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LAUREA MAGISTRALE IN BIOLOGIA MARINA

INFLUENCE OF NUTRITIONAL COMPOSITION OF
BROODSTOCK DIET ON MEAGRE, *Argyrosomus regius*,
REPRODUCTIVE PERFORMANCE AND EGG QUALITY

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Riassunto

L'Ombrina bocca d'oro (*Argyrosomus regius*) è emersa come nuova specie candidata all'acquacoltura nel bacino Mediterraneo solo nell'ultimo decennio. Precedentemente era conosciuta come specie dalle carni molto pregiate solo grazie alla sporadica cattura durante l'attività di pesca (FAO © 2005-2011; Monfort, 2010). Avendo un elevato tasso di crescita e un eccellente indice di conversione alimentare, l'Ombrina bocca d'oro è una specie promettente per l'acquacoltura. E' oltretutto un pesce magro con basso contenuto di grasso mesenterico e intramuscolare, lipidi costituiti da un'elevata percentuale di acidi grassi polinsaturi, alto livello di proteine (Piccolo *et al.*, 2008). Possiede un'alta capacità di adattamento alle diverse condizioni ambientali e un'alta resistenza allo stress (Monfort, 2010). Se l'allevamento di questa specie continuerà ad espandersi, sarà necessario aumentare le conoscenze in diversi campi, ad esempio i fabbisogni nutrizionali nei diversi stadi di crescita. Per continuare ad avere le ottime caratteristiche di questa specie allevata e fatta riprodurre in cattività bisogna porre attenzione alla qualità delle uova. Inoltre, è molto utile per un allevatore determinare la qualità delle uova prodotte e prevedere la sopravvivenza larvale prima di investire su strutture, personale e tempo (Mylonas *et al.*, 2004). La qualità delle uova è definita dall'insieme delle caratteristiche che determinano la loro capacità di sopravvivere, quindi le uova di buona qualità mostrano bassa mortalità ai diversi stadi di sviluppo (Bromage, 1995; Lahnsteiner *et al.*, 2008). Molti criteri possono essere utilizzati per valutare la qualità: dimensione e morfologia delle uova, parametri fisici e chimici, galleggibilità delle uova pelagiche, stato citogenetico, successo di fecondazione, sopravvivenza di embrioni e larve. Ma si deve ricordare che in un allevamento un buon criterio di valutazione dovrebbe essere applicabile nelle primissime fasi di sviluppo e facile da utilizzare (Kjørsvik, 1990). E' molto difficile stimare accuratamente la qualità delle uova prima della fecondazione, quindi tra i migliori criteri di valutazione c'è sicuramente la sopravvivenza a diversi stadi di sviluppo associata al monitoraggio di eventuali deformità (Bonnet *et al.*, 2007; Bobe & Labbé, 2010). La qualità delle uova è influenzata da molti fattori, ciascuno dei quali ha un effetto altamente variabile non sempre ben caratterizzato (Bobe & Labbé, 2010). Entrambi i gameti, oociti e spermatozoi, contribuiscono alla qualità delle uova, ma agiscono in tempi e modi diversi. Gli effetti paterni agiscono molto presto nell'ontogenesi, e riguardano densità e motilità degli spermatozoi, mentre gli effetti materni si rivelano dopo, contribuendo alla sopravvivenza embrionale (Lahnsteiner *et al.*, 2008). Guardando solo all'influenza materna, le uova possiedono sia caratteristiche intrinseche che estrinseche che ne determinano la qualità. Nei fattori intrinseci sono inclusi i geni embrionali e materni, l'età e lo stato del sistema endocrino materno, i nutrienti materni contenuti nell'uovo. Nei fattori estrinseci sono inclusi i fattori ambientali come la temperatura, il fotoperiodo, la salinità, gli inquinanti, la colonizzazione batterica, le pratiche di allevamento, lo stress, la nutrizione (Bobe & Labbé, 2010; Lahnsteiner *et al.*, 2008).

Nel presente studio viene presa in considerazione la nutrizione dei riproduttori, un fattore molto importante dal momento che una dieta non bilanciata o una restrizione nella disponibilità di un componente biochimico può influenzare il sistema endocrino dell'asse ipotalamo-ipofiso-gonadico e la formazione delle uova, riducendo sia la loro qualità che la fecondità della femmina. Alcune

componenti della dieta dei riproduttori sono necessarie per assicurare un normale sviluppo dell'embrione e aumentare la qualità delle uova, come le vitamine, i carotenoidi e gli acidi grassi essenziali (EFA) (Brooks *et al.*, 1997; Watanabe & Vassallo-Agius, 2003). Carotenoidi e vitamina E hanno un ruolo determinante nella qualità delle uova come antiossidanti, dal momento che proteggono i lipidi insaturi dall'ossidazione, eliminando le specie reattive dell'ossigeno e proteggendoli dall'azione dei radicali liberi (Faulk & Holt, 2008; Scabini *et al.*, 2011). Anche il contenuto e la qualità delle proteine nella dieta è importante, perché hanno un ruolo strutturale, funzionale ed energetico, oltre a un ruolo importante nella fecondazione e nel normale sviluppo embrionale. La qualità delle proteine dipende dal loro profilo aminoacidico e dal coefficiente di digeribilità per ogni specie (Fernández-Palacios *et al.*, 1997). La composizione in lipidi e acidi grassi della dieta è il maggior fattore determinante il successo della riproduzione e la sopravvivenza della prole (Izquierdo *et al.*, 2001). Acidi grassi essenziali, come la serie n-3 e n-6, hanno un ruolo fondamentale nella nutrizione delle specie marine, perché queste ultime non sono capaci di allungare gli acidi grassi a corta catena (Watanabe & Vassallo-Agius, 2003). Gli acidi grassi liberi saturi e monoinsaturi mobilizzati possono essere catabolizzati per la biosintesi di lipoproteine delle uova, mentre gli acidi grassi polinsaturi (PUFA), specialmente l'acido docosaesaenoico (DHA), vengono incorporati nella vitellogenina. L'acido arachidonico (AA) e l'acido eicosapentaenoico (EPA), assieme al DHA, giocano un importante ruolo strutturale come componenti di fosfolipidi e sono associati alla fluidità di membrana (Sargent, 1995; Izquierdo *et al.*, 2001). Il ruolo principale del DHA è la formazione delle membrane, principalmente neurali. Essendo che cervello e occhi costituiscono gran parte della massa corporea embrionale e larvale, il DHA ha un ruolo critico nella loro formazione (Sargent, 1995; Nocillado *et al.*, 2000). La seconda importante funzione degli EFA è il loro ruolo come precursori degli eicosanoidi. Acido arachidonico ed EPA sono coinvolti nelle funzioni cellulose-mediate e sono precursori delle prostaglandine: l'AA forma principalmente prostaglandine altamente attive biologicamente (PGE₂), prodotte in risposta a fattori esterni di stress, mentre l'EPA forma prostaglandine a bassa attività biologica e blocca l'azione steroidogena di AA e PGE₂ assieme al DHA. Quindi l'embrione è altamente vulnerabile a un rapporto dietetico materno (n-6)/(n-3) di PUFA troppo elevato durante la gonadogenesi. Un eccesso della serie n-6 porterebbe ad un aumento della suscettibilità della larva con amplificazione delle normali risposte allo stress (Sargent, 1995; Izquierdo *et al.*, 2001). Quindi non solo i livelli assoluti di DHA ed EPA sono importanti, ma anche il rapporto n-6/n-3 PUFA, tenendo conto che entrambi sono diversi tra le specie (Bromage, 1995).

In questo quadro è inserito il presente studio, che ha come obiettivo la valutazione degli effetti di due diverse diete sulla qualità delle uova prodotte dall'Ombrina bocca d'oro, comparando fecondità, successo di fecondazione, sopravvivenza embrionale e larvale a diversi stadi di sviluppo. Questo lavoro di tesi è stato condotto presso l'Istituto di Acquacoltura dell'Hellenic Centre for Marine Research (HCMR) a Creta (Grecia) e si inserisce in un progetto più vasto intitolato "Sviluppo di metodi per la riproduzione e l'allevamento dell'Ombrina bocca d'oro (*Argyrosomus regius*) come misura per accrescere la competitività dell'acquacoltura con l'introduzione di nuove specie" (ESPA 2007-2013, Collaboration). Gli obiettivi di questo programma sono lo studio di tutti gli aspetti

del ciclo vitale di questa specie in allevamento, in modo da ottimizzare i protocolli di gestione dei riproduttori, l'allevamento larvale, le fasi di nursery e di crescita in gabbie a mare.

La presente sperimentazione è stata condotta su Ombrine bocca d'oro mature di 6 anni, divise il 16/12/2010 in due gruppi, ciascuno composto da 10 individui (5 maschi e 5 femmine). I pesci, ridotti in entrambi i gruppi a 9 all'inizio dell'esperimento a causa di qualche evento di mortalità sono stati nutriti manualmente fino a sazietà apparente tutti i giorni. Prima del 21/02/2011 l'alimentazione fornita consisteva in una dieta di mantenimento mentre dal 21/02/2011 in avanti si sono somministrate le due diete sperimentali, costituite una da un alto e l'altra da un basso contenuto nutritivo (Tab. 1 e 2, pag.22). Allo scopo di monitorare lo stadio di maturazione sessuale, i pesci sono stati esaminati una volta al mese, partendo da marzo. Previa anestesia, nelle femmine è stata effettuata una biopsia ovarica mentre dai maschi è stato prelevato un campione di sperma. I pesci sono stati trattati con impianti di un agonista dell'ormone rilasciante le gonadotropine (Gonadotropin Releasing Hormone agonist-GnRH_a) a rilascio controllato. L'inserimento a livello del muscolo dorsale di questi impianti è stato effettuato in un momento di maturazione specifico dei riproduttori: quando gli oociti prodotti dalle femmine hanno raggiunto un diametro maggiore di 540 μm e tutti i maschi erano in fase di spermiazione. Dopo l'induzione riproduttiva, i collettori delle uova di cui era fornita ogni vasca sono stati esaminati giornalmente. Tutte le uova rilasciate sono state raccolte ogni mattina in un secchio da 10-l, riempito con acqua di mare. Da questo, allo scopo di valutare fecondità e successo di fecondazione, è stato prelevato un campione di 10 ml e osservato allo stereoscopio in un disco di vetro con solco circolare, creato apposta per determinare lo stadio di sviluppo delle uova, la fecondità e il successo di fecondazione (Fig. 5, pag.24). La fecondità relativa è stata determinata come numero totale di uova prodotte sul peso totale medio delle femmine trattate con l'impianto di GnRH_a il 23/05/2011. Il successo di fecondazione è stato determinato come numero di uova vitali sul numero totale di uova prodotte. Per determinare invece la sopravvivenza embrionale e larvale ai diversi stadi è stata usata la procedura di Panini *et al.*, 2001, utilizzando piastre da microtitolo con 96 pozzetti. Ogni giorno le uova vitali raccolte nel campione venivano trasferite, una per pozzetto, nella micropiastre (in due repliche) (Fig. 6 e 7, pag.25-26). Le piastre da microtitolo venivano poi mantenute per 7 giorni in un incubatore a temperatura controllata e giornalmente venivano controllati allo stereoscopio lo sviluppo embrionale e larvale, e veniva registrato il numero di embrioni/larve vive e morte. Con questi dati è stato possibile valutare gli altri parametri di qualità delle uova: la sopravvivenza embrionale a un giorno, il successo di schiusa, la sopravvivenza larvale a 5 e a 7 giorni. La sopravvivenza percentuale è stata stimata usando il rapporto fra il numero di embrioni/larve vive a quel determinato stadio e il numero di individui sopravvissuti allo stadio di sviluppo precedente.

I pesci hanno iniziato a deporre le uova due giorni dopo l'impianto di GnRH_a, il 25/05/2011, e hanno concluso il 13/06/2011 nel gruppo High Nutrient (HN), il 27/06/2011 nel gruppo Low Nutrient (LN). La fecondità relativa giornaliera ha mostrato un'ampia variazione in entrambi i gruppi, con un picco il secondo giorno di deposizione delle uova e un calo marcato i quattro giorni

successivi (Fig. 8, pag.28). Non si sono riscontrate differenze significative nella fecondità relativa media tra i due gruppi, ma in entrambi il valore di questo parametro è stato più alto durante la prima settimana dopo l'impianto di GnRH rispetto alla seconda e alla terza (Fig. 9, pag.29). Il successo di fecondazione è stato relativamente alto durante tutta la stagione riproduttiva, sebbene nel gruppo LN c'è stata una maggiore variabilità (Fig. 8, pag.28). Quando considerato su base settimanale, il successo di fecondazione medio giornaliero è diminuito significativamente durante lo studio ed è stato più alto per il gruppo HN (Fig. 9, pag.29). Le uova incubate nelle piastre da microtitolo a 18.8°C schiudevano solitamente il secondo giorno dopo la raccolta delle uova. La sopravvivenza embrionale un giorno dopo la raccolta delle uova non ha mostrato nessuna differenza significativa tra i due gruppi o tra settimane. Non si sono riscontrate differenze significative tra gruppi o settimane nemmeno nel successo di schiusa (Fig. 10, pag.30). Nella sopravvivenza larvale a 5-giorni invece si sono osservate differenze significative sia tra gruppi che tra settimane: la sopravvivenza media a 5-giorni durante la terza settimana nel gruppo HN è stata significativamente più bassa di quella nel gruppo LN (Fig. 11, pag.31-32). Anche nella sopravvivenza larvale a 7-giorni si sono evidenziate differenze significative sia tra gruppi che tra settimane, ma senza interazione tra gruppi e settimane come nella sopravvivenza a 5-giorni: la sopravvivenza media a 7-giorni è stata significativamente più elevata nel gruppo LN rispetto al gruppo HN, e in generale il valore della sopravvivenza media a 7-giorni è stata significativamente più bassa durante la terza settimana rispetto alla prima e alla seconda (Fig. 11, pag.31-32). Per quanto riguarda le correlazioni tra i parametri di qualità delle uova, le analisi di regressione hanno indicato una correlazione significativa solo tra fecondità relativa e successo di fecondazione (Fig. 12, pag.33) e tra la sopravvivenza larvale a 5-giorni e la sopravvivenza larvale a 7-giorni (Fig. 13, pag.33-35). In tutti gli altri parametri esaminati non si sono evidenziate correlazioni significative (Fig. 13, pag.33-35).

La nutrizione influenza la qualità delle uova in diversi modi. Livello, composizione e origine di lipidi e proteine sono considerati i fattori più importanti, essendo i principali componenti del tuorlo delle uova. Nella dieta dovrebbe sempre esserci un bilancio appropriato tra queste due componenti, perché i lipidi hanno una forte influenza sulla richiesta proteica dei pesci. La dieta HN utilizzata in questo studio ha un più alto contenuto di lipidi e proteine (18% e 59% rispettivamente) comparata alla dieta LN (15% lipidi, 49% proteine). Diversi studi hanno dimostrato che entrambi i livelli di lipidi e proteine possono influenzare significativamente la qualità delle uova. Ma nel presente studio i riproduttori di entrambi i gruppi hanno mostrato fecondità relativa, sopravvivenza embrionale a un giorno dalla raccolta delle uova e successo di schiusa comparabili. Una possibile spiegazione a quanto osservato potrebbe essere che il livello di proteine incluso nella dieta sia sufficientemente elevato e che una volta raggiunto un valore soglia richiesto dai riproduttori di questa specie un'inclusione maggiore di proteine non porti a nessuna differenza significativa in questi parametri. Anche per quanto riguarda i lipidi, considerando sempre fecondità relativa, sopravvivenza embrionale a un giorno dalla raccolta e successo di schiusa, potrebbe essere che una differenza di soli 3 punti percentuali non causi differenze significative in questi parametri tra i due trattamenti. Differenze significative potrebbero essere osservate con una differenza maggiore nel

contenuto lipidico. Se anche questo non portasse a nessun miglioramento nei parametri considerati, significherebbe che un maggior contenuto lipidico è inutile, quindi potrebbe esserci un minor utilizzo dei sottoprodotti dell'acquacoltura e un maggior risparmio per gli allevatori.

