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New developments and biological insights into the farming of *Solea senegalensis* reinforcing its aquaculture potential

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Abstract

Senegalese sole was one of the earliest identified candidate species with high potential for aquaculture diversification in the South of Europe. Its culture has been possible, and commercially attempted, for several decades but intensive production has been slow to take off. This has been explained mostly by serious disease problems, high mortality at weaning, variable growth and poor juvenile quality. However, a strong and sustained research investment that started in the eighties has led to a better understanding of the requirements and particularities of this species. More recently, better management and technical improvements have been introduced, which have led to important progress in productivity and given a new impetus to the cultivation of Senegalese sole. As a result, the last 5 years have marked a probable turning point in the culture of sole towards the development of a knowledge-driven, competitive and sustainable industry. This review will focus on the main technical improvements and advances in the state of knowledge that have been made in the last decade in areas as diverse as reproductive biology, behaviour, physiology, nutritional requirements, modulation of the immune system in response to environmental parameters and stress, and characterization and mitigation of the main disease threats. It is now clear that Senegalese sole has important particularities that differentiate it from other current and candidate marine aquaculture species, which bring about important challenges, some still unsolved, but also notable opportunities (e.g., a nutritional physiology that is better adapted to dietary vegetable ingredients), as will be discussed here.

Key words: aquaculture industry, diversification, flatfish, Soleidae.

Introduction

Two Soleidae, Senegalese sole (*Solea senegalensis* Kaup, 1858) and common sole (*Solea solea*, Linnaeus, 1758) have been identified as being among the most interesting and promising species for marine commercial aquaculture diversification in Europe for at least four decades now. As a result, sole has attracted a lot of research interest and considerable investments have been made to develop rearing methodologies during the last 30 years (Howell 1997; Dinis *et al*. 1999). Nevertheless, production of common sole in Central and Northern Europe (mostly in the Netherlands and in the United Kingdom) and of Senegalese sole in Southern Europe has failed to reach successful commercial development until recently (FAO 2011). This can be mostly explained by a high incidence of diseases, particularly Pasteurellosis and Flexibacteriosis, which have plagued quite a few farms. In addition to high vulnerability to diseases, other problems that were reported initially were difficulties in weaning onto formulated diets leading to low growth and high juvenile mortalities, a decreased growth rate at high stocking densities, pigmentation abnormalities and malformations related to eye migration (Dinis *et al*. 1999). Consequently, poor fry quality and high mortality rates during the weaning stage have led to juvenile scarcity for on-growing purposes. On the upside, several advantages have been identified: natural spawning of wild-caught broodstock readily occurs in captivity in close relation with the temperature cycle; larval growth is fast and larval rearing is easily accomplished at high survival rates; and the market value and demand for these species are high and not completely met by fishery catches (Howell 1997; Dinis *et al*. 1999). Most of these aspects have been comprehensively reviewed a decade ago by Imsland *et al*. (2003). However, in recent years there have been important technical developments in rearing and feed technologies and advances in the state of knowledge of the biology and nutritional requirements of the species in captivity, which have given a new impetus to the culture of these flatfish species. This is particularly the case for Senegalese sole which in the last 5 years has shown important signs of expansion and has attracted substantial investment in Southern Europe to intensify its production (Howell *et al*. 2011; APROMAR 2013), in spite of the actual climate of financial restraint. Here we will review the current state of biological knowledge and technical improvements achieved through research conducted during the last decade, pinpointing where scientific advances have been made on early bottlenecks to help build a stronger and more competitive sole farming industry. The emphasis on *S. senegalensis* in the Iberian Peninsula is justified by what has been an unbalanced research and commercial investment in *Solea* spp. across Europe in recent years but, given the close relationship between both species, some of the basic knowledge presented here could also be relevant to common sole cultivation in Central and Northern Europe.

Senegalese sole market and culture

Senegalese sole is a flatfish of high commercial value and demand not only in the Iberian Peninsula but also in the wider European market, indistinguishable by consumers from common sole, considered as the same species in marketing statistics, and often sold together under the name “sole”. These two species are commonly fished along the Eastern Atlantic coast, and common sole also along the Mediterranean basin, in gillnets or trawling nets, but most catches are from the North sea (Bjørndal & Guillen 2014). The biological and culture requirements of these two species are somewhat different (Dinis 1986; Imsland *et al*. 2003) and Southern Europe countries have selected Senegalese sole for farming mainly due to the species higher growth rates (Howell *et al*. 2009), and also higher temperature requirements (Howell 1997). It is reared commercially in Spain and Portugal, where existing facilities were adapted for sole culture (Imsland *et al*. 2003).

Market

Fisheries landings of sole showed a 43% reduction from 1995 to 2012 and the average size of wild sole has also been declining, while consumers prefer larger fish, with market prices proportional to size (Bjørndal & Guillen 2014). On the other hand, average prices of different sole products (fresh, frozen and farmed) in commercialization statistics from one of the most important fish markets in Spain, the MercaMadrid, have been quite stable from 2002-2013, with a tendency for fresh sole prices to increase since 2010 (Bjørndal & Guillen 2014). Furthermore, sole consistently attains high prices in European markets. These factors, associated with Southern European markets being saturated for the main aquaculture species, seabream (*Sparus aurata*) and seabass (*Dicentrarchus labrax*), have fuelled interest in sole for marine fish farming diversification.

Global flatfish aquaculture production has increased from 26,300 tonnes in 2000 to 148,800 tonnes in 2008, the world leading producer being China, which mainly produce turbot (*Scophthalmus maximus*), Japanese flounder (*Paralichthys olivaceus*) and tongue sole (*Cynoglossus semilaevis*), while Spain is the main producer in Europe, particularly of turbot (FAO 2011). Soleidae presently represent only a small fraction of global flatfish production and, according to EUMOFA, among the 7,752 tonnes of flatfish produced in 2011 in the EU, 157 tonnes were from sole. Production of Senegalese sole in the South of Europe, although still low due to the limited number of farms, has increased exponentially from only 60 or 11 tonnes produced in 2005 in Spain and Portugal to 194 or 100 tonnes in 2012, respectively. France has also started producing sole in 2010 (142 tonnes), almost duplicating production (220 tonnes) by 2012 (Fig. 1; APROMAR 2014, FEAP 2014). For 2013, the Spanish Marine Aquaculture Producers Association reported another important increase in sole production (343 tonnes, 300 of which produced in Galicia, and 3.9 million juveniles) and, with several new farms being currently under construction, further important expansion is anticipated in the coming years (APROMAR 2014). Spain is the largest market for sole in Europe, with a limited domestic supply meaning that most of the consumed sole is imported. Other important markets in terms of total supply (domestic production plus imports) are France, Italy and the United Kingdom (Bjørndal & Guillen 2014). In addition, the sole production market is being expanded to Asia; in 2013 the only sole hatchery operating in Portugal and producing eggs year-round sold juveniles (7g weight) to Asia, and increased sales are expected in 2014.

Current production costs for this species have been estimated at 9.62 €/kg, with just over 39% being accounted for by juvenile costs (3.77 €/kg) (Bjørndal & Guillen 2014). These costs are however expected to decrease considerably (possibly down to 7 €/kg) as the industry expands, due to economy of scale, better domestication and lower feed conversion ratios, as commercial cultivation experience builds up and it becomes advantageous for feed companies to produce specialised sole diets. Price of farmed sole in wholesale markets has only been available for two years. The farmed product attained a price of 12.25 €/kg in MercaMadrid in 2013, which is only slightly higher than wild small (under 500g) sole given that farmed sole have been commercialized at a similar small size, while larger fish are preferred by consumers and sold at substantially higher prices (Bjørndal & Guillen 2014). Therefore, although there are still many challenges to address, a recent thorough economical analysis by Bjørndal & Guillen (2014) concludes that market prospects for expansion of sole farming are good.

Production systems

Until recent years, Senegalese sole aquaculture was linked to the salt marshes ("esteros") in the South of Spain (Cádiz and Huelva, Andalusia) and South of Portugal (Algarve) as an added value product in polyculture with semi-intensive seabream and seabass cultivation (Ferreira *et al*. 2010; Yúfera & Arias 2010). Wild sole juveniles were passively trapped in the ponds or these were stocked with cultured juveniles bought from hatcheries. Sole juveniles would rely on the occurrence of natural prey in the bottom of the ponds with little additional investment by the producers or, in some cases, the natural prey was supplemented to an unknown extent by commercial feed left-over by seabream and seabass. In this culture system not only sole juveniles were present, but also other naturally occurring species (e.g., eel and mullets) could also be found. Survival of soles at the end of the on-growing process was very variable, depending on the pond sediment condition and seabream/seabass density (Quental-Ferreira *et al.* 2010). In these conditions, Senegalese sole can grow from 24 to 393g in 16 months and with 99% survival (Quental-Ferreira *et al.* 2010).

Nowadays, although salt marshes are still used for semi-intensive aquaculture, the trend is for more intensive on-growing systems using fibreglass or concrete tanks, or shallow raceways (for a more detailed description of the different culture systems see Imsland *et al*. 2013), using commercial feeds and maintaining highly controlled environments. More recently, recirculation aquaculture systems (RAS) have been implemented and now most of the new sole farms in Spain and Portugal use this type of system for a better control of environmental conditions, especially water temperature and nitrogen wastes. This has been an important technological development resulting in increases in production as sole are quite sensitive to environmental changes and the stable environment provided by recirculation technologies, particularly the tight temperature control, can give increased growth and survival.

Culture conditions and growth

The current general parameters that are being used or should be considered for sole culture and related existing data on growth performance are as follows:

Temperature: Typical rearing temperature of Senegalese sole is either following natural thermoperiod or maintaining constant around 20ºC. Although higher growth occurs at temperatures ranging from 20 to 25ºC, temperatures above 22ºC entail higher risk of pathological outbreaks (Cañavate 2005).

Salinity: Sole juveniles are partially euryhaline and can tolerate salinities from 5 to 55 (Arjona *et al*. 2007). However, growth was shown to be depressed at a salinity of 15 compared to 25 and 39, with some differences being also noted in terms of ingestion, metabolite levels, energy metabolism and cortisol response at salinities between 25 and 39, with slightly better results at 39 (Arjona *et al*. 2009).

Photoperiod: Sole has a nocturnal activity pattern, with locomotor activity peaking in the first part of the dark period (Bayarri *et al*. 2004) and a higher metabolic rate during the dark phase (Castanheira *et al*. 2011). However, broodstock appeared to habituate to husbandry routines and had the peak of activity during the day from 14:00-18:00, which coincided with feeding at 15:00 (Carazo *et al*. 2013). Aquaculture facilities for indoor on-growing use mostly a 12hL:12hD photoperiod and/or some shading in the tanks to keep light at the surface between 80 and 350 lux (Salas-Leiton *et al*. 2008; Boluda Navarro *et al*. 2009).

Feeding: Senegalese sole displays a nocturnal feeding pattern with 77-85% of feed demand, when using self-feeders, occurring at night (Boluda Navarro *et al*. 2009). However, in most farms food is offered in 4-8 meals during the day, or more frequently over the 24h using automatic feeders. Contrary to other flatfish species like turbot, Senegalese sole has a strict bottom-feeding behaviour.

Stocking density: Densities of up to 30 kg m^{-2} have been tested with no effects on growth (Salas-Leiton *et al*. 2008) although high densities (26.6 kg m^{-2}) might affect the shape of the fish (Ambrosio *et al*. 2008). A relationship has been found between high stocking densities and stress in the fish (Costas *et al*. 2008; Salas-Leiton *et al*. 2010), but it is unclear if this is due to density *per se*, or rather to deteriorating water quality.

There is little information regarding the growth potential of Senegalese sole in commercial culture given that the growth data available is taken from rearing experiments designed to test nutritional requirements or on-growing conditions (a summary is presented in Table 1, taken from Sánchez-Fernández 2012). On the other hand, a major issue is that Senegalese sole typically shows large size variation under culture, increasing with time and leading to high differences between fast and slow growers. Although Senegalese sole does not show any aggressive behaviour of large fish over smaller fish, a hierarchical structure exists. Salas Leiton *et al*. (2011), in a study of the effect of grading on growth, found that all the populations of fish (graded or not) tended to reach the same size variation after 60 days of culture, and suggested that group heterogeneity improves overall growth, associated with efficient social arrangements. Therefore, it was concluded that the hierarchical structure of the population is caused by fish size distribution and not the contrary.

Broodstock management

Sexual differentiation

Senegalese sole females grow faster and mature later than males (Dinis *et al*. 1999; Imsland *et al*. 2003) and thus female-based stocks would be an advantage for the culture of this species (Viñas *et al*. 2013). A recent study has demonstrated the influence of temperature on sex differentiation in sole, suggesting the potential applicability of environmental sex manipulation to sole aquaculture (Blanco-Vives *et al*. 2011). This work showed that sole larvae grown under three different thermoregimes, a thermophase-cryophase (TC) thermoregime (daily temperature fluctuation from 21ºC during the day to 18ºC during the night), an inversed thermoregime (CT: 18ºC day to 21ºC night) and a constant 19.5ºC thermoregime, exhibited highly different sex ratios, with the proportion of females being 70% for the TC, 20% for the CT and 40% (approximately the normally observed sex ratio in sole culture) in the constant thermoregime group.

Spawning of wild sole

Industrial Senegalese sole culture has been so far based on the spawning of wild-origin broodstocks (Dinis *et al*. 1999; Anguis & Cañavate 2005; Martin *et al*. 2014). Wild adult sole have been caught in coastal waters, often coastal lagoons on the southern Atlantic coast of Spain and Portugal, and transported to aquaculture or research facilities. After a period of quarantine and acclimation to captivity, groups of wild broodstocks can spawn naturally within the same year (Dinis *et al*. 1999; Imsland *et al*. 2003). Optimal holding conditions for successful spawning of wild breeders have been described (Table 2). The principal spawning period is during spring (February-March to June) with a less important spawning period in autumn (Howell *et al*. 2003; Anguis & Cañavate 2005). As in other species (Bromage *et al*. 2001), both photoperiod and temperature have been implicated in the control of the timing of spawning in Senegalese sole. However, to date there has been no published information on the use of artificial photoperiod regimes for the control and manipulation of spawning in Senegalese sole. Natural photoperiod has been most commonly recommended and used for successful spawning (Dinis *et al*. 1999; Howell *et al*. 2003; Anguis & Cañavate 2005) given that constant photoperiod was suggested to disrupt gonadal maturation (García-Lopez *et al*. 2006a; 2009). Nevertheless, the full role of photoperiod in Senegalese sole maturation has not been investigated and successful spawning has also been obtained under a constant photoperiod of 16hL:8hD (Martin *et al*. 2014). Perhaps more importantly, spawning was observed to be closely related to the temperature cycle (Dinis *et al*. 1999; Anguis & Cañavate 2005) and highest fecundities are between 16 and 21ºC (Anguis & Cañavate 2005). Through temperature control (oscillating temperatures between 16 and 18ºC) it has been possible to extend the natural spawning season with spawning every month from March to November, although with variable egg production (Martin *et al*. 2014; Table 2). Microsatellite analysis of parentage has demonstrated that only 8.7% to 51.7% of breeders actually participate in spawning (Porta *et al*. 2006; Martin *et al.* 2014), which leads to a sub-estimation of relative fecundity (eggs per kg of female biomass) of a broodstock. Individual females were observed to spawn with intervals from 1 to 56 days and fecundities were often over 100,000 eggs kg^{-1}.day^{-1} and on occasions such high fecundities from a single female were observed on consecutive days (Martin *et al*. 2014). These observations highlight the potential gains in egg production that could be made by management improvements leading to more breeders in a Senegalese sole broodstock participating in spawning.

