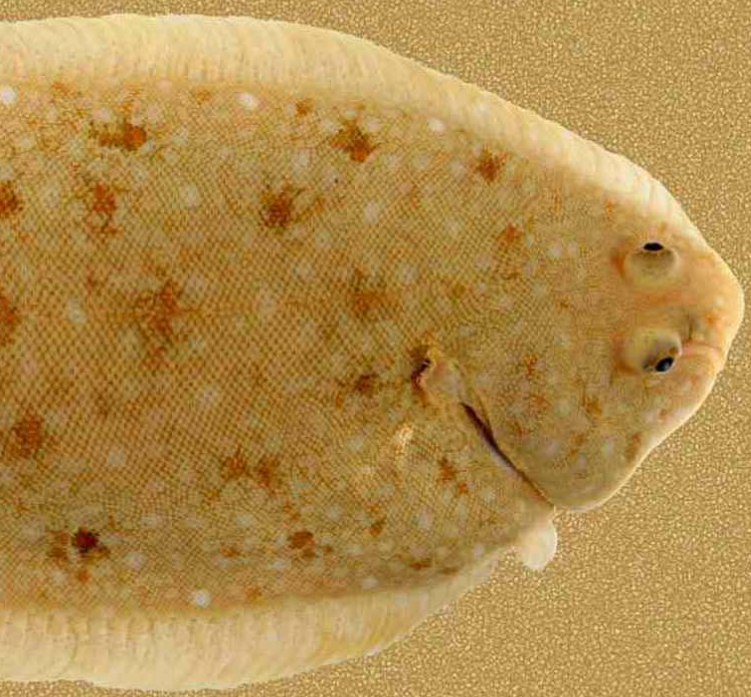
Improving growth performance of Senegalese sole postlarvae

Sofia Engrola



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Contents

Acknowledgements ---- v

Summary ---- vii

Resumo ---- xi

Chapter 1

General Introduction

1.1. Senegalese sole ---- 5

1.2. Feeding fish larvae ---- 6

1.3. Digestion in early life stages ---- 6

1.4. Sole weaning ---- 8

1.5. Early-weaning in co-feeding ---- 10

1.6. Protein utilization ---- 12

1.7. Sole quality ---- 13

1.8. This Thesis ---- 14

1.9. References ---- 14

Chapter 2

Effects of pre-weaning feeding frequency on growth, survival, and deformation of Senegalese sole, Solea senegalensis (Kaup, 1858)

2.1. Introduction ---- 29

2.2. Materials and Methods ---- 30

2.2.1. Fish and rearing ---- 30

2.2.2. Food and feeding regime ---- 31

2.2.3. Sampling ---- 31

2.2.4. Skeleton evaluation ---- 32

2.2.5. Data analysis ---- 32

2.3. Results ---- 32

2.3.1. Growth performance and survival ---- 32

2.3.2. Skeletal evaluation ---- 34

2.4. Discussion ---- 37

2.5. References ---- 38

Chapter 3

Improving weaning strategies for Senegalese sole:effects of body weight and digestive capacity

3.1. Introduction ---- 47

3.2. Materials and Methods ---- **49**
3.2.1. Larval rearing ---- 49
3.2.2. Postlarval rearing ---- 49
3.2.3. Experimental design ---- 50
3.2.4. Food and feeding regime ---- 52
3.2.5. Analytical methods ---- 53
3.2.6. Data analysis ---- 53

3.3. Results ---- **54**
3.3.1. Experiment 1 - Weaning strategies ---- 54
3.3.2. Experiment 2 - Effect of weight on digestive larval capacity ---- 56
3.3.3. Experiment 3 - Sole postlarvae early weaning ---- 59

3.4. Discussion ---- **62**
3.4.1. Does feeding regime affect digestive capacity in sole? ---- 62
3.4.2. Does weaning regime affect growth dispersion in sole? ---- 63
3.4.3. Is co-feeding important for weaning sole? ---- 63
3.4.4. Is early weaning possible in sole? ---- 64

3.5. Acknowledgements ---- **65**

3.6. References ---- **65**

Chapter 4

Co-feeding in Senegalese sole larvae with inert diet from mouth opening promotes growth at weaning ----

4.1. Introduction ---- **73**

4.2. Materials and Methods ---- **75**
4.2.1. Experimental design ---- 75
4.2.2. Food and feeding regime ---- 79
4.2.3. Analytical methods ---- 79
4.2.4. Data analysis ---- 81

4.3. Results ---- **83**
4.3.1. Pelagic phase ---- 83
4.3.2. Benthic phase ---- 83

4.4. Discussion ---- **91**
4.4.1. Co-feeding with inert diet at mouth opening promotes better growth in sole at weaning ---- 91
4.4.2. Maturation of the digestive tract was positively affected by inert diet ---- 93
4.4.3. Co-feeding regime did not impair the normal development of skeletal structures ---- 93
4.4.4. Co-feeding with inert diet from mouth opening, promotes better quality sole ---- 94

4.5. Acknowledgements ---- **95**

4.6. References ---- **95**

Chapter 5

Co-feeding of inert diet from mouth opening does not impair protein utilization by Senegalese sole larvae

5.1. Introduction ---- **107**

5.2. Materials and Methods ---- **109**
5.2.1. Larval rearing ---- 109
5.2.2. Protein utilization trials ---- 111
5.2.3. Analytical methods ---- 113

5.3. Results ---- **114**
5.3.1. Feed intake ---- 114
5.3.2. Protein utilization ---- 115

5.4. Discussion ---- **119**

5.5. Acknowledgements ---- **122**

5.6. References ---- **122**

Chapter 6

Senegalese sole larvae growth and protein utilization may be depressed when co-fed with inert diet depending on level of Artemia replacement

6.1. Introduction ---- **131**

6.2. Materials and Methods ---- **133**
6.2.1. Larval rearing ---- 133
6.2.2. Experimental design and sampling ---- 134
6.2.3. Protein utilization trial ---- 136
6.2.4. Analytical measurements ---- 136
6.2.5. Data analysis ---- 137

6.3. Results ---- **138**
6.3.1. Growth ---- 138
6.3.2. Feed intake ---- 139
6.3.3. Protein metabolism ---- 141

6.4. Discussion ---- **143**

6.5. Acknowledgements ---- **146**

6.6. References ---- **146**

Chapter 7

General Discussion

7.1. Feeding frequency during pre-weaning period affects weaning growth performance and survival ---- **155**

7.2. Weaning success depends greatly on sole weight at start weaning ---- **155**

7.3. Co-feeding with inert diet from mouth opening promotes better growth in sole at weaning ---- **157**

7.4. Maturation of sole digestive tract is promoted or impaired depending of feeding regime ---------- **158**

7.5. Feeding practices might have a positive impact on weaned sole quality ---------- **159**

7.6. *Artemia* intake is affected by feeding regime ---------- **160**

7.7. Sole digestibility is depressed during metamorphosis climax ---------- **161**

7.8. Sole larvae protein utilization may be depressed when co-fed with inert diet depending on level of *Artemia* replacement ---------- **162**

7.9. Final conclusions ---------- **163**

7.10. Further research ---------- **163**

7.11. References ---------- **164**

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Summary

Senegalese sole (*Solea senegalensis*) is a species of high commercial value whose wild catches are declining. Since the late 1990s, the high interest in culture of the species has led to major achievements in larval nutrition and rearing techniques, despite weaning success still being highly variable.

The onset of exogenous feeding is a critical moment in fish larvae life. Marine fish larvae have small body size and high metabolic demands due to high growth rates. At first feeding, the digestive tract of Senegalese sole, as many other fish species, develops from tubular to coiled and with several functional sections, namely the buccal-pharyngeal cavity, the esophagus, the immature stomach and the anterior and posterior intestine. Like in other marine fish, sole lacks a functional stomach at first feeding implying a high dependence from the pancreatic enzymes, like trypsin, lipase and amylase, for digestion.

In order to grow, larvae should eat and be able to digest the feed. Live preys, such as rotifers and *Artemia*, are normally offered to larvae in marine hatcheries at first-feeding. However the nutrient composition of these preys is inadequate to sustain optimal growth of fish larvae at later stages. Therefore, the importance of meeting the nutritional requirements of the larvae with a balanced feed in earlier phases is essential. Otherwise growth, food conversion efficiency, and even survival may be depressed. The development of an inert diet that is well ingested, digested and assimilated by larvae at mouth opening, has long been an objective of fish larvae researchers.

Early work, suggested that lower growth in fish larvae fed inert diets was related to low acceptance and attractiveness of inert diets, combined with poor ingestion, digestion and assimilation. It is usually recommended that the choice of the feeding strategy to adopt at weaning should be based on the postlarvae weight as it is a better indicator of the developmental stage and physiological status of the fish.

The aim of this Thesis is to provide a better insight into Senegalese sole growth performance during early life stages. It should contribute to the understanding of how sole growth is affected by different feeding practices and to develop appropriate feeding strategies in order to optimise growth and quality.

Since it is generally believed that a high feeding frequency maximizes growth in fish juveniles and larvae, **Chapter 2** tested two feeding frequencies with the same quantity of *Artemia* during a 13-day period just before weaning. The objective was to assess the impact of feeding frequency during the pre-weaning period on weaning success and postlarvae quality in Senegalese sole. The pre-weaning feeding frequency affected

weaning performance in sole. Semi-continuously (pulse) feeding, produced fewer but larger fish after weaning while, feeding twice daily lead to smaller fish with a higher survival rate.

Chapter 3 evaluated how weaning performance is affected by early introduction of inert diets and duration of the co-feeding period. Survival, growth and digestive enzymes activity were used as criteria. It was demonstrated that it is possible to wean sole postlarvae with two different feeding strategies, sudden weaning and *Artemia* co-feeding. However, the choice of the feeding strategy to adopt should be based on the postlarvae weight.

Given that in most marine species inert diets fed alone have a poor ability to sustain fish larvae growth and development, and the nutritional composition of live feeds is sub-optimal, **Chapter 4** evaluated if *Artemia* co-feeding with inert diet from mouth opening would affect growth performance and juvenile quality. The results demonstrated that offering inert diet to sole at mouth opening in a co-feeding regime promotes growth and better quality juveniles. Co-fed sole were larger and had a better tail condition at the end of the weaning period.

In **Chapters 5** and **6** the question of how sole larvae cope at the metabolic level with *Artemia* replacement, and how growth performance is affected was studied. **Chapter 5** investigated how different feeding regimes, live feed alone or co-fed with inert diet, influence protein utilization in Senegalese sole larvae using feed intake, protein digestibility, retention and catabolism as criteria. Co-fed sole presented higher feed intake and higher relative retention, catabolism and evacuation at the end of the study. It was also observed that digestibility was lower in co-fed sole during metamorphosis, and retention efficiency remains almost constant during early development.

As digestibility and protein retention are key issues in defining larval growth performance as well as survival rate, **Chapter 6** evaluated the effects of two levels of *Artemia* replacement by an inert diet on Senegalese sole growth performance, protein digestibility and protein retention efficiency. Sole growth decreased with increasing *Artemia* replacement in the feeding regime. Sole feed intake was relatively constant during ontogeny, increasing only in sole fed with high *Artemia* replacement after metamorphosis is completed. High *Artemia* replacement sole presented lowest protein digestibility and retention efficiency during metamorphosis climax. This suggests that sole growth performance is affected by protein utilization mostly during metamorphosis climax.

In **Chapter 7** different feeding strategies and regimes are discussed in relation to growth performance, digestion, protein utilization and sole quality. The present Thesis shows that it is possible to wean sole postlarvae with two different feeding strategies, sudden weaning and *Artemia* co-feeding. The choice of the feeding strategy to adopt should be based on the postlarvae weight. Nevertheless, in smaller postlarvae (around 2 mg DW) co-feeding strategy enhances digestive maturation and consequently promotes growth. This Thesis also shows that it is advantageous to offer a low proportion of inert diet to sole at mouth opening in a co-feeding regime, resulting in the production of larger and better quality postlarvae at later development stages. These results are explained by a high digestive capacity throughout development, with a noticeable reduction during metamorphosis climax, while protein retention efficiency remains almost constant during early development. However, a co-feeding regime with a high proportion of *Artemia* replacement at mouth opening reduces sole performance, with a lower protein digestibility and retention efficiency during metamorphosis climax.

Resumo

O Linguado do Senegal (*Solea senegalensis*) é uma espécie com um alto valor comercial cujas capturam têm vindo a diminuir nos últimos anos. O grande interesse na produção da espécie, desde os anos noventa, proporcionou grandes avanços na nutrição de larvas assim como na sua tecnologia de cultivo. Porém, apesar de todos os conhecimentos adquiridos, o sucesso do desmame do Linguado é ainda muito variável.

O início da alimentação exógena de uma larva de peixe é um momento crucial para o seu desenvolvimento. As larvas de peixes marinhos têm pequenas dimensões e grandes necessidade metabólicas devido às suas elevadas taxas de crescimento. Na altura da primeira alimentação, o tracto digestivo do Linguado do Senegal, assim como na maioria das larvas de peixe, é um tubo linear, embora ainda incompleto, com diversas estruturas, nomeadamente a cavidade buco-faríngea, o esófago, o estômago incipiente, o intestino anterior e o intestino posterior. Mais tarde o tracto digestivo continua a desenvolver-se até adquirir a sua estrutura adulta. Assim, como na maioria das espécies de peixes marinhos, o Linguado do Senegal não possui um estômago funcional à primeira alimentação, o que implica que possua uma grande dependência dos enzimas pancreáticos, como a tripsina, a lipase e a amilase, durante a digestão.

Para crescer, as larvas de peixe têm de comer e conseguir digerir o alimento ingerido. As larvas de peixes marinhos cultivadas em unidades de produção (maternidades) alimentam-se, assim que ocorre a abertura de boca, de presas vivas, nomeadamente de rotíferos e artémia. Porém, a composição nutricional destas presas não é a mais adequada para um óptimo desenvolvimento e crescimento larvar. Devido a este facto, a necessidade de encontrar um alimento larvar que colmate as necessidades nutricionais das larvas desde a abertura de boca, é essencial. Caso contrário, o crescimento, a conversão alimentar e mesmo a sobrevivência larvar poderão ser reduzidas. Um dos grandes objectivos da investigação em larvas de peixes marinhos tem sido o desenvolvimento de um alimento que possa ser ingerido, digerido e assimilado por larvas de peixes marinhos desde a abertura de boca.

Estudos recentes sugerem que o crescimento reduzido das larvas alimentadas com um alimento inerte está relacionado com a sua baixa aceitabilidade e atractividade, combinada com uma reduzida ingestão, digestão e assimilação. Normalmente é aconselhado que a estratégia de alimentação das larvas para o início do desmame tenha como base o seu peso e não a sua idade, pois este é considerado um melhor indicador do estado de desenvolvimento fisiológico das larvas.

O objectivo desta Tese foi o de proporcionar uma melhor percepção do potencial de crescimento do Linguado do Senegal durante o seu estado larvar. Esta deve contribuir para o conhecimento de como o crescimento do Linguado é afectado por diferentes prácticas alimentares e ajudar a desenvolver estratégias alimentares adequadas de modo a optimizar o seu crescimento e a sua qualidade.

Sendo geralmente aceite que elevadas frequências alimentares maximizam o crescimento em larvas e juvenis de peixes, no **Capítulo 2** foram testadas duas frequências alimentares, que dispensaram igual quantidade de artémia diariamente, ao longo de um período de 13 dias antes do início do desmame. O objectivo foi o de avaliar o impacto que as diferentes frequências alimentares, efectuadas antes do desmame, teriam no sucesso do mesmo, assim como na qualidade do Linguado do Senegal. Verificou-se que a frequência alimentar antes do desmame afecta o sucesso do mesmo. Uma frequência alimentar semi-contínua (por pulsos), produz menos mas maiores linguados no final do desmame, enquanto que uma frequência alimentar de duas alimentações ao dia, produziu maior número de linguados mas de menores dimensões.

No **Capítulo 3** foi avaliado como o desempenho das larvas durante o desmame é afectado pela introdução de alimento inerte e pela duração do período de co-alimentação. A sobrevivência, o crescimento e a capacidade digestiva das larvas foram utilizados como critérios de qualidade. Os resultados demonstraram que é possível realizar o desmame de Linguado com duas estratégias distintas, abrupta e em co-alimentação com alimento inerte. Porém verificou-se que a escolha da estratégia mais adequada deve ter como base o peso das larvas.

Tendo em consideração que a maioria dos peixes marinhos não crescem quando alimentados exclusivamente com alimento inerte, por este ter uma composição nutricional sub-óptima, no **Capítulo 4** avaliou-se como uma estratégia de alimentação em co-alimentação com alimento inerte, desde a abertura de boca das larvas, poderia influenciar o potencial de crescimento e a qualidade de juvenis de Linguado. Os resultados demonstraram que oferecer alimento inerte em co-alimentação com artémia a larvas de Linguado, desde a abertura de boca, promove o seu crescimento assim como a qualidade dos juvenis. Os linguados que foram co-alimentados eram maiores e tinham um melhor índice de condição de barbatana caudal no fim do desmame.

Nos **Capítulos 5** e **6** foi estudado como as larvas de Linguado ajustam o seu metabolismo proteico a uma estratégia de co-alimentação, e como esta afecta o seu crescimento. No **Capítulo 5**, procurou-se saber como diferentes regimes alimentares,

apenas presas vivas ou em co-alimentação com alimento inerte, influenciam o metabolismo proteico das larvas de Linguado. Como critérios de qualidade foram utilizados a ingestão de alimento, a digestibilidade da proteína, a eficiência de retenção da proteína e o catabolismo proteico. Os linguados co-alimentados apresentaram uma maior ingestão, assim como maior retenção relativa, catabolismo relativo e evacuação relativa. Foi também observado que a digestibilidade da proteína diminui durante a metamorfose dos linguados co-alimentados com alimento inerte, e que a eficiência de retenção proteica é praticamente constante durante o desenvolvimento larvar.

Sabendo que a digestibilidade e a retenção da proteína são factores chave na optimização do crescimento larvar assim como na sua sobrevivência, no **Capítulo 6**, foi avaliado o efeito de dois níveis distintos de substituição de artémia por alimento inerte, no regime alimentar. O seu impacto foi avaliado no crescimento, digestibilidade e eficiência de retenção da proteína. Observou-se que o crescimento das larvas diminui com o aumento da substituição de artémia por alimento inerte no regime alimentar. A ingestão de alimento foi constante durante o desenvolvimento larvar, aumentando nas larvas cujo o regime alimentar tinha maior substituição e após a metamorfose larvar estar completa. Um elevado nível de substituição de artémia por alimento inerte implicou uma redução da digestibilidade e eficiência de retenção da proteína pelas larvas durante o clímax da metamorfose. Os resultados sugerem que o potencial de crescimento das larvas é afectado pela utilização proteica, principalmente durante o clímax da metamorfose.

No **Capítulo 7** as diferentes estratégias e regimes alimentares são relacionados com o potencial de crescimento, a digestão, a utilização proteica e a qualidade do Linguado. Esta Tese demonstra que é possível realizar o desmame de Linguado adoptando duas estratégias diferentes, abrupta ou em co-alimentação. A escolha da estratégia mais adequada para cada caso deve ser apoiada no peso das larvas. Todavia, deve ser tido em consideração, que em larvas pequenas (menos de 2 mg de peso seco) um regime alimentar com presas vivas e alimento inerte fomenta a maturação do sistema digestivo e consequentemente um melhor crescimento. Esta Tese demonstra que alimentar as larvas desde a abertura de boca em regime de co-alimentação com alimento inerte promove um melhor crescimento assim como uma elevada qualidade das larvas em estadios tardios. Estas observações são fundamentadas na elevada capacidade digestiva das larvas durante o seu desenvolvimento ontogenético, apesar da redução durante o clímax da metamorfose, e por uma constante elevada eficiência de retenção da proteína durante o seu desenvolvimento.

Contudo, um regime alimentar de co-alimentação com alimento inerte em elevadas proporções reduz o potencial de crescimento das larvas, pois estas apresentam uma redução na digestibilidade e eficiência de retenção da proteína durante o clímax da metamorfose.

Chapter 1

General Introduction

1.1. Senegalese sole

Senegalese sole (*Solea senegalensis*) is a species of high commercial value whose wild catches are declining (Imsland et al., 2003). Since the late 1990s, the high interest in culture of the species has led to major achievements in larval nutrition and rearing techniques, despite weaning success still being highly variable (Conceição et al., 2007b).

Senegalese sole feeds basically on benthonic invertebrate, such as larvae from polychaets and bivalve mollusks that inhabits sandy or muddy bottoms of the continental shelf from the Gulf of Biscay to the coasts of Senegal (Quéro, 1984; Whitehead et al., 1986). Sole is a gonochoric species, and females mature at age 3+ with a total length of 32 cm (Dinis, 1986). Being an asynchronous fish, the same female may intercalate spawn with resting periods several times during the spawning season (Rodríguez, 1984; Dinis, 1986). Sole spawning season occur in nature from March to July, with most of the batches normally happening during May (Dinis, 1986). Eggs for farming this species are normally obtained from natural spawnings of wild broodstocks kept in captivity (Dinis et al., 1999), and are plantonic, golden color, and with a diameter between 0.87-1.00 mm (Lagardère, 1979; Rodríguez, 1984; Dinis, 1986). In contrast to most of marine fish eggs, sole eggs have a high number of small oil droplets mostly in the equatorial region with size between 0.02-0.05 mm (Lagardère, 1979). Larvae hatch after 24-48 h depending on the water temperature that might vary between 16-18ºC. Larvae hatch with an average size of 2.4±0.1 mm total length, but variability during the spawning season is high (Dinis et al., 1999). At first feeding, 2 days after hatching (DAH), larvae presents 3.0-3.3 mm of total length (Dinis et al., 1999) and a mouth gap of 350 μm (Parra and Yúfera, 2001). Thus, sole can eat directly *Artemia* nauplii as first prey (Magalhães and Dinis, 1996), though usually they are fed with rotifers for a few days (Dinis et al., 1999), in order to allow for HUFA enrichment.

Sole postlarvae differ from most other teleost fish species, even flatfish, because settled sole do not feed energetically in small bursts, preferring to graze continuously on *Artemia* or dry feed on the bottom of the tanks (Dinis et al., 2000). This feeding behaviour poses additional challenges in weaning sole species onto inert diets which had traditionally been a bottleneck in sole farming (Howell, 1997; Dinis et al., 1999). Nonetheless, weaned sole presents high growth during ongrowing in earthponds in policulture with gilthead seabream achieving a weight of 450g within one year (Dinis et al., 1999).

1.2. Feeding fish larvae

The onset of exogenous feeding is a critical moment in fish larvae life (Yúfera and Darias, 2007b). Marine fish larvae have small body size and high metabolic demands due to high growth rates (Houde, 1997). High growth rate is of vital importance for fish larvae as predation susceptibility decreases with increasing fish body size (Blaxter, 1988).

It is generally believed that a high feeding frequency maximizes growth in fish juveniles and larvae (e.g., Haylor, 1993), especially in pre-weaning stages when postlarvae are usually fed exclusively with *Artemia* metanauplii (Houde, 1989; Conceição, 1997). Growth rates vary greatly, and in many cases appear to be limited by food availability, as in Arctic charr (*Salvelinus alpinus*) (Miglavs and Jobling, 1989) and Japanese flounder (*Paralichthys olivaceus*) (Lee et al., 2000). Commercial hatcheries that produce marine fish generally supply food to the postlarvae several times during the day or even continuously. This procedure may enhance fish growth rates and decrease size variation, helping to shorten the time required to reach market size. More frequent meals reduce size dispersion in several species such as whitefish (*Coregonus lavaretus*) (Koskela et al., 1997) and greenback flounder (*Rhombosolea tapirina*) (Chen and Purser, 2001).

Fish larvae are known to present high growth rates when compared to older fish (Houde, 1989; Conceição et al., 1998a). In order to grow, larvae should eat and be able to digest the feed. Live preys, such as rotifers and *Artemia*, are normally offered to larvae in marine hatcheries at first-feeding. However the nutrient composition of these preys is inadequate to sustain growth of fish larvae at later stages (Planas and Cunha, 1999; Conceição et al., 2003). Therefore, the importance of meeting the nutritional requirements of the larvae with a balanced feed in earlier phases is essential because otherwise the growth, food conversion efficiency (Conceição et al., 2003), and even survival (Aragão et al., 2007) may be suppressed. As a result, the development of an inert diet that is well ingested, digested and assimilated by larvae at mouth opening, has long been an objective of fish larvae researchers.

1.3. Digestion in early life stages

At first feeding Senegalese sole, as many other fish species, developed the digestive tract from tubular to coiled and with several functional sections, namely the buccal-pharyngeal cavity, the esophagus, the immature stomach and the anterior and posterior intestine. Like

in other marine fish, sole lacks of a functional stomach at first feeding implying a high dependence from the pancreatic enzymes, like trypsin, lipase and amylase, for digestion (Ribeiro et al., 1999a).

Fish larval digestive capacity increases with age as demonstrated through tracer studies (Rust, 1995), and trials with graded levels of protein hydrolisates (Day et al., 1997). As sole is a flatfish, the spatial organization of the digestive system changes during metamorphosis (Ribeiro et al., 1999a). Sole metamorphosis starts at 10 days after hatching (DAH) reaching its climax around 15-16 DAH at 18-19ºC (Ribeiro et al., 1999a; Fernández-Díaz et al., 2001). The onset and the duration of sole metamorphosis are highly influenced by feed availability and type (Fernández-Díaz et al., 2001). Those authors fed sole from mouth opening with microcapsules or live feed alone, concluding that sole fed on live feed had a faster growth and the beginning of metamorphosis was earlier than microcapsules-fed sole.

Acid digestion is achieved with the formation of gastric glands, that according to (Ribeiro et al., 1999b) is around 27 DAH. However a true acid digestion pH < 4 is never reached in sole, that presents always values above 6.0 (Yúfera and Darias, 2007a) even in adult stage. De Groot (1971) observed that sole has one of the smallest stomachs and the longest intestines of flatfish species. Together this might imply different dietary requirements and rearing practices for sole (Yúfera and Darias, 2007a).

Senegalese sole presents digestive enzyme activity before onset of first feeding indicating that larvae are able to digest exogenous feed (Ribeiro et al., 1999b). Still, most of fish larva despite having a digestive enzyme activity it is not enough for a complete feed hydrolysis (Govoni et al., 1986). As earlier suggested by Ribeiro et al. (1999b) the higher trypsin and amylases activities observed in sole, when compared to European seabass (*Dicentrarchus labrax*) or gilthead seabream (*Sparus aurata*), are probably due to the higher rearing temperatures. The differences of enzymatic capacity usually detected at earlier development stages in fish larvae are temperature dependent (Rønnestad and Conceição, 2005). Furthermore, the introduction of inert diet alone from first feeding is unable to sustain larval growth in Senegalese sole (Cañavate and Fernández-Díaz, 1999). As suggested for European seabass, offering inert diet alone to fish larvae might delay the onset of pancreas secretory functions (Cahu and Zambonino-Infante, 1994). Therefore, as Senegalese sole has fully operational pancreatic mechanism around 21 DAH, Ribeiro et al. (1999b) suggested that weaning should be performed after this age.

Larval digestive tract maturation might be stimulated or impaired, depending on feeding regime, and a sub-optimal diet may cause mortalities (Cahu and Zambonino Infante, 2001). In fact, Fernández-Díaz et al. (2006) observed that Senegalese sole exclusively fed with microencapsulated diets had altered hepatic and gastrointestinal structures when compared to live feed sole. Furthermore, knowledge on the development of the digestive tract maturation is important to assess the larval nutrition needs and to develop adequate larval feeding protocols (Cahu and Zambonino Infante, 2001; Kolkovski, 2001; Koven et al., 2001; Zambonino Infante and Cahu, 2007). On the other hand, European seabass larvae were shown to adapt the digestive enzyme profile to diet composition (Zambonino-Infante and Cahu, 1994). The shift in the diet composition has also a direct impact on the digestive enzyme profiles of Atlantic cod (*Gadus morhua*) (Wold et al., 2007), sharpsnout seabream (*Diplodus puntazzo*) (Suzer et al., 2007) and white bream (*Diplodus sargus*) (Cara et al., 2003). The use of microalgae and inert diet from mouth opening increased the enzyme activity of trypsin and aminopeptidase in red drum (*Sciaenops ocellatus*) (Lazo et al., 2000). Maturation of the digestive tract of dorado (*Salminus brasiliensis*) was positively affected by a co-feeding regime with inert diet (Vega-Orellana et al., 2006).

1.4. Sole weaning

In the early 1980s, Métailler et al. (1983) observed that Dover sole (*Solea solea*) fed with a betaine and glycine supplemented inert diet could be weaned at 35 days after hatching (DAH) (110-130 mg wet weight) with survival rates higher than 65% (Table 1.1). The inclusion of protein hydrolysates in the inert diets during weaning was also positively correlated with weaning survival (Day et al., 1997).

Survival rates as high as 80% have been reported in Dover sole (Day et al., 1997; Palazzi et al., 2006). In contrast, weaning results with Senegalese sole, the species of interest for farming in Southern Europe, are less consistent. Some of the technologies developed for Dover sole were successfully applied to Senegalese sole. Dinis (1992) was able to wean *S. senegalensis* 31 days after hatching (DAH) with survival of 35.8% with a 9 days co-feeding period using an inert diet including attractants (Table 1.1). A co-feeding strategy was proposed for Senegalese sole (Cañavate and Fernández-Díaz, 1999; Ribeiro et al., 2002). Those authors were able to wean 30-40 DAH sole with survival up to 58%. The co-feeding period, when inert diet is co-fed with *Artemia* metanauplii, may change from few days to several weeks, and the amounts of *Artemia* metanauplii and

inert diet change gradually in an inverse manner. However, it should be performed carefully because mortality might be selective towards smaller postlarvae (Ribeiro et al., 2002). Still, one of the major problems in sole farming is the large variability in survival and growth dispersion between batches (Dinis, 1992; Conceição et al., 2007b).

Early work, suggested that lower growth in fish larvae fed inert diets was related to low acceptance and attractiveness of inert diets, combined with poor ingestion, digestion and assimilation (Koven et al., 2001). In addition, larval size, largely as a result of the gradual maturation of the larval digestive tract, has been considered the major determinant of weaning success in marine fish larvae. It is usually recommended that the choice of the feeding strategy to adopt at weaning should be based on the postlarvae weight as it is a better indicator of the developmental stage and physiological status of the fish (Verreth, 1994; Rosenlund et al., 1997).

Therefore, weaning of marine species might be accomplished with relative success with different strategies (Table 1.1). In European seabass, weaning can be accomplished from mouth opening but at expenses of low survival (Cahu et al., 1998). On the other hand a 14 day co-feeding period produces a two-fold increase in survival (Geurden et al., 1997) (Table 1.1). In Asian seabass (*Lates calcarifer*) survival rates had a four-fold increase when start of weaning was delayed two weeks (Table 1.1).

In Atlantic halibut (*Hippoglossus hippoglossus*) a shorter or longer co-feeding period produces similar survival rates, higher than 80% (Mæland et al., 1999; Næss et al., 2001; Hamre et al., 2005). In other flatfish, survival rates are always above 80%, when fish is weaned at younger ages with a longer co-feeding period (Hart and Purser, 1996; Lee and Litvak, 1996; Geurden et al., 1997).