In realtà, il successo di fecondazione, la sopravvivenza a 5-giorni e a 7-giorni dalla raccolta delle uova sono risultate significativamente influenzate dalle due diverse diete, ma in modo contraddittorio. Il successo di fecondazione ha indicato la dieta HN come quella che determina una miglior qualità delle uova; al contrario, la sopravvivenza larvale a 5 e 7-giorni durante la terza settimana ha indicato in tal senso la dieta LN. Un altro aspetto da considerare è che il successo di fecondazione ha evidenziato una differenza significativa tra settimane in entrambi i gruppi, ma nel gruppo HN ha mostrato un calo graduale, mentre nel gruppo LN un valore più basso durante la seconda settimana sia rispetto alla prima che alla terza. Anche l'andamento della sopravvivenza larvale a 5-giorni nel gruppo HN è stato discontinuo: il suo valore più alto si è osservato durante la seconda settimana, con un brusco calo durante l'ultima settimana. Nel gruppo LN invece questo parametro ha subito un leggero e non significativo calo graduale. Questo potrebbe significare che i pesci non hanno avuto tempo sufficiente per incorporare al meglio nelle uova i componenti delle due diverse diete, dato che la somministrazione è iniziata solamente 3 mesi prima del periodo riproduttivo. Mentre l'espressione nella qualità delle uova dei cambiamenti dietetici dei riproduttori dell'orata si verificano entro 15 giorni, l'Ombrina bocca d'oro potrebbe richiedere un periodo di tempo molto più lungo. Diversi studi riportano una somministrazione dei trattamenti 3 mesi prima della stagione riproduttiva, mentre altri studi un periodo molto più prolungato. In sintesi, ogni specie ha delle "finestre di opportunità" durante le quali una diversa alimentazione produce effetti significativi sulla qualità delle uova (Bromage, 1995). Sono necessari quindi maggiori studi sul periodo di somministrazione della dieta ai riproduttori di Ombrina bocca d'oro prima della stagione riproduttiva.

E' noto, come osservato precedentemente, che anche la composizione dei lipidi è importante. In questo studio però si conosce solo il livello lipidico incluso nella dieta, non i tipi di lipidi utilizzati. Dal momento che le due diete hanno diversi livelli di nutrienti ma ogni nutriente ha la stessa composizione in entrambi i trattamenti, il contenuto assoluto di acidi grassi della serie n-3 e n-6 è maggiore nella dieta HN rispetto alla dieta LN, mentre hanno uguale rapporto n-3/n-6. Generalmente, un più alto rapporto n-3/n-6 porta ad una miglior qualità delle uova, perché la serie n-3 include acidi grassi come il DHA, al cui contenuto è direttamente correlata la qualità delle uova, mentre la serie n-6 include AA, che come detto precedentemente va a produrre eicosanoidi coinvolti nella maggior sensibilizzazione allo stress. Va ricordato che i pesci marini non sono in grado di desaturare ed elongare gli acidi grassi a corta catena, come l'acido linoleico (18:2 n-6) e linolenico (18:3 n-3), per produrre i PUFA a lunga catena, perché gli enzimi $\Delta 5$ e $\Delta 6$ desaturasi ed elongasi hanno un'attività molto ristretta (Izquierdo, 2002; Sargent *et al.*, 2002). E' pertanto necessario somministrare a queste specie PUFA come AA, DHA ed EPA (Navas *et al.*, 1998), cercando sempre di mantenere un rapporto n-3/n-6 elevato. Alcuni studi tuttavia riportano che un livello eccessivo di lipidi portano ad un effetto negativo sulla qualità delle uova. Nel presente studio è ipotizzabile che la dieta HN abbia un livello troppo elevato di lipidi, che potrebbe aver portato inizialmente ad un aumento del successo di

fecondazione e successivamente ad una riduzione nella sopravvivenza larvale al 5° e 7° giorno. Potrebbe quindi essere che un'inclusione lipidica del 15% nella dieta sia migliore di un'inclusione del 18% per produrre uova di buona qualità in questa specie.

Un altro aspetto da considerare è il livello di sostanze antiossidanti presenti nella dieta. La facilità di ossidazione dei lipidi è proporzionale al numero di doppi legami che presentano. Quindi, l'aumento di PUFA deve essere bilanciato aumentando il contenuto di sostanze antiossidanti, specialmente vitamina E (Sargent *et al.*, 2002), la quale protegge i PUFA a lunga catena mantenendo la loro integrità e le loro concentrazioni, e di conseguenza anche la qualità delle membrane biologiche (Traber & Atkinson, 2007). Nel presente studio entrambe le diete somministrate hanno lo stesso livello di inclusione di vitamina E e quindi è possibile che il livello presente nel trattamento HN non sia sufficiente per proteggere il livello di lipidi.

Riassumendo, la formulazione di una dieta implica una scelta tra due approcci: 1-basare la formulazione solo sul valore nutrizionale, producendo una dieta molto costosa ma molto produttiva; 2-basare la formulazione solo sul costo totale della dieta e minimizzare i costi (Hardy & Barrows, 2002). Generalmente si cerca di produrre un mangime che permetta le più alte performance con il minor costo possibile, quindi, essendo più costosa l'inclusione nella dieta di livelli nutritivi più elevati, la decisione di incorporarli viene presa solo se porta effettivamente a una maggior qualità delle uova. Va tenuto in forte considerazione anche che l'acquacoltura deve diventare il più indipendente possibile dal patrimonio ittico selvatico, dal momento che molti stock stanno o sono già collassati. Per queste ragioni in questo studio si sono comparati gli effetti di due diversi livelli nutritivi.

In questo studio è stata valutata anche la correlazione tra fecondità relativa, successo di fecondazione, tasso di schiusa e sopravvivenza a diversi stadi di sviluppo per trovare indicatori pratici e precoci della qualità larvale. La fecondità relativa si è mostrata significativamente correlata al successo di fecondazione, ma questo dato non fornisce informazioni importanti sull'effettiva vitalità della prole ottenuta. Una correlazione positiva significativa importante è stata riscontrata tra la sopravvivenza larvale a 5 e 7-giorni, indicando che il successo di sopravvivenza a 5-giorni può essere utilizzato per la valutazione della qualità delle uova e quindi nella decisione se portare avanti o interrompere l'allevamento larvale del lotto valutato.

Concludendo, il presente studio introduce nuove informazioni sull'alimentazione dei riproduttori e sulla qualità delle uova dell'Ombrina bocca d'oro, fornendo un'ulteriore prova dell'idoneità di questa specie alla riproduzione in cattività. Tali informazioni sono utili per una corretta organizzazione di un centro di riproduzione commerciale, in particolare per quanto riguarda le esigenze dietetiche dei riproduttori, i programmi di produzione delle uova, la previsione di qualità delle uova e la sopravvivenza larvale. Ulteriori ricerche su riproduzione e richieste nutrizionali dei riproduttori di Ombrina bocca d'oro in cattività dovrebbero concentrarsi sulla composizione lipidica della dieta, il livello di inclusione di antiossidanti e il periodo di somministrazione della dieta prima della stagione riproduttiva.

Introduction

Description of *Argyrosomus regius*

Meagre (*Argyrosomus regius*, Asso 1801) is a teleost fish, belonging to the order Perciformes and the family Sciaenidae. Meagre is a large fish with elongated body, nearly fusiform (Whitehead *et al.*, 1984/1986). The terminal mouth is large and yellow-gold-orange inside, with well differentiated and strong teeth. Body colour is dark grey dorsally, with bronze traits on the sides and pearly-silver shades on the stomach. The fins are grey. Meagre have two adjacent dorsal fins, the first triangular and shorter than the second one, that leads to soft rays. The anal fin is short, as the ventral and pectoral fins, and it has a first short spiny ray and a second very thin one (FAO © 2005-2011). The caudal fin is large and truncated to S-shape (Whitehead *et al.*, 1984/1986). The lateral line is black and evident, extending onto the caudal fin (FAO © 2005-2011). Scales are ctenoid, except for some cycloid scales on the chest, snout and below the eyes (Whitehead *et al.*, 1984/1986). Total length is commonly 150 cm, but meagre can reach more than 200 cm long and a weight of 100 Kg (Froese & Pauly, 2011; Marine Species Identification Portal). Several branched appendages are present in the gas bladder, which can vibrate producing drumming sounds (a typical “grunt”), that can be heard up to 30 meters (Whitehead *et al.*, 1984/1986; FAO © 2005-2011).

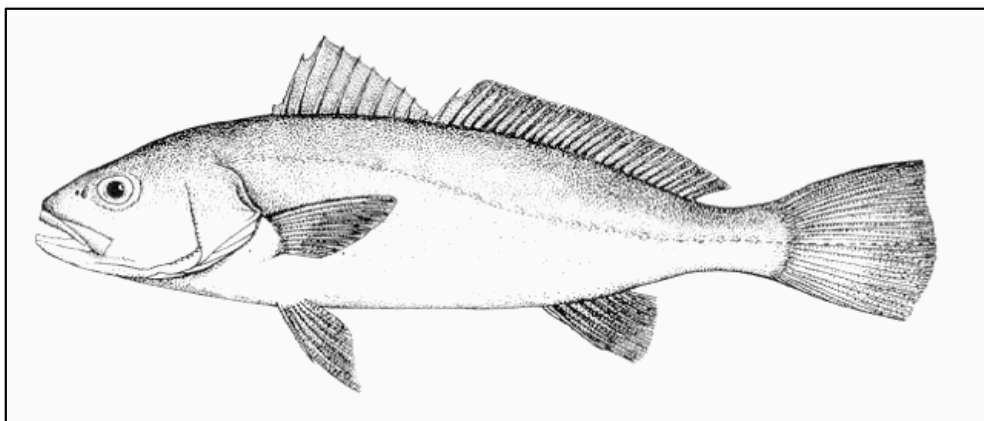


Figure 1. Morphological characteristics of meagre (FAO, 2011).

Habitat and biology

Meagre are found in the Mediterranean Sea, the Eastern Atlantic Ocean, between Senegal and the English Channel, and they are rarely found in Denmark, south Norway, Iceland and Black Sea (FAO © 2005-2011; Gonzáles-Quirós *et al.*, 2011). Meagre have also migrated to the Red Sea through the Suez Canal, doing the reverse Lessepsian migration (Chao, 1990).

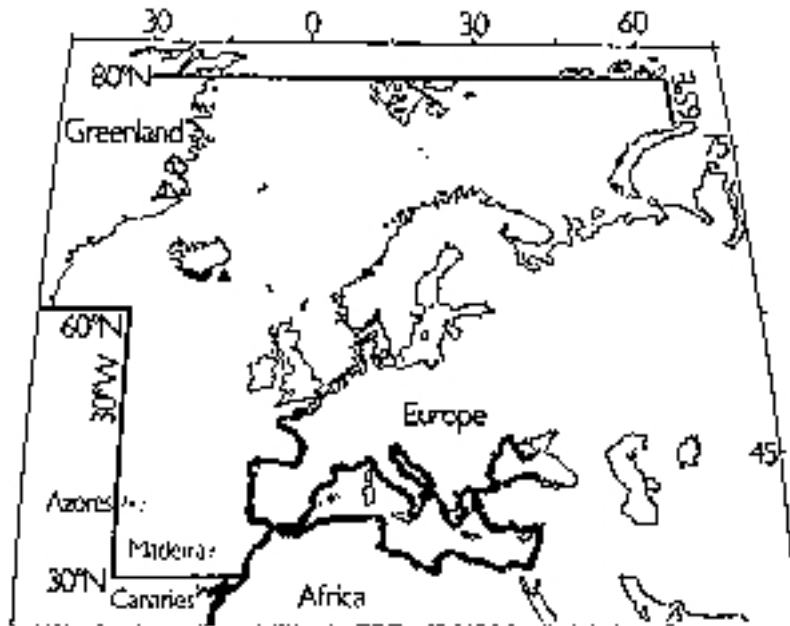


Figure 2. Distribution map of meagre (*Argyrosomus regius*) (Whitehead *et al.*, 1984/1986).

This species is euryhaline and benthopelagic, living at moderate depth ranging between 15 and 300 m, over sand and rocks (Froese & Pauly, 2011). Meagre are carnivores and their diet is based on atherinids, mugilids, clupeids and swimming crustaceans (Froese & Pauly, 2011; Marine Species Identification Portal). Females can live up to 19 years and males up to 16. They reach sexual maturity at 2-3 years of age, depending upon their range, and they undergo a reproductive migration towards the coast (Maybank, 2008) from April to July, in southern Mediterranean (Whitehead *et al.*, 1984/1986). They usually congregate inshore (Froese & Pauly, 2011) and penetrate into estuaries (FAO © 2005-2011) and salt-marshes. In these sites with high turbidity water they form large aggregations, facilitated by the emission from the males of long grunts (González-Quirós *et al.*, 2011).

Fertilization is external, with one clear seasonal spawning peak per year (Chao, 1990) and it seems to occur during in-pair courtship always associated with the production of these typical deep sound (Lagardère & Mariani, 2006; González-Quirós *et al.*, 2011), produced by pushing their abdominal muscles against the gas bladder. From mid-June until the end of July they leave estuaries to feed along the coast, where they remain in shallow water until the beginning of autumn. During winter meagre return to deeper water (FAO © 2005-2011). The three main spawning sites of meagre in the North Atlantic and the Mediterranean Sea are the Nile delta (Egypt), the Lévrier Bay (Mauritania) and the Gironde estuary (France) (Quéro, 1989a and 1989b). A 1.2 m female produces about 800,000 eggs and spawning occurs at 17-22°C.

Fertilized eggs are pelagic and they measure approximately 990 µm in diameter; depending on temperature, the lipid droplet is totally absorbed after 30 hours, whereas the yolk sac is almost consumed and the mouth is open at 96 hours. Juveniles leave the nursing areas (estuaries) at the end of summer, they

migrate to coastal waters, from 20-40 m, to spend the winter and they return again to their estuarine feeding areas starting from mid-May. Pelagic life is quite short (FAO © 2005-2011), juveniles leave the coastal areas and adopt a benthopelagic existence when they reach about 3.7 cm in length (Maybank, 2008). Water temperature is the most important factor that determines the trophic migration and reproduction of meagre. The best temperature for the growth of this species is between 17-21°C, with an acceptable range of 14-23°C (FAO © 2005-2011).

Fisheries of meagre

The production of meagre from capture fisheries in Europe is low, from a few hundred tones to 1500 tons in the different countries, supplemented by imports from Morocco. Wild meagre are common in western Europe, such as south Spain, south Portugal and west France, where they are very appreciated (FAO © 2005-2011), but for example now they are no longer caught in the Wadden Sea (southeastern part of the North Sea that laps Netherlands, Germany and Denmark) (Wolff, 2000a and 2000b). Indeed, although sciaenids have a relatively high fecundity, which would be of value when their stocks are heavily exploited (Powles *et al.*, 2000), meagre are an example of a species, belonging to this family, being highly vulnerable to over-fishing (Farmer *et al.*, 2005). It has a minimum population doubling time of 4.5 to 14 years (Maybank, 2008), it lacks the basic biological information and there are no specific regulations for meagre fishing (González-Quirós *et al.*, 2011). Hence, expanding aquaculture production is a possible solution to alleviate pressure on wild fisheries stocks and to avoid their collapse (Naylor *et al.*, 2000).