Spawning of G1 sole

A major constraint to the culture of Senegalese sole is that successful spontaneous spawning has not been obtained from generations (G1, G2...) of broodstock bred and reared in captivity. Therefore, the industry at present is threatened by its dependence on the capture of wild broodstock, which are in decline due to factors such as fishing pressure. Spawning from G1 broodstock has been infrequent and spawns were almost entirely of eggs that did not hatch (Agulleiro *et al*. 2007; Guzmán *et al*. 2008; Howell *et al*. 2009; Rasines *et al*. 2012; Norambuena *et al*. 2012a), and Carazo (2013) later observed that the eggs were not fertilized. Many industry and research centres have held G1 broodstocks under the same conditions as successfully spawning wild caught broodstock, both before and during the spawning period, with the same negative result. This has lead to the hypothesis that some aspect (developmental, nutritional, genetic or social / behavioural) in the early rearing (larval or juvenile) might be responsible for the failure of cultured broodstock to spawn viable eggs (Howell *et al*. 2009, 2011). A significant research effort detailed below has been directed to identify the problem and to develop solutions to preferably obtain spontaneous spawning in cultured breeders as in the wild broodstock, but also to develop strip spawning protocols that offer an immediate solution to the problem.

Reproductive behaviour

The reproductive behaviour of Senegalese sole has been studied using non-disrupting lighting and digital video recording (Carazo *et al*. 2013). Wild Senegalese sole in captivity exhibited a complex reproductive behaviour that was characterised by an increase in activity before paired synchronised spawning at the water surface (Carazo *et al*. 2011; Carazo 2013), which was similar to other species of flatfish, including common sole (Baynes *et al*., 1994). Conversely, Carazo (2013) identified that the eggs released from G1 broodstock were not fertilised by G1 males due to the absence of courtship behaviour. The observation of pre-spawning behaviour of wild breeders, that involved chasing (particularly between males) and fish resting on each other (particularly males on a female), combined with microsatellite paternity studies, indicated that spawning was dominated by a few individuals that exhibited fidelity to one another (Porta *et al*. 2006; Carazo 2013; Martin *et al*. 2014). Senegalese sole G1 breeders did not exhibit any aspect of the reproductive behaviour observed in wild breeders, either pre-spawning chasing or paired synchronised spawning (Carazo *et al*. 2011; Carazo 2013). Breeders from different origins were combined to establish that G1 and G2 females held with wild males produced viable spawns with typical reproductive behaviour while G1 males held with wild females did not produce viable spawns (Mañanós *et al*. 2007) and no courtship was observed (Carazo *et al*. 2011; Carazo 2013). These studies have unequivocally established that the reproductive problem is centred on G1 males that lack the reproductive behaviour necessary for successful spawning.

Broodstock nutrition

Achieving good spawning and egg / larval quality results with a specifically formulated broodstock diet is an important step for the development of the commercial aquaculture of sole, which requires consistent and sanitarily safe diets. The standard broodstock feeding regime for this species in captivity consists on natural food items, mostly polychaetes and frozen squid, mussels, and small shrimp, combined with broodstock diets formulated for other marine fish species. This leads to reasonably good spawning results, both in terms of quantity and viability of the eggs produced (Dinis *et al*. 1999; Imsland *et al*. 2003). However, the use of natural feeds poses important sanitary risks as these can be a source of pathogens or parasites, and in fact mortality outbreaks in common sole have been attributed to the live prey that was fed (Baynes & Howell 1993). Moreover, these wet feeds have variable supply and nutritional composition and contribute to the deterioration of water quality, and hence the goal is to completely replace them by formulated feeds. Recently, two new semi-moist diets specifically formulated for *S. senegalensis* broodstock have been tested during a period of two years, with very little polychaete supplementation (0.3% of weight once or twice a week). The most basic diet, formulated with high quality marine ingredients and containing 58.6% crude protein and 16.8% crude fat produced good results, comparable to the classical diet, in terms of egg quality and early larval performance, while egg fatty acid composition was improved when compared with previous spawnings from broodstock kept in the same facilities but fed the standard fresh feed diet (Morais *et al*. 2014a). Further supplementation of the broodstock diet with lipids (up to 23.5%), vitamins and long chain polyunsaturated fatty acids (LC-PUFA), mainly docosahexaenoic acid (22:6n−3, DHA) and eicosapentaenoic acid (20:5n−3, EPA), did not improve egg quality or larval performance up to 17 days post hatching (dph). Other studies have found that levels of essential fatty acids (EFA) affect gamete and larval quality (Fernandez-Palacios *et al*. 2011; Duncan *et al*. 2013) and a study on Senegalese sole wild males found that a formulated diet higher in DHA plus antioxidants significantly increased sperm quality, increasing both percentage of progressive spermatozoa and sperm velocity (Beirão *et al*. 2015).

Nutrition has also been implicated as a factor that contributes to the reproductive behavioural dysfunction in G1 broodstock. Norambuena *et al*. (2012a) found significant differences in proximate, lipid and fatty acid composition between wild and cultured breeders. The body composition of cultured breeders reflected the diet in captivity, with significantly lower levels of the EFA arachidonic acid (20:4n−6, ARA) in liver, testes and muscle, of DHA in liver and male muscle, of EPA in male muscle, and of cholesterol in liver. However, cultured breeders that were fed ARA at 2.3-3.2% of total fatty acids (TFA) (Norambuena *et al*. 2013a) or that demanded ARA at 3% TFA (Norambuena *et al*. 2012b) had similar ARA levels in the liver and gonads to those found in wild fish. These differences in nutritional state appeared to affect pathways for the production of prostaglandins (PGs) and possibly steroids given that cultured breeders presented significantly lower levels of total 2-series PGs and higher levels of 3-series PGs compared to wild breeders (Norambuena *et al*. 2012c). On the other hand, cultured breeders fed increasing contents of ARA (0.7-6.0% TFA) exhibited significantly increasing plasma levels of testosterone (T) and 11-ketotestorone (11KT) and significantly decreasing plasma levels of 3-series PGs, while levels of 2-series PGs were similar independently of dietary ARA composition (Norambuena *et al*. 2013b). When ARA content was ≥3.2% TFA no differences were observed between levels of 2-series and 3-series PGs. The 2-series PGs have been described to be more active or potent than the 3-series PGs (Tocher 2003) in stimulating steroid production (Wade & Kraak 1993) and acting as pheromones in the control of reproductive behaviour in cyprinids (Sorensen & Stacey 2004). Therefore, nutritional manipulation of ARA levels was shown to stimulate the endocrine system in ways that could affect reproductive success and behaviour and hence special attention should be given to the levels of this fatty acid in the formulation of broodstock diets.

Although to date no study has been performed to demonstrate whether feeding G1 fish diets with the EFA levels (and ratios) expected in the diet of wild fish can restore the complete reproductive failure in cultured Senegalese sole, it is believed that the root of the problem may not be simply nutritional.

Reproductive endocrinology

Further basic knowledge on the physiology and endocrinology of sole reproduction has been obtained in the last years, particularly with the characterization of pituitary gonadotropins (GTHs) and the study of their expression in sole males and females (Cerdà *et al*. 2008a; Guzmán *et al*., 2009a). In addition, the recent development of recombinant gonadotropins will enable further examination of the endocrine system in the near future (Chauvigné *et al*. 2012). On the other hand, a good deal of information became available on the major sex steroids and their relation with gonad development and spawning. The releasing profiles of sex steroid hormones has been described in sole broodstocks of wild (García-López *et al.* 2006b; Guzmán *et al.* 2009a) and cultured origin, including G1 (García-López *et al*. 2006c, 2007; Guzmán *et al*. 2008, 2009a,b; Oliveira *et al*. 2010) and G2 (Bayarri *et al*. 2011) generations. Plasma levels of the main androgens (11KT and T) and estrogens (estradiol, E2), in males and females, respectively, were highly correlated with gonad development and spawning time and are useful non-lethal markers to estimate the stage of gonad maturation of the broodstock. In males, T and 11KT plasma levels were low during the resting period (summer), increased during gametogenesis (winter) and peaked just before the spawning time (early spring) (García-López *et al*. 2006b,c; Bayarri *et al.* 2011). A very similar annual profile was observed for E2 in females (García-López *et al*. 2006b, 2007; Guzmán *et al*. 2008; Bayarri *et al*. 2011) and for the maturation inducing steroid (MIS) 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) in both sexes (García-López *et al*. 2006b,c; Bayarri *et al*. 2011), which presented a peak that was slightly delayed with respect to those of T, 11KT and E2. These results were similar to those obtained in other fish species and are in agreement with the known role of T, 11KT and E2 in the regulation of gametogenesis and that of MIS in controlling final gonad maturation and probably reproductive behaviour (Mañanós *et al*. 2008).

Studies have found that the holding environment can disrupt the endocrine reproductive profiles. Constant temperatures and photoperiod negatively affected steroid profiles and in particular constant temperature significantly reduced steroid levels (García-López *et al*. 2006a) and in females inhibited the steroid rhythm observed under a natural fluctuating temperature regime (Oliveira *et al*. 2009). On the other hand, wild males maintained in a long-term isolation from females presented lower androgen levels at the peak of the reproductive period, suggesting the absence of male-female communication had disrupting effects on the endocrine system (Cabrita *et al*. 2011). In addition, evidence of relevant female-to-male communication in sole reproduction was obtained after treating the females with MIS and registering a significant increase in sperm viability, velocity and motility in surrounding males (Cabrita *et al*. 2011).

Two aspects should be highlighted from these studies. Under the optimal reproductive conditions for Senegalese sole (Table 2), the synthesis and release of all relevant reproductive hormones, including steroids, vitellogenin (Guzmán *et al*. 2008), gonadotropins and neuropeptides (Guzmán *et al*. 2009a), seems to be correct and well correlated with gonad development, with similar profiles in both cultured and wild soles. Therefore, no evident endocrine failure has been detected in cultured breeders with respect to wild soles. Nevertheless, in spite of this similarity, plasma levels of reproductive hormones seem to be consistently lower in cultured G1 (and G2) stocks compared to wild soles. These observations could be related with the lower degree of gonad development and gamete production in cultured broodstocks, particularly in males. Future research will be necessary to definitively link dysfunctions of the endocrine reproductive axes with failed reproductive performance of the cultured broodstock.

Exogenous hormonal induction of reproduction

Hormonal induction of reproduction has been extensively studied in cultured sole broodstock and shown to be partially successful in both sexes. In females, different administration methods of gonadotropin-releasing analogue (GnRHa) have been tested. Injection of GnRHa, at doses of 5-25 µg kg^{-1} body weight (BW), induced a transient elevation of plasma E2 and T (Agulleiro *et al*. 2006; Guzmán *et al*. 2009b), slightly stimulated final oocyte maturation (Guzmán *et al*. 2009b) and induced egg spawning at 2-3 days after treatment (Agulleiro *et al*. 2006). Comparatively with the injection method, treatment of females with GnRHa slow-release delivery systems, including implants and microspheres, highly stimulated oocyte maturation, induced a higher and prolonged secretion of steroids and a higher number of spawns after a single treatment, thus increasing total egg production (Agulleiro *et al*. 2006; Guzmán *et al*. 2009b). However, spawning obtained after hormonal induction, as observed for non-induced natural spawning of cultured soles, were unfertilized in all experiments, and therefore the procedure is of limited interest for industrial application.

In the case of males, several hormones and administration methods have been tested to stimulate spermiation and sperm production. Treatment of males with GnRHa injection induced a transient elevation of androgens, but was ineffective to stimulate testis maturation or increase milt volume (Agulleiro *et al*. 2006; Guzmán *et al*. 2011a,b) and increases in sperm quality were also transient (Cabrita *et al*. 2011). The effectiveness of GnRHa was slightly increased by the use of slow-release implants, which induced higher release of steroids and a slight stimulation of spermatogenesis and milt volume (Agulleiro *et al*. 2007; Guzmán *et al*. 2011b). Combined treatment of GnRHa with 11-ketoandrostenedione, an 11KT precursor (Agulleiro *et al*. 2007) or pimozide, a dopamine antagonist (Guzmán *et al*. 2011a), slightly increased the potency of the GnRHa treatments. Treatment with a multiple injection protocol with human chorionic gonadotropin (hCG) also induced steroid release and proved to be more potent than GnRHa treatments to induce spermatogenesis and sperm production (Guzmán *et al*. 2011b). However, even if these combined treatments were effective to stimulate spermatogenesis and sperm production in males, with no detrimental effects on sperm quality, the increased volume produced was still insufficient and thus could only be considered as a minor success in terms of potential applicability in aquaculture production.

Gamete quality and quantity

To date few studies have compared gamete quality between cultured and wild females. Reared females held with wild males have produced viable spawns (Mañanós *et al*. 2007; Carazo *et al*. 2011; Carazo 2013) and Rasines *et al*. (2012, 2013) demonstrated that cultured breeders could be induced to ovulate and that ova could be stripped and fertilised. Although these studies did not explore the influence of female gamete quality in Senegalese sole reproductive dysfunctions, they indicate that cultured female gamete quality has not been a restrictive factor. On the other hand, sperm characteristics have been recently studied (Beirão *et al*. 2009, 2011), after the detection of low sperm volume, low cell concentration and production in both types of male breeders - wild and G1 (Cabrita *et al*. 2006). Senegalese sole males can produce motile sperm all year round with specific peaks of high spermiation and of high percentage of fluent males, which usually coincide with the female breeding season. However, considerable variation was observed in terms of sperm profiles in males maintained under the same conditions. Sperm volume collected ranged from 5 to 20 µl in G1 broodstocks and 10 to 80 µl in wild-captured broodstocks. Cell density and sperm production (total spermatozoa per stripping) ranged from 0.7 to 1.2×10^9 spz mL^{-1} and 20×10^6 in G1 males to values of $1–2\times10^9$ spz mL^{-1} and $40–60\times10^6$ spermatozoa for the wild-captured males, respectively. These results demonstrate that sperm production in this species is very low and variable, and that wild males produce sperm of higher quality (Cabrita *et al*. 2006). Therefore, sperm quality and quantity seems to be at least one of the reasons explaining the reproduction constraints of Senegalese sole. Recent studies have focused on gathering basic knowledge on the testis and sperm production (García-López *et al*. 2005; Marín-Juez *et al*. 2011, 2013; Chauvigné *et al*. 2014) and these and further studies will help provide solutions to improve sperm production.

