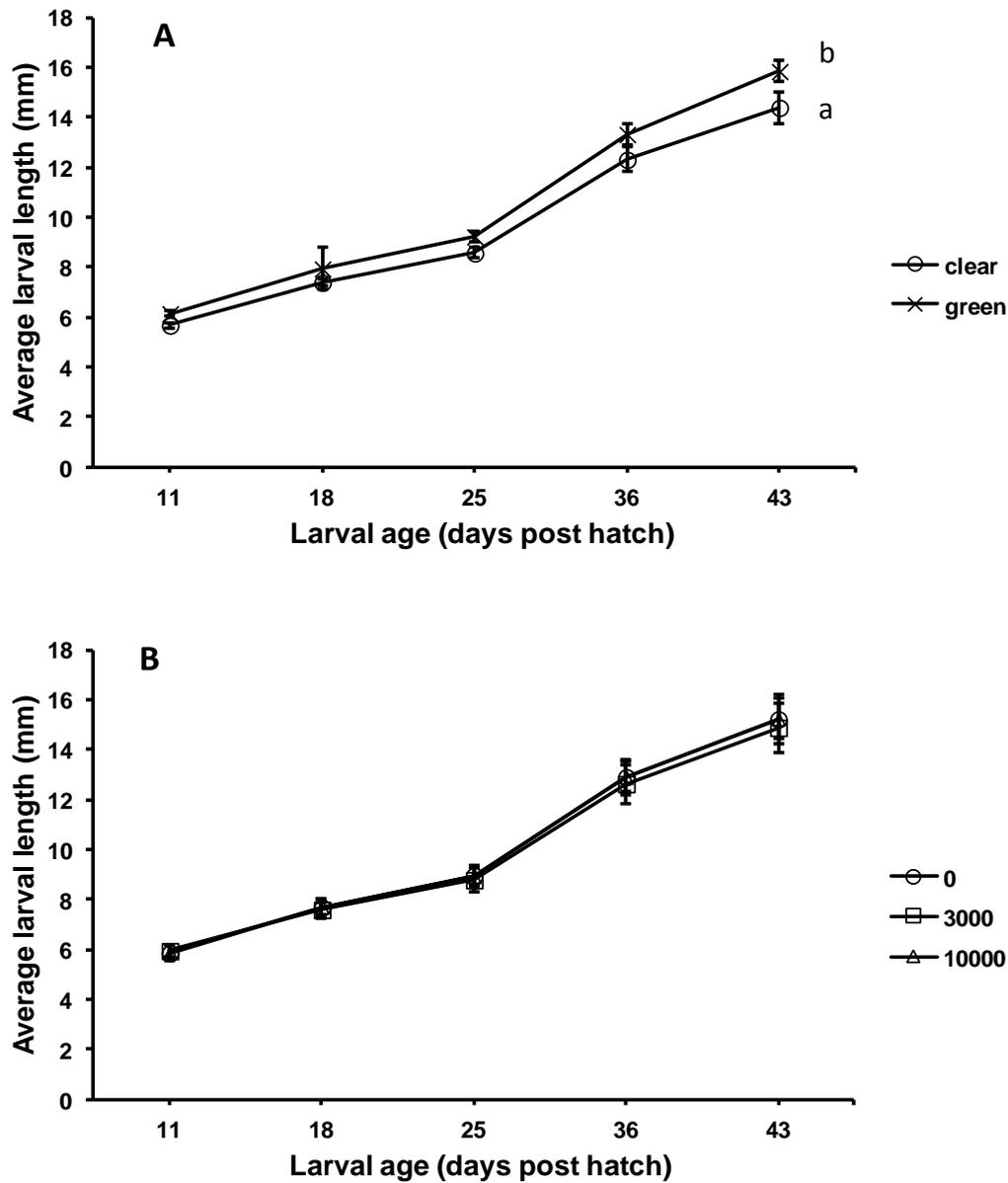


Figure 4: Larval growth in standard length (SL) for 11, 18 and 25 dph, total length (TL) for 36 and 43 dph of *Latris lineata* larvae and post-larvae fed rotifers enriched with graded doses of retinyl palmitate and reared in green or clearwater, A) according to water type (12 tanks per treatment), and B) according to vitamin level (eight tanks per treatment). Values are mean  $\pm$  SD ( $n = 20$  larvae per tank for 11, 18, 25, 36 and 43 dph). Different letters indicate significant differences among means (ANOVA,  $P < 0.05$ ).

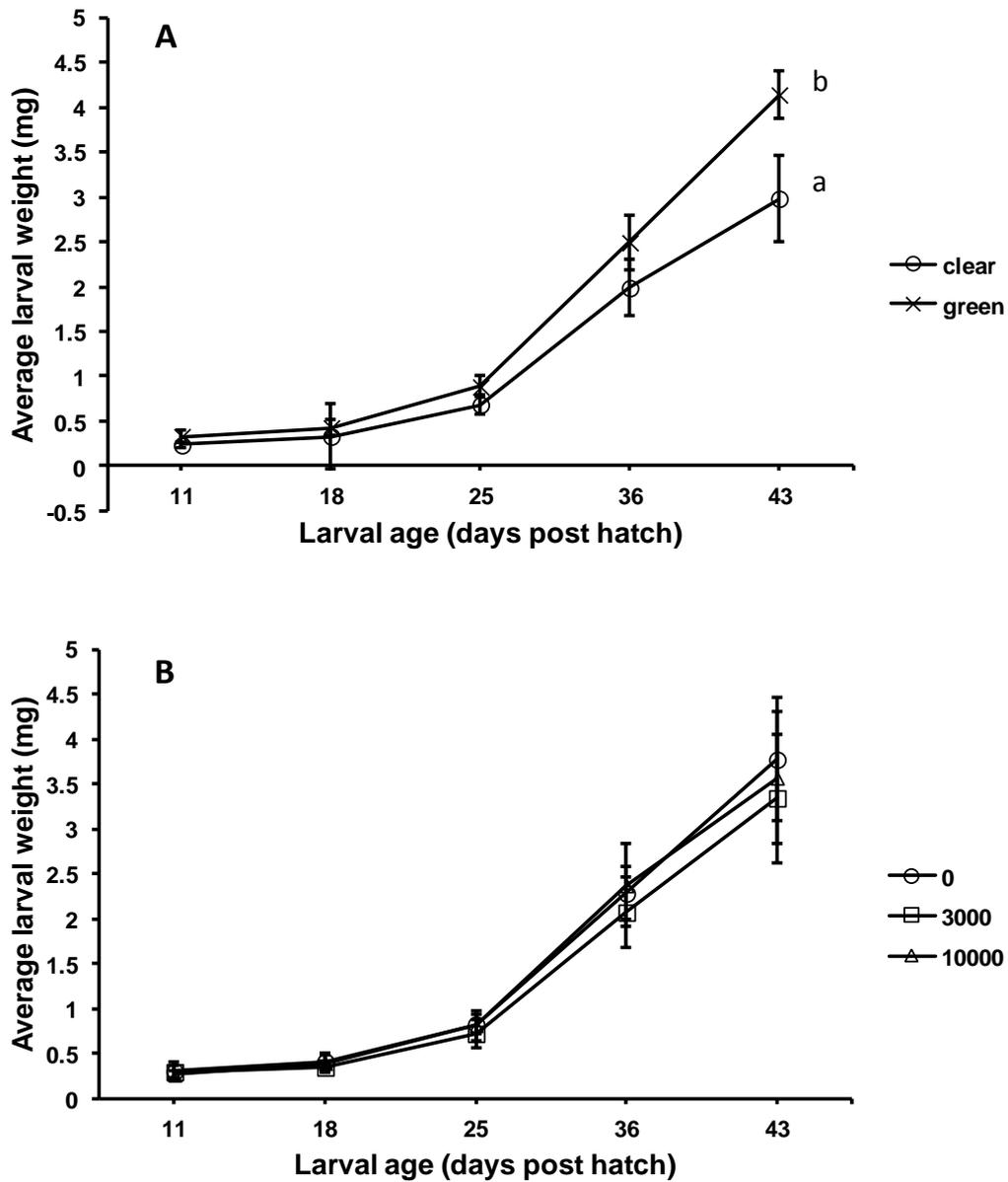


Figure 5: Increase in dry weight of *Latris lineata* larvae and post-larvae fed rotifers enriched with graded doses of retinyl palmitate and reared in green or clearwater, A) according to water type, n = 12 replicate tanks, and B) according to vitamin level, n = 8 replicate tanks. Values are mean  $\pm$  SD. Different letters indicate significant differences among means (ANOVA,  $P < 0.05$ ).

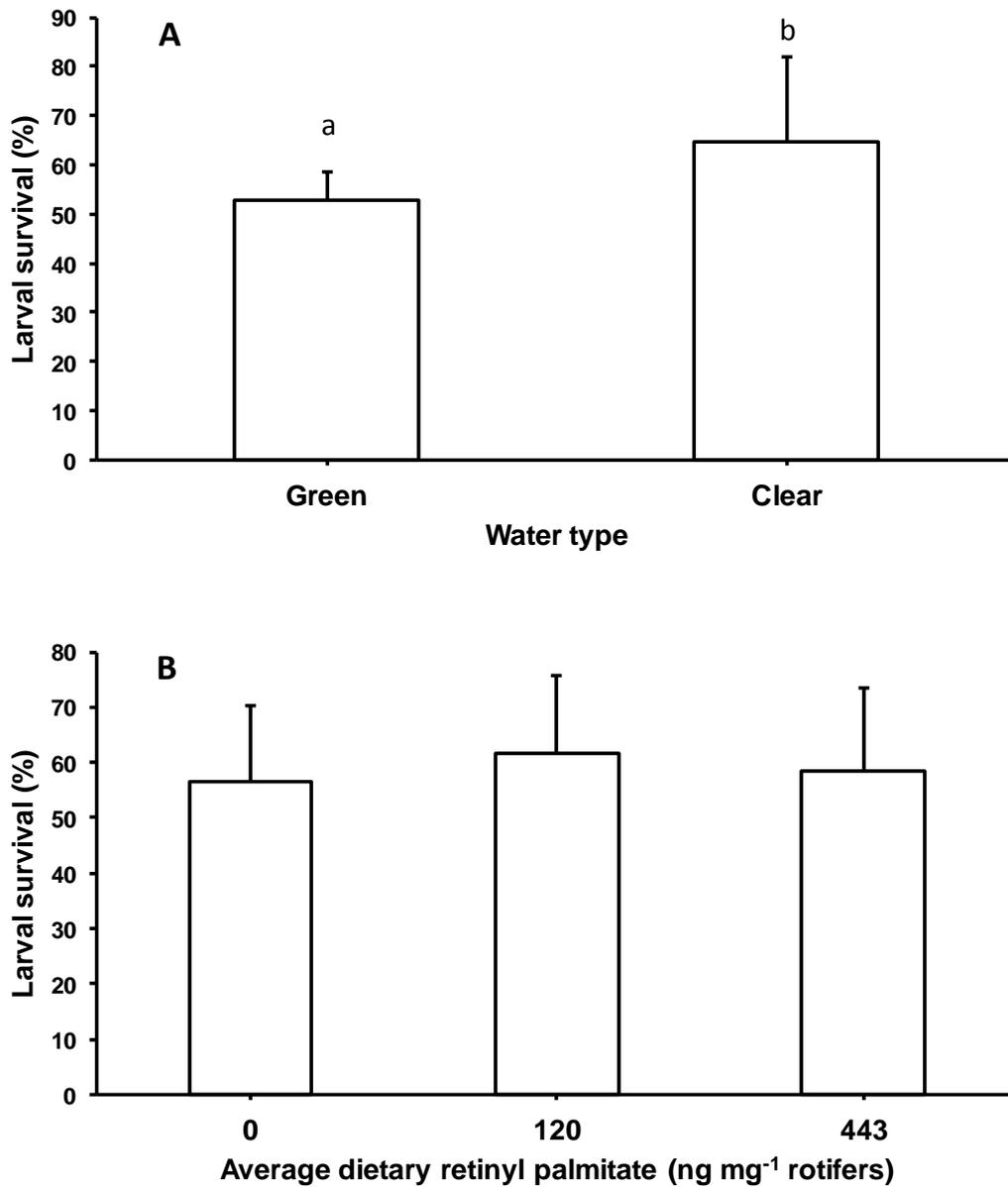


Figure 6: Survival of *Latris lineata* post-larvae at 43 dph fed rotifers enriched with graded doses of retinyl palmitate and reared in green or clearwater, A) according to water type, n = 12 replicate tanks, and B) according to vitamin level, n = 8 replicate tanks. Values are mean + SD. Different letters indicate significant differences among means (ANOVA, P < 0.05).

#### 4.4.2.2 Jaw malformation

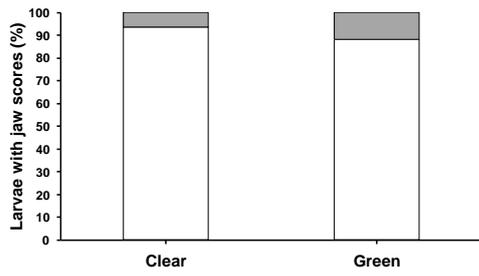
The different types of jaw malformations observed were very similar to those described in Chapter 3. Larvae displaying more than one malformation were scored 2 or 3. Malformations appeared around 11 dph, and then the prevalence and severity of malformations continued to increase until the end of the experiment. Water type significantly affected the prevalence, severity and types of jaw malformations while different dietary retinyl palmitate doses did not affect jaw malformations (Table 5 and Fig. 7). In younger larvae, jaw malformation was significantly higher in larvae reared in greenwater than those reared in clearwater. Lowered hyoid arch on 11 and 18 dph and different types of lower jaw malformations on 25 dph were significantly higher in larvae reared in greenwater (Table 5). As larvae grew older, on 36 and 43 dph, larvae with open jaws were significantly greater in clear than in greenwater (Table 5). Open jaws and different lower jaw malformations were the most common types of malformations by 43 dph, when open jaws were significantly higher in clearwater and different lower jaw malformations were significantly higher in greenwater (Table 5). By the end of the study, jaw malformation severity (score 3) was significantly lower in greenwater (Fig 7A). There was no significant interaction between water type and rotifer enrichment with different retinyl palmitate doses in the prevalence, severity and types of jaw malformation in *L. lineata*. There was a significant correlation between larval survival and jaw malformation, where higher survival was associated with lower jaw malformations ( $R^2 = 0.48$ ,  $P = 0.013$ ).

Table 5: Mean percentage of different types and time of occurrence (dph) of jaw malformations with respect to the water type (CL = clearwater; GR = greenwater) in *Latris lineata* larvae and post-larvae fed rotifers enriched with graded doses of retinyl palmitate and reared in green or clearwater. 12 replicate tanks per treatment, 20 larvae per tank on 11, 25 and 36 dph and 50 larvae per tank on 18 and 43 dph. Values are mean  $\pm$  SD. Different letters within the same column indicate significant differences among means within days (ANOVA,  $P < 0.05$ ).

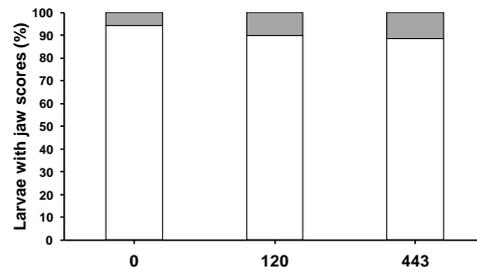
Larval age	Prevalence of different jaw malformations (%)			
	Lowered hyoid arch	Open	Lower jaw	Upper jaw
<b>11 dph</b>				
CL	3 $\pm$ 3 <sup>a</sup>	0 $\pm$ 0	4 $\pm$ 5	0 $\pm$ 0
GR	8 $\pm$ 7 <sup>b</sup>	0 $\pm$ 0	5 $\pm$ 5	0 $\pm$ 0
<b>18 dph</b>				
CL	0 $\pm$ 1 <sup>a</sup>	0 $\pm$ 1	7 $\pm$ 6	0 $\pm$ 1
GR	3 $\pm$ 3 <sup>b</sup>	0 $\pm$ 1	10 $\pm$ 3	1 $\pm$ 1
<b>25 dph</b>				
CL	3 $\pm$ 5	28 $\pm$ 14	24 $\pm$ 12 <sup>a</sup>	1 $\pm$ 2
GR	5 $\pm$ 8	22 $\pm$ 13	38 $\pm$ 10 <sup>b</sup>	0 $\pm$ 0
<b>36 dph</b>				
CL	1 $\pm$ 3	25 $\pm$ 12 <sup>b</sup>	36 $\pm$ 16 <sup>a</sup>	3 $\pm$ 5
GR	1 $\pm$ 2	10 $\pm$ 10 <sup>a</sup>	65 $\pm$ 16 <sup>b</sup>	2 $\pm$ 3
<b>43 dph</b>				
CL	2 $\pm$ 2	48 $\pm$ 12 <sup>b</sup>	51 $\pm$ 10 <sup>a</sup>	5 $\pm$ 2
GR	1 $\pm$ 2	23 $\pm$ 11 <sup>a</sup>	63 $\pm$ 10 <sup>b</sup>	4 $\pm$ 5

11 dph

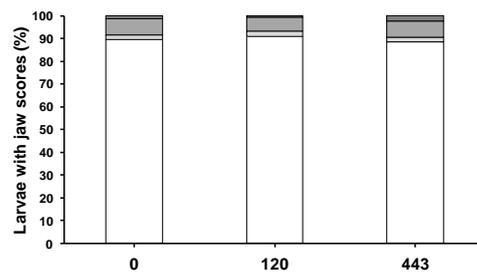
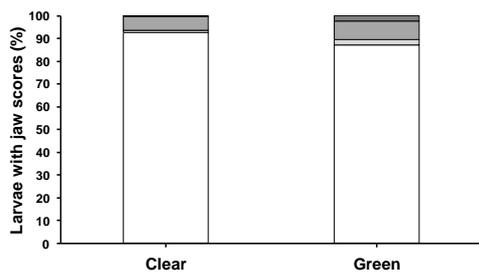
**A**



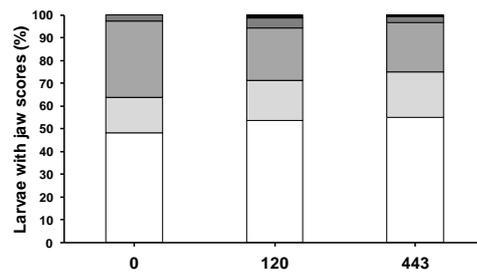
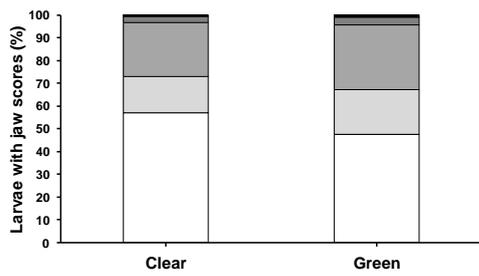
**B**