While live preys are a high digestibility protein source for fish larvae, other protein sources such as fish meal probably have low digestibility (Rønnestad and Conceição, 2005). In addition, purified model proteins as salmon serum or algal protein have been shown to have in fact a low digestibility by fish larvae (Rønnestad et al., 2001; Tonheim et al., 2004). Hence, as suggested by Rønnestad and Conceição (2005) the complexity of the dietary nitrogen is the key issue for an optimal larval growth performance.

Table 1.1 – A review of weaning strategies and performances for Senegalese sole and some other marine fish species.

Species	Ref.	Weaning period (days)	Start weaning (DAH)	Temperature (ºC)	RGR (%/day)	Survival (%)
Centropomus paralelus	Alves et al., 2006	10	30	25	7	99
Cynoglossus semilaevis	Chang et al., 2006	31	6	21	5	63
Dicentrarchus labrax	Geurden et al., 1997	14	39	23	5	88
	Cahu et al., 1998	0	6*	19	8	35
Gadus morhua	Baskerville-Bridges and Kling, 2000	7	17	10	7	23
	Callan et al., 2003	14	7	10	8	23
	Fletcher et al., 2007	38	25	11	10	12
Hippoglossus hippoglossus	Mæland et al., 1999	30	120mg	13	5	86
	Hamre et al., 2001	17	160mg	12	4	96
	Næss et al., 2001	7	160	12	2	96
	Kvåle, 2007	0	63 dpff	12	3	57
Melanogrammus aeglefinus	Hamlin and Kling, 2001	7	42	11	10	65
Lates calcarifer	Curnow et al., 2006b	14	20	26		65
	Curnow et al., 2006a	14	6	29		14
Pleuronectes americanus	Lee and Litvak, 1996	7	47	15	10	92
Rhombosolea tapirina	Hart and Purser, 1996	20	23	16	3	82
Scophthalmus maximus	Geurden et al., 1997	4	42	23	5	96
	Roselund et al., 1997	7	21	21	13	99
Solea senegalensis	Dinis, 1992	9	31	18		36
	Cañavate and Fernández-Díaz, 1999	7	43	20	3	39
	Ribeiro et al., 2002	39	36	20	2	58
Solea solea	Person Le Ruyet et al., 1980	45	35	18	5	40
	Gatesoupe and Luqet, 1982	5	11	19	7	49
	Métallier et al., 1983	3	35		5	85
	Day et al., 1997	0	60	15		91
	Rueda-Jasso et al., 2005	3	50	16	7	78
	Palazzi et al., 2006	24	30	18	3	79
Sparus aurata	Yúfera, et al., 1999	0	8	20	4	70
	Robin and Vicent, 2003	0	3*	20	6	26

Note that in column "Weaning period (days)" 0 day refers to sudden weaning and 3 or more days refer to duration of the co-feeding period. Growth is expressed as relative growth rate (RGR, % day), determined between beginning and end of weaning period, using the formula $(e^{(g)}-1) \times 100$ where $g = (\ln_{final}\ wt - \ln_{initial}\ wt)/(time)$ (Ricker, 1958). dpff: days post first feeding; * age at mouth opening.

1.5. Early-weaning in co-feeding

Inert diets are nutritional balanced, ready to use, and have a long shelf life. However, in most marine species compounds diets fed alone have a poor ability to sustain fish larvae growth and development (e. g. Cañavate and Fernández-Díaz, 1999; Robin and Vincent, 2003). The low performance usually observed when delivering inert diet from mouth

opening to marine fish larvae might be due to sub-optimal diet composition and the larval poor ability to modulate its digestive enzymes (Cahu and Zambonino Infante, 2001).

Thus, *Artemia* replacement regimes, i.e. a co-feeding regime with live prey and inert diet during a period prior to weaning, are a feeding strategy widely used in fish larval rearing since inert diets are easier to use and have a stable composition, while composition of live feed can vary according to culture/enrichment conditions. On the other hand, *Artemia* replacement diets can improve survival and growth performance of several marine fish larvae even in early larval stages (Holt, 1993; Rosenlund et al., 1997; Baskerville-Bridges and Kling, 2000; Alves et al., 2006) and may enhance digestive maturation as suggested by several authors (Kolkovski et al., 1993; Kolkovski et al., 1997b; Rosenlund et al., 1997; Baskerville-Bridges and Kling, 2000). This feeding strategy is also known to stimulate feeding rates in Dover sole (Knutsen, 1992) and gilthead seabream (Kolkovski et al., 1997a), and to pre-condition larvae onto inert diet (Hart and Purser, 1996; Brown et al., 1997; Callan et al., 2003; Curnow et al., 2006a; Fletcher et al., 2007).

The importance of the early feeding regimes was showed in fish species such as Asian seabass (Curnow et al., 2006b). These authors showed that depending on the combination of types of inert diet and live prey, larger larvae could be produced. In Atlantic cod, a co-feeding strategy produced a two-fold increase in larval weight, in comparison to an inert diet strategy (Fletcher et al., 2007). Alves et al. (2006) noticed that doubling the co-feeding period in fat snook (*Centropomus parallelus*) would promote a two-fold increase in larval length. In tongue sole (*Cynoglossus semilaevis*) the addition of inert diet to live feed alone promotes a two-fold increase in larval weight (Chang et al., 2006). In greenback flounder a 20 day co-feeding period promotes higher growth than a 5 day period (Hart and Purser, 1996).

Nowadays suitable larval inert diets are available for several marine fish species that can be used from mouth opening. That is the case of European seabass (Cahu and Zambonino Infante, 2001), gilthead seabream (Yúfera et al., 2000; Robin and Vincent, 2003), red seabream (*Pagrus major*) (Takeuchi, 2001) and red drum (Lazo et al., 2000). Nevertheless, in most marine fish species, a co-feeding regime during an extended period is still needed to sustain larval growth at earlier stages. Therefore, marine fish larvae can utilize compound diets from mouth opening if the diet composition takes into account the digestive specificity of fish in early stages of development (Cahu and Zambonino Infante, 2001).

1.6. Protein utilization

Larval protein metabolism and consequently growth performance can be affected by several factors: higher feed intake has lead Pacific herring (*Clupea harengus pallasi*) to higher growth rates despite having lower protein retention efficiency, due to the positive net balance between intake and retention (Boehlert and Yoklavich, 1984); African catfish (*Clarias gariepinus*) larvae increased absorption rates and retention efficiency at higher temperature and consequently grew faster than at lower temperatures (Conceição et al., 1998b); and Senegalese sole postlarvae fed with soy protein concentrate diet presented a higher amino acid catabolism but growth was not impaired probably due to a higher dietary protein intake (Aragão et al., 2003). In short, digestibility and protein retention are key issues in defining larval growth performance as well as survival rate. In fact, high digestibility has been shown to correlate with better growth and survival rate in Western Atlantic seabream (*Archosargus rhomboidalis*) larvae (Houde and Schekter, 1983).

Growth and survival are the most common and practical criteria to determine if a feeding regime is suitable or not for a given fish species. In addition, feed intake in fish larvae is in general determined by visual counting of ingested prey (Haylor, 1993; MacKenzie et al., 1999), or particles of inert diet (Yúfera et al., 1995). This makes estimation of feed intake quite time consuming and often inaccurate. Therefore, tools using tracer nutrients have been proposed to determine the impact of a feeding regime in fish larvae (see review by Conceição et al., 2007a). In recent years, new techniques improved quantification of feed intake and protein utilization. The used of alginate-based inert diet, where is possible to extract and measure chlorophyll was a step forward in the direct assessment of larvae feed intake (Kelly et al., 2000). The use of stable (e.g. ^{13}C or ^{15}N), or radio tracer labelling molecules (e.g. ^{14}C or ^{35}S), has improved and simplified the quantification of feed intake and nutrient utilization (e.g. Boehlert and Yoklavich, 1984; Rust, 1995; Conceição et al., 2001; Kvåle et al., 2006; Gamboa-Delgado et al., 2008; Jomori et al., 2008). Using a methodology based on radiolabelled *Artemia* protein (Morais et al., 2004a) it is possible to determine feed intake, and how the ingested protein is digested, retained and catabolized by fish. The use of such a methodology in distinct larval phases allows an understanding of the larvae digestive development and how larvae are copping at the metabolic level. Hence, Morais et al. (2004b) observed that Senegalese sole larvae have high *Artemia* protein digestibility (73-83% of intake), between 12 and 35 DAH. This indicates that sole have a high digestive capacity for digesting live preys since young ages.

1.7. Sole quality

Skeletal abnormalities are a severe problem in aquaculture production as they affect fish appearance, thus reducing market value (Koumoundouros et al., 1997). Reared Senegalese sole larvae were reported to have a deformity rate of 44% (Gavaia et al., 2002). Most of deformities are in the caudal vertebrae, what might indicate an impact of rearing conditions. Nutritional factors can cause alterations in the normal development of the skeleton leading to structural abnormalities in adult fish (Hilomen-Garcia, 1997; Cahu et al., 2003; Lall and Lewis-McCrea, 2007). Some advances have been made in terms of individual nutrient requirements for normal skeletal formation. Safe levels of vitamin A were investigated for normal skeletogenesis in Japanese flounder (Dedi et al., 1995; Takeuchi et al., 1995) and to the pigmentation success in turbot (*Scophthalmus maximus*) (Estevez and Kanazawa, 1995). The minimum levels of vitamin K that promotes a normal skeletal growth and mineralization has been recently determined for haddock (*Melanogrammus aeglefinus*) (Roy and Lall, 2007). The incorporation of vitamin C and highly unsaturated fatty acids (HUFA) in *Artemia* was shown to diminish opercular deformities in milkfish (*Chanos chanos*) (Gapasin et al., 1998). The supplementation of phosphorus (Uyan et al., 2007), and the substitution of native fish meal proteins by fish meal hydrolysate (Zambonino Infante et al., 1997) led to a decrease of deformities in fish.

Malpigmentation is a common problem in flatfish hatcheries that decreases the market value of the fish (Bolker and Hill, 2000). Senegalese sole is normally beige in the ocular side of the body. In commercial hatcheries, during early development stages sole sometimes develop a dark coloration (Ruane et al., 2005). In turbot malpigmentation was related to lower growth and high mortalities (Munro et al., 1994). The key factor for malpigmentation appears to be larval nutrition and usually offering diets supplemented with fatty acids and vitamin A reduces malpigmentation rates (Bolker and Hill, 2000). In Senegalese sole an increase in ratio of arachidonic acid (ARA)/eicosapentaenoic (EPA) and ARA/docosahexaenoic (DHA) produces a higher number of fish with malpigmentation (Villalta et al., 2005).

Fin erosion has been identified as a quality indicator for sole, when production started in European hatcheries. Fin erosion problems are commonly reported in Atlantic salmon (*Salmo salar*) (Noble et al., 2008) and Atlantic cod (Hatlen et al., 2006), and normally imply welfare problems and have an economic impact. In Senegalese sole rearing is a common problem, usually caused by direct contact with the bottom of the tanks. Generally, is diminished if substrate is added and if tank hygiene is optimal.

1.8. This Thesis

The aim of this Thesis is to provide a better knowledge of Senegalese sole growth performance during early life stages. It should contribute to the understanding of how growth is affected by different feeding practices and to develop appropriate feeding strategies in order to optimise growth, survival and quality of sole postlarvae.

A general overview of the main factors affecting fish larvae rearing is done in **Chapter 1**. In **Chapter 2** the effect of different feeding frequencies before weaning are related to quality and weaning success in Senegalese sole. In **Chapter 3** different weaning strategies and initial sole weights were tested and evaluated in terms of growth and digestive enzyme profiles. The growth performance of sole larvae co-fed from mouth opening with inert diet was analyzed in **Chapter 4**, where enzymatic activity and larval quality was also studied. **Chapter 5** and **6** describes how sole larvae cope at the metabolic level with *Artemia* replacement, and how growth performance and protein utilization are affected by a co-feeding regime. Finally, in **Chapter 7** different feeding strategies and regimes are discussed in relation to growth performance, digestion, protein utilization and sole quality.

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Chapter 2

Effects of pre-weaning feeding frequency on growth, survival, and deformation of Senegalese sole, *Solea senegalensis* (Kaup, 1858)

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Abstract

Despite much interest in the production of Senegalese sole (*Solea senegalensis*) in southern Europe, weaning of this species onto artificial diets is problematic and varying results are obtained. The aim of this study was to test two feeding frequencies during a 13-day pre-weaning period and assess their impact on the growth and survival of Senegalese sole. Postlarvae were fed *Artemia* metanauplii with a peristaltic pump every hour for 12 hours per day or twice daily (morning and late afternoon). Both groups were suddenly weaned onto a commercial diet for an additional 30 days. At the end of the experiment, the relative growth rate and final dry weight were significantly higher and the survival significantly lower in the 12-hour treatment than in the twice-daily treatment. The feeding frequency had no effect on condition factor. The incidence of deformities was about 80% in both treatments.

Keywords: Senegalese sole, *Solea senegalensis*, Growth, Feeding frequency, Malformations.

2.1. Introduction

It is generally believed that a high feeding frequency maximizes growth in fish juveniles and larvae (e.g., Haylor, 1993), especially in pre-weaning stages when postlarvae are usually fed with *Artemia* metanauplii exclusively (Houde, 1989, Conceição et al., 1997). Growth rates vary greatly, and in many cases appear to be limited by food availability, as in Arctic charr (Miglavs and Jobling, 1989) and Japanese flounder (Lee et al., 2000). Animals compete intraspecifically for resources and it is generally assumed that more competitive individuals with high feeding ranks have higher growth rates (Damsgård et al., 1997). Commercial hatcheries that produce marine fish generally supply food to the postlarvae several times during the day or even continuously. This procedure may enhance fish growth rates and decrease size variation, helping to shorten the time

required to reach market size. More frequent meals reduce size dispersion in several species such as whitefish, *Coregonus lavaretus* (Koskela et al., 1997) and greenback flounder, *Rhombosolea tapirina* (Chen and Purser, 2001).

Skeletal abnormalities are a serious economic problem in aquaculture as they affect fish appearance and survival, reducing market value (Koumoundouros et al., 1997a). Nutritional factors have been indicated as possible causes for alterations in the normal development of skeletal structures that lead to abnormalities in adult fish (Takeuchi et al., 1995; Gapasin and Duray, 2001).

Studies of husbandry techniques in flatfish show that feeding amounts, frequencies, and durations significantly impact growth and homogeneity (Carter et al., 1996; Shelverton and Carter, 1998; Verbeeten et al., 1999; Chen and Purser, 2001). Senegalese sole (*Solea senegalensis*) postlarvae differ from other species not only because they settle on tank bottoms well before weaning but also because they display peculiar feeding behavior; settled Senegalese sole do not react readily to supplied food and prefer grazing on *Artemia* or dry diets from tank bottoms (Dinis et al., 2000). Despite the high potential of Senegalese sole as an aquaculture species, only few studies have been done in relation to larvae rearing conditions (Esteban et al., 1995; Dinis et al., 1999) and weaning periods (Marin-Magan et al., 1995; Cañavate and Fernández-Díaz, 1999).

The aim of this study was to test two feeding frequencies with the same quantity of the same food during a 13-day pre-weaning period and assess the impact of feeding frequency on weaning success and postlarvae quality in Senegalese sole, *S. senegalensis*.

2.2. Materials and Methods

2.2.1. Fish and rearing

Senegalese sole larvae were reared until the beginning of the experimental period as described by Dinis et al. (1999). Newly hatched larvae were reared in a 200 L cylindro-conical tank in a closed recirculating system at a density of 100 larvae per liter. Larvae were fed rotifers (*Brachionus rotundiformis*) enriched with microalgae, *Isochrysis galbana* and *Tetraselmis chuii*, three days after hatching. At five days, *Artemia* sp. nauplii (Be 480 strain, INVE Aquaculture) were added to the diet. Rotifers were gradually reduced until day 8. After 10 days, *Artemia* metanauplii enriched with *I. galbana* and *T. chuii* were provided.

At 20 days, postlarvae were transferred to six 50 L white flat-bottom plastic tanks (surface area 0.5 m^2) in a closed recirculating system of 3000 L at 3000 individuals/m^2.

Artemia metanauplii (RH strain, INVE Aquaculture) enriched with Super SELCO (INVE Aquaculture) were supplied to the postlarvae.

Environmental parameters were measured daily. Temperature and salinity averaged 20.9±0.6°C and 32.0±1.0‰, respectively. Dissolved oxygen was around 6.45±0.7 mg/L. A photoperiod of 12h light:12h dark was produced by overhead fluorescent tubes. Tanks were cleaned and dead fish were removed and counted daily.

2.2.2. Food and feeding regime

The pre-weaning period started 26 days after hatching to avoid possible stress effects due to transfer from the cylindro-conical tanks to the flat-bottom tanks and ensure that fish were eating normally. Treatments were randomly assigned to the tanks, with three replicate tanks per treatment. In the pulse treatment, postlarvae were fed *Artemia* metanauplii by peristaltic pump every hour for twelve hours per day. In the second treatment, two meals were provided (morning and late afternoon). Both treatments received the same daily ration per fish. Between days 30 and 37, the metanauplii supply was gradually changed from live to frozen until, on days 37 and 38, postlarvae were fed frozen metanauplii exclusively. The postlarvae were weaned on day 40, after a one-day fast, and given AgloNorse no. 2 (0.6-1.0 mm) until day 63 and AgloNorse no. 3 (1.0-1.6 mm) afterward. The experiment ended on day 69. In both treatments, the inert diet was supplied by automatic feeders every hour for 18 hours a day. Throughout the experiment, it was attempted to feed close to satiation, based on the predicted maximum growth. Daily adjustments were based on visual inspection (to avoid excess uneaten food).

2.2.3. Sampling

At the end of the pre-weaning period, samples of twenty 40-day postlarvae were taken from each tank (60 postlarvae per treatment). At the end of the experiment, thirty 69-day postlarvae were sampled from each tank (90 postlarvae per treatment). The postlarvae were measured (total length) and kept frozen at -80ºC for dry weight determination. On day 69, samples taken for skeleton evaluation were fixed overnight in 4% formaldehyde buffered to pH 7.4 with PBS. Fish were counted at the end of the weaning period to determine survival.

2.2.4. Skeleton evaluation

Specimens for skeleton evaluation were submitted to a double staining procedure using Alcian Blue 8GX to stain cartilage and Alizarin red S to stain bone, according to the procedure described in Gavaia et al. (2000). Specimens were preserved in glycerol until observation. Structural development was determined on the axial skeleton based on Gavaia et al. (2002) and eye migration was determined.

2.2.5. Data analysis

Relative growth rate (RGR, %/day) at the end of the experiment was calculated by the formula: $(e^{(g)}-1) \times 100$ where $g = (\ln_{final}$ wt - $\ln_{initial}$ wt$)/($time$)$ and e = Napier's constant (2.71). The coefficient of variation (CV) was determined as: (treatment standard deviation/treatment mean) x 100 to determine inter-individual weight and length variation among fish in the same treatment at the end of both periods. Food conversion ratio (FCR) was determined as: feed supplied to the tank/(final wt - initial wt) x number of fish per tank, where the proportion of food wasted (e.g., flushed out or dissolved) was considered negligible. The condition factor (K) was calculated as: (fish wt/total length^3) x 100. Data are presented as arithmetic means with standard deviations. One-way ANOVA was used to test differences between treatments. Differences were considered significant when $p<0.05$. When significant differences were found, Tukey's Honest Significant Difference (HSD) test was used to determine if the treatments differed significantly at $p<0.05$. All statistical analyses were carried out using the Statistica 5.1 and SigmaPlot packages software.

2.3. Results

2.3.1. Growth performance and survival

The mean dry weight at the end of the pre-weaning period (40 days) did not significantly differ between treatments and was 13.7±4.0 mg in pulse-fed fish and 13.9±3.0 mg in fish fed twice daily. At the end of the experiment (69 days), however, postlarvae from the pulse feeding regime were significantly larger (76.2±2.1 mg) than those fed twice daily (64.1±7.7 mg).

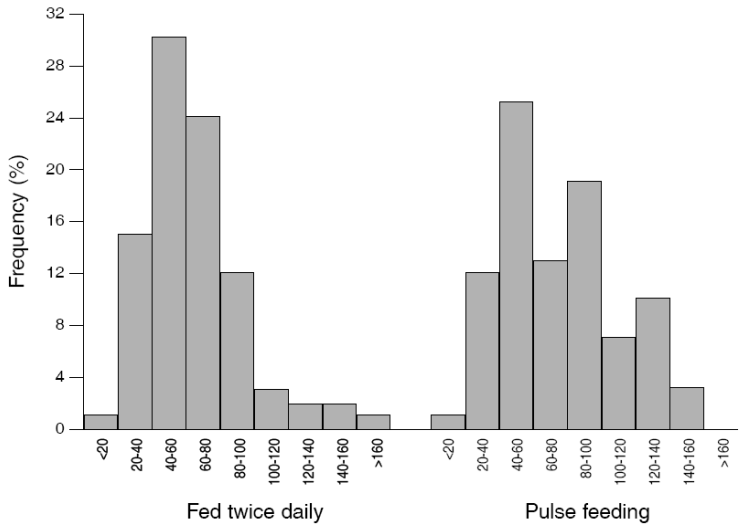

Fig. 2.1 - Distribution of fish weight (n = 90) by treatment at the end of the experiment (69 days).

Table 2.1 – Survival, coefficient of variation (CV) for weight and length, condition factor (K), food conversion ratio (FCR), and relative growth rate (RGR) of Senegalese sole at weaning period (40 days) and at the end of the experiment (69 days).

	Pulse feeding	Fed twice daily
40 days		
Survival (%)	**100**	**100**
CV (Dry Weight**)**	**29.4±0.8**[a]	**21.8±3.2**[b]
CV (Length**)**	**7.8±0.4**	**9.4±3.8**
K	**1.0±0.1**	**0.9±0.1**
69 days		
Survival (%)	**44.3±15.2**[a]	**69.8±14.8**[b]
FCR	**1.3±0.2**[a]	**1.7±0.2**[b]
RGR (%)	**6.3±0.7**[a]	**5.6±0.7**[b]
CV (Dry Weight)	**45.0±6.7**	**44.7±8.4**
CV (Length**)**	**15.1±2.2**	**16.0±3.6**
K	**1.3±0.0**	**1.3±0.2**

Values with different superscript significantly differ ($p<0.05$).

The twice daily feeding frequency significantly increased survival and the food conversion ratio at 69 days, while there was no significant difference between treatments in condition factor. The coefficient of variation of weight in the fish fed twice a day was significantly lower than for the pulse-fed fish at 40 days, but there was no significant difference at 69 days (Table 2.1).

These results are visible in the distribution of fish weights (Fig. 2.1). There was no significant difference in the coefficient of variation of length at either 40 or 69 days. Accordingly, the relative growth rate at 69 days was significantly higher in the pulse feeding treatment than in the twice-daily treatment (Table 2.1).

2.3.2. Skeletal evaluation

The most common deformities on caudal and pleural vertebra were the fusion and compression of vertebral centra, affecting adjacent neural arches and spines, however parapophysis was rare. The preural vertebrae 1-4 that contribute to the caudal fin internal skeleton were commonly fused or deformed, in some cases with an absence of neural or hemal arches. In the hypuralia, only hypurals 1-5 and the parhypural were affected.

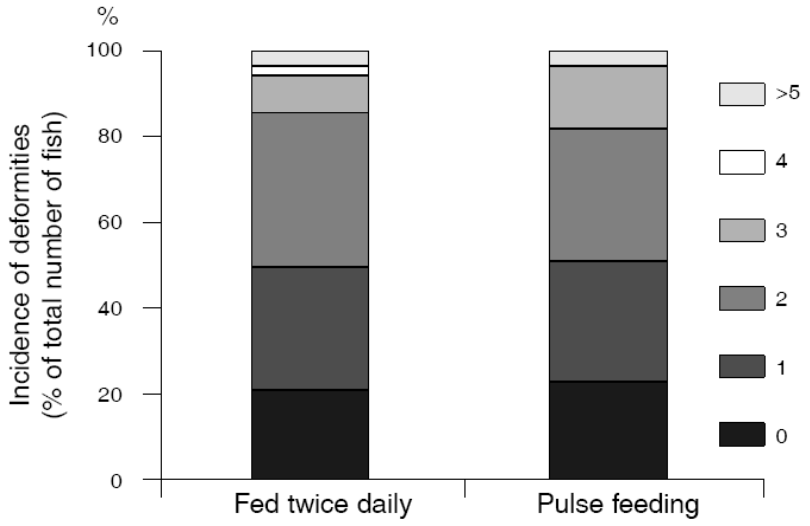

Fig. 2.2 - Distribution of the number of deformities per fish at the end of the experiment (69 days).

Abnormality in the hypuralia usually involved fusion of hypurals 1-2 or hypurals 3-5 and, sometimes, absence of any of these structures. The caudal, dorsal, and anal fins were rarely affected, with only minor malformations of pterigophores and rays that were

either shortened or abnormally bent. Cases of abnormal eye migration (uncompleted or no migration) were rare. There was no significant difference between treatments in number of skeletal abnormalities (Fig. 2.2), however the number of fish considered normal was low (~20%) in both treatments. In both treatments, some 60% of the fish had one or two deformities while fish with more than two deformities were less common.

Analysis of double stained specimens showed that the most affected structures were the caudal vertebra, adjacent arches, and spines. There were no significant differences in abnormalities between treatments, with the exception of the neural and hemal spines (Fig. 2.3). The number of deformed pleural vertebrae 1-4 was comparable to the neighboring area, presenting fusions between vertebral centra and malformations in the adjacent arches and spines.

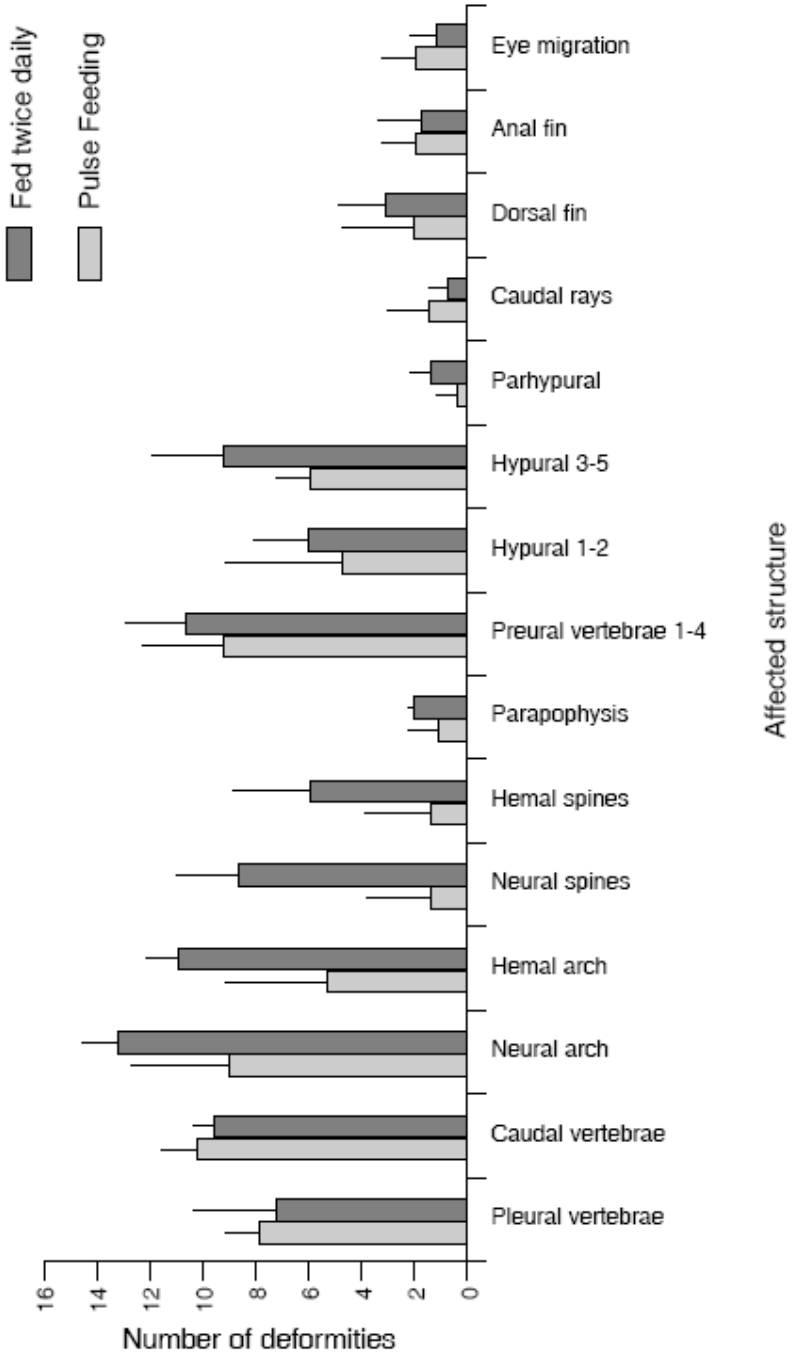

Fig. 2.3 - Distribution of abnormalities by affected structure (n = 90) at the end of the experiment (69 days).

2.4. Discussion

At the end of the pre-weaning period, the dry weight of the postlarvae was the same in both treatments and higher than values reported earlier (Cañavate and Fernández-Díaz, 1999). However, there was a significant difference in the coefficient of variation of the weight. The pulse-fed postlarvae had a greater weight distribution than those fed twice daily. A high coefficient of variation in fish may lead to aggressive behavior and/or reduced availability of food to less competitive animals (Jobling and Wandsvik, 1983). Despite the absence of apparent aggressive behavior in our study, the high coefficient of variation suggests that a feeding hierarchy may have existed. It is unlikely that a lack of food availability caused the high coefficient of variation since the fish were fed in slight excess. The smallest fish in the pulse treatment were probably those that died during the weaning period, suggesting that they may have been under some sort of stress, nutritional or other, as suggested by Jobling (1982). Experiments with turbot indicate that larger juveniles cause stress to smaller fish, preventing them from obtaining a normal feed intake (Carter et al., 1996).

At the end of the experiment, survival rates were high compared to those in other studies (Cañavate and Fernández-Díaz, 1999). The high survival rates were possibly a result of different rearing and weaning techniques. At the end of our pre-weaning period, postlarvae were larger and suddenly weaned instead of fed both commercial feed and *Artemia* metanauplii as in Cañavate and Fernández-Díaz (1999). The pulse-fed sole had significantly lower survival than the twice-daily sole, but significantly better growth and food conversion. During feedings, Senegalese sole usually respond passively, similar to Arctic charr (Linnér and Brännäs, 2001). In these two species growth results were similar: fish fed more frequently grew better. Size-selective mortality in the pulse treatment may partly explain the absence of a difference in coefficient of variation for weight at the end of the experiment, in contrast to the end of the pre-weaning period.

