Universidade do Algarve
Faculdade de Ciências e Tecnologia
Masters in Aquaculture and Fisheries
Master Thesis

Feeding strategies to improve growth in clownfish juveniles (*Amphiprion ocellaris*)

Maria Ana Morais

Faro, 2019

Feeding strategies to improve growth in clownfish juveniles (*Amphiprion ocellaris*)

Maria Ana Morais

Dissertation for the attainment of the Master's degree in Aquaculture and Fisheries

This thesis was supervised by:

Dr. Sofia Engrola, CCMAR

Dr. Jorge Dias, SPAROS Lda.

Faro, 2019

Feeding strategies to improve growth in clownfish juveniles (*Amphiprion ocellaris*)

Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam na listagem de referências incluída.

Universidade do Algarve, 30 de setembro de 2019

_____

Acknowledgements

I would like to thank Dr. Sofia Engrola and Dr Jorge Dias for accepting me as their MSc student and giving me the opportunity to perform this study, for which I will cherish for life. Specially, Dr Sofia whose motivation, dedication, patience, rectifications and time spent in transferring her scientific knowledge, helped me complete this dissertation.

I want to make a special thanks to Dr Carmen Navarro and Rita Colen for all the support, scientific knowledge, incentive and technical assistance and availability in assisting me whenever needed during this thesis, without them it wouldn't be possible.

My sincere thanks to all members from the Aquagroup team, who received me with affection and hospitality, being always available to help me, especially to Miguel Cabano, Raquel Carrilho and André Lopes, for guiding and helping me at the various stages of this project.

I would also like to thank Mr. Jorge Corga for all his availability, guidance and knowledge in the ornamental field which helped a lot during the experimental period.

A special thanks to my boyfriend Miguel Patusco, who always supported me through this process and gave me the strength to move forward.

This dissertation wouldn't be possible without my family, especially my parents and brothers, who always supported me and allowed me to further my education and always gave me strength and advice I needed during my academic path.

Thank you all!!

Abstract

The ornamental trade has been growing in the last decade. Traditionally, fish are collected from the wild, causing the destruction of reef habitats and wild populations. Consequently, ornamental aquaculture is a viable strategy to a healthy and sustainable ecosystem. Ornamental fish value is mainly based on skin colour. However, the main problem in reared fish is the loss of colour due to carotenoid deficiency in diets. Therefore, nutrition is a valuable tool to improve quality of the reared ornamental fish. The dietary inclusion of marine biomasses and alternative new ingredients could overcome this situation. The objective of the present study was to assess how innovative diet formulations could improve performance of *Amphiprion ocellaris* juveniles, as well as to standardize a feeding protocol that promotes fish quality, based on growth and colour. A stock of 180 juveniles were divided in 9 tanks in a recirculation aquaculture system and each diet was assigned to triplicate tanks. Fish were fed by hand to apparent satiety, several times a day, with the respective diets. Diet A contained high levels of traditional marine protein sources and was supplemented with synthetic astaxanthin whereas diets B and C comprised a marked increase of emergent raw materials at the expenses of traditional marine protein sources and without the addition of synthetic astaxanthin. The impact of experimental diets was determined on juvenile key performance indicators at the end of the experimental period. Fish fed with diet A presented a better overall performance nonetheless in the ornamental market the main focus is pigmentation that determines fish value. Consequently, fish fed with diet B presented better results in terms of the desired pigmentation in the fish. Further studies on this topic are required to validate the observed effects over a longer feeding period and over specific phases of the life cycle.

Key words: *Amphiprion ocellaris*, ornamental aquaculture, nutrition, alternative ingredients, colour

Resumo

O mercado ornamental aumentou na última década. Tradicionalmente, as espécies ornamentais são recolhidas na natureza, o que causa a destruição dos habitats de recifes e das suas populações selvagens. Consequentemente, a aquacultura ornamental será a estratégia viável para a manutenção de um ecossistema saudável e sustentável. O valor das espécies é baseado principalmente na sua cor. No entanto, um dos principais problemas de peixes produzidos em aquacultura ornamental é a perda de cor devido à deficiência de carotenóides nas suas dietas. Assim, a nutrição pode ser uma ferramenta valiosa para melhorar a qualidade dos peixes ornamentais. A inclusão de biomassas marinhas e de novos ingredientes alternativos poderá superar essa situação. O objectivo do presente estudo é avaliar como as formulações inovadoras podem melhorar o desempenho de juvenis de *Amphiprion ocellaris*, bem como padronizar um protocolo de alimentação que promova a qualidade do peixe, com base no crescimento e na sua cor. Um grupo de 180 juvenis foi repartido aleatoriamente em 9 tanques num sistema de aquacultura de recirculação e cada dieta foi atribuída aos tanques em triplicado. Os peixes foram alimentados à mão até à aparente saciedade, várias vezes ao dia, com as respectivas dietas. A dieta A continha elevados níveis de fontes tradicionais de proteínas marinhas sendo suplementada com astaxantina sintética, enquanto as dietas B e C incluíram um aumento acentuado de várias matérias-primas emergentes de modo a substituir as fontes tradicionais de proteínas marinhas e sem a adição de astaxantina sintética. O impacto das dietas experimentais foi determinado em vários indicadores-chave de desempenho no final do período experimental. Os peixes alimentados com a dieta A apresentaram melhores resultados em termos de crescimento, porém o foco do mercado ornamental é a pigmentação, sendo o factor determinante para o valor do peixe. Consequentemente, foi observado que os peixes alimentados com a dieta B apresentaram melhores resultados em termos da pigmentação desejada. Será necessário explorar em profundidade este tópico de modo a validar as observações realizadas, através de estudos com maior duração ou em fases de desenvovlimento mais precoces.

Palavras-chave: *Amphiprion ocellaris*, aquacultura ornamental, nutrição, ingredientes alternativos, cor

Abbreviations

ΔE- perceptible colour differences

a- red-green chromaticity

ABW- average fish weight

ALP- alkaline phosphatase

AMPN-aminopeptidase

ANOVA-analysis of variance

b- yellow-blue chromaticity

BW_i- initial body weight

BW_{int} – intermediary body weight

BW_f – final body weight

C_{ab}-chroma

Chy- chymotrypsin

CieLAB- colorimetric method

cm – centimetres

DAH- days after hatching

DGI - daily growth index

DHA- docosahexaenoic acid

DMSO- dimethyl sulfoxide

FAO - Food and Agriculture Organization

FB- final biomass

FCR- feed conversion rate

FI – feed intake

FDW - fish dead weight

g- grams

H- hue

h- hours

HSI- hepatosomatic index

HSD- Honest Significant Difference

IB- initial biomass

k- condition index

L*- lightness

L- litters

LAS -Leica Application Suit

LIP-lipases

LW- wet liver weight

m- meters

min-minutes

mg- milligrams

ml- millilitres

mM- millimolar

mm- millimetres

n- number

nd- number of dead fish

nm- nanometre

p- p-value

ppm- parts per million

RAS- recirculation aquaculture system

S%- survival rate

SGR- specific growth rate

TL_i- Total initial length

TL_{int} . intermediary total lenght

Tl_f- Total final length

Try - trypsin

µl - microliter

µm- microliter

VSI – viscerosomatic index

VW – viscera wet weight

WG - weight gain

USA- United States of America

US- United States of America dollars

ºC- degrees Celsius

Contents

List of Figures

List of tables

1. Introduction

1.1 *Status of Amphiprion ocellaris in aquaculture*

The ornamental fish industry is growing economically worldwide being valued at approximately US$15 billion (Rhyne *et al*., 2017). Therefore, the need for ornamental fish has grown in the last decade. For many decades ornamental animals were collected from the wild, often with the use of destructive fishing methods such as cyanide and dynamite (Olivotto *et al.*, 2017; Pomeroy *et al*., 2006; Wabnitz *et al*., 2003). These evasive fishing techniques, due to the high demand of the ornamental market, have put at risk nearly 80% of southeast Asia coral reefs and 60% of the Pacific, being now considered one of the most threatened habitats of the world (Pomeroy *et al*., 2006). More than a decade ago marine ornamental aquaculture was identified as an alternative to reduce the pressure and destruction of coral reef ecosystems (Pomeroy *et al*., 2006).

The ornamental industry depends on around 1400 species, however only 30- 40 species are currently produced in aquaculture (Balamurugan *et al*., 2018). Consequently, there is a need to develop production protocols to improve ornamental aquaculture (Olivotto *et al*., 2017). The ornamental industry is still in its infancy. Therefore, the sector still faces several problems related to broodstock management, embryo development, egg hatching and the proper inert diets suitable for the different production stages (Calado *et al*., 2017).

One of the main issues of the ornamental industry is the lack of knowledge regarding nutrition of tropical fish, due to the disproportionate level of research dedicated to the aquaculture production of fish for Human consumption when compared to the ornamental industry (Vargas-Abúndez *et al*., 2019). Even though, there are commercial diets available for ornamental fish they are usually too expensive for large scale productions (Vargas-Abúndez *et al*., 2019).

Clownfish are probably the most popular seawater fish species, due to its popularity in the Disney movie “Finding Nemo” which increased the demand for this species (FishLore.com, 2013). Consequently, *Amphiprion ocellaris* became one of the most produced fish in the ornamental industry (Avella *et al*., 2007). The market prices, depending on species, may range from 10 to 600 or more dollars per fish. In addition, the fish value will be influenced by phonotypical characteristics, like colour and size, and country where it is sold and produced. Therefore, there is a huge commercial potential in breeding and producing this ornamental fish species.

1.2 *Clownfish species*

Tropical ornamental fish are one of the world´s most popular attractions due to their capacity to adapt to live in confinement and their diverse and different patterns and colorations (Ghosh *et al*., 2012). Clownfish belong to *Pomacentridae* family and subfamily *Amphirioninae* and about 30 species are divided in two genera *Amphiprion* and *Permnas* (Dhaneesh *et al.*, 2009). Some of the most popular and used species of clownfish are *Amphiprion ocellaris* (false percula clownfish) (Fig.1), *Amphiprion percula* (percula clownfish) (Fig.3), *Amphiprion frenatus* (tomato clownfish) and *Amphiprion akallopisos* (skunk clownfish*).*

Amphiprion ocellaris and *Amphiprion percula* are very similar phenotypically, but may be distinguished from one another through the following characteristics: different dorsal number of dorsal spines (*A. percula* has 10 whereas *A. ocellaris* 11); the spinous anterior of *A. ocellaris* is taller; *A. ocellaris* never has a thick black margin around the white bars and the distribution of these two species does not overlap (Fautin *et al*., 1992).

In the wild both species live in tropical reef habitats and have a symbiotic relationship with anemones, which provide them with protection and sometimes even food (Buston, 2003; Fautin *et al*., 1992). Clownfish show preferences for host anemones, *Amphiprion ocellaris* is usually found in *Heteractis magnifica* and *Stichodactyla mertensii*, whereas *Amphiprion percula* in *Heterac*tis *crispa* and *Heteractis magnifica*, both species are present in *Stichodactyla gigantean* (Fautin *et al*., 1992). Clownfish prefer tropical temperatures ranging from 26 to 30 ºC (Kumar and Balasubramanian, 2009; Madhu *et al*., 2006).

1.2.1 *Amphiprion ocellaris*

The *Amphiprion ocellaris* (Fig.1) are native from the Indo-Pacific region (Fig. 2) and reach a maximum size of 8 cm in the wild and 5 cm in an aquarium (Pomeroy *et al*., 2006). Its colour is usually bright orange with three white bars, the middle one with forward projecting bulge and bars with a very narrow to almost non-visible black margins (Fautin *et al*., 1992).

Figure 1 - A Western Clown Anemonefish, *Amphiprion ocellaris*, at Dompu, Bima, Nusa Tenggara Barat, and Indonesia. Source: Mark Rosenstein (http://fishesofaustralia.net.au/home/species/1275)

This non-migratory fish has a depth range of 1-15m (Allen, 1991) and are the most commonly kept clownfish in aquaria, representing the ideal marine experimental model due to its easy maintenance in laboratory conditions, regular spawning and its genome is partially available in GenBank (Vargas-Abúndez *et al*., 2019).

