UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA
Instituto Universitario de Sanidad Animal
y Seguridad Alimentaria

MEJORA DEL BIENESTAR DEL LENGUADO SENEGALÉS (Solea senegalensis), MODULACIÓN NUTRICIONAL DE LA RESPUESTA DE ESTRÉS Y SISTEMA INMUNE

Vanesa Benítez Dorta

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Grupo de Investigación en Acuicultura

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Anexo I

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Anexo II

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

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A mis padres

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Resumen

Es de sobra conocido que el estrés y una dieta poco equilibrada inciden seriamente en el bienestar de los peces. En la actualidad se tiende a sustituir el aceite de pescado (FO) por otros aceites de origen vegetal (VO) en los piensos formulados, debido a su elevada demanda y baja disponibilidad en el mercado, lo cual incrementa drásticamente los costes de producción. Por ello, el objetivo que se planteó para el desarrollo de esta tesis fue evaluar el efecto de la utilización de aceite de lino (LO) y aceite de soja (SO) en la dieta sobre el bienestar y sistema inmune de juveniles de lenguado senegalés (*Solea senegalensis*). Así, en el Capítulo 3 se determinaron los efectos derivados de la sustitución total de FO por VO en la dieta, en términos de crecimiento, composición lipídica, perfil de ácidos grasos y expresión de genes relacionados con estrés, en hígado, músculo e intestino de juveniles de lenguado sometidos a un estrés por persecución, mientras que en el Capítulo 4 se estudió el efecto de un cambio de temperatura sobre el cortisol plasmático y la expresión de genes relacionados con la respuesta de estrés en hígado, intestino, músculo, branquias y cerebro. Y por último, en los Capítulos 5 y 6 se estudió la expresión de genes relacionados con el sistema inmune en intestino de juveniles de lenguado en estado basal y post estrés.

Los resultados obtenidos en el Capítulo 3 mostraron que el uso de SO o LO en la dieta del lenguado senegalés, es capaz de modificar los perfiles de ácidos grasos en músculo, hígado e intestino, e inducir una reducción de ácidos grasos poliinsaturados de cadena larga, mientras, la relación de ácido araquidónico/ácido eicosapentanoico (ARA/EPA) y los niveles de ácido docosahexaenoico (DHA) se mantienen constantes. A su vez, en el desarrollo de dicho estudio se secuenciaron por primera vez los receptores de glucocorticoides (GR), tipo 1 (GR1) y tipo 2 (GR2), para el lenguado senegalés. Y en relación a los resultados relativos a la expresión de genes de respuesta a estrés, en este capítulo se evidenció cómo el uso de VO en la dieta disminuyó la expresión relativa de la proteína de estrés térmico 70 (HSP70) en intestino, observándose a su vez un aumento en la expresión relativa de la proteína de estrés térmico 90 AA (HSP90AA) en el hígado de los lenguados alimentados con LO. También se observó un

incremento de la expresión relativa del GR1 en músculo y una disminución de la expresión relativa del GR2 en intestino, además de un incremento de la expresión relativa de la HSP70 en músculo y una disminución de la expresión relativa de la proteína de estrés térmico 90 AB (HSP90AB) en el hígado de los lenguados sometidos a estrés por persecución, independientemente del tipo de aceite dietético utilizado.

En el Capítulo 4 se mostró cómo un incremento gradual (1 hora) de 5ºC sobre la temperatura ambiental indujo un aumento de la expresión de los GRs y las HSPs en tejido hepático y cerebro de lenguado senegalés durante las primeras 24 horas. También se observó una elevación de la expresión del gen de proteína de unión a la hormona liberadora de corticotropina (CRHBP) en cerebro una semana después de comenzar el estrés térmico, además de un aumento de la relación HSP90/GR en hígado y branquias.

En relación a los genes correspondientes a la respuesta inmune y los receptores de eicosanoides descritos en los Capítulos 5 y 6, se observó que la sustitución total de FO por SO en dietas para el lenguado senegalés indujo un incremento de la expresión intestinal de genes relacionados con el sistema inmune; incluyendo interleuquinas, ciclooxigenasas, componentes de la vía clásica del complemento y de la respuesta viral y receptores "toll-like", en estado basal. También se observó que la sustitución total de FO por SO o LO en dietas para el lenguado senegalés indujo una disminución de la expresión intestinal del gen EP4. Mientras que el efecto combinado del uso del SO con un estrés agudo por persecución ocasionó una falta de respuesta generalizada en términos de cambios en la expresión de genes inmunes. Sin embargo, el efecto combinado del uso de LO con un estrés agudo por persecución, provocó un incremento de la expresión intestinal de genes relacionados con el sistema inmune y del gen EP4.

En resumen, esta tesis mostró que el uso de VO en la dieta de juveniles de lenguado senegalés modifica la expresión de genes relacionados con la respuesta a estrés. Esta información debe tomarse en cuenta para futuras formulaciones alimentarias en esta especie, y aunque se deban realizar más estudios para determinar el efecto del uso de VO sobre el sistema inmune del lenguado senegalés, en general, la sustitución total de FO por SO mostró un efecto

negativo sobre el estado inmunológico de los juveniles de lenguado, mientras que el uso de una dieta formulada con un 100 % de LO tendió a equilibrar este estado.

Abstract

It is well known that stress and an unbalanced diet seriously affect the welfare of several fish species. At present, there is increasing interest in replacing fish oil (FO) by vegetable oils (VO) in formulated feeds, due to its high demand and low availability in the market, which increases production costs dramatically. Therefore, the aim of this thesis was to evaluate the effect of different dietary (linseed oil, LO; soybean oil, SO) on welfare and immune system of juvenile Senegalese sole (*Solea senegalensis*). In Chapter 3, the effects of total substitution of FO by VO in the diet, in terms of growth, lipid composition, fatty acid profile and expression of stress-related genes, were determined in liver, muscle and intestine of Senegalese sole juveniles submitted to stress by persecution. In Chapter 4, the effect of temperature change was studied on plasma cortisol level and expression of genes related to stress response in liver, intestine, muscle, gills and brain. Finally, in Chapters 5 and 6, the expression of several genes related to the immune system in the gut of juvenile Senegalese sole baseline and post stress were studied.

The results obtained in Chapter 3 showed that the use of SO or LO in the diet of Senegal sole, affects the fatty acid profile in muscle, liver and intestine, inducing a reduction in long chain polyunsaturated fatty acids, while the ratio of arachidonic acid / eicosapentaenoic acid (ARA / EPA) and the levels of docosahexaenoic acid (DHA) remain constant. In this study, the glucocorticoid receptor (GR), type 1 (GR1) and type 2 (GR2), were sequenced for the first time for Senegalese sole. Regarding the stress genes expression, the use of VO in the diet decreased the relative expression of heat shock protein 70 (HSP70) in intestine and the full replacement of FO by LO increased the relative expression of heat shock protein 90 AA (HSP90AA) in liver. Also, Senegalese sole submitted to persecution stress showed, regardless of the type of dietary oil used, an increase of GR1 and HSP70 relative expression in muscle, a decrease of GR2 relative expression in intestine and a decrease of heat shock protein 90 AB (HSP90AB) relative expression in liver.

In Chapter 4, a gradual (in 1 hour) increase of 5 ° C in water temperature induced increasing expression of several genes related to stress: an increase of GRs and HSPs expression in liver and brain of Senegalese sole during the first 24 hours, the increase of the corticotropin releasing hormone binding protein (CRHBP) gene expression in brain one week after starting the heat stress and an increase in HSP90/GR ratio in liver.

Regarding to the immune response genes and eicosanoid receptors described in Chapters 5 and 6, the total replacement of FO by SO in Senegalese sole diet induce an increase of immune genes expression in intestine, including interleukins, cyclooxygenase, components of the classical complement pathway and viral response and "toll-like" receptors in basal state. Furthermore, it was also observed that the total substitution of FO by SO or LO in Senegalese sole diet induced a decrease in intestinal EP4 gene expression. The combined effect of an acute stress by persecution and the use of SO diet induced a general absence of response in terms of changes in expression of immune genes. However, the combined effect of LO diet with an acute persecution stress caused an intestinal increased expression of the immune system related genes and EP4 gene in gut.

As a conclusion, this thesis evidenced that the use of VO diets in juvenile Senegalese sole modifies the expression of genes related to stress response and this information must be taken into account in the formulation of feeds for this species. Although further studies are needed to fully understand the effect of VO on the immune system of Senegalese sole, in general, the total substitution of FO by SO showed a negative effect on the immune status of this species, while a diet with a 100 % of LO tends to balance this state.

Abreviaturas

100FO	Aceite de pescado como única fuente lipídica
100LO	Aceite de lino como única fuente lipídica
100SO	Aceite de soja como única fuente lipídica
ACTH	Hormona adrenocorticotropa
ALA	Ácido alfa-linolénico; 18:3 n-3
AMPc	Adenosín monofosfato cíclico
ANOVA	Análisis de varianza
ARA	Ácido araquidónico; 20:4 n-6
BHT	Butil hidroxitolueno
c1ql4	C1q-proteína 4
c2	Complemento C2/ Factor beta
c3	Complemento C3
c4a	Complemento C4-1
c4b	Complemento C4-2
c5	Complemento C5
c9	Complemento C9
CA	Catecolaminas
cck3	Quimioquina C-C_CK3
ccl19	Quimioquina CC 19
ccl20	Quimioquina CC 20
ccl25	Quimioquina CC 25
cd4	Antígeno de diferenciación CD 4
cd8a	Antígeno de diferenciación CD 8 Alfa
cd8b	Antígeno de diferenciación CD 8 Beta
cDNA	Ácido desoxirribonucleico complementario
cfh	Factor H del Complemento
COX	Ciclooxigenasa
COX1A	Ciclooxigenasa 1a
COX2	Ciclooxigenasa 2
CRH	Hormona liberadora de la corticotropina
CRHBP	Proteína de unión al CRH
cxcl10	Quimioquina CXC 10

cxcl13	Quimioquina CXC 13
cxcr2	Receptores CXC 2
DHA	Ácido docosahexaenoico; 22:6 n-3
DHGLA	Ácido dihomo-υ-linolénico; 20:3 n-6
DPA n-6	Ácido n-6 docosapentaenoico, 22:5 n-6
DPA	Ácido docosapentaenoico 22-5 n-3
ED50	Concentración nanomolar en el que la respuesta es la mitad del máximo
eef1a1	Factor de elongación Eucariótico 1A1
EFA	Ácidos grasos esenciales
Elovl	Ácido graso elongasa
EP	Receptor de eicosanoides
EPA	Ácido eicosapentanoico; 20:5 n-3
ER	Retículo endoplasmático
F	Cebador forward
Fad	Ácido graso desaturasa
FAME	Ésteres metílicos de los ácidos grasos
FCR	Tasa de conversión alimenticia
FID	Detector ionizador de llama
FO	Aceite de pescado
gapdh2	Gliceraldehído-3-fosfato deshidrogenasa 2
GAS	Síndrome general de adaptación
GC	Glucocorticoides
GR	Receptor de glucocorticoides
GR1	Receptor de glucocorticoides 1
GR2	Receptor de glucocorticoides 2
GRE	Elemento de respuesta a glucocorticoides
HPI	Hipotálamo-Pituitario-Interrenal
HSC	Hipotálamo-Simpático-Cromafín
HSP	Proteínas de choque térmico
HSP70	Proteínas de choque térmico 70
HSP90AA	Proteínas de choque térmico 90AA
HSP90AB	Proteínas de choque térmico 90AB
HUFA	Ácidos grasos altamente insaturados
ICCM	Instituto Canario de Ciencias Marinas