The total number of individuals with skeletal abnormalities in both groups (~80%) was much higher than the 44% obtained for the same structures in postlarvae and juveniles in earlier studies (Gavaia et al., 2002). One explanation for the differences between studies may be different feeding regimes. In Gavaia et al. (2002), no rotifers were offered as first feeds and A. metanauplii were enriched only with microalgae. Similar figures and variability were obtained for *Paralichtys olivaceus* seedlings where 30-60% had malformations in the caudal complex (Hosoya and Kawamura, 1998). In *Sparus aurata*, the number of hatchery reared individuals with deformed caudal complex

and vertebral column can reach 100% (Boglione et al., 2001). Although a high number of skeletal abnormalities were observed in our study, survival was comparable to previous studies of this species (own unpubl. results). Earlier studies suggest that malformations are induced in the early embryonic and larval stages, although the causes and mechanisms are not well understood (Koumoundouros et al., 1997b). Most axial skeleton structures appear, and probably acquire deformities, prior to and during metamorphosis (around 10-18 days after hatching). Therefore, differences due to dietary treatment would not be expected. The absence of significant differences between treatments in frequency of abnormalities indicates that skeletal abnormalities do not interact with pre-weaning feeding frequency to define selective mortality during the subsequent period.

In summary, the present study indicates that pre-weaning feeding frequency affects weaning performance in Senegalese sole. Pulse feeding produces fewer but larger fish while feeding twice daily leads to smaller fish with a higher survival rate. This suggests that mortality during weaning is selective (higher amongst smaller fish) under a pulse feeding regime but is unaffected by skeletal abnormalities.

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Chapter 3

Improving weaning strategies for Senegalese sole:

effects of body weight and digestive capacity

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Improving weaning strategies for Senegalese sole: effects of body weight and digestive capacity

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Abstract

To optimise Senegalese sole weaning strategies three experiments were performed. The first trial tested four weaning strategies with 10-mg sole. *Artemia* fed sole grew threefold less than fish fed an inert diet. Sudden weaning (abrupt change from *Artemia* to inert diet), and weaning with co-feeding produced larger sole than did a late weaning treatment; delayed weaning negatively affected fish growth. In the second experiment, the digestive capacity of early weaned 1-, 2- and 4-mg sole was investigated. The highest growth was observed in sole weaned at 4 mg. Digestive enzyme profiles suggest that sole have an adaptation period to inert diets, with reduced feed intake. This adaptation period is inversely proportional to postlarvae weight. The third experiment examined weaning with co-feeding at different weights (2, 5 and 11 mg). These studies demonstrate that sole of 5-10 mg can be weaned, with high survival rates. Based on the digestive enzyme profiles, the early introduction of inert diets in co-feeding with *Artemia* seems to affect intestinal processes in smaller postlarvae. This study also suggests that trypsin and alkaline phosphatase may be used as indicators of nutritional status in sole of less than 5 mg.

Keywords: Senegalese sole; *Solea senegalensis*; Weaning; Growth; Digestive enzymes; Early-weaning.

3.1. Introduction

Sole has been considered a promising candidate for marine aquaculture in Europe since the nineties. Despite high interest in its aquaculture potential, there have been few published studies related to larval rearing conditions and weaning performance for *Solea solea* L. (*S. solea*) and *Solea senegalensis* Kaup (*S. senegalensis*). Sole postlarvae differ from most other Teleost fish species because they settle to the bottom of tanks and display a peculiar feeding behaviour. Settled sole do not feed energetically in small

bursts, preferring to graze continuously on *Artemia* on the bottom of the tanks (Dinis et al., 2000). This feeding behaviour poses additional challenges in weaning sole species onto inert diets, and weaning has traditionally been a bottleneck in sole farming (Howell, 1997; Dinis et al., 1999).

In the early 1980s Métailler et al. (1983) observed that *S. solea* fed with a betaine and glycine supplemented inert diet could initiate weaned at 35 days after hatching (DAH) (110-130 mg wet weight) with survival rates higher than 65%. The inclusion of protein hydrolysates in the inert diets during weaning was also positively correlated with survival (Day et al., 1997). Survival rates as high as 92.5% have been reported (Day et al., 1999), and recent studies give similar rates (79% to 89%; Palazzi et al., 2006). In contrast, weaning results with *S. senegalensis*, the species of interest for farming in Southern Europe, are less consistent. Some of the technologies developed for *S. solea* were successfully applied to *S. senegalensis*. Dinis (1992) was able to wean *S. senegalensis* 30 days after hatching (DAH) with survival of 35.8% with a co-feeding regime using an inert diet including attractants. Increased feeding frequency during the pre-weaning phase has been shown to produce larger but fewer juveniles after weaning (Engrola et al., 2005). Still, one of the major problems is the large variation in survival and growth dispersion between batches (Dinis, 1992). Although weaning of sole postlarvae from live feed onto inert diets can be done with fish weighing 15 mg (dry weight) without co-feeding (Engrola et al., 2005), smaller sole (7-9 mg dry weight) showed promising results with a 27 day co-feeding period (Cañavate and Fernández-Díaz, 1999).

Early work, suggested that lower growth in fish larvae fed inert diets was related to low acceptance and attractiveness of inert diets, combined with poor ingestion, digestion and assimilation (Koven et al., 2001). In addition, larval size, largely as a result of the gradual maturation of the larval digestive tract, has been considered the major determinant of weaning success in marine fish larvae. Therefore, marine fish larvae can utilize compound diets from mouth opening if the diet composition takes into account the digestive specificity of fish in early stages of development (Cahu and Zambonino Infante, 2001). It has also been suggested that co-feeding strategies can improve survival and growth performance of several marine fish larvae even in early larval stages (Holt, 1993; Rosenlund et al., 1997; Baskerville-Bridges and Kling, 2000; Alves et al., 2006).

The aim of this study was to evaluate how early introduction of inert diets and duration of the co-feeding period could affect sole weaning performance using survival, growth and digestive enzymes activity as criteria.

3.2. Materials and Methods

3.2.1. Larval rearing

Prior to the experimental period all the fish were reared according to what has being described for the species by Dinis et al. (1999). In short, newly hatched larvae were reared in a 200 L cylindro-conical tank in a closed recirculation system with an initial density of 100 larvae L^{-1}. Larvae were fed at 3 days after hatching (DAH) with rotifers (*Brachionus rotundiformis* T.) enriched with DHA Protein SELCO (INVE Aquaculture, Belgium). At 5 DAH larvae were also fed *Artemia* nauplii (AF Strain, INVE Aquaculture, Belgium). Rotifers were gradually reduced until the 8 DAH. *Artemia* metanauplii (RH Strain, INVE Aquaculture, Belgium) enriched with DC DHA SELCO (INVE Aquaculture, Belgium) were provided to the larvae after 10 DAH. Between the 12 and 14 DAH live *Artemia* metanauplii were gradually changed to frozen *Artemia* metanauplii. The *Artemia* was harvested, washed in seawater, counted, and frozen in a -20ºC freezer. Just before feeding, it is thawed in seawater. This water is then removed, and new seawater added before feeding to the postlarvae tanks. From 14 DAH onwards sole postlarvae were fed exclusively with frozen *Artemia* metanauplii until the beginning of the experiments.

3.2.2. Postlarval rearing

All the experiments were carried out in a 3000 L closed recirculating system provided with a mechanical filter, a submerged biological filter, a protein skimmer and a UV sterilizer. Water temperature was 21 ºC and a photoperiod of 12L:12D was maintained in all experiments. The experimental units consisted of white flat bottom plastic tanks of 3.8 L (23 x 33 x 5 cm) for Experiments 1 and 2, and 50 L (50 x 100 x 10 cm) for Experiment 3.

Temperature, dissolved oxygen and salinity were measured daily during the experiments. Tanks were cleaned every day in the morning before feeding and dead fish were counted and removed.

3.2.3. Experimental design

3.2.3.1. Experiment 1: Effect of feeding strategies on weaning success

This experiment was planned to study the growth, growth dispersion and survival during postlarvae weaning with different feeding protocols. This experiment started with 40DAH, 10 mg dry weight (DW) postlarvae, and consisted of four treatments run in triplicate. There were 160 postlarvae per tank. Treatments were ART: *Artemia* metanauplii during the whole experiment, CO: co-feeding for 20 days, SWE: sudden weaning at 40 DAH, and SWL: sudden weaning at 60 DAH. In the control treatment (ART), postlarvae were fed frozen enriched *Artemia* metanauplii throughout the experimental period. For the co-feeding (CO) treatment, 5% of the total daily enriched *Artemia* metanauplii was replaced by inert diet for 20 days and then completely replaced by inert diet throughout the rest of the experiment. Sudden weaning (SW) means fish were fed inert diet exclusively after one day fasting. In the early sudden weaning (SWE) treatment postlarvae were weaned at the start of the experiment while in late sudden weaning (SWL) treatment postlarvae were weaned 20 days later (Fig. 3.1).

The postlarvae where transferred to the tanks two weeks before the beginning of the experiment for acclimatising, during this time sole were fed frozen enriched *Artemia* metanauplii. At the beginning of the experiment, 40 DAH and 60 DAH, samples were taken to evaluated weight and length. For each sample 20 postlarvae were randomly sampled from every tank for a total of 60 postlarvae per treatment. Experiment 1 lasted 52 days. During the experiment the water temperature was 20.97±0.83 ºC (means±S.D.) and the dissolved oxygen was 94.49±5.32% of saturation. The salinity was maintained at 31.99±1.40 gL^{-1}.

3.2.3.2. Experiment 2: Effect of body weight on larval digestive capacity

This experiment was designed to determine if difficulties in early weaning of sole are due to a lack of digestive capacity. When the postlarvae reached ~1 mg DW they were transferred to 4 L white flat bottom plastic tanks and were acclimatised for four days before beginning of Experiment 2. Each tank was stocked with 220 sole postlarvae. Enriched frozen *Artemia* metanauplii were supplied to the postlarvae until the weaning started.

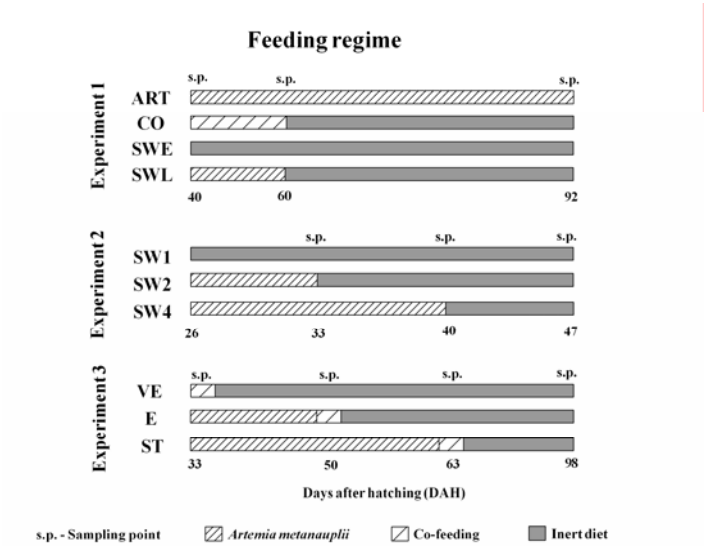

Fig. 3.1 - Summary of feeding regime for postlarvae during three experiments. In Experiment 1, treatments were ART: *Artemia* metanauplii during the whole experiment, CO: co-feeding during 20 days, SWE: sudden weaning at 40 DAH and SWL: sudden weaning at 60 DAH. In Experiment 2 treatments were SW1: sudden weaning at 26 DAH, SW2: sudden weaning at 33 DAH and SW4: sudden weaning at 40 DAH. In Experiment 3, treatments were VE: weaning with co-feeding at 33 DAH, E: weaning with co-feeding at 50 DAH and ST: weaning with co-feeding at 63 DAH. Note that fish weights were not the same for a given fish age in the different experiments (see text).

The experimental design consisted of three treatments in triplicate in which postlarvae were abruptly weaned at weights of either 1 mg DW (SW1 treatment), 2 mg DW (SW2 treatment), or 4 mg DW (SW4 treatment) (Fig. 3.1). The experiment lasted 21 days and growth, growth dispersion and enzymatic capacity were evaluated. From each tank, 15 postlarvae (45 postlarvae per treatment) were randomly sampled for weight and length evaluation at the beginning of the experiment once a week throughout the experiment. Pools of 30 postlarvae per replicate were sampled for digestive enzyme activity once a week.

During the experiment postlarvae were reared at 19.79±1.20 ºC water temperature, and salinity of 36.00±0.00 gL^{-1}. Dissolved oxygen in the water was at 94.36±2.89% of saturation.

3.2.3.3. Experiment 3: Sole postlarvae early-weaning

The experiment was designed to study the effect of a short co-feeding period (5 days) on the performance of early weaned sole postlarvae. At 20 DAH (~ 1 mg DW) postlarvae were transferred to 50 L white flat bottom plastic tanks to acclimatise and gain weight

until the beginning of the experiment. The first weaning treatment started when the postlarvae reached 2 mg DW (Very early-VE). The second treatment started weaning at 5 mg DW (Early-E), and the last treatment (Standard-ST) started weaning at 11 mg DW (Fig. 3.1). The co-feeding period lasted 5 days; in the first three days, 66% of total food weight was inert diet plus 33% *Artemia* metanauplii, and in the remaining two days 80% was inert diet plus 20% *Artemia* metanauplii. Each treatment had three replicates with a total of 1500 fish per replicate. Samples of 45 postlarvae per treatment were taken at the beginning of each treatment (33, 50 and 50 DAH) and at the end of experimental period (98 DAH postlarvae) to determined dry weight and length of sole postlarvae. For enzymatic digestive capacity assays, 90 postlarvae were sampled in the first two sampling points (30 and 50 DAH), 60 postlarvae in the third, and 15 postlarvae at the end of the experiment.

During the experiment water temperature was maintained at 21.39±0.08 ºC and salinity at 32.14±1.26 gL^{-1}. Dissolved oxygen in the water was at 95.59±1.74% of saturation.

3.2.4. Food and feeding regime

In all experiments fish were fed close to satiation (Engrola et al., 2005), based on predicted maximum growth and daily adjustments based on visual inspection (to avoid large excess of uneaten food). AgloNorse (EWOS, Norway) diet used in Experiments 1, 2 and 3 had 59% protein, 20% lipid, 4% carbohydrate, less than 10% moisture, between 9-15% ash, and 1% fiber. Gemma Micro (Trouw, France) (0.3mm) diet used in Experiment 3 had 55% protein, 15% lipids, and 13.5% ash, according to the producer data.

In Experiment 1 *Artemia* metanauplii were offered to the postlarvae two times per day, once in the morning (10.00h) and again in the late afternoon (18.00h). The inert diet used, AgloNorse no. 2 (0.6 – 1.0 mm) was continuously supplied by automatic feeders for 22 hours a day in the middle of the tanks.

In Experiment 2 *Artemia* metanauplii were offered to the postlarvae two times per day (10.00h and 18.00h). The inert diet used in this experiment was AgloNorse no. 2, continuously supplied by automatic feeders for 22 hours a day.

In Experiment 3 *Artemia* metanauplii were offered to the postlarvae three times per day (10.00, 14.00 and 18.00h). During the co-feeding period inert diet was supplied half an hour before the *Artemia* feeding. The Gemma Micro (0.3 mm) and AgloNorse no.1 (0.2-0.6 mm) diets were fed by hand 5 times a day (09.30, 11.30, 13.30, 15.30 and

17.30h). AgloNorse no.2 was semi-continuously supplied by automatic feeders for 18 hours a day, 5sec each hour, for a total of 18 meals per day. The inert diets were offered to the sole postlarvae based on their weight.

3.2.5. Analytical methods

The postlarvae were measured (total length) and kept frozen at -20ºC for dry weight determination. The postlarvae were freeze-dried and weighted on a balance of 0.001mg of precision.

For enzymatic determination in Experiment 2, the assays were performed on whole body postlarvae given the small size of the fish. In Experiment 3, the whole postlarvae were also used, except for last sampling point where sole were dissected on ice to obtain the anterior (IA) and posterior segment (IP) of the digestive tract.

Postlarvae were homogenised in 5 volumes (w/v) of ice-cold distilled water. Trypsin activity was measured using Nα-Benzoyl-DL-arginine-p-nitroanilide (Bapna) as the substrate (Tseng et al., 1982). Amylase activity was measured using starch as the substrate (Métais and Bieth, 1968). Alkaline phosphatase activity was measured using p-Nitrophenylphosphate (pNPP) as the substrate (Bessey et al., 1946), and leucine-alanine peptidase activity was measured using leucine-alanine as the substrate (Nicholson and Kim, 1975). Pepsin activity was determined at pH 2 using bovine haemoglobin as a substrate and the method of Anson (1938), in the anterior segment.

Enzyme specific activities were expressed as μmoles of substrate hydrolysed min^{-1} per mg of protein (i.e. Umg $protein^{-1}$) at 37ºC for alkaline phosphatase and leucine-alanine peptidase, and 25ºC for trypsin. Amylase specific activity was expressed as the equivalent enzyme activity that was required to hydrolyse 1mg of starch in 30 min at 37ºC per mg of protein. Pepsin activity was expressed as specific activity with 1U representing 1 mM equivalent of tyrosine liberated per minute per mg of protein at 37ºC. These temperatures used are optimal for enzymatic assays in mammals but should allow a comparison of the relative enzymatic adaptation to the treatment of each postlarvae group. Protein was determined by the Bradford method (Bradford, 1976).

3.2.6. Data analysis

All data of dry weight (mg), total length (mm) and specific activity of digestive enzymes from sole postlarvae are means ± standard deviation (S.D.) of treatment replicates (n = 3). In all experiments growth, expressed as relative growth rate (RGR, %/day), was

calculated, between sampling points and at the end of the weaning periods, using the formula: (e^{g}-1) x 100 with g = [(ln final weight – ln initial weight)/time] (Ricker, 1958). The coefficient of variation (CV) was calculated using the formula: (treatment standard deviation/treatment mean) x 100 and used to determine the inter-individual weight and length variation among fish of the same treatment. Condition factor (K) for each treatment was calculated using the formula: (fish weight/total $lenght^{3}$) x 100. Data are presented as arithmetic means ± standard deviations. One-way ANOVA was used to test differences between treatments. Differences were considered significant when $P<0.05$. When differences were found ($P<0.05$), Tukey´s Honest Significant Difference (HSD) test was used to determine which specific treatments differed significantly. All statistical analysis was carried out using the Statistica 5.1 package software (StatSoft, Tulsa, USA).

3.3. Results

3.3.1. Experiment 1 - Weaning strategies

3.3.1.1. Growth results

At the beginning of the experiment the postlarvae had a dry weight of 9.22±1.84 mg and a total length of 17.55±1.75 mm. The coefficient of variation for weight and length respectively were 19.92% and 8.50%. Timing of weaning had a significant effect ($P<0.05$) on sole postlarvae growth at 60 DAH and 92 DAH (Table 3.1, Fig. 3.2), with fish that were fed with inert diet later (SWL) being significant smaller than postlarvae fed with inert diet at 10 mg of dry weight (SWE and CO treatments).

Growth dispersion was not affected by the applied feeding strategies. Values for CV weight where between 56.77% (ART), and 31.31% (SWL), while for CV length where between 17.96% (CO) and 9.54% (ART).

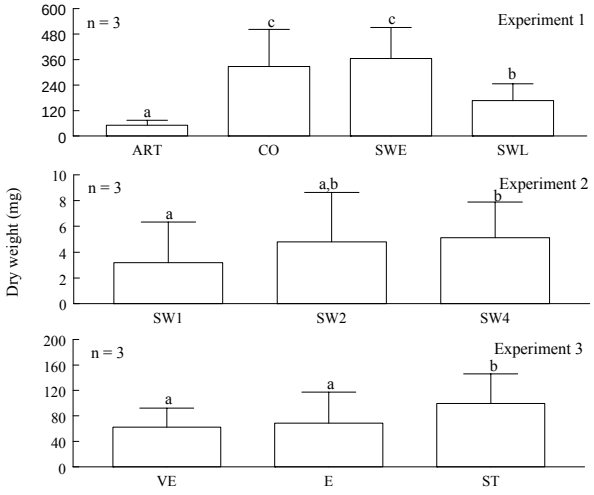

Figure 3.2 - Final dry weight of sole juveniles at the end of Experiments 1, 2 and 3. In Experiment 1 40 DAH sole were weaned with different weaning strategies: *Artemia* diet (ART), co-feeding during the first 20 days of experiment (CO), sudden weaning at 40 DAH (SWE) and sudden weaning at 60 DAH (SWL). In Experiment 2 sole were sudden-weaned at 26 DAH (SW1), 33 DAH (SW2) and 40 DAH (SW4). In Experiment 3 sole were weaned with co-feeding strategy at 33 DAH (VE), 50 DAH (E) and 63 DAH (ST). Values are means (±S.D.) of treatment replicates (n = 3). Values with different letters are significantly different (P<0.05). Please note that duration of experiments varied (52, 21 and 65 days for Experiments 1, 2 and 3, respectively).

At the end of the experiment sole postlarvae that were fed exclusively with *Artemia* metanauplii (ART) were three-to seven-folds smaller than the postlarvae that ate inert diet during the weaning period (Fig. 3.2). Sole postlarvae grew significantly (P<0.05) faster in the weaning strategies where inert diet was supplied earliest (SWE and CO treatments). As soon as postlarvae from SWL started to eat inert diet (60 DAH) their relative growth rate was significantly higher than postlarvae from the remaining treatments, demonstrating compensatory growth between the 60 and 92 DAH (Table 3.1).

Despite the fact that both coefficient of variation (weight and length), increased during the weaning period, weaning strategies did not have a significant effect on the growth dispersion parameters. Values for CV of weight where between 53.38% (CO), and 40.05% (SWE), while for CV of length where between 21.77% (CO) and 13.72% (SWE) (P>0.05).

Table 3.1 – Mean dry weight (mg), total length (mm), and relative growth rate (%) of sole postlarvae in Experiment 1: *Artemia* diet (ART), co-feeding during the first 20 days (CO), sudden weaning at 40 DAH (SWE) and sudden weaning at 60 DAH (SWL).

	Treatments			
	ART	CO	SWE	SWL
60DAH				
Dry weight (mg)	13.99±4.57^{a}	38.91±20.00^{b}	50.90±16.44^{c}	12.74±3.99^{a}
Total length (mm)	19.50±1.85^{a}	24.97±4.48^{b}	28.28±3.66^{c}	18.93±2.23^{a}
RGR (%)	2.28±0.79^{a}	6.68±1.27^{b}	8.51±0.39^{b}	1.89±1.14^{a}
$CV_{(weight)}$ (%)	56.77	51.41	32.30	31.31
$CV_{(length)}$ (%)	9.54	17.96	12.94	11.78
92DAH				
Dry weight (mg)	51.23±22.82^{a}	327.69±174.92^{c}	365.28±146.30^{c}	167.40±78.59^{b}
Total length (mm)	26.62±4.26^{a}	45.15±9.83^{c}	48.57±6.66^{d}	38.65±6.55^{b}
RGR (%)	3.91±0.31^{a}	6.90±0.93^{b}	6.35±0.37^{b}	8.40±0.30^{c}
$CV_{(weight)}$ (%)	44.54	53.38	40.05	46.95
$CV_{(length)}$ (%)	16.00	21.77	13.72	16.96
Survival (%)	97.80±1.93	98.44±1.36	98.76±0.84	94.59±2.95

Results are given as mean (±S.D.), of treatment replicates (n = 3). Different superscript letters indicate statistical differences ($P<0.05$, Tukey's Test) between postlarvae from different treatments at the same age.

The survival rate was not affected by the weaning strategies that the sole postlarvae experienced, and ranged between 94.59±2.95% (SWL) and 98.76±0.84% (SWE) (Table 3.1).

3.3.2. Experiment 2 - Effect of weight on digestive larval capacity

3.3.2.1. Growth results

Initial weight at weaning had a significant impact on postlarvae final dry weight (Fig. 3.2) and length (Table 3.2) with sole from SW1 being significantly smaller than sole from SW4. Early weaning had a significant impact on relative growth rate of postlarvae during the experimental period (Table 3.2).

The smaller postlarvae (SW1 and SW2) that were weaned onto inert diet had negative growth during the first two- and one-weeks respectively, after the weaning started.

Table 3.2 – Mean dry weight (mg), total length (mm), relative growth rate (%), and survival (%) of sole postlarvae in Experiment 2: sudden weaning at 26 DAH (SW1), sudden weaning at 33 DAH (SW2) and sudden weaning at 40 DAH (SW4).

	Treatments		
	SW1	SW2	SW4
33 DAH			
Dry weight (mg)	0.85±0.33^{a}	1.98±0.75^{b}	2.01±0.97^{b}
Total length (mm)	8.51±1.49^{a}	10.29±1.47^{b}	10.31±1.98^{b}
RGR (%)	-0.61±3.72^{a}	9.63±2.31^{b}	10.02±1.54^{b}
$CV_{(weight)}$ (%)	38.83	37.97	48.09
$CV_{(length)}$ (%)	17.47	14.30	19.16
40 DAH			
Dry weight (mg)	0.81±0.26^{a}	1.47±0.40^{b}	4.44±1.16^{c}
Total length (mm)	7.98±1.06^{a}	10.18±1.50^{b}	12.98±1.39^{c}
RGR (%)	-0.67±2.98^{a}	-4.11±2.49^{a}	12.17±2.07^{b}
$CV_{(weight)}$ (%)	31.57	27.10	26.11
$CV_{(length)}$ (%)	13.26	14.71	10.71
47 DAH			
Dry weight (mg)	3.18±3.16^{a}	4.79±3.84^{a,b}	5.12±2.77^{b}
Total length (mm)	11.03±3.75^{a}	13.45±3.31^{b}	14.88±2.43^{b}
RGR (%)	21.29±6.47^{a}	16.58±10.08^{a,b}	2.13±1.36^{b}
$CV_{(weight)}$ (%)	99.52	80.04	54.13
$CV_{(length)}$ (%)	34.03^{b}	24.64^{a}	16.34^{a}
Survival (%)	38.46±3.75^{a}	39.65±7.03^{a}	90.06±10.01^{b}

Results are given as mean (±S.D.), of treatment replicates (n = 3). Different superscript letters indicate statistical differences ($P<0.05$, Tukey's Test) between postlarvae from different treatments at the same age.

Values for CV of weight where very high, ranging between 99.52% (SW1) and 54.13% (SW4). The dispersion of length was also high, with postlarvae from SW4 (16.34%) being significantly more homogeneous than postlarvae from SW1 (34.03%), but not different from postlarvae from SW2 (24.64%). At the end of the experiment the survival rate of sole postlarvae in SW4 treatment was significantly higher than the other two treatments SW2 and SW1 (Table 3.2).

3.3.2.2. Enzymatic capacity

No significant differences in postlarvae trypsin specific activity were determined between treatments in experiment 2 (Fig. 3.3A). An almost twofold decrease in amylase specific activity in the postlarvae was observed between the first two sampling points from treatment SW1 (Fig. 3.3B). In the first sampling point amylase activity was significantly higher ($P<0.05$) in the postlarvae from treatment SW1 than for SW2 and SW4. No significant differences in the specific activity were found between the treatments in the two remaining sampling points ($P>0.05$).

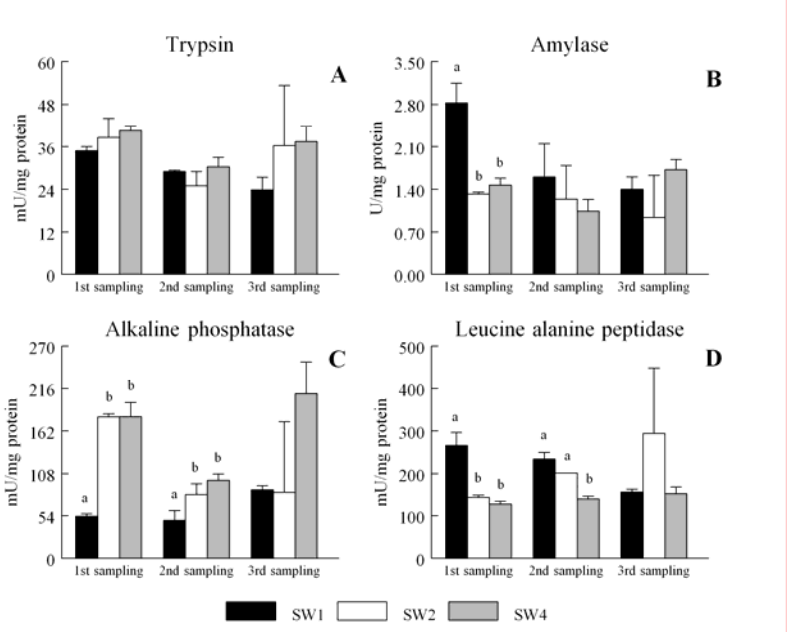

Fig. 3.3 - Specific activity of trypsin (A), amylase (B), alkaline phosphatase (C) and leucine-alanine peptidase (D) in whole sole postlarvae at three sampling points (33, 40, 47 DAH) during Experiment 2:sudden weaned at 26 DAH (SW1), 33 DAH (SW2) and 40 DAH (SW4). Values are means (±S.D.) of 30 postlarvae per treatment replicate (n = 3). Values with different letter in the same sampling point are significantly different (P<0.05).

A two to three-fold decrease in alkaline phosphatase specific activity was observed in SW2 and SW4, respectively, between the first and second sampling point (Fig. 3.3C). The enzyme activity in postlarvae from SW1 was always significantly lower at 33 and 40 DAH ($P<0.05$) when compared to postlarvae from SW2, and SW4 treatments.

The specific activity of leucine-alanine peptidase (Fig. 3.3D) at the first sampling point was significantly higher ($P<0.05$) in the group that was fed inert diet for a week (SW1) than in the postlarvae that were still eating *Artemia* metanauplii (SW2 and SW4). At the second sampling point postlarvae from both treatments that were fed inert diet (SW1 and SW2) showed similar results compared to the postlarvae that were fed live food (SW4). At the last sampling point the SW1 and SW4 treatments had very similar results.

3.3.3. Experiment 3 - Sole postlarvae early weaning

3.3.3.1. Growth results

Weaning weight of sole postlarvae significantly ($P<0.05$) affected larval growth and survival. Sole postlarvae from the VE treatment had similar weights to those of postlarvae from E treatment throughout the experimental period (Table 3.3 and Fig. 3.2). At the end of the experiment sole postlarvae from ST had significantly higher ($P<0.05$) dry weight and total length than the postlarvae from VE and E treatments. Condition factor was affect by the early weaning calculated from data in Table 3.3. At the end of the experiment sole postlarvae from the E treatment had a significantly higher condition factor than the postlarvae from VE and ST treatment. When sole postlarvae were eating inert diet AgloNorse no.1, postlarvae from VE and E had relative growth rates (RGR) of 1.79±2.36% and 1.92±0.95%, respectively, and the postlarvae from ST treatment, eating *Artemia* metanauplii, had RGR of 3.65±1.17%. At the end of the experiment all the postlarvae were eating inert diet AgloNorse no.2 and showed similar RGR, between 6.45±1.53% (ST) and 6.61±1.01% (VE). There were significant differences in survival between all the treatments (Table 3.3), with the best survival rate obtained with the postlarvae from E treatment, 38.61±3.13%. At the end of the experiment the coefficient of variation for weight and length were not affected by the studied parameters. Values for CV weight where between 70.81% (E), and 46.55% (SWE), while for CV length where between 26.50% (E) and 14.00% (VE) ($P>0.05$).