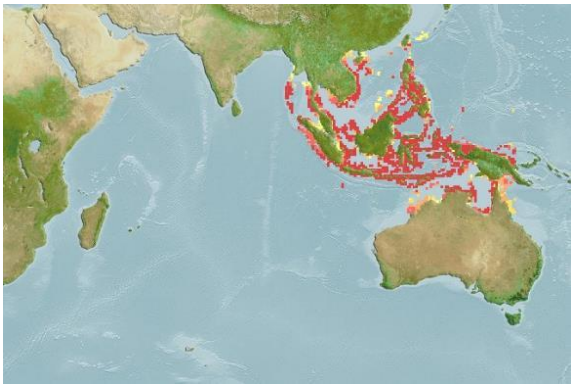

Figure 2 - Distribution Map for *Amphiprion ocellaris* (Clown anemonefish). Source: https://www.aquamaps.org/receive.php?type_of_map=regular#

1.2.2 *Amphiprion percula*

The *Amphiprion percula* (Fig. 3) is a tropical reef fish indigenous to Northern Queensland and Melanesia waters (Fautin *et al*., 1992) (Fig.4). It is described has a bright orange coloured fish with three white bars often bordered with black with variation of width and its middle with a forward projection bulge, it can reach a maximum length of 8 cm (Fautin *et al*., 1992).

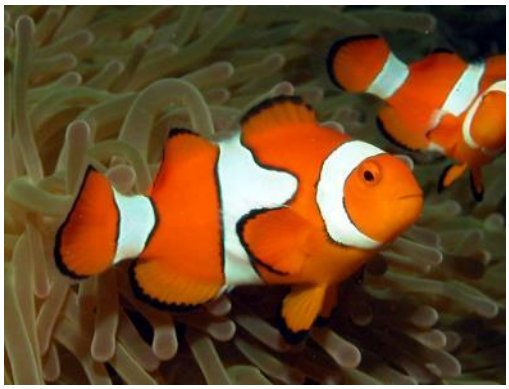

Figure 3 - An Eastern Clown Anemonefish, Amphiprion percula, at Tufi, Papua New Guinea. Source: Dave Harasti. License: All rights reserved (http://fishesofaustralia.net.au/home/species/317)

As well as *Amphiprion ocellaris*, *Amphiprion percula* is a non-migratory fish that can be found at a depth range of 1 - 15 m (Lieske and Myers, 1994).

Figure 4 - Distribution Map for Amphiprion percula (Orange clownfish). Source: https://www.aquamaps.org/receive.php?type_of_map=regular#

1.3 *Life cycle*

Amphiprion ocellaris are protandrous hermaphrodites, which means that juvenile fish will mature first as male and after as female (Khoo *et al*., 2018). This sex change occurs depending on the social hierarchy of the group and may not be reversible (Fig. 5) (Khoo *et al*., 2018).

This species lives in social groups (Barbasch and Buston, 2018), characterized by a large dominant female and a smaller subordinate male (Buston and Wong, 2014) that form a monogamous breeding pair while the rest of the group is formed by non-breeders (Khoo *et al*., 2018). Since the female is larger, it is usually more vulnerable to predation (Yasir and Qin, 2007). Consequently, when the dominant female dies or is removed from the group, the dominant male will assume the female´s position and one of the non-breeding individuals will

take his place forming a new dominant breeding couple (Fig.5) (Barbasch and Buston, 2018; Khoo *et al*., 2018).

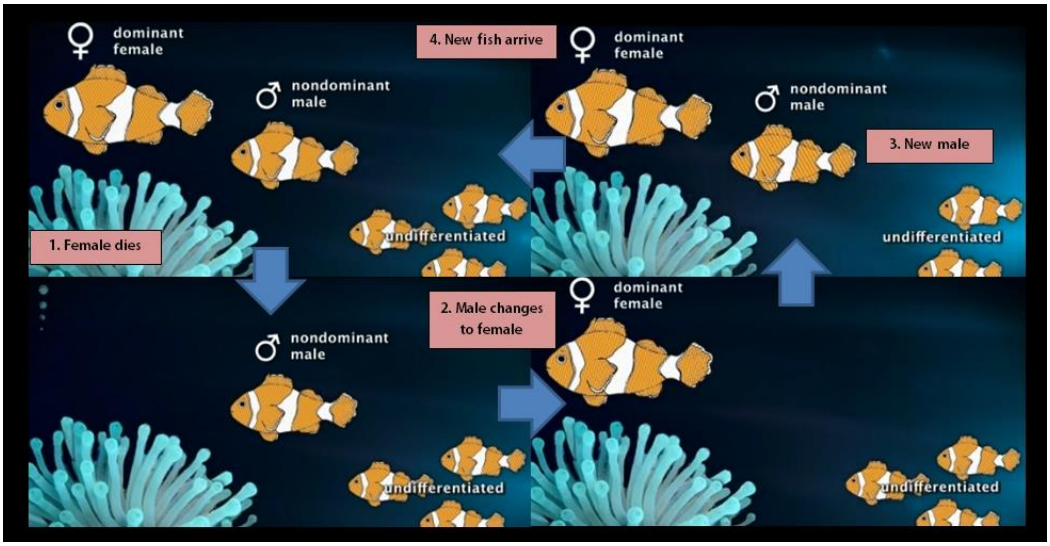

Figure 5 - Sex change cycle in Clownfish; how group structure influences sex change in clownfish. Source: http://blog.maldivescomplete.com/2015/01/22/maldives-qi-part-5/

Reproduction occurs during the all year and every month at least 1-2 spawns are produced by the breeding pair during the morning time (in captivity conditions) (Balamurugan *et al*., 2018; Buston and Elith, 2011; Dhaneesh *et al*., 2012; Dhaneesh *et al*., 2009). It is observed pair formation and sexual maturity after 3-4 month (Kumar *et al*., 2012; Madhu *et al*., 2006) and fish may reach weight between 6-7 g (Dhaneesh *et al*., 2012)

Before spawning, courtship behaviours like biting, chasing and cleaning the bottom of the tank or host anemone are observed, which indicate that the pair is ready to spawn (Balamurugan *et al*., 2018). After this ritual the female will deposit the eggs on a clean surface and the male will release the sperm to fertilize the eggs (Balamurugan *et al.*, 2018). The clutch of eggs is laid by the breeding pair up to three times per lunar month (Buston, 2004; Buston and Elith, 2011) and parental care is observed in the form of tending, mouthing the eggs to remove debris and dead eggs, and fanning the eggs with the fins to oxygenate the clutch (Barbasch and Buston, 2018). This species produce 100–300 eggs per spawn and usually males tend to the eggs until hatching (Wittenrich and Turingan, 2011).

1.3.1 *Early development*

Pomacentrid fish lay demersal eggs attached to submerged objects, whose shape may vary from oval to capsule depending on the species (Dhaneesh *et al.*, 2009) . The main particularities of anemonefish eggs are that they are capsule shaped, highly pigmented and rich

in carotenoids in the yolk sac which results in a whitish-orange to purple in colour appearance (Dhaneesh *et al*., 2012; Dhaneesh *et al*., 2009; Yasir and Qin, 2007). Egg incubation of *Amphiprion ocellaris* is around 7 to 8 days post fertilization under incubation temperature of 27 - 28C^{o} *(*Madhu *et al.,* 2006*;* Olivotto *et al*., 2011; Yasir and Qin, 2007).

After hatching, *Amphiprion ocellaris* larvae appear mostly transparent and depends on yolk-sac content until mouth opening. The developmental changes that occurs during the switch from endogenous feeding (larvae relies on the yolk sac for nutrition) to exogenous feeding (where the yolk sac is depleted and larvae starts to eat live prey), is one of the most critical transitional periods in the larvae life history, since there are usually quite high mortality rates (Wittenrich and Turingan, 2011).

After 9-11 days after hatching (DAH) the milky white band starts to appear (Fig. 1) (Ghosh and Kumar, 2015), and on the 14-16 DAH the larvae present a full coloration (Fig. 1). The pelagic phase is one of the most important in reef fish development and it is characterized by a series of developmental changes in morphology, physiology, and behaviour, during these stage larvae spend several weeks in the open ocean before settling down in benthic habitats (Wittenrich and Turingan, 2011). This phase ends around 25 DAH, where the larvae transforms into to a juvenile (Ghosh and Kumar, 2015).

1.4 *Clownfish nutrition /diet formulation*

The *Amphiprion* species, in the wild, are omnivorous and opportunistic feeders, whose diets include a high proportion of feed of vegetable origin (algae), followed by plankton, larvae and crustaceans (Allen, 1972; Galetto and Bellwood, 1994). Consequently, since their natural diet is rich on plankton it is important to include in formulated diets, high levels of protein as well as polyunsaturated fatty acids, which contain the necessary lipids for optimal growth and gonadal production (Díaz-Jiménez *et al*., 2019). Not a lot of studies have been made to date in regards to the specific nutritional requirements of juveniles *Amphiprion ocellaris*, but according to Díaz-Jiménez *et al.*, 2019, the recommended protein/lipid proportion would be of 430/100 g/kg of protein/lipid for optimal growth, nonetheless specific requirements of proteins/lipids depend on the fishes energy needs and developmental stage.

Consequently for aquaculture production of this species, the improvement of the diets nutritional quality are the most important factors that influence welfare, growth, colour and survival (Balamurugan *et al*., 2017; Sales and Janssens, 2003).

1.4.1 *Marine vs alternative ingredients*

Due to their positive impact on the composition of the final product the use of fishmeal and fish oil as a aquafeed ingredient has been a common practice in the formulation of diets (Henriques *et al*., 2014). Fishmeal is a good source of protein (60-72%), essential amino acids, nucleotides and phospholipids, whereas for fish oil it is a good natural source of essential polyunsaturated fatty acids (Salin *et al.*, 2018; Shepherd and Jackson, 2013). However, the main problems with these ingredients are the increase in price and the negative impact on wild pelagic fish stocks (overfishing for production of fish meal and oil), since 17% of the total capture fisheries was used for fish meal production (FAO, 2018). As a result, research for more economic and sustainable aquafeed ingredients has been developed (Lunger et al., 2007; Makkar et al., 2014).

Various potential substitutes for fish meal and oil such as plant proteins have been studied as a sustainable alternative. Even though they may present a good alternative, agricultural crops in feeds may have negative effects on Human food security, since it is in direct competition for resources, therefore the best alternatives would be to find other options that do not directly influence the human food chain (Olsen, 2011). Due to this fact, meat by-products, insect meal (Gasco et al., 2018) and microbial proteins (such as algae) (Matassa et al., 2016; Tibaldi et al., 2015) have been a target of research for new innovative aquafeed ingredients.

1.4.2 *Microalgae as a feed ingredient*

Microalgae's popularity has increased as animal feed supplement since they are natural source of pigments, antioxidants and other bioactive compounds in addition to their basic nutritional value (Spolaore *et al.*, 2006). Moreover, microalgae biomass contain low to medium protein levels (ranging from 43 to 60% of dry matter), depending on species, culture and harvesting conditions, being even considered as a valuable feed substitute (Becker, 2007; Biller and Ross, 2011).

The use of microalgae as a replacement for marine ingredients has been observed in various fish species, such as European sea bass (Tibaldi *et al*., 2015), Nile tilapia (Velasquez *et al.*, 2016), Atlantic salmon (Miller *et al*., 2007) and kenyi cichlids (Karadal *et al*., 2017). The use of dry microalgae such has *Schizochytrium spp.*, which is rich in docosahexaenoic acid (DHA) has been recognized has a successful partial substitute for fish oil in diets in marine species, such as the Atlantic salmon, where no significant differences were found in growth and FCR; and DHA content was higher in fish fed with algae oil when compared with fish fed with

fish oil (Miller *et al.*, 2007). It was also observed, in different fish species, that fishmeal and fish oil could be partially replaced (about 20 to 40%) by different types of microalgae such has *Isochrysis*, *Chlorella* meal, *Nannochloropsis sp.*, *Tretraselmis* sp. and *Spirulina* without having a negative impact or even improving fish growth in various species (Haas *et al.*, 2016; Salin *et al.*, 2018; Tibaldi *et al.*, 2015; Velasquez *et al*., 2016).

Microalgae are not only a source of protein and lipids but also a source of natural carotenoids being used as a natural colouring agent (Gouveia *et al*., 2006). Moreover, according to Rodriguez-Garcia and Guil-Guerrero, 2008, extracts of microalgae have higher antioxidant activity when compared to the antioxidants commonly used.