IFAPA	Instituto de Investigación y Formación Agraria y Pesquera
ifnc	Interferon C
il10	Interleuquina 10
il10rb	Receptor beta de la interleuquina 10
il11a	Interleuquina 11 alfa
il12b	Interleuquina 12 beta
il15	Interleuquina 15
il15ra	Receptor alfa de la interleuquina 15
il17c	Interleuquina 17 c
il17ra	Receptor alfa de la interleuquina 17
il1b	Interleuquina 1 beta
il1rl1	Receptor 1 de la interleuquina 1
il6	Interleuquina 6
il8b	Interleuquina 8 tipo b
il8c	Interleuquina 8 tipo c
irf1	Factor regulador de interferón 1
irf10	Factor regulador de interferón 10
irf2	Factor regulador de interferón 2
irf3	Factor regulador de interferón 3
irf7	Factor regulador de interferón 7
irf8	Factor regulador de interferón 8
irf9	Factor regulador de interferón 9
IUSA	Instituto Universitario de Sanidad Animal y Seguridad Alimentaria
JACUMAR	Junta Asesora de Cultivos Marinos
LA	Ácido linoleico; 18:2 n-6
LC-PUFA	Ácidos grasos poliinsaturados de cadena larga
LO	Aceite de lino
LOX	Lipoxigenasa
LPS	Lipopolisacáridos
LTB4	Leucotrieno B4
LTB5	Leucotrieno B5
lyc1	Lisozima C 1
lyc2	Lisozima C 2
lyg1	Lisozima G 1

lyg2	Lisozima G 2
lyg3	Lisozima G 3
MANOVA	Análisis multivariante de la varianza
MAPK	Proteín quinasas activadas por mitógenos
MF	Fragmento múltiple heurístico
MR	Receptor de mineralcorticoides
MSH	Hormonas estimulantes de los melanocitos
mx	Proteina mx inducida por interferón
MyD88	Factor de diferenciación mieloide 88
n-3	Ácido graso omega 3
n-6	Ácido graso omega 6
NF-kB	Factor de nuclear kappa B
NPO	Área preóptica hipotalámica
PAMP	Patrones moleculares asociados a patógenos
PGE_2	Prostaglandina E2
PGES	Prostaglandinas sintetasas
pglyrp1	Proteína de reconocimiento de péptidoglicano 1
PKA	Proteina quinasa dependiente de AMPc
PLA	Fosfolipasa A
PLA2	Fosfolipasa A2
POMC	Propiomelanocortina
PUFA	Ácidos grasos poliinsaturados
R	Cebador reverse
RIA	Radio inmuno ensayo
RNA	Ácido ribonucleico
SD	Desviación estándar
SDA	Ácido estearidónico; 18:4 n-3
SGR	Tasa de crecimiento específico
SNC	Sistema nervioso central
SO	Aceites de soja
sPLA2	Fosfolipasa A2 secretora
TGF- β	Factor de crecimiento transformante beta
tlr1	Receptor de células Toll 1
tlr5s	Receptor de células Toll 5 S

tlr8b	Receptor de células Toll 8 B
tlr9	Receptor de células Toll 9
tnfa	Factor de necrosis tumoral alfa
TRH	Hormona liberadora de tirotropina
TXB2	Tromboxano B2
ubi	Ubiquitina
ULPGC	Universidad de las Palmas de Gran Canaria
VO	Aceites vegetales
Δ6D	Delta-6 desaturasa

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INTRODUCCIÓN

Figura 1.1: Principales factores que afectan la salud y el bienestar de los peces y su interrelación con las condiciones ambientales. 1
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CAPÍTULO 1

INTRODUCCIÓN

1. Introducción

1.1 Nutrición, bienestar y salud en peces

El estrés y una dieta poco equilibrada inciden seriamente en el bienestar de los peces, provocando un aumento en los costes de producción (Conte, 2004). El bienestar de un animal se puede valorar a través de factores vitales susceptibles de ser afectados por el estrés (Fisheries Society of the British Isles [FSBI], 2002) (Fig. 1.1); privación de agua o alimentos y malnutrición (I), cambios medioambientales (II), enfermedades, lesiones y deterioro funcional (III), comportamiento y restricciones interactivas (IV) y sufrimiento físico y mental (V) (Mellor y Stafford, 2001).

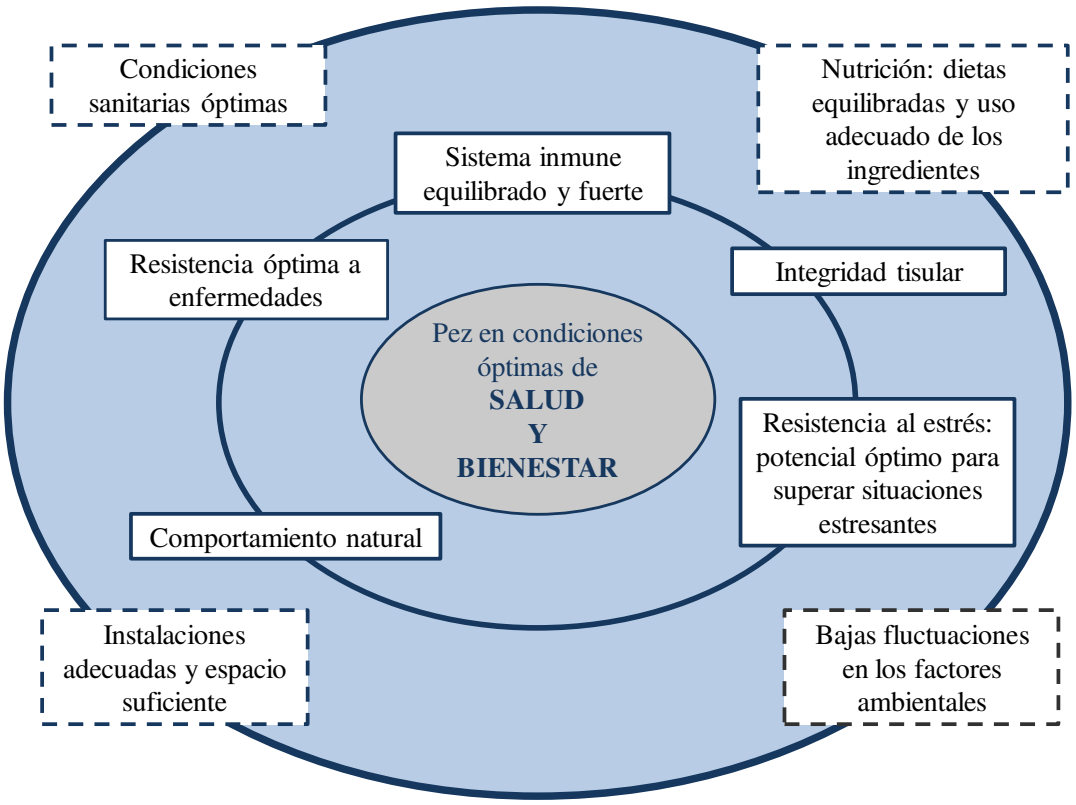

Figura 1.1: Principales factores que afectan la salud y el bienestar de los peces y su interrelación con las condiciones ambientales. Figura extraída de Montero e Izquierdo (2010).

Se requiere de un gran esfuerzo para entender las necesidades nutricionales de cada especie (FSBI, 2002), no sólo en términos de crecimiento y eficiencia alimenticia, sino también en términos de bienestar y salud (Rosenberg, 1994). En este sentido, la falta de suministro de aceites y harina de pescado a nivel mundial ha provocado la aparición de varias iniciativas donde se investiga la posibilidad de utilizar las proteínas y aceites vegetales como alternativa a las proteínas y aceites derivados de la pesca marina (Tacon y Meitan, 2008). El único inconveniente es la incapacidad que poseen los peces marinos para convertir ácidos grasos poliinsaturados (PUFA) de 18C en ácidos grasos altamente insaturados (HUFA) de 20C o 22C ya que éstos carecen o tienen reducida la expresión de la enzima delta-5 desaturasa (Mourente y Tocher, 1993) o presentan una capacidad limitada para elongar ácidos grasos de estas características (Ghioni *et al*., 1999).

Además del suministro directo de ácidos grasos esenciales (EFA) en la dieta de los peces marinos también se hace preciso establecer un balance adecuado de ácidos grasos omega 3 (n-3)/omega 6 (n-6) que se reflejará en la salud del pez (Simopoulos, 2008) a través de la función y la estructura de las células inmunitarias, la señalización celular, la producción de eicosanoides e incluso la respuesta inmune como un todo que depende directamente de la función y eficacia de los ácidos grasos (Calder, 2006; Yaqoob y Calder, 2007).

1.2 Consideraciones generales del estrés en peces

Con el fin de satisfacer la creciente demanda de proteína animal la acuicultura se apoya continuamente en nuevas técnicas para aumentar el rendimiento de la producción. Sin embargo, cuanto más se intensifican estas prácticas, mayor es el nivel de estrés en el animal, definiéndose dicho estado como la condición en la cual el equilibrio dinámico del organismo animal u homeostasis se perturba como resultado de una situación intrínseca o extrínseca denominada factor estresante (Chrousos y Gold, 1992).

En cultivo intensivo se debe considerar la existencia de agentes estresantes inherentes a la biología del propio animal practicándose unas correctas condiciones de cultivo (revisado por Dinis *et al*., 1999; Salas-Leiton *et al*., 2010), manejo y manipulación (Costas *et al*., 2011) y una alimentación adecuada (Guerreiro *et al*., 2012).

Los principales órganos implicados en la respuesta al estrés en peces son los ejes neuroendocrinos involucrados en la captación y utilización de oxígeno, la movilización de reservas energéticas, la re-administración de energía utilizada para el crecimiento y la reproducción, y la supresión de las funciones inmunes (revisado por Barandica y Tort, 2008).

En peces se observa una íntima comunicación entre los sistemas nervioso, endocrino e inmune (Tort, 2011). En mamíferos esta relación se debe principalmente a que las señales moleculares sintetizadas por células y tejidos de estos tres sistemas son similares y en algún caso idénticas (Turnbull y Rivier, 1999). Esta estrecha interrelación es fundamental entre otras cosas para hacer frente de manera coordinada y eficaz a las situaciones de estrés (Engelsma *et al*., 2002). El conjunto de cambios observados en estos tres sistemas orgánicos así como la respuesta a una situación de estrés se conoce genéricamente como síndrome general de adaptación (GAS) o ley de Seyle.

Ante situaciones de estrés entre peces y mamíferos existe una gran similitud a la hora de actuar aunque se hallan diferencias en cuanto a los órganos implicados y el tiempo de respuesta, siendo éste último un factor importante para la recuperación de la homeostasis. El GAS se compone de distintas etapas según la duración del agente estresante (Roberts, 1981; Maule *et al*., 1989; Anderson, 1990; Wedemeyer *et al*., 1990; Barton, 1997):

- La 1ª fase o de alarma se define como el intento de huida por parte del animal. Es una respuesta a nivel neuroendocrino donde se induce la liberación de catecolaminas (CAs) (adrenalina y noradrenalina) y cortisol al torrente sanguíneo.