Table 3.3 – Mean dry weight (mg), total length (mm), relative growth rate (RGR, %), and survival (%) of sole postlarvae in Experiment 3: weaning with co-feeding at 33 DAH (VE), weaning with co-feeding at 50 DAH (E) and weaning with co-feeding at 63 DAH (ST).

	Treatments		
	VE	E	ST
Start weaning weight (mg)	2.11±0.69	5.23±1.70	11.85±5.13
50 DAH			
Dry weight (mg)	5.26±3.17^{a}	5.23±1.70^{a}	7.34±3.34^{b}
Total length (mm)	14.18±3.21^{a}	14.62±1.74^{a}	15.87±3.35^{b}
RGR (%)	5.41±1.51	5.44±1.00	7.31±2.47
$CV_{(weight)}$ (%)	60.19	32.50	45.44
$CV_{(length)}$ (%)	22.62	11.87	21.10
63 DAH			
Dry weight (mg)	6.50±3.50^{a}	6.68±2.97^{a}	11.85±5.13^{b}
Total length (mm)	16.22±3.28^{a}	15.70±2.91^{a}	19.13±3.53^{b}
RGR (%)	1.79±2.36	1.92±0.95	3.65±1.17
$CV_{(weight)}$ (%)	53.88^{b}	44.48^{a,b}	43.30^{a}
$CV_{(length)}$ (%)	20.23	18.56	18.43
98 DAH			
Dry weight (mg)	62.35±29.87^{a}	68.68±48.63^{a}	99.73±46.43^{b}
Total length (mm)	30.33±4.25^{a}	28.59±7.58^{a}	33.58±5.54^{b}
RGR (%)	6.61±1.01	6.49±1.67	6.45±1.53
$CV_{(weight)}$ (%)	47.91	70.81	46.55
$CV_{(length)}$ (%)	14.00	26.50	16.50
Survival (%)	17.96±1.41^{a}	38.61±3.13^{b}	28.45±3.59^{c}

Results are given as mean (±S.D.), of treatment replicates (n = 3). Different superscript letters indicate statistical differences ($P<0.05$, Tukey's Test) between postlarvae from different treatments at the same age.

3.3.3.2. Enzymatic capacity

3.3.3.2.1. Whole body postlarvae (33, 50 and 63 DAH)

The trypsin specific activity from postlarvae of VE treatment tended to decrease throughout the experimental period (Fig. 3.4A). In all treatments the values of the enzyme activity tended to decrease when the postlarvae started to eat inert diet. Significant differences ($P<0.05$) were found for the third sampling point, where values from VE and E treatment were lower than in postlarvae from treatment ST.

In the amylase (Fig. 3.4B), alkaline phosphatase (Fig. 3.4C) and leucine-alanine peptidase (Fig. 3.4D) specific activity no significant differences were found between treatments.

3.3.3.2.2. Dissected postlarvae (98 DAH)

The enzymes (trypsin and amylase) were determined in the anterior and posterior segments of the digestive tract of dissected fish. Pepsin assays were performed on the anterior segment of digestive tracts of dissected juveniles.

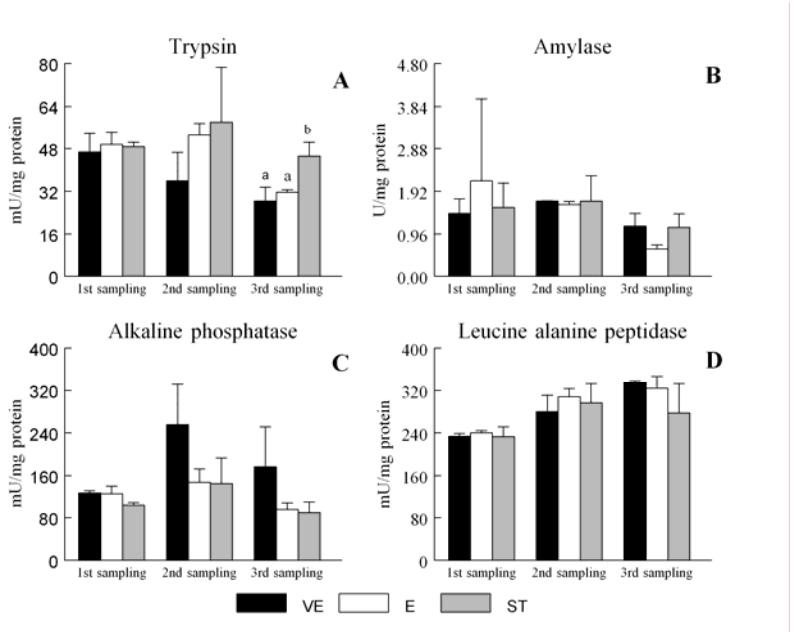

Fig. 3.4 - Specific activity of trypsin (A), amylase (B), alkaline phosphatase (C) and leucine-alanine peptidase (D) in whole sole postlarvae for the first three sampling points (33, 50 and 63 DAH) in Experiment 3: weaning with co-feeding strategy at 33 DAH (VE), 50 DAH (E) and 63 DAH (ST). Values are means (±S.D.) for 30 postlarvae per treatment replicate (n = 3). Values with different letter in the same sampling point are significantly different ($P<0.05$).

Trypsin specific activity determined in postlarvae anterior segment was VE: 4.25±1.03 mU/mg protein, E: 4.19±0.55 mU/mg protein and ST: 4.13±0.18 mU/mg protein while in posterior segment was VE: 0.55±0.16 mU/mg protein, E: 0.41±0.13 mU/mg protein and ST: 0.46±0.18 mU/mg protein. Amylase specific activity observed in the anterior segment was VE: 2.23±1.08 U/mg protein, E: 2.09±0.32 U/mg protein and ST: 2.65±1.00 U/mg protein while in posterior segment was VE: 5.61±4.35 U/mg protein, E: 4.25±1.02 U/mg protein and ST: 4.54±0.78 U/mg protein. Specific activity for both enzymes were not significantly ($P>0.05$) affected by any of the treatments.

Pepsin specific activity determined in sole juveniles (VE: 36.32±15.08 μU/mg protein, E: 33.07±13.07 μU/mg protein and ST: 41.90±8.98 μU/mg protein) was not significantly ($P>0.05$) affected by treatments.

3.4. Discussion

3.4.1. Does feeding regime affect digestive capacity in sole?

The specific activity of trypsin was significantly affected by feeding regime in Experiment 3 where postlarvae fed with *Artemia* showed higher activity. An increase of specific activity of trypsin should be expected when feeding sole postlarvae with inert diet, since inert diets usually have a higher content of protein and would therefore induce higher levels of enzyme activity (Tseng et al., 1982). The decrease of trypsin activity along the experimental period may indicate that the sole postlarvae were not eating properly. Although no significant differences were found among treatments in Experiment 2, there was a tendency for the trypsin specific activity to decrease after the first week of inert diet when the postlarvae are smaller (SW2) indicating that food intake probably diminished with the shift in food type, the same pattern was observed in European seabass by Cahu and Zambonino-Infante (1994). The same pattern also occurs in total enzyme activity (results not shown).

The higher activity of alkaline phosphatase in the SW4 sole (Experiment 2) probably indicates better developmental and nutritional status of postlarvae as observed by Ribeiro et al. (2002). In the week prior to start weaning, these postlarvae showed a decrease in alkaline phosphatase activity while eating *Artemia*, suggesting that this was not the most suitable feed. The absence of a similar pattern in postlarvae from SW2 together with the values of leucine-alanine peptidase during the second week of the experiment suggests a delay in the enterocyte maturation as suggested by Ribeiro et al.

(2002). Since the inert diet used was the same in all treatments this delay could be due to a lower food intake by the postlarvae.

In short, feeding regime seems to affect digestive enzymes profiles in sole. In addition, trypsin and alkaline phosphatase appear to be good indicators of nutritional status in sole. This confirms previous observations (Ribeiro et al., 2002; Fernández-Díaz et al., 2006) that sole digestive capacity can be influence by food type. The analysis of the digestive enzyme profiles of sole postlarvae (Fig. 3.3 and Fig. 3.4) indicates that smaller sole (around 2mg DW) with a co-feeding strategy (Experiment 3), had an improved digestive capacity compared to sudden-weaned sole (Experiment 2).

3.4.2. Does weaning regime affect growth dispersion in sole?

None of the weaning regimes significantly affected sole growth dispersion. Similar results were observed in Asian seabass (Curnow et al., 2006), fat snook (Alves et al., 2006) and Dover sole (Rueda-Jasso et al., 2005). All the values were similar to those reported for the species (Engrola et al., 2005), with the exception of the postlarvae from SW1 and SW2 (Experiment 2) where the observed values were very high. Individual capacity to cope with shifts in food type may explain these results. One of the reasons for high growth dispersion appears to be reduced availability of food, as in Artic charr (Jobling and Wandsvik, 1983), greenback flounder (Carter et al., 1996) and Japanese flounder (Lee et al., 2000). Nevertheless this it is very unlikely, since in the present study fish were fed slightly in excess.

3.4.3. Is co-feeding important for weaning sole?

Co-fed sole grew more than *Artemia*-fed fish, which agrees with findings for Atlantic halibut (Rosenlund et al., 1997). Nevertheless, the best weaning results in Experiment 1 where achieved when postlarvae were sudden weaned at 10 mg dry weight, and not when they were co-fed. This result supports a previous study (Engrola et al., 2005) where sudden weaning was proposed but it is in contradiction to earlier studies performed with the same species where a co-feeding strategy was suggested (Cañavate and Fernández-Díaz, 1999; Ribeiro et al., 2002). The different results may arise from different zootechnical techniques (Cañavate and Fernández-Díaz, 1999), start weaning weights (Ribeiro et al., 2002), and recent improvements in inert diet quality, such as the inclusion of protein hydrolysates (Zambonino Infante et al., 1997; Tonheim et al., 2005). The results of Experiment 3 do not support the idea that a short co-feeding period enhances

larval growth and survival rate. This is in apparent contradiction to observations for other species that a co-feeding regime would improve larval nutrition and may pre-condition larvae to better accept the inert diet (Rosenlund et al., 1997; Vega-Orellana et al., 2006). This apparent difference between sole and other species may arise from the peculiar passive feeding behaviour of sole. *Artemia* co-feeding may have a "distracting" effect on sole postlarvae. Nevertheless, in smaller postlarvae (around 2 mg DW) co-feeding strategy may enhance digestive maturation as observed in Experiment 3, and suggested by several authors (Kolkovski et al., 1993; Kolkovski et al., 1997; Rosenlund et al., 1997; Baskerville-Bridges and Kling, 2000). In this case co-feeding with inert diet during an extended period starting during the pelagic phase of sole might improve current larval quality and weaning success.

3.4.4. Is early weaning possible in sole?

This study demonstrates that it is possible to wean sole postlarvae between 5 and 10 mg of dry weight, with high survival rates. By having a treatment in Experiment 1 where the sole postlarvae were only fed with *Artemia* metanauplii (ART) it was shown that *Artemia* is not the most suitable feed for Senegalese sole after 10 mg of dry weight. Rueda-Jasso et al. (2005) in Dover sole and Brown et al. (1997) in Atlantic wolffish (*Anarhichas lupus*) observed similar feeding patterns, after a certain time live prey does not meet the energy requirements of the larvae as they grow. Digestive enzymatic capacities of sudden-weaned and co-fed postlarvae indicate that with the existing inert diets it is still difficult to successfully wean postlarvae of 1 or 2 mg DW.

In Experiment 2 it was possible to verify that sole postlarvae have an adaptation period to the inert diet. This period is larger in smaller postlarvae (Table 3.2). Growth rate increases in the last period in the SW1 and SW2 fish, being apparently higher compared to the SW4 sole in the same period. This may result from a growth depression during the adaptation of SW4 sole to inert diet, as observed during the sudden weaning periods for SW1 and SW2 fish. In addition, the increase in growth rate of SW1 and SW2 sole may result from selective mortality of (smaller) fish unable to adapt to inert diet in these treatments.

The results of the present experiments demonstrate that it is possible to wean sole postlarvae with two different feeding strategies, sudden and *Artemia* co-feeding. The choice of the feeding strategy to adopt should be based on the postlarvae weight. This is in agreement with observations (Verreth, 1994; Rosenlund et al., 1997) that larval weight

rather than larval age is a better indicator of the developmental stage and physiological status of the postlarvae.

3.5. Acknowledgements

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Chapter 4

Co-feeding in Senegalese sole larvae with inert diet from mouth opening promotes growth at weaning

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Abstract

The aim of this study was to determine if sole larvae co-fed with inert diet at mouth opening would perform better than larvae fed with live prey and if such a feeding regime would produce better quality juveniles. The experiment was separated in two phases: pelagic and benthic. In the pelagic phase, treatments consisted of the standard feeding regime (rotifer and *Artemia* feeding), the standard feeding regime and inert diet, and rotifer for a longer period than the standard feeding regime until larvae reached 9 days after hatching (DAH). By the end of the pelagic phase, when the postlarvae were 20 DAH, sole that were co-fed with inert diet from mouth opening, were significantly smaller in weight than postlarvae fed exclusively with live prey. Sole digestive maturation was improved by co-feeding the inert diet. Survival rates, skeletal deformities and quality evaluation were not affected by the feeding regimes. In the benthic phase, the postlarvae from standard feeding regime (pelagic phase) were separated in two treatments: standard live *Artemia* metanauplii until weaning and standard frozen *Artemia* metanauplii until weaning. Remaining treatments were the follow up of treatments from the pelagic phase. At the end of the experiment i.e. 68 DAH, the postlarvae co-fed with inert diet from mouth opening were significantly larger than all the postlarvae from remaining feeding regimes.

The results of the present study demonstrate that it is possible to offer inert diet to sole at mouth opening in a co-feeding regime and to produce better quality postlarvae. Co-fed sole were larger and had a better tail condition at the end of the weaning.

Keywords: Senegalese sole; Growth; Quality; Digestive enzymes; Early-weaning; Deformity.

4.1. Introduction

Fish larvae are known to present high growth rates when compared to older fish (Houde, 1989; Conceição et al., 1998). The importance of solving the nutritional requirements of

the larvae with a balanced feed in earlier phases is essential because otherwise the growth, food conversion efficiency (Conceição et al., 2003), and even survival (Aragão et al., 2007) may be lower. The development of an inert diet that is well ingested, digested and assimilated by larvae at mouth opening, has long been an objective of fish larvae researchers. Currently *Artemia* replacement diets are a feeding strategy widely used in fish larviculture since inert diets are easier to use and have a stable composition, while composition of live feed can vary according to culture/enrichment conditions. The importance of the early feeding regimes was observed during weaning of barramundi (Curnow et al., 2006b). These authors showed that, depending on the combination of types of inert diet and live prey, larger larvae could be produced. In Atlantic cod, a co-feeding strategy produced a two-fold increase in larval weight, in contrast to an inert diet strategy (Fletcher et al., 2007). Alves et al. (2006) noticed that doubling the co-feeding period in fat snook would promote a two-fold increase in larval length. This feeding strategy is also known to stimulate feeding rates in Dover sole (Knutsen, 1992) and seabream (Kolkovski et al., 1997), and to pre-condition larvae onto inert diet (Hart and Purser, 1996; Brown et al., 1997; Callan et al., 2003; Curnow et al., 2006a; Fletcher et al., 2007).

Larval digestive tract maturation can be affected by the inert diet, and a sub-optimal diet may cause mortalities (Cahu and Zambonino Infante, 2001). Furthermore, knowledge on the development of the digestive tract maturation is important to assess the larval nutrition needs and to develop adequate larval feeding protocols (Cahu and Zambonino Infante, 2001; Kolkovski, 2001; Koven et al., 2001; Zambonino Infante and Cahu, 2007). The development of the digestive tract and the impact of the feeding regimes in the digestive enzyme capacity, have been studied in several species. The shift in the diet composition had a direct impact on the digestive enzyme profiles of Atlantic cod (Wold et al., 2007), sharpsnout seabream (Suzer et al., 2007), and white bream (Cara et al., 2003). The use of microalgae and inert diet from mouth opening increased the enzyme activity of trypsin and aminopeptidase in red drum (Lazo et al., 2000a). A co-feeding of live feed and inert diet at 5 DAH improved the digestive maturation of dorado (Vega-Orellana et al., 2006). The type of inert diet during weaning period may delay or promote digestive maturation in Senegalese sole (Ribeiro et al., 2002; Engrola et al., 2007).

Skeletal abnormalities are a severe problem in aquaculture production as they affect fish appearance, thus reducing market value (Koumoundouros et al., 1997).

Nutritional factors can cause alterations in the normal development of the skeleton leading to structural abnormalities in adult fish (Hilomen-Garcia, 1997; Cahu et al., 2003; Lall and Lewis-McCrea, 2007). Some advances have been made in terms of individual nutrient requirements for a normal skeletal formation. Safe levels of vitamin A were investigated for other flat fish and related to the developmental problems during skeletogenesis in Japanese flounder (Dedi et al., 1995; Takeuchi et al., 1995) and to the pigmentation success in turbot (Estevez and Kanazawa, 1995). The minimum levels of vitamin K that promotes a normal skeletal growth and mineralization has been recently determined for haddock (Roy and Lall, 2007). The incorporation of vitamin C and HUFA in *Artemia* was shown to diminish opercular deformities in milkfish (Gapasin et al., 1998). The supplementation of phosphorus (Uyan et al., 2007), and the substitution of native fish meal proteins by fish meal hydrolysate (Zambonino Infante et al., 1997) led to a decrease of deformities in fish.

Senegalese sole is a species of high value for aquaculture in Southern Europe. The current knowledge about biology and ecology (Imsland et al., 2003), as well as nutritional physiology (Conceição et al., 2007) was reviewed recently. A pulse fed strategy during the pre-weaning phase has proved to produce larger but fewer juveniles after weaning (Engrola et al., 2005), a period that still presents some challenges. The choice of the feeding strategy to adopt at weaning should be based on the postlarvae weight as it is a better indicator of the developmental stage and physiological status of the postlarvae (Engrola et al., 2007).

The aim of this study was to determine if sole larvae co-fed with inert diet from mouth opening would present better growth performance, and if such larvae would develop into better quality juveniles.

4.2. Materials and Methods

4.2.1. Experimental design

4.2.1.1. Pelagic phase

The experimental phase was designed to study the effect of partial substitution of live prey by inert diet in terms of growth, digestive maturation, and larval quality. Treatments consisted of the standard feeding regime (Standard, ST), standard feeding regime and inert diet (*Artemia* replacement, ArtR), and rotifers until larvae reached 9 days after hatching (DAH) (Rotifers until 9 DAH, Rot9), as described in Table 4.1. Eggs from a single batch were obtained from natural spawning of Senegalese sole broodstock

maintained at the University of Algarve (Faro, Portugal). Newly hatched larvae were reared in 200L white cylindro-conical tanks in a semi-closed recirculation system of 2100L with a density of 104 larvae/L, and with three replicates per treatment. On the first day of the experiment, the water renewal stopped in one of the replicates for ArtR treatment, so this treatment was run in duplicate. The experimental system was equipped with a mechanical filter, a submerged biological filter, a protein skimmer and a UV sterilizer. Larvae were fed according to feeding regimes (Table 4.1) until 20 days and had one day of transition between different diets. At 2 DAH, larvae from all treatments were fed rotifers (*Brachionus rotundiformis*) enriched with DHA Protein Selco (Inve, Belgium). Larvae from ArtR treatment were also fed with inert diet. At 5 DAH, larvae from ST and ArtR treatments were also fed with *Artemia* sp. nauplii (Inve, Belgium). Rotifers were fed until 5 DAH in the ST and ArtR treatments, and 9 DAH for larvae from the Rot9 treatment. From 9 DAH onwards, *Artemia* sp. metanauplii enriched with Easy DHA Selco (Inve, Belgium) and AgloNorse Microfeed (Ewos, Scotland) were provided to the larvae in ST and ArtR treatmenst, and from 10 DAH in the Rot9 treatment. Larvae from ST and Rot9 were fed exclusively with enriched *Artemia* sp. metanauplii from 11 DAH. The experimental phase lasted 19 days.

From each tank, 30 larvae (a total of 90 larvae in ST and Rot9, and 60 in ArtR) were randomly sampled for weight and length evaluation at 2, 5 and 9 DAH. Pools of 60 larvae per replicate were sampled for digestive enzymes at the same ages. At the end of the pelagic phase 20 postlarvae per replicate were sampled for dry weight.

Fish length, pigmentation pattern, color and tail condition were determined. Pools of 20 and 30 postlarvae per replicate were sampled for digestive enzyme activity and skeleton evaluation, respectively.

Environmental parameters were measured daily. Temperature and salinity averaged 19.9±0.4ºC (mean±SD) and 37.2±0.8‰, respectively. Dissolved oxygen in water was 91.0±0.8% of saturation. A photoperiod of 12h light (L): 12h dark (D) cycle and a light intensity of 900 “lux” was used and provided by overhead fluorescent tubes. Lights came on at 09:00 and first prey addition was at 11:30.

Table 4.1 – Feeding regimes of Senegalese sole larvae during the pelagic phase (2 to 19 days after hatching, DAH) of the experiment. ST: Standard feeding regime; ArtR: *Artemia* replacement feeding regime and Rot9: Rotifers until 9 DAH feeding regime.

	Treatment									
	ST			ArtR				Rot9		
DAH	Rot	Na	Meta	Rot	Na	Meta	Inert diet	Rot	Na	Meta
2	3			3			6	3		
3	4			4			8	4		
4	5			5			11	5		
5	6	2		6	2		15	6	2	
6		4			4		32	8	4	
7		6			5		43	10	6	
8		8			6		58	10	8	
9		4	4		3	3	78	12	4	4
10			6			3	159			6
11			8			4	129			8
12			10			5	158			10
13			12			6	193			12
14			12			6	236			12
15			14			7	288			14
16			14			7	251			14
17			16			8	291			16
18			16			8	338			16
19			18			9	393			18

Rot: Rotifers; Na: *Artemia* nauplii; Meta: *Artemia* metanauplii and Inert diet: Proton diet. Rotifers are expressed as 'number of rotifers / ml / day', *Artemia* are expressed as 'number of *Artemia* / ml / day' and inert diet daily ration are expressed as 'mg /1000 larvae / day'.

4.2.1.2. Benthic phase

This experimental phase was designed to determine if the feeding regimes during the pelagic and benthic phase of sole may influence postlarvae quality and consequently weaning performance. The benthic phase of the experiment was carried out in a 500 L semi-closed recirculating system equipped with a mechanical filter, a submerged biological filter, a protein skimmer and a UV sterilizer. A photoperiod of 12h L: 12h D

and a light intensity of 400 “lux” were maintained during all the experimental phase. Lights came on at 09:00 and first prey addition was at 10:00. The experimental units consisted of 12 white flat-bottomed fiber glass tanks of 21 L (width 30 cm x lenth 70 cm x height 10 cm). Each tank was stocked with 630 postlarvae, corresponding to a density of 3000 individuals/m^2, with three replicates per treatment in a total of 12 tanks.

Treatments were assigned to tanks randomly. Postlarvae from ST treatment (pelagic phase) were separated in two treatments: Standard live *Artemia* metanauplii until weaning (STL); Standard frozen *Artemia* metanauplii (STF) until weaning. Remaining treatments were the follow up of treatments from the pelagic phase, frozen *Artemia* metanauplii (50%) and inert diet (60%) until 40 DAH (ArtR), and frozen *Artemia* metanauplii until weaning (Rot9). At 20 DAH postlarvae from treatments STL, STF and Rot9 were fed 200 *Artemia* metanauplii / fish. Ration was then daily increased until 3000 *Artemia* metanauplii / fish at 40 DAH. In treatment ArtR, at 20 DAH were fed 100 *Artemia* metanauplii / fish (50% of total daily ration) and 0.24 mg (60% of total daily ration in dry matter basis) of inert diet. This proportion of inert diet (in total daily ration, dry matter basis) was daily increased until 40 DAH in treatment ArtR, when sole postlarvae were fed 1500 *Artemia* metanauplii / fish and 5.1 mg of inert diet / fish. The frozen *Artemia* was harvested, washed in seawater, counted, and frozen in a -20ºC freezer. Just before feeding, it was thawed in seawater. This water was then removed, and new seawater added before feeding to the postlarvae tanks (STF, ArtR and Rot9). Postlarvae from STL, STF and Rot9 were sudden weaned at 40 DAH, meaning fish were fed inert diet after fasting one day, when their weights were within 5-10 mg dry weight (DW) as recommended by Engrola et al. (2007). At 40 DAH postlarvae from ArtR were exclusively fed with inert diet. Tanks were carefully cleaned every morning before feeding and dead fish were counted and removed. The experimental phase lasted 48 days.

At the beginning of the weaning, 40 DAH and at the end of the experiment 68 DAH, postlarvae samples were taken to evaluate dry weight (n = 20, per replicate), enzymatic capacity (n = 15, per replicate) and skeletal quality evaluation (n = 15, per replicate). In the postlarvae sampled for weight, total length and quality indicators were also determined. Tail condition, color and pigmentation pattern were assessed as quality indicators. Tail condition has long been described in sole (Flüchter, 1979) and usually is the early indicator of massive mortalities in Senegalese sole, specially in sizes ranging from 60 mg (weaned fish) to 5 g (own unpublished observations). Sole were counted at the end of the experimental phase to determine survival.

During the experiment, postlarvae were reared at 20.9±0.7ºC water temperature, and salinity of 37.6±0.5‰. Dissolved oxygen in the water was at 93.0±0.4% of saturation.

4.2.2. Food and feeding regime

During the study, fish were fed close to satiation, based on predicted maximum growth and daily adjustments based on visual inspection (Engrola et al., 2005) to avoid a large excess of uneaten food.

In the pelagic phase, live preys were offered to the larvae three times per day, once in the morning (11.30h), early afternoon (14.00h) and in the late afternoon (17.00h). The inert diet, Proton (Inve, Belgium) (100-200µm) was hand feed without mixing water twice a day, once in the morning (11.00h) and in the late afternoon (16.30h). In the ArtR treatment, inert diet was supplied half an hour before the live prey feeding.

In the benthic phase before weaning started, *Artemia* metanauplii were offered to the postlarvae three times per day (10.00h, 14.00h and 17.00h). Postlarvae from ArtR between 20 and 40 DAH, were hand fed with AgloNorse no. 1 (EWOS, Scotland) (0.2-0.6mm), twice a day (10.00h and 17.00h). The inert diet used during weaning was AgloNorse no. 2 (EWOS, Scotland) (0.6-1.0mm), semi-continuously (cycles of 2h of feeding followed by one hour break) supplied by automatic feeders for 24 hours a day.

The Proton (Inve, Belgium) diet used had 54% protein, 12% lipids, and 7% moisture. The AgloNorse (EWOS, Scotland) diet used had 59% protein, 20% lipid, 4% carbohydrate, less than 10% moisture, between 9-15% ash, and 1% fiber, according to the manufacturer's data.

4.2.3. Analytical methods

The postlarvae were measured (total length) and kept frozen at -20ºC for dry weight determination. The sole total length was determined with the help of the image processing and analysis program UTHSCSA Image Tool. During sampling it was taken a photo from each sole of each replicate, afterwards the length was determined with the help of the software. (v. 3.0, C.D. Wilcox, S.B. Dove, W.D. McDavid, and D.B. Greer, University of Texas Health Science Center, Texas, USA). The postlarvae were freeze-dried and weighed with 0.001mg precision.

For enzymatic determination, the assays were performed on whole body of pelagic larvae given the small size of the fish. In the benthic phase, the sole postlarvae

were dissected on ice to assay the abdominal segment of the digestive tract. Sole were homogenized in 5 volumes (w/v) of ice-cold distilled water. Trypsin activity was measured using Nα-benzoyl-DL-arginine-p-nitroanilide (Bapna) as the substrate (Holm et al., 1988). Amylase activity was measured using starch as the substrate (Métais and Bieth, 1968). Alkaline phosphatase activity was measured using p-nitrophenylphosphate (pNPP) as the substrate (Bessey et al., 1946), and leucine-alanine peptidase activity was measured using leucine-alanine as the substrate (Nicholson and Kim, 1975). Enzyme specific activities were expressed as μmoles of substrate hydrolysed min^{-1} per mg of protein (i.e. U mg $protein^{-1}$) at 37ºC for alkaline phosphatase and leucine-alanine peptidase, and 25ºC for trypsin. Amylase specific activity was expressed as the equivalent enzyme activity that was required to hydrolyse 1mg of starch in 30 min at 37ºC per mg of protein. Protein was determined by the Bradford method (Bradford, 1976).

The samples taken for evaluation of the fish skeleton were fixed over night in 4% formaldehyde buffered to pH 7.4 with PBS. The fish were submitted to a double staining procedure, using Alcian Blue 8GX to stain cartilage and Alizarin red S to stain bone according to the procedure previously described (Gavaia et al., 2000). Fish were preserved in glycerol until observation. The evaluation of the normal development of structures was determined on the axial skeleton based on the description of Gavaia et al. (2002) and was grouped as described in Table 4.2.

The use of the parameters for the determination of larval quality, such as color and tail condition, implies a human factor. This quality evaluation was always made by two persons for reduction of possible bias. Sole pigmentation pattern was ranked in complete and not developed pigmentation of the body and in two colors (beige and grey) at 20, 40 and 68 DAH. Color may have several nuances for beige (normal color) and grey so the determinations were always made with the agreement of both researchers. In the tail condition factor, the same protocol was used. Sole tail condition (% of complete tail presented) was done at the same age and was ranked as follows: 0% total absence of complete tail, 25% presence of a quarter of the complete tail, 50% presence of half of the complete tail, 75% presence of three quarters of the complete tail and 100% presence of complete tail.

4.2.4. Data analysis

All data of dry weight (mg), total length (mm) and specific activity of digestive enzymes from sole postlarvae are means ± standard deviation (S.D.) of treatment replicates (n = 3) for ST and Rot9 treatments, and n = 2 for ArTR treatment during the pelagic phase. During the benthic phase all data from sole postlarvae are means ± standard deviation (S.D.) of treatment replicates (n = 3). Growth, expressed as relative growth rate (RGR, %/day), was calculated, at the end of the pelagic and benthic phase, using the formula: $(e^{g}-1) \times 100$, with g = [(ln final weight – ln initial weight)/time] (Ricker, 1958). Condition factor (K) for each treatment was calculated using the formula: (fish weight/total length^3) x 100. One-way ANOVA was used to test differences between treatments. Differences were considered significant when $P<0.05$. When differences were found ($P<0.05$), Tukey´s Honest Significant Difference (HSD) test was used to determine which specific treatments differed significantly. Postlarvae quality analysis was performed using the chi-square test. All statistical analysis was carried out using the Statistica 5.1 package software (StatSoft, Tulsa, USA).

Table 4.2 – List of considered skeletal anomalies in sole postlarvae grouped by region and analyzed structures, and possible anomalies.

