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Effects of different protein to carbohydrate ratio in pellets for rearing European cuttlefish (*Sepia officinalis*, Linnaeus 1758) juveniles

Universidade do Algarve

Faculdade de Ciências e Tecnologia

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Trabalho efetuado sob a orientação de: António V. Sykes, PhD

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Abbreviation List

CH	Carbohydrates
DG	Digestive gland
DWi	Dry weight initial
DWf	Dry weight final
AGR	Absolute growth rate
SGR	Specific growth rate
BW	Body weight
DGI	Digestive gland index
AFR	Absolute feeding rate
SFR	Specific feeding rate
IF	Corrected ingestion
FCR	Feed conversion rate
FE	Feed efficiency
MWW	Mean wet weight
B	Biomass

Abstract

Being highly sought-after fishery resources, cephalopods potential for aquaculture has been a research topic for several decades. The diversity within the class means that each species has its own unique requirements under a culture environment, this poses a great obstacle to overcome. One of these bottlenecks is achieving a nutritional balance in artificial diets which promotes growth, welfare and directly influences the economic viability of cephalopod culture. In this study, which is part of the Sepiacul project, three different artificial diets were tested, each containing different protein to carbohydrate ratios (55:10, 51:15 and 47:20). These diets were tested on *Sepia officinalis*, against a control group, which was fed frozen grass shrimp (*Palaemonetes varians*). Cuttlefish used were reared in an open-water system, in the Ramalhete (Ria Formosa, South of Portugal – 37º00′22.39″N; 7º58′02.69″W) pilot station. Pelleted diets did not promote growth and even caused weight loss, resulting in negative absolute and specific growth rates and feeding efficiency. Artificial diets were accepted on day 1 in all treatments, however, cannibalism started occurring by the end of the trials second week, forcing trial shut down. Histology analysis was performed by removing the digestive gland and processing it. Afterwards four different parameters were studied to determine differences between treatments in the digestive gland: existence of structural damage; cell organization; presence of select structures; existence of carbohydrate/glycogen clusters. No difference was found between artificial diet treatments and control ($p > 0.05$). However, digestive gland index was found to have a significant difference between pelleted treatments and control ($p < 0.05$), with control digestive glands weighing far more than the pelleted diet counterparts. One of the factors that is believed caused artificial diets not to promote growth is related to raw material processing, more specifically the protein portion.

Keywords: Cuttlefish, *Sepia officinalis*; Aquaculture, Artificial diet, Carbohydrates, Protein, Ratio;

Resumo

Cefalópodes são, tanto na Europa como no resto do Mundo, recursos pesqueiros importantes que nos últimos anos têm visto um decréscimo na apanha anual e um aumento da pesca ilegal (apanha de animais abaixo do tamanho legal), estas tendências tornam a hipótese de introduzir esta classe de animais à produção em aquacultura algo cada vez mais necessário. Nas últimas décadas, a aquacultura tem vindo a mostrar ser uma alternativa à pesca. Só em 2016 produziram-se 80 milhões de toneladas de peixe, crustáceos e moluscos em aquacultura enquanto a captura gerou 93 milhões de toneladas.

A produção de choco em aquacultura é tópico de estudo há várias décadas, devido não só ao mercado que existe em torno destes animais, como também ao estilo de vida "live fast, die young" dos mesmos. Apesar de já ter sido fechado o ciclo de vida em cativeiro, existem ainda vários obstáculos que interferem com o avanço para uma escala maior.

Entre estes obstáculos ou "bottlenecks" temos o que mereceu a atenção do presente estudo (pertencente ao projeto Sepiacul), a formulação de uma dieta artificial atendendo às necessidades alimentares específicas do choco *S. officinalis*. Idealmente, terá de sustentar e promover o crescimento do animal sem repercussões negativas (canibalismo, comprometer a saúde do animal). Assegurando também uma alternativa mais económica face ao que é usado neste momento para alimentar os chocos, a camarinha *Palaemonetes varians*.

O objetivo deste estudo é criar uma dieta artificial capaz de sustentar juvenis de choco, promovendo o seu crescimento e bem-estar. Os animais, durante a experiência, permaneceram em tanques ligados a um circuito de água do mar da estação piloto do Ramalhete, pertencente à Universidade do Algarve. Estes tanques de fibra de vidro com uma capacidade de 250 L encontravam-se no exterior da estação, a administração de oxigénio foi feita através de uma pedra difusora e dois "airlifts", estruturas cujo objetivo é difundir oxigénio pelo tanque. A quantidade de água administrada, através de uma fonte de fluxo regulável, foi 48 l/h. A abertura dos tanques foi coberta com redes verde-escuro circulares de modo a proteger os animais neles contidos da luz solar.

A dieta artificial foi concebida no CCMAR por António V. Sykes e produzida pela SPAROS Lda., Olhão, Portugal. Estudou-se o efeito de três (3) razões diferentes para a dieta artificial (55:10, 51:15 e 47:20, proteína e hidratos de carbono respetivamente), com um controlo (alimentado a *P.*

varians). Cada um dos tratamentos foi triplicado, resultando num total de 12 tanques, cada um com 15 chocos (180 chocos no total).

De forma a avaliar a dieta artificial estudou-se o crescimento, recorrendo a taxas de crescimento absoluto e específico, taxas de conversão de alimento e eficiência do mesmo. De modo a verificar o crescimento diário teórico usou-se também o índice de biomassa (%). Foi feita também a análise comportamental do animal, verificou-se as interações do animal com a dieta artificial e relativas ao bem-estar do animal, tais como a interação entre indivíduos. De forma a verificar a deposição de nutrientes (especificamente hidratos de carbono) na glândula digestiva, recorreu-se à análise histológica que também compreendeu uma análise estrutural. Por último, testou-se também a estabilidade e o índice de desagregação da dieta artificial na água, de forma a verificar se esta permanecia tempo suficiente na coluna de água e a percentagem de pellet que se perdia ao longo do tempo.

A recolha de dados foi feita com amostragens semanais nas quais o peso de todos os animais em experiência eram determinados, e mais tarde usados de modo a calcular os índices e taxas acima referidos. No caso da análise comportamental, os dados foram recolhidos através de câmaras (uma por tanque, e por tratamento) que eram colocadas 10 minutos antes do alimento ser dado.

Relativamente à histologia, de modo a realizar uma análise comparativa foram estabelecidos quatro parâmetros, análise do índice da glândula digestiva, integridade celular e estrutural, organização extracelular, presença de diferentes tipos de células selecionadas, presença de aglomerados de hidratos de carbono e glicogénio.

A dieta foi aceite no primeiro dia de experiência, um resultado inédito que se traduz num passo na direção certa para criar uma dieta artificial de sucesso. Também se verificou um ligeiro aumento da quantidade de alimento consumida pelos chocos nas dietas artificiais, tanto como no controlo, ao longo do tempo.

A experiência foi abortada, e como previsto, foi realizada uma amostragem final para fins histológicos, no final da segunda semana, na qual 3 chocos de cada tanque foram selecionados aleatoriamente. Estes foram mortos através de overdose de anestesia (100g /L of $MgCl_26H_20$) e foi retirada a glândula digestiva dos mesmos, totalizando em 27 amostras.

Análise do índice da glândula digestiva mostrou ser significativamente diferente ($p < 0.05$) entre os tratamentos alimentados a ração e o controlo. Isto é devido à diferença que houve no crescimento entre os tratamentos, em que o controlo teve um peso médio final superior com 48.23

± 10.08 g (incremento de 87% da biomassa nos tanques de controlo), comparando com os tanques alimentados com dieta artificial 55:10 com 23.04 ± 5.03 g (decréscimo de biomassa de 11%), 51:15 com 25.78 ± 5.39 g (decréscimo de 4%), 47:20 com 22.89 ± 5.16 g (decréscimo de 12%).

A causa do canibalismo e da falta de crescimento provém, provavelmente, do perfil nutritivo da dieta artificial não ser adequado às necessidades do animal, não devido à formulação da dieta, mas sim ao processamento das matérias-primas usadas, especificamente a porção proteica. De forma a não promover a desnaturação e alteração da estrutura da proteína o processamento ideal é feito a menos de 60 ºC ou por liofilização a -50 ºC, ambos estes processos desidratam a proteína sem provocar danos estruturais, mantendo a capacidade nutritiva da mesma.

Introduction

With the decline of finfish stocks, the world fisheries turned their heads to cephalopods with the number of catches increasing almost eight-fold in the past century. Most of the large industrial cephalopod fisheries are concentrated in the Northwest/Central Pacific, the Northwest African coasts, the Mediterranean and Northwest Atlantic (Iglesias *et al.* 2014). Cephalopods are currently highly valuable commercial fishery resources with annual world catches landing at 4.7 million tonnes in 2015 (Fishery and Aquaculture Statistics, 2017). However recent FAO studies report a decrease in overall cephalopod catches from 2016 onwards, with decreases up to 1.2 million tonnes (FAO, 2018).

The absence of management measures for most European cephalopod fisheries reflects the lack of attention paid to the fishing of non-traditional species and the idea that cephalopods are resilient to overfishing (Pierce and Portela, 2014). Despite their resilience though, many of cephalopod stocks are either at maximum potential or maximally sustainably fished (FAO, 2018).

Cuttlefish specifically are one of the most valuable species in Mediterranean and Asian markets and its catches (alongside with other cephalopods) have seen a steady increase since the 1990's and though landings came out short in 2016 and 2017, the demand keeps increasing in countries such as Japan, the U.S.A and southern European countries like Spain (FAO, 2018).

Besides their importance for human consumption, cuttlefish are also useful research models not only in the field of medical and biological research, but also in physiology, neuroscience, nutritional biochemistry, molecular biology and immunology, due to their nervous system and sensory organs (Koueta *et al.* 2014; Sykes *et al.* 2014).

The introduction of a new species in aquaculture requires a series of preliminary studies related to the biology, ecology and physiology of the species. For cuttlefish specifically, part of this work as already been done.

The potential of the cuttlefish has been recognized by several authors such as Barnabé and Martínez (1996) and Sykes *et al*, (2006a) due to their high growth rates, short life spans and early sexual maturity. It has been successfully reared in several aquaculture experiments throughout European countries, such as Portugal (Sykes *et al.* 2006a) and in the USA (Castro *et al.* 1993) by the NRCC (National Resource Center for Cephalopods). *S. officinalis* (Linnaeus 1758) is among the species of cephalopods with the greatest interest for European markets and for introduction in aquaculture. If made possible, cuttlefish culture may have a large impact on

fisheries production in the near future, given that small animals (that are also highly sought after) may be obtained after 45-60 days (Sykes *et al.* 2014).

The bottlenecks in cuttlefish culture identified by Sykes *et al*, (2006a) and Villanueva *et al*, (2014) are, lack of full control of reproduction in captivity; the dependence on live prey during the hatchling stage; the lack of an adequate artificial diet for all life stages. The lack of sufficient knowledge on cephalopod nutrition, a key factor for proper growth and survival under captive conditions (Navarro *et al.* 2014; Valverde *et al.* 2013), is therefore partly stopping the jump to a more industrial scale.

Until now, mysids were used as food in the hatchling stage, as it promoted less mortality and higher growth rates. This was further improved when Sykes *et al*, (2012a) fed hatchlings using only frozen grass shrimp through all stages, enabling this kind of diet throughout all stages and not just from juveniles to sexually mature animals (Sykes *et al.* 2006a).

Sykes *et al*, (2006b) stated that the particular metabolism of cephalopods needs to be taken into consideration if a successful artificial diet is to be made. This means the nutritional balance of the diet must be optimal to sustain the existing metabolic costs, so that the animal is able to perform an optimal distribution of surplus energy to somatic growth and, at later stages, to reproduction (Wells and Clarke, 1996)**.**

The existing information regarding these animals metabolism comes down to two contrasting theories, Lee (1995) and Boucher-Rodoni and Mangold (1995) consider that under normal feeding conditions, both growth and energy use the protein fraction as fuel; Storey *et al*, (1983) and Hochachka (1995) consider that the carbohydrate fraction is used as the energy source and the protein fraction exclusively for growth.