- La 2ª fase o de resistencia sucede cuando el estrés persiste y el animal trata de adaptarse., disminuyéndose los niveles de CA y liberándose cortisol a nivel sanguíneo y tisular.
- La 3ª fase o de agotamiento aparece cuando el estrés persiste en el tiempo y tanto los niveles de cortisol como el metabolismo se encuentran aumentados interfiriendo así en procesos fisiológicos que pueden acabar con la muerte del animal.

1.2.1 Ejes neuroendocrinos del estrés

Los peces no poseen una glándula adrenal como tal sino un conjunto difuso de células interrenales situadas en el riñón anterior, debido a lo cual, el eje principal de respuesta endocrina a estrés se denomina eje hipotálamo-pituitario-interrenal (HPI). En peces se observa una clara interacción entre los estímulos derivados del estrés y las funciones de las células del eje HPI (Weyts *et al*., 2001), además de existir un segundo eje fisiológico llamado eje hipotálamo-simpático-cromafín (HSC) también implicado en la respuesta a los factores estresantes. Los factores estresantes se perciben inicialmente en el hipotálamo a través de los sensores del sistema nervioso central (SNC) donde ambos ejes son estimulados (Weyts *et al*., 2001). Las fibras colinérgicas del sistema simpático y un leve aumento tardío (retraso en minutos) de la liberación de las hormonas glucocorticoides (GCs) consiguen que se estimule la síntesis y liberación de las CAs en la circulación a través del eje HSC (Fig. 1.2). En peces teleósteos el principal origen de las CAs circulantes son las células cromafines dispuestas en la pared de la vena cardinal posterior del riñón anterior (revisado por Nilsson, 1984; Reid *et al*., 2001). Dichas células se encargan de activar la respuesta cardiovascular, respiratoria y metabólica con el fín de aliviar los efectos perjudiciales asociados con los factores estresantes de caracter agudo (Perry *et al*., 2004). La hormona liberadora de la corticotropina (CRH) se considera la primera hormona liberada por las neuronas hipotalámicas de la región preóptica del

eje HPI, encuentrándose en peces junto a la hormona liberadora de tirotropina (TRH) (Pepels *et al*., 2002). Con un aumento de la CRH se provoca la liberación de otras hormonas como las endorfinas y los péptidos derivados de la propiomelanocortina (POMC) (Matteri y Becker, 2004), siendo las células corticotropas y melanotropas las encargadas de secretar hormona adrenocorticotropa (ACTH), hormonas estimulantes de los melanocitos (MSH) y endorfinas. La liberación de la ACTH desde la pituitaria se estimula a partir de la TRH y la CRH para inducir la producción y liberación del cortisol a través de las células interrenales (Pickering *et al*., 1991; Wendelaar-Bonga, 1997; Mommsen *et al*., 1999; Pepels *et al*., 2002).

En peces teleósteos se considera que los glucocorticoides median la respuesta al estrés (Fuller *et al*., 2000) mientras que los mineralcorticoides, predominantemente la aldosterona, regulan la presión sanguínea principalmente controlando la retención de sodio por el riñón. Los efectos de ambas clases de corticosteroides están mediados por dos receptores intracelulares, el receptor de mineralcorticoides (MR) y el receptor de glucocorticoides (GR), con cierto parecido farmacológico entre ellos (Greenwood *et al*., 2003) y a los que el cortisol se une e induce actividad transcripcional, siendo dicha actividad mucho mayor en el GR que en el MR (Ducouret *et al*., 1995).

La mayoría de los peces posee un MR y dos isoformas de GR (GR1 y GR2). El GR2 parece ser más sensible a los bajos niveles de cortisol plasmático mientras que el GR1 es sensible sólo ante niveles muy altos de cortisol, como los acontecidos bajo un estrés agudo (Stolte *et al*., 2008).

Existe cierta discrepancia entorno a la acción que ejerce el cortisol en la regulación del GR, mientras que algunos estudios muestras que estos receptores se regulan a la baja (Pottinger y Pickering, 1990; Maule y Schreck, 1991; Pottinger *et al*., 1994; Shrimpton y Randall, 1994; Shrimpton, 1996; Shrimpton y McCormick, 1998; Shrimpton y McCormick, 1999) otros trabajos como los realizados por Sathiyaa y Vijayan (2003) muestran como sucede al contrario. En estos últimos se sugiere la existencia de una retroalimentación negativa entre el contenido de RNAm del GR y su expresión proteica, se podría decir, tal y como se sugiere en estudios

realizados por Terova y colaboradores (2005), que el efecto del estrés sobre el RNAm del GR puede ser o bien específico de la especie y/o en función del tipo, la intensidad y la duración del factor estresante, formando parte fundamental del sistema de retroalimentación entre el SNC y el cortisol.

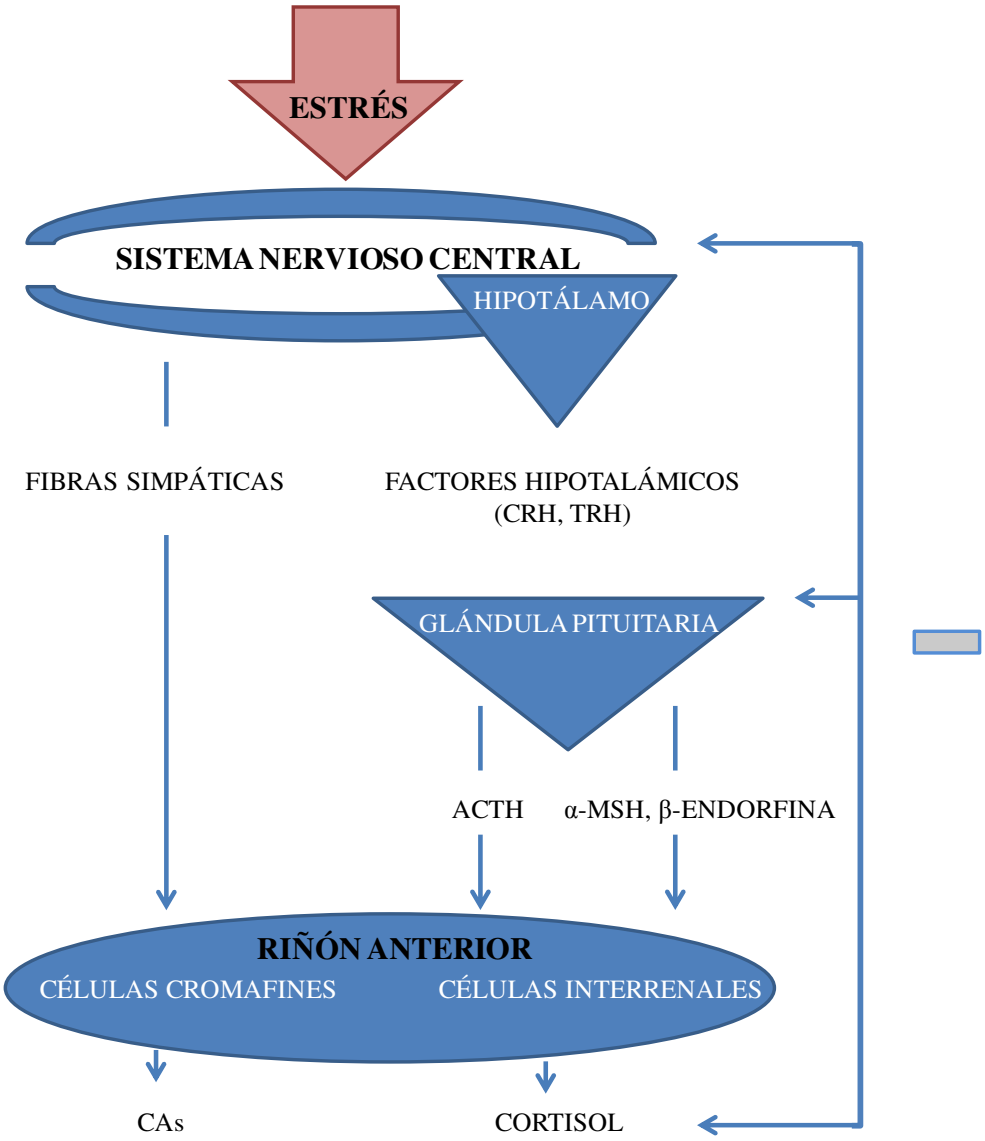

Figura 1.2: Principales elementos neuroendocrinos implicados en la respuesta al estrés en peces representados por; el eje hipotálamo-símpatico-cromafín (HSC), productor de catecolaminas (CAs) y el eje hipotálamo-pituitaria-interrenal (HPI), productor de cortisol. Figura extraída de Wendelaar-Bonga (1997).

1.2.2 Indicadores de estrés

1.2.2.1 Cortisol

El cortisol se identificó como la principal hormona corticosteroide en peces hace más de 30 años (Donaldson, 1981), considerándose la aldosterona como una hormona mineralocorticoide que sólo se detecta en cantidades muy pequeñas y carece de importancia fisiológica (Bern, 1967; Sangalang, 1994), y estableciéndose, como creencia general, que el cortisol actúa de igual manera como glucocorticoide o mineralocorticoide en peces teleósteos (Bern y Madsen, 1992; Wendelaar-Bonga, 1997; Mommsen *et al*., 1999; Stolte *et al*., 2008).

El cortisol se sintetiza a partir del colesterol en las células interrenales del riñón anterior una vez éstas han sido estimuladas por la cascada de hormonal del eje HPI; es fácilmente medible en la respuesta primaria al estrés (neuroendocrina) y se cree que el control endocrino de su secreción se lleva a cabo principalmente por la POMC derivada de la ACTH (Sumpter *et al*., 1986; Wendelaar-Bonga, 1997). En este sentido, la cascada de cortisol en peces es similar a la acontecida en mamíferos y otros grandes vertebrados, excepto por dos diferencias: la CRH se traslada desde el hipotálamo hasta la pituitaria por contacto neural directo en vez de por sangre y porque el tejido interrenal es el encargado de producir el cortisol en lugar de la glándula adrenal (Sumpter, 1997).