Region	Analyzed structures (anomalies)
A. Cephalic (1st–2nd vertebra; carrying epipleural ribs)	**Head (vertebral fusion/malformation)**
B. Pre-haemal or Pleural	**Pleural vertebrae (fusion/malformation)**
(With open haemal arches carrying epipleural or pleural ribs, without haemal spine)	**Pleural neural arch (malformed)**
	Parapophyse (malformed)
C. Haemal or Pre caudal	**Caudal vertebrae (fusion/malformation)**
(With haemal and neural arches closed by spines)	**Caudal neural arch (malformed)**
	Caudal hemal arch (malformed)
D. Caudal	**Pre-ural 1-3 (fusion/malformation)**
(With haemal and neural arches closed by modified spines)	**Urostyle (malformed)**
	Neural arch modified (malformed)
	Hemal arch modified (malformed)
	Epural (deformed, absent, fused, supernumerary)
	Hypural 1-5 (deformed, absent, fused, supernumerary)
	Parahypural (deformed, absent, fused)
E. Anal fin	**Anal fin rays (deformed, absent, fused, supernumerary)**
	Anal pterygophores (deformed, absent, fused, supernumerary)
F. Caudal fin	**Caudal fin rays (deformed, absent, fused, supernumerary)**
G. Dorsal soft rays	**Dorsal rays (deformed, absent, fused, supernumerary)**
	Dorsal pterygophores (deformed, absent, fused,

	supernumerary)
H. Pelvic fin	**Pelvic fin (malformed)**

4.3. Results

4.3.1. Pelagic phase

4.3.1.1. Growth

Feeding regimes had no significant effect on larval growth in the second and third samplings points, 5 and 9 DAH respectively; none of the three treatments was affected in growth, condition factor or RGR by the feeding regimes (Fig. 4.1). By the end of the pelagic phase, when the postlarvae were 20 DAH, sole that were co-fed with inert diet from mouth opening (ArtR treatment) were significantly smaller in weight than postlarvae fed exclusively with live prey (ST and Rot9 treatment) (Fig. 4.1). Relative growth rate was not affected by the different feeding regimes and averaged 22.20%/day. Survival at 20 DAH was not affected by the feeding regimes and was 47.39 ± 0.98 %, 49.35 ± 13.26 % and 49.35 ± 1.20 %, in Rot9, ArtR and ST treatments respectively.

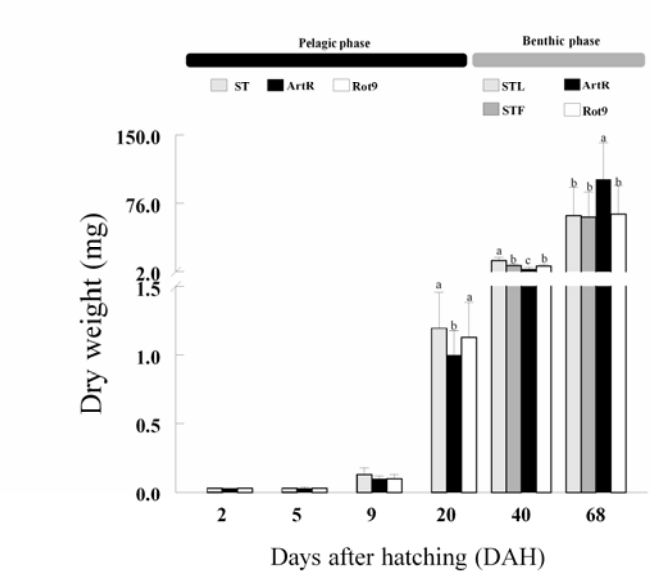

Fig. 4.1 – Sole dry weight during the pelagic phase (2 to 20 DAH) and the benthic phase (40 to 68 DAH) of the experiment. Refer to text and Table 4.1 for description of treatment abbreviations. During the pelagic phase, values are means (± SD) of treatment replicates (n = 3 for ST and Rot9 treatment, and n = 2 for ArtR treatment). During the benthic phase, values are means (± SD) of treatment replicates (n = 3). Different letters indicate statistical differences (P < 0.05, Tukey's test) between postlarvae from different treatments at the same age. Y-axis scale is linear but interrupt to better clarity of the results.

4.3.1.2. Enzymatic activity

The feeding regimes had no effect on the larvae trypsin specific activity (Fig. 4.2A) during this phase. Trypsin specific activity decreased in larvae from ArtR and Rot9 between 2 and 5 DAH. A two-fold increase of the activity occurs from 5 to 9 DAH, followed by a decrease to 20 DAH.

Larvae amylase specific activity (Fig. 4.2B) was not affected by the feeding regimes. The activity in larvae had a two-fold increase between 2 and 5 DAH in ST treatment and then decrease until the end of this phase in all treatments.

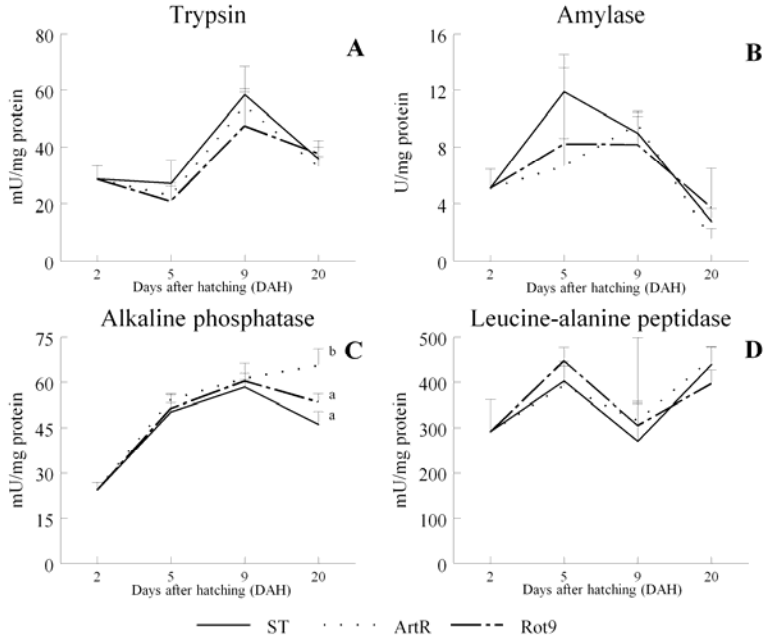

Fig. 4.2 – Specific activity of trypsin (A), amylase (B), alkaline phosphatase (C) and leucine-alanine peptidase (D) in whole sole during the pelagic phase (2 to 20 DAH) of the experiment. Refer to text and Table 4.1 for description of treatment abbreviations. Values are means (± SD) of treatment replicates (n = 3 for ST and Rot9 treatment, and n = 2 for ArtR treatment). Different letters indicate statistical differences (P < 0.05, Tukey's test) between postlarvae from different treatments at the same age.

A twofold increase of specific activity of alkaline phosphatase (Fig. 4.2C) occurred between 2 and 5 DAH larvae, followed by a small increase until 9 DAH. At the end of this phase larvae from ArtR treatment had significantly higher activity than larvae from other treatments.

Values of leucine-alanine specific activity increased between 2 and 5 DAH (Fig. 4.2D), from 5 to 9 DAH the activity decreased, and at 20 DAH increased once more. The specific activity of this enzyme was not affected by the feeding regimes.

4.3.1.3. Skeletal evaluation

Feeding regimes had no significant effect in the distribution of skeletal deformities per structure or on the incidence of deformities per postlarvae at the end of the pelagic phase (Tables 4.3 and 4.4).

Postlarvae from all treatments presented similar patterns of skeletal deformities. Less than 20% of postlarvae had no deformities and less than 10% presented 5 or more deformities. The higher values were present in the rank of 2 deformities per postlarvae, and values were between 33.6 and 28.9% in ST and ArtR, respectively. Most of the observed deformities affected the caudal vertebrae, followed by the haemal or pre-caudal vertebrae area. The caudal fin did not present any deformities.

4.3.1.4. Quality evaluation

Sole pigmentation was incomplete in all the larvae at the end of the pelagic phase, 20 DAH, and without any significant difference between the feeding regimes (Table 4.5).

Feeding regimes had a significant positive effect on larvae color (Table 4.5). Treatment ArtR had more beige larvae (95%) than the remaining treatments. A longer feeding protocol of rotifers produces a higher number of grey larvae. Tail condition was not affected by treatment (Fig. 4.4), and the majority of the fish presented a complete tail at the end of the pelagic phase.

4.3.2. Benthic phase

4.3.2.1. Growth

The feeding regimes had a significant impact on postlarvae weight at 40 DAH (Fig. 4.1). The postlarvae that consumed live *Artemia* (STL treatment) were significantly larger than postlarvae that ate frozen *Artemia* (STF treatment) (Fig. 4.1). The postlarvae that had been fed with rotifers or *Artemia* alone in the pelagic phase presented a significantly

higher weight when compared to the postlarvae that were co-fed with inert diet from mouth opening (Fig. 4.1).

At the end of weaning, 68 DAH, postlarvae that were co-fed with inert diet from mouth opening were significantly larger than all the postlarvae from the remaining feeding regimes (Fig. 4.1). Postlarvae that were sudden weaned presented similar weight at the end of the weaning period (Fig. 4.1). Postlarvae from ArtR and STF treatments showed a significantly higher condition factor (results not show). The RGR values ranged between 5.52%/day and 11.47%/day in postlarvae from STL and ArtR respectively. Survival rates from 20 to 68 DAH were not affected, and averaged 13.09±7.66%, 24.15±8.14%, 22.45±5.48% and 25.11±4.68%, in STL, STF, ArtR and Rot9 treatments, respectively.

4.3.2.2. Enzymatic activity

The postlarvae alkaline phosphatase specific activity (Fig. 4.3A) showed a twofold increase between the sampling points, 40 and 68 DAH, but was not significantly affected by the feeding regimes.

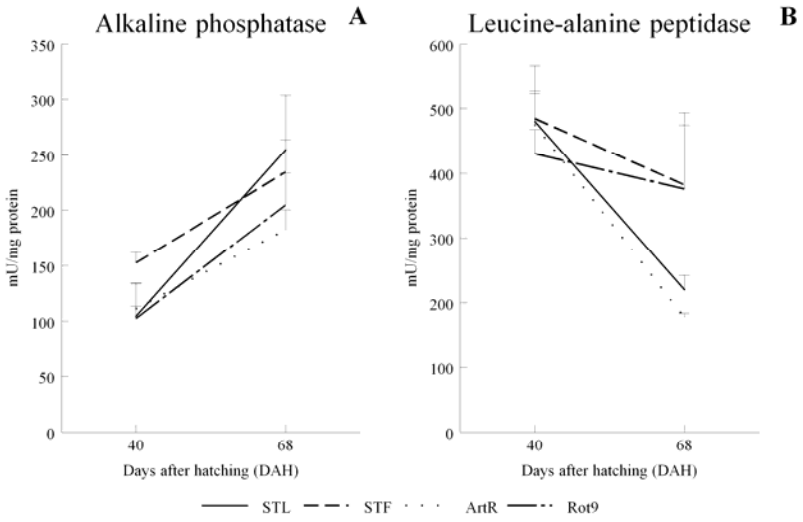

Fig. 4.3 – Specific activity of alkaline phosphatase (A) and leucine-alanine peptidase (B), in dissected sole postlarvae during the benthic phase, (40 and 68 DAH). Refer to text and Table 4.1 for description of treatment abbreviations. Values are means (± SD) of treatment replicates (n = 3). There were no significant differences between treatment means within

day.

The postlarvae leucine-alanine peptidase activity (Fig. 4.3B) was not significantly influenced by the feeding regimes. However leu-ala activity showed a tendency for a strong decrease in postlarvae from STL and ArtR that was not observed in the postlarvae from STF and Rot9.

4.3.2.3. Skeletal evaluation

Feeding regimes had no effect on the distribution of skeletal deformities per structure of sole at 40 and 68 DAH (Table 4.3). Most of the observed deformities where in the caudal vertebrae, followed by the haemal or pre caudal vertebrae area. The cephalic vertebrae and pelvic fin did not present any deformity.

The frequency of skeletal deformities at 40 DAH (Table 4.4), show that postlarvae from STF treatment were significantly different from postlarvae of ArtR. 34.9% of the postlarvae from STF had no deformities, while sole from ArtR presented 15.5%. Postlarvae from Rot9 showed significant differences from postlarvae of STL and STF treatments. Postlarvae at 68 DAH presented a higher value of absence of deformities than at 40 DAH. Values ranged between 42.2% in ArtR and 34.9% in Rot9.

4.3.2.4. Quality evaluation

Postlarvae pigmentation was complete at 40 DAH (Table 4.5). The feeding regimes had no impact on postlarvae pigmentation, at 40 and 68 DAH. Almost all of the postlarvae at 40 DAH had beige color (Table 4.5), without significant differences between feeding regimes. At the end of the experiment, 68 DAH, almost all postlarvae regardless of the treatment were grey.

Postlarvae tail condition was affected by the feeding regimes at 40 DAH (Fig. 4.4). Sole from ArtR and Rot9 were significantly better compared to sole in STL. The presence of a complete tail (100%) was only observed in a few postlarvae from STL (8.6%) and ArtR (10.0%). At the end of the experiment sole tail condition from ArtR and Rot9 were significantly better compared to STL and STF postlarvae.

Table 4.3 – Frequency (%) of sole skeletal deformities on each affected structure at the end of the pelagic phase (20 days after hatching, DAH), beginning of weaning (40 DAH), and at the end of the benthic phase (68 DAH).

Structure	20 DAH			40 DAH				68 DAH			
	ST	ArtR	Rot9	STL	STF	ArtR	Rot9	STL	STF	ArtR	Rot9
A. Cephalic vertebrae	**0.0**	**0.0**	**0.6**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
B. Pre haemal or Pleural vertebrae	**5.2**	**1.7**	**4.7**	**0.0**	**3.6**	**3.4**	**0.0**	**0.0**	**5.5**	**4.4**	**4.4**
C. Haemal or Pre caudal vertebrae	**31.8**	**29.9**	**33.7**	**28.1**	**20.2**	**20.2**	**29.6**	**20.8**	**21.8**	**26.1**	**35.3**
D. Caudal vertebrae	**50.0**	**54.7**	**48.3**	**43.8**	**57.1**	**48.3**	**44.4**	**62.5**	**52.7**	**41.3**	**38.2**
E. Anal fin	**5.2**	**6.0**	**4.7**	**13.5**	**6.0**	**5.6**	**4.9**	**4.2**	**9.1**	**13.0**	**5.9**
F. Caudal fin	**0.0**	**0.0**	**0.0**	**8.3**	**10.7**	**13.5**	**14.8**	**8.3**[a]	**0.0**[b]	**0.0**[b]	**1.5**[b]
G. Dorsal soft rays	**7.8**	**7.7**	**7.6**	**6.3**	**2.4**	**9.0**	**6.2**	**4.2**	**10.9**	**15.2**	**14.7**
H. Pelvic fin	**0.0**	**0.0**	**0.6**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**

During the pelagic phase, ST: Standard feeding regime; ArtR: *Artemia* replacement feeding regime and Rot9: Rotifers until 9 DAH feeding regime. During the benthic phase, STL: Standard live *Artemia* metanauplii until weaning feeding regime; STF: Standard frozen *Artemia* metanauplii until weaning feeding regime; ArtR: *Artemia* replacement feeding regime and Rot9: Rotifers until 9 DAH feeding regime. Values are observation of 90 postlarvae at 20 DAH, 45 postlarvae at 40 and 68 DAH per treatment. Values with different letters are significant different ($P < 0.05$) between treatments for the analyzed structure at the same age.

Table 4.4 - Frequency (%) of the incidence of deformities per fish at the end of the pelagic phase (20 days after hatching, DAH), beginning of weaning (40 DAH), and at the end of the benthic phase (68 DAH).

	0 defs.	1 def.	2 defs.	3 defs.	4 defs.	≥ 5 defs.
20 DAH						
ST	**17.9**	**26.1**	**33.6**	**8.2**	**7.5**	**6.7**
ArtR	**11.1**	**25.2**	**28.9**	**17.8**	**13.3**	**3.7**
Rot9	**14.9**	**23.4**	**30.1**	**14.5**	**11.5**	**5.6**
40 DAH						
STL	**24.6**	**22.4**	**16.3^{bc}**	**18.4**	**10.1^{a}**	**8.3**
STF	**34.9**	**21.6**	**10.8^{c}**	**10.8**	**10.8^{a}**	**11.2**
ArtR	**15.5**	**26.6**	**29.4^{ab}**	**8.3**	**13.7^{a}**	**6.8**
Rot9	**22.7**	**23.0**	**35.6^{a}**	**8.3**	**2.2^{b}**	**8.3**
68 DAH						
STL	**38.2**	**25.5**	**12.0**	**6.0**	**12.0**	**6.4**
STF	**35.8**	**26.3**	**14.7**	**13.0**	**4.9**	**5.3**
ArtR	**42.2**	**13.1**	**9.9**	**17.4**	**13.8**	**3.6**
Rot9	**34.9**	**26.1**	**15.1**	**8.8**	**11.3**	**3.9**

defs.: deformities; def.: deformity; Refer to text and Table 4.3 for description of treatment abbreviations. Values are observation of 90 postlarvae at 20 DAH, 45 postlarvae at 40 and 68 DAH per treatment. Values with different letters are significant different ($P < 0.05$) between treatments for the number of deformities at the same age.

Table 4.5 - Frequency (%) of pigmentation (complete and not developed) and color (beige and grey) on postlarvae at different ages (20, 40 and 68 days after hatching, DAH).

	20 DAH			40 DAH				68 DAH			
	ST	**ArtR**	**Rot9**	**STL**	**STF**	**ArtR**	**Rot9**	**STL**	**STF**	**ArtR**	**Rot9**
Pigmentation											
Complete	0.0	0.0	0.0	96.7	91.7	96.7	100.0	96.7	98.3	100.0	100.0
Not developed	100.0	100.0	100.0	3.3	8.3	3.3	0.0	3.3	1.7	0.0	0.0
Color											
Beige	75.0[a]	95.0[b]	56.7[c]	100.0	100.0	100.0	96.7	0.0	0.0	0.0	3.3
Grey	25.0	5.0	43.3	0.0	0.0	0.0	3.3	100.0	100.0	100.0	96.7

Refer to text and Table 4.3 for description of treatment abbreviations. Values are observation of 90 postlarvae at 20 DAH, 45 postlarvae at 40 and 68 DAH per treatment. Values with different letters are significant differences ($P < 0.05$) between treatments for the same age.

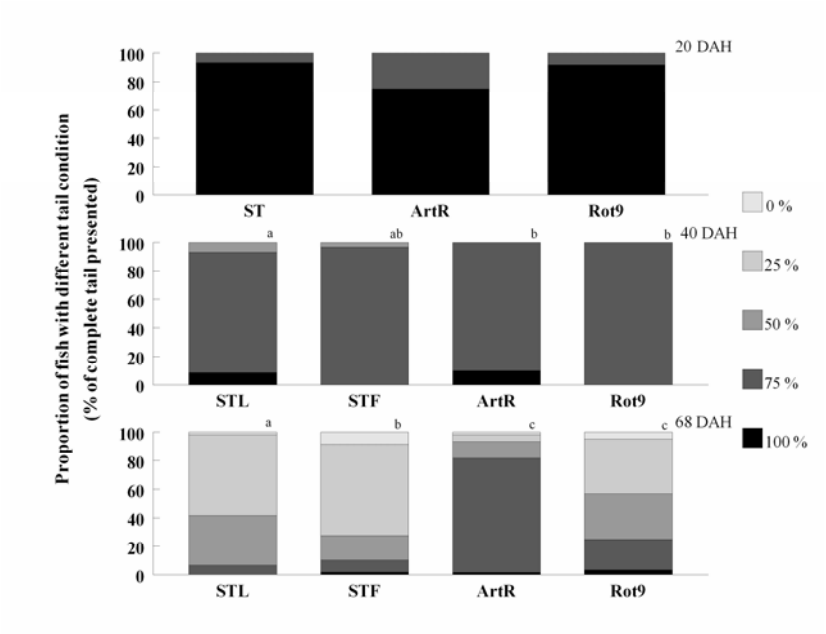

Fig. 4.4 - Distribution of the proportion of fish with different tail condition at 20 DAH, at 40 DAH, and at the end of the experiment, 68 DAH. Refer to text and Table 4.1 for description of treatment abbreviations. Different letters indicate statistical differences (P < 0.05, Chi-square test) between different treatments at the same age.

The best results were observed in postlarvae from ArtR where 80% of the sole had 75% presence of a complete tail.

4.4. Discussion

The effect of feeding regime on growth, enzymatic activity and larval quality of sole was evaluated in this study. Feeding regime had no or little effect on these parameters at 20 DAH. Nevertheless at the end of the experiment, 68 DAH, postlarvae that were co-fed with inert diet from mouth opening presented the best performance and quality.

4.4.1. Co-feeding with inert diet at mouth opening promotes better growth in sole at weaning

The results of this study demonstrate that co-feeding sole with inert diet from mouth opening will produce better quality fish at weaning. Sole postlarvae from ArtR treatment were larger and presented a better tail condition than postlarvae from remaining treatments. The previous work of Cañavate and Fernández-Díaz (1999) already indicated that sole larvae could be co-fed with inert diet at mouth opening. Nevertheless the sole

weight observed by those authors at the end of the experiment was twofold smaller than in the present study.

Weaning started when the postlarvae were between 5-10 mg DW as recommended by Engrola et al. (2007). Postlarvae weight at 68 DAH was several times higher than in previous studies (Cañavate and Fernández-Díaz, 1999; Ribeiro et al., 2005) but similar to the studies of Engrola et al. (2005; 2007). Several reasons may explain the better results recently reported, like different zootechnical conditions, improved feeding and weaning strategies, and different inert diets. Inert diets have experienced major improvements during the past years. Not only in physical properties, such as shape, size, sinking properties, color and leaching, but also in chemical properties, ingredients, inclusion of hydrolyzed protein and attractants, digestibility, amongst others (Kvåle et al., 2006). In addition, the inclusion of hydrolyzed protein in larval feeds promotes growth, and increases survival in European seabass (Zambonino Infante et al., 1997), Dover sole (Day et al., 1997) and Atlantic halibut (Tonheim et al., 2005). So the higher growth observed in the present study is a combination of several improvements made in the past years. Survival was lower in the present study when compared to previous sole weaning experiments (Cañavate and Fernández-Díaz, 1999; Engrola et al., 2005; 2007). However, it was similar among treatments, implying that the tested feeding regimes had no effect on the observed survival rates. Mortality occurred mostly two weeks after weaning started (40 DAH) in all treatments.

At the end of the pelagic phase, co-fed sole were smaller than sole fed live prey. These results contradict observations made in Atlantic halibut (Rosenlund et al., 1997) and Atlantic cod (Fletcher et al., 2007), where a co-feeding strategy of *Artemia* and inert diet can produce comparable growth when compared with *Artemia* alone. However at the end of weaning, postlarvae from ArtR treatment were larger than the remaining sole, suggesting that they were pre-conditioned onto inert diet (Vega-Orellana et al., 2006) and presented a faster maturation of the digestive tract (Cahu and Zambonino Infante, 1997). These results agree with the recent findings by Engrola et al. (2007), where the authors suggested that a co-feeding strategy in earlier stages, sole smaller than 2 mg DW, may promote growth during weaning. As observed by the authors when small postlarvae are sudden weaned they presented a period where growth is suppressed. On the other hand, 5 to 10 mg DW sole postlarvae fed exclusively with live prey until weaning presented similar growth when sudden weaned or in a co-feeding regime (Engrola et al., 2007) at the end of the weaning period.

4.4.2. Maturation of the digestive tract was positively affected by inert diet

The pattern of enzyme activity described for other species such as European seabass and red drum (Lazo et al., 2000b; Zambonino Infante and Cahu, 2001), Japanese flounder (Bolasina et al., 2006), and yellowtail kingfish (*Seriola lalandi*) (Chen et al., 2006), was also observed in the present study, as previously illustrated in the same species, Senegalese sole, by Martínez et al. (1999) and Ribeiro et al. (1999). An increase of enzyme activity until the beginning of metamorphosis, which starts around 11 DAH in sole, is followed by a constant decline. This decline is not caused by a reduction in enzyme synthesis but as result of larval growth (Zambonino Infante and Cahu, 2001).

During the pelagic phase only the alkaline phosphatase activity was affected by the feeding regimes. Postlarvae from ArtR presented higher activity than other postlarvae at the end of the pelagic phase. Brush border purification was not done in this study, due to insufficient size of the sample; nevertheless according to Cahu et al. (2000) and Ribeiro et al. (2002) the pattern of variation alkaline phosphatase brush border activity resembles the activity determined in the homogenate, despite the observed difference in magnitude of values. Therefore, as suggested by these authors the higher value presented by ArtR postlarvae might indicate an earlier intestinal maturation and a faster development of digestive capacity. At the end of weaning no differences were observed in the postlarvae enzymatic activity. This may indicate that digestive structures were fully developed and that sole had acquired an adult mode of digestion, as suggested by Hamza et al. (2007).

4.4.3. Co-feeding regime did not impair the normal development of skeletal structures

No negative effects of the feeding regimes were observed on the frequency of deformities of the axial and appendicular skeleton of the sole during the experiment. The highest incidence of skeletal deformities during the pelagic phase could be related to the importance of the described structures in the swimming and feeding performance of the postlarvae, particularly in early stages of their development as reported for other species like red seabream (Kohno et al., 1983), rabbit fish (*Signatus guttatus*) (Kohno et al., 1986) or Asian seabass (Kohno et al., 1996).

The decrease in the frequency of skeletal deformities over the benthic phase may be related to the existence of a selective pressure on the postlarvae, against those with skeletal deformities that impaired their normal growth and survival (Andrades et al.,

1996; Sadler et al., 2001). The study of the incidence of skeletal deformities per individual indicated that at the end of the pelagic phase, no significant differences existed between treatments. Likewise, there were no significant differences in the sole skeletal deformities between treatments at 40 DAH or at the end of the experiment (68 DAH). Thus, the co-feeding regime applied in ArtR was able to ensure suitable nutrition to sole regarding normal development of skeletal structures.

The average deformity rate among the treatments at 68 DAH was 62%. This value is lower than the rate of about 80% of fish with deformities obtained by Engrola et al. (2005), and higher than the value of 44% reported by Gavaia et al. (2002) for the same species. The results of the present study are comparable to those found in Japanese flounder reared in captivity (Hosoya and Kawamura, 1995; Hosoya and Kawamura, 1998), for which skeletal deformities such as deformed hypural, fused spines or central fusions have been detected in 30–60% of the total number of deformities observed in the caudal complex. A decrease in the frequency of skeletal deformities was observed in the present study at the end of the experiment. The decrease in incidence of deformities with the increasing age of the specimens observed in or study is in agreement with the results obtained for Atlantic halibut larvae up to 59 days post first feeding (DPFF) (Lewis and Lall, 2006) and for sharpsnout seabream where deformities were compared between juveniles at 173 DAH and adults after one year in cage rearing (Favaloro and Mazzola, 2000).

4.4.4. Co-feeding with inert diet from mouth opening, promotes better quality sole

The sole's pigmentation was not influenced by the different feeding regimes tested throughout the experiment. Between 40 and 68 DAH, all feeding regimes supplemented the sufficient nutrients for normal pigmentation. At 40 DAH, postlarvae already showed a high frequency of complete pigmentation.

At the end of the pelagic phase, larvae fed on ArtR treatment showed higher frequency of beige color than larvae fed on other treatments (ST and Rot9). It has been proposed that these differences in color of the larvae at 20 DAH are related to different levels of stress in larvae. Ruane et al. (2005) showed that for the same age, sole larvae with a darker color had significantly higher cortisol levels than sole with lighter color.

Tail condition was affected by the feeding regimes. Larvae that were co-fed with inert diet from mouth opening presented a better tail condition. In the present study tail condition was used as an indicator of fin erosion and nutritional status. Fin erosion

problems are also commonly reported in other species, such as Atlantic salmon (Noble et al., 2008) and Atlantic cod (Hatlen et al., 2006), and normally imply welfare problems and have an economic impact. Several factors can promote fin erosion, among others, aggressive behavior and restricted feeding (Damsgård et al., 1997). No direct observation of sole behavior was done in the present study and all fish were fed to satiation. In the present study, the better tail condition observed in the co-fed sole is interpreted as an indication of better nutritional status and physiological condition. Flatfish living on smooth bottom without substrate can develop wounds on the blind side even if the other environmental components in the tank are optima.

In conclusion, a co-feeding strategy with inert diet starting during the pelagic phase of sole larval rearing can improve postlarval quality. The results of the present study demonstrate that offering inert diet to sole at mouth opening in a co-feeding regime promotes growth and better quality juveniles.

4.5. Acknowledgements

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Chapter 5

Co-feeding of inert diet from mouth opening does not impair protein utilization by Senegalese sole larvae

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Co-feeding of inert diet from mouth opening does not impair protein utilization by Senegalese sole larvae

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Abstract

In most marine species inert diets alone have a poor ability to sustain fish larvae growth and development. Furthermore, results of co-feeding inert diets and live prey are variable, what may be related to the effect of inert diets on digestive maturation and subsequently protein utilization. The aim of the present work was to investigate how different feeding regimes, live feed alone or co-fed with inert diet, influence protein utilization in Senegalese sole larvae. Feed intake, protein absorption, protein retention and protein catabolism were estimated in sole from 8 to 35 days after hatching (DAH), using ^{14}C-labelled *Artemia* protein and posterior incubation in metabolic chambers. Postlarvae that were co-fed with inert diet from mouth opening ate more than postlarvae fed *Artemia* alone in most sampling ages. Sole *Artemia* protein digestibility ranged between 56.97% (16 DAH) and 81.32% (22 DAH). Sole larvae that were fed a second meal presented a slightly, though significant, higher digestibility than sole fed a single meal. Digestibility was lower in co-fed sole during metamorphosis climax, and similar between treatments at other developmental stages. Retention efficiency remains almost constant during early development, and was not affected by feeding regime. In short, co-feeding of inert diet from mouth opening does not impair protein utilization by Senegalese sole larvae.

Keywords: Feed intake; Protein metabolism; Digestibility; *Solea senegalensis*; Weaning.

5.1. Introduction

In most marine species compounds diets fed alone have a poor ability to sustain fish larvae growth and development (e. g. Cañavate and Fernández-Díaz, 1999; Robin and Vincent, 2003; Curnow et al., 2006a). The low performance usually observed when delivering inert diet from mouth opening to marine fish larvae might be due to sub-optimal diet composition and the larval poor ability to modulate its digestive enzymes (Cahu and Zambonino Infante, 2001). Therefore, feeding regimes based on a co-feeding strategy have been proposed for farmed species, such as dorado (Vega-Orellana et al.,

2006), Asian seabass (Curnow et al., 2006b), pikeperch (*Sander lucioperca*) (Hamza et al., 2007), and Atlantic cod (Rosenlund and Halldórsson, 2007).