Mediterranean finfish marine aquaculture is principally based on two species, European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) (Quéméner *et al.*, 2002). In addition, the European market for seafood is characterized by a dramatic deficit and it's extremely dependent upon external supplies (Monfort, 2010). One of the main strategies to ensure future expansion of Mediterranean aquaculture, besides production cost reduction and market enlargement, is the diversification of the production with new species. The diversification of reared fish species is necessary also for different reasons, among which the different range of environmental conditions that an aquaculturist can meet, a high increase in rearing yields and the possibilities to reduce risks of disease outbreak. At present, 25 marine fish species are being investigated in the Mediterranean region. The selection of new candidate species must take into consideration both biological and market criteria. Ideally, new candidate species for farming should combine low production cost and high market price. Nowadays, there is an increased interest among aquaculturists around the world for fast growing species, such as cobia (*Rachycentrom canadum*), greater amberjack (*Seriola dumerili*), meagre, common dolphin fish (*Coryphaena hippurus*) and different species of groupers (*Epinephelus* sp.) and tuna (*Thunnus* sp.) (Quéméner *et al.*, 2002).

Meagre culture started only recently, in the late '90s in the Mediterranean Basin, with juvenile production in Sète (France) and the growth in Orbetello lagoon (Italy). Their culture then spread to other Mediterranean countries (Spain, Portugal, Turkey, Greece, Egypt, Malta) and currently many hatcheries

throughout the Mediterranean are producing juveniles. The total aquaculture production was a few tones in 2000, 4000 tons in 2008 and over 10,000 tones in 2010, underlining the appearance of a new cultured species on the market (Monfort, 2010).

Meagre culture

Farmed meagre come from intensive production, conducted both in land-based tanks and sea cages. The supply of juveniles comes from hatcheries around the Mediterranean region, including Spain, France, Greece, Turkey and Cyprus. Juveniles from 3 g to 20 g are normally stocked into small ponds or cages, where they are usually kept for three months until they reach 100 g. Ongrowing techniques are similar to those used for European sea bass and gilthead sea bream. Actually meagre are mainly farmed in sea cages and they are fed with an extruded pellet with 45-48% protein and 20-24% lipid (Monfort, 2010; FAO © 2005-2011).

Meagre are a promising aquaculture species due to their high growth rate and excellent feed conversion ratio (FCR). Indeed they can reach around 1 Kg per year, depending on culture conditions, and they have a FCR of 0.9-1.2, depending on the feed. Meagre are a particularly lean fish, even when grown intensively (Piccolo *et al.*, 2008), and their meat quality is very good. This is due to the low presence of mesenteric and intramuscular fat (Piccolo *et al.*, 2008), long shelf life (FAO © 2005-2011) and their high protein levels and low lipid content, a high percentage of which is composed of polyunsaturated fatty acids (PUFA). PUFA are mainly represented by a high proportion of n-3 series and by low n-3/n-6 ratio values. Meagre are even characterized by high adaptation capacities to environmental conditions and by high resilience against stress factors (Monfort, 2010). Indeed they can tolerate wide ranges of temperature and salinity, adapting to different latitudes and rearing conditions (Cittolin *et al.*, 2008; Chatzifotis *et al.*, 2010).

Two major fields must be developed if meagre farming is going to expand: the control of juvenile quality and the low demand, due to lack of sufficient familiarity of meagre products to the public (FAO © 2005-2011). Other studies are necessary to increase knowledge on the real potential of the species, on the characteristics of the meat quality as affected by different diets (Piccolo *et al.*, 2008), on the nutritional requirements of this species, in particular on dietary lipid requirement, and to standardize larval rearing protocols (Roo *et al.*, 2010; Chatzifotis *et al.*, 2010).

Reproductive cycle and egg quality

Meagre are a gonochorist species that remains sexually undifferentiated until nine months of age.

The sexual differentiation becomes histologically recognizable starting from six months of age (Schiavone *et al.*, 2008) and usually occurs earlier in females than in males; indeed primary oogonia can be detected in ovaries of 9-month-old fish, while primary spermatogonia cannot be detected in fish younger than 11 months.

Meagre reach puberty at 2 and 3 years of age for males and females, respectively (Schiavone *et al.*, 2008 and 2011) and exhibit an asynchronous oocyte development pattern. The asynchronous type of ovarian development is exhibited by those species that produce multiple spawns through an expanded period of time (several months), normally on a daily basis. In this type, all classes of oocytes (from early vitellogenesis to late maturation) can be found in the ovary at any moment of the reproductive cycle. There are no batches of oocytes growth (Mañanós *et al.*, 2008). Natural spawning of meagre broodstock under captivity has not yet been reported and the production of viable eggs has only been achieved through artificial reproduction with hormone administration (Duncan *et al.*, 2008). Usually they are allowed to spawn spontaneously, without the necessity of stripping and artificially fertilize the eggs before incubation. This is due to their sensitivity to handling stress and because eggs are not release all at once but in multiple small batches (Mañanós *et al.*, 2008).

The success of reproduction depends on the successful progression through each stage of gametogenesis, which leads to the production of good quality gametes (Mañanós *et al.*, 2008). The quality of the gametes is defined as their ability to produce a fertilized egg and a normal embryo (Bobe & Labbé, 2010). But most of the studies talk about egg quality, that is defined as those characteristics of eggs which determine their capacity to survive (Bromage, 1995); so good quality eggs exhibit low mortalities at fertilization, eyeing, hatching and first feeding larvae (Lahnsteiner *et al.*, 2008). It is useful to be able to determine egg quality produced in a particular batch and predict the larval survival, before investing facilities, manpower and time in incubating eggs and rearing larvae (Mylonas *et al.*, 2004). Several types of criteria can be used (Bobe & Labbé, 2010), remembering that a good practical criterium for the determination of egg quality should be both possible to identify early in development and be simple to use (Kjørsvik, 1990). Quality criteria of eggs could be summarized in: egg size and morphology, physiochemical and biochemical parameters, buoyancy of pelagic eggs, cytogenetic status, fertilization success and embryonic survival.

- Egg size and morphology

The size and appearance of unfertilized eggs can tentatively be used to evaluate or estimate the overall developmental potential of the eggs after fertilization. The size of the eggs was sometimes considered to be beneficial for the future development of the embryo, especially in ecological studies. However, despite the large variations in egg weight that can be observed in fish within the same species, very little data can support this hypothesis (Bobe & Labbé, 2010).

The appearance or morphology of unfertilized eggs is also sometimes used to estimate developmental potential of the egg (Giménez *et al.*, 2006; Bobe & Labbé, 2010) or in pollution studies, due to their sensitivity to environmental variables (Shields *et al.*, 1997). The earliest cells (blastomeres) of an embryo are undifferentiated, and they form the basis for the differentiating embryo. Thus, a deviation (or defect) in these cells will influence the further development of the embryo more strongly than will defects occurring in single cells later in development. Good quality eggs are generally described as transparent (except for

oil droplets), perfectly spherical with clear, symmetrical early cleavages (Kjørsvik, 1990). Major morphological changes can routinely be observed in specific cases, but the experimental procedures are laborious, so not applicable to routine checking in commercial hatcheries (Shields *et al.*, 1997), and above all significant differences in egg quality are also observed that cannot be linked to simple morphological criteria (Bobe & Labbé, 2010).

- Physiochemical and biochemical parameters

Egg quality could also tentatively be predicted using indirect measurements such as physiochemical parameters of ovarian or coelomic fluid in which the eggs are bathed. Several studies have shown that the drop in coelomic fluid pH during post-ovulatory ageing may be due to the presence of egg content in the fluid, such as vitellogenin fragments. Hence, low pH values of ovarian fluid has been associated with reduced egg quality (Bobe & Labbé, 2010). On the contrary, it was shown in rainbow trout (*Oncorhynchus mykiss*) that no significant linear regression between pH and embryonic survival could be identified. Thus egg quality cannot be accurately predicted using ovarian fluid pH in all the species, especially when low egg quality is not induced by post-ovulatory ageing or when other factors are involved (e.g. temperature) (Aegerter & Jalabert, 2004; Bobe & Labbé, 2010).

Biochemical evaluation of egg quality could also be possible, even before fertilization, because certain components are known to be “essential” for a healthy egg, and these components have to be present in certain amounts to satisfy biological demands (Kjørsvik, 1990). For example, a study on Asian sea bass (*Lates calcarifer*) egg quality found that specific lipid and protein components, such as docosahexaenoid acid, of the eggs correlate with egg viability indicators, suggesting that these data are useful in establishing egg quality criteria, and formulating larval and broodstock diet (Nocillado *et al.*, 2000). Unfortunately, there are species-specific needs in term of nutrition, and the possibility of finding common biochemical quality criteria for fish eggs may therefore be questionable (Kjørsvik, 1990).

- Buoyancy of pelagic egg

The buoyancy of pelagic eggs was also currently used to identify good quality eggs, since it is often better for eggs able to develop normally (Kjørsvik, 1990), while poor quality eggs usually sank in the water column (Lahnsteiner *et al.*, 2008). Indeed in a recent study on cobia eggs, a positive correlation was found between the proportion of floating eggs and hatch rate (Faulk & Holt, 2008). So the proportion of floating eggs was suggested as a predictor of hatch rates, but not necessarily larval growth or survival through 15 days post hatching (dph), since these two last stages were not different. However also the buoyancy does not hold true for all species (Kjørsvik, 1990; Brooks *et al.*, 1997; Barbaro *et al.*, 1999; Bobe & Labbé, 2010). Moreover, estimate egg quality with this criteria is difficult, because eggs with reduce viability are still buoyant, or in any case they sink very slowly (Barbaro *et al.*, 1999).

- Cytogenetic status

Some species may have more resistant DNA than others (Bobe & Labbé, 2010). Indeed, clear correlations between survival and cytogenetic status are found for several species. Chromosome abnormalities at the early embryonic stage seem to be one of the best indicators of sublethal damage to the embryo, as they give a very efficient measure of the “health” status in a batch of eggs (Kjørsvik, 1990). But the effects of broodfish genetics on gamete quality remain poorly documented (Bobe & Labbé, 2010).

- Fertilization success and embryonic survival

Fertilization success usually indicates the percentage of fertilized eggs (Lahnsteiner *et al.*, 2008) and it is a useful parameters to detect poor egg quality (Kjørsvik, 1990). Fertilization success is probably one of the earliest estimators (Bobe & Labbé, 2010) and it is used also in studies of nutritional requirements of broodstock. However, fertilization success is not necessarily reflective of good survival and development in later embryonic stages, so alone it is not a reliable criterion of egg quality (Kjørsvik, 1990; Laine & Rajasilta, 1999; Bobe & Labbé, 2010).

Embryonic survival at specific developmental stages is one of the most common method of characterizing the ability of the fertilized egg to develop successfully. Survival can thus be assessed at specific stages such as the eyed stage, hatching, and yolk-sac resorption stage, which can be monitored in most fish species (Bonnet *et al.*, 2007). For example, in a study on the European sea bass hatching success and 4-day larval survival exhibited a significant positive correlation with 1-day embryo survival (Mylonas *et al.*, 2003 and 2004). In another study, conducted on common dentex (*Dentex dentex*), was found that larval survival at days 3 and 5 post-hatching can be more useful as indicators of egg quality, because they indicate the quality of the endogenous reserves contain in the yolk sac and the intrinsic survival potential of the larva (Giménez *et al.*, 2006). In addition, it is also noteworthy that monitoring survival at successive developmental stages can be extremely valuable to characterize the timing of embryonic mortalities that can significantly differ between different experimental treatments (e.g. broodstock breeding conditions) (Bonnet *et al.*, 2007; Bobe & Labbé, 2010).

Thus, apart from markers of extremely low quality, it is still very difficult to accurately estimate the quality of the gametes prior to fertilization. Hence, the only biologically relevant way to accurately estimate gamete quality is to perform fertilization and/or monitor embryonic and larval survival. Furthermore, monitoring embryonic malformation or larval deformities will further help characterize gamete quality. Specific malformations are associated with specific gamete quality problems induced by specific factors (Bonnet *et al.*, 2007; Bobe & Labbé, 2010). It is thus important not only to record survival throughout development but also to monitor embryonic and larval deformities to completely assess gamete quality (Bobe & Labbé, 2010).

Factors affecting egg quality

Egg quality is influenced by many factors and the relative effects of each factor on it can be highly variable and are not always well characterize (Bobe & Labbé, 2010). Hence, egg quality often varies greatly and in a non-controllable way. In larvae originating from high quality egg batches, viability and stress resistance may be better than in larvae from low quality egg batches. So egg quality control is very important. Both gametes contribute to egg quality (Blaxter, 1969), but they operate at different times and in different ways. Paternal effects are produced very early in ontogeny, regarding spermatozoan density and motility (Lahnsteiner *et al.*, 2008; Mylonas *et al.*, 2003a), whereas maternal effects reveal later, contributing to embryonic survival. Since females are responsible for the synthesis of the yolk and other substances that are indispensable for a normal larval development, research studies have mainly focused on the maternal contribution to egg quality (Lahnsteiner *et al.*, 2008). Eggs possess both intrinsic and extrinsic characteristics that determine egg quality. Dealing only with the maternal contribution, intrinsic factors are: egg genes, maternal age and genes, maternal nutrient contained inside the yolk, endocrine status. Extrinsic factors that will influence egg quality are: environmental factors, such as temperature, photoperiod, salinity, pollutants and bacterial colonization, husbandry practices, stress and diet.

Intrinsic factors

Regarding egg genes, little is yet known about transcription of the genes in fish oocytes or the mechanism of regulation and translation of mRNAs. Hundreds of genes are transcribed and RNAs translated during embryo development. Some are vital to development, whereas others are less crucial (Brooks *et al.*, 1997). Inappropriate expression of these genes, either in strength or timing, probably leads to problems during embryogenesis, and hence to a “poor-quality egg”. Most, possibly all, of the other factors considered to affect egg quality do so by influencing gene expression and RNA translation in the egg (Brooks *et al.*, 1997). Among these, the maternal factors are essential for the earliest stages in development for all animal species, including fish species such as zebrafish (*Danio rerio*), goldfish (*Carassius auratus*) and medaka (*Oryzias latipes*). Maternal factors are indispensable prior to the activation of the zygotic genome at the midblastula transition, namely the blastula stage during which occurs normally the initiation of the zygotic gene program, but they have also been shown to have important functions after this transition. The transition itself from maternal to zygotic control of development appears to be governed at least partially by maternal cues (Lyman-Gingerich & Pelegri, 2007).

Maternal influence on egg quality is moreover very important because specialized materials such as amino acids, lipids, carbohydrates, calcium, vitamins and metals are derived from a number of maternal sources and must be incorporated during the growth of the oocyte in the ovary. Yolk proteins, lipids and carbohydrates constitute the bulk of material in fish eggs, but also other molecules like vitamins and metals are equally necessary for producing a viable egg (Brooks *et al.*, 1997). Most of these “building blocks” originate from the

uptake of vitellogenin (VTG), a complex molecule, and they are stored for a future use by the developing embryo during the vitellogenesis, the principal event responsible for the enormous growth of oocytes. If an egg does not contain a particular compound, or contains an inappropriate amount of a compound, it will not be able to sustain development of a viable embryo (Brooks *et al.*, 1997). The correct uptake and deposit of these materials depend on the condition of females, and principally from their nutrient status, as demonstrate in a study on the Baltic herring (*Clupea harengus membras*) eggs, where the fertilization process and embryonic mortality were found dependant on the condition of females (Laine & Rajasilta, 1999).