El cortisol, como corticosteroide mayoritario en peces, es poseedor de importantes funciones osmorreguladoras, metabólicas e inmunes (Wendelaar-Bonga, 1997; Mommsen *et al*., 1999). Se puede afirmar que está involucrado de manera activa en una respuesta natural y adaptativa acontecida tras un estrés (Ellis *et al*., 2012). La concentración plasmática de cortisol se utiliza como indicador de estrés en múltiples estudios sobre la capacidad y resistencia de los peces ante situaciones que perturban su equilibrio homeostático:

- Cambios de temperatura (Foss *et al*., 2012)

- Concentración de oxígeno en el agua (Salas-Leiton *et al*., 2010; Castanheira *et al*., 2011; Li *et al*., 2011; Cook y Herbert, 2012)
- Presencia de agentes químicos en el agua (Oliva-Teles *et al*., 2012)
- Estresantes sociales (Sørensen *et al*., 2012)
- Señales de alarma procedecentes de peces estresados (Moreira y Volpato, 2004)
- Agentes biológicos (Salas-Leiton *et al*., 2012)
- Altas densidades de cultivo (Costas *et al*., 2008; Li *et al*., 2012).
- Estrés ocasionado por manejo (Foss *et al*., 2012)

1.2.2.2 Otros indicadores de estrés

Además del cortisol, también se conocen otros marcadores hormonales del estrés:

- CRH y ACTH: se sitúan al final de la cascada HPI junto al cortisol y actúan como precursores hormonales involucrados en la respuesta al estrés. La CRH interviene en tejidos diferentes de la pituitaria y también puede activar mensajeros inmunes tales como las citoquinas e inducir acciones secundarias sin el cortisol (Verburg-Van Kemenade *et al*., 2009).
- Cortisona: otro potente corticosteroide que en ocasiones se encuentra a niveles mayores que el cortisol en sangre (Pottinger y Moran, 1993).
- CAs (adrenalina y noradrenalina) (Mazeaud y Mazeaud, 1981): se liberan en las células cromafines y, de manera similar, en la vena post-cardinal y sus ramificaciones (Grassi-Milano *et al*., 1997), siendo la principal diferencia respecto al cortisol, que la liberación de CAs es desencadenada por circuitos nerviosos (en lugar de por una cascada de mensajeros químicos) y, por lo tanto, acontece de manera inmediata. Esta circunstancia hace prácticamente imposible obtener una concentración basal en la sangre sin que las CAs se encuentren

afectadas por el propio muestreo. Por tanto, las CAs en los peces han recibido mucha menos atención que el cortisol pero poseen un mayor mérito en su investigación (Pottinger, 2010).

1.2.3 Reguladores fisiológicos del cortisol

1.2.3.1 Receptores de glucocorticoides

Los efectos del cortisol en la célula se regulan a través del GR, que es miembro de una gran familia de receptores nucleares y actúa como ligando dependiente de factores de transcripción, controlando y regulando la expresión génica (Mommsen *et al*., 1999). En el citosol, el GR se encuentra en forma no activada dentro de un complejo multiproteico (Fig. 1.3), junto con varias proteínas de choque térmico (HSPs) tales como la HSP70 y 90, cuyas funciones son el ensamblaje, la funcionalidad y el transporte de los recursos genéticos (Pratt y Toft, 1997). Las HSPs se encuentran asociadas al GR hasta que aparece una señal hormonal, como el cortisol, que induce un cambio en este último, resultando en una conformación con menor afinidad por las HSPs. Esta acción permite que el GR se disocie y se oriente a los sitios de activación de transcripción, siendo el receptor capaz de translocarse en el núcleo y unirse a la región específica de ADN, el elemento de respuesta a glucocorticoides (GRE), donde se regula la transcripción de los genes en respuesta a los glucocorticoides (Vijayan *et al*., 2005; Aluru y Vijayan, 2009).

La caracterización molecular del GR en peces se inició con una clonación realizada en trucha arcoíris, *Oncorhynchus mykiss* (Ducouret *et al*., 1995). Desde entonces, la caracterización molecular de este gen se lleva a cabo en varias especies de peces como la tilapia, *Oreochromis mossambicus* (Tagawa *et al*., 1997), el falso halibut del Japón, *Paralichthys olivaceus* (Tokuda *et al*., 2005) y la dorada, *Sparus aurata* (Acerete *et al*., 2007); demostrándose la presencia de

una segunda isoforma del GR en diferentes estudios realizados en trucha arcoíris (Bury *et al*., 2003) y Astatotilapia Burtoni, *Haplochromis burtoni* (Greenwood *et al*., 2003).

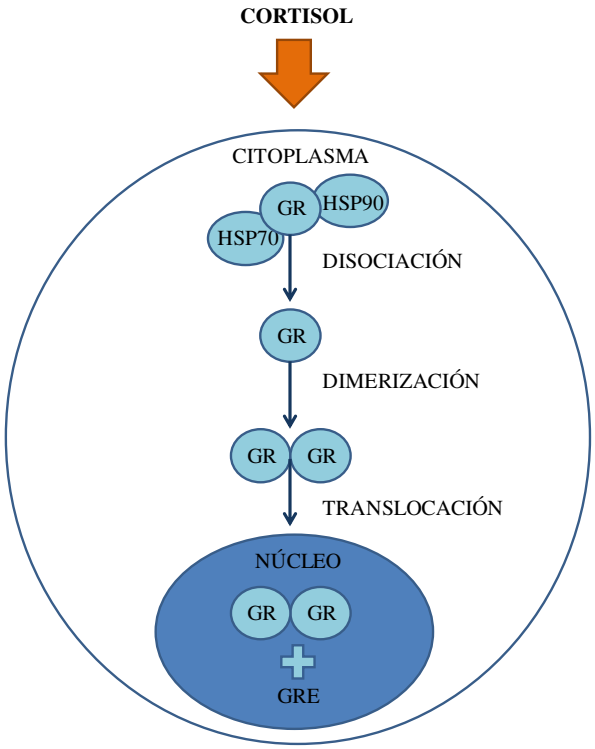

Figura 1.3: Activación del GR por medio del cortisol

Los análisis funcionales realizados en ambos GRs de trucha arcoíris han mostrado que el GR2 se caracteriza por una mayor sensibilidad al cortisol en comparación con el GR1. Si se comparan los niveles de cortisol ED50 (concentración nanomolar en el que la respuesta es la mitad del máximo) para cada una de las formas del GR en líneas celulares transformadas (Bury *et al*., 2003) y, la concentración de cortisol circulante en peces (Wendelaar-Bonga, 1997) (Fig. 1.4), se obtiene que los niveles plasmáticos para ambos GRs están por encima de los valores de cortisol ED50 en peces estresados. En peces no estresados, los niveles para el GR1 se sitúan por debajo de los del cortisol ED50, mientras los del GR2 se sitúan por encima, lo que indicaría que el cortisol podría no unirse al GR1 en condiciones basales o de estrés leve, y que tanto el GR1 como el GR2 se pueden movilizar en condiciones de estrés.

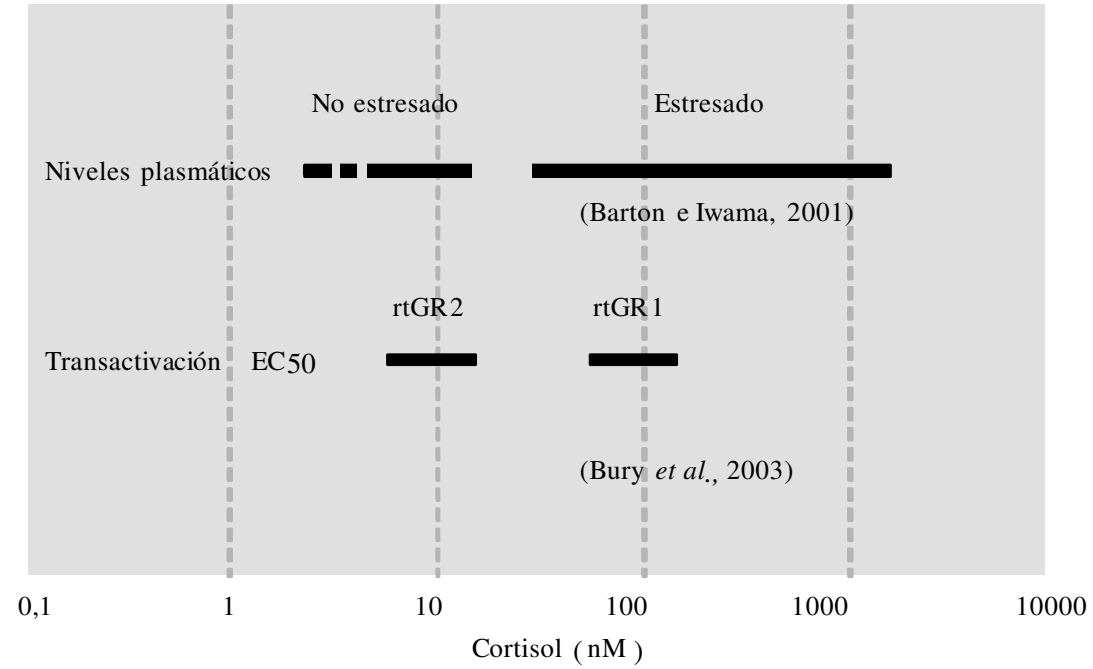

Figura 1.4: Sensibilidad de los receptores de glucocorticoides 1 y 2 (rtGR1 y rtGR2) frente al cortisol en comparación con los niveles de cortisol en plasma reportados en salmónidos. ED50 es la concentración nanomolar en la que la respuesta es la mitad del máximo. Figura extraída de Prunet y colaboradores (2006).

Además, diversos estudios han comprobado que tanto el tipo como la intensidad con la que se presenta el estrés, pueden afectar a la expresión de los GRs en los diferentes tejidos (Greenwood *et al*., 2003). Por ejemplo, a través de ellos el cortisol promueve la adaptación de los teleósteos frente a cambios de salinidad en órganos osmorreguladores como las branquias y el tracto gastrointestinal (Takahashi y Sakamoto, 2012). Tras inyecciones de lipopolisacáridos (LPS) en dorada se ha observado, también, que la expresión del GR en intestino y riñón aumenta 6 h y 12 h post infección, respectivamente (Acerete *et al.,* 2007). Esto quizás se deba a que dichos órganos están involucrados en la respuesta inmune innata en peces (Tort *et al.,* 2004).

1.2.3.2 Proteínas de choque térmico

Tanto en mamíferos como en peces se señala a las HSPs como indicadores de estrés más rápidos que el GR, y pertenecientes a una familia de proteínas de bajo peso molecular

(Abukhalaf *et al*., 1994); siendo su respuesta variable en función del tejido, tipo de HSPs, organismo, estado de desarrollo y agente estresante (Iwama *et al*., 2004). Estas proteínas son esenciales para la activación de los receptores hormonales del núcleo e interacción con las señales moleculares del ciclo de vida y muerte celular (Harris y Bird, 2000).

En estado normal, las HSPs 70 y 90 se encuentran unidas al GR, y una vez activadas, juegan un papel fundamental en el plegado y ensamblaje de otras proteínas celulares; implicándose en la regulación del sistema inmunitario, la apoptosis y los procesos inflamatorios (revisado por Roberts *et al*., 2010). También desarrollan un papel relevante en la termotolerancia (Feige *et al*., 1996), promueven la supervivencia de los organismos sometidos a patógenos crónicos y neoplasias (revisado por Roberts *et al*., 2010), reaccionan ante agentes contaminantes, cambios bruscos medioambientales o acciones propias del manejo (Vijayan *et al*., 1997, 1998; Manchado *et al*., 2008).