Strip spawning and artificial fertilization

Artificial fertilization in sole is now feasible after the standardization of hormone-based protocols to induce ovulation in females, which allowed obtaining eggs through manual stripping (Liu *et al*. 2008; Rasines *et al*. 2012). A single GnRHa injection (25 µg kg^{-1} BW) induced ovulation in 70-85% of the treated females at 41-44 h post-treatment, at a water temperature of 16ºC (Rasines *et al*. 2012, 2013). The timing of ovulation was temperature-dependent, and only females at advanced stages of maturation were responsive to the treatment. Relative fecundity of Senegalese sole females after hormonal induction and egg stripping was around 100,000-150,000 eggs kg^{-1} BW (Rasines *et al*. 2013) and highest fertilization and hatching rates were obtained within 3 h after ovulation (Rasines *et al*. 2012). Therefore, using stripped eggs and sperm from G1 sole, an artificial fertilization protocol, based on that used for other flatfishes, has been successfully used for the production of fertilized eggs and viable larvae (Rasines *et al*. 2012, 2013). Nevertheless, fertilization and hatching rates were low, highly variable, and most importantly, unpredictable, with typical values around 30% and 15%, respectively. However, fertilization and hatching rates as high as 84.6% and 61.6% have been achieved (Rasines 2013), which confirms the potential of this technique. Future experiments are necessary to determine what are the critical parameters to optimize in order to render artificial fertilization a predictable and reliable tool.

As described above, one of the main problems in artificial fertilization has been the variable quality and particularly the low quantity of sperm collected. The several hormone induction methods that have been attempted resulted in small improvements (Agulleiro *et al*. 2006, 2007; Guzmán *et al*. 2011a,b; Cabrita *et al*. 2011), but the volumes obtained were generally not sufficient for aquaculture production. In addition, it is conceivable that the small testis size with gonadosomatic index of <0.15% (García-López *et al*. 2005) cannot be expected to produce large quantities of sperm. Therefore, in combination with artificial fertilization, cryopreservation of quality-controlled sperm could be used as a tool to guarantee that fish farms are able to store this material and have enough sperm of the necessary quality to fertilize the egg batches when necessary. Several protocols have been tested, mostly adapted from other species such as turbot. Briefly, sole sperm has been stored using the Mounib extender with 10% DMSO, packaged in 0.5ml straws and cryopreserved using a slower cooling rate (straws set at 5cm from the surface of liquid nitrogen, N_2) (Rasines *et al*. 2012) or packaged in 0.25 ml straws and cooled at a faster rate (2cm from the N_2 surface) (Cabrita, unpublished). Both protocols yielded good results in terms of fertility and sperm motility, but more research is required to optimize the procedure and to ensure that it does not affect progeny quality.

Larval nutritional physiology

As a candidate species for aquaculture, the easiness of larval and post-larval rearing of Senegalese sole compared to other marine species was one of the most remarkable and attractive aspects of this species from the beginning. For that reason, culture protocols were established early on (Dinis *et al*. 1999; Imsland *et al*. 2003; Conceição *et al*. 2007) and are nowadays fairly standardized, with post-larvae being routinely produced with good growth and at high survival rates. In spite of this, considerable research is still centered during these early life stages given that suboptimal rearing or nutritional conditions could have profound effects on later juvenile quality. Therefore, some of the problems observed during the nursery and on-growing stages, such as difficulties in weaning, variable growth rates, malformations and maybe even susceptibility to diseases, can potentially be improved by a better knowledge of larval nutritional physiology.

Digestive physiology and feeding

As in other vertebrates the digestive function in teleosts is highly associated to the particular anatomical characteristics and feeding habits that are very variable among species. As a flatfish, the Senegalese sole undergoes a remarkable transformation during the second week of life that strongly affects its behaviour, feeding and digestive physiology

(Fernández-Díaz *et al*. 2001). Conversely, metamorphosis may be affected by feeding and nutritional conditions (Yúfera *et al*. 2005; Villalta *et al*. 2008a; Engrola *et al*., 2009b; Engrola *et al*., 2010). A fair amount of studies have been conducted on the digestive physiology of this species, which have set the basis for a comprehensive morphological and functional characterization of its gastrointestinal ontogeny (see Conceição *et al*. 2007 for a thorough revision of earlier literature). The development of the gut and other organs involved in feeding and digestion is well known in Senegalese sole (Sarasquete *et al*. 1996; Ribeiro *et al*. 1999a; Fehri-Bedoui *et al*. 2000; Padrós *et al*. 2011) and follows the general pattern observed in other marine fish with altricial larval development and particularly that observed in other pleuronectids. However, this species has some important particularities. For instance, the mouth opens relatively early in Senegalese sole, at two days after hatching at 19-20ºC, and the mouth gape is wide enough to allow the ingestion of large zooplanktonic prey from first feeding. On the other hand, this species is characterized by having the smallest stomach and the longest intestine among the different families of flatfishes (De Groot 1971) and there is evidence that it does not have a good capacity for acid digestion, even after the gastric glands are formed in the juvenile (Yúfera & Darias 2007). Therefore, gastric digestion seems to be residual and most of the digestion occurs in the long intestine under an alkaline environment. This is likely associated to its omnivorous diet, although crustaceans, bivalves and polychaetes are the preferred prey of juvenile and adults (Cabral 2000; Sá *et al*. 2003).

Several studies have examined the activity of the main digestive enzymes during the larval development of Senegalese sole and showed that sole larvae are ready to digest from first feeding (Martínez *et al*. 1999; Ribeiro *et al.* 1999b; Moyano *et al.* 2001). Zymogen granules are present at the opening of the mouth and the activity of pancreatic (trypsin, amylase and lipase) and intestinal enzymes (alkaline phosphatase and leucine-alanine peptidase) exhibits the expected pattern found in most altricial marine fish species (reviewed in Conceição *et al*. 2007; Rønnestad *et al*. 2013). The alkaline proteases working in the developing gut exhibit a notable change by the time of metamorphosis (Moyano *et al.* 2001). Furthermore, settled post-larvae undergo a process of enzymatic maturation of the intestine which occurs between 21 and 27 dph at 22-25ºC (Ribeiro *et al*. 1999b) with an increase in the alkaline phosphatase activity and a decline of the leucine-alanine-peptidase activity concomitant with the development of the brush border membrane of the enterocytes. A recent study has shown that the maturation of the digestive system (measured as an increase in N-aminopeptidase/leucine-alanine-peptidase and alkaline phosphatase/leucine-alanine-peptidase ratios) was highly affected by fatty acid formulation used for the *Artemia* enrichment (Boglino *et al*. 2012a). The alkaline phosphatase activity, reflecting the degree of maturation of the digestive tract, was also previously seen to be affected by an early co-feeding of inert diet (Engrola *et al*. 2007; 2009a). Trypsin activity, on the other hand, appeared to be poorly affected by the feeding regime in sole larvae and post-larvae (Engrola *et al*. 2007; 2009a). However, in sole juveniles the amount of protein in the diet and the origin of the protein source, animal or vegetal, affected respectively the amount and composition of the alkaline proteases secreted in the intestine lumen (Rodiles *et al.* 2012). Therefore, proteolitic activity seems less dependent on diet composition during the pelagic phase and early post-larval stages while juveniles appear to be able to modulate the proteolitic activity in response to changes in dietary protein levels and sources (Sáez de Rodrigáñez *et al.* 2011; Rodiles *et al.* 2012).

Recently, genes for several digestive enzymes have been sequenced, which will allow more in depth studies looking into molecular mechanisms. Manchado *et al.* (2008), using high throughput transcriptomic techniques, identified six different trypsinogen genes grouped in three groups. Group I or anionic trypsinogens (*ssetryp1a*, *ssetryp1b* and *ssetryp1c*), group II or cationic trypsinogen (*ssetryp2*) and group III or psychrophilic trypsinogens (*ssetryp3* and *ssetrypY*). These showed a different pattern of expression before and after the eye migration stage, with *ssetryp1a* variants and *ssetryp2* transcripts exhibiting higher expression during pre-metamorphosis while *ssetryp3* and *ssetrypY* transcripts were more abundant in juveniles. This change had been previously noted in the zimograms of proteases (Moyano *et al*. 2001). In another study, as noted previously, Gamboa-Delgado *et al.* (2011) found that trypsin and chymotrypsin activities showed a similar pattern in Senegalese sole during early development independently of the dietary treatment but, however, increases in *ssetryp1* transcription were observed at different timings when feeding on rotifers from first feeding or when larvae were fed directly on *Artemia* nauplii (4 or 5 dph, respectively) at 16-22ºC. Therefore, there is evidence that the type of diet might modulate trypsinogen gene expression during early larval development, even if this is not reflected in trypsin activity.

Other recent studies have started looking at daily rhythms of feeding and digestion, as this information is important for optimizing species- and developmental stage-specific feeding protocols. Feeding incidence is high from the opening of the mouth, being 80% of larvae hatched from an egg batch able to feed actively in the first day of feeding and practically the totality of larvae in the second day (Navarro-Guillén *et al*. 2015). Although nocturnal habits have been described in juveniles and adults (Bayarri *et al*. 2004), we have only recently gained insight on the daily rhythms of activity during the larval and post-larval stages, which show a clear switch from diurnal to nocturnal when the larvae finishes the eye migration phase and starts the benthic life (Blanco-Vives *et al*. 2012). This has been related to feeding, as results show that during the early pelagic stage sole larvae exhibit a clear diurnal feeding pattern with maximum gut content at sunset hours while after the start of benthic life post-larvae may feed during the whole day (Navarro-Guillén *et al*. 2015). In another experiment with 35 dph post-larvae fed on *Artemia* with ^{14}C-labelled protein, a daily feeding and digestive rhythm was observed independent of the illumination cycle. In this experiment, post-larvae fed at 10:00, 14:00, 18:00 and 04:00h presented higher *Artemia* protein digestibility than sole fed at 23:00h, when *Artemia* intake was lower. In addition, higher protein retention and lower catabolism occurred in the hours with lower ingestion (18:00 and 23:00h) (Navarro-Guillén *et al*. 2014a). Finally, recent results on daily rhythm of digestion in sole larvae showed that trypsin activity had a daily pattern following that of the gut content, while lipase and amylase activities presented completely independent patterns (Navarro-Guillén *et al*. 2015).

Fatty acid and lipid requirements

The lack of suitable inert microdiets, with good acceptability and the possibility to manipulate nutrient composition with high precision and reproducibility, has not enabled performing detailed studies on lipid and fatty acid requirements in fish larvae. Therefore, the only available studies are with live prey, either non-enriched or after enriching with different commercial products or oil emulsions, which present important constraints in terms of the accuracy and reproducibility of the factors that can be analysed.

Available evidence suggests that, just as in juvenile stages (see below), high total lipid levels should be avoided in Senegalese sole larvae and post-larvae diets (Morais *et al*. 2005a,b; Conceição *et al*. 2007).

Senegalese sole larvae have particularly low requirements for LC-PUFA (reviewed in Conceição *et al*. 2007). In fact, post-metamorphosed larvae could be grown on diets containing negligible DHA and low EPA levels, such as non-enriched live prey, without obvious detrimental effects (Morais *et al*. 2004a, 2005a,b; Villalta *et al*. 2005a). From the studies that have been performed so far there is the indication that Senegalese sole larvae perform well, with good growth and survival up to 40 dph, with dietary EPA levels as low as 0.5% TFA (when DHA was provided at 4.3% TFA), while growth was decreased at higher (10.7 and 20.3% TFA) levels of EPA (Villalta *et al*. 2008a). Regarding DHA, Villalta *et al*. (2005a) observed a better growth (only significantly for total length, up to 36 dph) with 7.7% TFA of DHA than with 4.4% or 14.7% TFA but, nonetheless, differences were not significant in the treatment fed *Artemia* containing no DHA (all treatments contained 1.7% TFA of EPA). On the other hand, in a study by Morais & Conceição (2009), growth and metabolic results suggested that DHA requirements of Senegalese sole post-larvae, after settlement, are probably not higher than 3% TFA. Fine studies on EFA requirements are still missing but Boglino *et al*. (2012a), when testing different *Artemia* commercial enrichment products to determine the most suitable for Senegalese sole larval development until 38 dph, concluded that larval growth was highest when using a product which led to *Artemia* metanauplii containing intermediate values of ARA, EPA, DHA, total PUFA and monounsaturated fatty acids (32.7, 0.7, 3.1, 9.5, 51.1 and 32.7% TFA, respectively). Conversely, the treatment inducing the lowest growth and delayed morphogenesis was the one with highest levels of EPA (7.6% TFA).

Nevertheless, there is evidence suggesting that LC-PUFA requirements are higher in the early larval (pelagic) stage, which is probably a critical period associated with extensive organogenesis and tissue remodelling culminating in metamorphosis, which in flatfishes involves dramatic functional and morphologic changes (Morais & Conceição 2009; Dâmaso-Rodrigues *et al*. 2010). Therefore, EFA requirements need to be examined independently in the pre- and post-metamorphic stages. In addition, it should be kept in mind that the estimation of requirements may vary depending not only on the relative amounts of EFA (particularly DHA/EPA ratio) but also on the available dietary energy (Villalta *et al*. 2005a, 2008a; Boglino *et al*. 2012a). A recent study (Navarro-Guillén *et al*. 2014b) has shown that supplementation of DHA (up to 4% of TFA) to emulsions based on vegetable oils (supplying higher levels of better energy-yielding substrates than typical enrichment products) improved larval growth and survival, suggesting that a correct balance needs to be found between dietary energy and EFA.