However, egg quality is affected by other intrinsic aspects, such as the age of the females and their endocrine status. The age of the females seems to affect the viability period after ovulation, as well as the quality of the eggs in most species. Middle-aged females seems to produce the best eggs, but this rule is not without exceptions (Kjørsvik, 1990; Brooks *et al.*, 1997). For example, in sharpnose sea bream (*Diplodus puntazzo*) an aging affect seems possible, resulting in a decrease in egg quality after females exceeded 4 years of age (Papadaki *et al.*, 2008). Dealing with the endocrine status, fish larvae are strongly affected by the action of different hormones during development, among which thyroid hormones and cortisol. These hormones could be synthesized *in situ* at any time after fertilization, but it seems more probable that they are supplied to the egg before fertilization, in which case they must enter by maternal transfer (Brooks *et al.*, 1997). It was possible to establish a correlation between thyroid hormone levels and egg quality for some species. Significant amounts of cortisol were also observed in eggs and embryos. However, besides these observations no evidence exists that cortisol affects egg quality (Lahnsteiner *et al.*, 2008).

Extrinsic factors

Many investigations have led to the identification of external or rearing factors that can significantly impact gamete quality, such as domestication level, adequacy of rearing system and broodstock management conditions (Bobe & Labbé, 2010). Environmental factors that may affect egg quality in farmed fish include the physicochemical conditions of the water in which the eggs are incubated (Kjørsvik, 1990; Brooks *et al.*, 1997): temperature, photoperiod, salinity, but also pollutants and microorganisms.

Water temperatures during reproductive season and incubation of the eggs are particularly important in affecting the rate of development and egg quality (Swift, 1965; Brooks *et al.*, 1997; Bobe & Labbé, 2010), as shown in different studies on perch (*Perca fluviatilis*), pike (*Esox lucius*), rainbow trout and Atlantic mackerel (*Scomber scombrus*) (Swift, 1965; Aegerter & Jalabert, 2004; Mendiola *et al.*, 2006). For example, in salmonid species high temperatures significantly increased the incidence of embryonic deformities and triploid fry (Aegerter & Jalabert, 2004).

Since in many fish species the decrease of egg quality observed has often been attributed to suboptimal temperature, separating the specific effects of temperature and photoperiod on egg quality is difficult (Bobe & Labbé, 2010). Indeed it was shown in rainbow trout that artificial photoperiod resulted in a

significant decrease of egg quality even if the water temperature was kept at 12°C during the reproductive season (Bonnet *et al.*, 2007; Bobe & Labbé, 2010). Hence, the photoperiod-induced manipulation of spawning can negatively impact egg quality (Bobe & Labbé, 2010) and the importance of this negative effect seems highly dependent on the type of photoperiod regime used (Brooks *et al.*, 1997; Bobe & Labbé, 2010). Manipulation of photoperiod often results in the modification of other parameters throughout oogenesis, so its effect is difficult to study. However, it seems also that suboptimal temperature around spawning time will further increase any negative impact of the photoperiod regime on the overall egg quality (Bobe & Labbé, 2010).

Another environmental factor to consider is salinity, that may also affect egg quality. It is capable of modifying the physiological effects of temperature on embryo development in both marine and brackish-water fish (Kinne & Kinne, 1962; Brooks *et al.*, 1997). Regarding pollutants, fish oocytes and eggs are particularly sensitive to a variety of environmental contaminants. From their exposure to these substances, malformations and impaired development and viability may result (Brooks *et al.*, 1997).

In this view and considering these aspects, rearing environment must be well designed for the farmed species and always fully controlled in all its parameters. A characteristic failure of most aquatic environments is moreover the large numbers of microorganisms, which are present in the water. There are further significant increases in these numbers following the culture of fish eggs and larvae in the same environment. The enhancement of microbial growth probably occurs as a result of the increased numbers of surfaces for microbial colonization and the various lipid and protein components of fish eggs. These provide an excellent source of nutrients and their glycoprotein-rich chorion encourages microbial attachment, such as the fungus *Saprolegnia* and different species of bacteria (Bromage, 1995).

Turning to husbandry practices, they should be appropriate by optimizing broodstock stress, spawning induction, egg post-ovulatory ageing parameters and nutrition.

In many ways broodfish are far more tolerant of stress than fry or juvenile fish, possibly by virtue of their age, size and metabolic requirements and reserves. However, during reproduction the fish have a much lower threshold of effect of stress (Bromage, 1995). The effects of broodstock stress, such as exposure to emersion, confinement, crowding, handling, capture and transportation, on gamete quality are different depending on the type and intensity of stressor, the species and the period when the stressor is applied. It seems that stress applied during oocyte growth can result in significantly smaller egg size, while stress applied during the final maturation stage can result in advanced ovulation (Brooks *et al.*, 1997).

Stress associated with captivity, the absence of appropriate environmental conditions in culture facilities or other extrinsic factors can block the reproductive axis, inhibiting neuroendocrine secretions from the brain and thus inhibiting reproductive success. So, egg quality is affected by hormones not only during embryonic and larval development, but in all the reproductive cycle, during which hormones play a very important role. The synchronized secretion of Gonadotropin-Releasing Hormone (GnRH) from the brain, pituitary

Gonadotropins (GTHs) and gonad steroid hormones throughout the reproductive axis and their coordinated action is essential for successful spawning of viable gametes (Mañanós *et al.*, 2008). Major reproductive dysfunctions observed in captive female fish are classified in three main types, each one physiologically different: the inhibition of vitellogenesis; the inhibition of oocyte maturation, which causes atresia of post-vitellogenic oocytes; the inhibition of spawning only, with ovulated oocytes retained in the ovarian or abdominal cavity. The application of hormonal treatments has effectively resolved reproduction in many species exhibiting the second dysfunction type (Mañanós *et al.*, 2008), and it is actually widely used in aquaculture (Bobe & Labbé, 2010). Solution of the first dysfunction type is under investigation, whereas reproduction of species with the third one can be achieved through artificial fertilization, after manual stripping of eggs and sperm (Mañanós *et al.*, 2008). Hormonal induction of spawning is used also to synchronize ovulation for practical reasons in other species (Bobe & Labbé, 2010). A great deal of information is thus available on hormonal treatments and their efficiency in term of ovulation rate. In contrast, the effect of hormonal therapies on egg quality compared to naturally spawning broodfish is less documented and sometimes limited to embryo survival at early stages (Avery *et al.*, 2004; Bonnet *et al.*, 2007; Bobe & Labbé, 2010; Mylonas *et al.*, 2010). These effects may have delayed consequences on embryonic development (Bonnet *et al.*, 2007; Mylonas *et al.*, 2010), besides impact on embryo survival. However, appropriately employed hormonal therapies do not usually have a negative effect on egg quality (Barbaro *et al.*, 1997; Mylonas *et al.*, 2010). In order to achieve a successful reproduction and good egg quality, different aspects must be considered: the developmental stage of the gonads at the time the hormonal therapy is applied, the type of hormonal therapy, the possible stress induced by the manipulation necessary for hormone administration and the latency period between hormonal stimulation and stripping for *in vitro* fertilization, in the case of artificial insemination (Mylonas *et al.*, 2010). Paying attention to these aspects, good egg quality is obtained, as shown in studies on European sea bass, gilthead sea bream and dusky grouper (*Epinephelus marginatus*) (Barbaro *et al.*, 1997; Mylonas *et al.*, 2003; Marino *et al.*, 2003).

Egg post-ovulatory ageing occurs between the release of the oocyte from the follicle at ovulation, and spawning. During this time period, the oocyte undergoes an overall decrease in its ability to be fertilized, through morphological and biochemical changes, and to subsequently develop into a normal embryo. This process is called “over-ripening”. The decrease in egg quality occurs more or less rapidly depending on the species and it is also highly dependent on external factors and subject to high inter-female variability (Bobe & Labbé, 2010).

Difference in egg quality as a consequence of diet, especially the diet’s lipid content, is one of the most researched aspects concerning egg quality and viability (Brooks *et al.*, 1997). In fact broodstock nutrition is an important factor since it can significantly impact reproductive performance, which includes many types of parameters such as timing of reproduction season, fecundity, plasma levels of reproductive hormones and spawning frequency. (Kjørsvik, 1990; Brooks *et al.*, 1997; Izquierdo *et al.*, 2001; Bobe & Labbé, 2010).

Food restriction generally reduces total fecundity and may delay maturation and decrease the proportion of maturing fish (Kjørsvik, 1990;

Izquierdo *et al.*, 2001). Besides the feeding ratio, broodstock diet has to be appropriately formulated also because a nutrient imbalance or a restriction in the availability of a biochemical component can influence the brain-pituitary-gonad endocrine system and the egg formation, reducing fecundity and egg quality (Izquierdo *et al.*, 2001). Thus, some components of broodstock diet are necessary to ensure a normal development of the embryo and improve egg quality, such as vitamins, carotenoids, essential fatty acids, among others (Brooks *et al.*, 1997; Watanabe & Vassallo-Agius, 2003).

Carotenoids constitute one of the most essential pigment classes in fish, important for a normal embryonic and larval development (Izquierdo *et al.*, 2001), as shown in a study on gilthead sea bream where an increase in dietary carotenoids from 40 to 60 mg Kg⁻¹ significantly improved egg fertilization success (Scabini *et al.*, 2011). Carotenoids have to be absorbed from the diet, since fish are unable to synthesize them, and they are deposited in various body tissues, including the gonads (Christiansen & Torrissen, 1997). Carotenoids have a wide variety of functions, including protection from adverse lighting conditions, a provitamin A source and antioxidant functions including singlet oxygen quenching (Izquierdo *et al.*, 2001).

Similar to carotenoids, vitamin E plays a determining role in egg quality as dietary antioxidant, protecting unsaturated lipids from oxidation (Izquierdo *et al.*, 2001; Faulk & Holt, 2008). From the oxidation of n-3 highly unsaturated fatty acids (HUFA) derive free radicals that can cause severe damage to the different components of cell membrane, impairing its correct functioning. Vitamin E, together with carotenoids and vitamin C, are thus strong scavengers of active oxygen species and have been shown to have a protective role against the action of free radicals (Izquierdo *et al.*, 2001; Scabini, *et al.*, 2011). Indeed α -tocopherol requirements have been shown to increase with polyunsaturated fatty acid content in diet for gilthead sea bream broodstock (Izquierdo *et al.*, 2001). In another experiment on gilthead sea bream, the lowest fertility and larval survival was reported in eggs from broodstock fed the lowest dietary levels of α -tocopherol (Izquierdo *et al.*, 2001; Watanabe & Vassallo-Agius, 2003).

Vitamin C content of broodstock diets affects the survival of embryo not only for its scavenger activity but also because it is necessary for the synthesis of collagen during embryo development. Another important requirement during gonadal maturation and spawning is the vitamin A, despite the fact that little is known about it. It is considered important for embryo and larval development due to its important role in bone development, retina formation and differentiation of immune cells (Izquierdo *et al.*, 2001).

Other dietary nutrients that have been found to affect the reproductive performance of marine fish include dietary protein intake (Izquierdo *et al.*, 2001). Indeed, besides their important roles as structural, functional and energetic constituents of tissues and organs in fish body, the proteins have particularly important roles for fertilization and normal development of the embryo (Fernández-Palacios *et al.*, 1997). For example, reduction of dietary protein levels together with an increase in dietary carbohydrates levels reduced egg hatchability in sea bass (Cerdá *et al.*, 1994; Izquierdo *et al.*, 2001). Also the quality of the protein source is important, and it depends both on its amino acid profile and digestibility coefficient for each particular fish species. It has been shown in gilthead sea bream that the squid protein based diets have a superior nutritional

value, related to the higher apparent digestibility coefficients of its protein (Fernández-Palacios *et al.*, 1997).

Lipid and fatty acid composition of broodstock diet have been identified as major dietary factors that determine successful reproduction and survival of offspring (Izquierdo *et al.*, 2001). Essential fatty acids such as n-3 and n-6 HUFA play a very important role in marine broodstock nutrition, because marine fish are incapable of elongating the shorter chain fatty acids (Watanabe & Vassallo-Agius, 2003). Fatty acids are mobilized from the neutral lipid reserves of fish adipose tissue during gonadogenesis and transferred via the serum to the liver where they are assembled into the egg-specific lipoprotein, vitellogenin. More than half of the free fatty acids mobilized, preferentially saturated and monounsaturated fatty acids, can be catabolized to provide metabolic energy for egg lipoprotein biosynthesis. The remainder, preferentially n-3 PUFA and especially 22:6(n-3), or docosaexaenoic acid (DHA), are incorporated into the phospholipid-rich vitellogenin, which is transferred via the serum to the developing eggs (Sargent, 1995). Generally, arachidonic acid (AA), or 20:4(n-6), and eicosapentaenoic acid (EPA), or 20:5(n-3), with DHA, play an important structural role as components of phospholipids in fish biomembrane and are associated with the membrane fluidity (Izquierdo *et al.*, 2001).

The chief role of DHA is the formation of membranes, primarily the brain and the eyes (Nocillado *et al.*, 2000). Indeed there is an unusual richness of DHA in neural membranes, indicating that this fatty acid has a critical role in the formation of the brain and the eyes, which constitute a large fraction of the embryonic and larval body mass (Sargent, 1995).

The second important function of essential fatty acids is their role as precursor of eicosanoids. Arachidonic acid and EPA are involved in cell-mediated functions and are precursor of prostaglandins. Highly biologically active eicosanoids (prostaglandins from series II, PGE₂) are formed principally from AA. These eicosanoids are produced physiologically in response to a range of external stressors (Sargent, 1995). Instead, EPA forms prostaglandins with low biological activity (from series III) and moreover, together with DHA, blocked the steroidogenic action of both AA and PGE₂ (Izquierdo *et al.*, 2001). Hence, embryo is vulnerable to an over-high maternal ratio of dietary (n-6)/(n-3) PUFA during gonadogenesis, an excess of (n-6)PUFA in broodstock fish will be readily transferred to the egg and result in an elevated production of eicosanoids during subsequent embryogenesis and larval development. This will cause an increase in the susceptibility of the resulting larvae to external stressor, exaggerating normal stress responses (Sargent, 1995; Izquierdo *et al.*, 2001).

Concluding, not only the absolute levels of DHA and EPA are important, but also the n-6/n-3 PUFA ratio, considering that requirements and ratios will be probably different in different species (Bromage, 1995).

Purposes of this study

The objective of this work was to study the effects of two different diets on the egg quality produced from meagre broodstock, by comparing fecundity, fertilization success, embryonic and larval survival at different stages.

This work was part of a larger program titled “Development of methods for reproduction and rearing of meagre (*Argyrosomus regius*) as a measure to enhance the competitiveness of aquaculture with the introduction of new species” (ESPA 2007-2013, Collaboration). The objectives of the program were to study all aspects of the lifecycle of meagre in culture, in order to optimize protocols for broodstock management, larval rearing, nursery and grow out in sea cages. The present thesis work adhered to this research project and was carried out in the Institute of Aquaculture of the Hellenic Centre for Marine Research (HCMR) in Crete, Greece.