Paradójicamente, en el daño celular se ocasionan dos respuestas contrapuestas: la apoptosis, que se define como un tipo de muerte celular donde se reparan los daños celulares evitándose la inflamación; y la respuesta a estrés, en la cual se previenen los daños celulares o se facilita la recuperación de la célula. El destino de una célula se determina a partir del tipo de interacción entre estas dos vías, ocasionándose un efecto profundo sobre las consecuencias biológicas del estrés. La apoptosis se puede medir por medio de factores internos como los niveles elevados de cortisol circulante (Bury *et al*., 1998; Laing *et al*., 2001). Así, en estudios *in vitro* realizados en trucha arcoíris, se observa que estas mismas acciones podrían estar alentadas por el GR (Van der Salm *et al*., 2002). Al mismo tiempo, la capacidad de liberación de glucosa por estimulación del cortisol es suprimida en aquellas células donde las HSP70 se encuentran acumuladas. Esta disminución de su capacidad de reacción se podría correlacionar con la baja expresión del GR (Boone *et al.,* 2002). Con un aumento en la expresión de las HSP70 se puede llegar a bloquear la apoptosis a través de la inhibición de las proteínas caspasas (Beere, 2004) y re-naturalizar las proteínas dañadas antes de que la célula tome la decisión de suicidarse o no a través de un proceso apoptótico.

En el citosol, el GR se encuentra de manera inactiva formando un complejo multiproteico junto con varias HSPs como la 70 y la 90, cuyas funciones incluyen el ensamblaje, la funcionalidad y el transporte de recursos génicos (Pratt y Toft, 1997), jugando un papel importante en el proceso para adquirir termotolerancia (Kregel, 2002; Fangue *et al*., 2006).

En concreto, la HSP70 es esencial en el ensamblaje y mantenimiento de este complejo (Pratt y Welsh, 1994; Hutchison *et al*., 1994). Por otra parte, estudios realizados por Whitesell y Cook (1996) sugieren que la unión entre la HSP90 y el GR estabiliza el complejo multiproteico tras una degradación proteolítica, apoyándose esta teoría en estudios posteriores realizados en ratas, donde se examinaron los efectos del cadmio sobre la asociación de la HSP90 con el GR (Dundjerski *et al*., 2000).

El GR se puede unir a hormonas en ausencia de HSPs, pero existen evidencias de que estas últimas aumentan la capacidad de unión al receptor de esteroides, facilitando la translocación nuclear en el complejo receptor e incrementando su vida media (Pratt y Welsh, 1994; Czar *et al*., 1997; Smith *et al*., 1998).

En análisis realizados sobre tejido hepático procedente de truchas arcoíris hipercortisolémicas se demostró que los niveles de HSP70 libres se encuentran disminuidos mientras los niveles de HSP70 unidos a GR se incrementan tras la exposición a un shock térmico; concluyéndose que, probablemente, la capacidad del cortisol o el estrés para promover la asociación de las HSP70 con el GR actúe como un mecanismo adaptativo para mejorar la funcionalidad del GR y garantizar así una respuesta más eficiente ante el estrés (Basu *et al*., 2003).

Aunque las HSPs tienen una vida media relativamente corta, sus niveles permanecen elevados en todo el organismo mucho después de que el factor estresante se haya eliminado, lo que indica su papel en la adaptación a largo plazo, incrementando la tolerancia al estrés (Morimoto y Santoro, 1998) y jugando un papel importante en el mantenimiento de la homeostasis (Iwama *et al*., 1998).

1.2.3.3 Hormonas del eje hipotálamo-pituitario-interrenal

Los niveles de cortisol en estado basal y bajo estrés se diferencian ampliamente entre y dentro de especies (Pottinger, 2010), provocándose inicialmente un proceso adaptativo durante el estrés, que puede revertirse si el estrés se hace crónico (Barton y Iwama, 1991), causándose en este último supuesto, una activación prolongada del eje HPI (Varsamos *et al*., 2006). Tras una situación de estrés, la información sensorial estresante se transporta al área preóptica hipotalámica (NPO) provocándose la liberación de la CRH que, a su vez, desencadena la liberación de la ACTH a la circulación general, la cual activa las células interrenales en el riñón anterior para que se produzca y secrete cortisol. Este cortisol se encarga de redistribuir el flujo energético cuando el organismo se enfrenta al agente estresante, garantizándose así la homeostasis (Wendelaar-Bonga, 1997). A su vez, el cortisol puede actuar inhibiendo la transcripción de la CRH para conseguir con ello modular la síntesis de receptores de la CRH y proteínas de unión de la CRH (CRHBP). Los primeros median las acciones de la CRH mientras que las últimas interfieren en la liberación de cortisol mediante el bloqueo de la CRH (Flik *et al*., 2006; Westphal y Seasholtz, 2006). Se cree que las células productoras de la CRH en el núcleo preóptico hipotalámico juegan un papel crucial en los procesos de adaptación ante factores estresantes.

Además del papel que ejecuta como regulador de la CRH, el cortisol también se implica en la síntesis y liberación de la POMC y la ACTH. En varios estudios realizados en peces planos se sugiere que la POMC ejerce una retroalimentación negativa sobre los niveles de cortisol, participando así en la respuesta adaptativa de estos peces tras una situación de estrés (Palermo *et al*., 2008; Wunderink *et al*., 2011).

1.2.4 Efectos ocasionados por el estrés en el sistema inmune

La comunicación entre el sistema inmune y el endocrino es un fenómeno evolutivo conservado que se ocupa de transmitir información acerca de la actividad inmunológica al cerebro, el cual intervendrá en la regulación de la respuesta inmune (revisado por Verburg-Van Kemenade *et al.,* 2011). En peces teleósteos la inervación parasimpática directa del sistema inmune es posible gracias a que los receptores de CAs del sistema simpático suprarrenal se encuentran presentes en las células inmunitarias (Roy y Rai, 2008). Para interpretar estas interacciones, muchos investigadores se basan en la observación de cambios de expresión génica en diferentes regímenes de estrés en peces a través de chips de ADN, determinándose que existe una respuesta inmune conservada ante la exposición a un factor estresante (Momoda *et al*., 2007; Wiseman *et al*., 2007; Cairns *et al*., 2008). En mamíferos, las interacciones entre el estrés y el sistema inmunológico están bien establecidas y se consideran adaptativas (Munck *et al*., 1984; Sapolsky *et al*., 2000). Por el contrario, muy pocos estudios se han ocupado de examinar el papel del cortisol en la modulación inmune de los genes de respuesta a estrés *in vivo* en peces, con el fin de comprender las conexiones mecánicas entre ambos.

Tal y cómo se ha comentado con anterioridad, tras una situación de estrés, la NPO se sensibiliza y como resultado se libera CRH y, consecutivamente, la ACTH. Esta última no sólo activa las células interrenales que liberan cortisol sino que también, al igual que la CRH, posee receptores que se expresan en órganos como el timo y el riñón en fases iniciales del desarrollo de peces como la lubina (Mola *et al*., 2005). Esto es indicativo de su efecto directo sobre estos órganos clave del sistema inmune. Además, se ha demostrado que la administración de ACTH incrementa la expresión de algunas citoquinas, como el factor de necrosis tumoral alfa (tnfa) y la interleuquina 6 (il6), e inhibe la expresión de la interleuquina 1 beta (il1b), en macrófagos de dorada cultivados *in vitro* (Castillo *et al*., 2009) y, también, es capaz de inducir un incremento de la actividad de estallido respiratorio en fagocitos de trucha (Bayne y Levy, 1991). De esta manera, se sugiere que los primeros mediadores en la activación del eje HPI pueden tener

algunos efectos estimulantes sobre los mecanismos inmunes, reforzándose así la respuesta frente a un estrés agudo, mientras que el cortisol, como producto final, se encontraría ejerciendo una acción inhibidora o supresora de dicha respuesta.

Diversos estudios realizados en peces han utilizado cultivos celulares con macrófagos procedentes del riñón anterior para investigar el mecanismo de las interacciones entre el estrés y el sistema inmunológico a partir de la estimulación con cortisol (Huising *et al*., 2005; MacKenzie *et al*., 2006) o el uso de LPS (unos potentes estimuladores de la respuesta inmune innata) (Iliev *et al*., 2005; Holen *et al*., 2011), con el propósito de caracterizar las propiedades inmuno estimulantes de diferentes patrones moleculares asociados a patógenos (PAMP), incluyendo la inducción de la expresión génica del factor de necrosis tumoral 2 (tnf2), la il1b y la COX2. La influencia de la COX2 dentro del sistema inmune se desarrolla a través de la síntesis de prostaglandinas que tienen variedad de funciones importantes en todos los estados de inflamación (Vane *et al*., 1998). En el papel de las prostaglandinas durante la respuesta inflamatoria de los mamíferos se incluyen la vasodilatación y el incremento de la permeabilidad vascular por su interacción con la histamina y la bradiquidina, además de la disminución de las funciones leucocíticas por reducción del estallido respiratorio y la proliferación de linfocitos (Secombes *et al*., 2001).

1.3 Los aceites vegetales en la dieta para peces

1.3.1 Implicaciones del uso de aceites vegetales en dietas para peces

La dieta y el estado nutricional se consideran factores fundamentales para una explotación piscícola. Ambos factores resultan determinantes para alcanzar un estado fisiológico óptimo en los peces, no sólo por la influencia que ejercen de manera directa sobre el estado energético del animal, sino también por la acción realizada de manera indirecta sobre la

resistencia de los peces al estrés (Ganga *et al*., 2006, 2011a, 2011b; Alves-Martins *et al*., 2011, 2013) y su respuesta inmune (Montero *et al*., 2010).

En la actualidad, el aceite de pescado (FO) contenido en los piensos acuícolas debe ser sustituido por otros tipos de aceites de origen marino o terrestre debido a su limitada disponibilidad e incapacidad para cubrir su creciente demanda a nivel mundial (Tacon y Meitan, 2008). Debido a esto, en los últimos años se ha ido realizando un esfuerzo cada vez mayor al utilizar fuentes de lípidos alternativas y sostenibles, incluyendo diferentes aceites vegetales (VO) (Turchini *et al.,* 2009). Aunque la sustitución de FO al 60-75% con fuentes de lípidos alternativos no parece afectar al crecimiento de los peces ni su utilización por parte del organismo en casi todas las especies de peces (siempre que los requerimientos de EFA estén cubiertos), la sustitución total de FO por VO puede inducir un efecto negativo sobre el crecimiento de la mayoría de especies de peces marinos estudiados. Además, estos efectos negativos se ven influenciados por varios factores como la temperatura del agua, el tipo de aceite usado, la cantidad de harina de pescado que se utiliza en la dieta y/o el tamaño del pez (Glencross y Rutherford, 2010).

Los requisitos específicos de EFA son diferentes entre especies marinas y de agua dulce (Sargent *et al*., 2002). Esto se debe a que los peces de agua dulce pueden convertir PUFA de 18C a HUFA de 20C ó 22C por medio de reacciones de elongación y desaturación, por lo que sus requerimientos de EFA se suplen con los PUFA (ácido alfa-linolénico, ALA; 18:3n-3 y ácido linoleico, LA; 18:2n-6) contenidos de manera natural en los VO de la dieta. Los peces marinos no pueden realizar tal conversión ya que carecen o tienen reducida la expresión de la enzima delta-5 desaturasa (Mourente y Tocher, 1993), o bien presentan una capacidad limitada para elongar ácidos grasos de 18C hasta 20C (Ghioni *et al*., 1999). Esta circunstancia obliga a los peces marinos a ingerir diariamente unas cantidades mínimas de ácido araquidónico (ARA; 20:4n-6), eicosapentanoico (EPA; 20:5n-3) y docosahexaenoico (DHA; 22:6n-3) (Tocher, 2003, 2010), que se hacen imprescindibles a la hora de obtener una alta eficiencia alimenticia acompañada de elevados niveles de crecimiento.