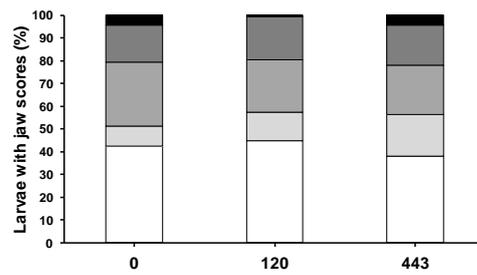
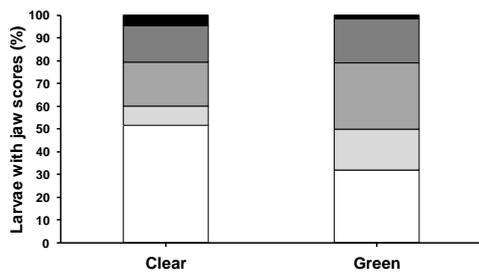
18 dph



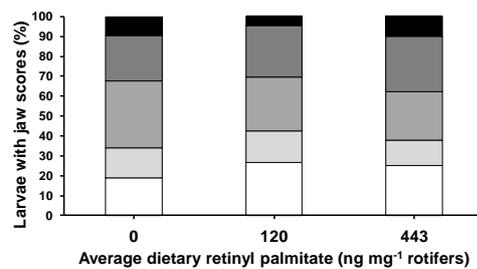
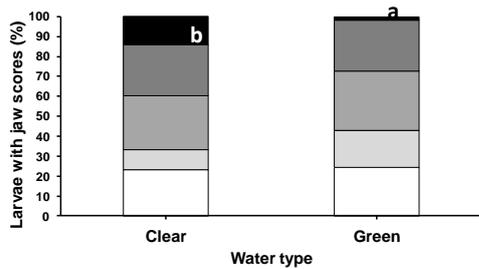
25 dph



36 dph



43 dph



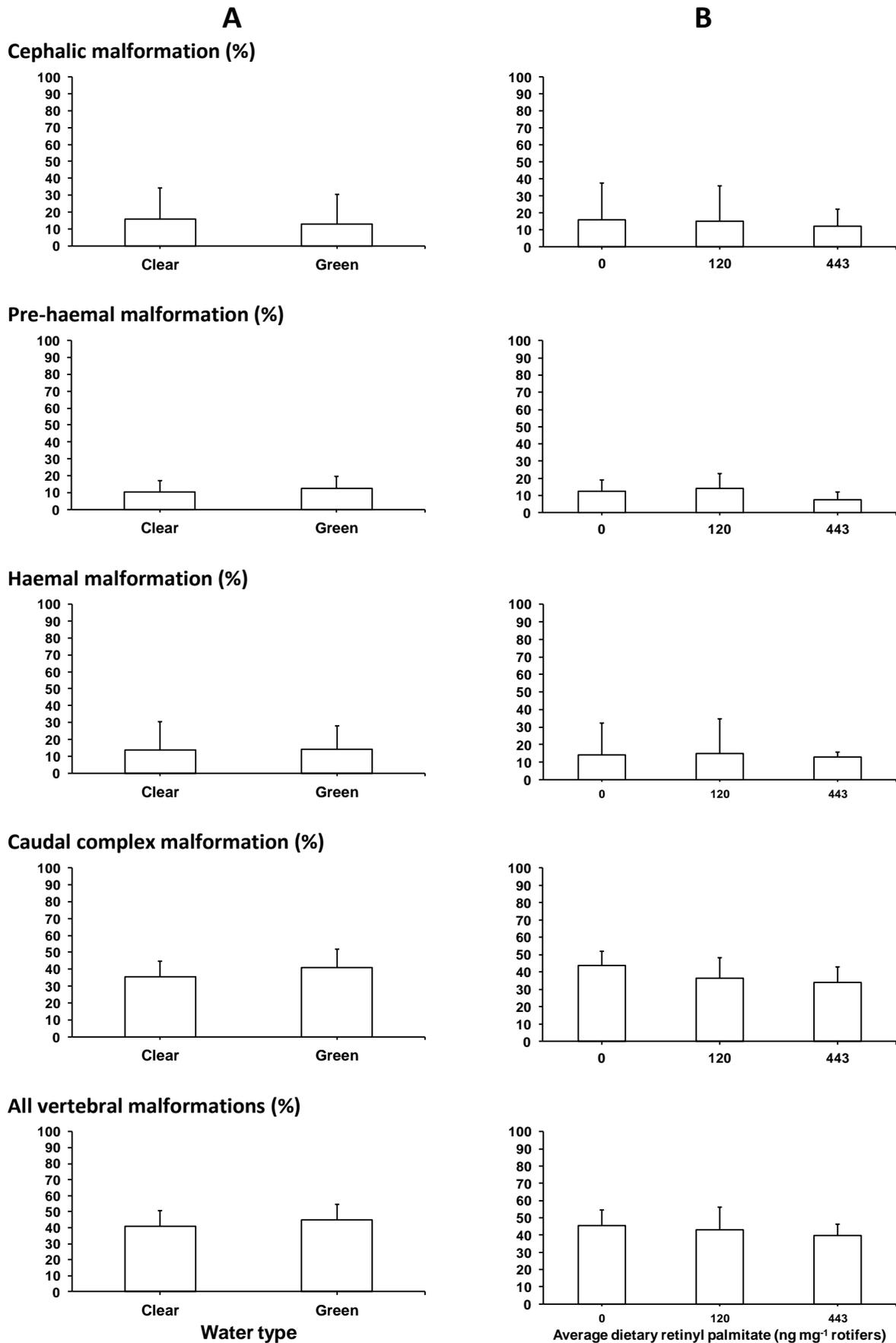
0 0.5 1 2 3 Jaw malformation score

#### 4.4.2.3 Vertebral column malformation

Water type and different retinyl palmitate doses had no significant effect on the prevalence of skeletal malformation in any region of the vertebral column in *L. lineata* post-larvae on 43 dph (Fig. 8). Different types of vertebral malformations were as described in Chapter 3. By 43 dph,  $43 \pm 10\%$  of the post-larvae across experimental tanks showed malformation in at least one region of the vertebral column. Malformation in the caudal region was the most pronounced type of malformation, average of  $38 \pm 10\%$ . While pre-haemal and haemal malformations were the least common, average of  $11 \pm 7\%$  and  $14 \pm 12\%$  across treatments. There was no significant interaction between water type and rotifer enrichment with different retinyl palmitate doses on prevalence of vertebral malformations in *L. lineata* post-larvae by the end of the experiment.

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Figure 7: Prevalence, severity and development of jaw malformation in *Latris lineata* larvae and post-larvae fed rotifers enriched with graded doses of retinyl palmitate and reared in green or clearwater, A) according to water type, n = 12 replicate tanks, and B) according to vitamin level, n = 8 replicate tanks. 20 larvae per tank on 11, 25 and 36 dph and 50 larvae per tank on 18 and 43 dph. Bar shading represents jaw malformation index as shown in legend. Descriptions of the jaw scores are as mentioned in Chapter 3 text. Different letters indicate significant differences among mean prevalence of severe jaw malformations (score 3) at 43 day post hatch (dph) (ANOVA,  $P < 0.05$ ).



## 4.5 Discussion

The present study is the first to my knowledge to evaluate the effect of greenwater combined with dietary VA on the total incorporation of retinoids in marine finfish larvae and the subsequent effect on larval performance and skeletal malformations. *Latris lineata* larvae were reared in green or clearwater and fed enriched rotifers with three doses of VA from 6 to 19 dph. Retinoid inclusion in *L. lineata* larvae fed VA enriched rotifers was not affected by the water type. However, the retinoid content at 18 dph larvae was positively correlated with total retinol content in the rotifers they fed on, where it increased significantly with increasing dietary total retinol. This result agrees with Fernández et al. (2009) and Villeneuve et al. (2005) who found that in *D. labrax*, and *S. senegalensis*, larval body VA content was correlated with the dietary VA given to the larvae, being particularly high in larvae fed 646,800 IU VA kg<sup>-1</sup> DW extracted diet (24.7 times lower than our highest treatment) and 13,333,000 IU VA kg<sup>-1</sup> experimental emulsion (1.2 times lower than our highest treatment), respectively. There was no significant difference in VA content of the larvae with increasing VA in equivalent treatments in Chapter 3 (0, 3,000 and 10,000 mg retinyl palmitate l<sup>-1</sup>). In a statistical comparison of the greenwater tanks between the two experiments, there was no significant effect of VA on larval retinoid content. The variation within treatments (among tanks) was responsible for the lack of significant effects between experiments, and the rotifer VA content was proportionally higher (12-15%) in the current study which would have contributed to the trend in differences in larval VA content among experiments.

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Figure 8: Prevalence of vertebral column malformations in *Latris lineata* larvae at 43 dph fed rotifers enriched with graded doses of retinyl palmitate and reared in green or clearwater, A) according to water type where n = 12 replicate tanks, and B) according to vitamin level where n = 8 replicate tanks. n = 50 larvae per tank. Values are mean (%) + SD. No significant differences among treatments or interaction between water type and different retinyl palmitate doses.

Chapter 2 showed that the concentration of retinoids in previously enriched rotifers decreased over time when reared in clearwater and increased in greenwater, although losing or accumulating VA depends on the previous doses of enrichment. Despite that, the rotifers maintained the significant difference between their previous doses of enrichments for at least the first 4 h in both clear and greenwater (Chapter 2). The results of the present study agree with the general findings of Chapter 2, where the doses of VA in enriched rotifers were different for each treatment level and further, this was reflected in the concentration of VA in the larval body and was not influenced by the water type. Vitamin A enriched rotifers were offered to the larvae at 09:00 for 8 h daily. The morning feed is when the larvae are most hungry as before that time, feeding was not possible because between 01:00 and 08:00, the lights were off and *L. lineata* larvae are visual predators requiring light to feed (Cobcroft et al., 2001a). In addition, the rearing water in the tanks was flushed from 23:00 to 08:00 to remove any uneaten live prey. Consequently, larvae fed generously on the VA enriched rotifers when they were offered to them at 09:00, which was reflected in their retinoid content being correlated with that of the rotifers.

Rearing *L. lineata* larvae in greenwater significantly improved the growth in length and weight, although the survival was significantly higher in clearwater, while VA enriched rotifers did not significantly affect the growth or survival. This result agrees with a study by Cobcroft et al. (2001a), in which greenwater increased the feeding capabilities of *L. lineata* larvae through improvement of the photopic visual sensitivity. This is mainly because the turbidity induced by the greenwater affects the larval distribution in the water column and enhances the visual contrast of rotifers which allows them to catch more live prey (Lazo et al., 2000; Naas et al., 1992; Shaw, 2006). Rotifers enriched with VA did not affect the growth parameters in *L. lineata* larvae, which is in agreement with the results in Chapter 3. Vitamin A doses used in the present study (0, 3,000 and 10,000 mg retinyl palmitate l<sup>-1</sup>) are within the range used in Chapter 3. Similarly, increasing doses of VA in species such

as *S. senegalensis* and Summer Flounder, *Paralichthys dentatus*, did not affect growth and survival (Fernández et al., 2009; Martinez et al., 2007).

Jaw malformations are an issue in the culture of *L. lineata*, and they can be detected as early as 9 dph (Battaglione and Cobcroft, 2007 and unpublished data; Cobcroft et al., 2001b). By the end of the study, the prevalence and types of jaw malformation in *L. lineata* larvae and post-larvae reared in clear or greenwater and fed VA-enriched rotifers were not affected by the range of VA tested, where 62% of the post-larvae on 43 dph displayed jaw malformation. However, the severity of jaw malformation (score 3) by 43 dph was affected by the water type where it was significantly lower in larvae reared in greenwater (2%) compared with those reared in clearwater (14%). Jaw malformation prevalence was not affected by dietary VA in the current study, agreeing with the results of Chapter 3, where increasing dietary VA between 0 and 10,000 mg retinyl palmitate L<sup>-1</sup> emulsion during the rotifer feeding period did not affect the jaw malformation incidence in *L. lineata* larvae when reared in greenwater. The absence of an effect of VA on jaw malformations, despite the high prevalence of malformations, also concurs with Martinez et al. (2007), where the overall frequency and severity of jaw defects (42% slight and 54% severe) increased when Summer Flounder, *P. dentatus* larvae were exposed to different VA doses (retinoic acid) for 10 days but no dose response was observed. However, the absence of a significant effect for VA on jaw malformations in *L. lineata* contrasts with the results reported for *D. labrax*, where the prevalence of jaw malformation (including hyoid arch and lower jaw) increased with increasing the doses of VA (Mazurais et al., 2009). *Dicentrarchus labrax* larvae fed on extracted diets containing 3,152 and 9,402 IU VA kg<sup>-1</sup> DM from 9 to 45 dph had significantly lower jaw malformations than the larvae fed on diets containing 20,755 to 155,200 IU VA kg<sup>-1</sup> DM (Mazurais et al., 2009).

The potential reason for the absence of a VA effect on the jaw malformation in the current study, despite the significant difference of VA incorporation in the larvae is the overriding effect of physical factors or rearing conditions, rather than nutritional factors (at least not VA). Cobcroft and

Battaglione (2009) found that the jaw malformation in *L. lineata* post-larvae was correlated with walling behaviour that was in turn affected by tank wall colour. Larval tendency for walling on the tank sides can be reduced by applying the greenwater technique due to the modified lighting conditions induced by the turbidity (Bristow and Summerfelt, 1994; Bristow et al., 1996; Cobcroft et al., 2001b). In the current study, the severity of jaw malformations (score 3, larvae showing multiple severe malformations) was significantly lower in greenwater than clearwater mainly due to the application of turbidity (greenwater) in culture conditions during the rotifer feeding period that reduced the walling behaviour. In addition, the percentage of normal larvae (score zero) on 18 dph were significantly higher in clearwater (93%) than in greenwater (87%), because larvae reared in clearwater were significantly smaller (shorter and lighter) than those reared in greenwater. Larvae reared in clearwater were less developed than those larvae reared in greenwater and malformation was not yet apparent. It should be noted that the timing of ossification can be affected by dietary VA (Fernández et al., 2008, 2009), which could also impact differences in observed malformations in fish of different sizes, although ossification was not investigated in my study. Following further development at the end of the experiment, 43 dph, there was no significant difference in the percentage of post-larvae with normal jaws reared in clear and greenwater with an average of 24%. Thus, there was an incremental increase in the percentage of larvae displaying jaw malformations. The relationship between low survival and high jaw malformation in clearwater-reared larvae but not in greenwater, may be associated with the smaller and less developed larvae in clearwater that have not finished metamorphosis, and so have not passed an important mortality window compared with greenwater treatments. It appears that the smaller larvae in clearwater are susceptible to conditions in individual tanks (rather than a VA effect) that may induce jaw malformation and be linked to lower survival. This implies that there is a window during the developmental period of the larvae that might be targeted to avoid the onset of malformations. This window might be affected by the rearing conditions resulting in walling behaviour which led to physical damage to different jaw parts (Cobcroft and Battaglione, 2009; Cobcroft et al., 2001b, 2012).

Vertebral column malformation in *L. lineata* larvae and post-larvae occurs under intensive culture conditions but there is no published data on the prevalence (Cobcroft and Battaglione, unpublished data; Trotter et al., 2001). The VA experiments in this thesis provide the first information on the prevalence of vertebral malformation in cultured *L. lineata* post-larvae. In the current study, the prevalence of vertebral column malformation in *L. lineata* was not affected by the water type or VA-enriched rotifers with an average of 43% on 43 dph. In other studies, the prevalence of vertebral column malformation is affected by the VA concentration in the diet and hence in the larvae (Dedi et al., 1995; Fernández et al., 2008, 2009; Hernandez-H et al., 2006).

In Chapter 3, the prevalence of vertebral column malformations in *L. lineata* post-larvae fed on VA-enriched rotifers and reared in greenwater was affected and correlated with the retinoid content of the larvae. This contradiction of results can be explained by the dietary VA dose fed to the larvae in the current study. In Chapter 3, larvae were reared in greenwater during the rotifer feeding period and fed eight increasing doses of VA that ranged from 0 to 10,000 mg retinyl palmitate l<sup>-1</sup>. There was no significant difference in the prevalence of vertebral malformations between the larvae fed 0, 1,500, 3,000 and 10,000 mg<sup>-1</sup> l retinyl palmitate with an average of 35% malformations (Chapter 3). However, the average prevalence of skeletal malformation in the current study was 43%. This elevation in the percentage of post-larvae displaying malformations in the current study could be explained by the fact that there was a different batch of experimental emulsions used in each experiment. These experimental emulsions were designed nominally to contain the same concentration (doses) of retinyl palmitate as those in Chapter 3. However, chemical analysis revealed that the concentration of retinyl palmitate was 15% and 10% higher in the 3,000 and 10,000 mg retinyl palmitate l<sup>-1</sup> respectively, compared with the concentration of retinyl palmitate in Chapter 3. This elevation of retinyl palmitate concentration in the experimental emulsions was reflected in the enriched rotifers (12% higher for 3,000 mg l<sup>-1</sup>, and 13% higher for 10,000 mg l<sup>-1</sup>) than Chapter 3. In addition, whilst there was no retinyl palmitate detected in the control treatments (0

mg retinyl palmitate l<sup>-1</sup> emulsion), total retinol was 15% higher in the current study than in Chapter 3.

## 4.6 Conclusion

*Latris lineata* retinoid content increased significantly with the increasing retinoid content in the enriched rotifers they fed on, although the prevalence of skeletal malformation was not affected by the doses of dietary VA examined. Water type, clear or greenwater, did not affect the incorporation of retinoids by larvae. The most obvious effect for the greenwater was the higher growth and survival in larvae reared in greenwater compared to clearwater. Greenwater also affected the severity of the jaw malformation in 43 dph *L. lineata* post-larvae, where it was significantly lower than in larvae reared in clearwater, but did not affect the prevalence of vertebral column malformation. Finally, the study supports the hypothesis that jaw malformation in *L. lineata* larvae and post-larvae is more strongly linked to physical factors rather than nutrition.

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# Chapter 5

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**Effect of dietary vitamin A during  
*Artemia* feeding on performance  
and skeletal abnormality of Striped  
Trumpeter *Latris lineata* larvae  
and post-larvae**

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## 5.1 Abstract

Skeletal malformation is a limiting factor that has an impact on the quality of hatchery produced fish. Several nutritional studies have found that excess or reduced dietary vitamin A (VA) causes skeletal malformations in marine fish larvae. Chapter 3 results have shown that the prevalence of vertebral column malformation was affected in Striped Trumpeter (*Latris lineata*) post-larvae when fed enriched VA rotifers from 6 to 18 dph (days post-hatch), while growth, survival and jaw malformation (incidences and severity) were not affected. This Chapter examines the effect of VA during later stages of development: *Artemia* feeding period (from 19 to 44 dph). The larvae were reared in a 3000 l tank and fed a common diet of Algamac-3050 enriched rotifers at a feeding density of 10 ml<sup>-1</sup> once daily from 2 to 16 dph. At 16 dph larvae were transferred to 24 x 300 l tanks. *Latris lineata* larvae were fed *Artemia* enriched with one of six doses of retinyl palmitate (VA) four times per day at 09:00, 13:00, 17:00 and 21:00 from 19 to 44 dph. Enriched *Artemia* fed at 09:00 and 17:00 were fresh-rinsed while the 13:00 and 21:00 feeds were cold-stored. Average VA in enriched *Artemia* per feed was 0, 6, 7, 27, 30 and 55 ng retinyl palmitate mg<sup>-1</sup> dry weight (DW) following enrichment with emulsions containing 0 (control), 795, 1,558, 3,174, 4,911 and 10,518 ng retinyl palmitate mg<sup>-1</sup> emulsion. The retinoid content of *L. lineata* at 44 dph was positively correlated with the enriched *Artemia* they fed on, increasing significantly with enrichment dose. By 44 dph, neither larval growth in length (15.91 ± 0.31 mm, mean ± SD) or dry weight (5.08 ± 0.32 mg), nor survival (26.1 ± 2.9%), were significantly affected by increasing dietary doses of retinyl palmitate. Unlike other studies, on a range of marine fish species treated with increased doses of dietary VA, retinyl palmitate enrichment in *Artemia* did not affect jaw malformation in *L. lineata*. By the end of the study, 54 ± 10% and 18 ± 6% of the post-larvae across treatments had short lower and open jaws, respectively. Severe jaw malformations affected 50 ± 11% of the post-larvae. The prevalence of vertebral column malformations in 44 dph post-larvae were also not affected by dietary VA. Across treatments, 58 ± 4% of the post-larvae had vertebral malformation. The results demonstrate that *L.*

*lineata* larval survival, growth and malformation were unaffected by dietary VA during the *Artemia* feeding period from, 19 to 44 dph. The study suggests that the window of influence for VA is during earlier development of bone structures.