Materials and methods

Broodstock

The experiment was carried out in the indoor facilities of the Institute of Aquaculture, Hellenic Centre for Marine Research (HCMR) in Crete, using 6-year-old mature meagre produced from wild broodstock in France, and reared at HCMR starting from the eggs. Fish were individually tagged with a 12-mm passive integrated transponder (PIT) tag (AVID, Inc., UK).

Fish were divided on 16/12/2010 in two groups, each group being composed of 10 individuals (5 females and 5 males). The mean (S.D.) weight was 5.54 (0.82) Kg for the males and 5.75 (1.04) Kg for the females. Due to some mortality, both groups were composed of 9 individuals at the onset of the spawning induction experiment. One group was composed of 4 females and 5 males from 11/03/2011 and the other group was composed of 5 females and 4 males from the 11/05/2011.

Fish were maintained in two 10-m³ rectangular tanks, that were supplied with seawater from a well, and they were exposed to the same simulated natural photoperiod and modified seawater temperature over the experiment (Fig. 3). During the spawning induction period, the water temperature was maintained at 19°C.

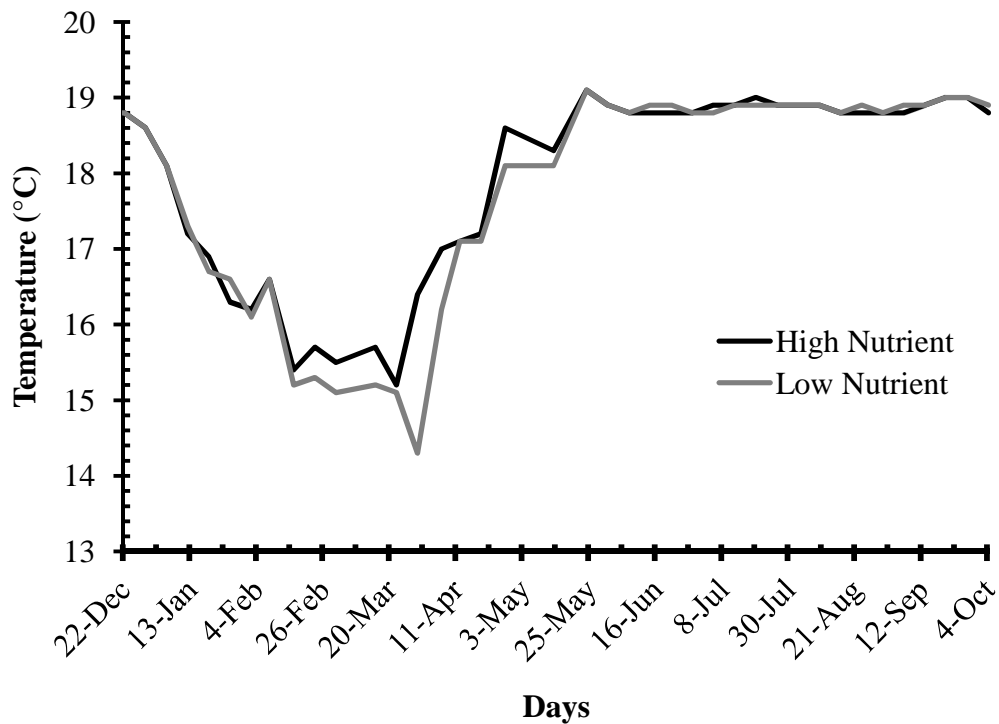


Figure 3. Water temperature (°C) in the meagre broodstock rearing tanks between 22/12/2010 and 4/10/2011.

The broodstock tanks were maintained on a flowthrough system until 17/12/2010, with a daily exchange of 100%. From 17/12/2010 until the 8/07/2011 the tanks were connected to a recirculation system consisting of an upwelling, plastic tubing mechanical and biological in order to control seawater temperature. With this system the water renewal was 11% day⁻¹. Oxygen saturation ranged between 83 and 100% and the pH was 7.4. Each tank was provided with an automatically overflow egg collector.

Dietary treatment

Fish were fed manually to apparent satiation every day. Before the 21/02/2011, they were fed a maintenance diet (Table 1) and from the 21/02/2011 onwards they were fed with two experimental diets made with high and low nutrient content (Table 2). Both feeds were extruded with a pellet size of 8 mm.

Table 1. Composition of the maintenance diet given to meagre broodstock during the reproductive quiescent period (Eco Feeds, Irida S.A., Greece).

Diet	Eco Feeds
Protein % (Nx6.25)	43
Fat %	17.0
Phosphorus %	1.1
Cellulose %	2.0
Vitamin A (IU/Kg)	12000
Vitamin D3 (IU/Kg)	1400
Vitamin E (mg/Kg)	180
Vitamin C stay (mg/Kg)	150
Inositol (mg/Kg)	150
Cholin (mg/Kg)	3000

Table 2. Composition of the Low Nutrient (LN) and High Nutrient (HN) experimental diets given to meagre broodstock during the reproductive period (Eco Feeds, Irida S.A., Greece).

Diets	Low Nutrient	High Nutrient
Moisture %	8.10	4.38
Oil %	15.22	17.54
Protein % (Nx6.25)	48.54	58.83
Fiber %	1.59	0.25
Ash %	9.82	11.22
Vitamin A (IU/Kg)	10000	10000
Vitamin D3 (IU/Kg)	2000	2000
Vitamin E (mg/Kg)	800	800
Vitamin C stay (mg/Kg)	1000	1000

Spawning induction treatment and sampling protocol

In order to monitor the stage of sexual maturation, the fish were sampled once per month, starting from March. For sampling, the water level in the broodstock tanks was reduced to 50 cm and clove oil was added at a concentration of 0.01 ml l⁻¹ for light sedation. When the fish were swimming slowly and begun losing equilibrium, they were taken one by one with a stretcher and transferred to a 340-l circular tank with clove oil at a concentration of 0.03 ml l⁻¹ for complete anesthesia. Once the fish were completely anesthetized they were identified by their PIT tag, and an ovarian biopsy and a sperm sample were obtained to evaluate reproductive status.

The ovarian biopsy was evaluated under a compound microscope for oocyte diameter and maturation stage. The spermiation status was evaluated according to an arbitrary scale (Papadaki *et al.*, 2008) and sperm quality was evaluated in the laboratory. Fish were treated with GnRH_a-loaded controlled-release implants (Mylonas *et al.*, 2010) when the females had oocytes > 540 µm in diameter and all the males were in spermiating condition. A dose of 250 µg of GnRH_a was used for females with a weight < 5.5 Kg, 400 µg for females with a weight > 5.5 Kg and 500 µg for all males. Fish were implanted in the dorsal musculature, at the level of the base of the second dorsal fin (Fig. 4). A total of 18 fish were implanted: five males and four females in the LN group and four males and four females in the HN group. Only one female in the HN group was not implanted because the maximum oocyte size was around 220 µm and the fish was not in vitellogenesis.



Figure 4. Implantation of GnRH_a delivery systems in meagre broodstock.

Evaluation of egg and larval quality

After spawning induction, the egg collectors were examined daily for eggs. All spawned eggs were collected every morning using a plankton net and put in a 10-l bucket, filled with seawater. In order to evaluate fecundity and fertilization, a sample of 10 ml was taken with a 10 ml volumetric pipet, put in a test tube and then placed in a glass dish with a circular groove (fig. 5).

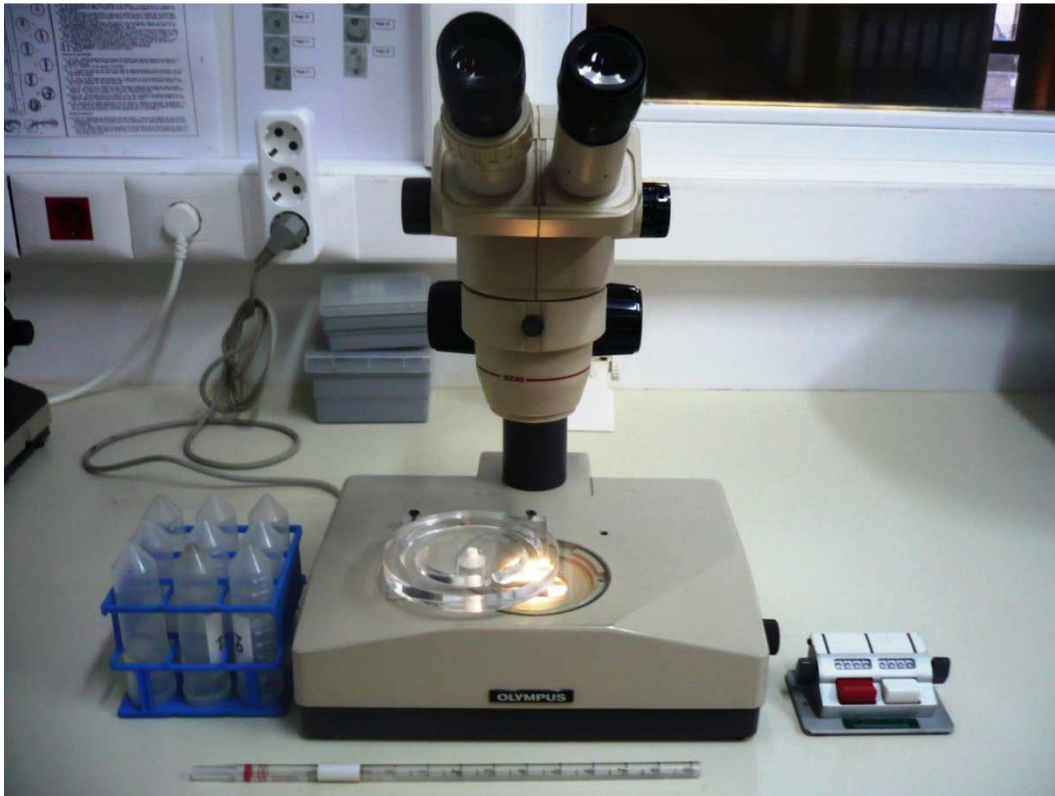


Figure 5. Stereoscope and glass dish with circular groove used for determine the stage of the eggs, the total fecundity and the fertilization success.

The stage of the eggs was determined and the number of live eggs was counted with a stereoscope. Relative fecundity was calculated as the total number of spawned eggs/total mean weight of GnRHa implanted females on 23/05/2011. The fertilization success was calculate as the number of live eggs/the total number of spawned eggs. In order to evaluate the quality of the eggs and larval survival, the following parameters were used: relative fecundity, fertilization success, survival of the eggs 24h after egg collection (1-day embryo survival), percentage of eggs hatched, survival of the larvae on day 5 and on day 7 after egg collection. These parameters were evaluated using the procedure of Panini *et al.* (2001) with some modifications, using 96 well microtiter plates (Fig. 6).



Figure 6. Fish larvae in microtiter plates, used to evaluate egg and larval quality.

Every day the eggs in the 10-l bucket were left inside for 5 minutes without agitation, in order to separate the fertilized eggs (floating) from the dead eggs (sinking); about 1000 floating eggs were taken with a little net and put in a 1000-ml beaker filled with the same seawater of the egg collector. In the laboratory sterilized seawater was put in a controlled-temperature incubator at the same temperature of the spawning tank. The beaker with the eggs was put in the same incubator for 10 minutes, to facilitate the separation of the live eggs from any dead eggs. The floating eggs were then taken with a 250- μ m mesh filter and washed twice with sterilized seawater, in order to reduce the risk of contaminations of bacteria or other pathogen to the eggs: the first time the eggs were transferred in a 250-ml beaker filled with sterilized seawater, the second time in a Petri dish, containing approximately 100-200 eggs. The Petri dish was placed under a stereoscope to be able to see the eggs, and the eggs were taken one by one with a 1000- μ l micropipette set on 200 μ l, fitted with a cut-off pipette tip. The eggs were transferred in a 96-well microtiter plates (in two replicates), one egg per well (fig. 7).

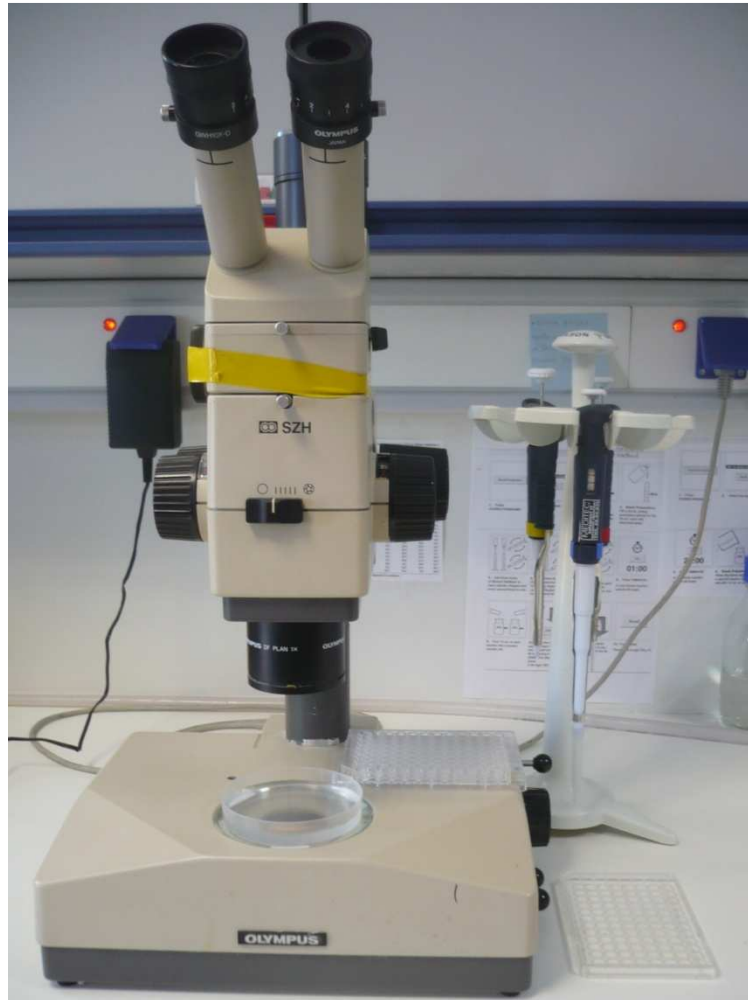


Figure 7. The transfer of the eggs in microtiter plates under the stereoscope.

The microtiter plates were covered with plastic lids to avoid any evaporation or contamination, placed in a controlled-temperature incubator and maintained for 7 days at 18.8°C. Once per day, embryonic and early larval development were evaluated with the stereoscope, and the number of live and dead embryos/larvae was recorded every day for 7 days. With this data the other egg quality parameters were evaluated: 1-day embryo survival was calculated as the number of eggs having live embryos/the number of fertilized eggs initially loaded in the microtiter plates, and it was evaluated the day after collection (1 day). Hatching success was calculated as the number of hatched larvae/the number of live 1-day embryos. Five-day larval survival was calculated as the number of live larvae 5 days after egg collection/the number of hatched larvae and 7-day larval survival was calculated as the number of live larvae 7 days after egg collection/the number of live 5-day larvae. Estimating survival % by using in the denominator, the number of individuals that survived to the previous developmental stage (Mylonas *et al.*, 1992) results in a more independent evaluation of survival within specific developmental stages, without the potential of a masking effect of the previous stage (Mylonas *et al.*, 2003).