Such unusually low requirements for LC-PUFA during the larval stages, compared to most marine fish species, were quite intriguing. However, a recent discovery revealed the physiological reason behind these observations, by uncovering a unique gene activity in the LC-PUFA biosynthesis pathway of this species. The classical pathway for the synthesis of DHA from EPA in vertebrates, known as the “Sprecher” pathway, involves two sequential elongations of EPA to 24:5n–3 followed by Δ6 desaturation and one round of peroxisomal β-oxidation (Voss *et al*. 1991). However, the extent to which a vertebrate species can produce LC-PUFA from lower chain precursors (C18 PUFA) varies according to its repertoire of fatty acyl elongase (Elovl) and desaturase (Fad) enzymes. In teleosts, a long standing paradigm was that species diverge according to the environment and/or trophic level they occupy and that marine species (contrary to freshwater/diadromous species) cannot biosynthesise LC-PUFA and hence have a strict dietary requirement for these fatty acids (Tocher 2010). For this reason, the discovery of a gene coding for a fatty acyl desaturase with Δ4 activity (*Δ4fad*) and which is able to produce DHA directly from desaturation of docosapentaenoic acid (DPA; 22:5n‒3), which is synthesised from EPA via elongation through *elovl5* (also functionally characterized in the same study), was an extraordinary breakthrough (Morais *et al*. 2012; Fig. 2). Furthermore, the expression of *Δ4fad* was shown to be transcriptionally regulated by dietary levels of LC-PUFA, being up-regulated when sole larvae were fed diets containing low levels of DHA (Morais *et al*. 2012; Navarro-Guillén *et al*. 2014b). What was even more surprising, considerable *elovl5* expression was already present in eggs, transcription of *Δ4fad* started just after hatching, and both transcripts peaked at the start of exogenous feeding (Morais *et al*. 2012). Furthermore, their levels in eggs and newly hatched larvae were significantly affected by the content of DHA in the broodstock diet, which in turn was reflected in the egg biochemical composition (Morais *et al*. 2014a). These results indicate a high degree of regulation suggesting a true biological relevance of this pathway in reducing LC-PUFA dietary requirements in this species.

Amino acids and protein requirements and metabolism

Early work on amino acid (AA) requirements of Senegalese sole (reviewed in Conceição *et al.* 2007) indicated that the ideal dietary AA composition changes along ontogenesis, probably tightly linked with metamorphosis (Aragão *et al.* 2004). These earlier studies also demonstrated that bioavailability of individual AA changes, and that early life stages of sole have a good capacity to regulate AA catabolism (Conceição *et al.* 2007).

As metamorphosis is a critical point in Senegalese sole development and is regulated by thyroid hormones, recent studies have looked at the importance of their precursors - the aromatic AA phenylalanine and tyrosine. Previous work by Aragão *et al.* (2004) showed that the contribution of aromatic AA to the larval profile decreased significantly after metamorphosis. Therefore, the fate of aromatic AA was followed by tube-feeding radiolabelled phenylalanine and tyrosine at different developmental windows, and the results showed a preferential retention of these AA during metamorphosis (Pinto *et al.* 2009), indicating that physiological requirements for these AA likely increase during this stage. In order to verify this, dietary aromatic AA supplementation at this stage was tested by means of short-term tube-feeding experiments (Conceição *et al.* 2010). The results suggested that tyrosine is probably conditionally indispensable for this species at metamorphosis, while dietary tyrosine supplementation may be beneficial to help larvae to cope with metamorphosis-related processes (Pinto *et al.* 2010a). In post-metamorphic fish, although no clear effects of dietary aromatic AA supplementation were observed under normal conditions, it was demonstrated that tyrosine supplementation helps the fish to maintain a normal tyrosine metabolism under stressful conditions (Aragão *et al.* 2010). Tyrosine, as mentioned before, is the precursor of thyroid hormones, but also of catecholamines, both mediating the stress response in fish.

In studies of live feed replacement by microencapsulated diets it was found that taurine supplementation increased larval growth potential and metamorphosis success in sole larvae (Pinto *et al.* 2010b). These effects were not observed during the pelagic phase, but a clear effect of earlier dietary taurine supplementation was observed in post-metamorphic larvae. Taurine is not used in protein synthesis but can be found freely in the cytosol. Among other factors, the antioxidant properties of taurine may be of special importance during sole metamorphosis. Later work revealed that larvae were fully able to uptake taurine in the digestive tract at the onset of metamorphosis and that metamorphosis was an important developmental trigger to promote taurine gut transport (Pinto *et al.* 2012).

Collectively, these results reemphasize that metamorphosis is a critical developmental stage in Senegalese sole, during which dietary AA profiles assume an extreme importance.

Recent modelling studies suggest that Senegalese sole are highly sensitive to dietary AA imbalances, which result in high AA losses and have a major impact in protein retention (Rønnestad & Conceição 2012). As sole larvae are still largely fed on live preys, which have an imbalanced AA profile (Aragão *et al.* 2004), and considering the high physiological requirements for some specific AA revealed in the latest studies, dietary supplementation with these AA seems paramount to maximize growth. The challenge now is to know how much and for how long these supplements should be provided. It has already been demonstrated in Senegalese sole juveniles that dietary AA supplementation with tryptophan during 14 days presents more benefits than during 28 days (Costas *et al.* 2012). Therefore, it is essential to evaluate different levels of dietary AA supplementation at specific developmental windows, so that the industry can accurately assess the cost/benefits of such an investment in growth and larval quality.

Estimates of *Artemia* protein digestibility in Senegalese sole larvae and post-larvae vary between 57% and 98% (Rønnestad *et al*., 2001; Morais *et al*., 2004b; Engrola *et al*., 2009b; Engrola *et al*., 2010; Campos *et al*., 2013a; Navarro-Guillén *et al*., 2014a). This variation can be explained by differences in developmental stage, temperature, time of feeding and feeding regime between studies. Some studies have addressed the effect of live prey replacement by inert diet at different developmental stages. In this respect, sole fed live prey alone or co-fed with 20% (low replacement) of inert diet showed a similar protein retention, always above 70%, except at 8 dph, when this was slightly reduced to around 65% (Engrola et al., 2009b). However, in sole co-fed with 58% of inert diet from mouth opening (high replacement), protein digestibility and retention efficiency were significantly reduced between 6 and 15 dph (Engrola et al., 2010). During the metamorphosis climax (14-18 dph) both live fed and co-fed sole had a lower protein digestibility than at younger or older ages (Engrola et al., 2009b; Engrola et al., 2010). This is probably explained by a reduced digestive capacity during the metamorphosis climax, as the retention efficiency remained almost constant throughout the experimental period at low replacement levels. Sole larvae do not seem to increase feed intake during this period (Parra & Yúfera, 2001; Engrola et al., 2010), and therefore protein deposition must be sustained by using energy reserves accumulated during the earlier stages (Parra & Yúfera, 2001).

Studies on the capacity of larvae to digest proteins of different complexities have been performed in view of the long-term aim of developing an inert diet that can be digested from mouth opening. These have shown that sole presents a poor capacity to digest complex proteins at mouth opening (Gamboa-Delgado *et al.*, 2008; Engrola *et al.*, 2009a). However, when testing peptide fractions, sole showed a high capacity to digest 1.0kDa peptide fractions at all developmental stages. In addition, the retention of a 7.0kDa peptide fraction was improved along development (Richard *et al.*, 2013). These studies support the suggestion that the complexity of the dietary nitrogen is a key issue that needs to be considered to improve marine larval growth (Conceição et al., 2011).

Finally, studies have shown that Senegalese sole protein metabolism and retention (hence growth) is highly modulated by thermal conditions during early developmental stages (Campos *et al.*, 2013b,c), as would be expected. It is noteworthy that larval rearing temperatures of 18 and 21ºC positively affected *Artemia* protein digestibility during metamorphosis, compared to larvae that were reared at 15ºC, indicating that the thermal history modulated physiological pathways (Campos *et al.*, 2013a). However, when the same larvae were analysed at an older age, *Artemia* protein digestibility was higher in larvae reared at lower temperature (15ºC), indicating that digestive capacity was no longer impaired and the improved digestibility could be at least partially responsible for the compensatory growth that was observed in larval weight (Campos *et al.*, 2013a).

On-growing diets

Nutrient requirements

Senegalese sole inhabits sandy or muddy bottoms of coastal or brackish areas, where it has a low-diversity diet consisting predominantly of polychaetes, but also small crustacean (such as tanaids and amphipods) and bivalve molluscs (Garcia-Franquesa *et al*. 1996; Cabral 2000). This dietary regime is likely characterized by higher protein and carbohydrate levels and lower total lipid contents than in the zooplanktonic food web. For instance, the biochemical composition of one of the main prey items in sole's diet, the polychaete *Nereis diversicolor*, varies from 47%-60% protein, 6.6%-19.3% lipids and around 8%-20% carbohydrates, depending on the season, in the Tagus estuary (Portugal) (Luis & Passos 1995). Nutritional studies in captivity have shown that sole diets should include a high crude protein level (53% dry matter, DM) to maintain good overall growth performance (Rema *et al*. 2008). The only available direct estimate for indispensable amino acids (IAA) requirement in sole refers to lysine (Lys), as Silva (2010) estimated an optimum dietary supply of 4.7 Lys 16 g^{-1} N for maximum protein accretion. More recently, Costas (2011) further refined the ideal protein profile in diets for juvenile sole by estimating the bioavailability of the IAA. In this study, the utilization of the 10 individual IAA was evaluated by tube-feeding Senegalese sole juveniles with compound feeds containing ^{14}C-labelled IAA as tracers. Results showed differences in digestibility, retention and catabolism between individual IAA, and bioavailabilities relative to lysine were calculated. High relative bioavailabilities were found for histidine, leucine, isoleucine, valine, methionine, threonine, phenylalanine and arginine, meaning that these IAA are retained more efficiently than lysine, while tryptophan had the lowest relative bioavailability among IAA (Costas 2011).

In most marine fishes, a significant protein sparing can be achieved by increasing digestible energy levels through an increase in fats and/or carbohydrates (Helland & Grisdale-Helland 1998; Kaushik 1998). However, contrary to most marine fish species, the ability of Senegalese sole juveniles to efficiently use high dietary lipid levels seems limited, in both juvenile (Dias *et al.* 2004; Borges *et al.* 2009; Guerreiro *et al.* 2012) and market-sized fish (Valente *et al.* 2011). Borges *et al*. (2009) clearly demonstrated a low lipid tolerance in this species, and recommended a dietary lipid inclusion of up to 8% for optimal growth and feed utilisation efficiency at a protein level of 57% (DM basis). Dietary lipids do not seem to be a good energy source for promoting growth in Senegalese sole as there is no clear evidence of a protein-sparing effect by increasing dietary lipid levels, even when the dietary protein level is below this species requirement (Mandrioli *et al.* 2012; Borges *et al.* 2013a). Irrespective of the rearing temperature (16 vs. 22°C), Guerreiro *el al*. (2012) also found that feed efficiency, N retention and energy retention were highest in sole juveniles fed a diet containing 55% protein and 8% lipids. The activity of enzymes involved in key metabolic pathways points towards a lack of metabolic adaptation to high lipid levels (Dias *et al.* 2004; Borges *et al.* 2013a). Recently, Mandrioli *et al*. (2012) showed that a concomitant increase in dietary lipids and decrease in dietary protein and was associated with a massive storage of unused lipid within sole hepatocytes. Similarly, Valente *et al*. (2011) reported moderate steatosis and some cellular necrosis in large-sized sole (>300g) fed high lipid levels. Moreover, Campos *et al.* (2010) observed a decrease in the expression of myogenic regulatory factors and myosins in the muscle of Senegalese sole fed increasing dietary lipid levels, supporting the hypothesis that high lipid levels somehow depress growth by reducing protein accretion.

Even though dietary lipids are not efficiently used as non protein energy sources in Senegalese sole, its digestion and absorption seems identical to other marine species (Dias *et al.* 2010; Borges *et al.* 2013b). Different dietary levels equally induced high lipid digestibility and intestinal lipase activity, while increased plasma concentrations were observed in fish fed high fat diets compared to those fed the low fat diet, demonstrating effective lipid absorption (Borges *et al.* 2013b). *S. senegalensis* is a lean fish (1–4g of fat/100g of flesh) with a scarce capacity to accumulate fat even when fed high lipid levels (Borges *et al.* 2009, 2014; Fernandes et al. 2012). Previous studies (Rueda-Jasso *et al.* 2004; Borges *et al.* 2009; Valente *et al.* 2011; Fernandes *et al.* 2012) reported that liver is the preferential site for fat deposition (5.5–37%). Furthermore, sole liver seems to have an important role in clearing plasma triglycerides, showing an increased expression of genes involved in lipid transport (microsomal triglyceride transfer protein, MTP), trafficking (fatty acid binding protein 11, FABP11) and fatty acid uptake (very low density lipoprotein receptor, VLDL-r) in juvenile fish fed high fat diets (Borges *et al.* 2013b).

As the recommended diets for Senegalese sole have a low lipid level (8%), most energy must be derived from protein and starch substrates. Marine carnivorous fish generally show a low ability to use dietary carbohydrates as an energy source. However, data from Borges *et al*. (2013a) and Guerreiro *et al*. (2014) showed that increased levels of dietary carbohydrates had no detrimental effects on the growth performance of Senegalese sole juveniles. Additionally, Borges *et al*. (2013a) reported that phosphofructokinase 1 (PFK-1) was up-regulated in sole fed a low fat:high starch diet, suggesting also a role for glucose as an energy source in Senegalese sole muscle. To investigate this further, these authors looked at the effect of high dietary lipid levels on glucose metabolism in sole (Borges *et al*. 2014a). Results showed that fish fed on high fat:low starch diet exhibited prolonged hyperglycemia and greater liver glucose-6-phosphatase activity than fish fed on low fat:high starch diet. Moreover, the level of major proteins involved in the insulin and nutrient signalling pathway (AKT, p70 ribosomal S6-K1 Kinase and ribosomal protein S6) were reduced in muscle of fish fed on high fat diets suggesting a possible insulin resistance state (Borges *et al*. 2014a). Further studies on the metabolic abilities of Senegalese sole to cope with high dietary carbohydrate levels are needed.

Alternative vegetable ingredients and nutritional value for human consumption

High-quality fish meal (FM) is still the major protein source currently used in sole diets. However, supplies of FM and fish oil (FO) are finite, and their replacement in aquafeed formulations with ingredients from more available plant sources is a major necessity (Tacon & Metian 2008). In this respect, Senegalese sole appears to have an important advantage with regards to other marine fish species, as it has been demonstrated to cope well with diets in which most FM (89%) was replaced by plant protein (PP) sources (Silva *et al*. 2010). Furthermore, FM could be totally replaced by a mixture of PP sources without any adverse effects on growth, feed or protein utilization provided that the dietary AA are balanced by the addition of small amounts of crystalline AA (Silva *et al.* 2009). After an intense research effort in recent years, it is now clear that replacement of marine-derived protein sources by practical PP ingredients in Senegalese sole feeds with minimal AA supplementation is feasible in both juvenile (Cabral *et al.* 2011) and large-sized fish

(Valente *et al.* 2011; Cabral *et al.* 2013). Present data indicates that Senegalese sole can efficiently use diets with up to 75% of FM replacement by PP sources, but growth rate and nutrient gain in juveniles mainly depend on the selection of adequate PP blends, rather than on the PP incorporation level (Cabral *et al.* 2011). The source of dietary protein modified both the amount and composition of the pancreatic proteases secreted into the intestinal lumen of juveniles, without reducing growth (Rodiles *et al.* 2012), which indicates the capability of Senegalese sole to modulate digestive protease secretion when dietary protein sources are modified. This is further supported by data on large-sized sole, revealing similar nutrient intake and utilization (including similar apparent digestibility coefficients (ADCs) of nutrients) in fish fed either PP or FM based diets (Cabral *et al.* 2013). It is also noteworthy that increasing FM replacement level has a positive environmental impact as it reduces nitrogen losses and fecal phosphorus (P) waste, as well as the FM used per kg of sole produced (Fi:Fo ratio) (Cabral *et al.* 2011; Cabral *et al.* 2013). Further studies on the digestibility of alternative ingredients are warranted to help select the most adequate PP combination for this species.