The existence of two opposing theories may be due to the physiological plasticity of the species, which is both temperature and food dependent (Villanueva *et al.* 2014). Studies done on *S. officinalis* from the English Channel populations showed the metabolism of lipids instead of protein or carbohydrates when facing prolonged starvation, bringing insight on how certain nutrients are allocated and used. Speers-Roesch *et al*, (2016) using animals produced in an aquaculture station, proposed a three-stage metabolic response when cuttlefish undergo starvation. Where, carbohydrates are conserved, lipids stored in the digestive gland are used up and protein reserves are only used as a last resort, once lipid stores fall below a critical level. In the same experiment, Lamarre *et al*, (2016) came to similar conclusions, where triglyceride stores were

depleted at fast rates (1/3 at 3 days of fasting and depleted at 12 days). Meaning that, when fasting, cuttlefish switch from an amino-acid based metabolism to a lipid-based metabolism.

The cuttlefish digestive gland termed "*hepatopancreas*" is a polyvalent organ similar to the vertebrate counterpart, the liver. The functionality of this organ has long been recognized, responsible not only for digestion (either through intracellular digestion or enzyme excretion) and excretory functions, but also for carbohydrate storage (Costa *et al.* 2014). Because of this, the digestive gland is a great indicator of a balanced nutrition and what nutrients are a priority throughout the animal's life.

Advancing to a more large-scale type of culture requires the existence of a diet, tailored specifically to the metabolic needs of the species. Paying attention not only to nutrient profiles, sources and proportions as well as to digestibility and attractiveness of the diet. This prevents any issues related to substantially lower feeding rates of the pellet as compared to a natural diet. Hence, understanding the different uses of each nutrient is key to manufacturing a suitable pelleted diet.

Cuttlefish are mainly composed of protein, ranging 81 ± 2 % of dry weight (Zlatanos *et al.* 2006). Since cuttlefish are mostly made up of protein, present high levels of protein assimilation and it is the largest contributor for somatic growth, it is vital that the artificial diet possesses a strong protein profile (Lee, 1995).

Given its importance as the main energy source for cuttlefish metabolism, there is a need for characterization of the amino acid pool at different life stages of reared animals (Lee, 1995; Lamarre *et al.* 2016). Supplementation of diets with essential amino acids, arginine, leucine and lysine, is of paramount importance (Domingues *et al.* 2005a; Valverde *et al.* 2013). Additionally, providing taurine as supplementation would prove beneficial not only due to its abundance in cuttlefish blood, but also because it plays a vital role in modulating cardiac function and metabolism in *S. officinalis* (MacCormack *et al.* 2016).

In the past lipid supplementation in diets for cuttlefish was neglected due to the low amount in their proximal composition (Lee, 1995) and past reports on their low capacity of metabolizing lipids (O'dor *et al.* 1984). Since then, some studies devoted some attention to its supplementation and how essential it is for cuttlefish development through its growth (Domingues *et al.* 2003).

Cuttlefish are unable to store lipids in the digestive gland and require high levels of phosphatidylethanolamine (PE) and cholesterol in their diets. This need of lipids and fatty acids is highest during the embryonic development (Almansa *et al.* 2006; Fluckiger *et al.* 2008) and, as

cuttlefish reach sexual maturity (Reis *et al*. 2016). In the same study it is also stated that supplying DHA/EPA/ARA (long-chain PUFA) is required for normal growth and to design an efficient artificial diet.

Carbohydrate supplementation, contrary to proteins and lipids, was mostly ignored in the past, since this nutrient represents a lower percentage of body composition when comparing to other macronutrients, it was generally accepted that cephalopods do not have a specific need for dietary carbohydrate and rarely use it as an energy source (Lee, 1995).

Until the existence of carbohydrate metabolism was suggested by Sykes *et al*, (2009). The maintenance of lipid levels throughout embryonic egg development may indicate a possible alternate source of energy such as protein or carbohydrate. O'Dor *et al*, (1984) states that cephalopods use protein as building blocks for growth and carbohydrates as an alternate energy for explosive actions, the latter being stored in small amounts as muscle glycogen (Navarro et al. 2014) and in the digestive gland. Lamarre *et al*, (2019) has recently shown that glycolysis stimulates protein synthesis in cuttlefish early juveniles, showing that action such as jetting can stimulate this process by approximately 25%.

As for minerals, the specific supplementation necessary for cephalopods in general is poorly understood. For octopuses, as a carnivorous species, the diet meets most of the elemental requirements, however, direct uptake from seawater has been shown to occur, an ion balance mechanism that is regulated by appendages of the digestive gland (Wells and Wells, 1989). Elements like copper (Cu) and sulphur (S) are regarded as essential, making the difference in diets, aiding in the maturation of hemocyanin and the formation of muscular proteins (Navarro *et al.* 2014). Others such as strontium (Sr) and cobalt (Co) appear to be incorporated by seawater uptake (Hanlon *et al.* 1989). The cuttlefish's cuttlebone is made of calcium, it's lack in an artificial diet may cause individuals to suffer malformation resulting from malnutrition. Making it an implicit supplement in any artificial diet (Navarro *et al.* 2014).

Vitamins are considered crucial, especially at earlier life stages during which they act as the main antioxidants, in order to prevent lipid oxidation, particularly α-tocopherol as it is degraded to protect PUFA from oxidizing (Sargent *et al.* 1997). Vitamin A also acts as a stimulus for cell growth, aids in maintaining resistance to foreign infections and is an essential building block for photo-pigments of the cephalopod retina (Blomhoff *et al.* 1992; Terakita *et al.* 1989). *S. officinalis, O. vulgaris* and *L. vulgaris* (Lamarck 1798) hatchlings were profiled to determine vitamin A and

E composition. Vitamin A values were found not to be very different from other marine molluscs and fish larvae, however, high contents of vitamin E were registered (Navarro *et al.* 2014).

Artificial diets for cephalopods have been subjected to numerous trials over the past few years to lower costs. Early diets were based on shrimp paste in Lee *et al*, (1991), other authors would later try using surimi-based diets (Castro *et al.* 1993; Domingues, 1999). These managed to maintain cuttlefish with some degree of acceptance, however with very low or even negative growth rates. In later studies such as Domingues *et al*, (2005b) and Ferreira *et al*, (2010), artificial diets (semi-dry pellets) were accepted by cuttlefish but causing negative growth, higher mortalities and events of cannibalism, contrary to a closer to natural diet.

Today, despite the state of existing knowledge, artificial diets are still under development, having at best been tried and accepted by juvenile cuttlefish. In early studies, juveniles have been noted to resort to cannibalism and ignore the pellet diet, however more recently, a first dried pelleted diet (developed during the Sepiatech project) was accepted and ingested by cuttlefish without cannibalism (Sykes *et al*. personal communication).

Despite these encouraging results, these diets success rate pale in comparison to more common alternatives such as the frozen grass shrimp which obtained much higher growth rates, food conversion rates and overall feeding rates. The use of ingredients such as squid meal (mollusc) and grass shrimp (crustacean) based meal can improve attractiveness (Domingues *et al.* 2005b, Sykes *et al.* 2006b). As shown in several studies, such as Martínez *et al*, (2014), the use of proteins from these sources may aid in a successful artificial diet.

Successful tested diets that were accepted and ingested do not allow for the introduction of the species in the aquaculture industry. The lack of knowledge regarding cuttlefish's specific nutritional requirements and balance remain as a major bottleneck to overcome to create a pelleted diet (Villanueva *et al.* 2014).

Since cuttlefish metabolism has been pinpointed as amino-acid based and to also use lipids and carbohydrates and not just protein as energy sources. It is important to find the ideal amounts of each of these nutrients in artificial diets. Past studies have supplemented large amounts of protein (60-90%) (Castro *et al.* 1993; Domingues *et al.* 2005b; Ferreira *et al.* 2010) and did not succeed. Perhaps testing a diet with lower protein profile and a stronger carbohydrate presence to offset this, as it has been shown to stimulate protein synthesis (Lamarre *et al.* 2019), will provide better results.

Determining the optimal dietary presence of protein and carbohydrates in a cuttlefish artificial diet is vital. The present study focused on testing out the growth of *S. officinalis* fed three different diets that possess different ratios of these nutrients (55:10, 51:15 and 47:20) and a control group fed on *P. varians.*

Materials and methods

Ethical statement and humane endpoints

As stated by Decreto de lei nº113/2013 of August 7th animals subjected to scientific experimentation or any procedure considered invasive or non-invasive are required to be maintained under conditions that will promote their welfare and all stressful interactions are to be minimized.

To prevent unnecessary animal suffering, endpoints to the experiment were created based on the Arrive guidelines. If any of the following conditions occurs, replicates of a diet were interrupted and individuals allowed to recover, being fed frozen grass shrimp:

If the new diet was not accepted after 10 days, the replicates of a given diet were interrupted and the individuals allowed to recover in the same tank whilst fed on frozen grass shrimp;

If cannibalism was observed to occur, the replicates of a given diet were interrupted and the individuals allowed to recover in the same tank whilst fed on frozen grass shrimp;

If cuttlefish mean wet weight (MWW) of a given tank loses more than 10% of the initial mean weight for that same tank, the replicates of a given diet were interrupted and the individuals allowed to recover in the same tank whilst fed on frozen grass shrimp;

If mortality was registered, if it was related eventually to food and was higher than that observed in the control group, the replicates of a given diet were interrupted and the individuals allowed to recover in the same tank whilst fed on frozen grass shrimp;

Experimental Setup

180 animals were placed in twelve, 250 L with 1 meter in diameter and 0.79 m^2 of bottom area, half-spherical, tanks painted black on the inside (Sykes *et al.* 2011), totalling 15 animals per tank.

Placed on the exterior of Ramalhete aquaculture research station, these tanks were equipped with two airlifts, one plastic air diffuser and one central filter (1 cm mesh), giving a total maximum water height of 43 cm.

The water inlet was set to provide the tank with 5 x 250 L every day, to do this, the water flow input was set to 48 L/hour. Water was pre-filtered through an industrial sand filter and decanted before entering the tanks.

Figures 3.1 and 3.2 show relative tank positioning in the station and the complete tank setup. As well as a pre-determined tank schematic done to prevent any skewness resulting from tank positioning.

Figure 3.1. Aerial view of tank positioning, red lines represents relative tank positioning (left), complete tank setup (right);

The trial consisted of three different stages, there was an acclimatisation process for all animals when transferred to the new tanks. During this period feed supplied was the same diet of grass shrimp used for their grow out until this point. The trial itself using the pelleted and control diets and lastly the recovery, this last stage occurred if any of the end points occurred.

Figure 3.2. Tank schematics and positioning, including supplied diet for each tank;

If none of the above-mentioned endpoints occur, the trial is set to last 45 days.

Rearing conditions and maintenance

Temperature and dissolved oxygen were also measured each day using a VWR probe DO220, calibrated each day according to salinity and pressure. Salinity was determined using a VWR probe EC300 (Table 3.1).

Table 3.1 Experimental conditions (probe data) throughout the trial;

Treatment	Control	55:10	51:15	47:20
Temperature (ºC)	23.73±1.54	23.74±1.58	23.66±1.47	23.69±1.54
Salinity (ppm)	36.67±0.40	36.67±0.40	36.67±0.40	36.67±0.40
Dissolved Saturated Oxygen (%)	95.16±3.96	95.87±4.01	97.02±3.86	96.78±3.97

Average temperature was 24.72 ± 1.86 ºC, being recorded through temperature chips, (Maxim-Dallas, Thermochron – DS1921G) placed on tanks 2, 4, 6, 7, 9 and 11. These were programmed to register temperature every hour for the entirety of the experiment duration as to obtain a consistent temperature profile (Figure 3.3). Maximum and minimum recorded temperatures were 29.0 ºC and 20.0 ºC, respectively, Table 3.2 shows values for all chips.