## 5.2 Introduction

Skeletal malformation is a limiting factor in the commercial scale production of some cultured marine fish species, although the occurrence and types of malformations varies according to species.

The most common regions that are affected are the jaw, opercula, vertebral column and fins (Barahona-Fernandes, 1982; Fraser and De Nys, 2011; Koumoundouros, 2010; Nagano et al., 2007).

In a survey of 18 marine fish hatcheries from around Australia, 44% indicated that skeletal malformations were a significant issue, with up to 70% malformation in some batches of cultured Yellowtail Kingfish, *Seriola lalandi*, and up to 95% in Striped Trumpeter, *Latris lineata* (Cobcroft and Battaglione, unpublished data). Other commercial species with documented skeletal malformations include European Sea Bass, *Dicentrarchus labrax*, Barramundi, *Lates calcarifer*, Red Sea Bream, *Pagrus major*, Gilthead Sea Bream, *Sparus aurata*, Japanese Flounder, *Paralichthys olivaceus* (Bogliione et al., 2001; Chatain, 1994; Fraser and de Nys, 2005; Hattori et al., 2003; Tomoda et al., 2006). Malformations add to the cost of farming by reducing survival, adding infrastructure and labour costs due to manual sorting of malformed fish that reduce marketability and decrease fish quality (Cahu et al., 2003; Koumoundouros et al., 2002, 1997). European research reviewed available scientific data and knowledge and identified three probable reasons for malformations: rearing temperature, tank environment such as the dissolved oxygen and water currents or quality, and nutrition (Cahu et al., 2003; Divanach et al., 1997; Haaparanta et al., 1997; Hattori et al., 2004; Lall and Lewis-McCrea, 2007; Sfakianakis et al., 2004). In aquaculture, the knowledge of larval fish nutrition and feeding is still incomplete, due in part to the huge number of aquatic species cultivated and variation in their physiology and behaviour (Teles et al., 2011). Amino acids, oxidized lipids, phospholipids, PUFAs, minerals and vitamins A, C, D and K have previously been linked with

malformations that appear both during the larval and juvenile stages (Cahu et al., 2003; Lall and Lewis-McCrea, 2007).

Retinoids, which are vitamin A (VA) related compounds, are a group of morphogenetic nutrients that include all the compounds that possess the same biological activity as retinol. Retinoids regulate a wide variety of functions including morphogenesis, cellular differentiation, tissue homeostasis and bone metabolism (Haga et al., 2002b; Ross et al., 2000). The biological activity of retinoids is expressed in international units (IU), where 1 IU of VA is equivalent to 0.3 µg retinol (Wolf, 1984). Fish like other living organisms, are not able to synthesize VA, they have to obtain it from the diet not only at the optimum dose but also in the proper chemical form (Fernandez and Gisbert, 2011; Guillaume, 2001).

Nutritional requirement studies of larval marine fish and the effect of different nutrients on skeletal development and performance parameters are usually examined through enrichment of live feed, i.e. either rotifers or *Artemia* (Copeman et al., 2002; Giménez et al., 2007; Roo et al., 2009).

Enrichment is through bioencapsulation where live prey is cultured in a medium rich in lipids, fatty acids or vitamins. The non-selective feeding behaviour of the live prey enables them to feed on the correctly sized diffused particles, incorporate them into the digestive tract and assimilate them into their bodies depending on enrichment periods and temperature (Conceição et al., 2010; Sorgeloos et al., 2001; Sweetman, 2004). Enrichment of *Artemia* with graded doses of VA or different VA metabolites is the best way to examine the effects of VA on normal development but is technically and resource challenging, and reported for relatively few species (Fernández et al., 2009; Takeuchi et al., 1998; Tarui et al., 2006).

Dietary VA has been extensively studied in flatfish where it has been found to affect pigmentation and skeletal malformation (Estevez and Kanazawa, 1995; Haga et al., 2002a; Martinez et al., 2007; Suzuki et al., 2000). Skeletal malformations in different vertebral regions, fins, opercular and caudal

fin complexes due to improper VA doses have been reported in other marine fish such as the *P. major*, *S. aurata* and *D. labrax* (Fernández et al., 2008; Hernandez-H et al., 2006; Mazurais et al., 2009). Cephalic malformations, particularly in the jaw, were reported with imbalanced VA doses in *P. olivaceus*, Summer Flounder, *P. dentatus* and *D. labrax* (Haga et al., 2002b, 2003; Martinez et al., 2007; Villeneuve et al., 2006).

*Latris lineata* is native to south-eastern Australia and New Zealand. Intensive research has been undertaken on this species since the early 1990's as a result of its selection as the best candidate for sea cage culture diversification in Tasmania (Battaglione and Cobcroft, 2007). Success has been achieved in controlling brood stock reproduction, egg incubation and parasite control (Battaglione and Cobcroft, 2007). Many larval rearing problems have been solved, which has resulted in higher and more reliable survival and growth. High incidences of jaw malformation in juveniles remain the largest impediment to successful commercial culture (Cobcroft and Battaglione, 2009; Cobcroft et al., 2001). Research has examined the early larval requirements for essential fatty acids during both rotifer and *Artemia* feeding periods (Bransden et al., 2005a, 2005b) and for vitamins C and E (Battaglione et al., unpublished data; Brown et al., 2005a, 2005b). Recently, jaw malformation was linked with walling behaviour that is affected by tank colour (Cobcroft and Battaglione, 2009). Green water, tank size and availability of live feed in the water column have also been implicated as factors influencing jaw malformation in *L. lineata* via their impact on walling behaviour (Battaglione and Cobcroft, 2007; Cobcroft et al., 2012).

The effect of increasing dietary retinyl palmitate doses on *L. lineata* larval performance parameters (growth and survival) and skeletal development, particularly jaws and vertebral column has been examined during rotifer feeding (Chapters 3 and 4). The larvae were fed rotifers enriched with increasing retinyl palmitate doses from first feeding up to 18 dph. Increasing retinyl palmitate doses resulted in no significant effects on larval growth or survival to 43 days post hatch (dph). The incidence and severity of jaw malformations was also not significantly affected. However,

malformations in the vertebral column were correlated with the VA content of the larvae. The safe dose of total VA inclusion during the rotifer feeding period was recommended to be more than 123 ng mg<sup>-1</sup> dry weight (DW) rotifers (Chapter 3).

The objective of the present research was to evaluate the effect of six graded doses of retinyl palmitate on *L. lineata* post-larvae during the *Artemia* feeding period, from 17 to 44 dph, on the larval performance parameters and skeletal development, focusing on the jaw and vertebral column.

## 5.3 Materials and methods

### 5.3.1 Egg incubation and rearing of larvae

Gametes were obtained from naturally spawning broodstock held under controlled photoperiod and ambient water temperature at the Institute for Marine and Antarctic Studies, Fisheries, Aquaculture and Coasts facility (IMAS-FAC), Hobart. Eggs from one female were fertilised with sperm from three males. Immediately after fertilization the embryos were disinfected in ozonated seawater at 0.9 ppm for 65 s, to reduce the risk of infection with pathogens (Battaglione and Morehead, 2006). The seawater used was filtered to 1 µm and ozonated to 700 mV ORP for ≥ 10 min, treated with UV at 254 nm, and carbon-filtered before distribution to the incubation unit or larvae tanks at 300-350 mV ORP.

The embryos were incubated in a 250 l conical tank at 14.1 ± 0.09 °C (values are mean ± SD, here and throughout). The incubator was flow-through at approximately 160 l h<sup>-1</sup>, photoperiod 14 h L: 10 h D, salinity ranged from 32.2 to 33.5 ppt, pH from 8.04 to 8.15 and dissolved oxygen from 99.7 to 106.2%. The central screen was 250 µm to facilitate removal of hatch by-products when the eggs hatched after 5 days. After more than 90% of the eggs had hatched, the temperature was slowly elevated by 0.1 °C h<sup>-1</sup> to reach 16 °C. Yolk sac larvae (4.62 ± 0.09 mm total length) were stocked into a larval culture tank 2 days post-hatch (dph) at 10 larvae l<sup>-1</sup>.

Larvae were reared from 2 dph until 16 dph in a cylindroconical 3000 l tank fitted with 390  $\mu\text{m}$  central screens. The temperature range was 15.9 to 16.4  $^{\circ}\text{C}$ , salinity range was 32.4 to 33.8 ppt, pH between 8.09 and 8.36, the dissolved oxygen was > 96% and photoperiod was 16 h L: 8 h D (lights on at 09:00 and off at 01:00). The tank was static from 2 dph (stocking) until 6 dph (first feeding) when the incoming flow of sea water was 750 l h<sup>-1</sup> from 23:00 to 08:00. Surface skimmers were used from 8 dph until 13 dph to remove the oily films from the water surface to facilitate swim bladder inflation (Trotter et al., 2005). The larvae were reared in green water from 6 dph until 19 dph, where 300 l of temperature-acclimated *Nannochloropsis oculata* was added to the tank at 08:30 (before the lights came on) to achieve a turbidity of 3 NTU. Moderate aeration was used during the light period.

Larvae were fed rotifers *Brachionus plicatilis* (Austrian strain) from 6 dph until 18 dph, once per day at 09:00 at a feeding density of 10 ml<sup>-1</sup> and then 5 ml<sup>-1</sup> on 17 and 18 dph. Rotifers were cultured on commercial paste algae (*Nannochloropsis sp.*, Reed Mariculture Inc. CA, USA) and enriched with Algamac-3050 (Aquafauna Biomarine Inc. CA, USA) prepared according to manufacturer's instructions for 8 h at 2 g per million rotifers at 500 rotifers ml<sup>-1</sup>. Enriched rotifers were rinsed, counted and introduced to the 3000 l tank or the 24 x 300 l tanks at the required density.

### 5.3.2 Experiment conditions

On 16 dph, 600 larvae were stocked into each of 24 x 300 l hemispherical tanks, with black base and marble coloured walls to reduce larval walling behaviour (Cobcroft and Battaglene, 2009). Larvae and post-larvae were reared in the experimental tanks from 16 dph until 44 dph where mean temperature was  $16.38 \pm 0.17$   $^{\circ}\text{C}$ , mean salinity  $32.35 \pm 0.23$  ppt, mean pH  $8.10 \pm 0.05$  and mean dissolved oxygen was  $102.18 \pm 2.46\%$ . The photoperiod was increased to 20 h L: 4 h D on 18 dph and 24 h L from 19 dph onwards to reduce downward nocturnal migration and associated mortalities (Bransden et al., 2005a). Light intensity was  $10.6 \pm 1.4$   $\mu\text{mol s}^{-1}$  at the water surface. The incoming

flow water exchange was  $104 \pm 1 \text{ l h}^{-1}$  for 9 h from 16 to 18 dph and then increased to 24 h from 19 dph. Water was recirculated for each tank through the 390  $\mu\text{m}$  central screens into 63  $\mu\text{m}$  bag screens in an external sump to filter out the uneaten prey, increasing the total flow to  $232 \pm 10 \text{ l h}^{-1}$  for 9 h on 16, 17 and 18 dph and for 24 h from 19 dph until 44 dph.

The effect of dietary VA on the performance, growth, survival and skeletal malformation was determined for the larvae during the *Artemia* feeding period. *Artemia* were enriched with six graded doses of retinyl palmitate and fed to the larvae from 17 dph to 44 dph (4 tanks per treatment). *Artemia* were fed four times each day at 09:00, 13:00, 17:00 and 21:00 at a density of  $0.25 \text{ ml}^{-1}$  per feed, except on 17 dph and 18 dph when the larvae were fed *Artemia* at 17:00 and 21:00 only. From 25 dph, the feeding density was adjusted according to the larval survival and prey consumption, with *Artemia* added to each tank ranging between  $0.1$  to  $0.2 \text{ ml}^{-1}$  per feed.

### 5.3.3 Enrichment

*Artemia* cysts (AAA, INVE Aquaculture Nutrition, UT, USA) were decapsulated and stored in brine at  $4^\circ\text{C}$  at the onset of the experiment, and hatched daily as required (Sorgeloos et al., 1977). *Artemia* (7 h after hatching) were enriched with experimental emulsions having graded doses of retinyl palmitate ( $1,600,000 \text{ IU g}^{-1}$ , MP Biomedicals, Australia); 0 (control), 7,50, 1,500, 3,000, 5,000 and 10,000 mg retinyl palmitate  $\text{l}^{-1}$  WW (wet weight) emulsion. The emulsion basic constituents were lipids 57%, vitamin E 4% and vitamin C 4% (prepared by Nutrakol, Mullalo, WA). Enrichment of *Artemia* ( $50 \text{ ml}^{-1}$ ) was for 24 h in complete darkness at  $0.6 \text{ g l}^{-1}$  enrichment.

After enrichment, *Artemia* were siphoned from each vessel, collected into 150  $\mu\text{m}$  screen bags, rinsed with sea water and suspended into 10 l sea water, then divided into two equal portions. There were two harvests per day. The first half of each harvest was counted and fed immediately according to the required density for the 09:00 and 17:00 feeds. The second half was stored at high density in 1 l pre-cooled sea water at  $4^\circ\text{C}$  for the 13:00 and 21:00 feeds. Samples from the four feed

times were taken at 23, 31 and 42 dph for later biochemical analysis. For simplicity, 09:00 and 17:00 feeds were referred to as 'fresh-rinsed' while the 13:00 and 21:00 feeds were the 'cold-stored'.

### 5.3.4 Sampling

Before stocking the larvae into the experimental tanks, 20 larvae from the 3000 l tank were anaesthetised in 0.06% 2-phenoxethanol. Their total length was measured using an eyepiece graticule fitted to an Olympus SZ stereomicroscope. Twenty other larvae were placed on pre-weighed filter paper, rinsed with 15 ml 0.5 M ammonium formate and re-weighed to record the wet weight. Filtered samples were stored at -80 °C before freeze-drying to determine larval dry weight.

On 24, 30, 37 and 44 dph, 20 larvae were siphoned from each tank before feed addition. These larvae were anaesthetised (as above) and examined under the microscope. They were scored for their total length, general condition and gross jaw malformation. After scoring, their wet and dry weight was recorded as described above.

On 44 dph, 20 post-larvae from each tank were fixed in 10% formaldehyde for later analysis of skeletal malformation. Whole larvae were cleared and stained for bone and cartilage using the method described by Taylor and VanDyke (1985). Recognition of jaw and skeletal malformations used the scoring system and morphological region identification described in Chapter 3.

Remaining post-larvae were siphoned from each tank at 44 dph and counted to calculate the final survival. To quantify the retinoid content of the post-larvae, all remaining post-larvae were rinsed with 0.5 M ammonium formate and stored in liquid nitrogen before storage at -80 °C, and analysed within nine months.

### 5.3.5 Biochemical analysis

Biochemical analyses were conducted according to methods described in Chapters 2 and 3. Retinoids were measured as total VA (the sum of retinyl palmitate, retinol and retinoic acid) and total retinol

using two different techniques modified from Moren et al. (2002 and 2004) and Takeuchi et al. (1998). Total VA and total retinol were measured in enrichment emulsions and live prey while only total retinol was measured in 44 dph post-larvae due to the small size and scarcity of larval samples. Samples were analysed using a Waters Model 600E liquid chromatograph system (Waters Corporation, Milford, MA, USA) supplied with a Waters Model 996 photodiode array (PDA) detector and 475 scanning fluorescence detector set for an excitation maximum at 325 nm and emission maximum at 470 nm. Peak areas were quantified using Waters Millennium software. Retinyl palmitate results were obtained using the PDA detector while retinol and retinoic acid were determined from the fluorescence detector. The concentration of retinoids in samples was calculated by comparing peak area to those of internal and external standards. Live feed samples and post-larvae were not freeze-dried and appropriate calculations were made to express the results as dry weight. Larvae, rotifer and *Artemia* dry weights were based on dry weight measurements for 44 dph post-larvae and different freeze-dried samples of live feed during the experiment. Data are presented as  $\text{ng mg}^{-1}$  DW (dry weight).

Total lipids and fatty acid methyl esters were determined using a modified version of Bligh and Dyer (1959) in live prey, according to methods described in Chapter 2. FAME were analysed by GC (Agilent Technologies 7890A) equipped with methyl silicone fused capillary column (15 m x 0.1 mm internal diameter, 0.1  $\mu\text{m}$  film thickness). Samples were injected in splitless mode at 120 °C using an Agilent Technologies 7683B injector with Helium as carrier gas. Acquired peaks were quantified with Agilent Technologies Chemstation software (Palo Alto, CA, USA). Preliminary peak identifications were done by comparing retention time data with authentic and internal standard methyl tricosanoate ( $\text{C}_{23:0}$ ). GC-Mass spectrometric (GC-MS) analyses were performed on a Finnigan GCQ plus GC-MS (Finnigan corp., San Jose, CA, USA) ion trap fitted with a capillary column.

### 5.3.6 Statistical analysis

Effects of vitamin treatments were determined using a three-way ANOVA for the enriched *Artemia* (fixed factors: time, storage and vitamin dose), two-way ANOVA for the lipid data (fixed factors: storage and vitamin dose) and one-way ANOVA for performance parameters, jaw and skeletal abnormalities. Mean percentage data were transformed by  $\arcsin \sqrt{x}$ , where  $x$  is the proportion, and the dry weight data was  $\log_{10}$  transformed to achieve homogeneity of variance and normal distribution. Levene's test was used to assess homogeneity of variance. Where significant treatment effects were found, a post hoc Tukey test was used to determine differences among means. Correlation was used to test the association between *Artemia* and post-larvae retinoid content. Significant differences were accepted at  $P < 0.05$ . Statistical analyses were performed using SPSS 13.0 (SPSS Inc.).