Some studies have reported a decrease in whole body lipid content of sole fed PP diets with the increasing replacement of FM (Silva *et al.* 2009; Cabral *et al.* 2013). However, another study has shown that high dietary PP levels can increase lipid storage in liver (Valente *et al*., 2011), while muscle lipid content is generally not affected by the inclusion of PP. On the other hand, the replacement of FM by PP, even at extremely high levels (75 and 100%), is still effective in producing a n-3 LC-PUFA rich product. Muscle fatty acid profile of fish fed PP sources showed higher levels of C18:2n-6 while levels of DHA, DHA/EPA and EPA/ARA ratios were not affected (Cabral *et al.* 2013).

Fish is the main dietary source of DHA and EPA for humans and these fatty acids are best known for preventing cardiovascular and inflammatory diseases (Simopoulos 1999). There is a general concern about a possible loss of health beneficial effects for human consumers when replacing FO, rich in EPA and DHA, by vegetable oils (VO) which lack these EFA. In Senegalese sole it was possible to substitute up to 100% of FO by either linseed oil and soybean oil (Benítez-Dorta *et al.* 2013) or blends of rapeseed, soybean and linseed oil (Borges *et al.* 2014b) without significantly compromising juvenile performance; of these, only the 100% soybean oil diet slightly reduced growth and feed utilization. Although such high substitutions altered muscle fatty acid profile, reflecting the dietary composition, a selective deposition and retention of DHA was observed, as this fatty acid was only slightly, but not significantly, reduced in comparison to FO fed fish (Benítez-Dorta *et al.* 2013; Borges *et al.* 2014b). Recommended daily intake (RDI) of EPA+DHA is estimated to be at least 0.25 g per day for healthy human individuals (EFSA 2010) and even sole fed on VO based diets can provide consumers with almost two times the RDI, confirming its good nutritional value (Borges *et al.* 2014b). Nevertheless, it should be noted that in all tested diets FM has largely contributed to total dietary fat content and therefore further research is necessary to evaluate vegetable diets during all production cycle and with simultaneous substitution of FM and FO in order to clarify the impact of these diets in Senegalese sole flesh quality. However, it is likely that the fatty acid profile could be re-established with a finishing diet, although this still needs to be demonstrated.

The above results are surprising for a marine species but, as mentioned previously, a fatty acyl elongase (*elovl5*) and a unique desaturase coding for a protein with Δ4 activity (*Δ4fad*) were discovered and functionally characterized in Senegalese sole and *in vitro* results suggested capability to synthesize DHA from EPA (Morais *et al.* 2012) in this species. In addition, other circumstantial evidence related to the expression of these genes and the fatty acid profile of post-larvae fed *Artemia* enriched with different VO emulsions supplemented with DHA supported this hypothesis and went further in suggesting that DHA might even be produced from C18 PUFA precursors, even though a gene coding for a Δ6Fad has not been found so far in this species (Navarro-Guillén *et al*. 2014b). However, the *in vivo* activity and biological relevance of this pathway could not be determined at the time. This was very recently addressed in a study where juvenile sole were fed isoproteic diets containing 8% or 18% total lipid supplied by either 100% FO or with 75% of the FO replaced by a VO blend. When incubating hepatocytes and enterocytes isolated from these fish with [1-^{14}C]18:3n-3 and [1-^{14}C]20:5n-3, it was found that there was no Δ6 desaturation of ^{14}C-labeled 18:3n-3 in either tissue and only elongation to 20:3n-3, and therefore the hypothesis of a potential capability of LC-PUFA synthesis from C18 PUFA was not substantiated. In contrast, substantial amounts of radioactivity from ^{14}C-labeled EPA were recovered in 22:5n-3, 24:5n-3 and DHA, and the levels of these metabolites were higher at lower dietary lipid level and clearly stimulated by raising dietary VO levels particularly in hepatocytes (Morais *et al*. 2014b). This unequivocally confirmed the existence of a true capacity to biosynthesize DHA from EPA in Senegalese sole which, furthermore, is modulated by dietary composition both in terms of lipid level and fatty acid composition.

Finally, considering the highly appreciated sensorial characteristics of Senegalese sole flesh, it is important to evaluate how this can be affected by vegetable-based diets. The sensory evaluation of cooked flesh slices showed that the replacement of FM by PP blends did not have a significant impact on the majority of volatile compounds (Silva *et al.* 2012; Moreira *et al.* 2014) or sensory descriptors (Cabral *et al.* 2013). Nevertheless, the long term impact of high PP incorporation levels on gut integrity, liver function and immune status still needs to be addressed.

Phenotypic abnormalities affecting fish quality

Skeletal deformities and, to a lesser extent pigmentary disorders, are two important factors that may reduce the productivity and profitability of Senegalese sole intensive production (Koumoundouros 2010; Fernández & Gisbert 2011; Boglione *et al*. 2013a). The percentage of fish with medium to severe anomalies and/or malpigmentation varies greatly, not only among different farms, but also among different batches within the same hatchery or even within the same batch of eggs (Boglione *et al*. 2013a; Darias *et al*. 2013a). Hatcheries that provide juveniles grade out malformed fish, while those farms that grow-out abnormal fish to market size have either to downgrade the product to a lower value or discard them before their commercialization. Moreover, fish with severe pigmentary disorders cannot be commercialized as these fish do not meet the quality standards set up for fishery products (Bolker *et al*. 2005; Hamre *et al*. 2007; Darias *et al*. 2103a). Therefore, losses during the whole production cycle may be substantial. Hatchery and nursery productions are mostly affected, since the majority of skeletal structures (Gavaia *et al.* 2002) and skin chromatophores (Darias *et al*. 2013b) develop and differentiate during the larval and post-larval stages.

Skeletal deformities

Regarding the etiology of deformities, skeletal anomalies are generally the result of genetic factors and/or the incapacity of homeorhetic mechanisms to compensate for stressful environmental conditions (Boglione *et al*. 2013a). In this context, the development of skeletal disorders has been linked to a poorly understood relationship between environmental and biotic factors affecting fish morphogenesis (see reviews in Lall & Lewis-McCrea 2007; Boglione *et al.* 2013a). Consequently, understanding the environmental needs and nutritional requirements of fish larvae is essential for improving husbandry and rearing practices, and reducing the incidence of skeletal disorders and associated economical costs. Although there is considerable information on the impact of environmental and genetic factors on the development of skeletal deformities in several farmed teleost species (Boglione *et al*. 2013b), most of the information gathered in Senegalese sole deals with the impact of nutrition on larval morphogenesis (Engrola *et al*. 2009a; Fernández *et al*. 2009; Fernández & Gisbert 2010, 2011; Boglino *et al.* 2012a,b,c), and there is only one report on the effects of environmental factors on skeletogenesis (Blanco-Vives *et al.* 2010). Larval nutrition during pre- and pro-metamorphosis is one of the key parameters affecting skeletogenesis during sole early development and several studies have shown that suboptimal levels and different forms of nutrients supplied in the diet are responsible for the appearance of skeletal deformities (Lall & Lewis-McCrea 2007; Fernández & Gisbert 2011; Boglione *et al.* 2013a). Most of these studies in Senegalese sole have focused on lipids, particularly EFA (Boglino *et al*. 2012a,b,c, 2013), and liposoluble vitamins, especially vitamin A and K (Fernández *et al*. 2009; Fernández & Gisbert 2010, 2011; Richard *et al*. 2014).

Different studies have shown a high incidence of skeletal deformities in hatchery-reared Senegalese sole, with values ranging from 44% (Gavaia *et al.* 2002) to 80% (Engrola *et al.* 2009a; Fernández *et al.* 2009; Boglino *et al.* 2012a). Most of the skeletal anomalies are typically found along the vertebral column and caudal fin complex of pro-metamorphic larvae and early juveniles. The vertebral column in Senegalese sole is generally composed of 45 vertebrae, divided in 8 prehaemal and 37 haemal vertebrae (including the urostyle). The haemal region of the vertebral column, especially the pleural vertebrae, is mostly affected by the fusion and/or compression of vertebral bodies, and by abnormalities of the vertebral arches (Engrola *et al.* 2009a; Fernández *et al.* 2009; Cardeira *et al.* 2012). Incomplete and complete fusion of vertebral bodies are not necessarily considered as different stages of the same anomaly, as not always incomplete fusions aggregate in older fish (Deschamps *et al.* 2009). Although these skeletal disorders are common in reared specimens (Gavaia *et al*. 2009; Engrola *et al*. 2009a; Fernández *et al*. 2009; Fernández & Gisbert 2010; Boglino *et al*. 2012a,c), they have also been identified in wild animals, but at a lower frequency (Gavaia *et al*. 2009).

Among the different body regions, the head is the area less affected by skeletal disorders in Senegalese sole. This differs from round finfish species, where jaw abnormalities are very common (Boglione *et al*. 2013a), and might be explained by differences in the timing of ossification. In Senegalese sole both jaws ossify soon after the onset at exogenous feeding (*ca.* 2 dph at 17-18ºC; Wagemans & Vandewalle 2001; Fernández *et al*. 2009), whereas in other species such as gilthead sea bream or European sea bass this process takes place at later stages of development (Koumoundouros 2010; Gisbert *et al*. 2014). In addition, lower incidences of jaw anomalies were observed when sole were reared at a thermal cycle of 22.1°C day/19.0°C night, instead of 19.2°C day/22.0°C night (Blanco-Vives *et al.* 2010). Studies in mammals have shown that light and temperature manipulation changes the pattern of osteoblast and bone mass proliferation in response to changes in circadian pattern, which might have affected the normal development of jaws in sole (Boglione *et al*. 2013a).

When different commercial enrichment products differing in biochemical composition and fatty acid profile were tested, the incidence of skeletal deformities was similar (71.9 - 79.3%). However, data from larval performance and degree of ossification suggested that levels of DHA, EPA and ARA of 9.5, 3.1 and 0.7% TFA, respectively, and (n−3)/(n−6) PUFA, DHA/EPA, ARA/DHA ratios of 5.2, 3.0, 0.1, respectively, were the most adequate, among the tested products, for proper larval development (Boglino *et al.* 2012a). So far most of the nutritional studies dealing with EFA have focused on the effect of ARA on skeletogenesis. Senegalese sole post-larvae fed high dietary ARA levels (7% TFA) from 8 to 50 dph tended to have a less mineralized skeleton in comparison with those animals fed lower doses of ARA (4.5% TFA) (Boglino *et al*. 2012b). Generally, lower mineralization has been shown to contribute to the development of skeletal disorders in fish, as less mineralized bones tend to be more fragile and prone to abnormal development or get more easily deformed (Boglione *et al*. 2013b). Although Boglino *et al*. (2012b) recommended 4.5% TFA as the optimal level of dietary ARA for proper growth and mineralization in Senegalese sole larvae during the *Artemia* feeding period, none of the tested dietary ARA levels (1.0, 4.5 and 7.0% TFA) affected the incidence of skeletal deformities. However, feeding Senegalese sole larvae with high levels of ARA (10.2 and 7.1% TFA in enriched rotifer and *Artemia* metanauplii, respectively) at different developmental stages resulted in differences in the incidence of vertebral column deformities (Boglino *et al*. 2012b). In brief, skeletal deformities affecting the vertebral column were 25-32% higher when larvae were fed high dietary ARA levels during both the rotifer and *Artemia* feeding periods (2-50 dph) than when these were fed only during the *Artemia* period (8-50 dph). These results suggest an important effect of ARA intake during the pre-metamorphic stage (3-10 dph) on the proper development of the vertebral axis. In addition, ARA levels also appeared to affect the processes of eye migration and cranial bone remodelling as sole juveniles fed high dietary ARA levels from mouth opening presented a higher incidence of cranial deformities (95.1 ± 1.5%). These were mainly associated to the impairment of eye migration, which resulted in various alterations including shape of the head, disposition of the eyes with regards to the vertebral column and mouth axes, and a shorter interocular distance. Furthermore, changes in the positioning of both eyes resulted in osteological differences for some of the skeletal elements from the splanchnocranium and neurocranium (Boglino *et al.* 2013). A possible explanation for these disorders could be an overproduction of ARA-eicosanoid derivates, like prostaglandin E2 (PGE_2) (Boglino *et al.* 2013) or leukotriene B4 (LTB_4) (Alves-Martins *et al*. 2012), which can disrupt the rate of bone mineralization, formation and resorption.

With respect to vitamins, there is extensive information on the effects of dietary vitamin A (VA) on skeleton morphogenesis of Senegalese sole as well as of other flatfish species (see review in Fernández & Gisbert 2011). In general, the incidence of vertebral deformities in Senegalese sole increased with dietary VA content, independently of the developmental stage (pre-, pro-, and post-metamorphic larvae) at which the VA doses were administered. However, the severity of these deformities was higher when high VA doses were given at early developmental stages (Férnandez & Gisbert 2011). In particular, high levels of VA (203,000 IU kg^{-1}) offered to larvae from 6 to 37 dph induced a lower length and an increase in deformed fish, as well as in the percentage of fish showing more than one deformity (Fernández *et al*. 2009). However, no jaw deformities were observed in any larval group, suggesting that the dietary VA imbalance occurred at a non-critical developmental stage for jaw skeletogenesis. Still, larvae fed the 203,000 IU VA kg^{-1} showed cranial deformities related to the opercular complex that was significantly correlated to deformed prehaemal vertebrae. In addition, when such high VA levels were administered at the pre-metamorphic stage (3-10 dph) the notochord segmentation process was disrupted and the percentage of post-metamorphic specimens with a supranumerary vertebra increased. In a subsequent study, Fernández and Gisbert (2010) observed that skeletal structures presented differential sensitivity to dietary VA imbalance in terms of their ossification. Senegalese sole larvae fed *Artemia* containing more than 42,666 IU kg^{-1} VA showed a significant increase in the incidence of deformities in most chondral structures (neural and haemal spines, epural and parahypural), whereas intramembraneous bone structures (haemal and caudal vertebrae centra) were only affected when larvae were fed the highest VA dose (203,000 IU total VA kg^{-1}). Comparing these studies in Senegalese sole with those in other flatfish species like Japanese flounder, summer flounder (*Paralichthys dentatus*) and Atlantic halibut, it becomes clear that flatfish larvae present different developmental timings, and it is likely that the same dietary VA imbalance offered at the same time after hatching may induce different skeletal deformities or, at least, different deformity incidences and severities for each species (Fernández & Gisbert 2011). Furthermore, retinoid requirements seem to be quite different between flatfish species; e.g., a safe level for Japanese flounder should be < 50,000 IU kg^{-1} VA (Dedi *et al.* 1995), whereas < 42,666 IU kg^{-1} VA is advisable for Senegalese sole (Fernández *et al*. 2009).