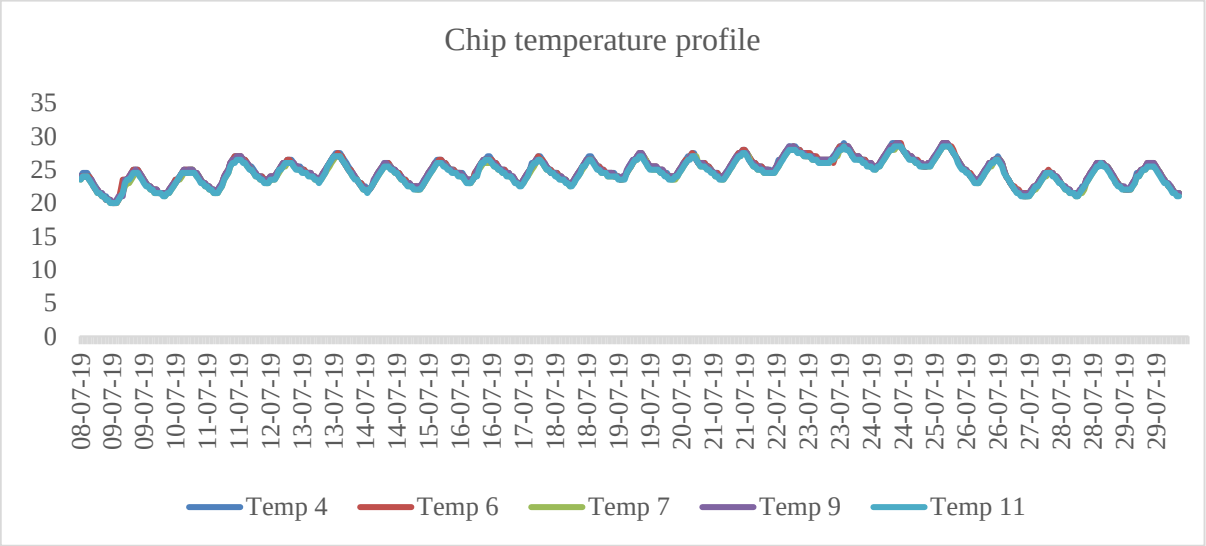

Figure 3.3 Temperature data profile gathered through chips;

Table 3.2. Data gathered in ºC for each chip;

Parameters	Tank 4	Tank 6	Tank 7	Tank 9	Tank 11
Mean	24.88	24.85	24.56	24.81	24.50
SD	1.88	1.86	1.83	1.83	1.83
Maximum	29	29	28.5	29	28.5
Minimum	20	20	20	20	20

Being on the exterior, tanks were exposed to natural light, as such they were covered with dark-green shading net to achieve approximately 100 lux. The covers were slightly elevated (2 cm's) as for natural air transfer to occur.

Diets

Three different diets were tested, in triplicates, with a control group being fed grass shrimp (*P. varians*). The three pelleted diet formulas were designed by António V. Sykes in CCMAR and produced by SPAROS Lda. located in Olhão, Portugal.

Protein sources for the diet varied in how they were prepared, shrimp (*P. varians*) meal was obtained via lyophilization (at -50ºC), whereas squid meal was heat treated (60 ºC). Mixing and milling (if necessary) of raw materials, cold extrusion (at 6 mm size), drying, coating (to avoid leaching) and lastly sieving were all done according to SPAROS's optimized parameters.

The dietary variations between different treatments were the different protein to carbohydrate ratios, shown below in Table 3.3. All diets contained the same vitamin and mineral supplementation as well as a similar amino acid profile.

Table 3.3 Basal mixture (% dry weight);

Diets	Ingredients	55:10	51:15	47:20
		%	%	%
Basal Composition	Squid meal	39.0	34.0	29.0
	Gelatin	5.0	5.0	5.0
	Shrimp meal	30.0	30.0	30.0
	Glucose	4.7	9.7	14.7
	Krill oil	1.4	1.4	1.4
	CaCO3	15.0	15.0	15.0
	Taurine	2.3	2.3	2.3
	Vit/Min premix	2.0	2.0	2.0
	Arachidonic oil	0.6	0.6	0.6

Animals and sampling

All animals used in this trial were reared in the same 1000 L tank, with conditions similar to those they would be subject to in this experiment.

A preliminary sampling was done, before trial start, to select animals with roughly the same size and weight for every treatment. Initial MWW for each tank was 23.4 g ± 4.55 g and mean initial tank Biomass was 351.5 ± 1.95 g.

Sampling was done weekly and, in the morning, after recovery of excess feed. Daily feeding was delayed until all cuttlefish were sampled and back in their respective tanks. Each cuttlefish was individually weighed, in a black plastic container placed on a KERN EW6000-1M scale. During sampling cuttlefish from each tank were placed in separate black buckets with one air diffuser. Weight data was gathered to enable analysis of growth and feed efficiency.

Feeding and feed processing

Each set of triplicate tanks with its own, different, diet. The first day feeding equals 5% of tank biomass. The next day feeding was determined according to the leftovers of the previous day (*ad libitum*), if any leftovers, either lower or maintain food values, depending on how much leftover there was (Sykes *et al.* 2012a).

Feeding was done daily, at 12h00, 10 minutes after the cameras were positioned as to record behaviour.

For the pelleted diets, the initial amount was exactly 15 pellets, one for each animal, as to try and avoid competition for the feed. These low initial values were due to the fact that cuttlefish are selective in what they eat, and will initially reject a pelleted diet, even if based on shrimp meal (Domingues *et al.* 2005b). When increasing this amount, to provide an equal chance of each cuttlefish receiving the same amount of feed, 1 more pellet was given per individual.

For control cuttlefish, the amount of feed supplied was predetermined each week depending on the biomass obtained in the last weekly sample.

Throughout the experiment, excess feed was recovered the morning after, before tank cleaning. The excess feed would dry for 24 hours in an oven set to 105º C, to remove most of the water content. Afterwards, each sample was left to cool inside an airless (air removed via vacuum pump) container filled with silica. Once cooled down excess feed was weighed using silicone recipients, this allowed to process feed acceptance per tank. Recovery of feed also allowed for analysis of feed intake during trial and feed efficiency. Feed processing and amount of excess feed was calculated according to Horwitz *et al,* (1980).

Food given, overall acceptance and excess food (before and after weight) were all registered daily.

Diet stability and sinking

To test diet stability, triplicates (1g each sample) of each diet were placed in falcon tubes containing saltwater for 30 minutes, 2 hours, 4 hours, 6 hours, and 24-hour trials. During which they were periodically inverted as to prevent the pellets from aggregating in the bottom for a long period. Afterwards pellets were dried at 105ºC and weighed, disaggregation rate was then calculated according to the following formula:

$$\text{Dissagregation rate} = ((DWf - DWi) / DWi) * 100$$

Where DWi and DWf correspond to the initial and final dry weights, respectively (Rodríguez-González *et al.* 2015).

For sinking, another set of 1g triplicates from each diet were used. Tests were performed in a 30 cm deep aquarium, filled with saltwater, to check how long it took for the pellet to sink. Footage was obtained using a Go Pro camera set to record at 720p at 60fps, compiled and input into a motion tracking software, Noldus's EthoVisionXT, this allowed us to track the pellet's sinking and the time it took to reach the bottom.

Arena, detection (brightness and pellet size) and trial settings (number of pellets in arena) were optimized as to detect only the currently sinking pellet. Total time the pellet took to sink (exact moment of entry in water to moment of reaching bottom of tank) was obtained directly from the raw data excel file obtained following the processing of the video by the software.

Behaviour

Behavioural data was captured by one camera (Sony FDR-X3000R) per tank (4 cameras total, 1 per treatment), to obtain video footage of feeding and animal response. A recording rotation was established so that no tank was used for gathering data in succession, and so that every treatment was recorded every day. This was done following a repeating 3-day schedule; day 1: tanks 1, 2, 3 and 4; day 2: tanks 5, 6, 7 and 8; day 3: tanks 9, 10, 11 and 12.

Cameras were placed 10 minutes before food was given and would remain underwater for 1h hour afterwards. The footage was used to verify diet attractability, ingestion and individual interaction with the aid of ethograms (Tables 3.4). Camera settings were optimized to obtain the desired footage, with the quality set to 1080p and 25 fps.

For each day of observations, behaviour related to feeding and to interaction between animals was recorded.

Table 3.4. Feeding ethogram based on Mather, 1986;

Feeding Ethogram			
Behaviour Type	Definition	Key	Observations
Feeding	Attack on pellet	1	Processed for each individual feed
Multiple feeding	Attacking 2+ pellets	2	
Immediate feeding			
Pick and drop		3	
Intimidation		4	
Attack		5	

Multiple feeding was defined as whenever a cuttlefish would pick up a pellet and immediately or within a short amount of time (whilst still eating/holding the other pellet or shrimp) pick up another.

Immediate feeding counts were any number of (individual) cuttlefish that immediately (within the first 30 seconds of footage) ate the pellet/frozen shrimp.

Pick and drop was defined as whenever a cuttlefish would pick up a pellet or shrimp and drop it before starting to eat it or half-way through eating.

Intimidation was defined as a cuttlefish charging another with the intent of making it drop the feed.

Attack behaviour was defined as whenever a cuttlefish attacked another over the feed.

Welfare was tracked as well as to check for any behavioural anomalies that could occur, either because of experimental conditions or due to the supplied feed (Table 3.5).

The ethograms presented were input into BORIS (behavioural tracking program), recovered data was then processed using the statistical analysis tools (time budget and event plot) present in the software with the aid of Microsoft Excel (2016), allowing for the analysis of total observations for each behaviour and linking each event to a time stamp.

Table 3.5. Welfare ethogram based on Cooke and Tonkins, 2015 and Fiorito *et al.* 2015;

Welfare Ethogram				
Behaviour Type		Definition	Key	Observations
Locomotor				
	Avoidance of movement	Avoidance of motion due to stress	5	Welfare compromised
	Pacing	Swimming with no clear purpose	6	Welfare compromised
	Jetting	Sudden and quick movement	7	Welfare compromised
	Arm waving	Front two arms lifted	8	Welfare compromised
	Moving stripe	A quick moving stripe throughout the body	9	Warning/ward off threat
Defense				
	Ink pseudomorph	Small release of ink	q	Welfare compromised
	Ink fully	Large release of ink	w	Welfare compromised
Texture				
	Papillated skin	Rough texture to skin	e	Welfare compromised

Tissue sampling and histology

In the last day of diet testing trials, tissue samplings were gathered from cuttlefish from each tank, note that since treatment 51:15 was interrupted earlier, samples from this treatment were not taken.

Three animals were taken from each tank (selected randomly), totalling 27 animals from all tanks, and killed for sampling using terminal anaesthesia (100g /L of $MgCl_2 6H_20$) (Sykes *et al.* 2012b), death confirmation was also performed via destruction of brain.

Each animal was numbered, according to the tank and treatment, photographed (side, dorsal, ventral, innards and both sides of the cuttlebone) and had their digestive gland removed, weighed

(digestive gland index), split into two equal portions (one of which for histology trials) and stored in falcons containing PFA (paraformaldehyde) (mass volume ratio of 1:4) (Garrido *et al.* 2017).

The digestive gland portions were then dehydrated in a progressive series of ethanol (70% - 95%), as used in Jiménez-Prada *et al,* (2014), shown below in Table 3.6.

Table 3.6. Dehydration of samples methodology;

Ethanol %	Number of washes	Duration of washing (hours)
70	4	3-4
90	3	3-4
	1	12
95	1	3-4

The embedding process was done using a Technovit 7100 kit, which involved inclusion of samples in preparation solution (100 ml of resin and 1g of hardener 1) mixed with ethanol at 95% in a 1:1 proportion, samples were then embedded in resin containing hardener II and readied for sectioning. This was done using a Microm HM 340 E, equipped with tungsten blades, set to obtain sections ranging from 6-8 μm (feed). Trimming was done at 10 μm.