## 5.4 Results

### 5.4.1 Biochemical analyses

#### 5.4.1.1 Retinoid content in experimental emulsions, live prey and larvae

Emulsions in the current study were designed to nominally contain 0 to 10,000 mg l<sup>-1</sup> retinyl palmitate. Retinyl palmitate was the predominant form of VA in the emulsions but a small retinol peak was observed that was difficult to quantify as it was masked by other higher concentration peaks of undetermined substances. The retinol peak was due to the base emulsion ingredients (lipid base contained tuna oil with 700 IU VA kg<sup>-1</sup>, NuMega certificate of analyses) prior to retinyl palmitate addition. Retinol was quantified using the saponification process and expressed as total retinol, which is the retinyl palmitate hydrolysed into retinol plus the retinol from the emulsion ingredients. Retinyl palmitate and total retinol in the emulsions increased significantly with increasing addition of retinyl palmitate (Table 1). Retinyl palmitate concentration was 94 to 102% of the nominal

concentration. The corresponding values for total retinol was 139 to 1456 ng total retinol mg<sup>-1</sup> emulsion, and there was a detectable quantity of total retinol (0.26 ng mg<sup>-1</sup>) in the 0 (control) emulsion.

Table 1: Retinyl palmitate and total retinol (saponification method) in experimental live prey enrichment emulsions. Values are mean  $\pm$  SD and n = 3. Different letters within the same column show significant differences among emulsions (ANOVA, P < 0.05).

Nominal concentration (mg retinyl palmitate l <sup>-1</sup> )	Retinyl palmitate ng mg <sup>-1</sup>		Total Retinol ng mg <sup>-1</sup>	
0	0.00	$\pm$ 0.00 <sup>a</sup>	0.26	$\pm$ 0.11 <sup>a</sup>
750	794.70	$\pm$ 5.68 <sup>b</sup>	138.81	$\pm$ 29.35 <sup>b</sup>
1500	1557.51	$\pm$ 26.03 <sup>c</sup>	290.24	$\pm$ 40.24 <sup>c</sup>
3000	3174.27	$\pm$ 38.43 <sup>d</sup>	552.51	$\pm$ 89.65 <sup>d</sup>
5000	4910.87	$\pm$ 132.52 <sup>e</sup>	875.97	$\pm$ 141.22 <sup>e</sup>
10000	10517.85	$\pm$ 49.76 <sup>f</sup>	1455.92	$\pm$ 160.75 <sup>f</sup>

In the enriched *Artemia*, retinyl palmitate was significantly different between the morning (09:00 and 13:00) and afternoon feeds (17:00 and 21:00), and between the fresh rinsed and cold stored feeds, while there were no significant differences for the retinol and retinoic acid (Table 2). Total VA content in the enriched *Artemia* was not significantly different between the morning and afternoon feeds but there was significant difference between the fresh rinsed feeds and the cold stored (Table 2).

To provide an average VA fed to the larvae at each feed time, the mean of the four feeds is shown in Table 3. Retinyl palmitate was the predominant form of VA with detectable quantities of retinol and retinoic acid. *Artemia* enriched with emulsions containing 795 and 1,558 ng retinyl palmitate mg<sup>-1</sup> had no significant difference in their retinyl palmitate content; nor did *Artemia* enriched with either 3,174 or 4,911 ng retinyl palmitate mg<sup>-1</sup>. Retinol content in the enriched *Artemia* was significantly different for all treatments while there was no significant difference in the retinoic acid content for the lowest three treatments and the two highest treatments. Total VA and retinol were significantly different in the enriched *Artemia* across treatments.

Table 2: Results of three-way ANOVA to test the effects of treatment, feeding time and storage on retinyl palmitate, retinol and retinoic acid content of enriched *Artemia*. Vitamin A treatment has 6 doses of increasing retinyl palmitate. Time is morning (09:00 and 13:00 feeds) and afternoon (17:00 and 21:00 feeds). Storage is fresh-rinsed (09:00 and 17:00 feeds) and cold-stored (13:00 and 21:00 feeds). \* indicates significant effect  $P < 0.05$ .

	Source of variation	F value	d.f.	P value
<b>Retinyl palmitate</b>	Vitamin Treatment	1575.77	5	0.000*
	Time	5.83	1	0.024*
	Storage	27.61	1	0.000*
	Treatment x Time	4.86	5	0.003*
	Treatment x Storage	5.15	5	0.002*
	Time x Storage	5.11	1	0.033*
	Treatment x Time x Storage	5.64	5	0.001*
<b>Retinol</b>	Vitamin Treatment	506.33	5	0.000*
	Time	1.02	1	0.322
	Storage	0.02	1	0.889
	Treatment x Time	0.90	5	0.495
	Treatment x Storage	6.25	5	0.001*
	Time x Storage	5.04	1	0.034*
	Treatment x Time x Storage	2.77	5	0.041*
<b>Retinoic acid</b>	Vitamin Treatment	141.86	5	0.000*
	Time	0.50	1	0.488
	Storage	2.28	1	0.144
	Treatment x Time	1.18	5	0.349
	Treatment x Storage	8.20	5	0.000*
	Time x Storage	2.13	1	0.157
	Treatment x Time x Storage	1.04	5	0.418
<b>Total VA</b>	Vitamin Treatment	1554.65	5	0.000*
	Time	1.49	1	0.234
	Storage	50.59	1	0.000*
	Treatment x Time	3.49	5	0.016*
	Treatment x Storage	0.89	5	0.501
	Time x Storage	3.01	1	0.096
	Treatment x Time x Storage	4.28	5	0.006*

Table 3: Average total VA content (retinyl palmitate, retinol and retinoic acid) and total retinol (saponification method) ng mg<sup>-1</sup> DW *Artemia* enriched with graded doses of retinyl palmitate and fed to *Latris lineata* larvae during one complete experimental day. Values are mean ± SD and n = 8 (2 from each feed time). Different letters within the same column show significant differences among dietary groups (ANOVA, P < 0.05).

Retinyl palmitate in emulsion	Retinyl palmitate		Retinol		Retinoic acid		Total VA		Total Retinol (retinyl palmitate + retinol)	
<b>0</b>	0.00	± 0 <sup>a</sup>	0.97	± 0.14 <sup>a</sup>	0.22	± 0.24 <sup>a</sup>	1.19	± 0.23 <sup>a</sup>	0.44	± 0.07 <sup>a</sup>
<b>795</b>	5.67	± 0.94 <sup>b</sup>	1.25	± 0.12 <sup>b</sup>	0.29	± 0.24 <sup>a</sup>	7.20	± 1.08 <sup>b</sup>	2.43	± 0.17 <sup>b</sup>
<b>1558</b>	6.60	± 1.61 <sup>b</sup>	2.20	± 0.43 <sup>c</sup>	0.74	± 0.71 <sup>ab</sup>	9.56	± 1.81 <sup>c</sup>	7.18	± 0.92 <sup>c</sup>
<b>3174</b>	27.10	± 4.61 <sup>c</sup>	3.44	± 0.46 <sup>d</sup>	1.19	± 0.32 <sup>b</sup>	31.74	± 4.70 <sup>d</sup>	9.91	± 0.93 <sup>d</sup>
<b>4911</b>	29.58	± 7.91 <sup>c</sup>	4.58	± 0.54 <sup>e</sup>	5.65	± 0.87 <sup>c</sup>	39.79	± 8.15 <sup>d</sup>	19.85	± 1.70 <sup>e</sup>
<b>10518</b>	54.67	± 10.46 <sup>d</sup>	6.89	± 0.38 <sup>f</sup>	7.44	± 2.02 <sup>c</sup>	68.97	± 12.14 <sup>e</sup>	44.06	± 3.77 <sup>f</sup>

By the end of the study, the total retinol content of *L. lineata* at 44 dph was positively correlated with the enriched *Artemia* on which they fed (Figs 1 and 2). Total retinol content was 2.5 times higher in larvae fed the highest VA treatment compared with control larvae and twice as high as those fed the *Artemia* enriched with the lowest VA dose ( $F_{5,18}=10.427$ ,  $P < 0.004$ ). There was no significant difference in the total retinol content of larvae fed the intermediate treatments.

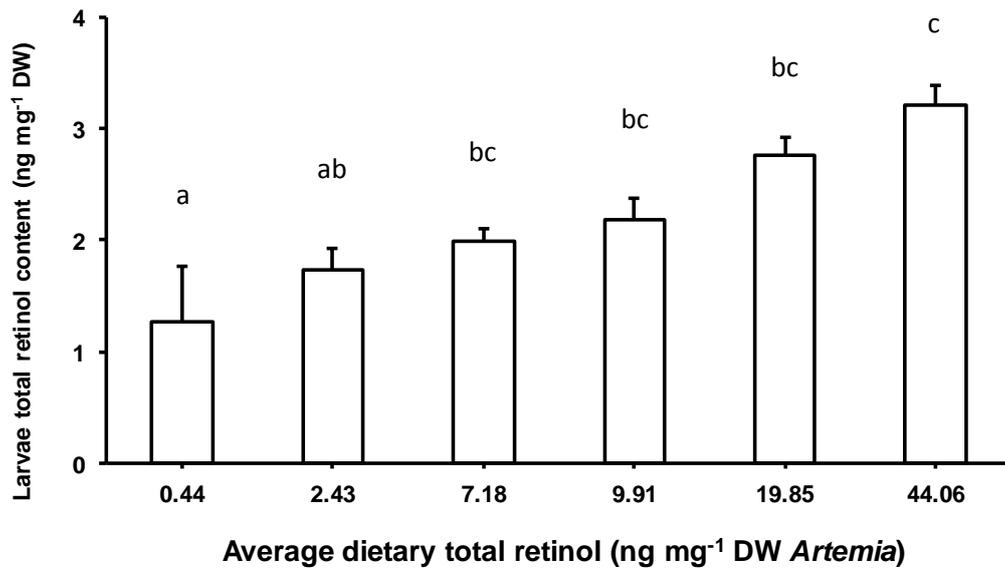


Figure 1: Total retinol in *Latris lineata* post-larvae at 44 dph fed *Artemia* enriched with graded doses of retinyl palmitate. Retinoid content is expressed as ng mg<sup>-1</sup> DW larvae. Values are mean  $\pm$  SD and n = 4 replicate tanks. Histograms not sharing a common letter indicate significant differences among means (ANOVA,  $P < 0.05$ ).

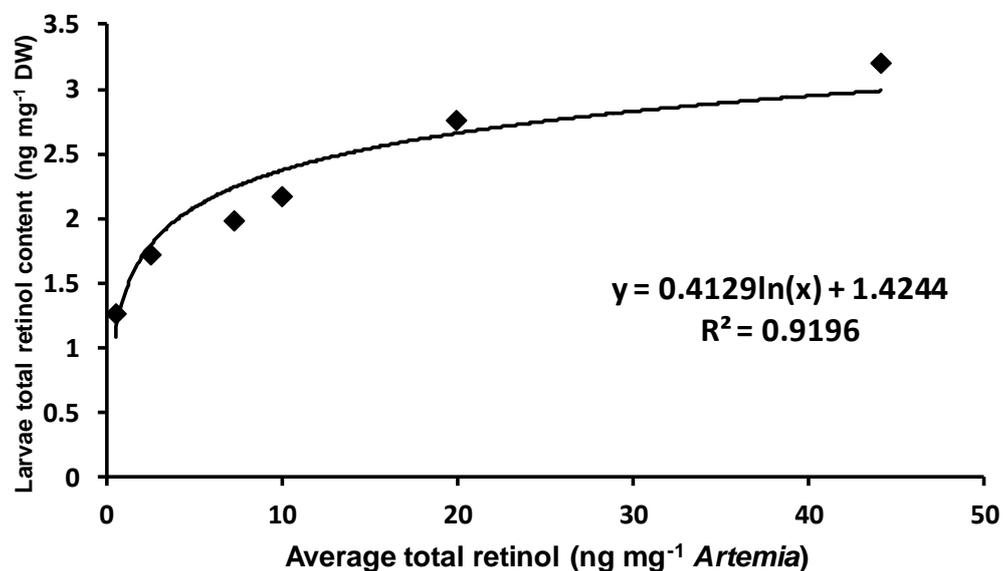


Figure 2: Logarithmic correlation between post-larvae total retinol content (ng mg<sup>-1</sup> DW) at 44 dph and total retinol content of the *Artemia* they were fed on ( $R^2 = 0.92$ ,  $P = 0.007$ ).

### 5.4.1.2 Lipid content of live prey

To determine if the lipid profile changed with different VA enrichments, *Artemia* from three treatments (0, 1558 and 3174 ng retinyl palmitate mg<sup>-1</sup>) were analysed. There was a significant difference in the eicosapentaenoic acid (EPA, 20:5 $\omega$ 3) and docosahexaenoic acid (DHA, 22:6 $\omega$ 3) content among each fresh rinsed dose and the corresponding cold-stored sample, where they increased by 11% and 14%, respectively in the cold-stored treatment. There was no significant difference in the arachidonic acid (ARA, 20:4 $\omega$ 6) and total fatty acid content among each fresh rinsed dose and the corresponding cold-stored sample. Statistical analysis among selected fatty acid groups and polyunsaturated fatty acids between fresh rinsed and cold stored *Artemia* are shown in Table 4.

For simplicity, and to reflect the overall diet provided to the larvae, averages of selected fatty acid groups and polyunsaturated fatty acids of fresh rinsed and cold stored feeds for the three VA treatments are shown in Table 5. There were no significant differences among the VA treatments in any of the fatty acid groups or polyunsaturated fatty acids except for the total monounsaturated fatty acid (MUFA) and total fatty acids (Total FA) where they were significantly higher in the 1558 than 3174 ng retinyl palmitate mg<sup>-1</sup> treatment, and intermediate in the 0 ng retinyl palmitate mg<sup>-1</sup> treatment.

Table 4: Results of two-way ANOVA to test the effects of treatment and storage on the lipid profile of enriched *Artemia*. Vitamin treatment has 3 doses of retinyl palmitate. Storage is fresh-rinsed and cold-stored.

	Source of variation	F value	d.f.	P value
<b>Total SFA</b>	Vitamin treatment	6.435	2	0.032*
	Storage	6.890	1	0.039*
	Vitamin treatment x Storage	2.776	2	0.140
<b>Total MUFA</b>	Vitamin treatment	7.732	2	0.022*
	Storage	0.295	1	0.606
	Vitamin treatment x Storage	3.117	2	0.118
<b>Total n:ω6</b>	Vitamin treatment	4.681	2	0.060
	Storage	3.949	1	0.094
	Vitamin treatment x Storage	2.363	2	0.175
<b>Total n:ω3</b>	Vitamin treatment	5.680	2	0.041*
	Storage	10.419	1	0.018*
	Vitamin treatment x Storage	1.536	2	0.289
<b>Total PUFA</b>	Vitamin treatment	5.378	2	0.046
	Storage	8.547	1	0.027*
	Vitamin treatment x Storage	1.748	2	0.252
<b>20:4ω6 (ARA)</b>	Vitamin treatment	8.348	2	0.018*
	Storage	5.850	1	0.052
	Vitamin treatment x Storage	1.991	2	0.217
<b>20:5ω3 (EPA)</b>	Vitamin treatment	6.865	2	0.028*
	Storage	11.099	1	0.016*
	Vitamin treatment x Storage	1.432	2	0.310
<b>22:6ω3 (DHA)</b>	Vitamin treatment	7.783	2	0.022*
	Storage	14.923	1	0.008*
	Vitamin treatment x Storage	1.360	2	0.326
<b>Total FA (mg g<sup>-1</sup>DW)</b>	Vitamin treatment	6.972	2	0.027*
	Storage	0.302	1	0.602
	Vitamin treatment x Storage	2.830	2	0.136
<b>22:6ω3 / 20:5ω3</b>	Vitamin treatment	17.824	2	0.003*
	Storage	34.131	1	0.001*
	Vitamin treatment x Storage	3.579	2	0.095

Table 5: Average of selected fatty acid groups and polyunsaturated fatty acids in *Artemia* ( $\text{mg g}^{-1}$  DW) enriched with 0, 1558 and 3174 ng retinyl palmitate  $\text{mg}^{-1}$ . Values are mean of 4 replicates  $\pm$  SD (2 from fresh feeds and 2 from cold stored). SFA = saturated fatty acid, MUFA = monounsaturated fatty acid, PUFA = polyunsaturated fatty acid and FA = fatty acid. Different letters within the same row show significant differences among dietary groups (ANOVA,  $P < 0.05$ ).

	Enrichment ( $\text{ng mg}^{-1}$ retinyl palmitate)					
	0		1558		3174	
Total SFA	32.80	$\pm$ 1.95	34.46	$\pm$ 3.26	30.45	$\pm$ 1.34
Total MUFA	64.93	$\pm$ 2.76 <sup>ab</sup>	68.80	$\pm$ 3.81 <sup>b</sup>	61.16	$\pm$ 3.16 <sup>a</sup>
Total n: $\omega$ 6	20.63	$\pm$ 0.69	22.36	$\pm$ 1.18	20.39	$\pm$ 1.67
Total n: $\omega$ 3	45.44	$\pm$ 3.26	51.60	$\pm$ 3.29	46.91	$\pm$ 4.86
Total PUFA	66.07	$\pm$ 3.86	73.96	$\pm$ 4.39	67.30	$\pm$ 6.51
20:4 $\omega$ 6	2.03	$\pm$ 0.10	2.34	$\pm$ 0.13	2.05	$\pm$ 0.20
20:5 $\omega$ 3	11.27	$\pm$ 0.80	13.10	$\pm$ 0.97	11.74	$\pm$ 1.33
22:6 $\omega$ 3	13.47	$\pm$ 1.42	16.25	$\pm$ 1.33	14.63	$\pm$ 1.95
Total FA ( $\text{mg g}^{-1}$ DW)	169.69	$\pm$ 5.71 <sup>ab</sup>	183.54	$\pm$ 9.30 <sup>b</sup>	164.22	$\pm$ 10.06 <sup>a</sup>
22:6 $\omega$ 3 / 20:5 $\omega$ 3	1.19	$\pm$ 0.42	1.24	$\pm$ 0.15	1.24	$\pm$ 0.28

## 5.4.2 Larval growth and survival

### 5.4.2.1 Larval development

*Latris lineata* larvae fed graded doses of retinyl palmitate during the *Artemia* feeding period were not affected by diet in terms of larval growth in total length (TL) (Table 6) or weight (Fig. 3) (ANOVA, DW 44 dph,  $F_{5,18} = 1.605$ ,  $P = 0.209$ ). Likewise, there was no effect of dietary VA on survival (Fig. 4) by the end of the experiment, 44 dph ( $F_{5,18} = 0.258$ ,  $P = 0.930$ ). Before starting the dietary treatments on 16 dph, average length was  $7.32 \pm 0.32$  mm and weight was  $0.31 \pm 0.06$  mg. By the end of the experiment, average length and weight of post-larvae on 44 dph were  $15.91 \pm 0.31$  mm and  $5.08 \pm 0.32$  mg, respectively, while the mean survival ranged from 22.6 to 29.9%.

Table 6: Larval growth in total length of *Latris lineata* larvae and post-larvae fed *Artemia* enriched with graded doses of retinyl palmitate. Values are mean  $\pm$  SD (n = 4).