Statistical analysis

The data of spawning and egg quality were analyzed with two-way Analysis of Variance (ANOVA), followed by Duncan's New Multiple Range test (DNMR) at a minimum significance of $P < 0.05$. Percentage data were Arcsine transformed prior to statistical analyses to normalize variances. The existence of a correlation between some egg quality parameters was examined with a Simple Regression analysis. Statistical analyses were done using a linear statistics software (SuperAnova, Abacus Concepts, Inc., USA). Results are presented as means \pm S.E.M. Spawns having a total fecundity $< 20,000$ eggs were excluded from the statistical analysis for different reasons: the eggs spawned on 31/05/2011 from the HN group were excluded because the total fecundity was of 4000 eggs and the eggs were in 22 somites embryo stage. Usually the eggs were in late blastula/early gastrula stage, so probably these eggs were spawned the day before. On 5/06/2011 the HN group and on 8/06/2011 the LN group had a small number of alive eggs; these data were excluded because was difficult separate the viable eggs from the dead eggs and because they were probably spawned from only one female. The eggs spawned from the LN group on 11/06/2011 and on 14/06/2011 were excluded because they had a very low value of fertilization success, as well as a total fecundity $< 20,000$ eggs. Finally, the eggs spawned on 27/06/2011 from the LN group were excluded from the statistical analysis because they were the only spawned eggs in the fifth week from both the broodstocks. The two groups did not spawn after sampling done on 14/06/2011.

Results

Daily relative fecundity and fertilization success

Fish began to spawn two days after the GnRH α treatment, on the 25/05/2011, and stopped on the 13/06/2011 in the HN group and on the 27/06/2011 in the LN group (Fig. 8). Fish spawned for 17 days in the LN group and for 18 days in the HN group. Daily relative fecundity showed a large variation in both the broodstocks, with a peak the second day of spawning (66,598 eggs Kg $^{-1}$ in the LN group and 57,432 egg Kg $^{-1}$ in the HN group) and a marked decrease four days afterwards. After the planned sampling of the fish on the 14th of June, no further spawns were obtained, with the exception of a single spawn on 27/06/2011 of the LN group. There were no significant differences (ANOVA, DNMR, $P < 0.05$) in the mean relative fecundity between the two groups. In both broodstocks, the mean relative fecundity during the first week after the GnRH α treatment was significantly higher compared with the second and the third week, when a dramatic decrease was observed (Fig. 9).

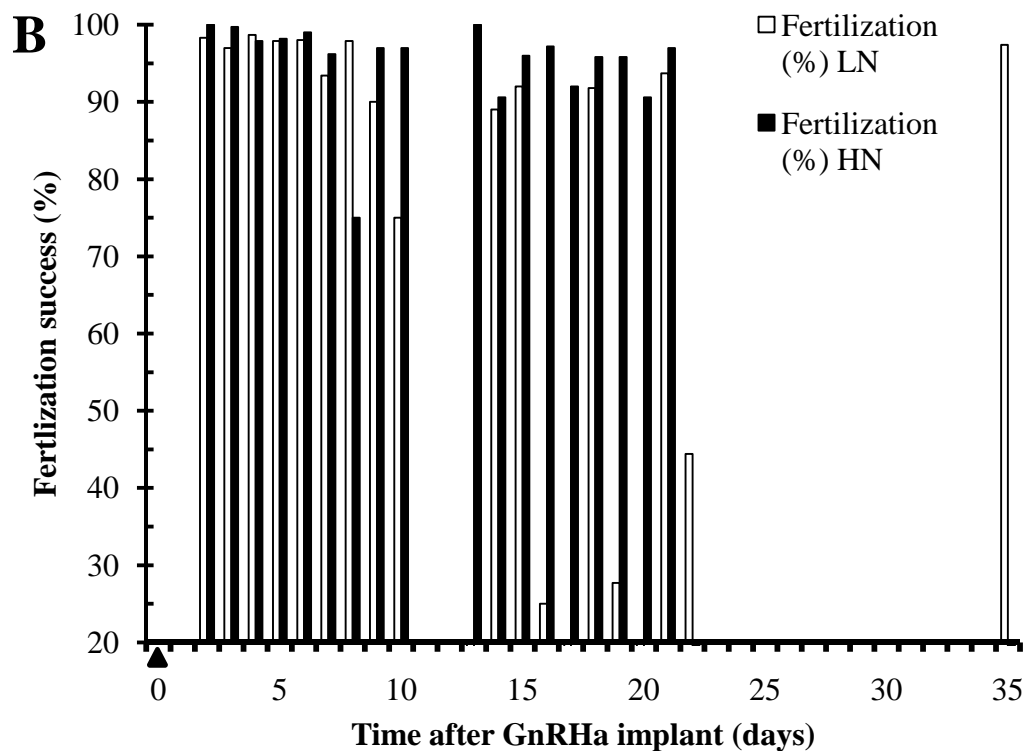
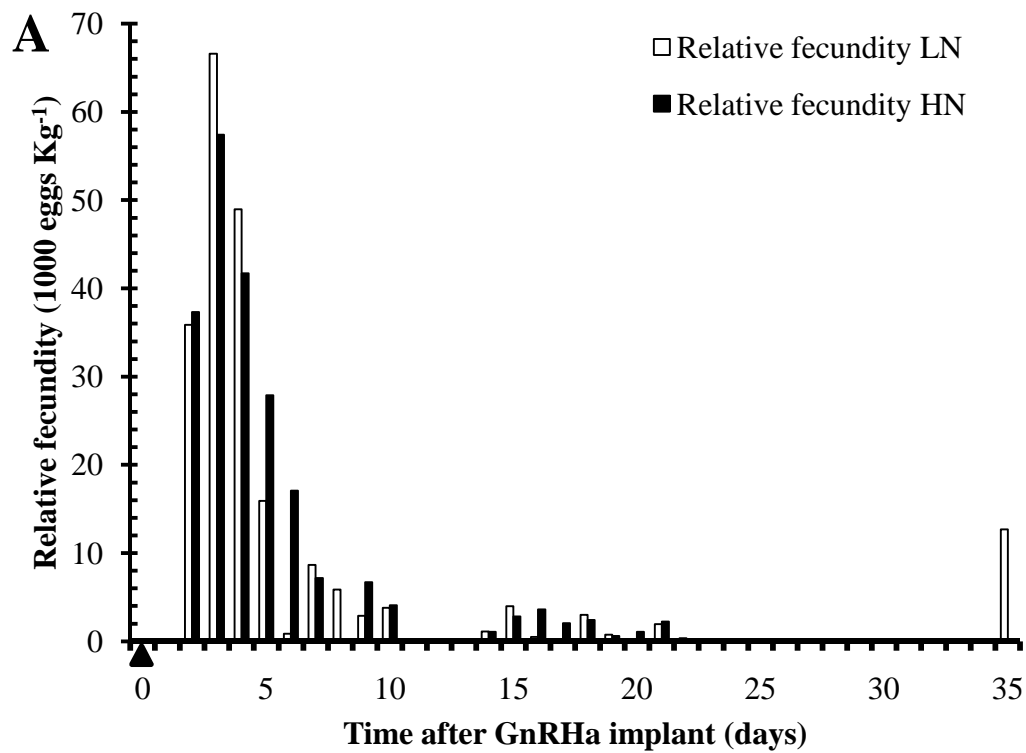


Figure 8. Daily relative fecundity in 1000 eggs Kg⁻¹ female biomass (A) and fertilization success (B) of two meagre broodstocks fed different diets (LN and HN) after GnRH α treatment (arrow heads). The total GnRH α -treated female biomass on 23/5/2011 was 24.4 Kg in the LN group and 29.6 Kg in HN group.

Fertilization success was relatively high throughout the reproductive season, although in the LN group there was much more variability, reaching a minimum value of 25% on the 16th day after treatment (Fig. 8). When considered on a weekly basis, the mean daily fertilization success decreased significantly during the study and was higher for the HN group (Fig. 9).

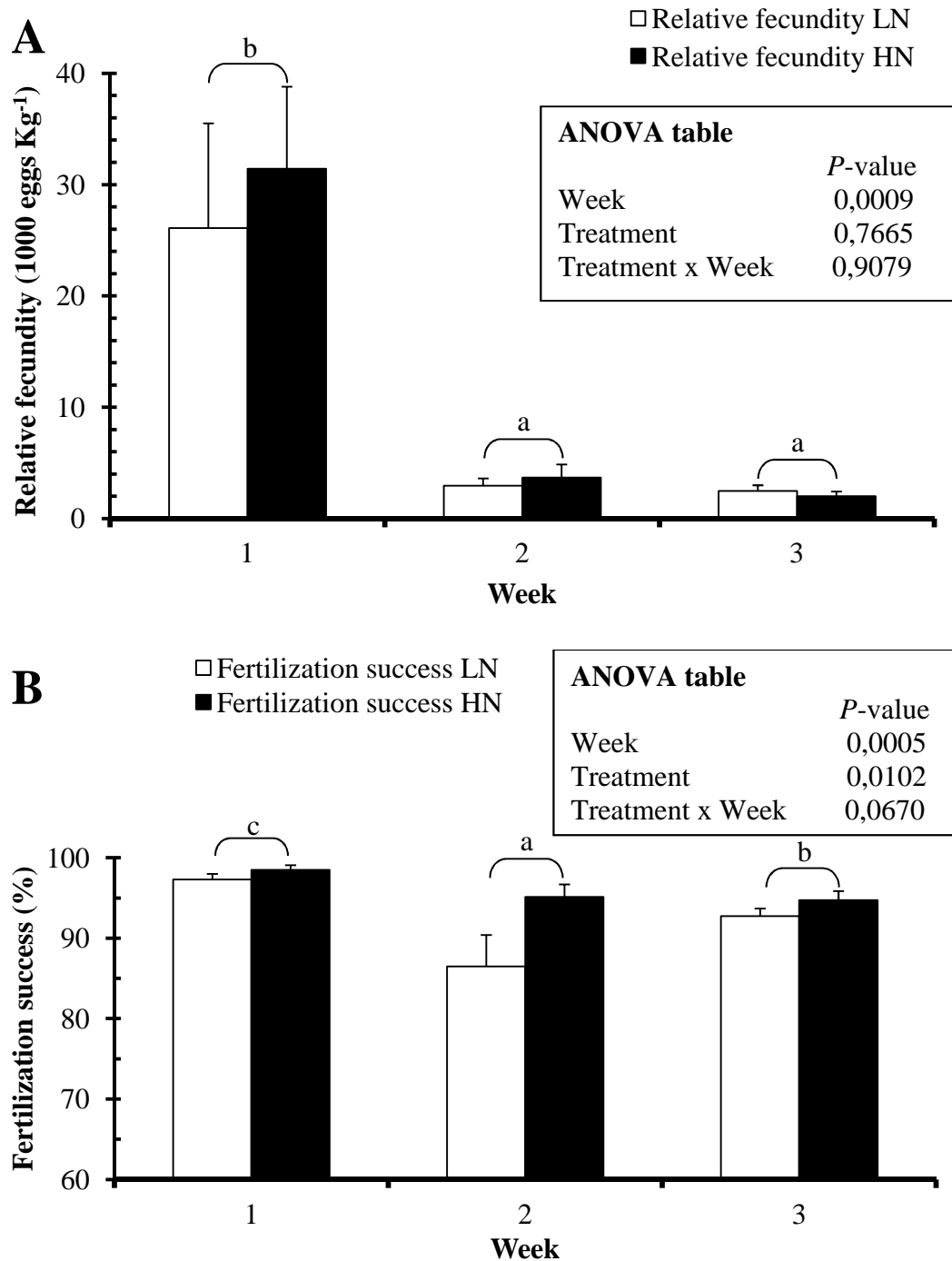


Figure 9. Mean (+ S.E.M.) daily relative fecundity (A) and fertilization success (B) during the three weeks after GnRHa treatment of two meagre broodstocks fed different diets (LN and HN). Significant differences among weekly means of both groups are indicated by different letter superscripts.

Egg quality parameters

The eggs incubated at 18.8°C in the microtiter plates hatched the second day after egg collection. Embryo survival one day after egg collection did not show any significant difference between the two diet groups or among weeks (Fig. 10). During the spawning period the mean embryo survival success in the LN group was of 98 ± 1.5 % and in the HN group it was 97 ± 1.4 %.

Also, there were no significant differences in the hatching success, neither among diets groups nor among weeks (Fig. 10). The mean hatching success during the spawning period for the LN group was 99.2 ± 0.7 %, for the HN group was 99.7 ± 0.1 %.