A minimum of 5 slides per sample (27 total) were taken, each slide held 2 cuts, totalling in 10 cuts per sample, 90 per treatment, 270 total cuts. Out of these samples, 10 slides for each treatment were used and studied on the microscope.

Microstructural analysis was done with haematoxylin/eosin staining (HE), enabling the visualization of nucleic acids (coloured blue by haematoxylin), elastic fibers, collagen and reticular fibers (coloured pink by eosin).

Periodic acid/schiff + haematoxylin (PAS+H) allowed us to spot glycogen and carbohydrate containing structures, tainting them in magenta (Ross and Pawlina, 2006). Half of the slides from each sample were dyed in HE, the remaining half on PAS+H.

Identification of structures was done according to Costa *et al*, (2014) using a Zeiss Azioskop microscope equipped with a Visicam 10.0, allowing for observation in a larger screen. In order to make a comparative analysis, four parameters were established. 1) structural integrity of cells, structures and marked elements; 2) cellular organization, if cells are displayed around lumen inside digestive tubules; 3) presence and amount of target cells (these are basal, digestive and boules) and structures (lumen, digestive tubule); 4) glycogen and carbohydrate clusters, viewable in PAS stained samples only.

The slides containing the best samples (samples that weren't torn or folded) were selected, stained and analysed on microscope.

Finding differences between the treatments using the set parameters was achieved via a point system based on Jorge *et al,* (2019) by noting if, 1) any structural damage existed in the sample such as destroyed cells (not resulting of cut or human manipulation); 2) whether or not cells were organized around the lumen and within digestive tubules; 3) lack of any of the structures identified;

Points were deducted every time, 1) rated 1 to 10 depending on if there was any structural damage or destroyed cells, points were deducted based on area size with damage; 2) 0.5 (value based on amount of samples (10)) points deducted if cells were not organized, per sample; 3) each structure not accounted for deducted 1 point (out of the possible 5 per sample).

By this system the best possible scores are, 1) 100% (no structural damage); 2) 10/10 and 3) 50/50.

To count the amount of basal, digestive and boules cells, 400x amplification images were sectioned into 4 equal parts, the total amount would later be compared between and within treatments.

Data collection and statistical analysis

Growth, ingestion and feed efficiency data, gathered from sampling and recovery of feed, were analysed using the following formulas; absolute growth rate (AGR (g/day)) = (Wf – Wi) / T, where Wi represents each tanks initial biomass, Wf the final biomass and T the time elapsed in days; specific growth rate (SGR: BW% / day) = (LnWf – LnWi) x 100 / T, where BW refers to individual body weight; B% (biomass change in a certain period, %) = [(Wf-Wi)/(Wi*t)]*100; digestive gland index (DGI: %) = DGW x 100 / Wf, here DGW refers to the digestive gland weight; corrected ingestion (or IF: wet weight in g) = (dry feed supplied (g) – uneaten dry feed in g x F) + (moisture feed supplied in g); absolute feed rate (AFR: g/day) = IF / T; specific feeding rate (SFR: BW%/day) = (AFR X 100) / Wa, where Wa is the average weight which is given by (Wi + Wf) / 2; feed efficiency (FE: %) = (Wf – Wi) x 100 / IF; feed conversion ratio (FCR) = IF / (Wf – Wi) (Rodríguez-González *et al.* 2015).

Analysis of data was done using IBM's SPSS v24, tables and graphs were obtained with Graphpad Prism v6 and v8.

In SPSS, equality of variances in the samples (homogeneity of variance $p > 0.05$) was tested using Levene's test and normality was checked using Shapiro-Wilk's test, these were used as they are more appropriate to studies in which the number of samples (n) is under 50.

Post-hoc tests were selected based on the number of samples as well, if the data was robust, Dunnett T3 was used as it is more appropriate for these sample sizes. If the data was parametric Tukey HSD was used as it also is the most powerful statistical test and more appropriate since we were looking for the pairwise differences between groups (Martin and Bridgmon, 2012). Non-parametric data was compared using the Kruskal-Wallis test.

Results

Temperature data gathered from chips and daily with the probe was compared to determine whether tanks were submitted to the same conditions, no significant difference was found ($p < 0.05$). No differences between salinity or dissolved oxygen levels were seen ($p < 0.05$).

Due to occurrence of cannibalism 10 days into the experiment in treatment 51:15 and 4 days later for the remaining artificial diets, all replicates were interrupted as was defined above in the humane endpoints. Nonetheless, some positive results were obtained with the data gathered.

Diet stability and sinking

No significant differences were found ($p > 0.05$) in disaggregation values between different pellets after analysis in SPSS. Disaggregation rates for each diet are shown in Table 4.1, below.

Table 4.1. Mean disaggregation rate (%) with standard deviation for each diet;

Time	55:10	51:15	47:20
30 minutes	6.45 ± 0.24	9.54 ± 0.47	10.9 ± 0.73
2 hours	11.31 ± 0.41	14.66 ± 0.40	15.82 ± 0.65
4 hours	17.55 ± 0.20	20.51 ± 0.75	23.05 ± 0.81
21 hours	27.45 ± 0.56	31.06 ± 0.72	33.25 ± 0.36
24 hours	29.99 ± 6.24	31.75 ± 0.44	34.26 ± 0.29

Regarding sinking, the time it took for pellets from different diets to reach the bottom once released was found to be significantly different ($p < 0.05$; Table 4.2), with the 55:10 diet sinking the slowest and 51:15 sinking the fastest. Where P1-P6 refer to the replicates used from each diet.

Table 4.2. Sinking values for the artificial pellets (in seconds);

Replicates	55:10	51:15	47:20
P1	4.50	3.46	3.12
P2	4.77	2.73	4.46
P3	3.93	2.87	3.86
P4	5.97	3.12	2.92
P5	6.68	2.85	2.78
P6	8.35	3.22	3.10
Average	5.70	3.04	3.37
SD	1.64	0.28	0.65

Growth data was divided into the three parts of the experiment, acclimatization, trial and recovery.

Growth: acclimatization

Preliminary sampling showed no significant differences existed for weight in cuttlefish selected for the trial ($p > 0.05$, 1-W-ANOVA).

Table 4.3 shows the statistical analysis done for the acclimatization period the experiment undertook. Despite every tank was still being fed the same diet (shrimp), they were still separated into the same treatment groups they would belong to during the trial, for the purpose of enabling comparisons.

Table 4.3 MWW, SGR, total biomass and B% comparison between tanks in acclimatization;

Variable	Test	Factor	SS	df	Statistic	P-value
MWW	Kruskal-Wallis	Diet	-	3	1.069	= 0.784
SGR	1-w-ANOVA	Diet	0.115	3	0.167	= 0.916
Biomass	1-w-ANOVA	Diet	22.409	3	0.090	= 0.964
B%	1-w-ANOVA	Diet	0.135	3	0.162	= 0.919

The values we see above in the table were recovered on the sampling that marked the start of the trial (day 0). No statistical differences were found between treatments ($p < 0.05$).

Complete acclimatization data is shown below, in Table 4.4.

Table 4.4 Complete acclimatization data for the analysed growth and efficiency parameters;

Index/Diet		Control	55:10	51:15	47:20
Growth	W (g)				
	Day 0	351.83±1.96	350.7±1.78	351.9±2.34	351.6±2.69
	Day 5	386.63±15.31	389.7±8.78	386.17±2.77	387.87±3.80
	AGR	6.96±2.83	7.78±2.00	6.85±0.57	7.25±1.10
	SGR	1.87±0.7	2.10±0.52	1.86±0.15	1.96±0.29
Efficiency					
	B%	1.98±0.79	2.22±0.57	1.95±0.17	2.06±0.32

Growth: trial

Regarding the trial itself all artificial treatments showed low feeding rates and negative growth. Pellet fed cuttlefish were seen to either lose or maintain weight which resulted in very low or even negative rates of FE as well as negative AGR and SGR. FCR was also seen to reach negative values in artificial diets, whereas in the control group it was 4.09±1.28 g of feed for each 1 g of animal weight. This was the case despite feed (both pellet and shrimp) being increased in a daily basis, since little was recovered each day.

Overall pelleted diet performances were not significantly different between each other in the growth, ingestion and efficiency parameters analysed, shown in Table 4.5.

Table 4.5. Complete growth, ingestion and feed efficiency data table from trial results;

Index/Diet	Control	55:10	51:15	47:20
Growth	W(g)	W(g)	W(g)	W(g)
Day 0	386.83±15.3	389.7±8.78	386.17±2.77	387.87±3.80
Day 7	516.40±18.78	374.17±5.85	378.87±5.52	377.07±7.85
Day 14	659.20±71.25	299.50±60.74	349±14.56	320.63±16.86
Gw (g)	272.57±85.37	-90.20±67.46	-37.17±17.14	-67.23±16.11
AGR (g/day)	19.47±6.10	-6.44±4.82	-3.72±1.71	-4.80±1.15
SGR (%BW/day)	3.79±1.06	-1.99±1.68	-0.40±0.48	-1.37±0.37
Ingestion	Control	55:10	51:15	47:20
AFRD (g/day)	18.71±1.31	2.52±0.60	2.73±0.29	2.74±0.19
AFRM (g/day	74.44±5.21	2.64±0.62	2.91±0.31	2.94±0.20
SFRD (g/day)	3.58±0.11	0.74±0.20	0.72±0.08	0.77±0.07
SFRM (g/day)	14.23±0.44	0.77±0.21	0.77±0.09	0.83±0.08
Efficiency	Control	55:10	51:15	47:20
B %	5.08±1.74	-1.64±1.20	-0.96±0.44	-1.24±0.30
FE (%)	25.92±7.11	-236.97±144.52	-128.58±61.44	-161.98±27.60
FCR (%)	4.09±1.28	-0.53±0.28	-0.97±0.61	-0.63±0.10

Gw, gained weight, MWW, mean wet weight, SGR, specific growth rate, AGR, absolute growth rate, AFRD, absolute feeding rate Dry, AFRM, absolute feeding rate moist, SFRD, specific feeding rate dry, SFRM, specific feeding rate moist, B, biomass, FE, feed efficiency, FCR, feed conversion rate. Diet denotes the variable of each pelleted diet given to a group of tanks.

The only exception being in MWW between 51:15 and both 47:20, 55:10 (Table 4.6). Although this may be because 51:15 tanks were subjected to recovery 4 days earlier than the remaining pellet fed tanks, spending less time under trial. Due to this, an extra sampling was done for these tanks, resulting indirectly in narrower gaps between sampling days. Formulas were adjusted accordingly to reflect the shorter period and this variable was considered during statistical analysis.

Table 4.6. Statistical analysis of the effects of different diets on different growth, ingestion and efficiency parameters for *S. officinalis* juveniles;

Data	Test	Factor	SS	df	Statistic	P-value	Post-hoc	H.G
MWW Trial	Welch	Diet	858.25	3	11.181	< 0.001	Dunnett T3	47:20, 55:10 < 51:15, C
	Mann Whitney	Time	96.536	1	1.008	0.376	-	-
MWW day 14	1W-ANOVA	Diet	1366.043	3	290.49	< 0.001	Tukey HSD	47:20, 55:10, 51:15 < C
SGR Trial	Kruskal-Wallis	Diet	-	3	13.34	0.004	Dunn	55:10, 51:15, 47:20 < C
	Mann Whitney	Time	-	1	32	0.02*	-	-
Biomass Trial	Welch	Diet	0.21	3	17.974	< 0.001	Dunnett T3	47:20, 55:10, 51:15 < C
	Mann Whitney	Time	0.001	1	0.077	0.784	-	-
B day 14	Welch	Diet	257,952.79	3	16.671	0.01	Dunnett T3	55:10, 47:20, 51:15 < C
B % Trial	Kruskal-Wallis	Diet	-	3	13.3	< 0.004	Dunn	55:10, 51:15, 47:20 < C
	Mann Whitney	Time	-	1	31.5	0.018	-	-
FE Trial	Welch	Diet	1.519	3	19.325	< 0.001	Dunnett T3	51:15, 55:10, 47:20 < C
	Mann Whitney	Time	0.566	1	5.62	0.027*	-	-
FE day 7	1W-ANOVA	Diet	1.282	3	40.705	< 0.001	Tukey HSD	55:10, 47:20, 51:15 < C
FE day 14	Welch	Diet	0.413	3	11.168	0.03	Dunnett T3	47:20, 51:15, 55:10 < C
FCR Trial	Welch	Diet	3.904	3	7.102	0.007	Dunnett T3	51:15, 47:20, 55:10 < C
	Mann Whitney	Time	0.331	1	1.422	0.246	-	-

H.G refers to homogeneous group, C refers to control, MWW, mean wet weight, SGR, specific growth rate, B, biomass, FE, feed efficiency, FCR, feed conversion rate. Diet denotes the variable of each pelleted diet given to a group of tanks; 1W-ANOVA refers to one-way ANOVA. Tests with p-value showing a significant difference were followed up with a post-hoc according to the nature of the test (parametric: Tukey HSD, Robust/Welch: Dunnett T3, non-parametric: Dunn), these were chosen due to being indicated for our sample size and their test power; *no conclusions can be withdrawn from Time analysis as there were not enough samplings (due to an abrupt end to the experiment) to support post-hoc analysis.