In conclusion, the use of microalgae in fish diets have various benefits that make them an interesting ingredient to be included in fish nutrition especially in the ornamental market where colour is one of the major factors of the industry.

1.4.3 *Insect meal as a feed ingredient*

As referred before fish meal is one of the most used resources in fish diet formulation, however the decline of wild fish catches and the increase demand in aquaculture has resulted in the decrease in availability and increase of price of this resource (FAO, 2018).

That said, other more sustainable and effective alternatives are necessary in regard to diet development. The use of insects as a sustainable protein rich feed is a feasible possibility (Sánchez-Muros et al., 2014). According to Gasco et al., (2018), insects are one of the most promising alternative sources for protein as well as can be a valuable source of compounds that may have positive effects on animal health reducing the use of antibiotics in production.

Insects are a rich source of amino acids, lipids, vitamins and mineral, as well as a natural food source for some species of fish (Henry et al., 2015). The most studied insect is the Black soldier fly (*Hermetia illucens*),whose larvae are rich in proteins (400g/kg) and lipids (300g/kg), and its protein has been successfully included has a feed ingredient not only in terrestrial animals as well in fish such as channel catfish, blue tilapia, rainbow trout, turbot and Atlantic salmon (Zhou et al., 2018).

Although, it is been shown to be a good sustainable alternative for protein source, insects only recently have been allowed to be included has an feed ingredient in animal diets. Being only included the ones derived from black soldier fly (*Hermetia illucens*), common housefly (*Musca domestica*), yellow mealworm (*Tenebrio molitor*), lesser mealworm (*Alphitobius diaperinus*), house cricket (*Acheta domesticus*), banded cricket (*Gryllodes sigillatus*) and field cricket (*Gryllus assimilis*) (Cabano, 2017).

1.5 *Fish colour and nutrition*

Skin colour is one of the most important quality parameters for ornamental fish market and their desirability (Gouveia *et al*., 2003; Gouveia and Rema, 2005). Colour influences ornamental fish commercial value being one of the most important factors for the ornamental industry. Additionally may have other purposes such as gender identification; age; indication of breeding or gestational phase; communication; identification; camouflage; defence mechanism and adoption to the environment (Balamurugan et al., 2017; Devi et al., 2015).

In captivity colour usually starts to fade, in order to prevent this, various natural and synthetic pigments might be used improve pigmentation patterns (Devi et al., 2015). Fish skin colour depends on chromatophores cells, present in the tissue. There are four main groups of pigments that can provide coloration in this cells, namely melanins, carotenoids, pteridines and purines (Devi et al., 2015). The deposition of these pigments is controlled by internal and external factors and may be altered by environmental conditions such has stress or physical changes (Izquierdo *et al*., 2005).

Carotenoids are the main pigment responsible for the coloration of *Amphiprion ocellaris*, ranging in hues from yellow to red (Devi et al., 2015; Ho et al., 2013). Since vertebrates are not able to synthesise the carotenoids, its absorption and deposition depends on the fish diet *(*Hekimoğlu *et al*., 2017; Ho *et al*., 2013). Several types of carotenoids are used in aquaculture, namely to enhance pigmentation in fish as well have some antioxidant properties (Ho *et al*., 2013). It is known that carotenoids of the lutein, tunaxathin and doraxanthin groups enhance yellow colour in fish; ß-carotene, zexanthin and canthaxanthin help improve orange hue and groups such as astaxanthin and erichineon are responsible for red coloration improvement (Nhan *et al*., 2019). Studies have shown that the astaxanthin is the main dietary pigment in clownfish aquaculture, since it is responsible for the orange-red coloration (Ho et al., 2013).

The use of synthetic astaxanthin it is a common practice as a supplement for pigmentation (Yasir and Qin, 2010). However, the use of synthetic carotenoids is expensive and limited, since it is quite difficult to not only incorporate them effectively as an aqua feed ingredient, as well has may contain carcinogens (Gupta et al., 2007; Hekimoğlu et al., 2017; Nhan et al., 2019). Therefore, the use of alternative and natural sources of carotenoids is quite important. Nowadays microalgae are one of the most used sources of natural carotenoids. The harvesting time of microalgae can determine the desired colour pigment, that said if the desired colour is green the harvest should be done very early whereas for orange-red, carotenogenesis

must progress over time and biomass harvesting must be performed on a later stage (Gouveia *et al*., 2006). According to Hekimoğlu *et al*., 2017, the addition of microalgae species such has *N. ocular*, *P.cruentum* and *Spirulina* involving natural pigment materials to the feeds increases the total amount of carotenoids in clown fish skin.

One of the most used methods to evaluate colour is the colorimeter method. Colour can be measured in different ways (instrumental or visual analysis). Perceived colours are often characterized by 3 dimensions: Lightness, Hue and Chroma (Fernández-Vázquez *et al.*, 2013). Where, Chroma is usually used to determine the degree of difference of a hue and considered a quantitative attribute of colour and it is measured in percentage from the centre of the cone (0) to the surface (100) (Fig. 6). Hue is the attribute that defines colour and it is considered a qualitative attribute and it is measured in angular degrees around the cone ending in red ranging from 0 to 360º(Fig. 6) (Balamurugan *et al*., 2017; Fernández-Vázquez *et al*., 2013). Luminosity is a measure of lightness and is measured in percentage, where it ranges from 0 (black) to 100 (white), across the height of the cone (Fig. 6) (Ho *et al*., 2013). Hue and Chroma parameters can be calculated in accordance with the recommendations of the International Commission on Illumination (CIE of a* and b*)(CIE, 1976) through the use of a* and b* coordinates (Sant'Ana, 2019). This method is also called colour machine vision system that matched colours to the standardized L*, a* and b* colour space values. Where a* has red-green chromaticity (green (-a*) and red (+a*)) and b* yellow-blue chromaticity (blue (-b*) and yellow (+b*)) (Fig.6) (Nhan *et al.*, 2019). Therefore trough the use of this method it is possible to evaluate fish pigmentation.

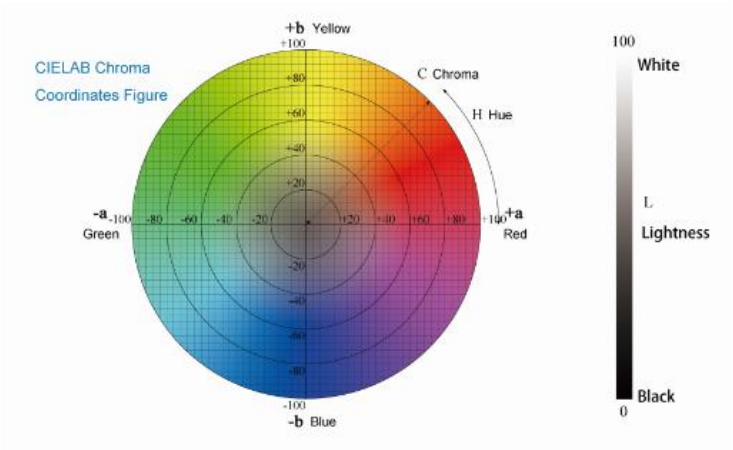

Figure 6 - Representation of Cielab colour space.

1.6 *Digestive enzymes*

The digestive enzymes located in the brush border section of the intestine are responsible for the digestion and absorption of nutrients where food is break down at the final stage and absorbed (Silva et al., 2010). Proteases are responsible for protein breakdown, being that proteases like trypsin and chymotrypsin are responsible for the hydrolysis of polypeptides, followed by aminopeptidase that further degrades protein into smaller peptides and amino acids (Natalia et al., 2004). Alkaline phosphatase is usually found in cell membrane where active transport occurs, therefore it is speculated that they play an important role in the absorption process of nutrients and its increase presence in the intestine should indicate a higher development of the digestive capacity of the fish (Cara et al., 2003). Lipase is one of the main lipolitic enzymes studied in fish and are secreted by the hepatopancreas in response to presence of triglycerides, being able to catalyse the hydrolysis of carboxy-ester bonds, not only this subtract but also cholesterol esters or fat-soluble vitamin esters (Infante and Cahu, 2007). Therefore this enzyme is one of the main keys in lipid digestion.

Digestive enzyme activity is an important and useful parameter to evaluate the structural and functional development of the digestive system as well as a way to determine digestive capacity and nutritional requirements and capacity of fish, allowing the development of feeding protocols specific for each species (He et al., 2012). Due to the new feed ingredient tendencies the characterization of proteases activity is important in several fish species in order to simplify diet formulation, prediction of inhibition by antinutritional factors (in plant-based ingredients) and suitable feeding regimes based on the rhythm of protease secretion (Natalia et al., 2004).

1.7 *Objectives*

The main objective of this thesis was to evaluate the impact of nutrition on key performance indicators of *Amphiprion ocellaris* juveniles, as well as, to identify a diet formulation for juveniles that promotes optimal growth and quality.

2. Methodologies

2.1 *Fish and experimental design*

The fish were reared in the LEOA facility at University of Algarve (Faro, Portugal). The stock of 186 fish were obtained from a company in Israel. Each tank initially contained 20 fish with fish density of 0.68g/L and mean weight of 0.36g and 27.7 mm length. Juveniles were placed in 9 rectangular (14x15x50 cm; 10.5 L) tanks in a recirculation aquaculture system

(RAS). The experimental system was equipped with a mechanical filter, a Biofilter, a protein skimmer and a UV sterilizer (Fig.7). Upon arriving, the fish were maintained at a photoperiod of 14:10h (L:D) cycle with light intensity of 600 lux, temperature average of 26-28 ±1 ºC, salinity 0.03 ±1 g/L, dissolved oxygen maintained above 95% of saturation and nitrogen compounds at 0 mg/L.

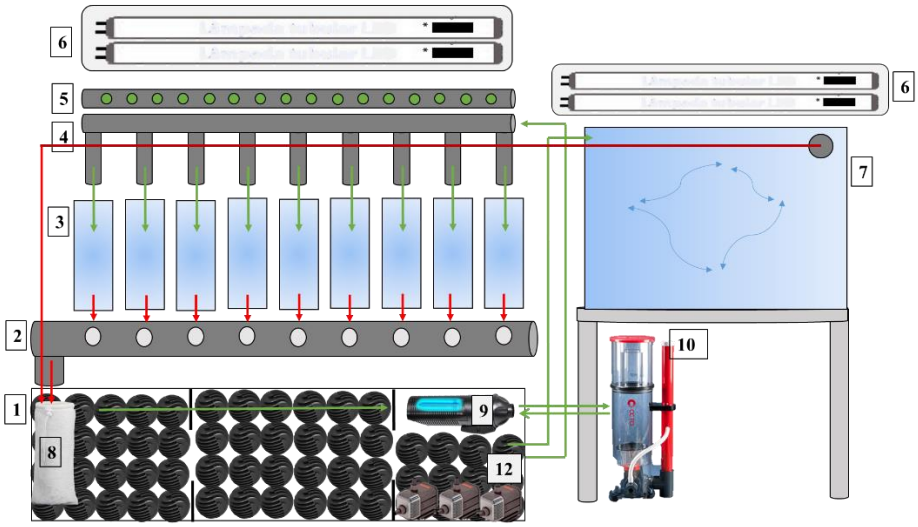

Figure 7 - Experimental system design. 1- Biofilter; 2- Water waste; 3- Larvae tanks; 4-Tank water supplier; 5- Aeration system; 6- Lights; 7- Water recirculation tank; 8- Mechanical filter; 9-UV light filter; 10- Skimmer.

A daily monitoring of environmental parameters of the rearing water and juvenile mortality was done; the tanks were siphoned daily in order to remove uneaten food and every two days cleaned with a sponge and filters cleaned regularly. Since it is a recirculating system, a gradual automatic water change was done daily in order to preserve water quality. Water renewal in the tanks started at 9 renewals/h and progressively increased has the fish grew, finishing in 11renewel/h, in order to promote better water quality in each tank.

The fish were fed *ad libitum* by hand several times a day to avoid feed losses from 30DAH to 90 DAH. During the night-time each replicate tank had an automatic feeder (Fish Mate), which dispensed feed every 3h during the night. Feed per tank was quantified during the experiment using a scale (0.001g; Precisa 1212M SuperPal-series). The inert diet size increased according with juvenile growth and mouth size as described in Table 1.