Retinyl palmitate ng mg <sup>-1</sup> DW <i>Artemia</i>	Larval length (mm) within age (dph)			
	24 dph	30 dph	37 dph	44 dph
<b>0</b>	8.50 $\pm$ 0.23	10.44 $\pm$ 0.49	12.93 $\pm$ 0.28	16.20 $\pm$ 0.48
<b>5.67</b>	8.63 $\pm$ 0.08	10.66 $\pm$ 0.34	13.22 $\pm$ 0.19	15.78 $\pm$ 0.17
<b>6.60</b>	8.52 $\pm$ 0.28	10.53 $\pm$ 0.28	13.13 $\pm$ 0.35	15.97 $\pm$ 0.19
<b>27.10</b>	8.84 $\pm$ 0.16	11.03 $\pm$ 0.45	13.41 $\pm$ 0.37	15.90 $\pm$ 0.41
<b>29.58</b>	8.53 $\pm$ 0.19	10.67 $\pm$ 0.31	13.17 $\pm$ 0.19	15.66 $\pm$ 0.43
<b>54.67</b>	8.67 $\pm$ 0.10	10.78 $\pm$ 0.39	13.06 $\pm$ 0.74	15.98 $\pm$ 0.21
<b>ANOVA</b>	F <sub>5,18</sub> = 1.903, P = 0.144	F <sub>5,18</sub> = 1.180, P = 0.357	F <sub>5,18</sub> = 0.644, P = 0.669	F <sub>5,18</sub> = 1.200, P = 0.348

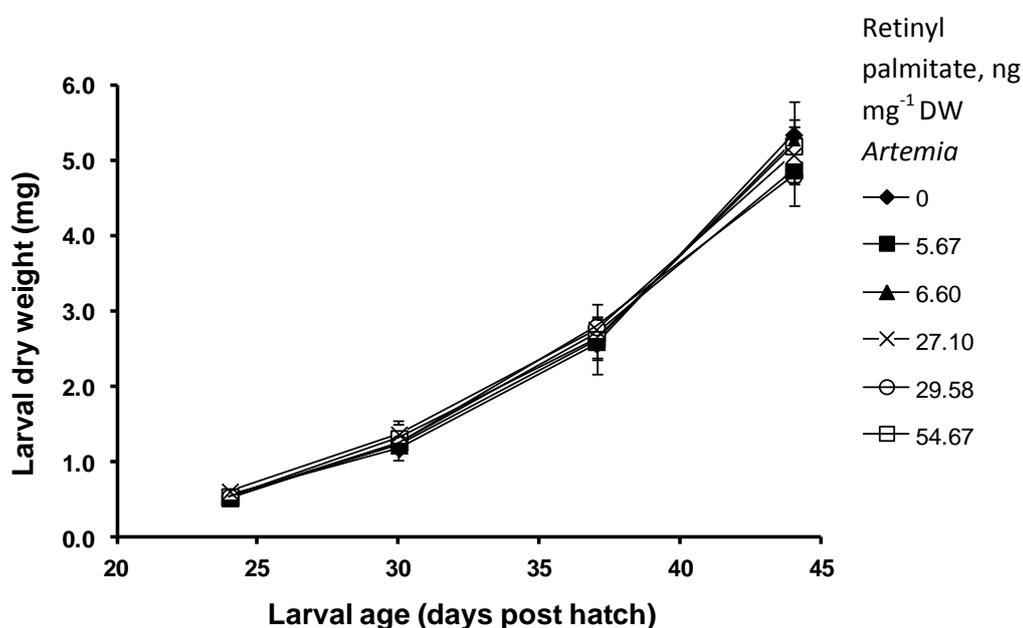


Figure 3: Increase in dry weight of *Latris lineata* larvae and post-larvae fed *Artemia* enriched with graded doses of retinyl palmitate. Values are mean  $\pm$  SD (n = 3 replicate tanks). No statistical differences among treatments (ANOVA, P > 0.05).

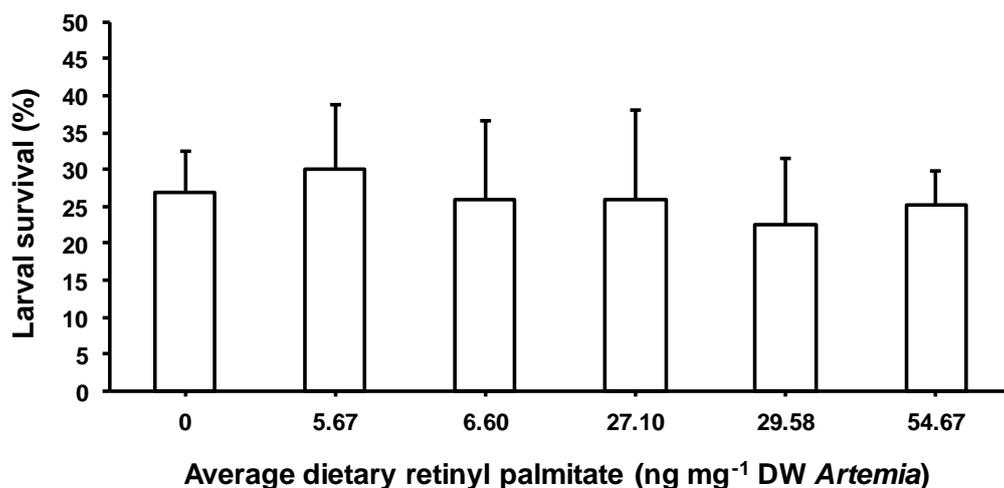


Figure 4: Survival of *Latris lineata* post-larvae from 16 to 44 dph fed *Artemia* enriched with graded doses of retinyl palmitate. Values are mean  $\pm$  SD (n = 4 replicate tanks). No statistical differences among treatments (ANOVA, P > 0.05).

#### 5.4.2.2 Jaw malformation

Different VA treatments did not significantly affect the incidence, severity or types of jaw malformation observed during the experiment (Table 7 and Fig. 5), (ANOVA, P > 0.05). The different types of jaw malformations observed were the same as those described in Chapter 3. Malformations appeared around 15 dph where incidence and severity continued to increase until the end of the experiment, although the incidence was not significantly different among treatments within age.

Short lower jaws were the most pronounced type of malformation with  $54 \pm 10\%$  across all treatments by the end of the experiment. Other lower jaw malformations included lower (dentary) bent up, or lower (dentary) bent right or left on 30 dph ( $2 \pm 2\%$ ) plus dentary thickened, abraded or twisted on 37 and 44 dph  $7 \pm 5\%$  and  $20 \pm 6\%$ , respectively. Open jaws were the second most common type of malformation among treatments with  $18 \pm 6\%$  on 44 dph. Different upper jaw (maxilla and premaxilla) malformations were the least observed by 44 dph ( $2 \pm 2\%$ ). No malformation was recorded for lowered hyoid arch by 44 dph.

Commercially important malformations (scores 1 + 2 + 3) were not affected by dietary VA, and were  $50 \pm 11\%$  across treatments at 44 dph (ANOVA,  $F_{5,18} = 1.182$ ,  $P = 0.356$ ) (Fig 5). Post-larvae with normal jaws (score 0) were  $22 \pm 7\%$  across treatments at 44 dph.

Table 7: Mean percentage of different types and time of occurrence (dph) of jaw malformations in *Latris lineata* larvae and post-larvae fed *Artemia* enriched with graded doses of retinyl palmitate. Four replicate tanks per treatment,  $n = 20$  on 24, 30 and 37 dph and  $n = 50$  on 44 dph. Values are mean  $\pm$  SD.

Larval age	Incidence of different jaw malformations				
	Lowered hyoid arch	Open jaw	Short lower jaw	Other lower jaw malformation	Upper jaw
24 dph	1 $\pm$ 5	1 $\pm$ 2	14 $\pm$ 7	0	0
30 dph	0	4 $\pm$ 4	46 $\pm$ 13	2 $\pm$ 2	0
36 dph	0	3 $\pm$ 4	56 $\pm$ 10	7 $\pm$ 5	5 $\pm$ 6
44 dph	0	18 $\pm$ 6	54 $\pm$ 10	20 $\pm$ 6	2 $\pm$ 2

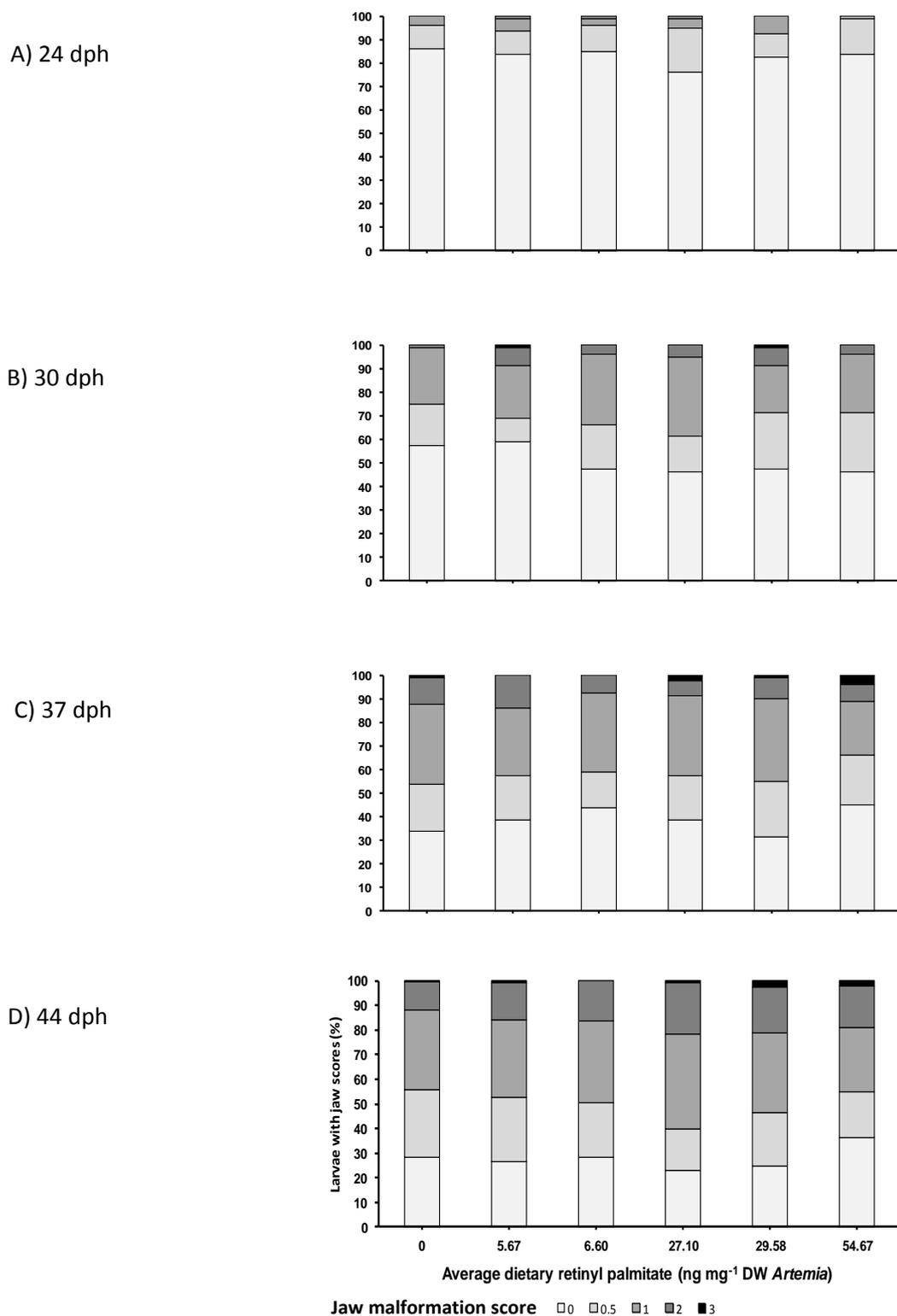


Figure 5: Incidence, severity and development of jaw malformation in *Latris lineata* larvae and post-larvae fed *Artemia* enriched with graded doses of retinyl palmitate. Bar shading represent jaw malformation index as shown in legend. Descriptions of the jaw scores are as in Chapter three. Four replicate tanks per treatment,  $n = 20$  larvae on 24, 30 and 37 dph and  $n = 50$  on 44 dph.

### 5.4.2.3 Vertebral column malformation

Different graded retinyl palmitate doses fed to larvae during the *Artemia* feeding period had no effect on vertebral column malformation (Fig. 6). Different types of vertebral malformations were observed and described as in Chapter 3. By 44 dph,  $58 \pm 4\%$  of the post-larvae across treatments showed malformation in at least one region of the vertebral column. There was no apparent pattern associated with the percentage of post-larvae showing malformation in any region of the vertebral column. Caudal and cranial regions were the most affected regions where the incidence was  $31 \pm 6\%$  and  $24 \pm 8\%$ , respectively. Malformation in the haemal region was lowest with an average of  $7 \pm 3\%$  of post-larvae affected across treatments.

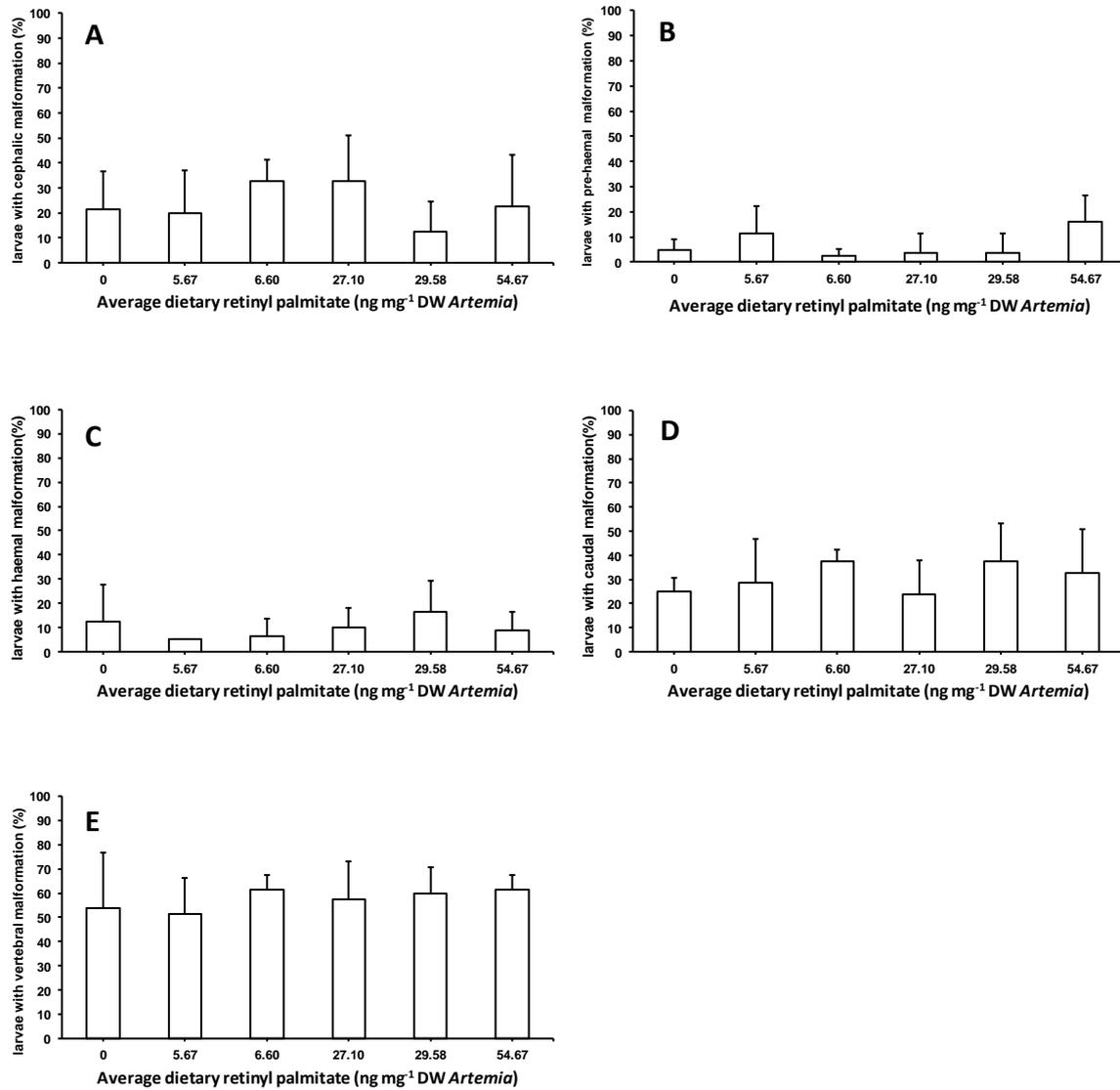


Figure 6: Percentage of skeletal malformations in *Latris lineata* post-larvae at 44 dph fed *Artemia* enriched with graded doses of retinyl palmitate, in A) cephalic (first two vertebrae) B) pre-haemal C) haemal and D) caudal complex regions of vertebrae and E) vertebral malformation in any region. Values are mean (%) + SD. Four replicate tanks, n = 20 larvae per tank. No statistical differences among treatments (ANOVA,  $P > 0.05$ ).

## 5.5 Discussion

Retinol and retinoic acid increased significantly in the enriched *Artemia* in parallel to the increasing doses of retinyl palmitate in the emulsions, which indicates that the *Artemia* were able to absorb, digest and metabolise retinyl palmitate in the emulsions as shown in other studies (Fernández et al., 2009; Giménez et al., 2007; Takeuchi et al., 1998; Tarui et al., 2006). My study utilised a similar 24 h method of enrichment of *Artemia* as described by Giménez et al. (2007). Although, the preliminary results (Chapter 2) showed that the concentration of total VA is significantly higher after 3 h of enrichment and that no significant difference in total VA content in *Artemia* occurs after 12, 18, 22 or 24 h of enrichment, *Artemia* were enriched for 24 h to ensure the lipid requirements of *L. lineata* were met (Bransden et al., 2004, 2005a).

*Artemia* cysts do not contain any VA, however, they have carotenoids in the form of canthaxanthin which is a source of VA (Olson, 1989; Stappen., 1996). Fish are able to convert canthaxanthin into  $\beta$ -carotene where  $6 \mu\text{g } \beta \text{ carotene} = 1 \mu\text{g retinol}$  (Olson, 1989; Ross and Ternus, 1993). In dehydrated cysts, the average cis and trans- canthaxanthin were  $152 \pm 52$  and  $134 \pm 42 \text{ ng mg}^{-1}$ , respectively while the corresponding concentration in hydrated cysts were  $177 \pm 60$  and  $112 \pm 35 \text{ ng mg}^{-1}$ , respectively (Nelis et al., 1987). Cis to trans- canthaxanthin ratio is associated with physical and biological characteristics of the cysts (Nelis et al., 1987). Although the concentration of canthaxanthin in the *Artemia* cysts was not measured in the current study, its contribution to the total VA in the enriched *Artemia* is not significant compared with the doses of enrichment.

Spinal malformation in *L. lineata* larvae or post-larvae has been linked to non-inflation of the swim bladder and parasites (Trotter et al., 2005; Grosse, 2003). There is no published data on the percentage of occurrence of spinal malformations despite considerable research on lipid and vitamin requirements. Accordingly, the VA experiments provide the first detailed information on the prevalence of vertebral malformation in cultured *L. lineata* post-larvae. The retinoid content of *L.*

*lineata* at 44 dph was positively correlated with the total retinol content in the *Artemia* they fed on, where it increased significantly with increasing dietary total retinol. However, this increase in the larval retinoid content did not appear to affect the skeletal malformation in any region of the vertebral column. By the end of the study, the average of all vertebral malformations was  $58 \pm 4\%$  across treatments. This is in contrast to studies on other fish species where increased VA has been correlated to increased skeletal malformation. For example, in Red Sea Bream, *Chrysophrys major*, *D. labrax*, *P. olivaceus* and Senegalese sole, *Solea senegalensis*, the prevalence of skeletal malformation increased with increasing VA concentration in the diet being 61, 80, 86, 100% for the highest treatment, respectively (note VA from and level are not the same among different studies) (Fernández et al., 2009; Hernandez-H et al., 2006; Tarui et al., 2006; Villeneuve et al., 2005).