Amount of feed supplied was seen increasing throughout the experiment in all treatments as seen below in Figure 4.1, a significant difference was found in amount required to feed tanks belonging to treatment 55:10 and the other 2 pelleted treatments ($p < 0.05$). Tanks belonging to 55:10 had a smaller demand for the supplied diet. Control fed cuttlefish had the highest demand for food, significantly different when compared to the pelleted treatments.

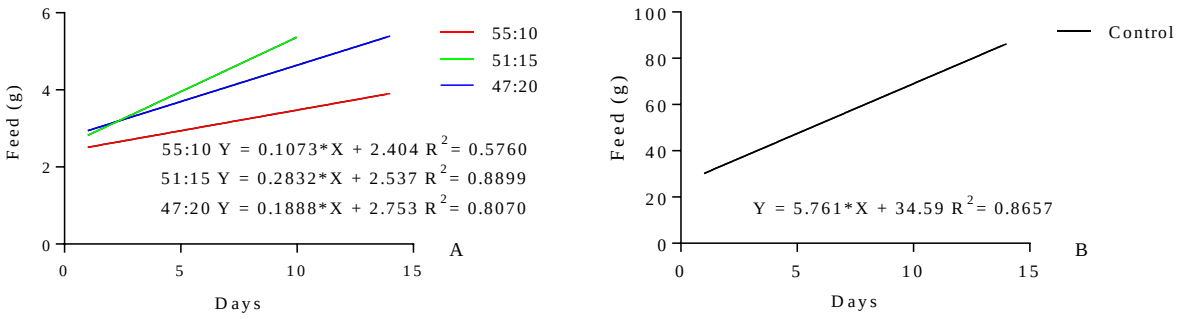

Figure 4.1. Dry (for both pelleted diets and grass shrimp) feed supplied in grams over time;

It's worth to note that most of the weight in shrimp is water content that is not deducted when calculating amount of feed given. Determining the amount water content in the shrimp used as feed (74.86%) allowed to calculate the dry weight of feed given and this was still higher than amount supplied to pelleted diets.

Growth: recovery

It's worth noting since tanks belonging to treatment 51:15 began recovery sooner, the recovery period was larger, granting more time to re-gain weight.

Statistical analysis of the recovery period was also done, to determine if whether animals managed to recover in a short period, demonstrated in Table 4.7.

Table 4.7. Comparisons in weight and growth between acclimatization (A) data and recovery (R) data;

Variable	Test	Factor	SS	df	Statistic	P-value	Post-hoc	H.G
MWW	1-W-ANOVA	Recovery	92.571	1	10.780	< 0.005	Dunnett T3	A < R
SGR	Welch	Recovery	2.115	1	1.648	0.218	-	-
Biomass	Welch	Recovery	23,486.669	1	3.779	0.70	-	-
B%	Welch	Recovery	1.656	1	1.187	0.292	-	-

The recovery period of 6 days (10 for 51:15 animals) showed no significant differences in biomass, B% and SGR. The only statistical difference came from animal MWW, with animals placed on recovery already weighing more than they did during acclimatization.

Complete recovery data is shown in Table 4.8, below.

Table 4.8 Complete recovery data for growth and efficiency parameters analysed;

Index/Diet		Control	55:10	51:15	47:20
Growth	W (g)				
	Day 0	516.70±69.53	219.83±59.53	349.0±14.56	246.37±24.49
	Day 5	712.37±147.82	241.20±72.64	448.80±20.39	257.0±65.06
	AGR	27.95±13.72	3.05±3.78	9.07±0.76	1.52±5.37
	SGR	4.47±1.55	1.25±1.29	2.29±0.14	0.34±2.30
Efficiency					
	B%	5.33±2.18	1.35±1.43	2.60±0.19	0.47±2.26

Behaviour and feeding

Acceptance of diet was seen right from the start in all diets and recorded from day 2 onward. There are no records of first day of feeding, this was caused by an error in camera positioning which didn't allow for visualization of footage. Despite this fact there was still pellet acceptance in day 1, cuttlefish were seen eating by two of the people involved in this study.

The amount of feed counts was tracked throughout the experiment in all tanks (Tables 4.9).

Table 4.9. Total feeding counts throughout the trial;

Days	Control	55:10	51:15	47:20
10-07-2019	66	9	2	-
11-07-2019	30	3	5	5
12-07-2019	88	7	9	2
13-07-2019	66	8	8	6
14-07-2019	46	0	4	4
15-07-2019	75	7	10	13
16-07-2019	55	11	20	14
17-07-2019	83	11	17	10
18-07-2019	59	6	53	28
19-07-2019	87	13	-	26
20-07-2019	90	20	-	11
21-07-2019	34	27	-	36
22-07-2019	69	25	-	29
Total	848	147	128	184
Average	65.23	11.31	14.22	15.33
SD	19.92	8.12	15.68	11.45

Note: the grey portion represents the time at which treatment was placed in recovery

A significant difference was found between the control and the remaining diets, but not between each other ($p < 0.05$; Welch test), control having the most counts by far. Pick and drop counts seem to increase as time passed (Table 4.10). In Figure 4.2, pick and drop counts dropped for control, and treatment 51:15 seemed to have a large increment through time, with the remaining pelleted treatments remaining stable. However, R square analysis shows there is no clear relation between time and pick and drop counts.

Table 4.10. Pick and drop data;

Days	Control	55:10	51:15	47:20
10-07-2019	5	0	0	0
11-07-2019	2	2	0	2
12-07-2019	3	2	0	1
13-07-2019	1	0	2	0
14-07-2019	1	0	0	0
15-07-2019	0	1	0	1
16-07-2019	0	1	11	3
17-07-2019	0	0	3	1
18-07-2019	1	0	14	4
19-07-2019	0	2	-	3
20-07-2019	1	0	-	1
21-07-2019	0	3	-	2
22-07-2019	0	3	-	0
Total	14	14	30	18
Average	1.08	1.08	3.33	1.38
SD	1.50	1.19	5.36	1.33

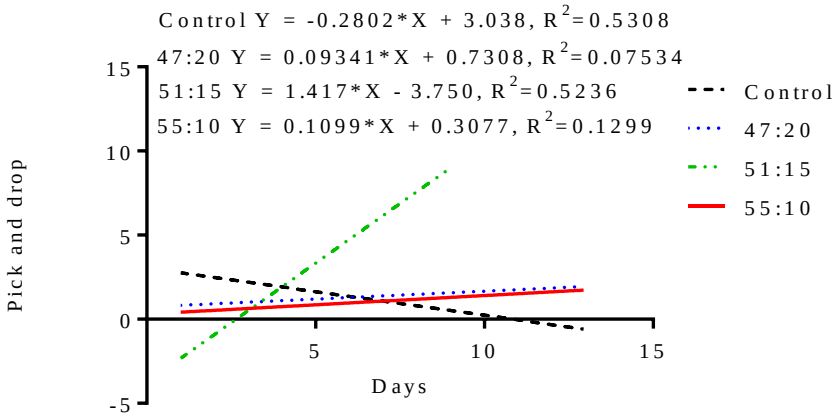

Figure 4.2. Pick and drop linear regression;

Feeding counts were corrected by subtracting the amount of 'pick and drop' behaviour counts to obtain a more realistic scenario, shown in Table 4.11.

Table 4.11. Total feeding counts corrected by subtracting pick and drop counts;

Days	Control	55:10	51:15	47:20
10-07-2019	61	9	2	-
11-07-2019	28	1	5	3
12-07-2019	85	5	9	1
13-07-2019	65	8	6	6
14-07-2019	45	0	4	4
15-07-2019	75	6	10	12
16-07-2019	55	10	9	11
17-07-2019	83	11	14	9
18-07-2019	58	6	39	24
19-07-2019	87	11	-	23
20-07-2019	89	20	-	10
21-07-2019	34	24	-	34
22-07-2019	69	22	-	29
Total	834	133	98	166
Average	64.15	10.23	10.89	13.83
SD	19.97	7.55	11.14	10.93

There were no differences between pelleted treatments in terms of acceptance, amount of feeding counts as shown on Figure 4.3, however this may not be completely correct to assume as 51:15 had fewer sample days.

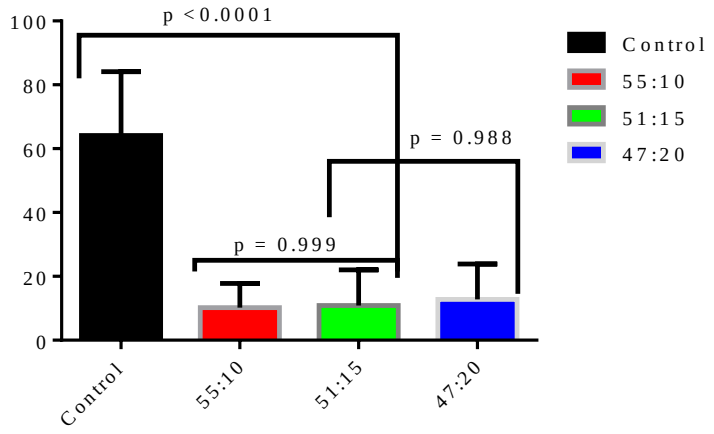

Figure 4.3. Mean feed counts (corrected with pick and drop data), no significant differences (1-way ANOVA) between pelleted treatments were detected;

Figure 4.4 shows a visual representation of feeding counts (corrected with pick and drop) through time, even though amount of feeding counts in pellet fed tanks increased slightly with time, results show it's in no way comparable to what cuttlefish fed shrimp ate in total. Ultimately R square analysis shows that no clear relation exists between feeding counts and time.

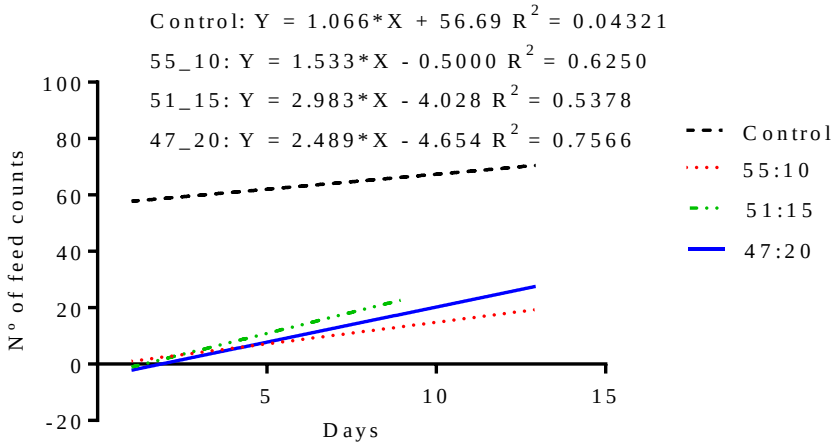

Figure 4.4. Linear regression with respective equations and R^2 for feeding counts (corrected), seen increasing throughout trial;

Multiple feeding behaviour (Table 4.12) was less common in artificial diets than in pelleted treatments as opposed to the control fed cuttlefish that always attacked in a more voracious fashion with multiple counts from day 1 onwards. It was more common to spot these occurrences in control fed tanks (Figure 4.5).