Table 1 - Clownfish feeding schedule.

Days after hatching (DAH)	Inert feed size
30-42 DAH	400-600 µm (50%) + 600-800 µm (50%)
43-53 DAH	400-600 µm (25%) + 600-800µm (75%)
54-90 DAH	600-800µm (100%)

2.2 *Experimental diets*

The experimental trial lasted 60 days and during that period, three experimental diets were assigned randomly to 9 tanks: Control- Diet A -standard feed (CTRL treatment), Diet B- (B treatment) and Diet C – (C treatment). The inert diets were formulated and processed by SPAROS Lda (Olhão, Portugal). Diet A was formulated with high levels of traditional marine protein sources (fishmeal, krill meal, squid meal, marine hydrolysates) and was supplemented with synthetic astaxanthin (Table 2); diets B and C comprised a marked increase of various emergent raw materials such as natural Artemia biomass, microalgae (*Nannochloropsis* sp., *Tetraselmis* sp., Spirulina, *Chlorella* sp.) and insect meal, at the expenses of traditional marine protein sources and without the addition of synthetic astaxanthin (Table 2). All diets were isonitrogenous and isoenergetic. The impact of experimental dietary formulations on several juvenile key performance indicators (growth, FCR, K, proximal composition, digestive capacity, colour) were determined at the end of the experimental period (90 days).

Table 2 - Formulation of the experimental diets (Diet A, Diet B and Diet C).

Ingredients, % **Diet CODES**	**Diet A**	**Diet B**	**Diet C**
MARINE proteins			
Fishmeal Fish hydrolysate Squid meal Shrimp meal Krill meal	59.00	32.25	20.00
Vegetable proteins			
Pea protein concentrate Wheat gluten	22.80	22.80	16.00
Artemia biomass		5.00	5.00
Microalgae biomass		17.50	30.00
Insect meal		10.00	20.00
Oils	9.20	7.95	5.20
Vitamins and minerals	1.50	1.50	1.50
Garlic extract		0.15	0.15
Spinach powder		0.10	0.10
Other additives	7.50	2.75	2.05
Total	**100.00**	**100.00**	**100.00**

2.3 *Sampling*

During the experimental period, juveniles were sampled at three distinct periods: initial (day 0, 30DAH), intermediate (day 30, 60DAH) and final (day 60, 90DAH). Samples were taken to analyse growth and performance indicators, colour, digestive capacity and diet utilisation.

At 30DAH fish were weighed in bulk and distributed in the tanks replicates in order to maintain an initial wet weight coefficient of variation around 10%. Initial body weight (BW_i, g) and total length (TL_i, cm) (Fig. 8) were determined in bulk (*n*=180). Fish were weight using a scale (0.001g; Precisa 1212M SuperPal-series; Switzerland) and length by photographing (Sony DSC-S85) the fish and measuring with ImageJ software program (https://imagej.nih.gov/ij/). Fish were euthanized by lethal anaesthesia at 500ppm (2-phenoxyethanol, VWR life Science), sampled for initial whole-body composition (*n*=5) and frozen at -20ºC for posterior analyses.

For the intermediate sampling, 60DAH fish from each replicate (*n*=3, *n*=9/treatment) were euthanized by lethal anaesthesia at 500ppm (2-phenoxyethanol, VWR life Science). Body weight (BW_{int}, g) and total length (TL_{int}, cm) (Fig. 8) were determined as described for the

initial sampling. The muscle, liver and digestive system were sampled and weight. Both individual fish and viscera were weight using a scale (0.0001g; Denver Instrument TB-215D; USA). All samples were frozen at -20ºC for posterior analyses (*n*=27). During the sampling, tank biomass was determined by bulk weight using scale (0.001g; Precisa 1212M SuperPal-series, Switzerland).

At the final sampling, 90DAH fish from each replicate (*n*=10, *n*=30/treatment) were euthanized by lethal anaesthesia at 500ppm (2-phenoxyethanol, VWR life Science). Body weight (BW_f,g) and total length (TL_f,cm) were determined individually. Length was measured by a ruler (Fig. 8) and weight individually in a scale (0.0001g; Denver Instrument TB-215D; USA). Liver and digestive system were sampled and weight (*n*=10, *n*=30/treatment). Fish for final whole-body composition (*n*=5, *n*=15/treatment) were frozen at -20ºC for posterior analyses as well as liver, digestive system and muscle (*n*=5, *n*=15/treatment). Each sampled fish was photographed (*n*=10, *n*=30/treatment) using Leica Application Suit (LAS) (Germany) microscope. Remaining fish from each tank were measured individually by ruler (Fig. 8) and weight in bulk using a using scale (0.001g; Precisa 1212M SuperPal-series; Switzerland).

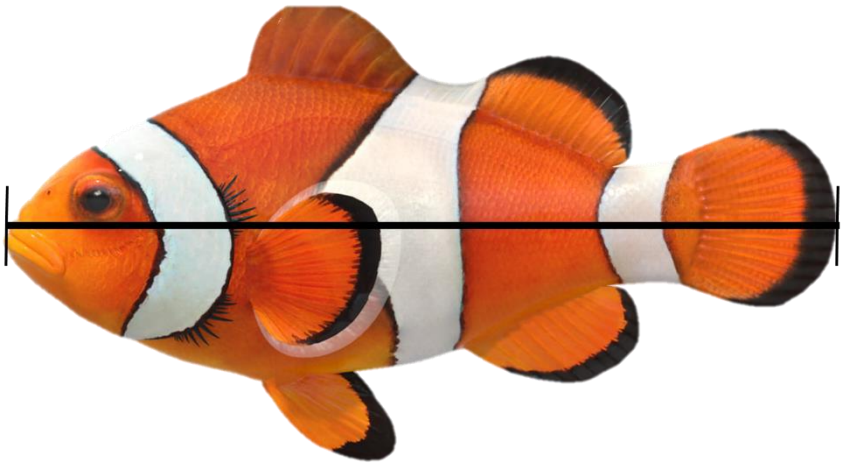

Figure 8 - Fish total length measurement was done from head to caudal fin.

2.4 *Biochemical analysis*

2.4.1 *Diet and Fish proximal composition*

- **Samples**

For proximal composition samples from fish and diets where freeze-dried and kept in dry conditions until further analysis. Fish samples where gathered from the initial sampling (day 0, 30DAH) (*n*=5) and final sampling (day 60, 90DAH) (*n*=5, *n*=15/treatment; *n*=4, *n*=12/treatment). Before grinding the samples, dry weight of each sample was weight in a scale (0.0001g; Denver Instrument TB-215D; USA) and registered. Diets were grinded using a pestle whereas fish using a small dispersing instrument (T10 basic Ultra-Turrax, Ika, China).

- **Protein analysis**

Protein was determined following the method of Lowry modified by (Rutter, 1967). This method is based on two reactions: the Biuret reaction, where peptide bonds of proteins, under alkaline conditions, react with cooper to produce Cu+ that reacts with the Folin reagent; the Foloin-Ciocalteau reaction where phosphomolybdotungstate is reduced to heteropolymolybdenum blue by copper- catalysed oxidation of aromatic amino acids. This reaction result in a strong blue colour depending on the tyrosine and tryptophan content. This method is sensitive down to about 0.01 mg of protein/ml and its best applied on concentrations ranging from 0.01-1.0 mg/ml of protein.

- **Lipid analysis**

Lipid analysis was determined using the modified method of Trondheim by (Bligh and Dyer, 1959). This method consists in the homogenization of wet tissue with a mix of chloroform and methanol. Followed by a dilution and water that separates the homogenate in 3 layers, with the chloroform layer containing the all the lipids. This purified lipid extract is obtained by the isolation of the chloroform layer. This layer is then evaporated to dryness and the weight of lipid residue is determined.

2.4.2 *Digestive enzymes*

The digestive enzymes analysed were proteases (trypsin, chymotrypsin and aminopeptidase), 18C-like lipase and alkaline phosphatase. The enzymatic activity was analysed using a florescent subtract specific to each enzyme. Enzyme activity of trypsin and

chymotrypsin was determined using the method described by Rotllant et al., 2008, whereas for aminopeptidase was according to Sanz and Toldrá, 2002. Enzymatic activity of lipases was determined according the method of modified Rotllant et al., 2008 and alkaline phosphatase through the Fernley and Walker, 1965 method.

- **Sample preparation**

The complete digestive tract of three individuals per tank (*n*=9 per treatment) were dissected, immediately frozen at −80°C and later freeze-dried. Enzyme extracts were prepared for enzyme activity measurement from these samples. Samples were manually homogenized in 1mL distilled water and centrifuged for 10 min at 4 °C at 650 g. The supernatants were measured for enzymatic activity. During sample preparation process, the samples were kept in cold conditions, in order to avoid enzymes denaturation and/or damage. The same process was done during the final trial as well, where the complete digestive tract of five individual per tank (*n*=9 per treatment) were dissected. All enzyme activities were expressed as RFU (Relative Fluorescence Units) per mg fish weight. Enzymes were analysed in micro plates using a micro plate reader (Synergy/HT, Biotek).

- **Proteases (Tripsin, Chymotripsin and Aminopeptidase)(TRY, CHY, AMPN)**

For trypsin, chymotrypsin and aminopeptidase analysis, the fluorogenic substrates Boc-Gln-Ala-Arg-7- methylcoumarin hydrochloride (BOC - SIGMA B4153), N-Succinyl-Ala-Ala-Pro-Phe-7-amido-4-metilcoumarin (SIGMA S9761) and N_{α}-Benzoyl-L-arginine-7-amido-4-methylcoumarin hydrochloride (SIGMA B7260), respectively, were diluted in dimethyl sulfoxide (DMSO), to a final concentration of 20 µM. For analysis, 190 µl of 50 mM Tris + 10 mM CaCl2 buffer (pH 8.5), 15 µl of the extract homogenate and 5 µl of the flourogenic substrate were added to the microplate. Fluorescence was measured at 355 nm (excitation) and 460 nm (emission).

- **Lipase (LIP)**

Lipase activity was evaluated using 4-methylumbelliferyl oleate (SIGMA 75164). Substrate was dissolved in phosphate buffer (pH 7.0) to a final concentration of 4.0 mM 15 µL of the extract homogenate was added to the microplate and mixed with 250 µL of 0.4mM substrate for the analysis. Fluorescence was measured at 355 nm (excitation) and 460 nm (emission).

- **Alkaline phosphatase (ALP)**

The substrate 4-Methylumbelliferyl phosphate disodium salt (4-MUP, M8168 Sigma) was diluted in borate buffer (ph 8.5) to a final concentration of 1mM. In the microplate, 100ul of substrate and 15ul of extract were mixed. Fluorescence was measured at 360 nm (excitation) and 440 nm (emission).

2.5 *Phenotypic analysis*

Pigmentation analysis was done using the Leica Application Suite (LAS) microscope in darkness to photograph each fish. Photographs were analysed with Adobe Photoshop CS6 and two squares (20x20mm) were determined, one below the initial part of the spiny dorsal fin (Square 1) (Fig. 9) and a second square the other between the soft dorsal fin and anal fin adjacent to the white middle band of the fish (Square 2) (Fig 10). Colour measurements were based on 4 points from each square to obtain CIELab coordinates to identify colour in a colour space. CIELab means International Commission on Illumination (CIE) and Lab means variables that will be measured, L*: lightness, a*: redness and b*: yellowness (http://www.cie.co.at/).