The different results of the current study with other studies in relation to malformations can possibly be explained by the developmental stage at which dietary VA was delivered. A higher percentage of *L. lineata* post-larvae developed a normal vertebral column by 43 dph when fed VA enriched rotifers from 6 to 18 dph (69%, Chapter 3) compared with only 43% in the current study, despite the fact that VA dose in Chapter 3 was twice as high (retinyl palmitate  $\geq 108.56 \text{ ng mg}^{-1} \text{ DW}$ ;  $\geq 173,696 \text{ IU VA kg}^{-1}$ ) as the maximum examined in the current study with *Artemia* (retinyl palmitate =  $54.67 \text{ ng mg}^{-1} \text{ DW}$ ; =  $87,472 \text{ IU VA kg}^{-1}$ ). Increasing VA in *Artemia* did not affect the vertebral column development possibly because the larvae from 17 to 44 dph were past a critical point in their bone development. Differentiation of the vertebrae in *L. lineata* commences in the cephalic region around 7 mm SL and progresses toward the caudal vertebrae by 11 mm SL, and ossification of the vertebrae commences in the cephalic region around 11mm SL (Cobcroft, unpublished data). Before starting the dietary treatments on 16 dph, average length was  $7.32 \pm 0.32 \text{ mm}$  suggesting that vertebral development in larvae is susceptible to dietary VA during the rotifer feeding. Vitamin A requirements for normal skeletal development depends upon the developmental stage (Haga et al. 2002a; Hernandez-H et al., 2006; Hernandez et al.,2004). Haga et al. (2002a) found that, the effect of VA on the mandible (jaw),

caudal and vertebral malformations of *P. olivaceus* is significantly higher when the larvae were fed with VA from 4 to 8 dph (rotifer feeding period) than from the *Artemia* feeding period (8 to 37 dph). Vitamin A affected the skeletal development during the larval stage (from 6 to 20 dph) but not the juveniles of *C. major* (Hernandez-H et al., 2006; Hernandez et al., 2004). The results for *L. lineata* demonstrate that larvae are more sensitive to the VA treatment when they are younger, during the differentiation stage of skeletal elements. In relation to the overall high prevalence of vertebral malformation by 44 dph (58%), the transfer of the larvae on 16 dph from the 3000 l tank to the experimental tanks may be a potential reason for the high incidence of vertebral malformation, as the larvae were likely stressed by the handling process, although vertebral malformation was not observed in another experiment with a similar transfer (Cobcroft and Battaglione, 2009). Therefore, it is more likely that nutritional requirement(s) of the larvae were not met by the VA-enriched *Artemia*, as larvae in the Cobcroft and Battaglione (2009) were all fed Algamac-3050 enriched *Artemia* and there was lower (or no) vertebral malformation found.

Jaw malformations are a major concern in the culture of *L. lineata*, they can be detected as early as 9 dph (Battaglione and Cobcroft, 2007; Cobcroft et al., 2001; Cobcroft and Battaglione, unpublished data). The current study found that while severity increased with age (from  $5 \pm 2\%$  on 24 dph to  $50 \pm 6\%$  on 44 dph), dietary intake of retinyl palmitate during the larval and post-larval stages of *L. lineata* did not affect the severity of jaw malformation. However, VA affects the formation of different skeletal elements including the jaw and different mouth parts in other marine fish such as *D. labrax*, *P. olivaceus*, *S. aurata* and *P. dentatus* (Fernández et al., 2008; Haga et al., 2002a; Martinez et al., 2007; Mazurais et al., 2009; Suzuki et al., 2000; Villeneuve et al., 2005). Unlike other species, jaw malformation in *L. lineata* larvae and post-larvae was not affected by increasing doses of retinyl palmitate in the *Artemia*. The lack of any effect in spite of incremental increases in total VA indicate that *L. lineata* jaw malformation was not affected by VA within the range tested in the *Artemia* feeding stage. Likewise, in Chapter 3, increasing doses of retinyl palmitate did not affect jaw

malformation in *L. lineata* larvae fed VA at an earlier developmental stage in enriched rotifers. Accordingly, VA does not appear to affect the onset or severity of the jaw malformations in *L. lineata* larvae and post-larvae within the dietary concentrations tested. It is likely that physical factors, rather than nutritional ones, are the main causative factors in the jaw malformation in *L. lineata* larvae and post-larvae, as described by Cobcroft and Battaglione (2009), where jaw malformation was correlated with walling behaviour that was affected by tank wall colour (see Chapter 3 for details).

Although increasing retinyl palmitate doses in rotifers did not affect jaw malformation in *L. lineata* larvae and post-larvae (Chapters 3 and 4), the percentage of post-larvae with commercially accepted jaws (score 0 + 0.5) was 50% on 44 dph in the current study compared with 19% on 43 dph in Chapter 3. The better jaw development in the current study is attributed to the earlier rearing being conducted at a lower stocking density in larger tanks with marbled colour walls. In the rotifer experiment (Chapter 3), yolk sac larvae were stocked into 300 l tanks with black walls from 1 dph at a density of 10 larvae l<sup>-1</sup>. In the current *Artemia* study, 2 dph yolk sac larvae were reared in 3000 l tanks and transferred to the 300 l tanks on 16 dph at density of 2 larvae l<sup>-1</sup>. It is likely that larvae in the current study came into less contact with the 3000 l tanks, due to the higher volume to surface area ratio and the marble background that modified 'walling' behaviour (Cobcroft and Battaglione, 2009).

*Latria lineata* post-larval survival and growth were not affected when the larvae were fed increased retinyl palmitate doses from 0 to 54.67 ng mg<sup>-1</sup> DW *Artemia* during the *Artemia* feeding period (54.67 ng mg<sup>-1</sup> is equivalent to 87,472 IU VA kg<sup>-1</sup> *Artemia*). Similar results have been described for *S. senegalensis* and Summer Flounder, *Paralichthys dentatus* (Fernández et al., 2009; Martinez et al., 2007). On the contrary, increased VA has been shown to affect survival and growth in other species e.g., *S. aurata*, Tilapia, *Oreochromis niloticus* and *C. major* (Fernández et al., 2008; Hernandez-H et al., 2006; Saleh et al., 1995). In *S. aurata*, feeding retinyl palmitate at 66.7 and 100.3 ng mg<sup>-1</sup> DW

rotifers (106,720 and 160,480 IU VA kg<sup>-1</sup> rotifers, respectively) during the rotifer feeding period, improved both survival and growth. While doses of retinyl palmitate as low as 29 ng mg<sup>-1</sup> diet (95,700 IU VA kg<sup>-1</sup> extracted diet) in *C. major* larvae improved growth and 5,000 IU VA kg<sup>-1</sup> in *O. niloticus* diet improved both survival and growth. The difference in the results among species demonstrates the species-specific nature of the effects of VA.

Similarly increasing VA doses during the rotifer feeding period (Chapter 3) did not affect *L. lineata* growth in length or weight and survival. In Chapter 3, average larval weight was 4.6 mg DW on 43 dph compared with 5.1 mg DW on 44 dph in the current study. The increase in the larval weight during the current study could be attributed to the lower survival and consequently differential survival of larger larvae and/or lowering stocking densities that increased access of survivors to food, facilitating higher growth. Survival was 26% from 16 to 44 dph and the average number of post-larvae per tank was 116 when larvae were fed VA-enriched *Artemia*, compared with 35% survival from 1 to 43 dph and 864 post-larvae per tank in larvae fed VA-enriched rotifer fed larvae (Chapter 3). A potential reason for the lower survival in the current study was that the VA-enriched *Artemia*, as explained previously, may not have met larval nutritional requirement(s), in this case to optimise survival or that PUFA levels are more determinant than VA.

## 5.6 Conclusion

*Latris lineata* retinoid content was positively correlated with the retinoid content in the *Artemia* they fed on. Increasing post-larval retinoid content did not affect the skeletal malformation in any region of the vertebral column, suggesting larvae are more sensitive to retinoids during the early developmental stages (rotifer-feeding). Increased dietary doses of retinyl palmitate during the *Artemia* feeding had no effect on *L. lineata* growth, survival, incidences and severity of jaw malformations. This study supports the hypothesis that nutritional (i.e., PUFA) and other environmental factors associated with walling behaviour, rather than VA nutrition, have an impact

on jaw malformations in *L. lineata*. Half of the post-larvae had normal or minor jaw malformation, appropriate for commercial purposes by the end of the experiment, higher than in similar studies, attributed to rearing in larger tanks from 2 to 16 dph, which decreased walling behaviour.

## 5.7 References

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# **Chapter 6**

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## **General discussion**

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## 6.1 Introduction

My study examines the effects of increasing doses of vitamin A (VA) through enrichment of live feeds, during two different larval developmental stages of Striped Trumpeter, *Latris lineata*; from first-feeding through to mid-flexion (rotifer feeding period, 6 to 18 days post hatch, dph) and mid-flexion through to post-larvae (*Artemia* feeding period, 17 to 44 dph). To my knowledge this has not been achieved in any one study for any other marine finfish. The specific aims of my study were to investigate the effects of VA on skeletal development, especially jaw malformation which has been a bottleneck in hatchery production, to determine suitable doses of VA for inclusion in live feeds and to determine if there is a critical window during development where the effect of VA is most pronounced.

The study first examined the enrichment characteristics of the live feeds, rotifers and *Artemia*, with VA and showed them to differ with enrichment time and with abiotic factors including light (Chapter 2). Rotifers displayed a higher retinoid inclusion pattern than *Artemia* for the same enrichment dose and it was technically difficult to maintain the same VA dose in both live feed species. Consequently, the effect of VA on *L. lineata* larvae was examined separately in rotifer and *Artemia* feeding periods using a similar dose-response approach in two experiments, with different ranges of dietary VA constrained by the live prey inclusion (Chapters 3 and 5). The effect of greenwater (microalgae) on the VA content of rotifers was found to be a potentially confounding factor and was examined in further detail (Chapter 4). To my knowledge this is the first study to investigate the effect of greenwater on VA requirements, despite greenwater being commonly used in marine finfish culture.

In Chapter 6 the results of my study are put into context with other studies, including the difficulty in management of lipid and vitamin enrichment of live feeds, the incorporation of retinoids into larvae and the effects on skeletal development. The chapter concludes with the constraints and limitations of the study and future directions for research.

## 6.2 Other studies with finfish larvae and dietary vitamin A

Despite the considerable amount of published work on VA, most studies have used a formulated diet to examine the effect of VA on finfish larvae (Georga et al., 2011; Hemre et al., 2004; Hernandez-H et al., 2006; Mazurais et al., 2009; Moren et al., 2004; Villeneuve et al., 2005; Yang et al., 2008).

Published studies on the effect of VA through enrichment of live feeds, especially rotifers (one paper), are less common (Dedi et al., 1998; Fernández et al., 2008, 2009; Takeuchi et al., 1998; Tarui et al., 2006). Clearly, it is easier to design and conduct requirement studies using formulated diets, relative to live feeds, because the formulated diet can be designed to contain a known concentration of VA. In addition, the VA compounds remain more stable and are not metabolized into other forms, being protected from light and heat which can cause VA denaturation (Barua and Furr, 1998).

Further advantages of formulated feeds are the lower resources and manpower required to run the experiments. Unfortunately, in common with many other marine fish, formulated diets have not been developed for *L. lineata* larval stages and they require live feeds. Live feeds need to be mass-cultured, enriched and fed to larvae daily.

Fernández et al. (2008) was the first to examine the dose effect of VA during the rotifer feeding period, with four increasing VA doses, on the performance of marine finfish larvae, Gilthead Sea Bream, *Sparus aurata*. Earlier studies had investigated one dose of VA fed to Japanese Flounder, *Paralichthys olivaceus* larvae through enrichment of rotifers (10 mg retinoic acid and 50,000 IU VA l<sup>-1</sup> enrichment) (Haga et al., 2002a; Miki et al., 1990). In Haga et al. (2002a), the effect of the same single VA dose during different developmental stages, from post-embryonic to metamorphosis, was examined. In the study, VA enriched rotifers were fed to *P. olivaceus* from 4 to 8 dph only. While Miki et al. (1990) examined the effect of different vitamins, including VA, on the reduction of albinism. Consequently, the present study is only the second to investigate the effect of graded VA doses during the rotifer feeding period. It is also the only study to examine eight doses of VA (as retinyl palmitate) in a dose-response experimental design.

### 6.3 Managing lipid and vitamin requirements

Unlike other marine finfish larvae, such as European Sea Bass, *Dicentrarchus labrax*, Red Sea Bream, *Chrysophrys major* (*Pagrus major*), and *S. aurata*, the current rearing method for *L. lineata* larvae is reliant upon live feeds, rotifers and then *Artemia* until 40 dph, when formulated diet starts and live feeds are fed until 50 dph (Battaglione and Cobcroft, 2007, 2010; Choa et al., 2010). Hence, to study the effect of VA from first-feeding in *L. lineata* larvae, enriched rotifers were essential. Chapter 2 revealed that rotifer enrichment with VA is optimal after 2 h. However, this relatively short enrichment period was not adequate for the incorporation of essential fatty acid in the rotifers to meet the requirements of *L. lineata* larvae, that are higher than many other marine finfish larvae (Chapters 2 and 3) (Bransden et al., 2003, 2004, 2005b). To meet the conflicting requirements for enrichment of lipids and dosed VA in rotifers two different sources of enriched rotifers were fed: VA dosed rotifers in the morning and lipid enriched rotifers in the afternoon (Chapter 3 and 4). By separating the enrichments, accurate measured doses of VA were fed to the larvae and they received adequate lipid nutrition. As discussed in Chapter 3 there is the possibility that feeding two sources of rotifers confounded the studies and may have masked the effects of VA. This would be the case if larvae differentially fed on the two sources of rotifers. The evidence available suggests that this was unlikely (see Chapter 3 and next section).

## 6.4 Incorporation of retinoids into larvae

My study is the first to quantify VA concentration in marine fish larvae fed increased doses of VA during the rotifer feeding period. Table 1 presents, to the best of my knowledge, all the published studies that measured VA in fish larvae. The main technical challenge in determining VA content in the larvae is their small size and difficulty in obtaining large numbers of larvae (sample quantity). Although two VA extraction methods were used to quantify the retinoids in the experimental emulsions and the live feeds in the current study, only one was used to determine the VA concentration in the larvae because there was an insufficient quantity of larvae (weight) in the samples to use the alternative extraction method. Different studies have used different extraction methods to quantify VA in the larvae fed *Artemia* or formulated diets; this is reflected in the way the larval VA concentration is reported (Table 1). Despite the differences in analytical methods, it appears that the concentration of VA increases in the larvae with increasing dietary VA, irrespective of the dietary route. However, the retinoid concentration in *L. lineata* larvae on 16 dph, following 10 days of feeding on rotifers incorporating 1,716 to 305,382 IU VA kg<sup>-1</sup>, showed a different trend, where the concentration of VA was lower, 5.71 ng retinol mg<sup>-1</sup> DW for the larvae fed on high dietary VA rotifers (> 53,823 IU VA kg<sup>-1</sup>) than at intermediate dietary VA (Chapter 3) (Table 1). The reason VA concentration in the larvae did not increase with increasing VA doses in the enriched rotifers is unclear. The possibility that the afternoon feed (Algamac-3050 enriched rotifers) masked the effect of VA in the high treatments is unlikely given that this was not observed in the larvae fed on lower treatments (3,069 and 7,656 IU VA kg<sup>-1</sup>) that incorporated an average of 11.08 ng retinol mg<sup>-1</sup> DW, which was 94% higher than the larvae fed on rotifers incorporating > 53,823 IU VA kg<sup>-1</sup>. In addition, there was no significant difference in the larval growth or survival between the treatments, which implies that there was no palatability preference for rotifers with different VA content among the larvae. A potential reason for this bell shaped incorporation of VA into the larvae is regulation of the proteins responsible for VA metabolism, although this needs further investigation. Nevertheless, it is

worth pointing out that although this is the first study to use the greenwater technique in an investigation of dietary VA (Table 2), the carotenoids in the live algae did not affect the VA content in the larvae (Chapter 4). Further research is needed to investigate the apparent disconnection between the VA concentrations in larval tissue and the rotifers on which they were fed to determine if it is a species-specific issue, related to the method of rotifer enrichment or a masking effect of using two sources of enriched rotifers. Of note, this issue was not apparent when *L. lineata* larvae were fed increased doses of VA through *Artemia*, where the concentrations of retinoids in the post-larvae were positively correlated with the doses of VA in the enriched *Artemia* they fed on (Chapter 5) (Table 1).

Table 1: Comparison of vitamin A content of marine finfish larvae fed vitamin A (VA) in fortified diets or live feeds. Studies are arranged in order of wet weight of larvae analysed for VA composition. Modified from Dedi et al. (1995), Fernández et al. (2009), Hernandez-H et al. (2006), Villeneuve et al. (2005), Chapters 3 and 5.