Table 4.12. Total multiple feeding counts, throughout the trial;

Days	Control	55:10	51:15	47:20
10-07-2019	7	0	0	0
11-07-2019	13	0	0	0
12-07-2019	16	2	0	0
13-07-2019	14	0	0	0
14-07-2019	13	0	0	1
15-07-2019	11	0	0	1
16-07-2019	7	2	3	1
17-07-2019	15	0	0	1
18-07-2019	6	0	0	3
19-07-2019	6	0	-	0
20-07-2019	12	1	-	0
21-07-2019	3	1	-	4
22-07-2019	4	3	-	1
Total	127	9	3	12
Average	9.77	0.69	0.33	0.92
SD	4.42	1.03	1.00	1.26

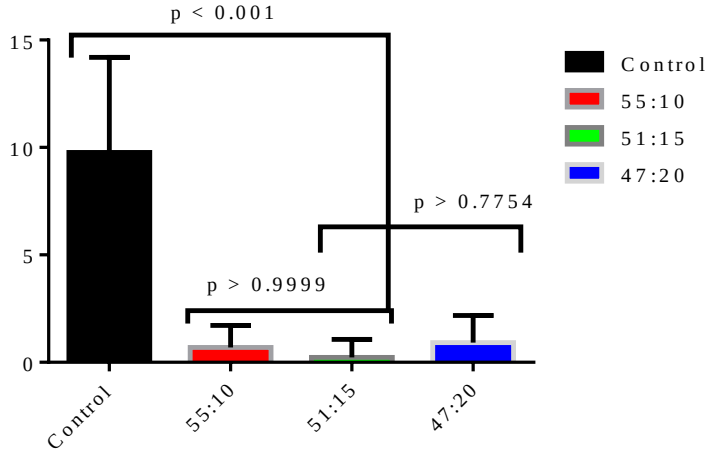

Figure 4.5. Multiple feeding counts analysis (Kruskal-Wallis multiple comparison test);

Like in feeding counts and corrected feeding counts, multiple feeding was mostly observed in control tanks, with no significant difference between the remaining pellet fed tanks.

Immediate feed was initially thought to grow very slightly in pellet fed tanks, as shown in Figure 4.6, however R square analysis shows there's no clear increase in this behaviour.

Similar to previous data, immediate feeding was mostly observed in cuttlefish fed shrimp and growing in time in the pelleted treatments, analysis found a significant difference between the control and the remaining diets ($p < 0.0001$).

In Figure 4.6, pellet fed cuttlefish immediate feeding seems to be increasing, with more cuttlefish going for pellets right after these were given, however R^2 analysis shows there is no correlation between time and this variable. Total immediate count data can be seen on table 4.13, below.

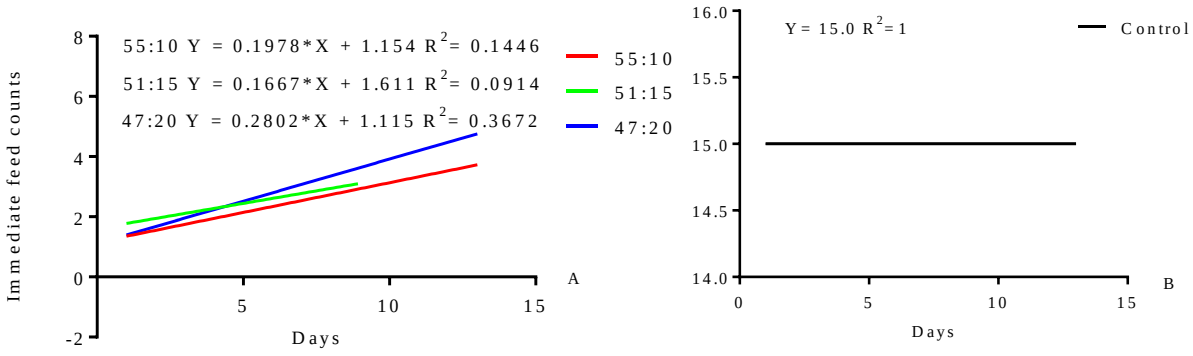

Figure 4.6. Linear regression with respective equations and R^2 for immediate feeding for each treatment;

Table 4.13. Immediate feeding data;

Days	55:10	51:15	47:20
10/07/2019	0	0	0
11/07/2019	3	2	4
12/07/2019	2	5	2
13/07/2019	3	2	3
14/07/2019	0	1	2
15/07/2019	1	4	2
16/07/2019	5	3	3
17/07/2019	5	2	1
18/07/2019	0	3	6
19/07/2019	3	-	3
20/07/2019	5	-	3
21/07/2019	1	-	6
22/07/2019	5	-	5
Total	33	22	40
Average	2.54	2.44	3.08
SD	2.03	1.51	1.80

Note: Control counts were always the maximum for this parameter, which is 15;

In footage obtained from control tanks every cuttlefish would immediately start moving towards the surface even before feed was given. Every animal was recorded eating in the first 30 seconds of the video, resulting in what is observable in Figure 4.6.

Welfare counts were much rarer, with most counts being caused by external stimuli (mostly placement of cameras or caretaker approach during feeding), showed in Figure 4.7. Pacing counts and large amounts of inking were the only behaviours that were not observed during any of the footage analysed.

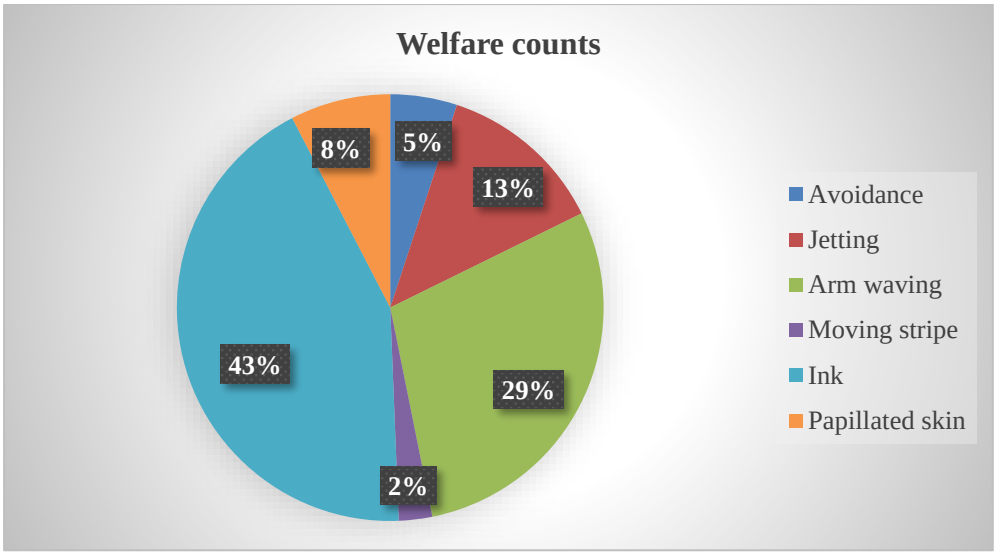

Figure 4.7. Total welfare counts registered throughout the full duration of the trial;

Lastly, intimidation and attacks were the least recorded behaviours (Table 4.14 and 4.15), occurring mainly immediately after feed was given, despite the availability of enough food for every cuttlefish.

No significant difference was found between treatments for either behaviours (intimidation $p = 0.1080$; attack $p = 0.4698$).

Table 4.14. Total intimidation counts;

Days	Control	55:10	51:15	47:20
10-07-2019	0	3	0	0
11-07-2019	1	0	0	0
12-07-2019	2	0	0	0
13-07-2019	2	0	0	1
14-07-2019	0	0	0	1
15-07-2019	2	0	0	1
16-07-2019	0	0	0	1
17-07-2019	0	0	0	0
18-07-2019	0	0	0	0
19-07-2019	1	0	-	2
20-07-2019	0	3	-	0
21-07-2019	0	0	-	3
22-07-2019	0	0	-	0
Total	8	6	0	9
Average	0.62	0.46	0.00	0.69
SD	0.87	1.13	0.00	0.95

Table 4.15. Total attack counts;

Days	Control	55:10	51:15	47:20
10-07-2019	0	0	0	0
11-07-2019	0	0	0	0
12-07-2019	0	0	1	0
13-07-2019	0	0	0	1
14-07-2019	0	0	0	0
15-07-2019	1	0	1	0
16-07-2019	0	0	0	0
17-07-2019	0	1	1	0
18-07-2019	0	0	0	0
19-07-2019	0	0	-	1
20-07-2019	1	0	-	0
21-07-2019	0	0	-	0
22-07-2019	0	0	-	0
Total	2	1	3	2
Average	0.153	0.076	0.333	0.153
SD	0.38	0.28	0.50	0.38

Histology

A significant difference was found between pelleted treatments and control in DGI ($p < 0.05$, 1-way-ANOVA, Tukey HSD), with control fed cuttlefish showing much larger digestive glands than pellet fed cuttlefish.

After analysis of both PAS and HE samples, no significant difference was found in (1) structural integrity between treatments nor in (2) cell organization and (3) presence of identified (Figure 4.8) cell/structures, shown in Table 4.16. 10 samples were selected of each diet to make comparative analysis; 1) each structurally compromised count would deduce 5% of the total; 2) each time cells weren't organized around the lumen or inside digestive tubules 0.5 points were deduced, if both were found to occur, 1 point; 3) each time an identified structure wasn't found, 1 point would be deduced from the total;

Table 4.16. Score table for each treatment;

Diet	Control	55:10	47:20
1)	100%	100%	100%
2)	10/10	9.5/10	9/10
3)	50/50	49/50	48/50

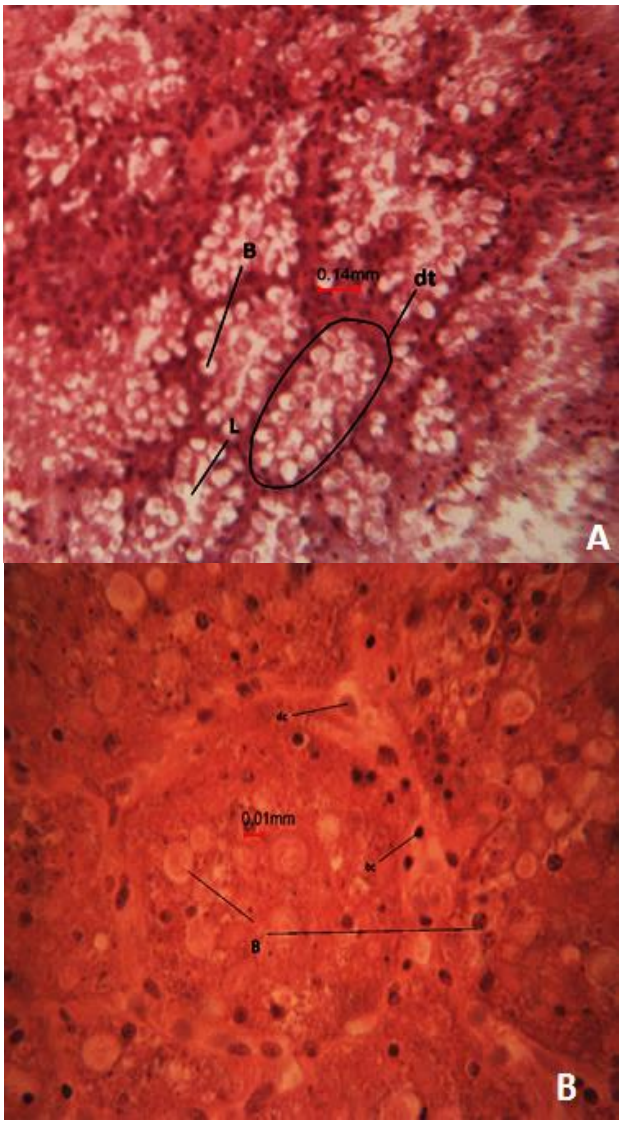

Figure 4.8. Structural analysis of *S. officinalis* section of digestive gland. Image A shows a 100x total amplification (10x10 ocular x objective) with HE tint. The dt refers to the digestive tubules, densely packed structures containing most other cells and components of the digestive gland, L the lumen is at the center of the dt*;* image B shows a 400x amplification (10x40) with HE tint. The bc and dc refer to basal cells and digestive cells respectively, lastly B are *boules*, which are responsible for intracellular digestion and can contain a variety of materials;

Figures 4.9 shows us examples from each diet of how our samples looked in general, it is possible to see every structure identified in Figure 4.7 along with how they are organized.