La riqueza de los aceites vegetales en PUFA n-6 y n-9 de 18C, los hace candidatos ideales para su inclusión en dietas destinadas a peces marinos (Turchini *et al.,* 2009; Sales y Glencross, 2011). Sin embargo, al ser una fuente pobre en ácidos grasos poliinsaturados de cadena larga (LC-PUFA), se corre el riesgo de provocar una reducción de sus efectos positivos sobre la salud de los peces (Turchini *et al.,* 2009), al afectarse su capacidad de resistencia frente al estrés (Montero *et al*., 2003; Jutfelt *et al*., 2007) mediante la alteración de los niveles circulantes de cortisol (Ganga *et al*., 2011a) y su liberación (Ganga *et al*., 2011b). Además, con el uso de VO pueden causarse alteraciones en la morfología y fisiología de los tejidos diana (Caballero *et al*., 2003, 2004, 2006), alterándose con ello el mantenimiento de las funciones de barrera del epitelio y la regulación de la respuesta inflamatoria en el tracto gastrointestinal (Oxley *et al*., 2010).

Adicionalmente, el uso de aceites vegetales en la dieta puede provocar un impacto negativo sobre la salud o el bienestar de los peces (revisado por Montero e Izquierdo, 2010; revisado por Oliva-Teles, 2012), reduciéndose la resistencia a patógenos y alterándose varios parámetros relacionados con el sistema inmunológico (revisado por Montero e Izquierdo, 2010; Montero *et al*., 2010), así como la producción de eicosanoides (Ganga *et al*., 2006). En contraposición, un uso abusivo de ARA en la dieta también puede alterar de manera negativa la respuesta inmune (Xu *et al*., 2010), dependiendo en última instancia de la abundancia de LC-PUFA n-3, como el EPA y el DHA (Calder, 2009).

En peces, la delta-6 desaturasa (Δ6D) es la enzima limitante implicada en la biosíntesis de PUFA a partir de sus precursores (Fig. 1.5). La actividad de la Δ6D en la regulación nutricional de la dorada fue desvelada por Seiliez y colaboradores (2003), tras observarse un aumento del 18:3n-6, en los peces alimentados con una dieta libre de HUFA. En larvas de dorada alimentadas con dietas en las que el FO fue sustituido totalmente por aceite de colza o de soja, se observaron niveles significativamente altos de 18:2n-9 y 18:3n-6, y aumentos de más de 6 veces en la expresión del mRNA de la Δ6D (Izquierdo *et al*., 2008). De la misma manera, en estudios realizados por Vagner y colaboradores (2007a, 2007b, 2009) se mostró cómo los

niveles de mRNA de la Δ6D son mayores en larvas alimentadas con bajos niveles de HUFA, junto con un aumento del contenido de 18:3 n-6 en los fosfolípidos. Debido a esta modulación se permite el control del metabolismo lipídico y el mantenimiento de la funcionalidad de la membrana celular (revisado por Vagner y Santigosa, 2011).

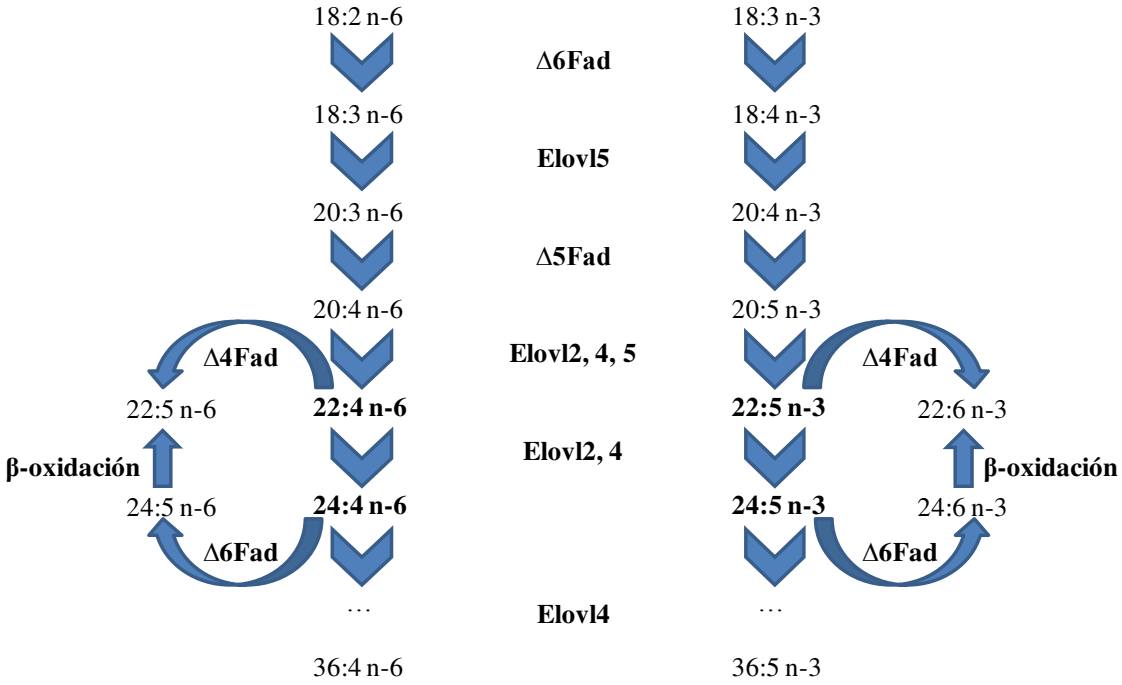

Figura 1.5: Biosíntesis de ácidos grasos altamente insaturados (LC-PUFA) en los vertebrados, incluyendo a los peces, y diferentes enzimas complementarias involucradas (Fad, ácido graso desaturasa; Elovl, ácido graso elongasa) (no necesariamente presentes en todas las especies). Figura extraída de Navarro-Guillén y colaboradores (2014).

La expresión del gen de la Δ6D y su actividad enzimática es mayor en peces alimentados con dietas de VO que en aquellos alimentados con dietas de FO, independientemente de su ciclo de vida en agua salada o agua dulce, lo cual es de esperar debido a la deficiencia de LC-PUFA en las dietas constituidas por VO (revisado por Vagner y Santigosa, 2011). Sin embargo, se observan discrepancias respecto a la expresión del mRNA de la Δ6D cuando se usa aceite de lino (LO) en la dieta (Izquierdo *et al*., 2008) debido, probablemente, a que este aceite no sólo contiene 18:2n-6 sino también altas cantidades de 18:3n-3, que pueden actuar como competidores en la elongación y desaturación de los PUFA de 18C. En studios realizados recientemente por Navarro-Guillén y colaboradores (2014) en post-larvas de lenguado senegalés

se sugiere la existencia de un único gen ácido graso desaturasa tipo 2, el cual posee una actividad Δ4 predominante, y que en condiciones de bajos niveles de DHA combinado con altos niveles de PUFAs de 18C, actuarían sobre los sustratos de la Δ6 y la Δ5 como recurso para lograr la biosíntesis del DHA a partir de sus prepulsores de 18C.

1.3.2 Efecto de los aceites vegetales sobre el estrés y el sistema inmune

Durante la práctica de la acuicultura se ocasiona un estrés fisiológico de manera regular pero no constante sobre los peces cultivados (Barton y Iwama, 1991). El resultado de este estrés constante o crónico se traduce en inmunosupresión (Tort *et al*., 2004) a través de hormonas glucocorticoides como el cortisol (Pickering y Pottinger, 1985, 1987). En peces se ha observado que la sustitución parcial de FO por VO en la dieta no afecta al crecimiento ni al índice de conversión de la ingesta (Torstensen *et al*., 2000; Bell *et al*., 2001, 2002; Caballero *et al*., 2002; Izquierdo *et al*., 2003, 2005; Montero *et al*., 2005), sin embargo, se sabe que el uso de dietas ricas en VO afecta seriamente la resistencia a estrés en peces marinos (Montero *et al*., 2003; Jutfelt *et al*., 2007), vinculándose a este tipo de dietas con una alteración en los niveles plasmáticos de cortisol post-estrés (Ganga *et al*., 2011a) y con la liberación de cortisol a partir de células interrenales cultivadas *in vitro* (Ganga *et al*., 2011b).

En peces marinos se confirma que la sustitución de FO por LO o aceite de soja (SO) altera las proporciones entre los ácidos grasos n-3 y n-6 y esto conlleva a una alteración en la composición de membrana del riñón anterior (Bell *et al*., 2001, 2002; Caballero *et al*., 2002; Izquierdo *et al*., 2003) afectándose, en consecuencia, su respuesta al estrés (Montero *et al*., 2003). Así mismo, la liberación de cortisol desde el riñón anterior, modulada a partir de los metabolitos de la COX y la LOX, se ve seriamente alterada (Ganga *et al*., 2011b). En concreto, con el uso de altos niveles de LO o HUFA n-3 en la dieta, se incrementan de manera significativa los niveles de cortisol tras ocasionarse un estrés agudo en diferentes especies de peces (Montero *et al*., 2003; Oxley *et al*., 2010). Mientras que, con el uso de SO en la dieta se

aumentan los niveles de cortisol de manera crónica, afectando a la salud y el bienestar de estos peces a largo plazo (Jutfelt *et al*., 2007). Estudios realizados en riñón anterior de dorada han mostrado que los HUFA libres son capaces de modular la secreción de cortisol (Ganga *et al*., 2006), de manera que su liberación por medio del tejido interrenal se estimula a partir del ARA, el EPA y el DHA contenido en las células, mientras que con el ácido dihomo-ϒ-linolénico (DHGLA; 20:3n-6) se inhibe. Además, también se expone que la ACTH inducida por la liberación de cortisol está mediada por los metabolitos de la ciclooxigenasa (COX) y la lipoxigenasa (LOX) (Ganga *et al*., 2006, 2011b). En concreto, la sensibilidad del eje hipotálamo-hipofisario-suprarrenal se modula a partir de la prostaglandina E2 (PGE_2) derivada del ARA, responsable de la liberación de cortisol en respuesta al estrés en mamíferos (Lands, 1991; Nye *et al*., 1997). Y posiblemente la PGE_2 también sea capaz de realizar esta acción de manera homóloga en el eje HPI de los peces (Gupta *et al*., 1985).

Se considera que los eicosanoides derivados del ARA son más abundantes y bioactivos, que aquellos obtenidos a través del EPA (Tocher, 2003; Calder, 2009). El uso de ARA en la dieta se relaciona con la resistencia a diversos factores estresantes como una exposición aguda a aireación (Van Anholt *et al*., 2004; Alves-Martins *et al*., 2013), manejo (Koven *et al*., 2001, 2003), agitación (Alves-Martins *et al*., 2012) o cambios en la salinidad del agua (Koven *et al*., 2003; Van Anholt *et al*., 2004). Sin embargo, estos y otros efectos derivados del uso del ARA en la dieta dependen, también, de la abundancia de LC-PUFA n-3 como el EPA y el DHA, que pueden inhibir su metabolismo, alterando así el perfil de síntesis de los eicosanoides (Calder, 2009), tal y como se comprueba en salmón atlántico, *Salmo salar* (Bell *et al*., 1993) y en rodaballo, *Psetta maxima* (Bell *et al.,* 1998), o se sugiere en estudios realizados en larvas de dorada (Alves-Martins *et al*., 2012). En postlarvas de lenguado senegalés se observó que el uso de proporciones bajas de ARA/EPA mejoraron la respuesta al estrés agudo por aireación, a pesar de haberse obtenido buenos resultados en crecimiento y supervivencia con diferentes porcentajes de ARA/EPA en la dieta (Alves-Martins *et al*., 2011). Además, un exceso de ARA

en la dieta puede ocasionar la aparición de efectos perjudiciales sobre el crecimiento, como se observó en juveniles de serránido japonés, *Lateolabrax japonicus* (Xu *et al*., 2010).