Weaning success of Senegalese sole (*Solea senegalensis*) is still a critical step given its variability (Conceição et al., 2007b). Senegalese sole weaning can be accomplished with different strategies, sudden or co-feeding (Cañavate and Fernández-Díaz, 1999; Engrola et al., 2005; Ribeiro et al., 2005). However, the choice of the feeding strategy to adopt should be based on postlarvae weight (Engrola et al., 2007); with sudden weaning normally leading to poor results in fish smaller than 25 mg wet weight. In fact, when early co-feeding regimes are applied growth retardation at the end of the pelagic phase is commonly observed (e.g., Engrola et al., submitted for publication). In most cases sole does not recover, with growth potential being impaired, until at least the early juvenile stage (own unpublished observations). Still, it has been shown that is possible to produce larger postlarvae at 68 days after hatching (DAH) and with better tail condition, when sole are co-fed with live prey and inert diet from mouth opening, compared to a live feed regime (Engrola et al., submitted for publication). Therefore, it remains to be established to what extent early co-feeding regimes might affect sole growth, digestive capacity, and in particular protein utilization. It has been proposed that intestinal maturation might be stimulated or irreversible impaired, depending on how co-feeding of live prey and inert diets is performed (Cahu and Zambonino Infante, 2001).

Growth and survival are the most common and practical criteria to determine if a feeding regime is suitable or not for a given fish species. In addition, feed intake in fish larvae is in general determined by visual counting of ingested prey (Haylor, 1993; MacKenzie et al., 1999), or particles of inert diet (Yúfera et al., 1995). This makes estimation of feed intake quite time consuming and often inaccurate. Therefore, tools using tracer nutrients have been proposed to determine the impact of a feeding regime in fish larvae (see review by Conceição et al., 2007a). In particular, using a methodology based on radiolabelled *Artemia* protein (Morais et al., 2004a) it is possible to determine feed intake, and how the ingested protein is digested, retained and catabolized by fish. The use of such a methodology in distinct larval phases allows an understanding of the larvae digestive development and how larvae are copping at the metabolic level. Hence, Morais et al. (2004b) observed that Senegalese sole larvae have high *Artemia* protein digestibility (73-83% of intake), between 12 and 35 DAH. This indicates that sole have a high digestive capacity for digesting live preys since young ages. Still, Morais et al. (2004b) mentioned that digestibility might be overestimated as the determinations were

based on sole given a single meal, with larvae being subsequently deprived of feed. When larvae are feed several meals (or continuously), a lower digestibility may occur, due to an increased evacuation rate (Boehlert and Yoklavich, 1984). In fact, Morais et al. (2004b) used a hot-chase approach, in which the possibility of such an overestimation cannot be excluded (Conceição et al., 2007a). However, this uncertainty may be removed using a cold-chase approach. This approach differs from the hot-chase, in that after feeding larvae the diet containing the tracer nutrient, one or more meals of an identical non-labeled (i.e., cold) diet is given (see Conceição et al., 2007a). The cold-chase approach is believed to reproduce a more realistic feeding regime.

The aim of the present work was to investigate how different feeding regimes, live feed alone or co-fed with inert diet, influence protein utilization in Senegalese sole larvae. Feed intake, protein digestibility, retention and catabolism were estimated in sole from 8 to 35 DAH. Both cold and hot-chase approaches were used. A comparison was also made between the two approaches at 16 DAH, in order to assess if digestibility and retention efficiency are influenced by a second meal.

5.2. Materials and Methods

5.2.1. Larval rearing

Eggs were obtained from natural spawning of wild Senegalese sole broodstock kept at the Ramalhete facility at the University of Algarve (Faro, Portugal). Newly hatched larvae were reared in 200 L cylindro-conical tanks in a closed recirculation system with an initial density of 100 larvae L^{-1}. Treatments were randomly assigned and run in triplicates. Treatments consisted on the standard live feed feeding regime (Standard, ST) and live feed co-fed with inert diet from mouth opening feeding regime (*Artemia* replacement, ArtR). At larvae mouth opening (2 DAH) rotifers (*Brachionus rotundiformis*) enriched with DHA Protein Selco (Inve, Belgium) were offered in both treatments (ST and ArtR), and remain until larvae had 5 DAH (Fig. 5.1). Larvae from ArtR treatment were also fed with inert diet Proton (100-200 μm-Inve, Belgium) from mouth opening.

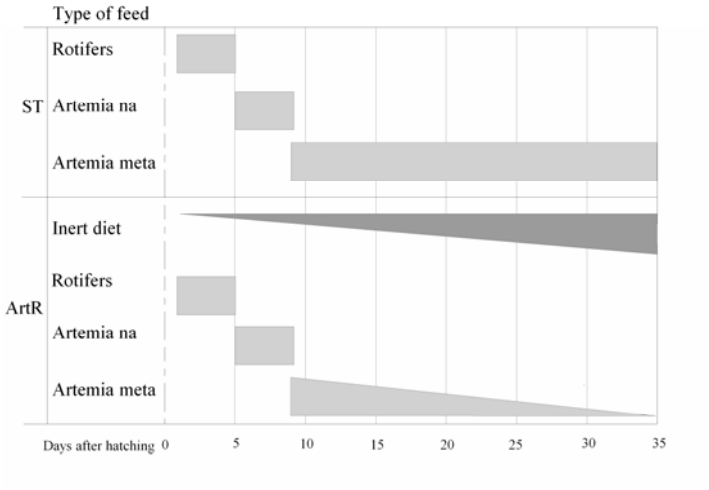

Fig. 5.1 – Feeding regime of sole larvae and postlarvae from 2 to 35 days after hatching (DAH). ST: Standard live feed feeding regime; ArtR: Live feed co-fed with inert diet from mouth opening feeding regime. *Artemia* na: *Artemia* nauplii; *Artemia* meta: *Artemia* metanauplii.

Artemia replacement treatment started with a 25% supplement of inert diet of total daily ration. From 5 to 8 DAH larvae from ST and ArtR treatments were also fed with *Artemia* nauplii (Inve, Belgium). From 9 DAH onwards, *Artemia* metanauplii enriched with Easy DHA Selco (Inve, Belgium) and AgloNorse Microfeed (Ewos, Scotland) were provided to the larvae in ArtR treatment. Larvae from ST were fed exclusively with enriched *Artemia* metanauplii from 11 DAH onwards. Larvae from ArtR treatment had a progressive reduction in *Artemia* concomitant with an increase of inert diet as described in Engrola et al. (submitted for publication), meaning that sole were being offered 45% *Artemia* metanauplii and 55% inert diet of the total daily ration at 19 DAH (Fig. 5.1). From 2 to 19 DAH pelagic sole were reared at 19.9±0.4 ºC (mean±SD) water temperature, and a salinity of 37.2±0.8‰. Dissolved oxygen in water was 91.0±0.8% of saturation.

Settled sole were obtained from eggs of natural spawning of wild Senegalese sole broodstock kept at the facilities of the IPIMAR–CripSul (Olhão, Portugal). Pelagic larvae were reared as described previously. Fish were transferred to white flat-bottomed fiber glass tanks of 21 L (width 30 cm x length 70 cm x height 10 cm), after settling at the bottom of the tank (19 DAH). Each tank was stocked with 630 postlarvae, corresponding to a density of 3000 individuals/m^2. The same feeding regimes (ST and ArtR) were maintained after settlement (Fig. 5.1). Settled sole from both treatments were fed with

frozen *Artemia* metanauplii enriched with Easy DHA Selco (Inve, Belgium) until the end of the experiment. *Artemia* metanauplii was harvest, washed in seawater, counted, and frozen a -20ºC freezer. Fifteen minutes before feeding, *Artemia* was thawed in seawater. Seawater was then removed, and new seawater was added before feeding to the sole tanks. Sole from ArtR treatment had a proportion of total daily ration of 45% frozen *Artemia* metanauplii and 55% inert diet until the end of the experiment. *Artemia* replacement sole were hand fed with AgloNorse no.1 (0.2-0.6 mm-Ewos, Scotland). Settled sole were reared at 20.9±0.7 ºC water temperature, and salinity of 37.6±0.5‰. Dissolved oxygen in the water was at 93.0±0.4% of saturation. The experiment ended at 35 DAH.

5.2.2. Protein utilization trials

5.2.2.1. *Artemia* [U-^{14}C] labelling

Artemia was radiolabelled with a [U-^{14}C] protein hydrolysate (1.85 MBq ml Amersham Pharmacia Biotech Ltd., UK) according to the method developed by Morais et al. (2004a). *Artemia* nauplii were enriched at a density of 200 *Artemia*/ml in a sealed incubation system at 28ºC, with a dose of 3.5µl of the [U-^{14}C] protein hydrolysate per ml of seawater.

The incubation system consisted in an aquarium with controlled temperature and an incubation bottle connected to a KOH trap to capture radiolabelled $^{14}CO_2$. The incubation lasted 14 hours. After this period *Artemia* metanauplii was washed several times, counted and samples (n = 4, 3ml each sample) were taken to measured the incorporated radiolabel. Samples of seawater of the beaker containing the radiolabelled *Artemia* were also taken (n = 4, 3ml each sample) to be able to correct for the ^{14}C present in the incubation seawater.

5.2.2.2. Sole metabolic trials

5.2.2.2.1. Trial 1

This trial was designed in order to assess if feeding regime could influence feed intake, and how larvae utilizes protein at early developmental stages, pre-metamorphic (8 DAH) and at metamorphosis climax (16 DAH) (Fernández-Díaz et al., 2001). A cold-chase approach was used, feeding larvae with radiolabelled *Artemia* (hot) followed by a second meal of non-labelled *Artemia* (cold). In addition, at 16 DAH a hot-chase approach was also used in order to assess if protein digestibility and retention efficiency are influenced

by a second meal. This age was chosen as this was the period when more significant differences would be expected, because larvae are at metamorphosis climax a challenging and sensitive life stage (Blaxter, 1988).

On the evening prior to the measurements of feed intake and protein utilization sole larvae were transferred to 1 L tanks at the radiolabelling laboratory (20±1 ºC) and deprived from feed for 16h. Measurements were conducted at two larvae ages: 8 DAH (ST, 0.15 mg DW and ArtR, 0.14 mg DW) and 16 DAH (ST, 0.71 mg DW and ArtR, 0.48 mg DW). At 8 DAH, 20 larvae from both ST and ArtR treatments were used for the cold-chase trial. At 16 DAH, 20 larvae for cold-chase and 10 larvae for hot-chase trials were used, in both treatments.

The cold-chase assays were performed in 8 and 16 DAH sole larvae. Larvae were allowed to eat the radiolabelled *Artemia* (hot) during 30 minutes; this period is a trade-off between the time necessary for a complete meal size and to avoid significant losses by larvae catabolism. After this period larvae were transferred to a tray with clean seawater (to eliminate any ^{14}C amino acids that could be present in the surface of the fish), and subsequently transferred to the incubation vial.

The incubation setup was described by Rønnestad et al. (2001). In brief, the incubation setup consists in sealed vials containing 7.5ml of seawater with gentle air flow where the postlarvae were placed. The air is forced through a capillary from the incubation vial to a CO_2 trap (5.0 ml of KOH, 0.5M). After a 24h incubation period each sole was rinsed with clean seawater and sampled for analysis. After sampling the incubation vials were resealed and HCl is injected gradually to the incubation vial (total of 1.0 ml of HCl, 1M), for diffusion of remaining CO_2 in the seawater to the CO_2 trap.

Cold-chase larvae were re-fed with non-labelled *Artemia* (cold) inside the incubations vials two hours after the incubation started. The amount of *Artemia* offered to the larvae was equal to the normal amount provided to larvae in the rearing tanks. The procedure of opening, feeding and resealing the incubation vials was performed as quickly as possible (<30 sec per incubation vial).

The hot-chase trials were performed in 16 DAH sole. Sole were fed in excess the radiolabelled *Artemia* during 30 minutes. After this period larvae were kept in the incubation setup during 24h.

5.2.2.2.2. Trial 2

The aim of this trial was to determine the effect of feeding regimes on the feed intake and protein utilization of sole postlarvae just after metamorphosis climax (22 DAH) and fully metamorphosed sole postlarvae (35 DAH) taking into account digestive enzymes profile (Ribeiro et al., 1999).

On the afternoon prior to the measurements, postlarvae from each treatment (22 DAH, *n* = 20; 35 DAH, *n* = 15), were randomly collected and transferred to the laboratory. Sole were stocked in 1L tanks, at a temperature of 20±1 ºC. Postlarvae were deprived from feed during 16h and feed intake and protein utilization was determined in the next morning. Measurements were determined using the hot-chase approach in sole of 22 DAH (ST, 1.2 mg dry weight (DW) and ArtR, 1.0 mg DW) and at 35 DAH (ST, 6.8 mg DW and ArtR, 3.9 mg DW).

5.2.3. Analytical methods

5.2.3.1. Radiolabel measurements

Vials containing incubation seawater or KOH (CO_2 traps) were added scintillation cocktail (Ultima Gold XR, Packard Bioscience) and counted for radioactivity (DPM, disintegrations per minute). Sole and *Artemia* were solubilized with a tissue solubilizer (Solvable, Perkin-Elmer) and incubated at 50ºC during 24h. After cooling, scintillation cocktail (Ultima Gold XR, Packard Bioscience) was added, and samples were counted for radioactivity (DPM).

Feed intake (FI) and protein utilization criteria of fed sole was determined at 8 DAH (ST, *n* = 20, ArtR, *n* = 20), 16 DAH (ST *n* = 30, ArtR, *n* = 30), 22 DAH (ST, *n* = 20, ArtR, *n* = 20) and 35 DAH (ST, *n* = 15, ArtR, *n* = 14). Feed intake (%BDW) after a single meal was determined as:

$$FI = [(R_{total}/SR_{Artemia})/DW_{fish}] \times 100$$

as described by Conceição et al. (1998), where R_{total} is the sum of the radioactivity in the incubation seawater, in the CO_2 trap and in fish (DPM), $SR_{Artemia}$ is the specific radioactivity in *Artemia* samples (DPM/mg *Artemia* DW), and DW_{fish} is the fish dry weight (mg).

Protein utilization was determined based on protein digestibility (D, %), retention efficiency (R, %), catabolism fraction (C, %), relative retention (rR, DPM/mg fish DW), relative catabolism (rC, DPM/mg fish DW), and relative evacuation (rE, DPM/mg fish DW). These estimates were determined as:

$D = [(R_{body} + R_{CO_2\ trap})/(R_{body} + R_{CO_2\ trap} + R_{water})] \times 100;$

$R = [R_{body}/(R_{body} + R_{CO_2\ trap})] \times 100;$

$C = [R_{CO_2\ trap}/(R_{body} + R_{metabolic\ trap})] \times 100;$

$rR = R_{body}/DW_{fish};$

$rC = R_{CO_2\ trap}/DW_{fish};$

$rE = R_{water}/DW_{fish}.$

where R_{body} is the total radioactivity in fish body (DPM), $R_{CO_2\ trap}$ is the total radioactivity per CO_2 trap (DPM), and R_{water} is the total radioactivity in the incubation seawater (DPM).

Fish that did not ingest any *Artemia* during the 30 minute feeding period were excluded from the analysis. All percentage data were arcsine ($x^{1/2}$)-transformed prior to analysis. One-way analysis of variance (ANOVA) was used to compare the feed intake and protein utilization in fed sole of different treatments at the same age (8, 22 and 35 DAH), and during sole ontogeny (8, 16, 22 and 35 DAH). Differences were considered significant when $P<0.05$. When differences were found Tukey's Honest Significant Difference (HSD) test was used to determine which specific age differed significantly. Two-way analysis of variance (ANOVA) was used to investigate the interactive effects of treatment and re-feeding on digestibility, retention efficiency, and catabolism fraction, and on relative retention, relative catabolism and relative evacuation of 16 DAH larvae in trial 1. When interactions were found ($P<0.05$), Newman-Keuls test was used to determine which specific treatments were significantly different.

5.3. Results

5.3.1. Feed intake

A total of 6 and 4 larvae from ST and ArtR treatments, respectively, did not ingest any radiolabelled *Artemia* during the FI measurements at 8 DAH, and were excluded from further analysis. Feed intake was two-fold higher in 8 DAH larvae from ArtR treatment, showing values of 12.37±8.17% BDW/meal (Fig. 5.2). The number of ingested *Artemia* per fish was 4.63±1.79 in ST and 8.45±5.58 in ArtR larvae. All the 16 DAH larvae ingested *Artemia* during the second measurement. Postlarvae from ArtR treatment presented a higher FI than ST sole at 16DAH, 10.80±2.81 and 7.75±2.45% BDW/meal (Fig. 5.2), respectively.

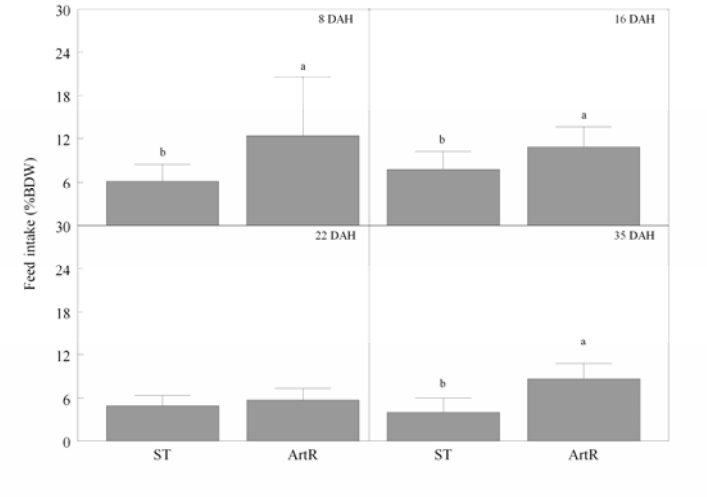

Fig. 5.2 – Feed intake of sole at 8, 16, 22 and 35 days after hatching (DAH). ST: Standard live feed feeding regime; ArtR: Live feed co-fed with inert diet from mouth opening feeding regime. Values are means ± SD (n = 14 to 20). Different letters indicate statistical differences (P<0.05, ANOVA) between sole from different treatments at the same age.

The number of ingested prey per sole was 25.99±6.75 *Artemia*/fish in ArtR and 22.47±9.66 *Artemia*/fish in sole from ST treatment. All the postlarvae from ST and ArtR treatments ingested *Artemia* at 22 and 35 DAH. At 22 DAH postlarvae from ST (4.95±1.41% BDW/meal) and ArtR (5.75±1.60% BDW/meal) presented a similar feed intake (Fig. 5.2). The number of ingested *Artemia* by sole was 29.69±8.45 and 28.76±8.02 for ST and ArtR, respectively. Postlarvae from ArtR (8.66±2.13% BDW/meal) presented significantly higher FI than ST postlarvae (4.05±1.95% BDW/meal) at 35 DAH (Fig. 5.2). Postlarvae from ST treatment ingested an average of 137.37±66.21 *Artemia*/fish, while sole from ArtR were eating 168.65±73.11 *Artemia*/fish.

5.3.2. Protein utilization

Protein digestibility and retention efficiency of 8 DAH sole larvae were not affected by feeding regime. Larvae from both treatments presented similar values of digestibility, 72.35±5.11% in ST and 70.33±5.81 in ArtR (Fig. 5.3). Retention efficiency was 65.90±5.32% and 64.44±14.90% in larvae from ST and ArtR, respectively (Fig. 5.4). However at 8 DAH values of relative retention (DPM/mg of fish) were 2 fold, relative catabolism 1.6 fold, and relative evacuation 2.3 fold, higher in ArtR larvae than in ST larvae, respectively (Fig. 5.5). Values were 27168.43±18604.09 DPM/mg of fish for

relative retention, 11144.53±6547.82 DPM/mg of fish for relative catabolism, and 18402.83±13462.45 DPM/mg of fish for relative evacuation.

Two-way ANOVA revealed that feeding regime (P<0.001), and re-feeding (P<0.001) but not the interaction of both factors (P=0.991), significantly affected the digestibility in 16 DAH larvae (Fig. 5.3 and Table 5.1). Highest digestibility was observed in larvae from ST treatment when re-fed, 65.30±2.88% (Table 5.1) at 16 DAH. However, ArtR larvae presented a decrease of only 0.06 fold in digestibility compared do ST larvae. Larvae that were fed a single meal presented a 0.06 fold higher retention efficiency (P=0.004) independently of treatment (P=0.719) or interaction of both factors (P=0.755) (Table 5.1).

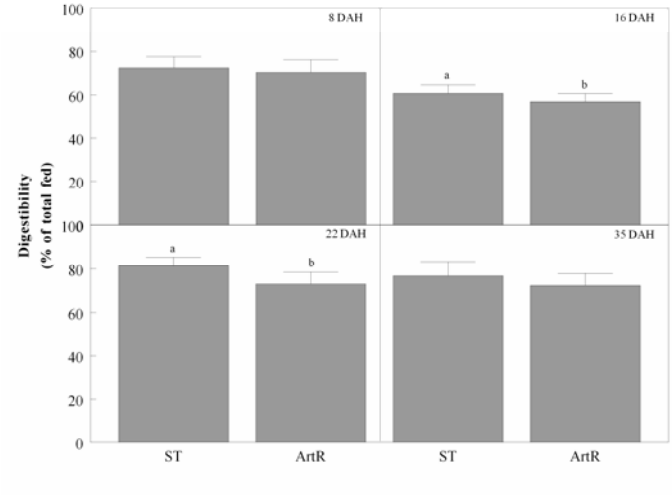

Fig. 5.3 – *Artemia* protein digestibility (radiolabel in the sole body and CO_2 trap in relation to total radiolabel fed) determined in sole at 8, 16, 22 and 35 days after hatching (DAH) after 24h of incubation. ST: Standard live feed feeding regime; ArtR: Live feed co-fed with inert diet from mouth opening feeding regime. Values are means ± SD (n = 14 to 20). Different letters indicate statistical differences (P<0.05) between sole from different treatments at the same age (8, 22, and 35 DAH). Two-way analysis of variance (ANOVA) was used at 16 DAH to determine treatment effect (P<0.05, ANOVA).

Larvae relative retention and relative evacuation (DPM/mg of fish) was influenced by feeding regime (P<0.001; P=0.016), and the interaction of the factors (P=0.003; P<0.001), but not by re-feeding (P=0.961; P=0.125) (Fig. 5.5 and Table 5.1). As a result, larvae from ArtR that were fed a single meal presented a higher relative retention (13615.58±1796.80 DPM/mg of fish) and higher relative evacuation (12514.29±2928.76 DPM/mg of fish) (Table 5.1). The lowest relative retention was

observed in larvae from ST fed one single meal (7973.00±2003.75 DPM/mg of fish) (Table 5.1).

In general, ArtR larvae fed one single meal presented the highest values of relative retention and relative evacuation, while the lowest values were observed in larvae from ST fed one single meal (Table 5.1).

After a 24 h incubation period, protein digestibility of 22 DAH postlarvae was significantly affected by feeding regime.

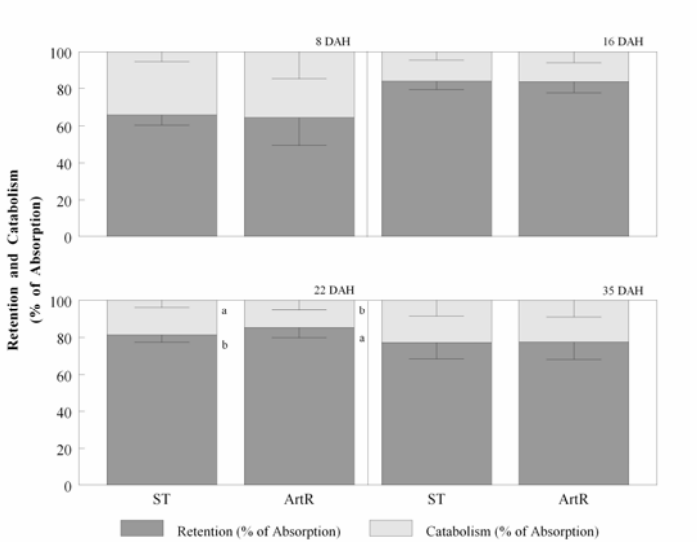

Fig. 5.4 – Retention efficiency of *Artemia* protein (radiolabel in the sole body in relation to absorbed label) and catabolism fraction (radiolabel in the CO_2 trap in relation to absorbed label) determine in sole at 8, 16, 22 and 35 days after hatching (DAH) after 24h of incubation. ST: Standard live feed feeding regime; ArtR: Live feed co-fed with inert diet from mouth opening feeding regime. Values are means ± SD (n = 14 to 20). Different letters indicate statistical differences (P<0.05, ANOVA) between treatments at the same age (8, 22, and 35 DAH). Two-way analysis of variance (ANOVA) was used at 16 DAH to determine treatment effect (P<0.05).

However, ArtR postlarvae presented only a 0.10 fold lower digestibility (72.97±5.49%) than ST postlarvae (81.32±3.94%) (Fig. 5.3). ST sole that presented highest digestibility also presented the lowest retention efficiency, 81.32±3.94% and the highest catabolism, 18.76±3.94% (Fig. 5.4). Relative evacuation (DPM/mg of fish) was significantly higher in ArtR postlarvae (13870.74±4062.70 DPM/mg of fish) than in ST postlarvae (8279.93±2854.72 DPM/mg of fish) (Fig. 5.5).

Table 5.1 –Two-way analysis of variance (ANOVA) for protein metabolism of 16 days after hatching (DAH) sole larvae (Trial 1), fed one (1x, hot-chase) or two (2x, cold-chase) meals of a standard live feed feeding regime (ST) or live feed co-fed with inert diet from mouth opening (ArtR).

	ST		ArtR		Feeding regime (FR)	Meal number (Mn)	FR x Mn
	1x	2x	1x	2x			
Digestibility **(% of total fed)**	**60.81 ± 3.82^{ay}**	**65.30 ± 2.88^{ax}**	**56.97 ± 3.65^{by}**	**61.57 ± 4.00^{bx}**	**P<0.001**	**P<0.001**	**P=0.991**
Catabolism fraction **(% of Absorption)**	**16.13 ± 4.43^{y}**	**20.71 ± 5.79^{x}**	**16.38 ± 5.91^{y}**	**21.99 ± 6.51^{x}**	**P=0.631**	**P=0.002**	**P=0.748**
Retention efficiency **(% of Absorption)**	**83.87 ± 4.43^{x}**	**79.29 ± 5.79^{y}**	**83.62 ± 5.91^{x}**	**78.01 ± 6.51^{y}**	**P=0.719**	**P=0.004**	**P=0.755**
Relative Evacuation **(DPM / mg of fish)**	**6209.41 ± 1851.49^{r}**	**6914.83 ± 2403.98^{r}**	**12514.29 ± 2928.76^{p}**	**9430.40 ± 3386.23^{q}**	**P<0.001**	**P=0.125**	**P=0.016**
Relative Catabolism **(DPM / mg of fish)**	**1610.60 ± 794.53^{by}**	**2732.57 ± 1403.50^{bx}**	**2754.70 ± 1314.24^{ay}**	**3237.64 ± 1382.18^{ax}**	**P=0.024**	**P=0.028**	**P=0.374**

Relative Retention **(DPM / mg of fish)**	**7973.00 ± 2003.75^{r}**	**10097.88 ± 2751.31^{q}**	**13615.58 ± 1796.80^{q}**	**11422.19 ± 2960.34^{p}**	**P<0.001**	**P=0.961**	**P=0.003**

Results are given as means ±SD (ST 1x, n= 10; ST 2x, n= 20; ArtR 1x, n = 10; ArtR 2x, n = 20). Different superscript letters indicate statistical differences by two-way ANOVA, in treatment (a, b), re-feeding (x, y), or interaction of both (p, q, r) on digestibility, retention efficiency, catabolism fraction of *Artemia* protein, or relative retention, relative catabolism and relative evacuation of 16 DAH larvae. Newman-Keuls test was used when interactions were found (P<0.05).

Older postlarvae, 35 DAH, were not affected by feeding regime in their digestibility and retention efficiency. The postlarvae retention efficiency was 77.27±9.31%, and 22.73±9.31% of the absorbed protein was catabolized (Fig. 5.4).

However, an influence of the feeding regime was observed in total DPM per mg of fish. ArtR sole were retaining, catabolizing and evacuating more than ST sole (Fig. 5.5). Larvae digestibility is affected by larval age. Independently of the treatment, sole presented significantly lower digestibility during metamorphosis climax-16 DAH, compared to other ages.

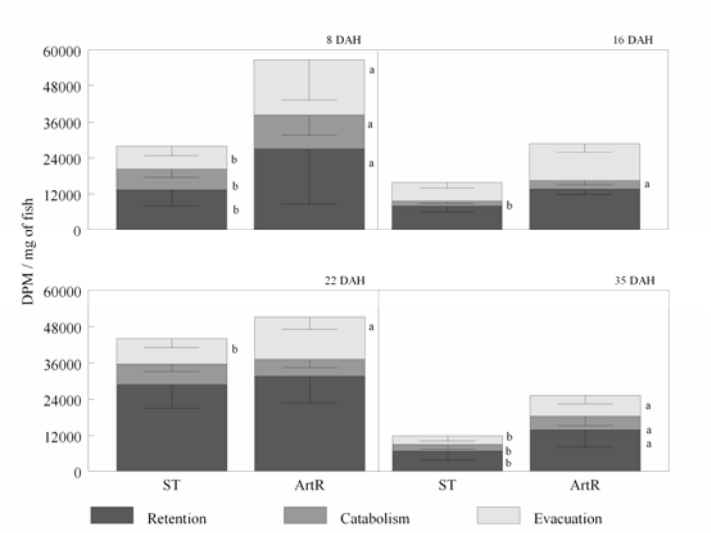

Fig. 5.5 – Relative evacuation (radiolabel in the incubation seawater), relative catabolism (radiolabel in the CO_2 trap) and relative retention (radiolabel in the sole body) (Total disintegrations per minute (DPM) per mg of fish) determined in sole at 8, 16, 22 and 35 days after hatching (DAH) after 24h of incubation. ST: Standard live feed feeding regime; ArtR: Live feed co-fed with inert diet from mouth opening feeding regime. Values are means ± SD (n = 14 to 20). Different letters indicate statistical differences (P<0.05, ANOVA) between of each compartment of different treatments at the same age (8, 22, and 35 DAH). Two-way analysis of variance (ANOVA) was used at 16 DAH to determine treatment effect (P<0.05).

5.4. Discussion

In the present study feed intake and protein utilization were affected by partially replacing *Artemia* with an inert diet from mouth opening. Co-fed sole presented higher feed intake and higher relative retention, catabolism and evacuation at the end of the study. Digestibility was lower in co-fed sole during metamorphosis, and retention efficiency remains almost constant during early development.

The results of the present study were obtained by feeding radiolabelled *Artemia* to sole that were eating live prey and to sole that were being co-fed with live prey and inert diet. *Artemia* is a protein source with high digestibility in fish larvae (Rønnestad and Conceição, 2005), and thereby using this methodology allows to compare sole protein utilization at its highest potential. The determination of larvae protein utilization using radiolabelled microdiets is important for the development of larval inert diets and improvements in rearing protocols but is beyond the scope of the present study. In addition, the incorporation of radioactive tracers in small particles of larval inert diets is technically difficult, and protein and amino acid leaching problems can easily occur (López-Alvarado et al., 1994; Yúfera et al., 2002; Kvåle et al., 2006; Nordgreen et al., 2007).

The present study is the first time that the advantages of the incubation setup often used in hot-chase studies (Rønnestad et al., 2001); the study of single individuals and the direct estimation of amino acid catabolism, are combined with the main advantage of the typical cold-chase method (Kolkovski et al., 1993; Morais et al., 2006); a more realistic feeding regime for accurate estimation of protein digestibility (Conceição et al., 2007a). Still, the use of modified cold-chase method proposed in this study is limited to species / life stages that will not be significantly disturbed by the operation of opening the incubation vials for re-feeding. Therefore, the cold-chase approach was not used for the sole at 22 and 35 DAH, once only minor differences were observed at 16 DAH between cold-chase and hot-chase results.