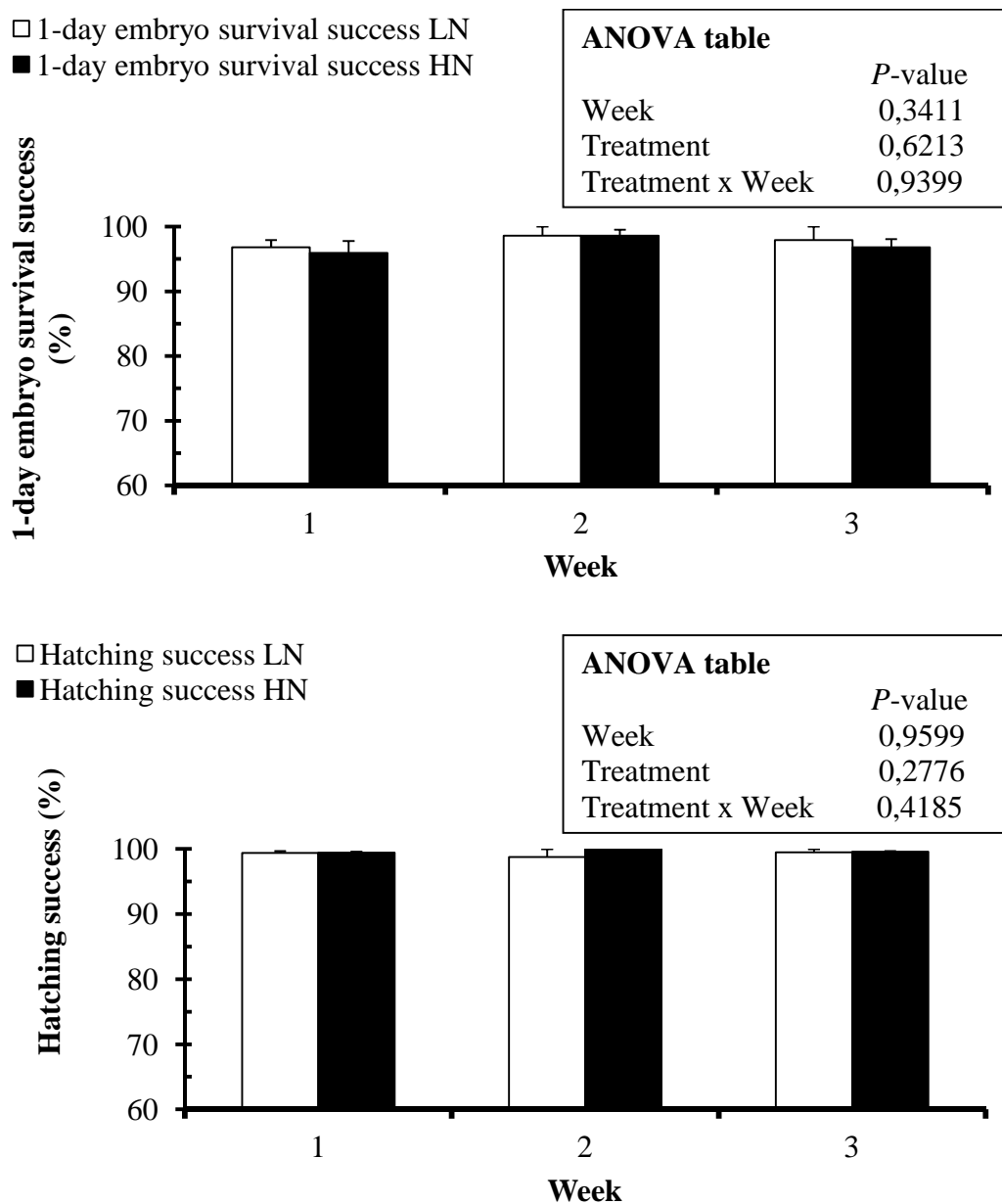
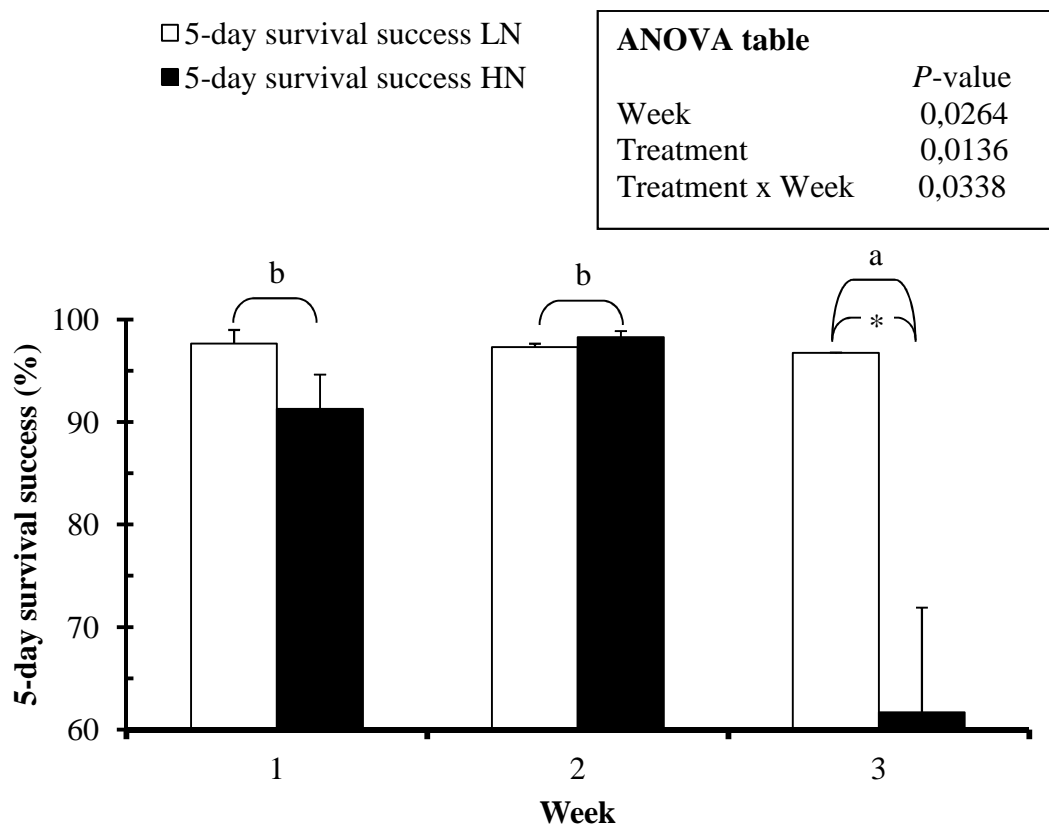


Figure 10. Mean (+ S.E.M.) values of embryo survival 1-day after collection success and hatching success of spawns from meagre broodstocks fed on different diets. No significant difference existed.

In the 5-day survival success there were significant differences between diet groups and among weeks after GnRHa treatment (Fig. 11). However, there was also a significant interaction between group and week ($P = 0.0338$), as mean 5-day survival success during the third week in the HN group was significantly lower compared with the LN group.

In the 7-day survival success there were significant differences among weeks and among groups, but there was not significant interaction between group and week. The mean 7-day survival success for the LN group was higher compared with the HN group, and the mean 7-day survival success during the third week was significantly lower compared with its values during the first and the second week (Fig. 11).



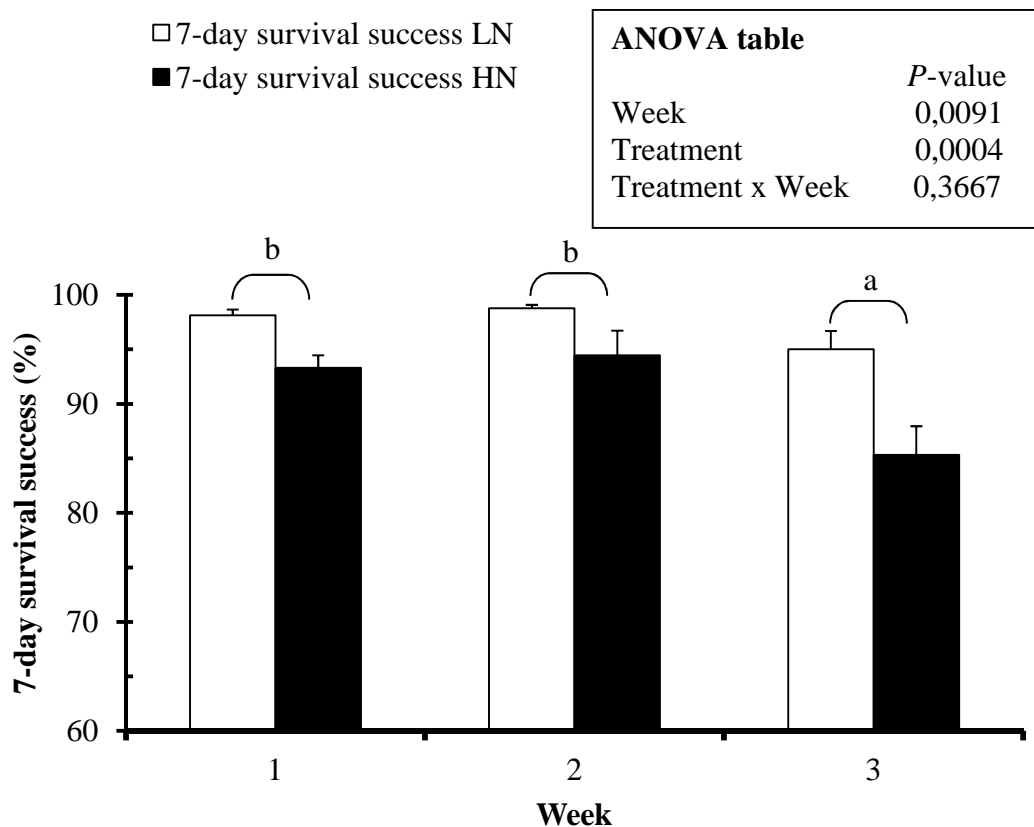


Figure 11. Mean (+ S.E.M.) values of 5-day survival and 7-day survival success of spawns from meagre broodstocks fed on different diets. Significant differences among sampling weeks of both treatments are indicated with different letter superscripts. The asterisk in the 5-day survival indicates a significant difference between the two diets on week 3 (Pair comparisons, $P < 0.002$).

Correlations between egg quality parameters

Regression analysis indicated that there was a significant correlation between relative fecundity and fertilization success, although the coefficient of correlation was rather low, due to the high variation of the data ($P = 0.0176$; $r^2 = 0.192$) (Fig. 12). The point at the extreme low range (75% fertilization, 4000 fecundity), corresponding at the eggs spawned on 2/06/2011, was subsequently removed from the analysis in order to see if the correlation was strong. Without this point the correlation among relative fecundity and fertilization success was still significant ($P = 0.0018$; $r^2 = 0.318$) so the point was maintained in the analysis.

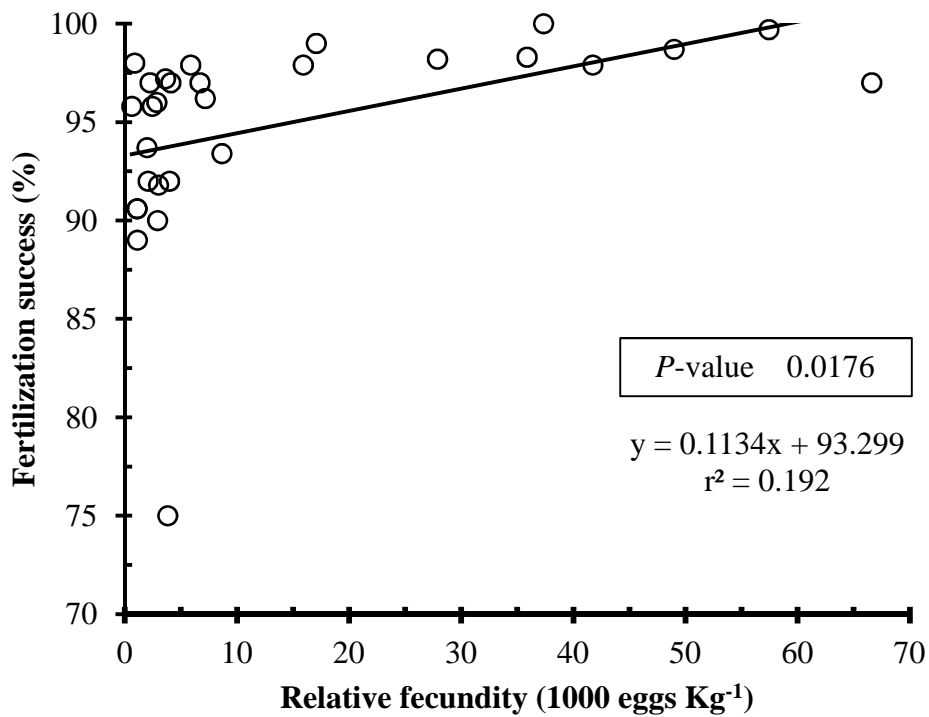
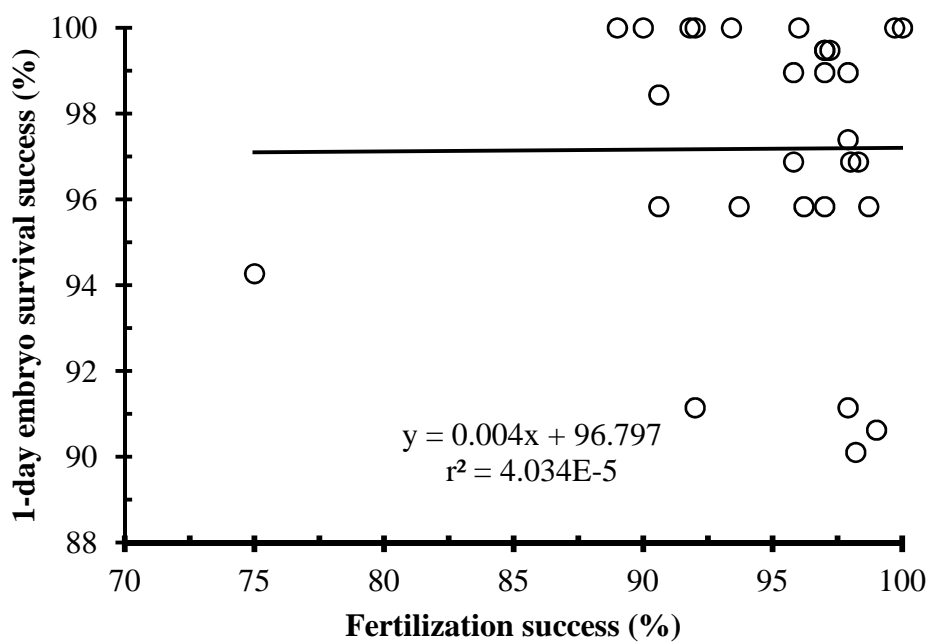


Figure 12. Linear correlation between relative fecundity and fertilization success of eggs obtained from meagre broodstocks during the 2011 reproductive season, induced to spawn with GnRH α .

There were no significant correlations in any of the parameters examined, with the exception of a significant correlation between the 7-day survival success and the 5-day survival success ($P = 0.0001$; $r^2 = 0.615$) (Fig. 13).



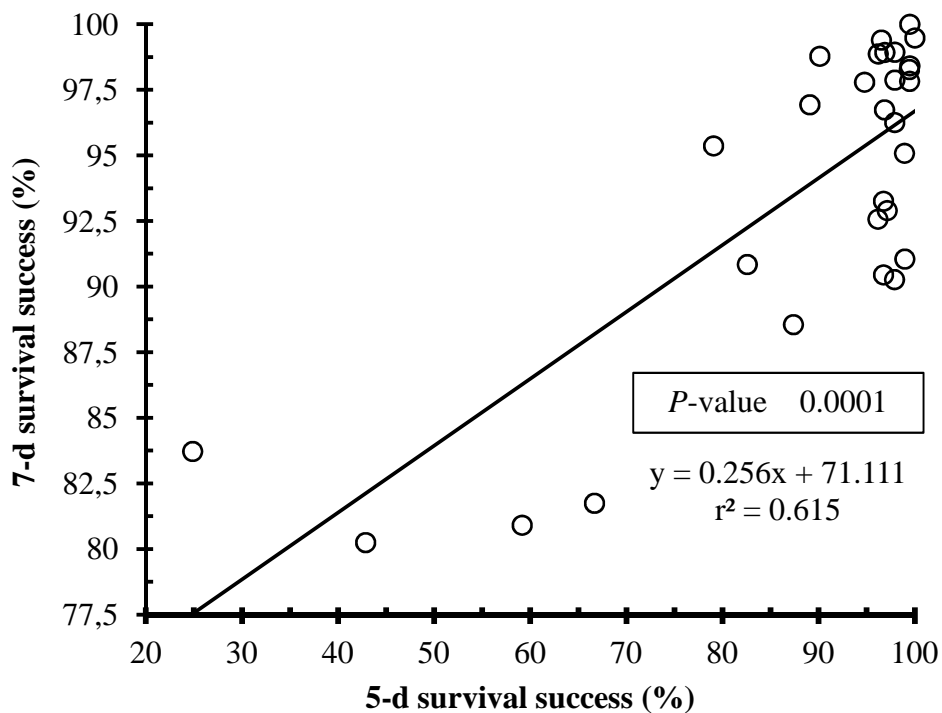
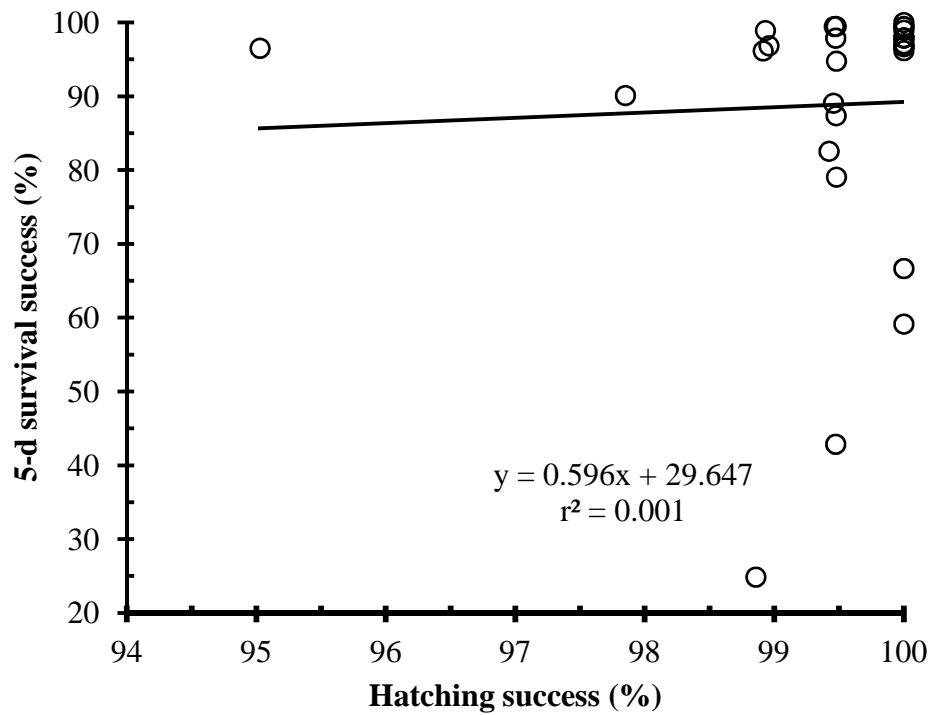


Figure 13. Linear correlation between various survival parameters (1-day embryo survival, hatching success, 5-day larval and 7-day larval survival) of eggs obtained from meagre broodstocks during the 2011 reproductive season, induced to spawn with GnRH α .