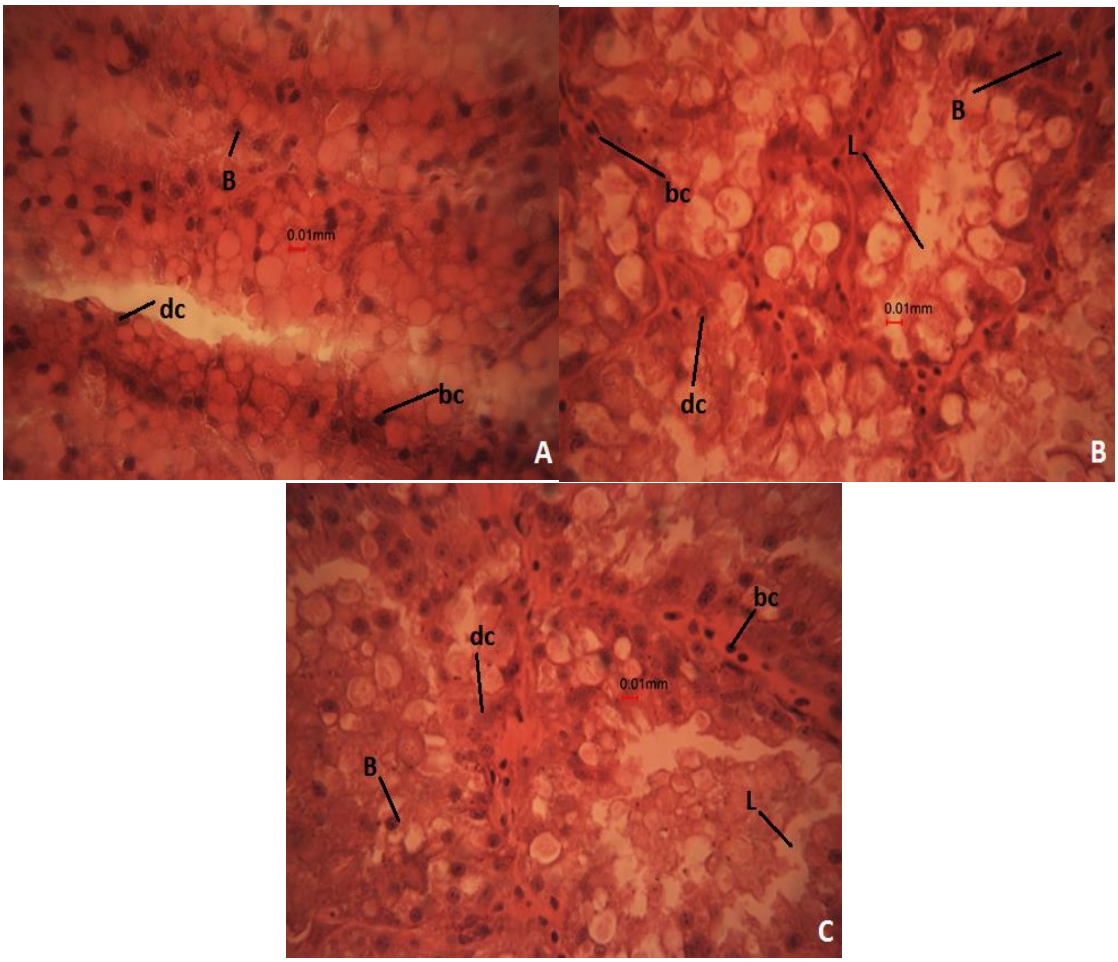

Figure 4.9. Image A: digestive gland of a cuttlefish individual fed on grass shrimp (400x amplification, HE stain), dc refers to digestive cells, bc to basal cells and B to boules; image B: 47:20 pellet fed digestive gland at 400x amplification, HE stain, L refers to lumen; image C: 55:10 pellet fed digestive gland at 400x amplification, HE stain;

The 4th point of comparison that was also considered was not included in the analysis of differences between treatments as PAS stain showed no HC or glycogen deposits in any of the analysed samples (on both control fed and pellet fed cuttlefish). Figure 4.10 shows examples of our PAS dyed samples with the same identified structures as in previous images.

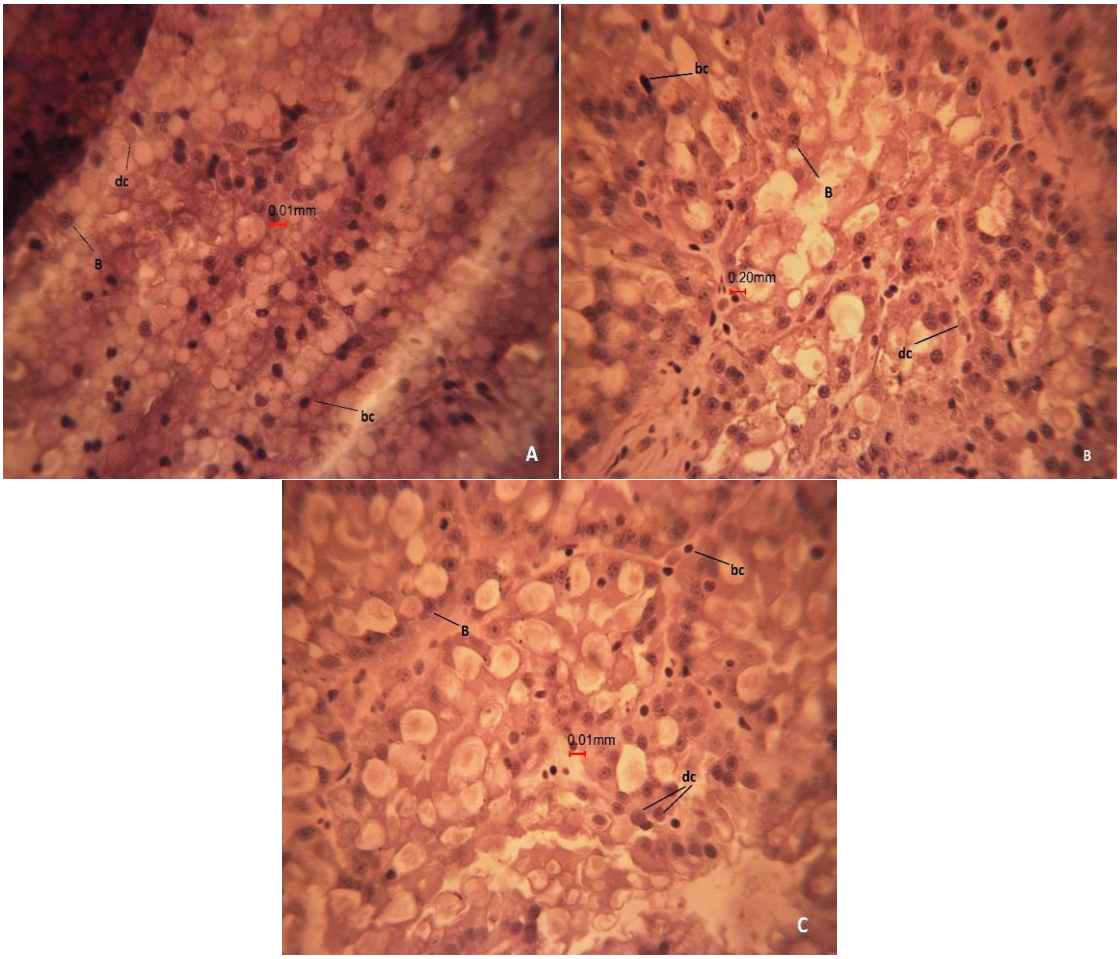

Figure 4.10. Image A: DG of a cuttlefish individual fed on grass shrimp (400x amplification, PAS stain), dc refers to digestive cells, bc to basal cells and B to boules; image B: 47:20 pellet fed digestive gland at 400x amplification, PAS stain; image C: 55:10 pellet fed digestive gland at 400x amplification, PAS stain;

Discussion

Disaggregation values were seen stabilizing around the 21 to 24-hour mark, when the pellet had lost, on average, about 30% of its contents to the surrounding environment.

Even though there are differences in protein to carbohydrate ratio between diets, the ingredients that grant the pellet its structural integrity (such as fish gelatin) were present in the same quantity in the diets, so similar disaggregation values were expected.

Sinking however was seen to be different between diets, with the one with most protein (55:10) sinking the slowest and the intermediate (51:15) the fastest. With the data recovered from sinking trials, we can't conclude that higher protein content correlates directly to slower sinking as there was no differences between 55:10 and 47:20.

However, cuttlefish fed pellet further into the trial were seen charging the pellet before it completely sank. This means that the pellet, despite the difference between sink times, remained in the water column long enough for animals to spot it and go for the pellet. As well most cuttlefish towards the end of the trial, identified the pellet as food.

Cuttlefish were successfully transferred to the tanks outside and adapted well to the outdoor tanks during acclimatization process. Conditions for these tanks (dissolved oxygen, temperature, salinity and feed) were equal to from their previous tank, with the only difference being the transition of interior to exterior of the station. All tanks displayed a healthy increase in cuttlefish MWW by an average of 30 g and positive growth rates almost comparable to those obtained in previous studies (Sykes *et al.* 2006a; Sykes *et al.* 2014).

Despite cannibalism occurring which ended the experiment early, loss of biomass was below 10% meaning this specific endpoint didn't occur, this may indicate that, despite the pelleted diet not being enough to prevent cannibalism, it was enough to satiate the animal to a point, or perhaps not enough time elapsed for significant weight loss to happen.

However, lacking the potential for growth to exist and to completely satiate the animal, it was increasingly probable for cannibalism to occur with time passing. Lack of growth in pelleted diets resulted in negative FE, AGR and SGR. In comparison with other studies that also tested artificial diets in cuttlefish; Castro *et al,* (1993) showed higher growth rates; Domingues *et al,* (2005b) also had negative growth rates and FCR but not as low as obtained on this trial; Ferreira *et al,* (2010) had even lower FCR's and similar negative growth rates.

Growth parameters did not show any differences between tested artificial diets, however treatments with lower protein concentration were more readily accepted with the animals being fed 47:20 and 51:15 diets requiring more feed than 55:10 fed animals.

The lack of differences in growth can be mainly attributed to the diet not fitting the necessary requirements for cuttlefish to grow. Although, this can't simply be blamed on the nutrient profile alone.

The fact that the pelleted diet may have been able to sustain the cuttlefish for a while, even though it wasn't perfect, may be due to a portion of the protein (shrimp meal) given was not treated as most meals (such as squid meal used in this trial) are in the industry (heat treated above 60ºC). But instead was lyophilized at -50ºC. It has been shown in past studies using *Octopus vulgaris*, that pelleted diets using raw materials that were treated via lyophilization and heat drying below 60 ºC, performed better in almost every single aspect, survival, growth and acceptance (Rodríguez-González *et al.* 2015).

The process of heating or boiling during raw material preparation causes protein denaturation, lipid oxidation and consequently amino acid, polar lipid and vitamin loss, this was found to be true by German (1999). Further proved when Domingues (2009) obtained similar growth rates and survival for cuttlefish fed shrimp (*P. varians*) and freeze-dried shrimp, but not on the same shrimp was heat treated, which resulted in lower survival and growth.