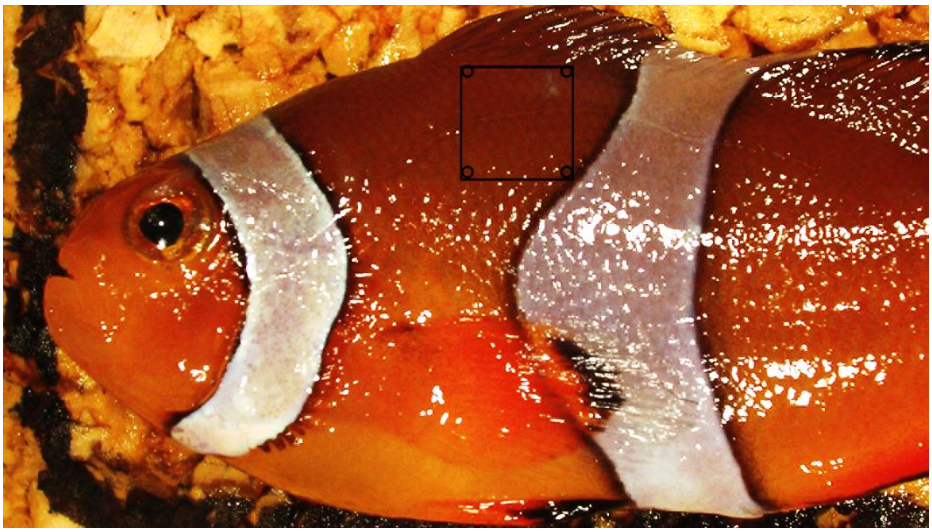

Figure 9 - Position of Square 1- below the initial part of the spiny dorsal fin; open circles represent sampling points for CIELab coordinates (L*, a*, b*).

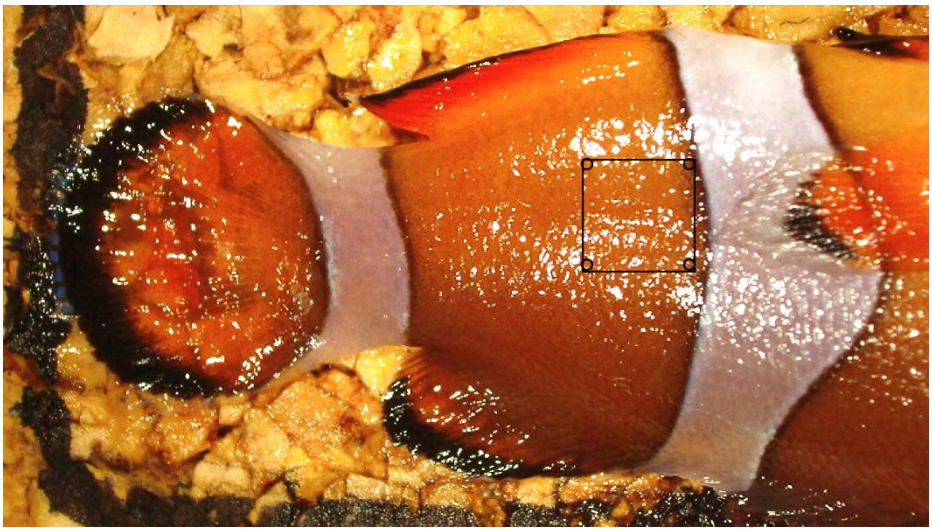

Figure 10 - Position of Square 2- between the soft dorsal fin and anal fin adjacent to the white middle band of the fish; open circles represent sampling points for CieLab coordinates (L*, a*, b*).

The CIELab colorimetric method, is based on a L*a*b* model for colour measurement, was used to determine the changes in skin pigmentation of fish due to dietary treatments. From a* and b* coordinates, hue $(H^{o})_{(1)}$ and Chroma $(C)_{(2)}$ parameters were calculated (Schloss et al., 2018):

(1) $H=\arctan(b^*/a^*)$ (2) $C_{ab}=\sqrt{(a^2+b^2)}$

To estimate perceptible colour differences $(\Delta E^*)_{(3)}$ among dietary treatments the (CIE, 1976) formula (based on the Euclidian distances between colours in CIELab space) was applied:

(3) $\Delta E^* = \sqrt{[(L_2*-L_1*)^2+(a_2*-a_1*)^2+(b_2*-b_1*)^2]}$, where 1 and 2 are the values determined in the different dietary treatments.

For both squares average values of colour coordinates were used for ΔE* calculation (Diet A vs Diet B; Diet A vs Diet C and Diet B vs Diet C) to evaluate colour differences. Where distances between colour were perceived has "irrelevant perceptual differences" ($\Delta E^* < 1$), "a slight perceptual difference ($1 < \Delta E^* < 2.3$) or a "clear perceptual difference" ($\Delta E^* > 2.3$) (Sant'Ana, 2019) .

For initial (0 days, 30DAH) and intermediate (30 days, 60DAH) samplings total length was determined by photographing using a photo camera (Sony DSC-S85) and each fish was measured through ImageJ software program. Whereas for the final (60 days, 90DAH) sampling they were measured using a ruler. Bulk weight was determined using a scale (Precisa 1212M SuperPal-series; Switzerland) and viscera (intestine and liver) and individual body weight using a scale (Denver Instrument TB-215D; USA).

2.6 *Data analysis*

The fish condition factor (K) was calculated using the final wet body weight (BW_f) and final body Total length (TL_f) (4):

$$(4)\ K=\frac{BW_f\,(g)}{[TL_f\,(cm)]^3}\ x\ 100$$

The hepatosomatic index (HSI) (5) and the viscerosomatic index (VSI) (6) were calculated using the wet liver (LW) and wet viscera (VW) weight, respectively divided by individual fish wet body weight (BW_f). The following formulas were applied:

$$(5)\ HSI=\frac{LW\,(g)}{BW_f\,(g)} \qquad (6)\ VSI=\frac{VW\,(g)}{WW\,(g)}$$

The daily growth index (DGI) was calculated using the difference in final body weight (BW_f) and initial body weight (BW_i), by the days of the experiment (7), as well as the specific growth rate (SGR (% day^{-1}))(8) and weight gain (WG)(%BW_i)(9).

$$(7)\ DGI=\left[\frac{BW_f^{\frac{1}{3}}\,(g)-BW_i^{\frac{1}{3}}\,(g)}{days\ (n)}\ x\ 100\right] \qquad (8)\ SGR=\frac{\ln BW_f\,(g)-\ln BW_i(g)}{days\ (n)}$$

$$(9)\ WG(\%\ BW_i)=\frac{(BW_f\,(g)-BW_{i(g)})}{BW_{i(g)}}\times 100$$

Voluntary feed intake (VFI, %ABW), will be calculated according to formula (10), where ABW is the average fish weight, whereas feed conversion rate (FCR) (11), using the feed intake (FI) and weight gain (WG), where fish dead weight (FDW), number of dead fish (n_d), initial body weight (BW_i) and initial and final biomass (IB) (FB), are used:

$$(10)\ VFI=\frac{crude\ feed\ intake}{\frac{ABW}{days\ (n)}}\times 100$$

Where, $ABW=\frac{BW_i\ (g)+BW_f\,(g)}{2}$

$$(11)\ FCR=\frac{FI(g)}{WG\,(g)}$$

Where, $WG=FB\,(g)-FI\,(g)+(FDW(g)-(BW_i\,(g)\times n_d))$

Survival rate (S, %) for each treatment was determined by direct counting of the individuals at the end of the experiment relative with the initially stocked fish (12).

(12) Survival (%)=(final fish number-initial fish number) x 100

2.7 *Statistical analysis*

Data was verified for normality (Shapiro-Wilk test) and homogeneity of variances before further analysis. If normality was observed a one-way ANOVA was done, followed by a post hoc Tukey´s Honest Significant Difference (HSD) test, if differences were observed. If normality was not observed a nonparametric test was done, followed by a Kruskal-Wallis post-hoc test if the null hypothesis was rejected. These analyses were used to determine the effect of the dietary treatments on growth performance, enzymes activities, skin pigmentation, and body and diet composition. Statistical significance was set at reliability level of 0.05 and all results were reported as arithmetic mean values ± standard deviation. SPSS software was used for statistical analyses and Excel (version 2010) for creating the charts and graphs. Data that was expressed in percentage lower than 100 was transformed using square root of arcsin (HIS, VSI, VFI, SGR, survival %, protein % and lipid%), whereas data higher than 100 was transformed using log10 (WG), prior to the statically analysis.

3. Results

3.1 Growth performance

At the intermediary sampling point (60 DAH) no effect on body weight (BW), specific growth rate (SGR), voluntary fee intake (VFI), total length (TL), condition index (K), viscerosomatic index (VSI) and hepatosomatic index (HSI) was observed on fish fed with either of the diets (Table 3). However, the feed conversion rate (FCR) was similar between fish fed with diets A and B (0.77± 0.03 and 0.69 ± 0.04 respectively). With fish fed with diet C presenting a lower FCR of 0.64 ±0.06 when compared to the fish fed with diet A ($P<0.05$) (Table 3).

Table 3 - *Amphiprion ocellaris* juvenile's performance indicators at 60 and 90 days after hatching (DAH).

	Diet A	Diet B	Diet C
60DAH			
Body weight (g)(BW)	0.76±0.18	0.74±0.17	0.74±0.12
Weight gain (%BW_i/day) (WG)	135.98±10.91	118.24 ± 14.57	108.42±17.81
Specific growth rate (%day) (SGR)	2.77± 0.15	2.51 ± 0.22	2.36 ± 0.28
Feed conversion rate (FCR)	0.77± 0.03^{a}	0.69 ± 0.04^{ab}	0.64 ± 0.06^{b}
Voluntary feed intake (%ABW/day; VFI)	3.37 ± 0.07	3.46 ± 0.18	3.54 ± 0.07
Feed Intake (g) (FI)	12.53 ±0.54	12.28±1.16	12.16±0.80
Total length (cm) (TL)	3.28 ± 0.34	3.07 ± 0.58	2.92±0.39
Condition index (K)	2.13 ±0.21	3.03 ±1.78	3.19 ±1.12
Hepatosomatic Index (HSI)	1.52 ± 0.71	2.23 ± 0.45	1.88 ± 0.30
Viscerosomatic Index (VSI)	6.22 ±1.19	7.09 ± 0.84	6.89 ± 0.57
90DAH			
Final body weight (BW_f)	1.00±0.32	1.00±0.23	0.92±0.24
Weight gain (%IBW/day)(WG)	150.57±7.65^{a}	124.59±18.25^{ab}	109.67±6.24^{b}
Specific growth rate (%day) (SGR)	1.77± 0.05	1.69±0.11	1.61±0.09
Feed conversion rate (FCR)	1.99±0.03^{c}	2.33±0.09^{b}	2.49±0.04^{a}
Voluntary feed intake (%ABW/day; VFI)	3.11±0.10^{b}	3.44±0.06^{a}	3.68±0.14^{a}
Feed Intake (g)(FI)	23.77±1.05	24.26±1.66	24.97±1.20
Total length (cm) (TL)	3.65±0.39	3.61±0.29	3.51±0.29
Condition index (K)	2.08±0.68	2.10±0.23	2.12±0.31
Hepatosomatic Index (HSI)	2.20±0.75	1.93±0.48	1.94±0.55
Viscerosomatic Index (VSI)	7.04±2.29	7.03±1.19	7.20±1.13
Survival rate (%)	100.00±0.00	96.67 ± 5.77	93.33 ± 2.89

Values are means ± standard deviation. Different superscript letters indicate statistical differences between replicates (Tukey's test, $P<0.05$).

Concerning fish growth performance indicators at 90DAH, statistical differences were observed in the fish regarding weight gain (WG), feed conversion rate (FCR) and voluntary feed intake (VFI). At 90DAH fish fed with diet A and B presented a higher weight gain (150.57±7.65 g and 124.29±18.25 g, respectively), when compared with diet C (109.67±6.24 g). Experimental diets had impact on feed conversion rate (FCR) of the fish, with fish fed with diet A having the lower FCR ($p<0.05$) (1.99±0.03). Voluntary feed intake (VFI), was significantly higher in fish fed with diets B and C (3.44±0.06 (%ABW/day) and 3.68±0.14 (ABW%/day)) when compared to diet A ($p<0.05$). All fish presented similar body weight (FBW), specific growth rate (SGR), feed intake (FI), final length (ST), condition index (K), viscerosomatic index (VSI) and hepatosomatic index (HSI) at the end of the experiment

independently of experimental diet. Survival rate was not affected by the dietary treatments (p>0.05).

3.2 Proximal composition

The protein content of fish at 90DAH was not affected by dietary treatments (Fig. 11) (p=0.21).

Regarding lipid content fish fed with diet B had a higher lipid % when compared to the ones fed with diet C. whereas fish fed with diet A had no differences in lipid content when compared to the ones fed with the alternative diets.