Discussion

Nutrition influences the egg quality in different ways. The lipid level, composition and source are considered the most important factors affecting the egg quality, but also the different content of protein, composition and source are important, since lipid and protein are the main components of egg yolk (Zakeri *et al.*, 2009). Moreover dietary lipid has a profound influence on the dietary protein requirement of fish, and the energy supplied from the catabolism of dietary protein or lipid sources supports growth and anabolism of fish (Zakeri *et al.*, 2009). A proper balance between dietary protein and lipid must be considered, since at inadequate energy level, dietary protein may be used as energy source; at adequate energy level, dietary protein can be spared for anabolic functions and at high protein concentration a proportion of this protein can be used as energy source (El-Sayed & Kawanna, 2008).

The HN diet used in this study had a higher dietary lipid level (18%) than the LN diet (15%). It had also a higher dietary protein level (59%) and a lower dietary fiber level (0.25%) than the LN diet (49% of protein; 1.6% of fiber). Hence, besides the influence of the dietary lipid level, the dietary protein level has also to be considered, as it may affect egg quality parameters. For example, in yellowfin sea bream (*Acanthopagrus latus*) it was found that the relative fecundity and the survival rate of 3 days post hatched larvae were affected significantly by both protein and lipid concentrations in the diet, whereas fertilization success and hatching success were not significantly affected. The best reproductive performance for yellowfin sea bream was achieved at 40% dietary protein and 20% dietary lipid concentrations, namely the lowest protein level and the intermediate lipid level (Zakeri *et al.*, 2009). But in the present study meagre broodstock of both the groups had comparable relative fecundity, 1-day embryo survival and hatching success. In these egg quality parameters no significant differences were found between the two dietary treatments. Hence, without considering the other egg quality parameters, these results seemed to indicate that there was no diet influence on egg quality of meagre broodstock.

Considering only the dietary protein level, a study on its effect on egg chemical composition in tilapia (*Oreochromis niloticus*) suggested that once a threshold dietary protein level is reached for the formation of post-vitellogenic oocytes, variations in the protein level would not affect the egg amino acid composition. This consideration is suggested since there were not any significant differences in the chemical composition of post-vitellogenic oocytes from different dietary regimes. The different treatments affected only puberty and maturation via dietary protein level effects on growth (Gunasekera *et al.*, 1995). So dietary protein level may not influence directly the egg quality, but undoubtedly dietary protein and lipid greatly influence in different ways the quality of the eggs produced from broodstock. However, a study on European sea bass established that artificial diets containing 51% of protein do not negatively affect egg quality (Cerdá *et al.*, 1994), confirmed also by another study on this species, where the reduction in egg quality was not attributed to diet protein, included in a concentration of 48-60% (Navas *et al.*, 1998). According to these researches, it might be assumed also in the present study that a dietary protein concentration of 49-59% achieves and exceeds the protein threshold required from meagre broodstock.

Considering now only the lipid, it was shown in a study on the effect of dietary lipid levels on growth of meagre juveniles that the best feed conversion ratio (FCR) was achieved by fish fed on the 17 and 21% lipid diets, while the 17% lipid diet achieved also the best protein efficiency ratio (PER) whereas the 13% lipid diet the worst, where PER is based on the wet weight gain of fish divided by its protein intake (all the diets had a 43% dietary protein level) (Chatzifotis *et al.*, 2010). Since fish fed the highest lipid diet had a reduced growth and PER, it may be that a difference in dietary lipid content of 3 percentage points does not cause significant differences in the relative fecundity, 1-day embryo survival and hatching success between the two dietary treatments of the present study. Significant differences in these egg quality parameters might be observed only with a greater difference in dietary lipid content between the two treatments, maintaining the same dietary protein level. If the difference in dietary lipid content does not result in a difference at least in one egg quality parameter, it means that include higher amount of lipid into the broodstock diet is useless, and it will lead to a reduced use of aquaculture byproducts and a greater saving for breeders.

On the other hand, in the present study fertilization success, 5-day and 7-day survival were significantly affected by the diets, but in a contradictory way. Fertilization success indicates that the high nutrient experimental diet led to a better egg quality than the low nutrient diet, since the mean daily fertilization success was higher in the HN group than the LN group. On the contrary 5-day and 7-day survival success indicated that the LN diet led to a better egg quality, since 5-day and 7-day survival success were higher in the LN diet than the HN diet during the third week.

The mean fertilization success is in agreement with previous studies, as a research on dietary lipid effects on black sea bass (*Centropristis striata*), where the mean fertilization success was significantly higher in the treatment with the highest total lipid concentration (32% of lipid content against 19% and 11% of the other two diets) (Bentley *et al.*, 2009). Beside the lipid levels, in the study on black sea bass it is important to consider that the diet with the highest amount of lipid was frozen Atlantic silversides (*Menidia menidia*), whereas the other treatments were two commercially prepared diets. Hence the proportion of n-3 series fatty acids and an overall dietary composition have to be take into account, because they were much better in the Atlantic silverside diet than in the other two diets, and they probably contributed to the higher fertilization success in the Atlantic silversides treatment (Bentley *et al.*, 2009).

As mentioned above, fertilization success was significantly different among weeks in both the treatments, but whereas in the HN group it was higher and with a gradually drop, in the LN group it was lower and without a drop from the value of the first week, through the second to the third ones. Indeed the fertilization success in the LN group was lower in the second week than the first and the third one. Also in the 5-day survival success of the HN group there was not a gradually drop. Indeed its value was higher in the second week than the first and third ones, and there was a severe drop during the third week. It could mean that fish had not enough time to absorb well and incorporate into the eggs the two broodstock diets, that were provided 3 months before the spawning period and throughout the reproductive season. Biochemical composition of organs involved

in reproduction are highly sensitive to the nutritional value of the diet, which affects egg quality, as demonstrated in gilthead sea bream (Harel *et al.*, 1994). As gilthead sea bream, meagre broodstock continue to eat during the reproductive and spawning season, so material deposited in the eggs probably originates from the diet provided. Whereas the expression of broodstock dietary changes in gilthead sea bream egg quality occurred within 15 days, meagre broodstock may need a much longer time period. Several studies reported administration of different treatments 3 months before the spawning season, for example in studies on the effects of dietary lipid on the spawning performance and egg quality of black sea bass (Bentley *et al.*, 2009), European sea bass (Navas *et al.*, 1998) and Japanese flounder (*Paralichthys olivaceus*) (Furuita *et al.*, 2002). But other studies reported that diets were supplied more than 3 months before the spawning time and throughout the reproductive season. For example in a research on the influence of nutritional composition of diet on egg quality of European sea bass the different diets were supplied 6 months before the reproductive season (Cerdá *et al.*, 1994), 4 months before in a study on the effects of protein and lipid concentrations in broodstock diets on egg quality of yellowfin sea bream (Zakeri *et al.*, 2009), 8 months before in a study on dietary lipid enhancement of egg quality in Atlantic halibut (*Hippoglossus hippoglossus*) (Mazorra *et al.*, 2003). A study on the impact of the lipid composition of broodstock diet on egg quality in the European sea bass observed that the administration of a high EFA diet during vitellogenesis (September-February) produced eggs of similar quality to those of the group fed the high EFA diet throughout the year. It demonstrated that the timing of the provision of dietary fatty acid is important and identified the period of vitellogenesis as the key time for the incorporation of essential fatty acids into the developing oocytes. Therefore the vitellogenic period appears to be the most influential time during which these important PUFA, especially DHA, are incorporated most effectively into the developing oocytes (Navas *et al.*, 1997). Summarizing, results from trials involving seasonal changes in ration indicate that there are “windows of opportunity” during which high and low feed rates produce significant effects on egg quality (Bromage, 1995). Thus, this consideration suggests further investigations also on the time period of broodstock diet’s administration prior to the reproductive season of meagre.

In the present study only the level of the lipid included in the treatments is known, whereas the types of lipids used is not noted. Since the two diets had different dietary nutrient levels but each nutrient had the same composition in both the treatments, the absolute amount of n-3 and n-6 series fatty acids were higher in the HN diet than the LN diet, whereas the n-3/n-6 ratio was the same in both the treatments. Generally, higher n-3/n-6 ratio lead to a better egg quality, since the n-3 series fatty acids include fatty acids such as DHA and EPA. The egg quality is directly related to their DHA content, since as mentioned before it is an important constituent of neural membranes, and a large fraction of the embryonic and larval body mass is constituted by the brain and the eyes. Marine fish are unable to desaturate and elongate short chain fatty acids, such as linoleic (18:2 n-6) and linolenic acids (18:3 n-3), to produce these long chain polyunsaturated fatty acids, since marine fish have a very restricted $\Delta 5$ and $\Delta 6$ desaturase and elongase activities, contrariwise to freshwater fish (Izquierdo, 2002; Sargent *et al.*, 2002). Therefore it is necessary to supply HUFA such as AA, DHA and EPA

through the diet (Navas *et al.*, 1998). It must be considered that not only the absolute dietary values for each of these EFA must be defined, but also optimum dietary ratios between them, since both factors will affect at least their incorporation into tissue lipids and hence membrane fluidity and function, the energy values obtained from their beta-oxidation and the production of metabolically active compounds (Izquierdo, 2002). Instead, regarding the protein to energy (P/E) ratio, it was higher in the HN diet. It is important, because, as mentioned above, the lipid has a sparing effect on the protein, so generally high lipid levels improve growth, feed and protein efficiency, especially the n-3 series fatty acids. Some studies reported that an excess of n-3 HUFA in broodstock diet has a negative effect on egg quality, for example in Japanese flounder (Furuita *et al.*, 2002). In another study on gilthead sea bream, four diets with different n-3 HUFA levels were administrated. Egg viability increased with the increase in n-3 HUFA level in broodstock diets, indicating the importance of the incorporation of these fatty acids into the eggs for the normal embryos development. On the other hand an excess of n-3 HUFA in the broodstock diet caused a reduction in larval survival at day 3 after hatching (Fernández-Palacios *et al.*, 1995). According to these results, it may be that the HN diet supplied in the present study had a too high level of lipid. This could explain the contradictory results of fertilization success, 5-day and 7-day survival success, since these last two parameters indicated that the LN diet led to a better egg quality. It could be that a dietary lipid level of 15% is better than a dietary lipid level of 18% to satisfy the requirements of meagre broodstock in order to produce eggs of good quality.

Looking to the lipid levels in the broodstock diets, the antioxidant substances levels included in the diets must be considered, because feeding very high levels of unsaturated lipid can increase the oxidative stress on fish consuming the diets. It is because the ease of peroxidation is proportional to the number of double bonds present. Thus, as the lipid content of the diet increases, the dietary n-3 HUFA levels also increase and the resulting increased unsaturation index of the diet must be balanced by an increasing dietary antioxidant content, especially vitamin E (Sargent *et al.*, 2002). Vitamin E is a strong antioxidant, since it protects long-chain polyunsaturated fatty acids, maintaining their integrity and their concentrations for important signaling events. As mentioned above an example of such a fatty acid is DHA, with 22-carbons and 6 double bonds. Since it is an omega-3 PUFA that is found in membrane phospholipids especially of the brain and the eyes, and they need high amount of antioxidant substances, the nervous system is a critical site harmed during vitamin E deficiency. Virtually all of the variation and scope of vitamin E's biological activity can be seen and understood in the light of protection of polyunsaturated fatty acids and the membrane qualities (fluidity, phase separation and lipid domains) that PUFA bring about (Traber & Atkinson, 2007). Both the dietary treatments had the same level of inclusion of vitamin E (800 mg/Kg). Hence it is possible that the vitamin E level included in the HN treatment is not enough in order to protect the lipid level. Furthermore the vitamin E level should be higher in the HN diet, since there is a higher amount of PUFA to protect from oxidation.

In summary, in general the process of diet formulation involves a difficult choice between two approaches that can be represented on the spectrum of feed cost. At one end of the spectrum is the approach of basing the formulation solely

on nutritional value, thereby producing a more expensive feed that is more productive. In the present study it may be represented by higher inclusion of dietary lipid level and vitamin E. The other end of the spectrum is the approach of basing the formulation solely on the total feed cost and attempting to minimize that cost. Usually, the type of feed being formulated will dictate which determinant, nutritional value or feed cost, is more important and the general goal is to support the highest performance at the lowest cost (Hardy & Barrows, 2002). It must also be considered that aquaculture must begin to be the most independent as possible from wild fisheries, since wild fisheries stocks are collapsing. For this reason in the present study the effect of two different dietary lipid levels is compared. The inclusion in the diet of higher lipid level is more expensive and the decision to incorporate higher amount of lipid is taken only if there is effectively a much higher performance of meagre broodstock corresponding to a higher egg quality.

The present study also considered the correlation between relative fecundity, fertilization success, hatching rate and survival at different stages, in order to find practical early indicators of larval quality. As mentioned before, the ability of the fertilized egg to develop successfully is often characterized by the embryonic survival at a specific stage (Bonnet *et al.*, 2007; Bobe & Labbé, 2010). Thus such quality criteria could be a useful predictive tool for potential viability of fish offspring obtained either in hatcheries or from wild fish (Kjørsvik *et al.*, 2003). The relative fecundity was significantly correlated to the fertilization success, but with a low coefficient of correlation. It is probable that a high relative fecundity will lead to a high fertilization success, but this consideration is not well supported, given the high variation of the data, and it is not so important for a hatchery, since this information does not predict the larval survival. A significant positive correlation was found between the 5-day and the 7-day survival success, indicating that the 5-day survival success may be applicable for egg quality assessment and it may be a useful predictive tool for evaluation of the potential juvenile production in meagre. This correlation suggest that within 5-day from egg collection may be taken the decision whether to carry on or abort the rearing of the larvae.

The present study introduces new information on the broodstock diet and the egg quality characteristics of meagre, on which few studies are present in the literature. It provides also further evidence of the suitability of meagre for reproduction in captivity. Such information is valuable for the proper organization of a commercial hatchery, specifically as it regards broodstock diet requirements, egg production plans, predictors of egg quality and larval survival. Further research on the reproduction and broodstock dietary requirements for the production of good egg quality of meagre in captivity should focus on the dietary lipid composition, the antioxidant level included in the diet, the protein to energy ratio, the time period of broodstock diet's administration prior to the reproductive season.

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