Por tanto, el uso de VO en la dieta de peces puede condicionar los niveles de cortisol. Existen evidencias que sugieren directa o indirectamente que el cortisol juega una importante función reguladora en la respuesta inmune (revisado por Wendelaar-Bonga, 1997); tal y como se ha observado en peces donde una elevación en los glucocorticoides reducen el número de linfocitos circulantes, disminuyéndose así la expresión de citoquinas, la proliferación linfocítica, la producción de anticuerpos y la fagocitosis, y alterándose, además, la actividad de la lisozima y el complemento (Harris y Bird, 2000; Engelsma *et al.,* 2002; Verburg-Van Kemenade *et al.,* 2009; Costas *et al*., 2011).

En multitud de estudios se ha examinado los efectos de inclusión de varios aceites en las dietas de los peces, concluyéndose de manera general que las diferentes funciones inmunológicas pueden verse comprometidas cuando el FO en la dieta es sustituido por VO (Kiron *et al*., 1995, 2011; Bell *et al*., 1996; Montero *et al*., 2008). Sin embargo, existen algunas publicaciones en las que se destaca lo contrario (Gjøen *et al*., 2004; Seierstad *et al*., 2009). Estas diferencias se deben a los niveles de sustitución utilizados en estas dietas: por lo general, altos niveles de sustitución de FO por un VO particular parecen afectar en mayor medida los parámetros inmunes, en comparación con porcentajes bajos de sustitución (Montero *et al*., 2003, 2008; Balfry *et al*., 2006; Lin y Shiau, 2007). Este hecho se debe a que la respuesta inmune se ve condicionada en concreto por los niveles de n-3 y n-6 de estos VO (Bell *et al*., 1996; Montero *et al*., 2003; Mourente *et al*., 2005a, 2005b, 2007). Se considera de manera general, que los VO ricos en n-3 PUFA afectan negativamente al sistema inmune de los salmónidos, mientras que en peces marinos, los VO ricos en n-6 PUFA parecen ser los más perjudiciales, de acuerdo con la importancia relativa de los ácidos grasos n-3 y n-6 en ambientes marinos o de agua dulce. Así, la actividad macrófaga se reduce en salmones alimentados con LO en sustitución del 50% de sus niveles de FO (J.G. Bell y D.R. Tocher, comunicación personal), mientras se observa el mismo efecto cuando se utiliza la SO en estas mismas proporciones de

sustitución en dorada (Montero *et al*., 2008) y un 100% de aceite de viborera, *Echium vulgare,* en bacalao del atlántico, *Gadus morhua* (Bell *et al*., 2006). En lucioperca, *Sander lucioperca,* alimentados con altos porcentajes de LO y SO también se redujo la respuesta inmunológica, fagocítica y linfocítica (Kowalska *et al.,* 2012).

1.3.3 Influencia de los aceites vegatales en la producción y acción de los eicosanoides

La composición en ácidos grasos de las membranas celulares se ve afectada significativamente por la composición en ácidos grasos de la dieta, determinándose así los tipos de PUFA con 20C disponibles para la síntesis de prostaglandinas. Los PUFA de la dieta son capaces de modular el sistema inmune a través de los eicosanoides derivados de los ácidos grasos de las membranas. Los eicosanoides son derivados oxigenados de los LC-PUFA y se generan desde los fosfolípidos de membrana por la acción hidrolítica de las fosfolipasas (PLA), en especial de la PLA2, proporcionando ácidos grasos libres como sustratos potenciales para la COX y la LOX (Rowley *et al*., 1995) (Fig. 1.6). Los ácidos grasos liberados desde los fosfolípidos de membrana se convierten en derivados cíclicos oxigenados, llamados colectivamente prostanoides, donde se incluyen prostaglandinas, prostaciclinas y tromboxanos; o en derivados lineales oxigenados como ácidos grasos hidroperoxi e hidroxi, leucotrienos y lipoxinas.

La COX es la principal enzima involucrada en la síntesis de prostaglandinas y está relacionada con la citoprotección gastrointestinal (Cho *et al*., 2004). Tiene una gran afinidad por el ARA produciendo 2 series de prostanoides que incluyen al menos 16 tipos de prostaglandinas tipo 2 y tromboxanos (Calder, 2002). Durante la acción de la LOX sobre el ARA se producen derivados hidroxi e hidroxiperoxi y leucotrienos de serie 4 (Calder, 2002). El EPA también es capaz de actuar como sustrato de la COX y la LOX, dando lugar a diferentes familias de eicosanoides: prostaglandinas y tromboxanos de serie 3 y leucotrienos de serie 5, además de

ácidos hidroxieicosapentanoicos (Calder, 2006). Otros ácidos grasos como el DHGLA o el DHA también actúan como sustratos de la COX y la LOX, produciendo derivados eicosanoides o docosanoides, respectivamente.

Las prostaglandinas y las prostaciclinas son eicosanoides que afectan particularmente al tono vascular y la permeabilidad, permitiendo la exudación del plasma sanguíneo y el edema tisular (Homaidan *et al*., 2002). Muchas PGE_2 poseen efectos antiinflamatorios que se llevan a cabo a través de los receptores de prostaglandinas EP2 y EP4. La participación de estos receptores de PGE_2 provocan un incremento intracelular del adenosín monofosfato cíclico (AMPc) mediante el acoplamiento a la proteína G (Faour *et al*., 2001; Fitzgerald *et al*., 2001). Juntas, estas señales participan en la generación de la respuesta final antiinflamatoria por medio de la supresión de factores de transcripción críticos para la expresión de muchas citoquinas, quimioquinas y metaloproteinasas de la matriz implicadas en la inflamación y la patogénesis inflamatoria (Min *et al*., 2002; Gomez *et al*., 2005). Durante la inflamación, la actividad del eje COX2/PGE_2 se autorregula mediante un circuito de retroalimentación positiva, primeramente implicando la mediación de la PGE_2 y la p38 MAPK, dependiente de la estabilización de la COX2 (Faour *et al*., 2001).

Como ya se ha comentado, los derivados del ARA son los eicosanoides más potentes, mucho más que los procedentes del EPA y el DHGLA. Por esta razón, toda la secuencia de estímulo extracelular tras la liberación de ARA desde los fosfolípidos de la membrana, por medio de la PLA2 citosólica dependiente de Ca^{+2} (revisado por Murakami *et al*., 2011), hasta la síntesis de eicosanoides, tanto por la acción de la COX, la LOX o la citocromo P450, se denomina "cascada de ARA" (Smith y Murphy, 2002). Por tanto, se considera que el ARA es el precursor predilecto de las prostaglandinas a través de la COX, mientras que el EPA y el DHGLA pueden llegar a modular la eficacia biológica de los eicosanoides derivados de éste (Horrobin, 1983; Bell *et al*., 1994; Ganga *et al*., 2005) debido a una inhibición competitiva de ambos por los sitios activos de la COX (Bell *et al*., 1996).

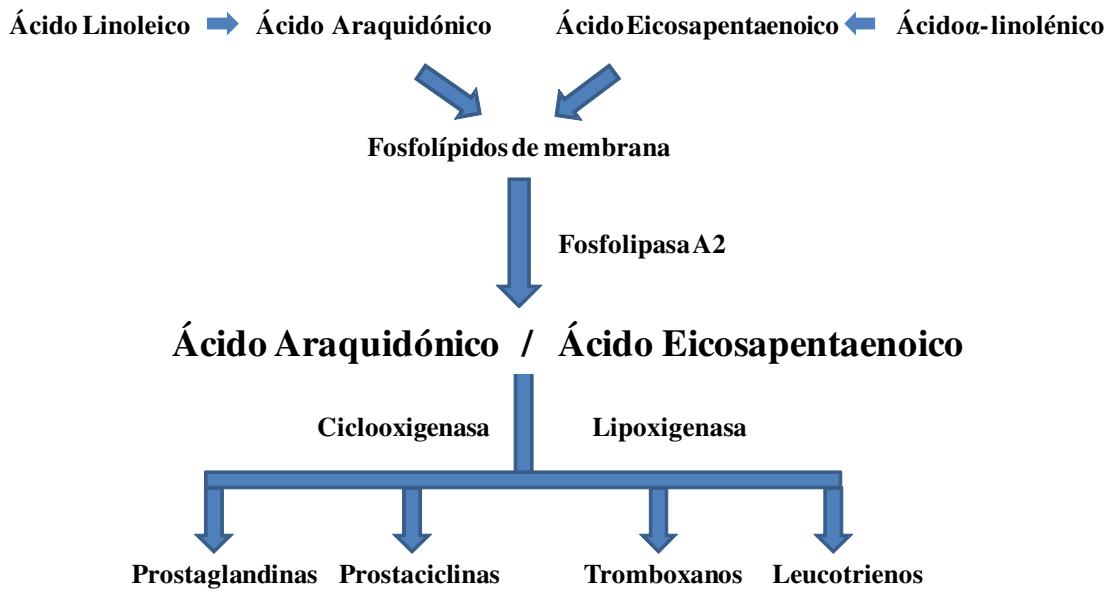

Figura 1.6: Bioconversión y efecto metabólico de los eicosanoides derivados del ácido araquidónico (ARA) y el ácido eicosapentaenoico (EPA).

Por tanto, con una dieta rica en n-6 PUFA se producen altos niveles de PGE_2, mientras que con altos niveles de n-3 PUFA en la dieta se eleva el número de PGE_3 (Ganga *et al*., 2005). De acuerdo a los estudios realizados por Bell y colaboradores (1993), los salmones atlánticos alimentados con dietas de aceite de girasol (rica en n-6 PUFA) producen más eicosanoides derivados del ARA que aquellos peces alimentados con dietas de LO o FO del hemisferio sur (con alto contenido en EPA). En base a esto, se puede inferir que el sistema inmune y los parámetros de salud en peces alimentados con dietas que incluyen altos porcentajes de VO (niveles de EPA más bajos que en dietas elaboradas con FO) pueden verse parcialmente afectados (Blazer, 1992; Thompson *et al*., 1996; Montero *et al*., 2003; Ganga *et al*., 2005).

La inflamación está coordinada localmente por medio de citoquinas, quimioquinas, neuropéptidos y eicosanoides en respuesta a un estrés agudo o crónico provocado en un tejido (Murray *et al*., 2004). James y colaboradores (2000) prueban que la adición de ácidos grasos n-3 reduce la síntesis de anticuerpos y citoquinas proinflamatorias, suprimiendo la respuesta

inflamatoria por reducción del ARA en las membranas y la síntesis de eicosanoides derivados de ésta. De manera que las prostaglandinas sintetizadas directamente desde el ARA, el EPA y el DHGLA en las membranas celulares, conforman un vínculo importante entre la nutrición lipídica y los tipos de respuesta inflamatoria (Calder, 2008).