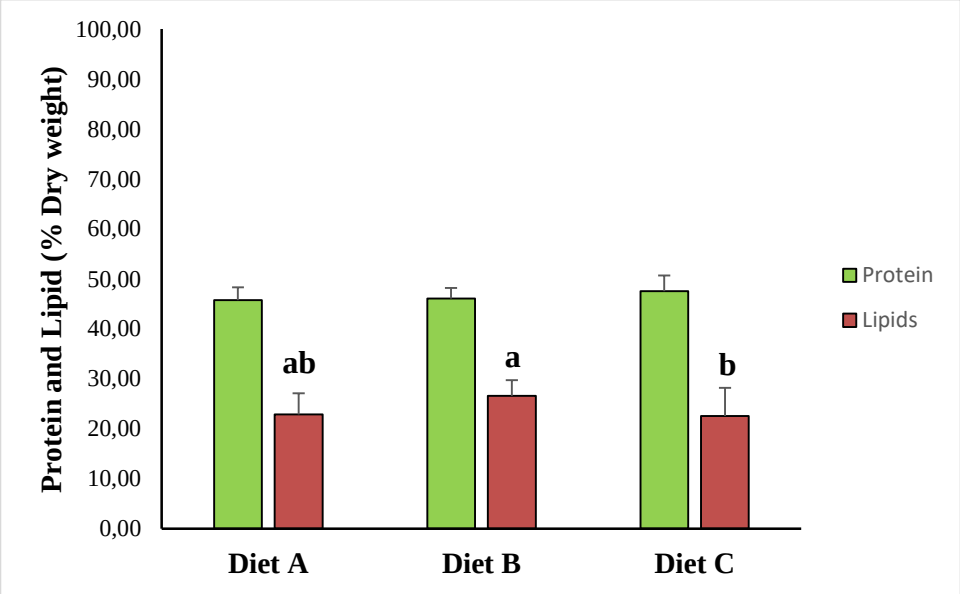

Figure 11 - Protein and lipid content of fish at 90DAH for fish fed with diet A, B and C. Values are means ± standard deviation. Data represented in bars with different superscript letters differ significantly. Absence of superscripts indicates no significant difference between treatments (P>0.05).

3.3 Digestive enzymes

Neither the alternative diets (B and C) nor control diet (A) had any influence on the digest enzymes (trypsin (TRY) (p=0.76), chymotrypsin (CHY) (p=0.81), aminopeptidase (AMPN) (p=0.53), lipase (LIP) (p=0.41) and alkaline phosphatase (ALP) (p=0.34)) of the fish at 60DAH (Table 4).

At 90 DAH, experimental diets had no effect on trypsin (TRY) and chymotrypsin (CHY) digestive enzymes of the fish (Table 4).

Table 4 - *Amphiprion ocellaris* juvenile's digestive enzymes at 60 and 90 days after hatching (DAH).

	Diet A	Diet B	Diet C
		60 DAH	
TRY	$3.57 \times 10^6 \pm 6.22 \times 10^6$	$7.20 \times 10^6 \pm 3.27 \times 16^9$	$3.74 \times 10^6 \pm 4.07 \times 10^6$
CHY	$4.50 \times 10^5 \pm 4.56 \times 10^5$	$2.58 \times 10^5 \pm 1.73 \times 10^5$	$4.79 \times 10^5 \pm 6.11 \times 10^5$
AMPN	$1.34 \times 10^4 \pm 5.68 \times 10^3$	$1.44 \times 10^4 \pm 6.15 \times 10^3$	$1.74 \times 10^4 \pm 1.05 \times 10^4$
LIP	$2.61 \times 10^4 \pm 2.29 \times 10^4$	$3.72 \times 10^4 \pm 2.40 \times 10^4$	$2.11 \times 10^4 \pm 1.18 \times 10^4$
ALP	$5.15 \times 10^5 \pm 2.34 \times 10^5$	$8.97 \times 10^5 \pm 8.59 \times 10^5$	$1.03 \times 10^6 \pm 6.90 \times 10^5$
90 DAH			
TRY	$5.33 \times 10^7 \pm 1.91 \times 10^7$	$7.64 \times 10^7 \pm 4.6 \times 10^7$	$5.44 \times 10^7 \pm 8.13 \times 10^6$
CHY	$6.51 \times 10^5 \pm 4.03 \times 10^5$	$4.90 \times 10^5 \pm 2.39 \times 10^5$	$3.61 \times 10^5 \pm 1.94 \times 10^5$
AMPN	$2.17 \times 10^4 \pm 9.54 \times 10^{3b}$	$3.77 \times 10^4 \pm 2.34 \times 10^{4b}$	$6.19 \times 10^4 \pm 1.48 \times 10^{4a}$
LIP	$2.39 \times 10^4 \pm 1.05 \times 10^{4a}$	$1.19 \times 10^4 \pm 6.75 \times 10^{3b}$	$6.83 \times 10^3 \pm 4.20 \times 10^{3b}$
ALP	$1.19 \times 10^7 \pm 2.69 \times 10^{6ab}$	$8.78 \times 10^6 \pm 4.23 \times 10^{6b}$	$1.31 \times 10^7 \pm 4.62 \times 10^{6a}$

Values are means ± standard deviation. Different superscript letters indicate statistical differences between replicates (Tukey's test, $P<0.05$). Enzyme activity is expressed as RFU (Relative Fluorescence Units) per mg fish weigh.

Fish fed with diet C presented a higher concentration of AMPN ($6.19 \times 10^4 \pm 1.48 \times 10^4$) when compared to the fish fed diets A or B ($2.17 \times 10^4 \pm 9.54 \times 10^3$ and $3.77 \times 10^4 \pm 2.34 \times 10^4$). Regarding enzyme lipase (LIP), fish fed with traditional diet (Diet A) had a significantly higher amount of enzyme activity ($2.39 \times 10^4 \pm 1.05 \times 10^4$) when compared to the alternative diets (Table 4). It was observed that ALP enzyme content in fish fed with diet A ($1.19 \times 10^7 \pm 2.69 \times 10^6$) was similar to fish fed with diets B and C ($8.78 \times 10^6 \pm 4.23 \times 10^6$ and $1.31 \times 10^7 \pm 4.62 \times 10^6$), however, diet C was significantly higher when compared to diet B ($p<0.05$).

3.4 Pigmentation

Fish skin colour was positively influenced in the Square 1 by the alternative diets (B and C) ($p<0.05$) (Table 5). It was observed that fish fed with diet B had a higher level of redness (a*) (25.83±1.92) and colour present (Chroma) (36.67±2.40), followed by diet C (23.71±2.22 and 34.51±1.78), when compared to the diet rich in traditional marine ingredients (diet A) 21.33±1.76 and 31.81±1.85, correspondingly. Regarding yellowness (b*), both alternative diets (diet B and C) presented a higher value (25.99±2.01 and 25.01±1.32) than diet A (23.54±1.63). Whereas for hue, fish fed with diet C (0.76±0.05) were has close to red and yellow, has diet A (0.74±0.05) and B (0.78±0.04), respectively.

Table 5 - Amphiprion ocellaris juvenile's colour 90 days after hatching (DAH).

	Diet A	**Diet B**	**Diet C**
Square 1			
L*	20.07 ± 1.58^{b}	21.12 ± 1.94^{a}	20.49 ± 1.09^{ab}
a*	21.33 ± 1.76^{c}	25.83 ± 1.92^{a}	23.71 ± 2.22^{b}
b*	23.54 ± 1.63^{b}	25.99 ± 2.01^{a}	25.01 ± 1.32^{a}
Chroma	31.81 ± 1.85^{c}	36.67 ± 2.40^{a}	34.51 ± 1.78^{b}
Hue	0.74 ± 0.05^{b}	0.78 ± 0.04^{a}	0.76 ± 0.05^{ab}
Square 2			
L*	34.09 ± 3.33	33.31 ± 3.39	33.67 ± 3.52
a*	25.34 ± 2.73^{b}	31.73 ± 3.18^{a}	29.86 ± 3.60^{a}
b*	35.78 ± 3.33^{b}	37.77 ± 3.63^{ab}	38.17 ± 3.77^{a}
Chroma	43.91 ± 3.56^{b}	49.37 ± 4.35^{a}	48.60 ± 3.74^{a}
Hue	0.62 ± 0.06^{b}	0.70 ± 0.04^{a}	0.66 ± 0.07^{a}

Values are means ± standard deviation. Different superscript letters indicate statistical differences between replicates (Tukey's test, $P<0.05$).

In the Square 2 fish colour was positively affected by the inclusion of new and emergent ingredients (B and C) ($p<0.05$) (Table 5). Both alternative diets (B and C) demonstrated in the fish a higher level of redness (a*) (31.73±3.18 and 29.86±3.60) as well as colour presence (Chroma) (49.37±4.35 and 48.60±3.74) than the traditional marine origin diet (A) (25.34±2.73 and 0.62±0.06 respectively). Lower levels of yellowness (b*) were observed in fish fed with diet A (35.78±3.33), having C a higher value (38.17± 3.17), nonetheless diet B presented the same level of yellowness has A and B. Both alternative diets (B and C) (0.70±0.04 and 0.66±0.07), regarding hue, are closer to yellow than diet A, which is closer to red (0.62±0.06).

Table 6 - Amphiprion ocellaris juvenile's perceptible colour differences under dietary treatments at the end of the experiment (90DAH).

	ΔE* Diet A vs Diet B	**ΔE* Diet A vs Diet C**	**ΔE* Diet B vs Diet C**
Square 1	5.23	2.82	2.42
Square 2	6.74	5.13	1.95

Perceptible colour differences (ΔE*) values are calculated on the formula based on the Euclidian distances between colours in CIELab space. Where ΔE* < 1-"irrelevant perceptual differences"; 1 < ΔE* < 2.3 -"a slight perceptual difference; ΔE* > 2.3-"clear perceptual difference".

In Square 1 there were clear perceptual colour differences between diet A and B (ΔE* Diet A vs Diet B=5.23), while between diet A and C, and Diet B and C differences where observed even though values are similar (ΔE*=2.82 and ΔE*=2.42, respectively) (Table 6).

In the Square 2 clear perceptual differences were observed between diet A and B, and diet A and C (ΔE*=6.74 and ΔE*=5.13, respectively). Whereas, no perceptual differences were observed in diet B and C (ΔE* Diet B vs Diet C=1.95) (Table 6).

4. Discussion

One of the main focuses of the ornamental market is the improvement of fish colour, since it is the main factor that determines fish price and quality. Fish pigmentation is controlled by the endocrine and nervous system, but dietary sources of pigments are the major players in determining fish colour (Hekimoğlu et al., 2017). The preferred colour in *Amphiprion ocellaris* by aquarists is reddish orange (Tanaka et al., 1992). Therefore, the use materials involving synthetic and natural carotenoid as a feed additive it is inevitable. The use of synthetic materials increases colour intensity of fish for a limited period as well may contain some carcinogens and are usually very expensive.

Microalgae have a rich carotenoid content, when produced under some conditions (Kop et al., 2010).

Some research has been done in this field, where the use of alternative and natural sources of carotenoids, such as microalgae have been shown to improve pigmentation not only in commercial fish such as European seabass, Atlantic salmon, Nile tilapia has well as in ornamental fish such as clownfish, goldfish, kenyi cichlids, among others. According to Gouveia et al., 2003, the supplementation of microalgae in feeds does not have a measurable effect growth or voluntary feed intake in freshwater fish. Whereas for the study performed by Hekimoğlu et al., 2017, showed that *Amphiprion frenatus* (tomato clownfish) fed with *Nannochloropsis oculata* (microalgae) and *Porphyridium cruentum* (macroalgae) had higher weight development due to the addition of these microalgae species.

The present study shows that the use of alternative diets, rich in emergent natural materials such as microalgae, insect meal, Artemia biomass and with no addition of synthetic astaxanthin (diets B and C) had a higher positive impact on fish colour when compared to the traditional marine protein sources diet (diet A) supplemented with synthetic astaxanthin. During this study through CIELAB colorimetric analysis it was determined that in both analysed sections of the fish, fish fed with the alternative diets have shown higher levels of redness (a*) and yellowness (b*) as well has higher colour presence (Chroma), whereas regarding hue fish fed with alternative diets where closer to yellowness than red, being more in the reddish orange colour which is the desired colour of *Amphiprion ocellaris*. Furthermore, perceptual colour differences (ΔE) where observed between fish fed with the alternative diets (B and C) with the traditional diet (A). Therefore, the inclusion of microalgae in diets of ornamental fish, may improve fish coloration, which in this market is the most desired trait.