Species Age (dph) Reference	Dietary route VA Form	Age (dph) <sup>o</sup>	Larval weight (mg)	Experimental emulsion or formulated diet nominal concentration (IU VA kg <sup>-1</sup> )	Extracted experimental emulsion (IU VA kg <sup>-1</sup> )	Enrichment dose in live feed Total VA (ng mg <sup>-1</sup> DW*)	Extracted formulated diet or live feed (IU VA kg <sup>-1</sup> )	Larval VA content (ng mg <sup>-1</sup> DW)			IU VA g <sup>-1</sup> larvae
								Retinyl palmitate	Retinol	Retinoic acid	
<i>Chrysophrys major</i> 5 to 20 Hernandez-H et al., 2006	Formulated diet (retinyl palmitate)	20	0.028	2,700			95,700 <sup>#</sup>			0.20	
			0.025	27,000	NA	NA	907,500	NA	NA	0.26	NA
			0.019	165,000			5,685,900			0.53	
<i>Latris lineata</i> 6 to 18 Chapter 3	Rotifers (retinyl palmitate)	16		0	0	6.94	1,716 <sup>#</sup>		4.92		
				160,000	109,120	11.38	3,069		11.74		
				480,000	299,648	19.04	7,656		10.42		
				1,200,000	851,440	33.75	22,011		9.56		
				2,400,000	2,243,712	68.95	53,823	NA	5.96	NA	NA
				4,800,000	4,272,128	123.23	114,708		5.66		
				8,000,000	7,693,360	240.31	174,009		5.93		
<i>Solea senegalensis</i> 6 to 27 Fernández et al., 2009	<i>Artemia</i> (retinyl palmitate)	15		1,666,000	2,112,000 <sup>o</sup>	11.1		7.5	0.70		
				3,333,000	3,344,000	13.4		9.0	0.70	NA	NA
				7,000,000	7,200,000	24.8	NA	9.0	0.80		
				13,333,000	20,656,000	60.9		15.0	0.90		
<i>Latris lineata</i> 17 to 44 Chapter 5	<i>Artemia</i> (retinyl palmitate)	44		0	0	1.19	1,452 <sup>#</sup>		1.27		
				1,200,000	1,271,520	7.20	8,019		1.73		
				2,400,000	2,492,016	9.56	23,694	NA	1.99	NA	NA
				4,800,000	5,078,832	31.74	32,703		2.18		
				8,000,000	7,857,392	39.79	65,505		2.76		
	16,000,000	16,828,560	68.97	145,398		3.21					

Species Age (dph) Reference	Dietary route VA Form	Age (dph) <sup>Ø</sup>	Larval weight (mg)	Experimental emulsion or formulated diet nominal concentration (IU VA kg <sup>-1</sup> )	Extracted experimental emulsion (IU VA kg <sup>-1</sup> )	Enrichment dose in live feed Total VA (ng mg <sup>-1</sup> DW*)	Extracted formulated diet or live feed (IU VA kg <sup>-1</sup> )	Larval VA content (ng mg <sup>-1</sup> DW)			IU VA g <sup>-1</sup> larvae
								Retinyl palmitate	Retinol	Retinoic acid	
<i>Paralichthys olivaceus</i> 10 to 40 Dedi et al., 1995	<i>Artemia</i> (retinyl palmitate)	40	87.3	0**			1,300		3.82 <sup>£</sup>		12.6
			91.4	2,000			45,200		4.42		14.6
			83.0	4,000	NA	NA	416,300	NA	10.12	NA	33.4
			80.8	6,000			585,500		10.94		36.1
			70.0	8,000			840,100		14.09		46.5
			54.0	10,000			1,282,900		16.76		55.3
<i>Dicentrarchus labrax</i> 7 to 42 Villeneuve et al., 2005	Formulated diet (retinyl acetate)	42	120	0***			39,600 <sup>#</sup>	350 <sup>¥</sup>	180 <sup>¥</sup>		
			150	5,000			42,900	350	180		
			160	25,000	NA	NA	102,300	350	180	NA	NA
			125	125,000			204,600	600	180		
			110	500,000			646,800	1100	300		

Ø, larval age at analysis,

\* Total VA include retinyl palmitate, retinol and retinoic acid,

#, changed to IU based on the relationship 1 µg retinol = 3.3 IU,

Ø, emulsion also contained retinol ranging from 0.0051 to 0.013 µg mg<sup>-1</sup> which was not included in the IU calculation (based on retinyl palmitate only),

\*\* these values are IU l<sup>-1</sup> enrichment,

£, values are calculated from IU,

\*\*\* values based on retinyl acetate equivalent to 500,000 USP units g<sup>-1</sup>,

¥, values are ng mg<sup>-1</sup> wet weight larvae,

dph indicates days post-hatch, NA indicates information not available.

## 6.5 Effect of vitamin A on skeletal development

Vitamin A has a significant and well documented role in the development of vertebrate skeletons (details in Chapter 1). Excess or deficiency of VA in fish larvae results in development of skeletal malformation in various parts of the skeleton including the skull, vertebral column, caudal fin and operculum (Table 2). My study found that dietary VA in live feeds affected the prevalence of skeletal malformations in *L. lineata* post-larvae in the vertebral column but not the jaws of *L. lineata* larvae or post-larvae (Chapters 3 and 5). Furthermore, the prevalence of vertebral column malformation was correlated with the larval retinoid content during the rotifer feeding period and was lower in the higher treatments (Chapter 3). I found that the daily requirement of dietary VA for *L. lineata* larvae to minimize the vertebral column malformations (to approximately 30%) was  $\geq 123$  ng total VA  $\text{mg}^{-1}$  DW rotifer, which is equivalent to  $\geq 35$  ng retinol  $\text{mg}^{-1}$  DW rotifer (Chapter 3). I also found that the water type did not affect the retinoid incorporation into the larvae, and accordingly, the prevalence of vertebral malformation was not affected by the water type. In contrast, the severity of jaw malformation was significantly reduced in greenwater (Chapter 4).

VA requirements for normal skeletal development vary greatly among different fish species. The United States National Research Council reported that the VA requirements for Rainbow Trout, *Oncorhynchus mykiss*, Channel Catfish, *Ictalurus punctatus*, Common Carp, *Cyprinus carpio* and Pacific Salmon, *Oncorhynchus* sp., are between 1,000 and 4,000 IU VA  $\text{kg}^{-1}$  (National Research Council, 1993). While the optimum level of VA in *Artemia* to minimize skeletal malformations in Senegalese Sole, *Solea senegalensis* and *P. olivaceus*, is < 44, 666 and 50,000 IU VA  $\text{kg}^{-1}$  DW, respectively (Dedi et al., 1995; Fernández et al., 2009). In Atlantic Halibut, *Hippoglossus hippoglossus*, Tilapia nilotica, *Oreochromis niloticus*, and Greasy Grouper, *Ephinephelus tauvina*, dietary incorporation of 8,333, 5,000 and 3,101 IU VA  $\text{kg}^{-1}$  DW, respectively, is the optimum for normal growth (Mohamed et al., 2003; Moren et al., 2004; Saleh et al., 1995).

Table 2: Selected published studies demonstrating different experimental designs examining the effect of VA on various marine finfish species. Different colours represent different dietary routes; Yellow, rotifers; Green, *Artemia*; Blue, formulated diet; Red, exposure in rearing water.

Fish species	Dietary VA (IU VA kg <sup>-1</sup> )	Larval rearing parameters					Larval performance			Skeletal malformation				References
		Duration of exposure (dph)	Photoperiod	Temperature (°C)	Light intensity	Size of vessel (l)	Water type	Growth (length and weight)	Survival	Cranial <sup>1</sup>	Operculum	Caudal fin	Vertebral column	
Vitamin A form, retinyl palmitate														
Gilthead Sea Bream, <i>Sparus aurata</i>	106,720 to 1,091,040*	4 to 20	12 L : 12 D	18 - 19	500 lx	100	CW	↑	↑	↑	↑	↑	↔	Fernández et al., 2008
Striped Trumpeter, <i>Latris lineata</i>	0 to 620,720*	6 to 18	16 L : 8 D	15.5 - 16.5	10.56 μmol s <sup>-1</sup> m <sup>-2</sup>	300	GW	no effect	no effect	no effect <sup>2</sup>	NA	no effect <sup>4</sup>	↔	Chapter 3
Striped Trumpeter, <i>Latris lineata</i>	0 to 708,800*	6 to 19	16 L : 8 D	15.1 to 16.3	6.44 μmol s <sup>-1</sup> m <sup>-2</sup>	300	CW and GW	no effect <sup>3</sup>	no effect <sup>3</sup>	no effect <sup>3</sup>	NA	no effect <sup>4</sup>	no effect <sup>4</sup>	Chapter 4
Senegalese Sole, <i>Solea senegalensis</i>	11,200 to 64,000*	6 to 37	12 L : 12 D	17 - 19	500 lx	100	CW	no effect	no effect	no effect	↑ <sup>5</sup>	↑	↑	Fernández et al., 2009
Japanese flounder, <i>Paralichthys olivaceus</i>	0 to 10,000***	27 to 31	NA	17.1 - 19.5	1,059 - 1,870 lx	100	CW	NA for weight but length ↓	no effect	NA	NA	↑	↑	Tarui et al., 2006
Striped Trumpeter, <i>Latris lineata</i>	0 to 87,472*	17 to 44	24 L	16.2 to 16.6	10.6 μmol s <sup>-1</sup> m <sup>-2</sup>	300	CW	no effect	no effect	no effect	NA	no effect <sup>5</sup>	no effect <sup>6</sup>	Chapter 5

Fish species	Dietary VA (IU VA kg <sup>-1</sup> )	Larval rearing parameters				Larval performance				Skeletal malformation				References
		Duration of exposure (dph)	Photoperiod	Temperature (°C)	Light intensity	Size of vessel (l)	Water type	Growth (length and weight)	Survival	Cranial <sup>1</sup>	Operculum	Caudal fin	Vertebral column	
Japanese flounder, <i>Paralichthys olivaceus</i>	1,300 to 1,282,900	10 to 40	12 L : 12 D	18.3 ± 0.2	1,000 lx	100	CW	NA	NA	NA	NA	↑	NA	Dedi et al., 1998
Red Sea Bream, <i>Chrysophrys major</i>	95,700 to 5,685,900**	5 to 20	11 L : 13 D	19.3 to 20.7	NA	30	CW	No effect for total length but weight ↓ <sup>7</sup>	no effect	Low incidence	NA	NA	↑	Hernandez-H et al., 2006
Vitamin A form, retinyl acetate														
European Sea Bass, <i>Dicentrarchus labrax</i>	39,600 to 646,800**	7 to 42	24 L	20	9 W m <sup>-2</sup>	35	CW	↔	↔	↑	↑	NA	Low incidence (less than 8%)	Villeneuve et al., 2005
European Sea Bass, <i>Dicentrarchus labrax</i>	3,152 to 155,200**	9 to 45	24 L	20	9 W m <sup>-2</sup>	35	CW	↔	no effect	↔	NA	↔	↓	Mazurais et al., 2009
Vitamin A form, retinoic acid														
Red Sea Bream, <i>Chrysophrys major</i>	32 to 1,640 <sup>‡</sup>	5 to 20	11 L : 13 D	19.3 to 20.7	NA	30	CW	↓	↔	Low incidence	NA	NA	↑	Hernandez-H et al., 2006

Fish species	Dietary VA (IU VA kg <sup>-1</sup> )	Larval rearing parameters				Larval performance				Skeletal malformation				References
		Duration of exposure (dph)	Photoperiod	Temperature (°C)	Light intensity	Size of vessel (l)	Water type	Growth (length and weight)	Survival	Cranial <sup>1</sup>	Operculum	Caudal fin	Vertebral column	
summer flounder, <i>Paralichthys dentatus</i>	Water containing 0 to 20 nM retinoic acid	10 days exposure	12 L : 12 D	21 to 23	600 lx	20	CW	no effect	no effect	no effect <sup>£</sup> ϕ	NA	↔ <sup>ϕ'</sup> ϕ	↔ <sup>ϕ</sup>	Martinez et al., 2007

CW, clearwater; GW, greenwater; NA, not available.

1, cranial malformation includes jaw, 2, jaw malformation was 82% on 43 dph, 3, greenwater affected growth, survival and cranial malformation, 4, vertebral column and caudal complex malformations were not affected by the range of VA used, 5, opercular malformation appeared only in larvae fed *Artemia* incorporating 64,000 IU VA kg<sup>-1</sup>, 6, effect of VA on vertebral column malformation was dependent on the stage of larval development, 7, weight was significantly less in larvae fed diet containing 172,300 IU VA kg<sup>-1</sup>.

\* Values represent retinyl palmitate in the enriched live feed (retinol and retinoic acid not included), \*\* extracted formulated diet, \*\*\* IU l<sup>-1</sup> enrichment.

¥, values represent ng retinoic acid mg<sup>-1</sup> diet; £, retinoic acid exposure increased overall frequency and severity of jaw malformations, but no dose response was observed; ϕ, result shown is for the pectoral fin; ϕ, result shown is only for severe malformations.

↑ Parameter increases with increasing VA dose treatments,

↓ Parameter decreases with increasing VA dose treatments,

↔ Significant effect of the VA treatment but not directional.

Despite this available data regarding the VA requirements of different fish species, direct comparisons are not accurate due to differences in the duration of each study, the initial weight of fish, the initial variation in body VA concentrations, dietary composition and experimental conditions (Mohamed et al., 2003). The assessment criteria used to quantify or identify the incidence of skeletal malformations is also potentially problematic, as it was not the same among different studies.

Despite the issues in the comparison of VA studies, it is worth noting that the incidence of vertebral column malformations, including the caudal fin complex in *L. lineata* post-larvae, was significantly lower with larger doses of VA, which is in agreement with the findings on *D. labrax* and *S. aurata* larvae (Table 2 and Figs. 1 and 2) (Fernández et al., 2008; Mazurais et al., 2009; Villeneuve et al., 2005). The dietary VA doses used to study the effect of VA on *D. labrax* skeletal development were lower in Mazurais et al. (2009) than in Villeneuve et al. (2005) (Table 2, Fig. 1). Mazurais et al. (2009) found that all of the vertebral column malformations observed in *D. labrax* were greatest (6 – 65%) in the larvae fed the diet containing the minimum VA dose (3,152 IU kg<sup>-1</sup> DW diet). Villeneuve et al. (2005) found that the incidence of vertebral column malformation across treatments was less than 8%, demonstrating that the higher doses of VA in that study ( $\geq 39,600$  IU kg<sup>-1</sup> DW diet) significantly decreased the incidence of skeletal malformation in *D. labrax* larvae compared with Mazurais et al. (2009). However, the results need to be interpreted with caution because the assessment criteria for the skeletal malformation were not the same in these two studies. In one study abnormalities in the whole vertebral column were included and in the other only transformation of the 12<sup>th</sup> pre-haemal vertebra to haemal. Similar trends were shown in *S. aurata* larvae where the incidence of vertebral column malformations (without caudal fin complex) was significantly higher in larvae fed on rotifers incorporating 160,480 IU kg<sup>-1</sup> DW than those fed on rotifers incorporating  $\geq 222,560$  IU kg<sup>-1</sup> DW (Fernández et al., 2008), although the amounts of VA were greater than the *D. labrax* studies and VA was delivered as the palmitate rather than acetate form (Fig. 2).

Effects of VA on vertebral development are associated not only with the vitamin dose but also with the developmental stage of the larvae (Haga et al., 2002a; Hernandez-H et al., 2006; Hernandez et al., 2004). Addition of VA into the diet of *C. major*, affected vertebral development during the larval stage (6 to 20 dph) but not in the juveniles, while in *P. olivaceus*, the effect was most pronounced when larvae were fed VA from 4 to 8 dph (rotifer feeding period) compared with dietary VA in the *Artemia* feeding period (8 to 37 dph). The dependence of VA effects on the larval developmental stage was evident in my study, where dietary VA affected the prevalence of vertebral malformation during the rotifer feeding period (6 to 18 dph) and not during the *Artemia* feeding period (17 to 44 dph) (Chapter 5). These studies imply that the window of sensitivity of larvae to VA, in relation to the development of the vertebral centra, is likely to be during early stages of development, at the end of rotifer feeding and early *Artemia* feeding, coinciding with early flexion. The effects of VA are probably mediated by the inhibition or acceleration of the proliferation of chondrocytes leading to skeletal malformation. Martinez et al. (2007) found that the prevalence and severity of skeletal malformations were significantly higher in the small larvae of Summer Flounder, *Paralichthys dentatus* (13.0 - 15.8 mm) than in the medium (16.0 - 19.9 mm) and bigger (20.0 - 28.0 mm) sized larvae at the end of their study. This indicates that malformations induced during development likely influenced larval growth, such that growth was compromised in fish with severe malformations. Similarly, *L. lineata* larvae reared in clearwater were significantly smaller and had a higher prevalence of severe jaw malformations, but not vertebral malformations, than those reared in greenwater (Chapter 4), although the culture water type was responsible for the difference in size and may not be directly associated with more severe jaw malformations (Chapter 3).

The prevalence of vertebral column malformation in *D. labrax* was not affected by high doses of dietary VA, < 8% across a wide range of treatments (Fig. 1) (Villeneuve et al., 2005). However, malformations in the neurocranium and splanchoanium (maxilla and operculum) were strongly correlated with the levels of VA in the larvae, i.e. dietary VA affected the incidence of malformation in the jaw and operculum regions but not the vertebral column within the levels tested (Villeneuve

et al., 2005). This result is in contrast to my study on *L. lineata* larvae, where the levels of dietary VA affected the prevalence of malformation in the vertebral column but not the jaws within the levels tested. The comparison among studies highlights the differences in species-specific VA effects and the possibility that one skeletal region might be affected by the dietary VA level and not necessarily the whole skeletal system (Table 2). Mazurais et al. (2009) concluded that the optimum dietary VA requirements depend on the skeletal elements under consideration, for example in *D. labrax*, the requirement to minimize jaw and hyoid malformations is  $< 9,402 \text{ IU kg}^{-1} \text{ DW diet}$  while the requirement to minimize the vertebral and fin malformations was  $\geq 9,402 \text{ IU kg}^{-1} \text{ diet}$ .

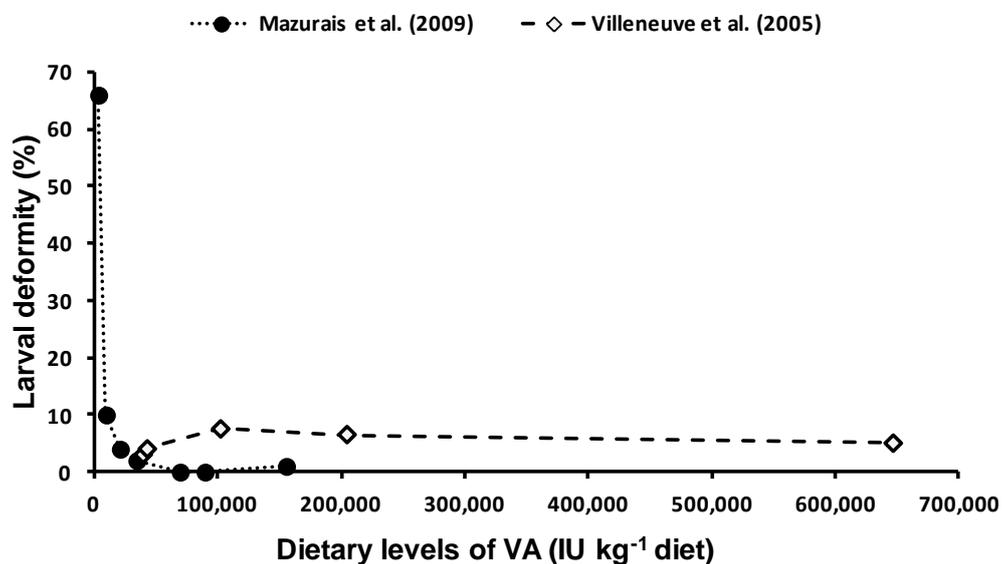


Figure 1: Prevalence of malformations in the vertebral column of *Dicentrarchus labrax* larvae fed increasing doses of vitamin A. •, represents the transformation of the 12<sup>th</sup> pre-haemal vertebra to haemal; ◊, represents the incidence of skeletal malformations in the vertebral column. Modified from Mazurais et al. (2009) and Villeneuve et al. (2005).