In the present study feed intake was affected by feeding regime (Fig. 5.2). Postlarvae that were co-fed with inert diet from mouth opening were eating more than postlarvae fed *Artemia* alone in most sampling ages. This might indicate that an *Artemia* replacement regime promotes appetite or that the larvae were at sub-optimal feeding status. In fact, the quantity of *Artemia* supplied daily was progressively reduced in the ArtR treatment, and although larvae were observed ingesting the inert diet, growth was depressed in younger stages (Engrola et al., submitted for publication). Therefore, it is not surprising that when fed *Artemia ad libitum*, as was the case of the metabolic trials, a higher feed intake was observed.

During metamorphosis climax (16 DAH) the digestibility of both the live feed and co-fed sole were lower than at younger (8 DAH) or older ages (22 and 35 DAH). Previous studies during sole larvae ontogeny did not detected such decrease in digestibility (Morais et al., 2004b). This might be due to different ages used. Morais et al.

(2004b) studied 12 (pre-metamorphic) and 22 DAH sole (after metamorphosis climax), while in the present study 16 DAH sole (metamorphosis climax) were also studied. Hence, lower protein digestibility is probably due to a reduction in larvae digestive capacity during metamorphosis climax. In previous studies a decrease in alkaline proteases activity during metamorphosis climax has been described in Senegalese sole (Martínez et al., 1999; Ribeiro et al., 1999). This may explain the decrease in growth rate and body energy content at this developmental stage (Parra and Yúfera, 2001). Also during metamorphosis climax of Japanese flounder, a decrease of digestive capacity is concomitant to a decrease in growth and lower feed intake (Bolasina et al., 2006).

Sole larvae that were re-fed (cold-chase) presented a slightly, though significant, higher protein digestibility than sole fed one single meal (hot-chase). This was an unexpected result, since a study with re-fed Pacific herring showed a decrease of digestibility when a second meal was provided (Boehlert and Yoklavich, 1984). A possible explanation for the present results is that sole were not satiated after re-feeding. However, as at the second meal ration size was six-fold higher than the total *Artemia* ingested at those ages that is unlikely. Therefore, the ingestion of a second meal two hours after the first might have stimulated the enzyme secretion as a reaction to feed intake and increased the digestibility of the first meal. It should be noted that herring larvae showed an increase in trypsin activity after being fed with inert polystyrene spheres indicating an automatic reaction to feed intake by the digestive system (Hjelmeland et al., 1988). Thus, digestibility of re-fed sole might indicate that an interval of two hours between meals is suitable for sole larvae. Still, the observed differences in digestibility are minor (0.06 fold), and at least for sole (e.g., Morais et al., 2004b) the hot-chase approach seems to provide realistic estimates.

Sole *Artemia* protein digestibility in the present study ranged between 56.97% (16 DAH) and 81.32% (22 DAH). Similar values were reported by Morais et al. (2004b) in 12, 22 and 35 DAH for the same species. Sole digestibility was significantly affected by feeding regime at 16 and 22 DAH. Values were 0.06 and 0.10 fold lower, respectively, in ArtR sole than in ST sole. Sole at 16 and 22 DAH are at metamorphosis climax, and just after it, respectively (Fernández-Díaz et al., 2001). During metamorphosis flatfish larvae suffer severe morphological changes to be able to change from pelagic to benthic mode of life (Blaxter, 1988). If a feeding regime is unable to allow larvae to accumulated energetic reserves that are going to be used during metamorphosis, their protein utilization might be affected. Studies made in sole from hatching to metamorphosis

observed that larvae energy content decreases during metamorphosis (Parra and Yúfera, 2001). Conjugating this with a reduction in digestibility is possible to explain the lower dry weight observed in sole at these ages (Engrola et al., submitted for publication), as retention efficiency remains almost constant throughout the study.

Relative evacuation (DPM/mg of fish), relative catabolism and relative retention were much higher in ArtR sole than in ST sole, both at 8 and 35 DAH. Combining this observation with the higher feed intake in ArtR sole observed at 8, 16 and 35 DAH might explain the larger sole in ArtR treatment at 68 DAH observed by Engrola et al. (submitted for publication). In Pacific herring it has also been shown that larvae with higher feed intake has higher growth rates, despite having lower protein retention efficiency, due to the positive net balance between these two processes (Boehlert and Yoklavich, 1984).

As suggested by Conceição et al. (1998) rearing fish larvae at optimal feeding regimes, might lead the larvae to improved condition, and subsequently to juveniles of better quality. This study showed that co-fed sole have high digestibility for *Artemia* protein, and that retention efficiency is not affected by feeding regime. Moreover, sole ArtR digestibility was lower than in ST sole, but still high within values for sole (this study and Morais et al., 2004b). Therefore, the lower growth at earlier stages in co-fed sole (Engrola et al., submitted for publication), is not due to a depressed digestive capacity, but probably to the fact that a suitable protein source was not available. In conclusion, co-feeding of inert diet from mouth opening does not impair protein utilization by Senegalese sole larvae.

5.5. Acknowledgements

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Chapter 6

Senegalese sole larvae growth and protein utilization may be depressed when co-fed with inert diet depending on level of *Artemia* replacement

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Senegalese sole larvae growth and protein utilization may be depressed when co-fed with inert diet depending on level of *Artemia* replacement

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Abstract

A large effort has been dedicated in the past years to the development of nutritional balanced inert diets for marine fish larvae in order to suppress the nutritional deficiencies of live feed. In this study growth performance, feed intake, protein digestibility and protein retention were measured for Senegalese sole, in order to provide insight into how protein utilization affects growth performance when *Artemia* is replaced by inert diet. Three feeding regimes were tested: ST - standard live feed; ArtRL - live feed and 20% *Artemia* replacement with inert diet (dry matter basis) from mouth opening; ArtRH - live feed and 58% *Artemia* replacement with inert diet from mouth opening. Feed intake and protein metabolism were determined at 6, 15 and 21 days after hatching using ^{14}C-labelled *Artemia* protein and posterior incubation in metabolic chambers. At the end of the experiment, sole fed exclusively with live feed were significantly larger than *Artemia* replacement sole. Protein digestibility decreases during sole ontogeny, and more sharply in ArtRH sole. Concomitantly retention efficiency increases during ontogeny but with a slight delay in ArtRH sole. Senegalese sole larvae growth and protein utilization may be depressed when co-fed with inert diet depending on level of *Artemia* replacement.

Keywords: Early-weaning; *Artemia* replacement; Feed intake; Protein metabolism; Digestibility; *Solea senegalensis*.

6.1. Introduction

Fast growth is of vital importance for larval fish as predation susceptibility decreases with increasing body size (Blaxter, 1988). In order to grow, larvae should eat and be able to digest the feed. Live preys, such as rotifers and *Artemia*, are normally offered to larvae in marine hatcheries at first-feeding; however the nutrient composition of these preys is inadequate to sustain growth of fish larvae at later stages (Planas and Cunha, 1999, Conceição et al., 2003). In fact, Engrola et al. (2007) observed that sole fed live feed alone were three- to seven-fold smaller than weaned sole, at 60 days after hatching

(DAH). In order to solve this constraint and improve larval performance, co-feeding regimes based on live feeds and inert diet, have been proposed for fish species such as red drum (Lazo et al., 2000), Asian seabass (Curnow et al., 2006), Atlantic cod (Fletcher et al., 2007) and Atlantic halibut (Hamre et al., 2001). However, while live preys are a high digestibility protein source for fish larvae, other protein sources such as fish meal probably have low digestibility (Rønnestad and Conceição, 2005). In addition, purified model proteins as salmon serum or algal protein have been shown to have in fact a low digestibility by fish larvae (Rønnestad et al., 2001, Tonheim et al., 2004). Hence, as suggested by Rønnestad and Conceição (2005) the complexity of the dietary nitrogen is the key issue for optimal larval growth performance.

Cahu and Zambonino Infante (2001) suggested that larval digestive maturation might be stimulated or impaired, depending on how co-feeding is performed. Knowledge of larval digestive maturation and the effect of co-feeding regimes in digestive capacity have been studied in several species. The shift in feed type had a direct impact on the digestive enzyme profiles of Atlantic cod (Wold et al., 2007), sharpsnout seabream (Suzer et al., 2007), and white bream (Cara et al., 2003). Maturation of the digestive tract of Senegalese sole (Engrola et al., submitted for publication-a) and dorado (Vega-Orellana et al., 2006) was positively affected by a co-feeding regime with inert diet. Nevertheless, in most marine fish species, including Senegalese sole, a co-feeding regime during an extended period is still needed to sustain larval growth at earlier stages (Engrola et al., submitted for publication-a).

Larval protein metabolism and consequently growth performance can be affected by several factors, among others, feed intake, Pacific herring has higher growth rates with higher feed intake, despite having lower protein retention efficiency, due to the positive net balance between these two processes (Boehlert and Yoklavich, 1984); temperature, African catfish larvae increased absorption rates and retention efficiency at higher temperature and consequently grew faster than at lower temperatures (Conceição, 1998); and protein sources, sole postlarvae fed with soy protein concentrate diet presented a higher amino acid catabolism but growth was not impaired probably due to a higher dietary protein intake (Aragão et al., 2003). In short, digestibility and protein retention are key issues in defining larval growth performance as well as survival rate. In fact, high digestibility has been shown to correlate with better growth and survival rate in Western Atlantic seabream larvae (Houde and Schekter, 1983).

Senegalese sole is a species of high commercial value and wild catches are declining for the species (Imsland et al., 2003). During the past years the high interest in culture of the species has led to major achievements in larval nutrition and rearing techniques, despite weaning success still being highly variable (Conceição et al., 2007b). Co-feeding with live feeds and inert diet from mouth opening has been shown to depress growth at sole earlier stages, but promotes better postlarval quality after full weaning (Engrola et al., submitted for publication-a). In fact, early introduction of inert diets in the feeding regime might delay or promote sole digestive maturation (Ribeiro et al., 2002, Engrola et al., 2007).

The aims of this study were to evaluate the effects of *Artemia* replacement by an inert diet on Senegalese sole growth performance, and understand how protein digestibility and protein retention efficiency may explain these effects. Protein metabolism was determined using ^{14}C-labelled *Artemia* protein and posterior incubation in metabolic chambers at key early development stages (Fernández-Díaz et al., 2001): pre-metamorphic (6 DAH), metamorphosis climax (15 DAH) and end of metamorphosis (21 DAH).

6.2. Materials and Methods

6.2.1. Larval rearing

Senegalese sole eggs were obtained by natural spawning of captive broodstock kept at the Ramalhete facilities (University of Algarve, Faro, Portugal). Newly hatched larvae were reared in a 100 L cylindro-conical fiber glass tanks in a closed recirculation system with an initial density of 100 larvae L^{-1}. The experimental system was equipped with a mechanical filter, a submerged biological filter, a protein skimmer and a UV sterilizer.

Photoperiod was set at 12 h light (L) : 12 h dark (D) cycle, and provided by overhead fluorescent tubes that produced an intensity of 900 Lux at the water surface. Green-water conditions were provided by daily addition of microalgae, *Tetraselmis suecica* and *Isochrysis galbana* to the rearing tanks. Environmental parameters were measured daily. Temperature and salinity averaged 19.6 ± 0.3 ºC (mean ± SD) and 35.9 ± 0.5 g L^{-1}, respectively. Dissolved oxygen in water was 91.9 ± 4.8% of saturation.

Table 6.1 – Feeding regimes of Senegalese sole larvae from 2 to 20 days after hatching (DAH), ST - standard live feed; ArtRL - live feed and 20% *Artemia* replacement with inert diet (dry matter basis) from mouth opening; ArtRH - live feed and 58% *Artemia* replacement with inert diet from mouth opening.

	Treatments								
	ST			**ArtRL**			**ArtRH**		
DAH	Rot	Na AF	Meta EG	Na AF	Na EG	Inert diet	Na AF	Na EG	Inert diet
2	5.0			2.0		0.1	1.0		0.2
3	5.0			2.0		0.1	1.0		0.2
4	2.0	2.0		4.0		0.1	2.0		0.3
5		4.0		4.0		0.1	2.0		0.4
6		5.0			4.0	0.2		1.3	0.3
7		6.0			3.0	0.2		1.2	0.5
8		4.0			4.0	0.3		0.9	0.6
9		4.0			4.0	0.4		1.1	0.8
10		2.0			1.0	0.5		0.3	0.8
11		11.0			6.0	0.6		2.0	0.9
12			12.0		7.0	0.8		2.0	1.1
13			13.0		7.0	0.9		2.0	1.4
14			6.0		3.0	1.1		0.7	1.7
15			10.0		5.0	1.2		1.3	1.8
16			11.0		6.0	1.5		1.4	2.2
17			13.0		7.0	1.8		1.6	2.7
18			14.0		7.0	2.2		1.6	3.3
19			14.0		7.0	2.7		1.8	4.0
20			12.0		6.0	3.2		1.5	4.8

Rot: Rotifers; Na AF: *Artemia* nauplii AF Strain; Na EG: *Artemia* nauplii EG Strain; Meta EG: *Artemia* metanauplii EG Strain and Inert diet: Proton diet. Rotifers are expressed as 'number of rotifers / ml tank volume / day', *Artemia* are expressed as 'number of *Artemia* / ml tank volume / day' and inert diet daily ration are expressed as 'mg / tank / day'.

6.2.2. Experimental design and sampling

Three feeding regimes were randomly assigned to 12 tanks: ST - standard live feed feeding regime (ST treatment); ArtRL - live feed and 20% *Artemia* replacement with inert diet (dry matter basis) from mouth opening feeding regime (ArtRL treatment); ArtRH - live feed and 58% *Artemia* replacement with inert diet (dry matter basis) from mouth opening feeding regime (ArtRH treatment) (Table 6.1). Each feeding regime was run in quadruplicate tanks. Larvae rearing lasted up to 20 DAH.

Larvae were fed rotifers (*Brachionus rotundiformis*) enriched with Rich Advanced® (Rich, Greece); *Artemia* AF nauplii (na) (Inve, Belgium) and *Artemia* metanauplii enriched with Easy DHA Selco® (Inve, Belgium), and AgloNorse® Microfeed (Ewos, Scotland) according to Table 6.1. Metanauplii supply was gradually changed from live to frozen *Artemia* between 14 and 20 DAH. *Artemia* replacement (ArtRL and ArtRH) larvae were fed *Artemia* AF Strain nauplii (Inve, Belgium) and inert diet Proton® (100-200 μm) (Inve, Belgium) at mouth opening (Table 6.1). From 6 DAH onwards *Artemia* EG Strain nauplii (Inve, Belgium), was offered to the larvae. At the end of the experiment inert diet account for 55% in ArtRL and 88% in ArtRH of the total daily ration of feed (dry matter basis). Sole were counted at the end of the experiment to determine survival.

During the experiment fish were fed to apparent satiation and daily adjustments were made based on visual inspection (to avoid large excess of uneaten feed). The daily amount of inert diet was divided in two doses (circa 50% each) and gradually increased per day (Table 6.1). Inert diet, Proton (100-200 μm) (Inve, Belgium), used had 54% protein, 12% lipids, and 7% moisture, according to the manufacturer's data. Live preys were offered to the larvae three times per day, once in the morning (11:00 h), early afternoon (14:00 h) and in late afternoon (17:00 h). Frozen *Artemia* metanauplii were harvested, washed in seawater, counted, and kept at -20 ºC. Fifteen minutes before feeding, *Artemia* was thawed in seawater. Seawater was then removed, and new seawater was added before feeding to the ST sole tanks. The inert diet was hand feed without prior hydration, in the morning (10:30 h) and in late afternoon (16:30 h). In the *Artemia* replacement treatments (ArtRL and ArtRH), inert diet was supplied half an hour before the live prey feeding until larvae had 11 DAH. After this period inert diet was semi-continuously (cycles of 2 h of feeding followed by one hour break) supplied by automatic feeders for 24 hours a day until the end of the experiment.

Dry weight was determined at 2 DAH (n = 3 in pools of 30 larvae), 15 (n = 60) and 20 DAH (n = 60). Length evaluation was determined at the end of the experiment, 20 DAH (n = 60). On the afternoon prior to the measurements of feed intake and protein utilization, sole from each treatment (n = 15), were randomly harvest from the tanks to a beaker and transferred to the radiollabeling laboratory and were stocked in 1 L tanks, at 20 ± 1 ºC. Sole were deprived from feed during 16h and measurements were performed in the next morning.

6.2.3. Protein utilization trial

6.2.3.1. *Artemia* [U-^{14}C] labeling

Artemia was radiolabelled with a [U-^{14}C] protein hydrolysate (1.85 MBq mL Amersham Pharmacia Biotech Ltd., UK) according to the method developed by Morais et al. (2004a). *Artemia* nauplii were enriched at a density of 200 *Artemia* mL^{-1} in a sealed incubation system at 28 ºC, with a dose of 3.3µl of [U-^{14}C] protein hydrolysate per mL of seawater.

The incubation system consisted in a controlled temperature aquarium (29 ± 1 ºC) with an incubation bottle connected to a KOH trap to capture the radiolabelled $^{14}CO_2$. The incubation was overnight and lasted 14 hours. After incubation *Artemia* metanauplii was washed several times, counted and samples (n = 4, 3 mL each sample) were taken to measured the incorporated radiolabel. Samples of the incubation seawater were also taken (n = 4, 3 mL each sample) to be able to correct for the ^{14}C present.

6.2.3.2. Sole metabolic trials

Sole of 6, 15, and 21 DAH were allowed to eat the radiolabelled *Artemia* during 30 minutes; this period is a trade-off between the time necessary for a complete meal size and to avoid losses by larvae catabolism. After this period fed sole were carefully transferred, one by one with a Pasteur pipette, through two tanks with clean seawater (to eliminate any ^{14}C amino acids that could be present in the surface of the fish), and subsequently transferred to the incubation vial.

The incubation setup was described previously by Rønnestad et al. (2001). Briefly, the incubation setup consists in sealed vials containing 7.5 mL of seawater with gentle air flow, where each incubated alone. The air is forced through a capillary from the incubation vial to a chemical trap (5.0 mL of KOH, 0.5 M), that entraps the $^{14}CO_2$. After a 24h incubation period each sole was rinsed with clean seawater and sampled for analysis. After sampling the incubation vials are resealed and HCl is injected gradually to the seawater (1.0 mL of HCl, 1 M), for diffusion of remaining CO_2 in the seawater to the KOH trap.

6.2.4. Analytical measurements

6.2.4.1. Growth

Fish for dry weight (DW) determination were kept frozen at -20 ºC, after they were freeze-dried and weighed with 0.001 mg precision. Fish total length was determined from

photographs. During sampling (20 DAH) a photo was taken from each sole; afterwards the length was determined with the help of using the image processing and analysis program UTHSCSA Image Tool (v. 3.0, C.D. Wilcox, S.B. Dove, W.D. McDavid, and D.B. Greer, University of Texas Health Science Center, Texas, USA).

6.2.4.2. Radiolabel measurements

Vials containing incubation seawater and KOH from the CO_2 trap were added scintiliation cocktail (Ultima Gold XR, Packard Bioscience) and counted for radioactivity (DPM, disintegrations per minute). Sole and *Artemia* were solubilized with a tissue solubilizer (Solvable, Perkin-Elmer) and incubated at 50 ºC during 24 h. After cooling scintiliation cocktail (Ultima Gold XR, Packard Bioscience) was added and samples were counted for radioactivity (DPM).

6.2.4.3. Feed intake and protein metabolism

Feed intake (FI) and protein utilization was determined at 6, 15, and 21 DAH in fed sole. Sole that did not ingest any live prey were eliminated from further analysis. Feed intake (% BDW) after a single meal was determined as:

$$FI = [(R_{total}/SR_{Artemia})/DW_{fish}] \times 100$$

as describe by Conceição et al. (1998), where R_{total} is the sum of the radioactivity in the incubation seawater, in the CO_2 trap and in fish (DPM), $SR_{Artemia}$ is the specific radioactivity per *Artemia* samples (DPM/mg *Artemia* DW), and DW_{fish} is the fish dry weight (mg).

Protein utilization was determined based on protein digestibility (D, %), retention efficiency (R, %), and catabolism fraction (C, %). These estimates were determined as:

$$D = [(R_{body} + R_{CO_2\ trap})/(R_{body} + R_{CO_2\ trap} + R_{water})] \times 100,$$

$$R = [R_{body}/(R_{body} + R_{CO_2\ trap})] \times 100,$$

$$C = [R_{CO_2\ trap}/(R_{body} + R_{CO_2\ trap})] \times 100,$$

where R_{body} is the total radioactivity in fish body (DPM), $R_{CO_2\ trap}$ is the total radioactivity per CO_2 trap (DPM), and R_{water} is the total radioactivity in the incubation seawater (DPM).

6.2.5. Data analysis

Data for DW (mg) and total length (TL, mm) are arithmetic means ± standard deviation (SD) of treatments replicates (n = 4). Growth was expressed as relative growth rate

(RGR, % day^{-1}), and determined between the beginning and the end of the experiment, 2 to 20 DAH. RGR was determined as: $(e^{g}-1) \times 100$ with $g = [(ln_{final\ weight} - ln_{initial\ weight})/time]$ (Ricker, 1958). The coefficient of variation (CV, %) was calculated as: CV = treatment standard deviation/treatment mean x 100 and used to determine the inter-individual weight and length variation among fish of the same treatment (60 fish per treatment).

All percentage data were arcsine ($x^{1/2}$)-transformed prior to analysis. One-way analysis of variance (ANOVA) was used to analyze the effect of feeding regime on dry weight, total length, RGR, survival, feed intake and protein utilization of sole of different treatments at the same age, as well during sole ontogeny (6, 15 and 21 DAH). Differences were considered significant when $P < 0.05$. When differences were found Tukey's Honest Significant Difference (HSD) test was used to determine which specific treatment or age differed significantly. All statistical analysis was carried out using the Statistica 5.1 package software (StatSoft, Tulsa, USA).

6.3. Results

6.3.1. Growth

At the beginning of the experiment, larvae had 0.03 ± 0.002mg of dry weight (Fig. 6.1). Feeding regimes had a significant ($P < 0.05$) impact on sole weight at 15 DAH (Fig. 6.1), with sole that were fed with a high *Artemia* replacement (ArtRH) being significantly smaller than sole fed live feed alone (ST treatment). At the end of the experiment, sole feed exclusively with live feed (ST) were significantly ($P < 0.05$) larger than sole eating live feed and inert diet (ArtRL and ArtRH) (Fig. 6.1). Total length was only negatively ($P < 0.05$) affected when sole was fed with the higher *Artemia* replacement regime.

Sole from ST grew significantly ($P < 0.05$) faster than sole from high *Artemia* replacement (ArtRH) (Table 6.2). Feeding regimes did not have a significant impact on coefficient of variation (dry weight and total length) (Table 6.2). Survival was not affected by feeding regimes and ranged between 30.10 ± 9.94% (ArtRH) and 37.93 ± 7.71% (ST) (Table 6.2).

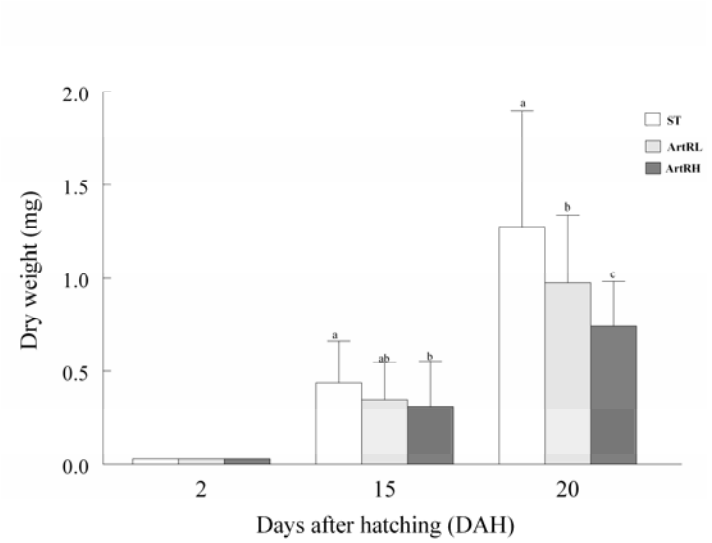

Fig. 6.1 – Senegalese sole dry weight during the experiment, ST - standard live feed; ArtRL - live feed and 20% *Artemia* replacement with inert diet (dry matter basis) from mouth opening; ArtRH - live feed and 58% *Artemia* replacement with inert diet from mouth opening. Values are means ± SD of sole dry weight at 2 DAH (n = 3, pooled samples of 30 larvae), 15 DAH (n = 15), and at 20 DAH (n = 15). Different letters indicate statistical differences (P < 0.05, Tukey's test) between sole from different treatments at the same age.

6.3.2. Feed intake

Offering inert diet from mouth opening had no effect in sole feed intake at 6 and 15 DAH ($P > 0.05$) (Fig. 6.2). Larvae from all treatments were eating similar amount of feed at 6 DAH, between 6.45 ± 4.30% BDW in ArtRH and 12.46 ± 8.63% BDW in ArtRL. ArtRH sole were eating 2.44 ± 1.63 prey $fish^{-1}$ while ArtRL were eating 4.92 ± 3.40 prey $fish^{-1}$. During metamorphosis climax, 15 DAH, sole were not affected in their feed intake by feeding regime ($P > 0.05$) (Fig. 6.2). Sole feed intake was 12.21 ± 2.26% BDW in ST, 16.10 ± 5.91% BDW in ArtRL, and 11.91 ± 6.50% BDW ArtRH feeding regime. The number of preys eaten per fish was between 18.76 ± 10.24 and 28.26 ± 10.37 in ArtRH and ArtRL, respectively. At late metamorphosis (21 DAH) significant differences ($P < 0.05$) were found in the sole feed intake (Fig. 6.2). Sole from ArtRH ate significantly more (22.59 ± 4.39% BDW) than sole from ArtRL (12.61 ± 3.22% BDW), and ST (10.74 ± 2.02% BDW).

Table 6.2 – Total length (mm), relative growth rate (RGR, %), coefficient of variation (CV, %) and survival (%) of Senegalese sole postlarvae at 20 DAH, ST - standard live feed; ArtRL - live feed and 20% *Artemia* replacement with inert diet (dry matter basis) from mouth opening; ArtRH - live feed and 58% *Artemia* replacement with inert diet from mouth opening.

	Treatments		
	ST	**ArtRL**	**ArtRH**
20 DAH			
Total length (mm)	**8.70 ± 1.81^{a}**	**8.16 ± 1.25^{a}**	**6.90 ± 0.93^{b}**
RGR (%)	**22.37 ± 1.03^{a}**	**20.44 ± 1.77^{ab}**	**18.78 ± 0.20^{b}**
$CV_{(weight)}$ (%)	**48.45**	**37.54**	**32.52**
$CV_{(length)}$ (%)	**20.78**	**15.30**	**13.40**
Survival (%)	**37.93 ± 7.71**	**33.97 ± 10.62**	**30.10 ± 9.94**

Results are given as means ±SD (n = 4). Different superscript letters indicate statistical differences (P < 0.05, Tukey's test) between postlarvae from different treatments.

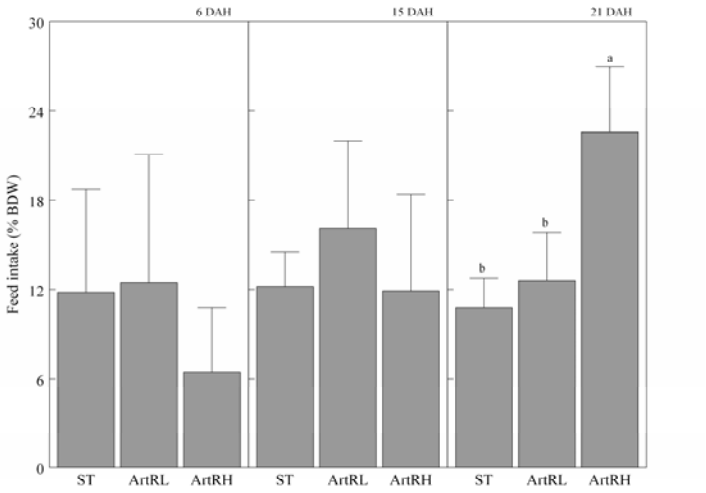

Fig. 6.2 – Feed intake of Senegalese sole at 6, 15, and 21 days after hatching (DAH), ST - standard live feed; ArtRL - live feed and 20% *Artemia* replacement with inert diet (dry matter basis) from mouth opening; ArtRH - live feed and 58% *Artemia* replacement with inert diet from mouth opening. Values are means ± SD of sole feed intake (n = 9 to 15). Different letters indicate statistical differences (P < 0.05, Tukey's test) between sole from

different treatments at the same age.

Number of ingested preys per fish was between 61.43 ± 15.71 in ArtRL and 83.70 ± 16.25 in ArtRH.

6.3.3. Protein metabolism

Digestibility of 6 DAH larvae was not affect (P > 0.05) by feeding regimes (Fig. 6.3). Larvae presented a protein digestibility of 83.08 ± 4.37%. During metamorphosis climax (15 DAH) larvae digestibility decreased with increasing *Artemia* replacement (P < 0.05) (Fig. 6.3). Larvae with the higher replacement (ArtRH) presented the lowest protein digestibility (69.04 ± 5.45%), while larvae fed live feed alone (ST) presented the highest digestibility (77.84 ± 3.73%). In 21 DAH postlarvae digestibility was similar between ST (76.83 ± 1.89%) and ArtRL (76.32 ± 1.39) and smaller (P < 0.05) in ArtRH, (73.78 ± 1.99%).

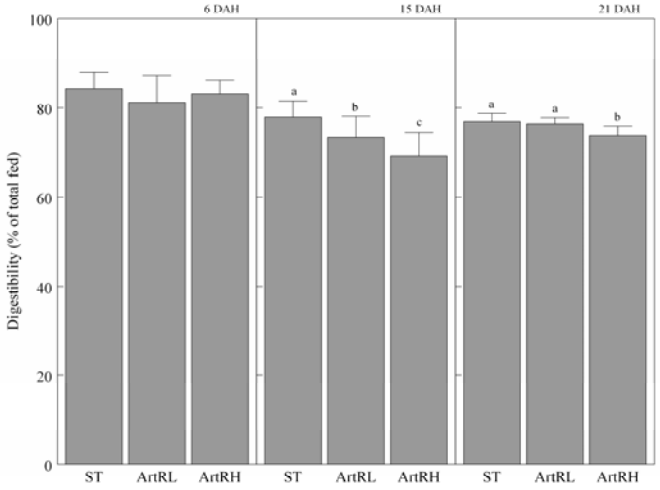

Fig. 6.3 –Protein digestibility (% of radiolabel in the sole and metabolic trap in relation to total radiolabel fed) in sole at 6, 15 and 21 days after hatching (DAH), after 24h of incubation. ST - standard live feed; ArtRL - live feed and 20% *Artemia* replacement with inert diet (dry matter basis) from mouth opening; ArtRH - live feed and 58% *Artemia* replacement with inert diet from mouth opening. Values are means ± SD of sole protein digestibility (n = 9 to 15). Different letters indicate statistical differences (P < 0.05, Tuckey's test) between sole from different treatments at the same age.