When raw materials are freeze dried and heat dried at around 57 ºC (below 60ºC), water is removed while maintaining the molecular structure and nutritional properties (Kennedy and Cabral, 1993) and as a result, Rodríguez-González *et al,* (2015) got all animals to accept the diets, grow, and produced faeces, with a 100% survival rate in all groups, which presented similar growth rates in weight throughout the experiment.

In the same study, freeze drying was found to alter total protein and ash content in the formula when comparing to the heat-treated meal, this difference was attributed to the different processing that the protein was subject to. It is the heat application that causes the structure of the protein itself to alter, causing denaturation which translates directly to a reduction in nutrient content (Lehninger, 1988).

Knowing this, and since the squid meal used in this trial was heat treated (at 60ºC or above), the total usable protein in each diet was not the one present in the ratios (55, 51 and 47), but much

lower. It would be interesting to verify the differences in growth, survival and even acceptance if this single ingredient was treated differently.

Regarding the different ratios studied in this trial, since a portion of the protein was less than optimal, we cannot withdraw conclusions regarding which ratio is best for growth and survival. Adding to this there were also no statistical differences between the pelleted treatments.

To know the optimal nutritional balance, first we must consider how each ingredient is treated and have the diet result in growth. Hence further trials are necessary to figure out which protein to carbohydrate ratio is best.

It's important to note that in this experiment, the amounts of protein used were relatively low when comparing to past studies in cephalopods. Past diets have used between 60-90% protein content (Castro *et al.* 1993; Domingues *et al.* 2005b; Ferreira *et al.* 2010) with cuttlefish and 55-95% protein (Martínez *et al.* 2014; Querol *et al.* 2013; Rodríguez-González *et al.* 2015) with two different species of Octopus (*Octopus vulgaris*, *Octopus Maya*) in more recent studies. The extremely high amounts used in the past were justified due to the nutritional constitution of a cephalopod, with protein being the most common, ranging 75-85%. Knowing this, it would be interesting to see how lower protein amounts affect cuttlefish growth and survival in future trials.

Recovery data was compared to acclimatization data and found no statistically significant difference between these time periods in SGR, tank biomass and B% ($p > 0.05$). There was however a slight difference in tank MWW ($p < 0.05$).

Despite artificial diets being a failure growth wise, data shows animals were able to fully recover back to values equal or better than those observed in acclimatization. Cuttlefish subject to the recovery period were able to regain lost weight and start growing once again. The ability to recover at this speed also reflects that, even though the diet was not enough to fully sustain the cuttlefish, it didn't cause any permanent damage that would impede growth when fed on a more appropriate diet.

In this study the protein portion was the problem, future artificial diet trials will have to consider proper care for all ingredients, lyophilization of protein and encapsulation of amino acids are examples of specific care when formulating diets as to not lose nutritional value in the pellet.

In the first day of feeding, when pellet was first given to each of the respective tanks, cuttlefish from every treatment were seen grabbing and eat the artificial diet. A great result that can be pinned on the formulation of the diet. Attractability is based on several variables, smell,

shape, taste (Darmaillacq *et al.* 2004) and consistency of the pellet. The use of protein, based on crustacean meals or mollusc meals, since suggested by (Martínez *et al.* 2014), has been a staple in the production of artificial diets for cephalopods, as it heavily contributes to diet attractability.

Another reason why we might have seen first day feedings is the use of freeze-dried shrimp powder, the same shrimp used for cuttlefish grow-out until trial start. The familiar odor and taste of shrimp might have triggered the initial curiosity for cuttlefish to assume that the pellet was food.

Despite having successfully formulated a diet that was accepted on day 1, the pattern of feed counts does not differ from other studies such as Castro *et al*, (1993), Domingues *et al*, (2005b), or Ferreira *et al*, (2010). Meaning there is only a slight growth over time in feed consumed, a result that can be improved upon, either by improving the diet to provide satiation or by improving attractability further.

The remaining mentioned variables, shape and consistency, may aid in a future endeavour to optimize diet attractability. The formulated pellets were much like the standard made semi-dry pellet, 6 mm balls that are harder to eat when compared to the soft innards of shrimp or mysids, and their unique shape. This may be a large disadvantage attractability wise, since cuttlefish are carnivorous predators who respond not only to odor queues but also visual ones (Messenger, 1968; Shashar *et al.* 2000). Perhaps a future study testing different consistencies or pellet shapes could help check if these variables would aid in artificial diet attractability. This should not be an immediate focus of study, since day 1 feeding was already achieved, and should be considered after obtaining an optimal nutrient profile that allows for growth.

Higher feed counts can also mean that, by having a non-optimal feed that doesn't abide to the specific needs of the cuttlefish, the animal will eat more in order to fulfil the need for nutrition.

It was possible to see that cuttlefish were less likely to go immediately for the pellet, sometimes checking what had entered the water, but then not picking up the pellet after a few more minutes, R square on figure 4.6 shows the correlation between immediate feeding and time is weak, meaning there's no evidence immediate feeding grew over time on pellet fed tanks. It's unlikely this is due to any satiation caused by the diet, since food was only given once every day and the artificial diets had such weak performances growth wise.

Regarding welfare, most of the counts spotted occurred whenever cameras were being set or whenever feed was being given. Some cuttlefish would react negatively to the presence of the caretaker and either ink or just remain still at the bottom of the tank. Further into the video we

could see cuttlefish gradually start swimming around and investigating the camera and other objects such as the airlifts, this curiosity is a sign of well-being (Fiorito *et al.* 2015). It is also arguable that reactiveness to outside stimuli (even if negative) is a sign of well-being as well, which is why avoidance of movement was also tracked (Cooke and Tonkins, 2015).

Despite each tank being supplied with enough food for every cuttlefish to have plenty to eat there were still attack and intimidation counts registered. This has been shown to be normal behaviour in cuttlefish in Mather (1986). But in a closed setting (like our tanks), a hierarchy that is based on size (but also on gender in later adult stages) does exist and determines which ones go for food immediately and which ones wait. Crowding may be the reason why these behaviours occur, yet cuttlefish are found to school when at younger stages (Cooke *et al.* 2019), only beginning to have agonistic behaviour towards each other when reaching the adult stage (Boal *et al.* 1999).

Since our observations weren't focused on determining a hierarchy and counts of these behaviours seemed more random than a hierarchical system would suggest, there is another valid explanation. Mather also states that some individuals are more likely to simply "bully" others out of eating initially, this is further validated by (Carere *et al.* 2015; Zoratto *et al.* 2018) that showed cuttlefish have personality variations that can vary and affect individual trade-offs and interactions. Both hypotheses might be correct, however with our data we cannot make assumptions regarding a hierarchy.

In every attack and intimidation case registered it is likely it wasn't due to lack of food as was initially thought.

Another interesting find was the overtime increase of pick and drop counts, seen mostly on pellet fed tanks. Unfortunately, past studies on cuttlefish feeding behaviour not only are scarce but also neglected this attitude towards the food. R^2 analysis for control (R^2 = 0.5308) and 51:15 (R^2 = 0.5236) confirms that we can't assume a relation between time and pick and drop counts.

Despite being the intermediate option between the pelleted diets, diet 51:15 was the first treatment to enter recovery, no differences were found in water quality parameters that could cause this. Tank positioning was also considered but ultimately disregarded as there was no difference between tanks relative to the station (all had the same exposure time to daylight, proximity to each other and other structures in the facility).

Diet 51:15 also had the least feeding growth over time (despite being supplied more feed, figure 4.1) and presented the most counts of pick and drop. It is likely that the first two facts are

associated, by eating less pellet than the other two diets, cuttlefish in 51:15 tanks started cannibalizing sooner because demand for food was higher. But the pick and drop counts can also be an indicator of something negative, especially because of the spike that occurred in the last days of tanks belonging to 51:15 diets. The amount of registered counts was caused by cuttlefish picking up, "munching", dropping and picking up again the same pellet repeatedly, without eating the pellet and just picking it apart. This could mean that by this stage cuttlefish were starting to slowly "reject" the diet, until on later stages, recurring to cannibalism.

Comparative analysis of histological samples showed no differences in all parameters screened ($p > 0.05$), structural integrity, cell organization and presence of identified cell/structures. However, no carbohydrate/glycogen clusters were found with PAS coloration. This is in accordance with past studies that also used cuttlefish reared in Ramalhete aquaculture station (Sykes *et al.* personal communication), in which there were also no traces of these nutrients in the digestive gland. The lack of presence of these clusters cannot be blamed on the failure of the pelleted diet as the control samples also did not have this nutrient stored.

Costa *et al,* (2014), saw, identified and described these same clusters using a similar PAS methodology, but the key difference between their trial and the present one is the source of cuttlefish. Whereas we used cuttlefish reared for several generations in an aquaculture system, Costa *et al,* used cuttlefish taken from the Sado estuary (SW Portugal) with a mean weight of 141 ± 41 g (compared to the smaller MWW of each of our treatments control, 55:10, 51:15, 47:20 – 48.23 ± 10.08; 23.04 ± 5.03; 25.78 ± 5.39; 22.89 ± 5.16, respectively). So, cuttlefish differed in both origin and size/weight. Cuttlefish of wild origin will have a substantially different diet even when compared to our control but will also have different ways of allocating the nutrients. It is possible that the methodology was not correctly done, however, there can be other explanations.

As previously stated, carbohydrates are used as an alternate energy source, stored in the form of glycogen in the muscles (O'do*r et al.* 1984). When protein and lipid reserves are being used for somatic growth, carbohydrates fuel explosive action such as fleeing from predators and capturing prey (Castro *et al.* 1992; Sykes *et al.* 2009). These actions are not a concern for cuttlefish grown on a controlled aquaculture environment, and even though explosive action such as jetting can still occur, it does so at a presumably smaller scale. Castro *et al,* (1992) also found that during the starvation process carbohydrates accounted for 2.4% of energy supplied from the digestive gland, the remaining amount came from lipid and protein sources.

The Portuguese sub-species of *S. officinalis*, from which our own derive, has been reported to be genetically different and to display a physiological plasticity which is temperature and food dependent (Navarro *et al.* 2014). Perhaps carbohydrate storage in the digestive gland depends on the sub-species or populations of cuttlefish, hence why Costa *et al.* (2014) was able to describe glycogen clusters and the same PAS methodology in this study didn't.

Past studies such as Ferreira *et al,* (2010) showed that artificial diets had digestive glands show lower concentrations of saturates and PUFA when compared to natural diets. In this study, lipid content wasn't studied, but it's interesting to see that the presence and amount of nutrients seem to change depending on the supplied diet and, despite the lack of nutrient reserves (in our case carbohydrate and glycogen), no differences in terms of structure and integrity were found in this study.

Digestive gland index was found to be significantly different between the two pelleted diets and control ($p < 0.05$, 1-way-ANOVA, Tukey HSD). Where 55:10 and 47:20 had lower DGI's than the control, this is a result similar to that of Querol *et al*. (2013) using octopus and *Ferreira et al,* (2010), using cuttlefish, where artificial diets obtained much lower DGI as well as cannibalism (with cuttlefish). This is simply due to cuttlefish fed a control diet grew, unlike cuttlefish that were fed an artificial diet that showed no growth and even loss of weight. It would be interesting to see if this was still the case after the suggested changes on protein processing were made.

Knowing all the protein portion must be treated in a way that it does not lose any of its nutritional properties, such as lyophilization, moving forward artificial diets should use ingredients prepared via this method. Despite not going as well as we had hoped, this study did obtain immediate acceptance, an important step to formulating a successful artificial diet. Future studies should consider similar diet attractability strategies in a similar formulation. Histology wise, it is also positive to see that the digestive glands of pellet fed cuttlefish were not negatively affected by the artificial diet, it would be interesting to verify if the carbohydrate/glycogen deposits were indeed due to differences between sub-species or the environment the cuttlefish grow in.

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