In a recent study of vitamin K, Richard *et al*. (2014) showed that live prey grown in a commercial enrichment emulsion supplemented with 250 mg kg^{-1} of vitamin K (phylloquinone) significantly improved larval growth performance, as well as the skeletal quality of post-metamorphic specimens. In particular, authors found that these fish had a lower incidence of deformities and a reduced percentage of fish with malformed/fused haemal and neural arches or spines, and fusions in caudal vertebrae. Dietary supplementation of vitamin K modulated the expression of proteins (MALDI-TOF-TOF analysis) involved in several biological processes including muscle contraction and development, cytoskeletal network, skin development, energy metabolism, protein chaperoning and folding, and bone development (Richard *et al*. 2014).

Flatfishes possess large dorsal and anal fins, supported by a large number of soft rays that form a semi continuous structure together with the caudal and anal fins, both in adult and larval fish. A significant number of anomalies affecting the fins of Senegalese sole have been observed, appearing mainly as fusions or anomalies of hypurals in the caudal fin and as anomalous pterygiophores in the dorsal and anal fins (Gavaia *et al*. 2002; Engrola *et al*. 2009a; Fernández *et al*. 2009; Fernández & Gisbert 2010). The cartilaginous elements, like those composing the internal skeleton of the dorsal, anal and caudal fins, appear to be more sensitive to high levels of VA or oxidized lipids than dermal bones (Fernández & Gisbert 2010; Boglino *et al*. 2014).

What is noteworthy in Senegalese sole is the higher incidence of osteological abnormalities in this species when reared under standard feeding protocols in comparison to other commonly produced species in the Mediterranean and Atlantic area, like gilthead sea bream or European sea bass (see review in Boglione *et al*. 2013a). Fernández *et al*. (2009) suggested that two different hypotheses might explain this. The first one considered that this flatfish species is more prone to develop skeletal disorders than other fish species under any rearing conditions, whereas the second hypothesis postulated that since the skeletal deformities observed in Senegalese sole were not lethal, higher final numbers of Senegalese sole specimens with deformities would be observed at the juvenile stage than in other species where deformities were lethal at early stages (see review in Koumoundouros 2010). Since both hypotheses are not mutually exclusive, determining which better explains the observations requires further developmental studies to identify the most sensitive periods of morphogenesis and their effect on the development of skeletal abnormalities, as well as the timing of appearance of the deformities and their impact on larval survival.

Pigmentation abnormalities

In flatfish species, pigmentation abnormalities are characterized by either a deficiency of pigment cells on portions of the ocular side (albinism, pseudoalbinism or hypomelanism), or excess pigmentation on the blind side (staining, spotting, or ambicolouration) (Bolker & Hill 2000). Pigmentary disorders, especially albinism and ambicoloration, can affect up to 61% of the reared fish of different flatfish species (Estévez & Kanazawa 1995; Estévez *et al*. 1999; Bolker & Hill 2000; Villalta *et al*. 2005b; Hamre *et al*. 2007; Guillot *et al*., 2012; Darias *et al.* 2013a; Boglino *et al*. 2014). However, pigmentation abnormalities are not as critical in Senegalese sole as it has been reported in other flatfish species (Seikai & Sinoda 1981; Bolker *et al*. 2005; Hamre *et al*. 2007). In fact, under current practical farming conditions malpigmentation in sole seldom occurs.

Most data regarding pigmentation disorders in Senegalese sole are from experimental studies in which pseudoalbinism was induced through a dietary excess of ARA (Villalta *et al*. 2005b, 2008b; Boglino *et al*. 2013; Darias *et al*. 2013a), although environmental rearing conditions and other nutrients like DHA, EPA and VA have been investigated as factors possibly related to the occurrence of albinism in hatchery production of other flatfish species (Bolker & Hill 2000; Hamre & Harboe 2008). Several studies have shown that when Senegalese sole larvae are fed high dietary ARA levels (7.1 – 10.2% TFA) post-larvae have a high incidence of pigmentary disorders (81.4 – 84.4% of pseudoalbinism), whereas only 0.3 to 0.9% of malpigmented fish were found when larvae were fed 0.1%

ARA (Villalta *et al.* 2005b, 2007; Boglino *et al*. 2013). A likely explanation is that high ARA levels, by altering the dietary and body ARA/EPA ratio, can modify the relative concentrations of ARA-derived eicosanoids in the developing organism, which might disrupt the morphogenesis of the skin resulting in pigmentary disorders. In addition, studies on Soleids have demonstrated that not only dietary levels of ARA, but also the timing of its administration in relation to the larval developmental stage (Lund *et al.* 2007, 2008; Boglino *et al.* 2014), as well as abiotic factors such as tank colour (Lund *et al*. 2010), can affect the incidence of pigmentary anomalies. In common sole, a strong light intensity (3600–4500 lx at the water surface) in combination with a transparent tank colour induced pseudoalbinism, even if on a much lower scale than the observed effects of ARA (Lund *et al*. 2010). Boglino *et al.* (2014) evidenced the existence of a “pigmentation window”, with greater larval sensitivity to high dietary ARA levels during pre- and pro-metamorphosis (2–15 dph) than post-metamorphosis (15–50 dph). This was correlated with the aspect and density of melanophores in the skin of the ocular side, which decreased in the order: normally pigmented individuals > pseudo-albino fish fed high ARA levels during postmetamorphosis > pseudo-albino fish fed high ARA levels during pre- and pro-metamorphic stages.

Morphological studies by Darias *et al*. (2013a) in Senegalese sole revealed that ARA did not affect larval pigmentation at the pre-metamorphic stage, but prevented chromatophore terminal differentiation at metamorphosis, leading to the appearance of pseudo-albinism. The authors concluded that the relative proportions between xanthophores and melanophores, cell proximity, size and shape were critical for the correct ontogeny of pigmentation. In this regard, those larvae later becoming pseudo-albino and pigmented individuals developed pigmentation in the same way but, once metamorphosed, the future pseudo-albinos began to show different relative proportions, allocation patterns, shapes and sizes of skin chromatophores. In sum, the amount of melanophores and iridophores in pseudo-albinos remained invariable during larval development, whereas normally pigmented specimens showed an increase in the population of melanophores and iridophores at post-metamorphosis (47 and 35 dph, respectively), which indicated that the new population of chromatophores that should appear after metamorphosis was not formed (or cells were not pigmented) in pseudo-albinos. Furthermore, a decrease in xanthophores was observed from 33 to 35 dph in pseudo-albinos, likely as the result of the degradation of already existent cells. Pseudo-albinos had also lost most of their leucophores, with those few remaining being located almost exclusively in the distal part of the fins (Darias *et al*. 2013a,b). In one of these studies, Darias et al. (2013a) has shed some light on the molecular regulation of some of the events leading to the establishment of the ARA-induced pseudo-albino phenotype, including the expression of several key pigmentation-related genes.

Regarding the establishment of dorsal-ventral pigmentation, pigment cell latent precursors are symmetrically located mainly along the dorsal and ventral margins of the flank during larval stages, and then migrate from these regions to the lateral sides. After late metamorphic stages, these precursors differentiate into adult-type chromatophores on the lateral asymmetrical sides. Since the asymmetric body plan, including eye migration, precedes adult pigment pattern formation (Watanabe *et al*., 2008), pigment asymmetry in flatfish seems to depend on an asymmetric organizational environment that may regulate survival, proliferation, distribution and differentiation of latent precursors into adult-type pigment cells. In this context, the agouti-signalling protein 1 (Asip1) has a key role in proper dorsal-ventral pigment patterning in juvenile and adult fish, since it induces the regulatory asymmetry involved in precursor differentiation into mature chromatophores.

Dorsal-ventral pigmentary disorders such as dorsal pseudoalbinism were explained as being the consequence of the expression of normal developmental pathways in an erroneous position, resulting in unbalanced *asip* production levels. These, in turn, generate a ventral-like differentiation environment (iridophore proliferation) in dorsal regions (Guillot *et al*., 2012; Darias et al., 2013a).

Health issues

Stress response

Senegalese sole response to acute stress is in line with that previously reported by Barton and Iwama (1991), being the highest increase in plasma cortisol within 0.5-1h after a stressful disturbance. However, Costas *et al.* (2011a) observed much higher cortisol concentrations than those reported in other studies with the same species (López-Olmeda *et al.* 2013) and those reported in several teleosts following air exposure (Waring *et al.* 1996; Arends *et al.* 1999; Jentoft *et al.* 2005; Cnaani & McLean 2009). Different cortisol values reported by Costas *et al.* (2011a) and López-Olmeda *et al.* (2013) are probably related to the duration of air exposure (3 min vs. 30 sec, respectively), suggesting that aquaculture-related handling procedures such as grading must be carefully supervised to avoid long-term air exposure and thus higher cortisol release.

Intensively farmed fish may encounter situations or events that they perceive as potentially threatening such as poor water quality, physical disturbances (i.e. transport, grading), or sub-optimal stocking densities and social environments (Pottinger 2008). Cortisol concentrations reported in chronically stressed Senegalese sole reared under comparable conditions are quantitatively similar (Aragão *et al.* 2008; Costas *et al.* 2008, 2012, 2013a; Salas-Leiton *et al.* 2010; Arjona *et al.* 2009), and appear to decrease in time, probably due to a negative feedback of cortisol at the level of the hypothalamus and pituitary axis, thus modulating adrenocorticotrophic hormone (ACTH) secretion and consequently cortisol production (Mommsen *et al.* 1999). Although it is widely accepted that chronic stressful conditions decrease growth in fish and may reduce immunocompetence leading to enhanced susceptibility to pathogens and parasites (Wendelaar Bonga 1997), most studies reported that growth in sole is not affected by high stocking densities and repeated handling stress (Aragão *et al.* 2008; Costas *et al.* 2008, 2012, 2013a; Salas-Leiton *et al.* 2010). Intensively reared Senegalese sole subjected to different high stocking densities (24 and 30 Kg m^{-2} initial stocking densities) initially showed decreased growth performance (after 30 and 40 days, respectively), developing thereafter a compensatory growth strategy resulting in no differences in growth after 60 days, compared to specimens reared at low density (Salas-Leiton *et al.* 2010; Andrade 2012). While most studies presented increased cortisol levels and/or energy metabolism in parallel with decreased immune parameters and/or eventual mortalities, Andrade (2012) observed no changes in Senegalese sole after 30 and 60 days suggesting that under farming conditions other chronic stressors related to water quality or rearing system may modulate the stress response. For instance, Pinto *et al.* (2007) reported that growth and nutritional state of Senegalese sole juveniles were adversely affected following chronic exposure to high environmental ammonia (497 mg L^{-1} NH_3) for 52 days. Similarly, Arjona *et al.* (2009) observed a decrease in growth and feed intake in Senegalese sole reared at salinities lower than 39‰, with the most profound effects observed at 15‰. Therefore, sudden salinity changes and increases in environmental ammonia represent a potential risk for Senegalese sole farms and must be avoided to increase productivity, while other intrinsic factors in intensive sole production such as repeated handling and high stocking density do not seem to be an issue provided that optimum rearing conditions are guaranteed.

Dietary effects, in particular the effect of ARA/EPA ratio, in the stress response of this species has also been investigated by Alves-Martins *et al*. (2011). These authors observed a quicker recovery from an air exposure acute stress, but not from a repeated stress (determined as the return of cortisol to basal levels) when post-larvae were fed a low ARA/EPA ratio (0.7). The same authors (Alves-Martins *et al*. 2013) later found that increases in dietary levels of ARA (up to 1.7% TFA) were positively associated with post-stress cortisol levels, and the highest tested ARA level (2.3% TFA) seemed to slightly enhance basal cortisol level and alter the response to stress in post-larvae at 35 dph.

On the other hand, differences in Senegalese sole plasma cortisol levels may also be related to genetic factors. Within a single strain or population, variation in stress responses also has a genetic component and some fish may be predisposed to consistently exhibit high or low cortisol responses to stressors (Tort *et al.* 2001). Therefore, selection of phenotypic characters (e.g. high- or low-cortisol responders) could be considered in order to improve production.

Immune response

The vertebrate innate immune system recognises pathogenic and non-pathogenic micro-organisms via germline encoded pathogen pattern recognition receptors (PRRs) that sense particular structures of the microorganisms (pathogen-associated molecular patterns, PAMPs) and initiate a well orchestrated immune response (Boltaña *et al.* 2011). The few existing studies on the Senegalese sole immune responses have mainly focused on innate immunity, particularly on the interactions between bacterial pathogens and parameters involved in responses to pathogens and stressful rearing conditions. Costas *et al.* (2013b) reported leucocyte responses to inflammation in both peripheral blood and peritoneal cavity following challenge with two *Photobacterium damselae* subsp. *piscicida* (*Phdp*) strains from different geographical origins, thus corroborating the hypothesis of cell migration to the inflammatory focus in fish. *In vitro* assays showed diverse innate immune responses of Senegalese sole phagocytes challenged with different strains of either *Phdp* (PC566.1 and PP3) or *Tenecibaculum maritimum* (ACC6.1 and ACC13.1), suggesting that PRRs from sole macrophages may have detected PAMPs associated with DNA structures unique for each bacterium isolate (Costas *et al.* 2013b,c). Lipopolysaccharide, an endotoxin found on the bacterial cell membrane, is considered to be the prototypical PAMP and was found to increase the level of expression of hepcidin antimicrobial peptide in both intraperitoneally injected Senegalese sole (Osuna-Jiménez *et al.* 2009) and primary head-kidney cells culture (Costas *et al.* 2013d). Mx protein, an interferon-induced protein that protects against viral infections, has also been studied in Senegalese sole (Fernández-Trujillo *et al.* 2008a). Fernández-Trujillo *et al.* (2008b) reported different Mx expression profiles following both poly I:C injection and solevirus inoculation. Additionally, the Senegalese sole innate immune machinery also increased following a mixed leucocyte reaction. The level of expression of interleukin (IL)-1β, IL-8, hepcidin antimicrobial peptide and g-type lysozyme increased after the incubation of blood leucocytes from three different individuals at 24 and 48 h (Costas *et al.* 2013d).