Another major market tendency is the reduction of fish meal and -oil in fish feeds through the inclusion of alternative sources such as insect meal and plant protein which are now emerging and showing huge potential as replacements for these finite and expensive ingredients (FAO, 2018).This alternative ingredients may also beneficial since most of them are already part of the natural diet of various species of fish. In this study the experimental diets are all isonitrogenous and isoenergetic, in order to evaluate the effects of the alternative ingredients in the fish diets. Diet A is composed by 59% of marine origin ingredients whereas B and C by 32.25 and 20 % respectively. The alternative diets (B and C) had 5% of Artemia biomass; 17.5 and 30 % of microalgae

biomass; and 10 and 20% of insect meal. The fish fed with the traditional diet presented an overall better performance indicators at the end of the trial when compared to the ones fed with the alternative diets. This was observed regarding weight gain (WG), feed conversion rate (FCR), and voluntary feed intake (VFI) of the fish. But even though fish fed with diet A presented a higher growth rate than fish fed with diet C, diet B had no differences between both. It was also observed that regarding fish fed with diet A had a lower FCR, followed by B and then C. Nonetheless in regards to VFI, fish fed with the alternative diets had higher values when compared to fish fed with diet A, even though this could be a positive aspect, fish did not show a lower FCR or higher WG, meaning that they "ate more and used less". Even though the use of insect meal and microalgae in diets as a substitute for fish meal have shown to maintain or improve growth performance in some fish(Li et al., 2017; Shi et al., 2017), the presence of chitin in insect meal, according to some authors, can affect the digestibility of nutrients influencing negatively fish growth (Belghit et al., 2019). Furthermore, results could be also justified but the increasing aggressiveness of the fish during the experiment, which could have influenced the feed intake of the fish during the trial. However, since the ornamental market main focus is colour it could be an advantage to have a diet that promotes a higher pigmentation incidence than overall growth.

Determination of sex in clownfish is determined by aggressiveness, since the strongest and most aggressive individuals transform into females, there is a possibility that this selective pressure may have caused the increase of juvenile aggressiveness in the tanks (Pradeep, 2010). Even though survival rate of the fish, was not affected by any of the diets some mortality was observed, that can be attributed to the increasing aggressiveness of the fish among each other, causing the need to add environmental enrichments to the tanks in order to decrease aggressiveness.

Enzymatic activity is one of the main parameters for the evaluation of fish digestive system maturity. At the end of this study it was observed differences between enzymatic content of aminopeptidase (AMP), lipases (LIP) and alkaline phosphatase (ALP). Fish fed with diet C had a higher AMP and ALP when compared to A and B, having fish fed with diet A no differences regarding ALP, with either of the fish fed with the other diets. Both these enzymes are useful to determine the absorptive capacity of the intestine. According to Vizcaíno et al., (2014), the addition of microalgae supplementation can increase AMP and ALP activity, this increase might contribute to overall efficiency of the digestive and absorptive processes. Moreover, it has also been

observed that increase of ALP in rock bream can indicate the maturation of the digestive system (He et al., 2012). Therefore fish fed with diet C presented a higher maturation of the digestive system, followed by the ones fed with diet B. Even though fish fed these diets had a higher maturation level of the digestive system when compared with diet A, it is possible that these alternative diets had a lower digestibility than the control diet, since it was observed a higher VFI with a high FCR and low WG in fish fed with the alternative diets. Concerning LIP enzyme activity it was observed that fish fed with diet A had a higher LIP content than fish fed diets B and C. This may be due to the source of lipids in diet A when compared with B and C. Diet A had a difference source of lipids than the alternative diets, therefore there is an hypothesis that high LIP content may be due to the existence of more complex lipid source in diet A, having the fish the need to have a higher LIP content to digest lipids.

Lipid content was higher on fish fed with diet B, with fish fed with the control diet having no differences in fish lipid content with fish fed both with B and C. Therefore, even though both diets were isoenergetic, fish fed with diet B had higher lipid retention when compared to diet C. Having diets B and A better energy capacity storage.

5. Conclusions

Even though fish fed with diet A had a better overall performance indicators, in the ornamental market the main focus is pigmentation that is the determinative factor for fish value. Consequently, fish fed with diet B presented better results in terms of the desired pigmentation in the fish. Further studies on this topic are required to validate the observed effects over a longer feeding period and over specific phases of the life cycle (e.g. larvae phase). It is also imperative to gain a better knowledge of natural compounds that can reduce aggressiveness during juvenile phase of *Amphiprion ocellaris* as well as increase the replacement rate of the ingredients traditional diets (fish meal) by the alternative sources (insect meal and algae) to observe the long term effects of this ingredients.

6. References

Allen, G.R., 1972. The anemonefishes: their classification and biology. TFH Publications Neptune City, p. 286.

Allen, G.R., 1991. Damselfishes of the world. Mergus Publishers, Melle, Germany, p. 271.

Avella, M.A., Olivotto, I., Gioacchini, G., Maradonna, F., Carnevali, O., 2007. The role of fatty acids enrichments in the larviculture of false percula clownfish *Amphiprion ocellaris*. Aquaculture 273, 87–95.

Balamurugan, J., Ajith Kumar, T.T., Kathiresan, K., Meenakumari, B., 2017. Determination of growth, colour and other traits in F1 hybrid of *Amphiprion percula* (male)× *A. ocellaris* (female). Aquac. Res. 48, 2989–3003.

Balamurugan, J., Kathiresan, K., Meenakumari, B., 2018. Interspecific Hybridization and Reproductive Biology of Marine Clownfishes, The Orange Clownfish *Amphiprion percula* and Clown Anemonefish *A. ocellaris*. N. Am. J. Aquac. 1–9.

Barbasch, T.A., Buston, P.M., 2018. Plasticity and personality of parental care in the clown anemonefish. Anim. Behav. 136, 65–73.

Becker, E.W., 2007. Micro-algae as a source of protein. Biotechnol. Adv. 25, 207–210.

Belghit, I., Liland, N.S., Gjesdal, P., Biancarosa, I., Menchetti, E., Li, Y., Waagbø, R., Krogdahl, Å., Lock, E.-J., 2019. Black soldier fly larvae meal can replace fish meal in diets of sea-water phase Atlantic salmon (*Salmo salar*). Aquaculture 503, 609–619.

Biller, P., Ross, A.B., 2011. Potential yields and properties of oil from the hydrothermal liquefaction of microalgae with different biochemical content. Bioresour. Technol. 102, 215–225.

Buston, P., 2004. Does the presence of non-breeders enhance the fitness of breeders? An experimental analysis in the clown anemonefish *Amphiprion percula*. Behav. Ecol. Sociobiol. 57, 23–31.

Buston, P., 2003. Social hierarchies: size and growth modification in clownfish. Nature 424, 145–146.

Buston, P.M., Elith, J., 2011. Determinants of reproductive success in dominant pairs of clownfish: a boosted regression tree analysis. J. Anim. Ecol. 80, 528–538.

Buston, P.M., Wong, M.Y.L., 2014. Why some animals forgo reproduction in complex societies. Am. Sci. 102, 290–297.

Cabano, M., 2017. Impact of fish meal replacement by alternative and sustainable ingredients in diets for gilthead seabream (*Sparus aurata* Linnaeus, 1758) juveniles. Universidade do Algarve.

Calado, R., Olivotto, I., Oliver, M.P., Holt, G.J.(Eds.), 2017. Marine ornamental species aquaculture. Wiley Blackwell, Oxford, U.K, 677 p.

Cara, J.B., Moyano, F.J., Cárdenas, S., Fernández-Díaz, C., Yúfera, M., 2003. Assessment of digestive enzyme activities during larval development of white bream. J. Fish Biol. 63, 48–58. https://doi.org/10.1046/j.1095-8649.2003.00120.x

CIE, O.J., 1976. Official recommendations on uniform color space, color difference equations and metric color terms. Colorimetry.

Devi, Nanthini K., Ajith TT Kumar, and T. Balasubramanian. 2016. Pigment Deficiency Correction in Captive Clown Fish, *Amphiprion ocellaris* Using Different Carotenoid Sources. Journal of FisheriesSciences. com 9, 72-79

Dhaneesh, K. V, Ajith Kumar, T.T., Shunmugaraj, T., 2009. Embryonic development of percula clownfish, *Amphiprion percula* (Lacepede, 1802). Middle-east J. Sci. Res. 4, 84–89.

Dhaneesh, K. V, Devi, K.N., Kumar, T.T.A., Balasubramanian, T., Tissera, K., 2012. Breeding, embryonic development and salinity tolerance of Skunk clownfish *Amphiprion akallopisos*. J. King Saud Univ. 24, 201–209.

Dhaneesh, K.V., Kumar, T.T.A., Swagat, G., Balasubramanian, T., 2012. Breeding and mass scale rearing of clownfish *Amphiprion percula*: feeding and rearing in brackishwater. Chinese J. Oceanol. Limnol. 30, 528–534.

Díaz-Jiménez, L., Hernández-Vergara, M.P., Pérez-Rostro, C.I., 2019. Protein/lipid ratio for the growth of juvenile clownfish, *Amphiprion ocellaris*. J. World Aquac. Soc. 1–13. https://doi.org/10.1111/jwas.12613

FAO, 2018. The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals.Rome. Rome.

Fautin, D.G., Allen, Gerald R, Allen, Gerald Robert, Naturalist, A., Allen, Gerald Robert, Naturaliste, A., 1992. Field guide to anemonefishes and their host sea anemones.(pages not available)

Fernández-Vázquez, R., Stinco, C.M., Hernanz, D., Heredia, F.J., Vicario, I.M., 2013. Colour training and colour differences thresholds in orange juice. Food Qual.

Prefer. 30, 320–327.

Fernley, H.N., Walker, P.G., 1965. Kinetic behaviour of calf-intestinal alkaline phosphatase with 4-methylumbelliferyl phosphate. Biochem. J. 97, 95–103.

FishLore.com, 2013. Saltwater Aquarium & Reef Tank Book [WWW Document]. URL https://www.fishlore.com/saltwater-aquarium-reef-tank-book.htm

Galetto, M.J., Bellwood, D.R., 1994. Digestion of algae by Stegastes nigricans and *Amphiprion akindynos* (Pisces: *Pomacentridae*), with an evaluation of methods used in digestibility studies. J. Fish Biol. 44, 415–428.

Gasco, L., Finke, M., Van Huis, A., 2018. Can diets containing insects promote animal health? J. Insects as Food Feed 4, 1–4.

Ghosh, S., Kumar, T.T., 2015. Band transformation secrets of Anemonefish Amphiprion ocellaris. Indian J. Geo Mar. Sci. 4, 902–904.

Ghosh, S., Kumar, T.T.A., Nanthinidevi, K., Balasubramanian, T., 2012. Reef fish Breeding and Hatchery Production Using Brackishwater, A Sustainable Technology with Special Reference to Clark's Clownfish, *Amphiprion Clarkii* (Bennett, 1830). Int. J. Environ. Sci. Dev. 3, 56–60.

Gouveia, L., Raymundo, A., Batista, A.P., Sousa, I., Empis, J., 2006. *Chlorella vulgaris* and *Haematococcus pluvialis* biomass as colouring and antioxidant in food emulsions. Eur. Food Res. Technol. 222, 362-367.

Gouveia, L., Rema, P., 2005. Effect of microalgal biomass concentration and temperature on ornamental goldfish (*Carassius auratus*) skin pigmentation. Aquac. Nutr. 11, 19–23.

Gouveia, L., Rema, P., Pereira, O., Empis, J., 2003. Colouring ornamental fish (*Cyprinus carpio* and *Carassius auratus*) with microalgal biomass. Aquac. Nutr. 9, 123–129.

Gupta, S.K., Jha, A.K., Pal, A.K., Venkateshwarlu, G., 2007. Use of natural carotenoids for pigmentation in fishes. Nat. Prod. Radiance 6, 46–49.

Haas, S., Bauer, J.L., Adakli, A., Meyer, S., Lippemeier, S., Schwarz, K., Schulz, C., 2016. Marine microalgae *Pavlova viridis* and *Nannochloropsis sp*. as n-3 PUFA source in diets for juvenile European sea bass (*Dicentrarchus labrax L*.). J. Appl. Phycol. 28, 1011–1021.