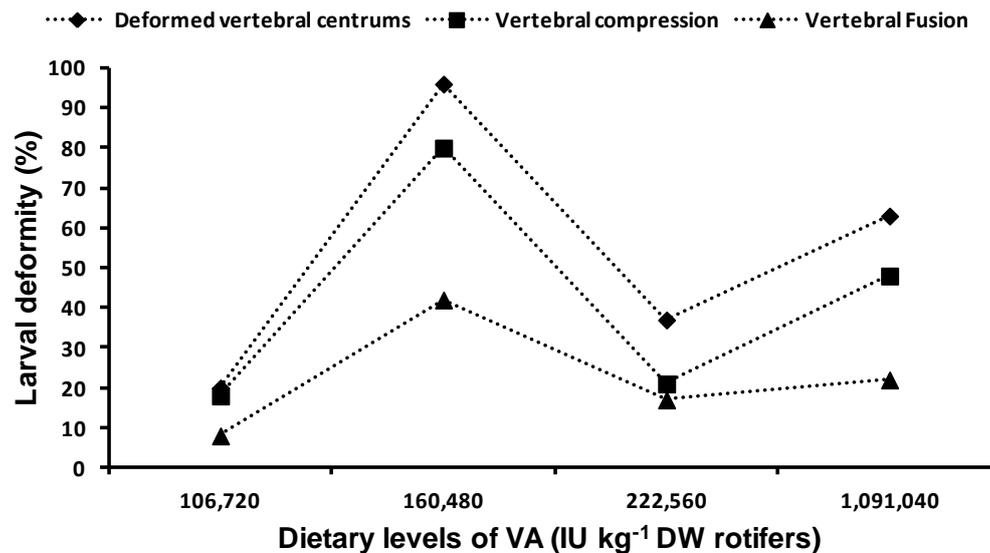


Figure 2: Prevalence of malformations in the vertebral column, without the caudal fin complex, of *Sparus aurata* larvae fed increased levels of vitamin A. The x axis represents the retinyl palmitate levels (without retinol and retinoic acid) in the enriched rotifers converted to IU VA based on the relationship 1 g = 1,600,000 IU. Modified from Fernández et al. (2008). Note x-axis is categorical data.

Vitamin A did not affect the jaw malformation of *L. lineata* during the live feed period, despite exposure to a wide range of dietary VA doses; 1,716 to 305,382 IU VA kg<sup>-1</sup> DW rotifers fed to larvae from 6 to 18 dph, and 1,452 to 145,398 IU VA kg<sup>-1</sup> DW *Artemia* fed from 17 to 44 dph (Table 2). There were low percentages of post-larvae with normal jaw structure at the end of each study across VA treatments (Chapters 3, 4 and 5) (Table 3), which were also lower (half or less) compared with those reported in previous studies (Cobcroft and Battaglione, 2009) (Table 3).

Different jaw elements in *L. lineata* start to differentiate around 6.3 mm standard length (SL) and continue differentiation and ossification until around 11.2 mm (Fig. 3) (Cobcroft et al., 2001).

According to the larval growth data in Chapter 3, the differentiation of different jaw elements would have started during the rotifer feeding period (average SL at 9 dph = 5.97 mm and 16 dph = 7.66 mm) and continued until the *Artemia* feeding period (average SL at 36 dph = 13.42 mm). In addition, according to the growth data presented in Chapter 5, the larvae started to feed on the dietary VA within the period of jaw differentiation (SL = 7.32 mm on 16 dph). It is apparent then that graded

dietary VA doses were fed to *L. lineata* larvae during the “window” of jaw development, which is usually a critically sensitive period, and yet no dose-response effect was recorded. Different marine fish species show diverse outcomes regarding the incidence of jaw malformation when fed or exposed to VA, for example, *P. olivaceus*, *D. labrax* and *S. aurata* larvae fed different VA levels displayed malformations in the jaw and different mouth parts, while in *P. dentatus*, exposure to retinoic acid in the rearing water, 0 to 20 nM for 10 days, increased the incidence, frequency and severity of jaw malformations but without a dose-response (Fernández et al., 2008; Haga et al., 2002b; Martinez et al., 2007; Mazurais et al., 2009; Suzuki et al., 2000; Villeneuve et al., 2005). In contrast, there was no skeletal malformation in the jaw apparatus and neurocranium of *S. senegalensis* larvae fed on dietary VA ranging from 11,200 to 64,000 IU VA kg *Artemia* from 6 to 37 dph (Fernández et al., 2009). Likewise for *C. major*, a low incidence of mouth malformation occurred when larvae were fed dietary VA ranging from 95,700 to 5,685,900 IU VA kg<sup>-1</sup> diet (Hernandez-H et al., 2006). These results highlight the apparent lack of a sensitive window to VA in jaw development of some species, including *L. lineata*, although the reason for that is not clear. A possible explanation is that the malformed structures in *L. lineata* are due to pre-existing malformations, prior to exogenous feeding, because some skeletal tissues commence development before first feeding, including the Meckel’s cartilage (Cobcroft et al., 2001).

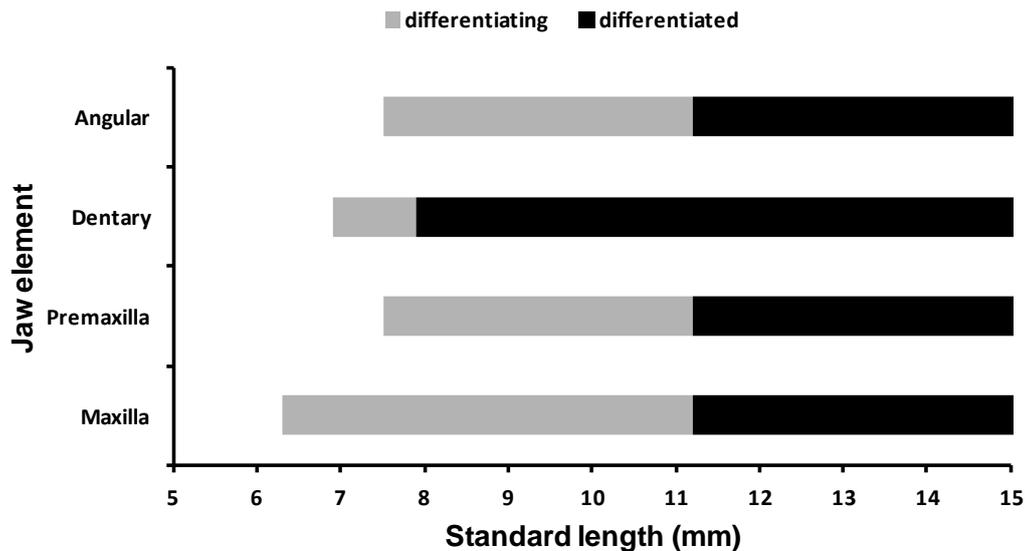


Figure 3: Developmental sequence of some jaw elements in *Latris lineata* larvae. Data modified from Cobcroft et al. (2001).

The high incidence of jaw malformation in *L. lineata* larvae and post-larvae has been an obstacle to the quality production of juveniles, with up to 95% in some batches (Battaglione and Cobcroft, 2007, 2010; Cobcroft and Battaglione, 2009; Cobcroft et al., 2001, 2012). Cobcroft and Battaglione (2009) found that the incidence of jaw malformation was correlated with walling behaviour, where the larvae swim into the tank walls with their head touching the tank surface, potentially causing damage to soft tissue and malformation in cartilage and bone elements of the jaw. Bristow and Summerfelt (1994) and Naas et al. (1996) explained that among the reasons causing this walling behaviour the water surface and tank wall were highly reflective and attractive to the larvae. Cobcroft and Battaglione (2009) demonstrated that the walling behaviour in *L. lineata* larvae was influenced and affected by the tank wall colours, being lower in black and marble coloured tanks. In Chapter 3, I mentioned that this walling behaviour was observed across all the treatments, although, the number of larvae walling per tank was not counted. The occurrence of walling behaviour, the stocking density (10 larvae l<sup>-1</sup>), the age of stocking into the 300 l tanks (1 dph), and the relatively high wall surface area to volume ratio in 300 l tanks are among the main possible factors that led to 5% of post-larvae with normal jaws (95% incidence of jaw malformations) in my study (Chapter 3) (Table

3). In comparison in Chapter 5, where the larvae were fed on dietary VA during the *Artemia* feeding period, the larvae were reared in 3000 l tanks until 16 dph, and this potentially decreased the larvae-wall interaction and hence the walling behaviour, leading to a higher percentage of post-larvae displaying normal jaws (28%) (Chapter 5) (Table 3).

In Chapter 4, the effect of water type (clear and greenwater) and dietary VA-enriched rotifers on the incidence of jaw malformation was evaluated. I found that although the water type did not affect the percentage of post-larvae displaying normal jaws (Table 3), the severity of jaw malformations (score 3) was significantly lower in greenwater (2%) compared with in clearwater (14%). Although the experiment in Chapter 4 was designed to mimic the larval rearing conditions of Chapters 3, where the larvae were fed dietary VA during the rotifer feeding period and reared in greenwater, the percentage of post-larvae with a normal jaw was higher in the experiment described in Chapter 4 than in Chapter 3 (24% compared with 5%) (Table 3). Changing the tank wall colour from black to marble may have been partly responsible for that increase in the percentage of post-larvae with normal jaws observed in Chapter 4, although only a small and non-significant benefit of marble over black was observed in a previous study (Cobcroft and Battaglione, 2009) (Table 3). Another, more likely factor that might have contributed to the elevation in the percentage of normal jaws is the possibility that the larvae used in the Chapter 4 experiment were of better quality. This is reflected in the higher survival by the end of the study (53%) compared with Chapter 3 (35%). Overall, the results of Chapters 3, 4 and 5 demonstrated that dietary VA did not affect the jaw malformation in *L. lineata* larvae or post-larvae. This thesis highlights the relatively high incidence of this malformation across all experiments, and that changing rearing conditions, specifically those affecting walling, such as the tank size, larval density and water type, could significantly reduce the incidence and severity of jaw malformations *L. lineata* larvae and post-larvae.

Table 3: Comparison between the current research and previous experiments with *Latris lineata* larvae in respect to different rearing conditions, larval growth parameters, survival and the incidence of post-larvae with a normally formed jaw (score 0) and vertebral column.

Rotifer phase parameters				Artemia phase parameters			Age* (dph)	Length			Weight (mg)	Survival (%)	Normal jaw (%)	Normal Vert. Column (%)	Reference
Water type	Tank size (l)	Tank wall colour	Enrichment	Tank wall colour	Enrichment	Standard (mm)		Fork (mm)	Total (mm)						
CW	2000	Black	Algamac-2000	Black	Algamac-3050	36	11.6	NA	NA	2.8	20	NA	NA	Brandsen et al., 2005a <sup>◊</sup>	
GW	300	Black	VA	Black	Algamac-3050	43	NA	NA	15.8	4.6	35	5	69 <sup>◊</sup>	Chapter 3 <sup>¥</sup>	
GW	300	Marble	VA	Marble	Algamac-3050	43	NA	NA	15.8	4.1	53	24	55	Chapter 4 <sup>¥</sup>	
CW	300	Marble	VA	Marble	Algamac-3050	43	NA	NA	14.4	3.0	65	23	59	Chapter 4 <sup>¥</sup>	
GW	3000	Marble	Algamac-3050	Marble	VA	44	NA	NA	15.9	5.1	26	28	43	Chapter 5 <sup>¥</sup>	
GW	3000	Marble	Algamac-2000	Black	Algamac-3050	44	NA	15.7	NA	7.8	71	60	NA	Cobcroft and Battaglione, 2009 <sup>¥</sup>	
GW	3000	Marble	Algamac-2000	Marble	Algamac-3050	44	NA	15.6	NA	7.5	58	52	NA	Cobcroft and Battaglione, 2009 <sup>¥</sup>	

CW, clearwater; GW, greenwater NA; not available; dph, days post hatch.

\* larval age at the end of the experiment and assessment of size, survival, and malformation;

◊, antibiotic was add to rearing water; ¥, ozonated rearing water;

φ, prevalence of normal vertebral column for larvae fed with dietary VA  $\geq 123$  ng total VA mg<sup>-1</sup> DW rotifer.

## 6.6 Greenwater effect on the vitamin A enriched rotifers

The greenwater technique is commonly used for rearing marine fish larvae, and the benefits of adding live microalgae directly into the rearing seawater were discussed in Chapter 4. To my knowledge this study was the first to examine the requirement for dietary VA during the rotifer feeding period in marine finfish larvae reared in greenwater (Chapters 3 and 4). I found that the retinoid concentration in the enriched rotifers was affected by the water type, the previous enrichment levels, and the time after transfer to the larval rearing water (Chapter 2). However, there was a significant difference in the total VA content of rotifers enriched with graded levels of VA for an important component of the larval feeding period in all experiments, for at least the first 4 h in clear water and until 4 to 8 h in greenwater (Chapter 2). *Latris lineata* larval retinoid content was not affected by the water type and was significantly correlated with the total VA in the enriched rotifers before adding to the tanks, indicating that even though rotifer VA changed in greenwater and over time, the graded VA levels were maintained in rotifers for sufficient time and resulted in the significant incorporation of different doses into larval tissues. Despite the finding, it is worth noting that the retinoic acid increased by 88% in the enriched rotifers held in greenwater from 6.24 ng DW<sup>-1</sup> rotifers at 4 h to 11.72 ng DW<sup>-1</sup> rotifers by 14 h. Retinoic acid is associated with the appearance of skeletal malformation in marine fish larvae and other vertebrates, as this is the most toxic form of VA (Haga et al., 2002a, 2002b; Martinez et al., 2007). A potential reason why *L. lineata* larvae were not affected by with the increase in retinoic acid was that the peak feeding of the larvae was soon after VA-enriched rotifers were added to the tanks in the morning, when the retinoic acid concentration in the rotifers was the lowest (Chapters 3 and 4). At 17:00, the Algamac-3050 enriched rotifers were added to the tanks, so in theory whatever left over rotifers (if any) from the morning VA-enriched feed would have been diluted by the addition of Algamac-3050 enriched rotifers. It is worth pointing out that the Algamac-3050 enriched rotifers added to the tanks at 17:00 would also have accumulated retinoic acid over a similar time to the VA-enriched rotifers (Chapter

2). However, Algamac-3050 enriched rotifers were only available to the larvae for 8 not 14 h due to the flushing of the tanks and the lights turning off (Chapters 3 and 4). Consequently, it appears the larvae were not affected by the elevation in the retinoic acid concentration over time. The results highlight that it is crucial when designing experiments with VA-enriched live feeds to consider changes in composition over time and be aware that retinoic acid may accumulate in prey in greenwater.

## 6.7 Constraints and limitation in the study

Research with marine finfish larvae using dose-response nutrition designs requires considerable resources, particularly due to the daily enrichment for live feeds (e.g. in Chapter 3, eight doses of VA in addition to afternoon enriched rotifers with Algamac-3050; in Chapter 5, six doses of VA in *Artemia*, harvested twice and fed out four times daily). In my study, it was also necessary to grow the larvae to a sufficient developmental stage to allow assessment of skeletal malformations, which required relatively long and labour intensive experiments with *L. lineata* up to 44 dph. In addition, the current rearing methodology available for *L. lineata* culture constrains larval nutritional research to live feeds, as the larvae do not perform well with microdiets, and the live feeds must meet the relatively high requirement of *L. lineata* for lipids. Consequently, I could not use microdiets, as in other VA studies, and it was necessary to have two rotifer diets in Chapters 3 and 4, with VA-enriched rotifers added in the morning and Algamac-3050 enriched rotifers in the afternoon.

Working with vitamin A analysis was also challenging, because I worked with two extraction methods that needed to be validated and modified for the analytical equipment in my laboratory. Preliminary research provided standardised and repeatable methods that could be used throughout the thesis, but was time consuming to establish. There were a large number of samples to extract (approximately 4,000 samples) to support a rigorous approach to measure VA in emulsions, enriched live prey and in larvae, which required a long time to process (nine months). The small number of

previous studies in VA that include a similar level of detail in marine finfish larvae are a testament to the technical difficulty of this work.

The combination of time requirements in the establishment of VA analysis methods, live feed enrichment methods, larval experimentation and subsequent biochemical analyses, reduced the time available for a comprehensive approach to investigating the specific types of skeletal malformations. However, I analysed the broad categories of skeletal malformations of relevance to commercial culture of *L. lineata*, focussing on jaw and regional vertebral malformations.

Other research has demonstrated that differential gene expression, particularly of retinoid receptors, is associated with VA nutrition and skeletal malformation. A companion study (Australian Research Council Linkage Grant LP0882042) conducted a limited investigation of gene expression in *L. lineata* from one dietary VA experiment and the gene expression methodology was beyond the scope of my research objectives.

In order to compare previous studies investigating VA and marine fish larvae, it was necessary to convert the retinoid values into a standard form, International Units (IU). There were different forms of VA used in previous studies and different analyses methods which made it challenging to compare values. However, I have done this to the best of my ability throughout the thesis and have included the calculation formulae (e.g. Table 1 in this Chapter).

## 6.8 Future directions for research

Based on the findings of my thesis, there are several research topics that could be investigated in future to improve our understanding of VA requirements and modes of action in developing marine fish larvae. These include:

- Study the effect of different doses of retinoic acid, which is the most active form of VA, on the skeletogenesis in *L. lineata*.

- Study the detailed development of normal skeleton formation in *L. lineata*. This would provide a foundation to examine malformations of the vertebral column, fins and throughout the skeleton, in response to dietary or environmental factors. This extended work could include assessment of the percentage of occurrence of various malformations and use more advanced methods (e.g. radiography).
- Examine the effect of VA on *L. lineata* organ morphogenesis (e.g. maturation of the digestive system), and skeletal differentiation and ossification.
- Compare the normal skeletal structure, body shape, types and prevalence of malformations occurring in wild and cultured *L. lineata* larvae, post-larvae and juveniles.
- Examine the effect of graded doses of VA to explore the mechanism for VA incorporation into larval tissues, particularly in relation to activity of transport proteins, cellular retinol binding proteins and cellular retinoic acid binding proteins.
- Examine the interactive effects of VA and lipid levels in *L. lineata* skeletal development (e.g. a fixed VA level and varied lipid concentration). This would allow a more detailed study of VA molecular pathways in *L. lineata*, and the relationship between different lipid levels and the recommended VA dose.
- Continue to explore the role of physical factors (e.g. tank hydrodynamics), which affect walling behaviour and are likely to impact the prevalence of jaw malformation in *L. lineata*.
- Examine the effect of greenwater in other marine fish species in relation to VA metabolism, including larval retinoid content. This may be particularly relevant in cases where uneaten rotifers are not flushed out, and therefore may accumulate VA, especially retinoic acid, over time. This could also impact species where graded doses of VA are known to impact skeletal malformation.

## 6.9 Conclusion

This is the first study to examine the effect of dietary VA on the prevalence of skeletal malformations in *L. lineata* post-larvae. I found that the delivery of dietary VA (daily inclusion of  $\leq 123$  ng total VA  $\text{mg}^{-1}$  DW rotifer, equivalent to  $\leq 35$  ng total retinol  $\text{mg}^{-1}$  DW rotifer) to *L. lineata* during the rotifer feeding period can minimize vertebral malformation to approximately 30%. Jaw malformations were not affected by the inclusion of dietary VA into larval diets and their prevalence and severity were influenced by other rearing conditions.

This study provides a fundamental knowledge base for future studies with dietary VA through enrichments of live prey, rotifers and *Artemia*, with retinyl palmitate, the dynamics of different VA compounds during the enrichment process, and after addition into the rearing tanks with respect to time. My study examined rearing marine finfish larvae in greenwater while feeding VA-enriched live prey, and demonstrated the importance of understanding the changes in the diet composition over time in future research. My study has achieved its aims and has contributed not only to the development of nutrition research in *L. lineata* but also to that in marine teleost larvae in general.

## 6.10 References

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