Protein retention efficiency and catabolism of 6 DAH sole were not affected (P > 0.05) by feeding regime (Fig. 6.4). Retention efficiency of 6 DAH larvae was 72.05 ±

6.48%, while catabolism was 27.95 ± 6.48%. The higher replacement regime (ArtRH) had a negative impact ($P < 0.05$) on 15 DAH larvae retention efficiency (Fig. 6.4). Larvae from this treatment presented the lowest retention (68.92 ± 8.74%) and highest catabolism (31.08 ± 8.74%) when compared with larvae from ST and ArtRL, (83.77 ± 3.76% and 79.33 ± 9.20%) of retention efficiency, and 16.23 ± 3.76% and 20.67 ± 9.20% for catabolism. Postlarvae from ST treatment (83.26 ± 5.05%) presented significantly higher retention efficiency ($P < 0.05$) than sole from ArtRH (78.23 ± 4.45%) at 21 DAH (Fig. 6.4).

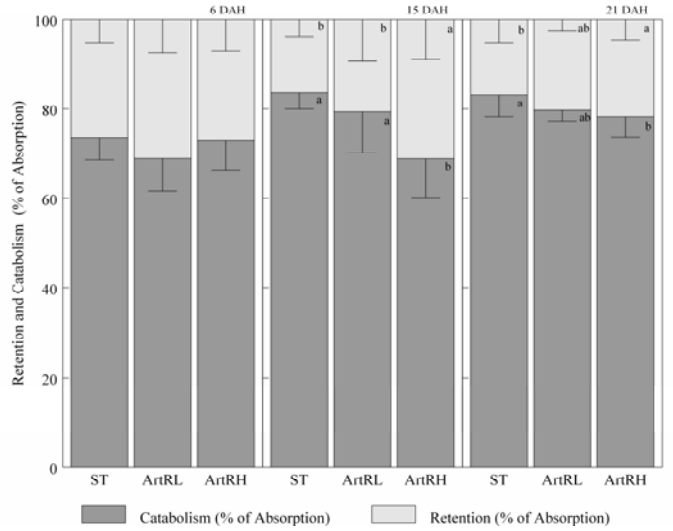

Fig. 6.4 – Protein retention (% of radiolabel in the sole in relation to digested label), and catabolism (% of radiolabel in the metabolic trap in relation to digested label) in sole at 6, 15, and 21 days after hatching (DAH), after 24h of incubation. ST - standard live feed; ArtRL - live feed and 20% *Artemia* replacement with inert diet (dry matter basis) from mouth opening; ArtRH - live feed and 58% *Artemia* replacement with inert diet from mouth opening. Values are means ± SD of sole retention and catabolism (n = 9 to 15). Different letters indicate statistical differences ($P < 0.05$, Tuckey's test) between treatments at the same age.

Protein digestibility and retention efficiency were significantly ($P < 0.001$) affected during sole ontogeny. An accentuated decrease was observed in larvae digestibility between 6 and 15 DAH in all treatments. Nevertheless, while larvae from ST and ArtRL decreased 0.06 fold and 0.12 fold, respectively, a decrease of 0.17 fold was observed in ArtRH larvae. Values remained about constant between 15 and 21 DAH sole. Concomitant with the decrease of protein digestibility, retention efficiency significantly ($P < 0.001$) increased from 6 to 15 DAH in ST and ArtRL treatments. ST and ArtRL

larvae presented an increase in protein retention efficiency of 0.16 fold and 0.10 fold; however ArtRH larvae presented a decrease of 0.04 fold between 6 and 15 DAH. The lowest values of digestibility and retention efficiency were observed at 15 DAH in ArtRH larvae, 69.04 ± 5.45% and 68.92 ± 8.74% respectively.

6.4. Discussion

The results of the present study show that sole growth decreases with increasing *Artemia* replacement in the feeding regime. Sole feed intake was relatively constant during ontogeny, increasing only in sole fed with high *Artemia* replacement (ArtRH) after metamorphosis is completed. High *Artemia* replacement sole presented lowest protein digestibility and retention efficiency during metamorphosis. Protein digestibility decreases during sole ontogeny more sharply in ArtRH sole. Concomitantly protein retention efficiency increases during ontogeny but with a slight delay in ArtRH sole. Sole growth performance is affected by protein utilization mostly during metamorphosis.

At the end of the experiment a significant negative impact of *Artemia* replacement in larval weight was observed. ArtRH sole weight was 42% lower in relation to ST fish. Sole dry weight at 15 and 20 DAH decreased with increasing levels of *Artemia* replacement (Fig. 6.1). Nonetheless, the larval weight observed in ArtRL sole of the present study was within the normal range observed in Senegalese sole. Sole fed live feed alone presents normally a dry weight higher than 1 mg DW around 20 DAH, while in a co-fed feeding regime values around 1 mg DW are common (Ribeiro et al., 1999, Parra and Yúfera, 2001, Cañavate and Fernández-Díaz, 1999, Fernández-Díaz et al., 2001, Engrola et al., submitted for publication-a). Feeding regimes where Senegalese sole larvae were fed exclusively with inert diet from mouth opening (Cañavate and Fernández-Díaz, 1999), or fed during a week with live feed and switched to inert diet (Fernández-Díaz et al., 2001, Yúfera et al., 2005, Gamboa-Delgado et al., 2008) have lead to growth arrest, poor survival rates or a combination of both. This indicates that the use of a negative control in the present study would have been of little use. Survival rate obtained in the present work was low comparing to previous values using similar feeding regimes (Cañavate and Fernández-Díaz, 1999, Engrola et al., submitted for publication-a). As survival rates were not influenced by feeding regime one can suggest that differences were due to egg quality.

Larval feed intake of the present study was higher than previously observed in Senegalese sole. Engrola et al. (submitted for publication-b) reported values between 6-

12% BDW in sole co-fed with *Artemia* metanauplii from 8 to 35 DAH. The *Artemia* replacement feeding regimes tested in the present study aimed at a complete substitution of *Artemia* metanauplii by nauplii, and a reduction to a minimum the *Artemia* nauplii by increasing the amount of inert diet without losing larvae quality. The results showed that sole from ArtRL treatment achieved a satisfactory weight (0.97 ± 0.37 mg DW) at 20 DAH without *Artemia* metanauplii in the feeding regime. *Artemia* nauplii are about 50% smaller and swim slower than *Artemia* metanauplii (Sorgeloos et al., 2001). These properties might facilitate the predation success by ArtRL sole and compensate the lower energy content with a higher feed intake.

In the present study feed intake was affected by feeding regime at the end of the experiment. Twenty one days old ArtRH sole ingested 1.9 fold more *Artemia* than ST and ArtRL (Fig. 6.2). This might indicate that an *Artemia* replacement regime promotes feed intake or that sole were somewhat fasted. However, it is very unlikely that the high *Artemia* replacement group (ArtRH) sole were fasted once these fish were growing at 18.78% day^{-1} (Table 6.2). Most likely the abundant presence of *Artemia* nauplii in the metabolic trial tanks promoted feed intake. It should be noted that the presence of *Artemia* nauplii in the rearing tanks increases inert diet intake in gilthead seabream larvae (Kolkovski et al., 1997a, Kolkovski et al., 1997b). On the other hand, the higher feed intake observed in 21 DAH ArtRH sole might be an attempt to restore energy reserves depleted during metamorphosis. Growth during sole metamorphosis climax, a highly energy demanding process, is sustained by using energy reserves accumulated during the earlier stages (Parra and Yúfera, 2001), as sole does not seem to increase feed intake during this period (Fig. 6.2; Parra and Yúfera, 2001).

Studies of larvae digestive capacity are usually limited by larval size, feed technology and low acceptance of inert diet (Tonheim et al., 2004, Conceição et al., 2007a). By feeding radiolabelled *Artemia* to larvae these constraints are overcome. Hence, feeding sole with a protein source with high digestibility, such as *Artemia* (Rønnestad and Conceição, 2005), one can compare sole protein utilization at its highest potential.

Sole *Artemia* protein digestibility ranged between 83.08 ± 4.37% (6 DAH) and 69.04 ± 5.45% (15 DAH ArtRH) (Fig. 6.3). The values are within normal values reported for the species (Morais et al., 2004b, Engrola et al., submitted for publication-b). Since sole feed intake was not affected at 15 DAH, the possibility that protein digestibility differences at this age are caused by variations in feed intake, as previously observed in

Pacific herring (Boehlert and Yoklavich, 1984), can be discarded. However, ontogeny of the sole digestive capacity was affected by feeding regime. A significant decrease, 0.06 fold and 0.12 fold, of sole protein digestibility was observed between 6 and 15 DAH, concomitant with a 0.16 fold and 0.10 fold increase of protein retention efficiency in ST and ArtRL sole, respectively. A similar pattern was previously observed in sole by Morais et al. (2004b) and Engrola et al. (submitted for publication-b). Those authors suggest that sole larvae might have the ability of compensate the lower protein digestibility by increasing the protein retention efficiency. However, the same pattern of protein digestibility and retention efficiency was not observed in sole reared with the high *Artemia* replacement regime (ArtRH). A decrease of 0.17 fold and 0.04 fold in protein digestibility and protein retention efficiency were observed between 6 and 15 DAH. Therefore, the lowest values of protein utilization are observed at 15 DAH ArtRH sole, i.e., during metamorphosis climax.

Metamorphosis is a challenging phase for a flatfish larva, allied to severe morphological changes, in a period of habitat shift (Blaxter, 1988). On the other hand, intestinal maturation might be stimulated but also irreversible impaired, depending on how co-feeding of live prey and inert diets is performed (Cahu and Zambonino Infante, 2001). Therefore, a possible explanation for the low protein utilization by ArtRH larvae is that sole were unable to cope at the metabolic level due to a depressed digestive capacity caused by high amount of *Artemia* replacement. In fact, Fernández-Díaz et al. (2006) observed that sole exclusively fed with microencapsulated diets had altered hepatic and gastrointestinal structures when compared to live feed sole. It should be noted that ArtRH sole were offered from 58% (2 DAH) up to 88% (20 DAH) of inert diet in the total daily ration (dry matter basis). So, the differences in larval growth performance and protein utilization might be the outcome of feeding protein sources with lower digestibility for marine fish larvae (Rønnestad and Conceição, 2005, Conceição et al., 2007b).

In conclusion, a co-feeding strategy enhances sole growth performance and protein utilization, although high *Artemia* replacement by inert diet depresses sole protein digestive capacity, protein retention efficiency, and thereby lead to lower growth. In addition, the present study shows that sole is able to adapt the protein metabolism to a low level of *Artemia* replacement what may promote growth at later stages (Engrola et al., submitted for publication-a).

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Chapter 7

General Discussion

7.1. Feeding frequency during pre-weaning period affects weaning growth performance and survival

Feeding *Artemia* to sole postlarvae with a semi-continuous (pulse) feeding frequency produces fewer but larger weaned fish while feeding twice daily leads to smaller fish with a higher survival rate after weaning (**Chapter 2**). However, pulse-fed postlarvae had a lower homogeneity in growth than those fed twice daily. A lower homogeneity in growth in fish may lead to aggressive behavior and/or feed intake in less competitive animals (Jobling and Wandsvik, 1983). Despite the absence of apparent aggressive behavior in sole, the high coefficient of variation suggests that a feeding hierarchy may exist. Experiments with turbot indicate that larger juveniles cause stress to smaller fish, preventing them from obtaining a normal feed intake (Carter et al., 1996) and consequently causing mortalities.

Senegalese sole usually respond passively during feeding, similar to Arctic charr (Linnér and Brännäs, 2001). In these two species growth results were similar: fish fed more frequently grew better. Size-selective mortality in the pulse feeding treatment may partly explain the absence of a difference in coefficient of variation for weight at the end of the experiment, in contrast to the end of pre-weaning period. In rainbow trout (*Oncorhynchus mykiss*), a species with aggressive feeding behavior, free access to self-feeders with low reward levels decreased feed intake and growth rate (Gélineau et al., 1998). Those authors suggested that the increase in feeding activity to compensate the lower nutrient intake was not sufficient to attain a suitable feed intake to sustain growth. Thus, as the total feed amount per day was the same in both treatments (**Chapter 2**), pulse fed sole were in constant feeding activity for lower feed amounts. Therefore, sole might adapt feeding activity in order to achieve satiation by increasing feed intake. This hypothesis is in agreement with findings of **Chapter 6** where sole fed with *Artemia* nauplii had a higher feed intake than sole fed with *Artemia* metanauplii (**Chapter 5**) probably due to the lower energy content of *Artemia* nauplii.

7.2. Weaning success depends greatly on sole weight at start weaning

Several reasons may explain the better weights after weaning recently reported: different zootechnical conditions, improved feeding and weaning strategies, and different inert diets. Inert diets have experienced major improvements during the past years. Not only in physical properties, such as shape, size, sinking properties, color and leaching, but also in ingredients, digestibility, amongst others (Kvåle et al., 2006). In particular, the inclusion

of hydrolyzed protein in larval feeds promotes growth, and increases survival in European seabass (Zambonino Infante et al., 1997), Dover sole (Day et al., 1997) and Atlantic halibut (Tonheim et al., 2005).

During this Thesis the best sole weaning results at later ontogenetic development where achieved when postlarvae were sudden weaned between 5-10 mg DW, and not when they were co-fed (**Chapter 3**). Postlarvae weight at 68 DAH was two-fold higher than in previous studies (Cañavate and Fernández-Díaz, 1999; Ribeiro et al., 2002) but similar to values determined in **Chapter 2** and **4**. These results supports the suggestion of **Chapter 2** where sudden weaning was proposed which is in contradiction to previous studies where a co-feeding strategy was suggested for sole (Cañavate and Fernández-Díaz, 1999; Ribeiro et al., 2002). Nevertheless, a co-feeding strategy may also give good results. However, it should be performed carefully because mortality might be selective towards smaller postlarvae (Ribeiro et al., 2002).

Weaning success also depends of the length of co-feeding period. The results of **Chapter 3** (Experiment 3) do not support the idea that a short co-feeding during late larval ontogeny enhances larval growth and survival rate. This experiment showed that five days of co-feeding in 2 mg DW postlarvae is not sufficient for fish to cope with weaning. This is in apparent contradiction to observations for other species that a co-feeding regime would improve larval nutrition and may pre-condition larvae to better accept the inert diet (Rosenlund et al., 1997; Alves et al., 2006; Vega-Orellana et al., 2006). This apparent difference between sole and other species may arise from the peculiar passive feeding behaviour of sole. *Artemia* co-feeding may have a "distracting" effect on sole postlarvae, with sole failing to adapt to inert diet.

Even when sudden weaning is applied to small sole (less than 2 mg DW) an adaptation period to inert diet is needed. This period of adaptation, normally with growth arrest, is larger the smaller postlarvae are (**Chapter 3** - Table 3.2,). Growth rate increases one or two weeks after weaning started in 1 and 2 mg sole (SW1 and SW2), being apparently higher compared to 4 mg sole (SW4) in the same period. This may result from a growth arrest during SW4 sole adaptation to inert diet, as observed during the sudden weaning periods for SW1 and SW2 fish. In addition, the increase in growth of smaller sole may result from compensatory growth, and/or from selective mortality towards (smaller) fish unable to adapt to inert diet.

Hence, results of the present Thesis demonstrate that it is possible to wean sole postlarvae with two different feeding strategies, sudden weaning and *Artemia* co-feeding.

The choice of feeding strategy to adopt should be based on the postlarvae weight. This is in agreement with observations (Verreth, 1994; Rosenlund et al., 1997) that larval weight rather than larval age is a better indicator of developmental stage and physiological status of postlarvae.

7.3. Co-feeding with inert diet from mouth opening promotes better growth in sole at weaning

The *Artemia* replacement with 20% of inert diet from mouth opening (ArtR in **Chapter 4**, and ArtRL in **Chapter 6**) promoted better sole growth and quality at weaning (**Chapter 4**). The previous work already indicated that sole larvae could be co-fed with inert diet from mouth opening (Cañavate and Fernández-Díaz, 1999). Nevertheless the sole weight observed by those authors at the end of the experiment was two-fold smaller than in the present Thesis (**Chapter 4)**.

Still, growth of sole fed the *Artemia* replacement (ArtR or ArtRL) was depressed compared to fish feed exclusively on live feed (**Chapters 4** and **6**). Sole fed live feed alone presents normally a dry weight higher than 1 mg DW around 20 DAH, while in a co-fed feeding regime values around 1 mg DW are common (**Chapters 4** and **6**; Cañavate and Fernández-Díaz, 1999; Ribeiro et al., 1999; Fernández-Díaz et al., 2001; Parra and Yúfera, 2001). Feeding regimes where Senegalese sole larvae were fed exclusively with inert diet from mouth opening (Cañavate and Fernández-Díaz, 1999), or fed during a week with live feed and switched to inert diet (Fernández-Díaz et al., 2001; Yúfera et al., 2005; Gamboa-Delgado et al., 2008) have lead to growth arrest, poor survival rates or a combination of both. Moreover, a high *Artemia Artemia* replacement (58% of inert diet from mouth opening, ArtRH) had a negative impact on sole growth (**Chapter 6**). Sole weight at 20 DAH was 42% lower in relation to fish fed live feed alone. Sole dry weight at 15 and 20 DAH decreased with increasing levels of *Artemia* replacement (**Chapter 6 -** Fig. 6.1).

At the end of pelagic phase co-fed sole were smaller than sole fed live prey (**Chapters 4** and **6)**. These results contradict observations made in Atlantic halibut (Rosenlund et al., 1997) and Atlantic cod (Fletcher et al., 2007), where a co-feeding strategy of *Artemia* and inert diet can produce comparable growth when compared with *Artemia* alone. However at the end of weaning, postlarvae from ArtR treatment (**Chapter 4**) were larger than the remaining sole. A faster growth in co-fed sole might be due to a pre-conditioning onto inert diet and a faster maturation of the digestive tract. Co-fed

dorado presented higher growth performance, survival and a faster maturation of the digestive capacity than sudden weaned fish (Vega-Orellana et al., 2006). These results agree with the results of **Chapter 3**, where a co-feeding strategy in sole smaller than 2 mg DW was suggested, to promote growth during weaning. Therefore, co-feeding with inert diet at mouth opening promotes better growth in sole at weaning. However, the amount of live feed co-fed seems critical and has to be fine tuned.

7.4. Maturation of sole digestive tract is promoted or impaired depending of feeding regime

The sole digestive capacity was evaluated in order to understand how fish enzymatic profile might explain growth performance. Sole postlarvae around 2 mg DW should be co-fed as the results of the present Thesis indicates. Co-fed sole presented an improved digestive enzyme profile than sudden weaned sole of the same weight (**Chapter 3**). An increase of trypsin specific activity should be expected when feeding sole postlarvae with inert diet, since inert diets usually have a higher content of protein and would therefore induce higher levels of enzyme activity (Tseng et al., 1982). The decrease of trypsin activity after weaning started may indicate that the sole postlarvae were not eating properly and that feed intake probably diminished with the shift in feed type. In European seabass larvae a similar pattern after start weaning was observed (Cahu and Zambonino-Infante, 1994).

On the other hand, in larger (4 mg) sudden weaned sole a higher activity of alkaline phosphatase was observed (**Chapter 3**). This may indicate a better developmental and nutritional status of the fish. A correlation between alkaline phosphatase segmental activity and larval growth was determined in sole during weaning (Ribeiro et al., 2002). Furthermore, these postlarvae showed a decrease in alkaline phosphatase activity while eating *Artemia*, in the week prior to start weaning, suggesting that *Artemia* was not the most suitable feed. The absence of a similar pattern in 2 mg sudden weaned postlarvae together with the values of leucine-alanine peptidase during the second week of the experiment suggests a delay in the enterocyte maturation. Sole postlarvae fed with inert diet were described to have a delay in the brush border activity due to adaptation to new feed type (Ribeiro et al., 2002).

Sole co-fed with inert diet from mouth opening presented a higher alkaline phosphatase activity than live feed sole around 20 DAH (**Chapter 4**). This higher value of alkaline phosphatase activity might indicate an earlier intestinal maturation and a faster

development of sole digestive capacity. In addition, trypsin and alkaline phosphatase appear to be good indicators of sole nutritional status as proposed by Ribeiro et al. (2002). This confirms previous observations (Ribeiro et al., 2002; Fernández-Díaz et al. (2006) that sole digestive enzyme profile can be influenced by feed type.

7.5. Feeding practices might have a positive impact on weaned sole quality

A better tail condition after weaning was observed in the sole co-fed with inert diet compared to fish fed live feed alone (**Chapter 4**). In addition at the end of the pelagic phase (20 DAH) sole co-fed with inert diet also seemed to be less stressed, as indicated by fish color (**Chapter 4**). These observations suggest that the sole co-fed with inert diet from mouth opening have better nutritional status and improved physiological condition.

The total proportion of individuals with skeletal abnormalities in **Chapter 2** (~80%) and **Chapter 4** (62%) was higher than the 44% previously determined in postlarvae and juveniles (Gavaia et al., 2002). One explanation for the differences between studies may be different feeding regimes. However, prey enrichment of the present studies was done with commercial products, which according to Villalta et al. (2005) have a well balanced HUFA content for sole development. Since, survival rate was not determined and no mention about larval rearing density is given in Gavaia et al. (2002), it is possible to speculate that during larval rearing mortality was selective towards deformed fish in that study. As pointed by those authors most deformities were vertebral fusion that might be caused by rearing conditions.

The results of the present Thesis are comparable to those found in Japanese flounder reared in captivity (Hosoya and Kawamura, 1995; Hosoya and Kawamura, 1998), for which skeletal deformities such as deformed hypural, fused spines or central fusions have been detected in 30–60% of the total number of deformities observed in the caudal complex. In gilthead seabream, the number of hatchery reared fish with deformed caudal complex and vertebral column can reach 100% (Boglione et al., 2001). The absence of differences among fish from different treatments indicating that the feeding regimes used were able to ensure suitable nutrition to sole regarding normal development of skeletal structures.

Sole's pigmentation was not influenced by offering inert diet from mouth opening or feeding live prey alone (**Chapter 4**). Since, flatfish pigmentation is a major concern in hatchery production a large effort was made in the past years to overcome some nutritional deficiencies. Thus, nowadays most of larval diets and enrichments present in

the market are nutritionally balanced to avoid malpigmentation problems (Bolker and Hill, 2000). As observed in Senegalese sole, live prey commercial enrichments usually ensure a normal pigmentation during the larval rearing (Villalta et al., 2005). Thus, all feeding regimes tested in the present had the necessary nutrients for normal pigmentation.

At 20 DAH sole postlarvae co-fed with inert diet had higher frequency of beige color than larvae fed live prey alone (**Chapter 4**). Several environmental factors, like light intensity, tank substrate, transportation, have been showed to play a critical role in flatfish color (Ellis et al., 1997; Venizelos and Benetti, 1999). It has been proposed that these differences in color of the larvae around 20 DAH are related to different levels of stress in larvae (Ruane et al., 2005). Those authors showed that sole larvae of the same age with a darker color had significantly higher cortisol levels than sole with lighter color. So, it appears that sole co-fed with inert diet were less stressed than the ones fed exclusively on live feed.

Sole larvae that were co-fed inert diet from mouth opening presented a better tail condition (**Chapter 4**). In the present Thesis tail condition was used as an indicator of fin erosion and nutritional status. Fin erosion problems are usually reported in aggressive feeding fish during restricted feeding, such as Atlantic salmon (Noble et al., 2008) and Atlantic cod (Hatlen et al., 2006). No systematic observation of sole behavior was done in the present Thesis. However, aggressive behavior between sole was never observed in the course of this Thesis, and seems unlikely due to sole behavior patterns. In addition, all fish were fed to apparent satiation. Normally fin erosion implies welfare problems and has an economic impact. Fin erosion has been identified as one of the major problems in farming sole. The better tail condition observed in the co-fed sole is interpreted as an indication of better nutritional status and improved physiological condition.

7.6. *Artemia* intake is affected by feeding regime

Sole larvae that were co-fed with inert diet from mouth opening had a higher *Artemia* intake than postlarvae fed *Artemia* alone (**Chapter 5** - Fig. 5.2). This might indicate that an *Artemia* replacement regime promotes appetite or that the larvae were at sub-optimal feeding status. In fact, the quantity of *Artemia* supplied daily was progressively reduced for co-fed fish, and although larvae were observed ingesting the inert diet, growth was depressed in younger stages (**Chapter 4**). Therefore, it is not surprising that when fed *Artemia ad libitum*, as was the case of the metabolic trials, a higher feed intake was observed.

On the other hand, in **Chapter 6** sole *Artemia* intake was only affected by feeding regime at 21 DAH. Sole co-fed with 58% of inert diet (ArtRH) ingested 1.9 fold more *Artemia* than live prey (ST) and those co-fed with 20% of inert diet (ArtRL) (Fig. 6.2). However, it is very unlikely that the high *Artemia* replacement (ArtRH) sole were fasted as proposed previously once these fish were growing at 18.78% day^{-1} (**Chapter 6 -** Table 6.2). Most likely the abundant presence of *Artemia* nauplii in the metabolic trial tanks promoted feed intake, and these results may be taken as an indication of improved appetite. It should be noted that the presence of *Artemia* nauplii in the rearing tanks increases inert diet intake in gilthead seabream larvae (Kolkovski et al., 1997a; Kolkovski et al., 1997b). On the other hand, the higher feed intake observed in 21 DAH ArtRH sole might be an attempt to restore energy reserves depleted during metamorphosis. Growth during sole metamorphosis climax, is sustained by using energy reserves accumulated during the earlier stages (Parra and Yúfera, 2001), as sole does not seem to increase feed intake during this period (**Chapter 6 -** Fig. 6.2; Parra and Yúfera, 2001).

7.7. Sole digestibility is depressed during metamorphosis climax

In the present Thesis, sole *Artemia* protein digestibility ranged between 56.97% (16 DAH – **Chapter 5**) and 83.08% (6 DAH – **Chapter 6**). The higher values are comparable to those reported by Morais et al. (2004) for the same species. During metamorphosis climax (14-18 DAH) the digestibility of both the live feed and co-fed sole were lower than at younger or older ages (**Chapters 5** and **6**). Previous studies during sole larvae ontogeny did not detected such decrease in digestibility (Morais et al., 2004). This might be due to different ages tested. (Morais et al., 2004) studied 12 (pre-metamorphic) and 22 DAH sole (after metamorphosis climax), while in the present studies, 15 and 16 DAH sole (metamorphosis climax) were also studied. Hence, lower protein digestibility is probably due to a reduction in larvae digestive capacity during metamorphosis climax. During metamorphosis flatfish larvae suffer severe morphological changes to be able to change from a pelagic to a benthic way of life (Blaxter, 1988). If a feeding regime is unable to allow larvae to accumulated energetic reserves that are going to be used during metamorphosis, protein utilization might be affected. Studies in sole from hatching to metamorphosis observed that larvae energy content decreases during metamorphosis (Parra and Yúfera, 2001). Conjugating this with a reduction in digestibility is possible to explain the lower growth observed in sole at these ages (**Chapters 4** and **6**), as retention efficiency remains almost constant throughout the study (**Chapters 5** and **6**). Also during

metamorphosis climax of Japanese flounder, a decrease of digestive capacity is concomitant to a decrease in growth and lower feed intake (Bolasina et al., 2006).

7.8. Sole larvae protein utilization may be depressed when co-fed with inert diet depending on level of *Artemia* replacement

Protein ingredients, such as fish meal, currently use in inert diets may be to complex for the immature digestive system of a fish larvae (Rønnestad and Conceição, 2005; Conceição et al., 2007), leading to low protein digestibility. It has been suggest that sole larvae might have the ability of compensate lower protein digestibilities by increasing the protein retention efficiency (Morais et al., 2004). In the present Thesis similar protein utilization was observed in sole fed live prey alone or co-fed with 20% of inert diet (**Chapters 5** and **6**). However, in sole co-fed with 58% of inert diet from mouth opening (ArtRH) protein both digestibility and retention efficiency decreased between 6 and 15 DAH (**Chapter 6**). As a result, the lowest values of protein utilization were observed at 15 DAH, i.e., during metamorphosis climax.

In addition, intestinal maturation might be stimulated but also irreversible impaired, depending on how co-feeding of live prey and inert diets is performed (Cahu and Zambonino Infante, 2001). In fact, sole exclusively fed with microencapsulated diets had altered hepatic and gastrointestinal structures when compared to live feed sole (Fernández-Díaz et al., 2006). It should be noted that ArtRH sole were offered from 58% (2 DAH) up to 88% (20 DAH) of inert diet in the total daily ration (dry matter basis), and had also lower growth compared to both sole fed live prey alone and co-fed with 20% of inert diet (**Chapter 6**). So, the differences in larval growth performance and protein utilization (**Chapter 6**) might be the outcome of an overall lower dietary protein digestibility. Therefore, when using co-feeding regimes with sole or other marine fish larvae, the relative amounts of live feed and inert diet co-fed seem critical. This may change in the future when less complex protein ingredients are included in larval inert diets.

7.9. Final conclusions

The present Thesis suggests the following conclusions:

- The pre-weaning feeding frequency affects weaning performance in Senegalese sole (**Chapter 2**). Semi-continuous (pulse) feeding produces fewer but larger fish while feeding twice daily leads to smaller fish with a higher survival rate. This most probably means that mortality during weaning is selective after a pulse feeding regime in the pre-weaning period, being higher amongst smaller fish,

- Sole weaning might be accomplished with two different feeding strategies, sudden weaning and a co-feeding period of *Artemia* and inert diet (**Chapter 3**). The choice of the feeding strategy to adopt should be based on the postlarvae weight.

- A co-feeding strategy with inert diet starting at first-feeding can promote growth and improve postlarval quality (**Chapter 4**).

- Protein digestibility is reduced during sole metamorphosis climax (**Chapter 5**), especially if a diet with a high proportion of complex proteins is used (**Chapter 6**).

- A co-feeding strategy with inert diet starting at first-feeding enhances sole growth performance through better protein utilization, although the relative amounts of live feed and inert diet co-fed are critical (**Chapters 5** and **6**).

7.10. Further research

The present Thesis aims for a better understanding of Senegalese sole larvae and postlarvae growth performance and might contribute for the improvement of feeding regimes in sole rearing. Nonetheless, the knowledge of the underlying mechanisms that affect fish larvae growth is not achieved only based on the effect of feeding regime on performance.

Tracers studies combined with different ingredients and feed types may be used in order to analyze how larvae cope at the metabolic level and related it to growth. A deep knowledge on the way that larva digests different proteins, and how afterwards the resulting amino acids are accreted into protein would be instrumental in this endeavor, and would be a major breakthrough in the development of optimized larval inert diets.

Larvae growth performance can be affected by several factors. As reviewed in **Chapter 1**, fish species, diet, temperature, feeding regimes and strategies alone or in combination might give different outputs. In addition, genetic breeding programmes may be a step forward on improvement of larval performance and quality. The challenge is to find the most suitable feeding regime for a certain genotype, for given species in a set of practical conditions.

7.11. References

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