He, T., Xiao, Z., Liu, Q., Ma, D., Xu, S., Xiao, Y., Li, J., 2012. Ontogeny of the digestive tract and enzymes in rock bream *Oplegnathus fasciatus* (Temminck et Schlegel 1844) larvae. Fish Physiol. Biochem. 38, 297–308.

https://doi.org/10.1007/s10695-011-9507-y

Hekimoğlu, M.A., Fırat, K., Saka, Ş., Süzer, C., Kop, A., Durmaz, Y., 2017. Effect of Supplemented Algal Carotenoid Diets on Skin Color of Tomato Clownfish, *Amphiprion frenatus*. Pak. J. Zool. 49, 663–668.

Henriques, J., Dick, J.R., Tocher, D.R., Bell, J.G., 2014. Nutritional quality of salmon products available from major retailers in the UK: content and composition of n-3 long-chain PUFA. Br. J. Nutr. 112, 964–975.

Henry, M., Gasco, L., Piccolo, G., Fountoulaki, E., 2015. Review on the use of insects in the diet of farmed fish: past and future. Anim. Feed Sci. Technol. 203, 1–22.

Ho, A.L.F.C., O'Shea, S.K., Pomeroy, H.F., 2013. Dietary esterified astaxanthin effects on color, carotenoid concentrations, and compositions of clown anemonefish, *Amphiprion ocellaris,* skin. Aquac. Int. 21, 361–374.

Infante, J.L.Z., Cahu, C.L., 2007. Dietary modulation of some digestive enzymes and metabolic processes in developing marine fish: applications to diet formulation. Aquaculture 268, 98–105.

Izquierdo, M.S., Kalinowski, C.T., Thongrod, S., Robaina, L., 2005. Nutritional needs for correct pigmentation in European red porgy (*Pagrus pagrus*). Nutr. Biotechnol. Feed food Ind. 307–313.

Karadal, O., Güroy, D., Türkmen, G., 2017. Effects of feeding frequency and Spirulina on growth performance, skin coloration and seed production on kenyi cichlids (*Maylandia lombardoi*). Aquac. Int. 25, 121–134.

Khoo, M.L., Das, S.K., Ghaffar, M.A., 2018. Growth pattern, diet and reproductive biology of the clownfish *Amphiprion ocellaris* in waters of Pulau Tioman, Malaysia. Egypt. J. Aquat. Res. 44, 233–239.

Kop, A., Durmaz, Y., Hekimoglu, M., 2010. Effect of natural pigment sources on colouration of cichlid. J. Anim. Vet. Adv. 9, 566–569.

Kumar, T.T., Balasubramanian, T., 2009. Broodstock development, spawning and larval rearing of the false clown fish, *Amphiprion ocellaris* in captivity using estuarine water. Curr. Sci. 97, 1483–1486.

Kumar, T.T.A., Gopi, M., Dhaneesh, K. V, Vinoth, R., Ghosh, S., Balasubramanian, T., Shunmugaraj, T., 2012. Hatchery production of the clownfish *Amphiprion nigripes* at Agatti island, Lakshadweep, India. J. Environ. Biol. 33, 623–8.

Li, S., Ji, H., Zhang, B., Zhou, J., Yu, H., 2017. Defatted black soldier fly (*Hermetia*

illucens) larvae meal in diets for juvenile Jian carp (*Cyprinus carpio* var. Jian): Growth performance, antioxidant enzyme activities, digestive enzyme activities, intestine and hepatopancreas histological structure. Aquaculture 477, 62–70.

Lieske, E., Myers, R., 1994. Coral reef fishesCaribbean, Indian Ocean and Pacific Ocean including the red sea. Harper Collins, London, p. 400.

Lunger, A.N., McLean, E., Gaylord, T.G., Kuhn, D., Craig, S.R., 2007. Taurine supplementation to alternative dietary proteins used in fish meal replacement enhances growth of juvenile cobia (*Rachycentron canadum*). Aquaculture 271, 401–410.

Madhu, K., Madhu, R., Krishnan, L., Sasidharan, C.S., Venugopal, K.M., 2006. Spawning and larval rearing of *Amphiprion ocellaris* under captive condition. Mar. Fish. Inf. Serv. Tech. Ext. Ser. 188, 1–5.

Makkar, H.P.S., Tran, G., Heuzé, V., Ankers, P., 2014. State-of-the-art on use of insects as animal feed. Anim. Feed Sci. Technol. 197, 1–33.

Miller, M.R., Nichols, P.D., Carter, C.G., 2007. Replacement of fish oil with *thraustochytrid Schizochytrium sp*. L oil in Atlantic salmon parr (*Salmo salar L*) diets. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 148, 382–392.

Natalia, Y., Hashim, R., Ali, A., Chong, A., 2004. Characterization of digestive enzymes in a carnivorous ornamental fish, the Asian bony tongue *Scleropages formosus* (Osteoglossidae). Aquaculture 233, 305–320.

Nhan, H.T., Minh, T.X., Liew, H.J., Hien, T.T.T., Jha, R., 2019. Effects of natural dietary carotenoids on skin coloration of false Clownfish (*Amphiprion ocellaris* Cuvier, 1830). Aquac. Nutr. 25, 662–668.

Olivotto, I., Chemello, G., Vargas, A., Randazzo, B., Piccinetti, C.C., Carnevali, O., 2017. Marine ornamental species culture: From the past to "Finding Dory." Gen. Comp. Endocrinol. 245, 116–121.

Olivotto, I., Di Stefano, M., Rosetti, S., Cossignani, L., Pugnaloni, A., Giantomassi, F., Carnevali, O., 2011. Live prey enrichment, with particular emphasis on HUFAs, as limiting factor in false percula clownfish (*Amphiprion ocellaris*, Pomacentridae) larval development and metamorphosis: molecular and biochemical implications. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 159, 207–218.

Olsen, Y., 2011. Resources for fish feed in future mariculture. Aquac. Environ. Interact. 1, 187–200.

Pomeroy, R.S., Parks, J.E., Balboa, C.M., 2006. Farming the reef: is aquaculture a solution for reducing fishing pressure on coral reefs? Mar. Policy 30, 111–130.

Pradeep, P.J., 2010. "Knowing Nemo". Prospect Malaysia Mag. 13, 65–68.

Rhyne, A.L., Tlusty, M.F., Szczebak, J.T., Holmberg, R.J., 2017. Expanding our understanding of the trade in marine aquarium animals. PeerJ 5, e2949.

Rodriguez-Garcia, I., Guil-Guerrero, J.L., 2008. Evaluation of the antioxidant activity of three microalgal species for use as dietary supplements and in the preservation of foods. Food Chem. 108, 1023–1026.

Rotllant, G., Moyano, F.J., Andrés, M., Díaz, M., Estévez, A., Gisbert, E., 2008. Evaluation of fluorogenic substrates in the assessment of digestive enzymes in a decapod crustacean *Maja brachydactyla* larvae. Aquaculture 282, 90–96.

Rutter, W.J., 1967. Methods in developemental biology, in: Academic Press NY. pp. 671–684.

Sales, J., Janssens, G.P.J., 2003. Nutrient requirements of ornamental fish. Aquat. Living Resour. 16, 533–540.

Salin, K., Arun, V., Nair, M., Tidwell, J., 2018. Sustainable Aquafeed, in: Hai, F.I., Visvanathan, C., Boopathy, R. (Eds.), Sustainable Aquaculture, Applied Environmental Science and Engineering for a Sustainable Future,. Springer, pp. 123-151 https://doi.org/https://doi.org/10.1007/978-3-319-73257-2_4

Sánchez-Muros, M.-J., Barroso, F.G., Manzano-Agugliaro, F., 2014. Insect meal as renewable source of food for animal feeding: a review. J. Clean. Prod. 65, 16–27.

Sant'Ana, A., 2019. Farmed fish as a functional food : fortification strategies with health valuable compounds. Universidade do Algarve.

Sanz, Y., Toldrá, F., 2002. Purification and characterization of an arginine aminopeptidase from *Lactobacillus sakei*. Appl. Environ. Microbiol. 68, 1980–1987.

Schloss, K.B., Lessard, L., Racey, C., Hurlbert, A.C., 2018. Modeling color preference using color space metrics. Vision Res. 151, 99–116.

Shi, X., Luo, Z., Chen, F., Wei, C., Wu, K., Zhu, X., Liu, X., 2017. Effect of fish meal replacement by *Chlorella meal* with dietary cellulase addition on growth performance, digestive enzymatic activities, histology and myogenic genes' expression for crucian carp *Carassius auratus*. Aquac. Res. 48, 3244–3256.

Shepherd, C.J., Jackson, A.J., 2013. Global fishmeal and fish-oil supply: inputs, outputs

and marketsa. J. Fish Biol. 83, 1046–1066.

Silva, F.C.P., Nicoli, J.R., Zambonino-Infante, J.L., Le Gall, M.-M., Kaushik, S., Gatesoupe, F.-J., 2010. Influence of partial substitution of dietary fish meal on the activity of digestive enzymes in the intestinal brush border membrane of gilthead sea bream, *Sparus aurata* and goldfish, *Carassius auratus*. Aquaculture 306, 233–237.

Spolaore, P., Joannis-Cassan, C., Duran, E., Isambert, A., 2006. Commercial applications of microalgae. J. Biosci. Bioeng. 101, 87–96.

Tanaka, Y., Yamamoto, A., Kamata, T., Simpson, K.L., 1992. Biochemical study on the carotenoids in the anemonefish, Amphiprion spp. Mem. Fac. Fish. Univ. 4, 1–8.

Tibaldi, E., Zittelli, G.C., Parisi, G., Bruno, M., Giorgi, G., Tulli, F., Venturini, S., Tredici, M.R., Poli, B.M., 2015. Growth performance and quality traits of European sea bass (*D. labrax*) fed diets including increasing levels of freeze-dried *Isochrysis sp*.(T-ISO) biomass as a source of protein and n-3 long chain PUFA in partial substitution of fish derivatives. Aquaculture 440, 60–68.

Vargas-Abúndez, A.J., Randazzo, B., Foddai, M., Sanchini, L., Truzzi, C., Giorgini, E., Gasco, L., Olivotto, I., 2019. Insect meal based diets for clownfish: Biometric, histological, spectroscopic, biochemical and molecular implications. Aquaculture 498, 1–11.

Velasquez, S.F., Chan, M.A., Abisado, R.G., Traifalgar, R.F.M., Tayamen, M.M., Maliwat, G.C.F., Ragaza, J.A., 2016. Dietary Spirulina (*Arthrospira platensis*) replacement enhances performance of juvenile Nile tilapia (*Oreochromis niloticus*). J. Appl. Phycol. 28, 1023–1030.

Vizcaíno, A.J., López, G., Sáez, M.I., Jiménez, J.A., Barros, A., Hidalgo, L., Camacho-Rodríguez, J., Martínez, T.F., Cerón-García, M.C., Alarcón, F.J., 2014. Effects of the microalga *Scenedesmus almeriensis* as fishmeal alternative in diets for gilthead sea bream, *Sparus aurata*, juveniles. Aquaculture 431, 34–43.

Wabnitz, C., Taylor, M., Green, E., Razak, T., 2003. From ocean to aquarium: the global trade in marine ornamentals. Cambridge, UK UNEP World Conserv. Monit. Cent. p. 10.

Wittenrich, M.L., Turingan, R.G., 2011. Linking functional morphology and feeding performance in larvae of two coral-reef fishes. Environ. Biol. fishes 92, 295–312.

Yasir, I., Qin, J.G., 2010. Effect of dietary carotenoids on skin color and pigments of

false clownfish, *Amphiprion ocellaris*, Cuvier. J. World Aquac. Soc. 41, 308–318.

Yasir, I., Qin, J.G., 2007. Embryology and early ontogeny of an anemonefish *Amphiprion ocellaris*. J. Mar. Biol. Assoc. United Kingdom 87, 1025–1033.

Zhou, J.S., Liu, S.S., Ji, H., Yu, H.B., 2018. Effect of replacing dietary fish meal with black soldier fly larvae meal on growth and fatty acid composition of Jian carp (*Cyprinus carpio* var. Jian). Aquac. Nutr. 24, 424–433.