Several humoral substances and cell secretions also contribute to the natural resistance of fish to pathogenic and infectious agents. These include complement, transferrins, anti-proteases, various lytic enzymes (e.g. lysozyme), lectins, C-reactive protein, interferon and enzyme inhibitors (Ellis 1999). Moreover, some of these factors, such as lysozyme and complement, appear to be more potent in fish than in mammals (Ellis 2001). Senegalese sole increased plasma lysozyme and peroxidase activities following challenge with *Phdp*, consistent with blood neutrophilia and monocytosis and the increase in neutrophils and macrophage numbers in the peritoneal cavity at 24 h post-challenge (Costas *et al.* 2013b). This study also showed different responses of the alternative complement pathway (expressed as ACH50) against two *Phdp* strains, being higher at 24 h than those reported for European seabass and gilthead seabream challenged with the same pathogen (Mauri *et al.* 2011). Therefore, ACH50 appears to be a mechanism of greater importance, among other possible responses, against this particular pathogen in Senegalese sole.

The neuroendocrine-immune network

The suppressive effect of stress on the immune system is highly disputable and does not necessarily translate into decreased resistance to infection in both mammals and fish (Dhabhar 2009; Verburg-van Kemenade *et al.* 2009). Depending on the duration and severity of the stressor, increased glucocorticoid levels may enhance innate and adaptive immune responses while similar hormone levels may suppress immune function. Acute and chronic stressful husbandry conditions induced different responses in several cell-mediated and humoral innate immune parameters of Senegalese sole. While plasma lysozyme activity decreased at 4 h after air exposure (Costas *et al.* 2011a) and after 18 days under high stocking density (Costas *et al.* 2013a), those levels increased in sole submitted to weekly handling for 14 and 28 days (Costas *et al.* 2012) or daily handling for 14 days (Costas *et al.* 2011b). Similarly, ACH50 was negatively correlated to plasma cortisol levels in specimens exposed to acute stress or reared at high density (Costas *et al.* 2011a, 2013a), whereas daily stressed sole for 14 days presented higher ACH50 values than control fish (Costas *et al.* 2011b). Senegalese sole respiratory burst responses to handling stress also showed different patterns. For instance, the release of reactive oxygen species decreased in stimulated macrophages from sole submitted to daily handling for 14 days, while nitric oxide production followed the opposite pattern (Costas *et al.* 2011b). Although chronically stressed specimens from the latter study showed a higher resistance to *Phdp* than undisturbed fish, Senegalese sole treated with dexamethasone (a potent glucocorticoid) for 14 days appeared to be more susceptible to the same pathogen (Salas-Leiton *et al.* 2012). The modulation of the innate immune system after a stress challenge was similarly observed in several teleosts. For instance, relevant genes associated with acute inflammation followed similar kinetics and an up-regulation was observed after acute stress or daily handling for 7, 14 and 28 days (Huising *et al.* 2003; Fast *et al.* 2008). In contrast, leucocytes respiratory burst activity decreased in specimens submitted to handling (Pulsford *et al.* 1994). Moreover, several *in vitro* studies demonstrated that cortisol alone inhibits lipopolysaccharides-induced expression of several immune-related genes (Saeij *et al.* 2003; Fast *et al.* 2008; Stolte *et al.* 2008; Castillo *et al.* 2009).

In vivo neuroendocrine-immune interactions are thus dependent on the actions of various hormones (e.g., catecholamines, cortisol, ACTH, β-endorphin) and cytokines (e.g., IL-1β, IL-6 and TNF-α), as well as on their interactions (Verburg-van Kemenade *et al.* 2009). This could explain the enhanced immune function frequently observed in Senegalese sole submitted to repeated handling (Costas *et al.* 2011b, 2012). Other hormones released during repeated stress responses may influence innate immune mechanisms at a higher degree, decreasing the suppressive effects of cortisol. In contrast, a different situation probably occurs during constant chronic situations such as high stocking density, where cortisol plays an immunosuppressive role. This hypothesis is supported by the increased susceptibility to opportunistic pathogens observed in Senegalese sole reared at high stocking density (Costas *et al.* 2008), in line with a general decrease in immune function (Salas-Leiton *et al.* 2010; Costas *et al.* 2013a). Although Andrade (2012) did not observe changes in the immune competence of intensively reared Senegalese sole during 60 days, especial attention must be given to rearing conditions and handling protocols in order to improve productivity, given that the immune system may be modulated. Further studies would be instrumental to unravel the mechanisms that Senegalese sole subjected to repeated handling may have adopted during resistance to bacterial challenge. These studies should consider leucocyte trafficking and redistribution, cytokine kinetics and the release of hormones other than cortisol.

Current and emergent diseases

One of the main factors that has historically hampered Senegalese sole farming has been the high incidence and intensity of diseases (Padrós *et al*. 2003; Toranzo *et al*. 2003). Although most of the diseases have been described in other species, sole seems quite prone to become infected by cohabitation with other fish species. In the early 90’s it was usual to find gilthead seabream, European seabass or turbot in the same facilities as Senegalese sole. This was a determining factor for the development of diseases in sole and for the economic viability of the farms, and was one of the reasons explaining the producer's hesitation in taking on the mass production of this alternative species. Nowadays sole are reared in specific facilities with controlled environmental factors, highly reducing the risk of transmission. Nonetheless, some diseases are still common in Senegalese sole production systems, possibly associated to the lack of appropriate standardised rearing techniques and sometimes due to poor husbandry or hygienic conditions of the tanks, or when the temperature exceeds 22°C (Cañavate 2005). Currently the main pathological problems are bacterial diseases, mainly Tenacibaculosis (formerly Flexibacteriosis, fin rot or black patch necrosis), Photobacteriosis (formerly Pasteurellosis) and Vibriosis.

Tenacibaculosis, which is mainly caused by *Tenacibaculum maritimum* (formerly *Flexibacter maritimum*) can cause significant morbility and mortality in fish farms in many countries, limiting the culture of economically important marine fish species (Santos *et al*. 1999). The presence of this pathogen in sole in Europe was first described in Scotland from common sole suffering from the so-called “black patch necrosis” (BPN) (Bernardet *et al*. 1990), probably the most important problem in the early culture attempts of this species because of its high incidence (McVicar & White 1979, 1982). Some years later, Cepeda and Santos (2002) isolated for the first time *T. maritimum* from Senegalese sole in southwest Spain, where it caused almost 100% mortality of the affected stocks. Affected sole usually display several external signs including eroded mouth, rotten fins and skin lesions. Although this disease was reported to be highly infectious, it seemed to be both prevented and controlled by providing a sand substrate in the rearing tanks (McVicar & White 1982). This is paradoxical given that the use of sand in sole farming has been considered adverse since deficient management and hygiene may favour the emergence of bacterial infections (Howell 1997). However, culturing these fish in smooth hard-bottomed tanks without sand has been associated to a series of harmful effects (Ottesen & Strand 1996; Ottesen *et al*. 2007). Recently, Vilar *et al*. (2012) described particularly severe ulcerative disease outbreaks in cultured Senegalese sole associated with *T. maritimum*. Grossly, the affected fish showed total loss of epidermis and dermis and extensive necrosis of the muscle layers. Many other environmental conditions such as higher temperatures, salinity, low water quality, excess of UV light and also management factors (i.e. high density and poor feeding) and host-related factors (stress, skin surface condition) have also been described associated to this disease (Avendaño-Herrera, 2005). Being a disease that may cause both skin lesions and systemic problems, combined treatments with external disinfectants (such as formalin or hydrogen peroxide) and oral treatments with antibiotics are usually used. Florfenicol, oxitetracycline, flumequine, potentiated sulfonamides and especially enrofloxacin were used for controlling *T. maritimum* outbreaks, although the rapid appearance of resistant strains has been described (Avendaño-Herrera, 2005). Other *Tenacibaculum* species such as *T. discolor* and *T. soleae*, have also been isolated from diseased Senegalese sole (Piñeiro-Vidal *et al*. 2008a,b), showing the typical signs observed in fish affected by *T. maritimum*. Regarding vaccination, there is nowadays a commercially available bacterin to prevent the disease caused by *T. maritimum* in turbot (Icthiovac TM®), which is applied by bath in fish from 1 to 2 g followed by a booster injection in fish from 20 to 30 g (Avendaño- Herrera *et al*. 2006). Although no licensed commercial vaccines are yet available for sole, autovaccines made using the strains isolated from the farms can also be used in this species.

Photobacteriosis, caused by *Photobacterium damsela* ssp. *piscicida*, is responsible for high losses in the aquaculture industry as it provokes massive mortalities in several marine fish species such as gilthead seabream (Toranzo *et al*. 1991), seabass (Balebona *et al*. 1992), and in the flatfish Japanese flounder (Fukuda *et al*. 1996), among others. Since it was first recorded in farmed Senegalese sole in southwest Spain (Zorrilla *et al.* 1999) several sole farms, mainly in the south of Spain, have suffered mortalities caused by this disease (Magariños *et al.* 2003). In most cases, peracute mortalities without apparent lesions are the most typical manifestation found mainly in juveniles. However, in subacute and chronic cases, external lesions of infected fish included only unspecific symptoms such as dark skin coloration and swelling of the abdominal cavity. This disease particularly affects Senegalese sole at temperatures above 18ºC and usually triggers severe acute cases in which mortality can be extremely high (Padrós *et al*. 2003).

Vibrioses affecting Senegalese sole are usually detected as secondary infections associated with an initial Tenacibaculosis, but often they can also be primary infections and its pathogenesis is still unclear (Padrós *et al*. 2003). *Vibrio harveyi* and *V. parahaemolyticus* are pathogenic bacteria which were described in an outbreak of farmed sole by Zorrilla *et al*. (2003), causing moderate mortalities in the south of Spain. Main external signs of the disease were skin ulcers and haemorrhagic areas near the fins and mouth (Zorrilla *et al*. 2003). Rico *et al*. (2008) also characterised strains of *V. harveyi* from diseased-farmed Senegalese sole in Spain from 2000 to 2004 and, recently, Gomez-Gil *et al*. (2012) isolated *Vibrio alfacsensis* from cultured sole in two regions of Spain.

There are several studies on vaccination against these diseases (e.g. Romalde *et al*. 2005) and a divalent vaccine against *P. damselae* subsp. *piscicida* and *V. harveyi* that provides short-time protection is being studied (Arijo *et al*. 2005). Although no vaccines for sole have been registered at present, autovaccines against *P. damsela* and *Vibrio* species have been used in some farms. In addition, recent studies on probiotics to control *Photobacteriosis* and different *Vibrio* species have given encouraging results (Garcia de la Banda *et al*. 2012; Tapia-Paniagua *et al*. 2012; Batista *et al*. 2013).

Recently, Magariños *et al*. (2011) reported *Aeromona salmonicida* subspecies *salmonicida* as the causative agent of a ‘typical’ furunculosis outbreak in cultured sole in a marine farm operating in a recirculation system in Galicia. Affected fish showed haemorrhagic areas at the base of the dorsal and ventral fins and, in some cases, ulcerative lesions on the ventral surface. Internally, peritoneal cavities were completely filled with ascitic fluid and livers were extremely pale and showed petechiae. In this particular case, soles were grown in a farm which also produced turbot, which pointed towards a potential crossed infection of this bacterium from one fish species to another.

Similarly, Castro *et al.* (2012) isolated *Edwarsiella tarda* in Senegalese sole growing in a farm which also produced turbot, which again pointed towards a potential crossed infection. Affected fish showed cutaneous lesions in the dorsal surface, tumefactions around the eyes and haemorrhages in their ventral surface, as well as internal lesions - abundant ascitic fluid, anaemic liver and kidney with petechial haemorrhages.

Regarding lesions in internal organs, granulomas in the kidney and spleen were observed in this fish species related to acid-resistant bacteria, Ziehl-Neelsen-positive stain (Francesc Padrós, pers. comm., 2013). These bacteria, resembling *Mycobacterium* spp., could represent a potential new hazard for cultured sole. Transmission of mycobacteria in fishes is poorly understood, but water and associated biofilms are natural habitats for *Mycobacterium* spp. (Pedley *et al*. 2004), and therefore recirculation systems may play an important role in its transmission in aquaculture.

Although progress has been made on nutrition and feeding, the lack of specific and standardised diets for some life stages of this species may also facilitate bacterial infections. With respect to this, bacterial enteropathy possibly associated with the administration of contaminated live food has been observed in larvae and juveniles (Padrós *et al*. 2003).

As viral diseases, betanodaviruses, the aetiological agents of the viral nervous necrosis or viral encephalopathy and retinopathy (VER), have also been detected in Senegalese sole (Starkey *et al*. 2001; Thiéry *et al*. 2004; Cutrín *et al*. 2007; Olveira *et al*. 2009; Hodneland *et al*. 2011). Although the typical nervous clinical signs and high mortalities are usually less common in sole than in other species, fish can show abnormal swimming behaviour and moderate to high mortalities (Hodneland *et al*. 2011). However, a serious episode of mortality associated with the presence of VER was detected in juvenile common sole reared in a farm where it had been previously detected in a batch of croaker, *Umbrina cirrosa* fry (Borghesan *et al.* 2003). Although this virus can be transmitted horizontally by contact between diseased and healthy fish, the main transmission route is vertical (Barja 2004), which highlights the importance of detecting broodstock carriers that could transmit the virus to the larvae through fertilised eggs. Currently there are no efficient treatments or commercial vaccines for nodavirus. The recommendation is that affected stocks are immediately removed and sacrificed when the virus is detected. However, there are some promising results from oral, bath and injection vaccination in other fish species such as the sevenband grouper, *Epinephelus septemfasciatus* (Lin *et al*. 2007; Kai & Chi 2008; Nishizawa *et al*. 2009; Yamashita *et al*. 2009).

Birnavirus and lymphocystis virus were also detected in cultured sole (Rodríguez *et al.* 1997; Toranzo *et al*. 2003; Alonso *et al*. 2005; Cano *et al*. 2010). The birnavirus was described as the agent causing 100% mortality in wild Senegalese sole broodstock introduced into a culture facility in southwest Spain, and its external signs were dark coloration, hyperactivity and uncoordinated swimming behaviour (Rodríguez *et al*. 1997). The characterisation of this virus indicated its similarity to infectious pancreatic necrosis virus (IPNV). However, both birnavirus and IPNV are widely distributed and affect many different aquatic organisms, and therefore its role as a primary pathogen in sole species is not so clear. Lymphocystis disease is caused by an iridovirus with a worldwide geographical distribution that involves a chronic disease characterised by papilloma-like lesions typically on the skin, fins and tail (Walker & Hill 1980). Although no viral haemorrhagic septicaemia virus (VHSV) outbreaks have been reported for the time being, López-Vazquez *et al*. (2011) demonstrated the susceptibility of Senegalese sole to a VHSV strain isolated from wild Greenland halibut, *Reinhardtius hippoglossoides*, and farmed turbot. On the other hand, in wild fish stocks which were held as future breeders, the presence of intracytoplasmic inclusion bodies in erythrocytes was detected causing low but continuous mortality (Padrós *et al*. 2003). Until now the presence of viral particles has not been demonstrated, although the existence of a process similar to VEN (viral erythrocytic necrosis), or similar processes described in other species, was not ruled out.

In recent years, systemic amoebic disease has become the main parasitic problem in cultured Senegalese sole. Although the condition was not associated to high mortalities, reduced growth and high morbidity was noted and fishes show protuberances on the skin surface in addition to unspecific signs of diseases (lethargy with sporadic and erratic swimming) (Constenla & Padrós 2010). Furthermore, the muscular lesions developed can later prevent the commercialization of the fish. *Endolimax piscium* (Archamoeba) is the causative agent of this amoebiasis (Constenla *et al*. 2014) causing a granulomatous inflammatory reaction mainly in muscle but also in different internal organs of the host. This parasite was also detected within the intestinal epithelium and submucosa in both lesioned fishes and apparently healthy fishes (Constenla & Padrós 2010). This location seems to be an initial stage in the development of the disease and, consequently, early detection of the parasite in the farm should be considered a priority for the management of this disease in sole culture, since there is no known effective treatment against these parasites. Amoeboid organisms similar to *Neoparamoeba* sp. have also been observed in cultured Senegalese sole causing a chronic proliferative mucoid inflammation in gills similar to amoebic gill disease in turbot, with epithelial hyperplasia and fusion of lamellae in the apical region of some filaments (Francesc Padrós, pers. comm., 2012). At present, the most effective treatment against these gill amoebae seems to be freshwater baths.

Some sporadic infections by protist parasites like flagellates or ciliates (*Amyloodinium*, *Cryptobia* and *Cryptocaryon*) have also been described (Padrós *et al*. 2003), usually in cases where sole were reared in ponds. Albeit infrequently, cases of massive parasitosis can eventually lead to high mortalities, especially in sole grown at high temperatures and in recirculation systems. Palenzuela *et al*. (2007) described an infection by *Enteromyxum scophthalmi* in sole cohabiting with infected turbot, and other *Myxozoa* such as *E. leei* and *Ceratomyxa* sp. have also been observed in sole. However, since sole are currently reared in specific facilities, it seems that infections by *Enteromyxum* spp. isolated from highly susceptible fish species such as gilthead seabream and turbot are not a real threat to sole farming, only a potential risk. Other internal parasites have occasionally been observed in cultured sole, such as mixosporidia found in renal tubules, xenomas of the microsporidian *Tetramicra* sp. in muscle and digenean metacercariae also encysted in the musculature (Padrós *et al*. 2003). Moreover, ectoparasites such as the leech *Hemibdella solea* (Hirudinae) have been identified on broodstock of Senegalese sole in the Virginia Institute of Marine Science (Gloucester Point, USA), but it does not seem to adversely affect the fish and can be controlled with low salinity (Dinis *et al*. 1999).

Finally, pathologies of apparently non-infectious origin have also been described by Padrós *et al*. (2003): cell necrosis of the subdermal adipose tissue, related to lipid peroxidation and/or excessive exposure to sunlight, characterized by yellowish areas at the base of dorsal and anal fins which can be associated to secondary bacterial infections; and kidney damage due to the deposition of minerals in the tubular lumen, similar to the processes of nephrocalcinosis. In addition, chronic exophthalmia due to the presence of retrobulbar and periocular bubbles were found in Senegalese soles from ponds under hyperoxic conditions (Salas-Leiton et al. 2009). Affected fish also showed bubbles in gills, causing lamellar obstruction, and under the skin all over the body, and deaths were attributed specially to asphyxia and tissue destruction (Salas-Leiton et al. 2009). Lastly, Senegalese sole seems to be very sensitive to changes in environmental and rearing conditions compared with other farmed species, and therefore even small changes could be more challenging for this species (Francesc Padrós, pers. comm., 2014).

Conclusion and future perspectives

This review clearly demonstrated that important progress has been made in the last decade towards developing a stronger and sustainable aquaculture industry for Senegalese sole, as a result of a strong and consistent research effort in several biological disciplines. The industry has now advanced from simply adapting culture protocols and methods that are routinely used in other marine aquaculture species as it is becoming increasingly clear that *Solea* spp. have important reproductive, behavioral, nutritional and physiological particularities that need to be taken into account in the rearing of these species. Some of these particularities result in challenges that are especially difficult to tackle, such as the reproductive difficulties of G1 stocks and high growth dispersion which, in spite of considerable advances in knowledge gained on the reproductive biology and behavior of Senegalese sole in captivity and on its digestive physiology and nutritional requirements, are still important bottlenecks for its cultivation. On the other hand, a major progress has been the change in production systems from predominantly earth ponds or salt marshes to dedicated recirculation systems, which has enabled dramatically improving disease issues in sole farms by eliminating contact with other fish species and enabling a much higher control of environmental parameters. It has also become apparent that many disease outbreaks occurred and were intensified when temperatures rise above 20-22ºC, which has led the industry to either procure areas in which the temperature can be easily maintained below these values or invest in RAS technologies. In addition, recent studies have started gathering information on the immune system of sole and how the immune response can be modulated or affected by a range of parameters, including the environment and stress. It is hoped that this knowledge will serve to improve aquaculture practices, leading to improved well being and reduction of susceptibility to diseases, as well as help develop prophylactic measures and products, such as vaccines. Moreover, recent research has uncovered an extraordinary capability of Senegalese sole to biosynthesise DHA from EPA which, possibly associated with lower requirements for LC-PUFA, explains at least partly the easiness of larval culture and high survival during the hatchery stage, as well as the good performance results and high flesh quality obtained when on-growing sole with diets containing high levels of PP and VO. At a time when the global aquaculture industry is struggling with a major sustainability issue related to the need to replace FM and FO in aquaculture feeds, this unique characteristic represents a very important selling point that differentiates Senegalese sole from other marine aquaculture species. On the other hand, a lot more is now known regarding the specific nutritional requirements of Senegalese sole and hopefully this will lead to larval dietary regimes reducing phenotypic abnormalities, as well as to the formulation and commercialization of improved weaning and on-growing species-specific diets. Advances in knowledge on the digestive physiology and feeding rhythms of this species are also noteworthy and should help improve weaning protocols and reduce growth dispersion.

Therefore, it is believed that there is now a good knowledge base that should have a strong impact in reducing many of the classical and specific bottlenecks in Senegalese sole culture. Future research is still required to achieve sustainable industrial production from G1 stocks of Senegalese sole. This will need a multidisciplinary approach to focus on two principal aspects: 1) the control and development of male reproductive behaviour from the larvae through to successful courtship and spawning, 2) the control of sperm production and sperm management. Much work has been done recently that has greatly increased the understanding of Senegalese sole reproduction and, furthermore, enabled substantial improvements to protocols that were, however, still insufficient to solve the reproductive dysfunction of cultured broodstocks. Behavioural research needs to examine social interactions, the bases of reproductive dominance and development of courtship behaviour. Endocrine research needs to aim at both the control of reproductive behaviour and sperm production. Chemical communication may also be involved and the olfactory system should be investigated. Research on larval development will be necessary to determine the influence of external factors (temperature, illumination, nutrition) on sexual differentiation and sex ratio of the population and on later potential effects on puberty and reproductive performance at adulthood. Research on optimized broodstock, weaning and on-growing diets will improve performance of farmed sole, make production more cost-effective, and further reduce quality and welfare problems.

Finally, a noteworthy aspect which will likely shape the next decade of research to come is the arrival of the "genomics era" to aquaculture in general and to flatfishes in particular. In recent years, a strong investment has been made to develop genomic resources for *Solea* spp. which will open up ample possibilities for molecular studies devoted to gain a deeper understanding of biological processes and with potential to also be used in future breeding programs, to identify molecular markers for traits of economical interest (Cerdà et al. 2008b; Cerdà & Manchado 2013; García-Cegarra *et al*. 2013). An important tool currently available to the academic community for gene sequence mining is the *Solea* transcriptome database (generated from different tissues, developmental stages or stimuli treatments) with a global assembly containing >1,560 million reads, >694K UniGenes and >337K SNPs (SoleaDB, http://www.juntadeandalucia.es/agriculturaypesca/ifapa/soleadb_ifapa/). This emergent area is expected to boost new research to uncover the molecular basis of many different physiological processes which might be at the root of some of the main biological bottlenecks that are currently holding up the industry.

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Figure legends

Figure 1 Schematic representation of the long-chain polyunsaturated fatty acid (LC-PUFA) biosynthesis pathway, including the complement of enzymes intervening in the different steps (not all are necessarily present in a same species). In bold and underligned are the two enzymes that have been found and characterized in Senegalese sole (Morais *et al*. 2012). Dashed arrows correspond to the Sprecher pathway (Voss *et al*. 1991).

Figure 2 Total production of farmed sole in the period 2005-2012 (in tonnes) in Europe (FEAP 2013), including different Spanish regions (APROMAR 2014).

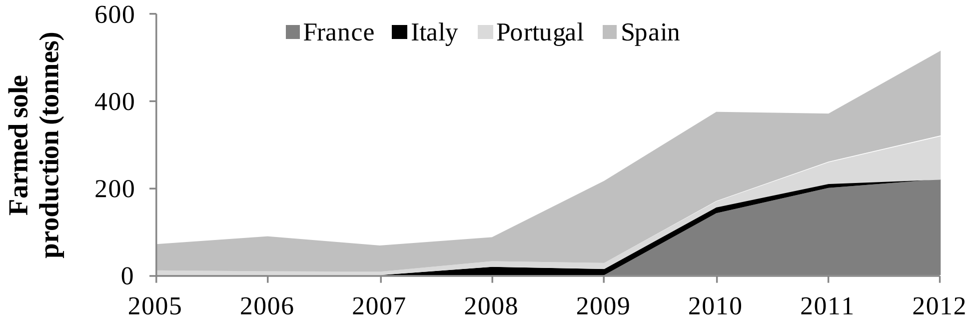
Table 1 Senegalese sole growth data according to bibliography. From Sánchez-Fernández (2012)

Initial weight (g)	Final weight (g)	SGR	FCR	Time (days)	Temp (ºC)	Protein (%)	Lipids (%)	Density (Kgm^{-2})	Authors
9.5	39.8	1.5	0.9	84	20	57	6	-	Silva *et al*. 2009
9.9	42.5-43.7	1.2	1.0-1.2	84	20	56	4-8	1	Borges *et al*. 2009
11.9	31.6-33	1.2	1.0	84	21	53-59	10-13	-	Rema *et al*. 2008
23.6	37.7-43.8	0.7-0.9	2.5-3.1	67	20	51	11-21	-	Rueda Jasso *et al*. 2004
39.7	-	0.3	0.8	21	22.5	68	15.7	-	Rubio *et al*. 2009
70	100	-	1.0-1.5	60	19.4	-	-	2-30	Salas Leiton *et al*. 2008
78.8	113-121.3	0.6-0.66	-	63	20	-	-	4-14	Costas *et al*. 2008
148.3	-	0.6-0.65	-	60	18.4	-	-	7-30	Salas Leiton *et al*. 2010
180	323-354	0.5-0.6	2.0-2.4	240	19	59	8-15	36	Valente *et al*. 2011
317	495.9	0.35	-	126	20	55	15	8.6	Ambrosio *et al*. 2008

Table 2 Holding conditions, breeder characteristics and egg production reported for naturally spawning captive wild Senegalese sole (*Solea senegalensis*) broodstock

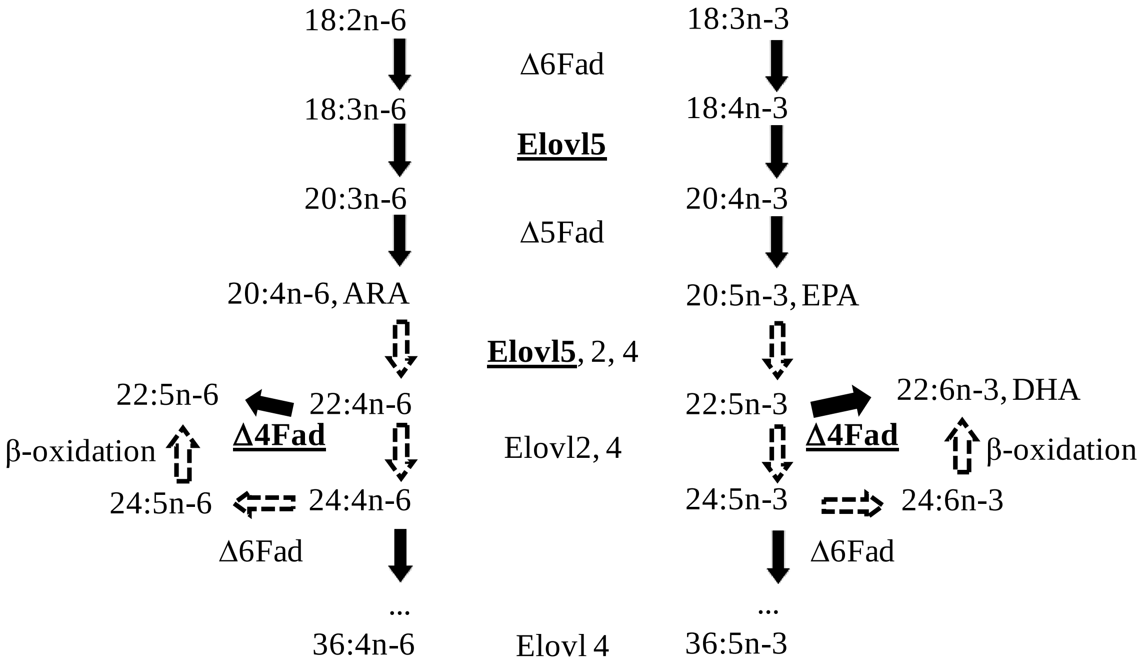
[redacted]

FIGURE 1



	2005	2006	2007	2008	2009	2010	2011	2012
France	0	0	0	0	0	142	200	220
Italy	0	0	0	19	14	14	10	0
Portugal	11	9	8	13	14	14	50	100
Spain	60	80	60	55	188	204	110	194
Andalucía	20	55	24	10	10	4		
Canarias			6	15	28	30	31	24
Galicia	20		30	30	150	170	79	170
Murcia	20	25						

FIGURE 2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18:3n-3
Δ6Fad
18:3n-6
18:4n-3
Elovl5
20:3n-6
20:4n-3
Δ5Fad
20:4n-6, ARA
20:5n-3, EPA
Elovl5, 2, 4
22:5n-6
22:4n-6
22:5n-3
22:6n-3, DHA
β-oxidation
Δ4Fad
Elovl2, 4
Δ4Fad
β-oxidation
24:5n-6
24:4n-6
24:5n-3
24:6n-3
Δ6Fad
Δ6Fad
...
...
36:4n-6
Elovl 4
36:5n-3