Por lo general, si los aceites vegetales de la dieta son ricos en LA (en dietas lipídicas constituidas con SO) o en ALA (en dietas lipídicas constituidas con LO) se potencia la síntesis de PGE_2 o PGE_3, respectivamente (Bell y Sargent, 2003; Gjøen *et al.,* 2004), y, en última instancia, se incrementa o disminuye la actividad inflamatoria. En vertebrados superiores, el leucotrieno B5 y la PGE_3 junto con otros eicosanoides derivados del EPA son frecuentemente menos potentes que sus equivalentes derivados del ARA, aunque también poseen importantes efectos antiinflamatorios mediados a través de las resolvinas de serie E (Serhan, 2006), al igual que las resolvinas derivadas del DHA (Marcheselli *et al*., 2003). Pero en algunos peces marinos, como la lubina y la dorada, los altos niveles de ALA en la dietas lípidicas ricas en LO, no afectan a la concentración de PGE_2 plasmático, quizás debido a la baja capacidad de síntesis desde el ALA hasta el EPA en estos peces marinos (Ganga *et al*., 2005; Mourente *et al*., 2005b). Aunque el efecto de los VO de la dieta sobre la producción de prostaglandinas siempre depende en última instancia del tipo de VO utilizado, su contenido en ácidos grasos, el porcentaje de sustitución sobre el FO y la habilidad de la especie para producir LC-PUFA. En general, la producción de prostaglandinas de serie 2 y 3 en los tejidos de los peces es proporcional a su porcentaje de EPA/ARA (Bell *et al*., 1994).

Aunque se asume, de manera general, que los eicosanoides derivados del ARA poseen potentes efectos proinflamatorios (Secombes, 1996), éstos también pueden actuar como antiinflamatorios, inhibiendo la producción de citoquinas inflamatorias y leucotrienos inflamatorios a partir de la PGE_2 e induciendo la producción de lipoxina A4 (revisado por Calder, 2009). Actualmente, en dietas de peces se está usando el ácido estearidónico (SDA; 18:4n-3), un PUFA raro de la serie n-3, que puede ser alargado y desaturado a EPA, con más eficacia que el ALA, y que ha sido ampliamente utilizado en seres humanos para tratar

enfermedades crónicas e inflamación (Coupland y Hebard, 2002). En larvas de lenguado senegalés se observa que el uso del SDA reduce la producción de prostaglandinas como consecuencia de la inhibición de la COX2 (Villalta *et al*., 2008), como ya se ha visto en mamíferos (Horia y Watkins, 2005). Es evidente que la aparente eficacia del SDA y sus metabolitos deben ser investigados por sus propiedades anti-ARA en el futuro, siempre considerando el equilibrio en la proporción n-3/n-6 en la dieta, la capacidad de desaturación/elongación y los niveles de prostaglandinas derivados del ARA y el EPA, que en última instancia están condicionados por la especie, y que conducirán a aumentar o disminuir la respuesta inflamatoria.

Actualmente, también se investiga la implicación de los metabolitos procedentes de la LOX5 en la síntesis de cortisol. En estudios realizados por Ganga y colaboradores (2011b) se estudió por primera vez la implicación de los derivados de la LOX5 en la modulación de la secreción de cortisol por el riñón anterior en peces, encontrándose resultados en consonancia con los obtenidos en otros estudios donde se propone la necesaria implicación de esta vía en la liberación de ACTH cuando se añade ARA a un cultivo de células de peces *in vitro* (Koven *et al*., 2003). Paralelamente, se ha demostrado que los metabolitos de la LOX5 procedentes del ARA también sirven para estimular la liberación de la hormona de crecimiento (GH) en células de la pituitaria anterior en ratas (Abou-Samra *et al*., 1986; Won y Orth, 1994). En mamíferos se investigó el papel que desarrollan los productos de la LOX5 en la secreción de ACTH y la esteroidogénesis suprarrenal (Wang *et al*., 2000; Yamazaki *et al*., 2001), observándose que otros eicosanoides como los metabolitos de epoxigenasa también podrían ser moduladores potenciales en el eje HPI en los peces, ya que se demuestra que pueden estimular la secreción de ACTH y de endorfinas en células de la pituitaria de ratas (Cowell *et al*., 1991; Won y Orth, 1994; Michl *et al*., 2000).

1.4 El lenguado senegalés como especie de interés acuícola

El lenguado senegalés, *Solea senegalensis* Kaup, 1858 (Pleuronectiformes, Soleidae) es un pez teleósteo plano de cuerpo asimétrico, con una forma oval alargada y comprimida, un lado ciego y dos ojos en el lado derecho. La principal característica que lo distingue del lenguado común, *Solea solea,* es que la membrana interradial de la aleta pectoral del lado de los ojos es de color negro (Fig. 1.7) (Desoutter, 1990; Nelson, 1994; Arellano y Sarasquete, 2005).

Se trata de una especie bentónica que vive en fondos arenosos o fangosos de la costa y lagos salobres, alimentándose de pequeños crustáceos, poliquetos y moluscos bivalvos (Desoutter, 1990; Arellano y Sarasquete, 2005). Su presencia en el atlántico se extiende desde el golfo de Vizcaya hasta las costas de Senegal, y también en las costas del mediterráneo occidental.

Desde los años 90, el lenguado senegalés ha sido considerado una especie prometedora para la acuicultura en el suroeste de Europa y países mediterráneos, por su alto precio, su gran demanda y sus bajos índices de captura (revisado por Imsland *et al.,* 2003; Conceição *et al.,* 2007). España, pionera en el cultivo de esta especie, es, con 194 toneladas producidas en 2013, el segundo país europeo con mayor producción de lenguado senegalés después de Francia. El engorde del lenguado senegalés se realiza en la actualidad desde instalaciones en tierra ubicadas en Galicia, Andalucía y Canarias. La producción a escala industrial ha estado frenada en los últimos años por dificultades técnicas relacionadas con el proceso de reproducción y aspectos sanitarios, que poco a poco van quedando atrás y posibilitan que la producción en 2013 se haya situado en las 194 toneladas, frente a las 65 toneladas registradas en 2005 (Rodríguez-Villanueva y Peleteiro-Alonso, 2014).

Figura 1.7: Lenguado senegalés, *Solea senegalensis*, Kaup 1858.

Se estima que el lenguado necesita un 60% de proteína en base al nivel de materia seca de la dieta (Rema *et al*., 2008). Actualmente, estas dietas se basan en harinas de pescado de alta calidad como principal fuente de proteínas. No obstante, la utilización de estas dietas a gran escala no es sostenible debido a sus altos costes de producción y su reducida disponibilidad (FAO, 2006). Por ello, varios estudios realizados recientemente han sustituido las proteínas procedente de la harina de pescado por otras de origen vegetal, consiguiéndose buenos resultados a nivel de crecimiento, alimentación y utilización proteica una vez que los aminoácidos de la dieta están en equilibrio (Silva *et al*., 2007, 2009).

Al igual que sucede con las proteínas obtenidas a partir de harina de pescado, el FO utilizado en las dietas de peces deben ser reemplazados por otros de origen vegetal debido a su limitada disponibilidad y su creciente demanda (Tacon y Metian, 2008). Aunque, cabe destacar que en especies como el lenguado senegalés (Dias *et al*., 2004) o el halibut, *Hippoglossus hippoglossus* (Caceres-Martinez *et al*., 1984; Berge y Storebakken, 1991; Aksnes *et al*., 1996; Grisdale-Helland y Helland, 1998; Hamre *et al*., 2003; Alves-Martins *et al*., 2007), no se necesitan altas proporciones lipídicas en la dieta para obtener un aumento del peso y de la utilización proteica, como sí ocurre en otras especies de peces planos como la platija,

Pleuronectes platessa (Cowey *et al*., 1975). Estudios realizados por Borges y colaboradores (2009) sugieren como máximo una inclusión lípidica del 8% en la dieta de juveniles de lenguado senegalés, para conseguir buenos resultados en crecimiento y utilización de nutrientes.

Sin embargo, el aspecto más llamativo del lenguado senegalés, respecto a sus necesidades dietéticas en ácidos grasos, es el aparentemente bajo requerimiento en LC-PUFA durante su etapa larvaria. Este hecho es muy raro en un teleósteo marino, en particular, refiriéndose a los niveles de DHA durante este periodo (Sargent *et al*., 1997). Recientemente, se ha llevado a cabo la caracterización funcional del ácido graso delta-4 desaturasa y del ácido graso elongasa 5 (ELOVL5) demostrándose la capacidad potencial de esta especie para sintetizar DHA a partir de EPA (Morais *et al*., 2012) e incluso desde ALA (Navarro-Guillén *et al*., 2014). Las larvas de lenguado senegalés se pueden cultivar con dietas que contienen cotas insignificantes de DHA y bajos niveles de EPA, tales como presas vivas no enriquecidas, sin obtenerse con ello efectos perjudiciales en su desarrollo (Morais *et al*., 2004; Villalta *et al*., 2005).

A pesar de los bajos requerimientos en LC-PUFA para esta especie, éstos son retenidos selectivamente en los tejidos formando parte de la estructura y funciones de la membrana celular (Tocher, 2003). Además, están implicados en la regulación de una multitud de vías metabólicas e inmunes a través de su papel como mensajeros secundarios y ligandos de factores de transcripción, y son potentes precursores bioactivos de los eicosanoides, los cuales poseen propiedades pro o antiinflamatorias (Jump *et al*., 1996; Calder, 2002; Riediger *et al*., 2009). Así mismo, se ha demostrado que a pesar de la tolerancia de las postlarvas de lenguado a un amplio rango de ARA/EPA en la dieta, en términos de crecimiento y supervivencia, éstas restablecen más rápidamente sus niveles basales de cortisol en respuesta a un estrés agudo cuando son alimentadas con bajas proporciones de ARA/EPA (Alves-Martins *et al*., 2011, 2013). Estos resultados proporcionan algunas pistas sobre el valor de los EFA en la señalización celular, y su implicación a largo plazo sobre el crecimiento, la inmunidad, y la capacidad para afrontar el estrés. Estos aspectos son fundamentales, por tanto, para el mantenimiento del bienestar de los

peces de piscifactoría bajo condiciones intensivas de cultivo, especialmente en este preciso instante, donde se busca la sustitución del FO por aceites alternativos.

1.5 Objetivos

El objetivo general de esta tesis es evaluar el efecto de la utilización de aceites vegetales en la dieta de juveniles de lenguado senegalés sobre el bienestar y sistema inmune, mejorando el conocimiento de los mecanismos implicados en la regulación de la respuesta a estrés por medio de los lípidos dietéticos y condiciones estresantes propias de la acuicultura. Para conseguir este objetivo general se abordaron los siguientes objetivos específicos:

1. Determinar los efectos derivados de la sustituición total de aceite de pescado por aceites vegetales en dietas para juveniles de lenguado senegalés, en crecimiento, composición lipídica y perfil de ácidos grasos, y expresión de genes relacionados con estrés, en hígado, músculo e intestino.
2. Estudiar el efecto de un cambio de temperatura sobre el cortisol plasmático y la expresión de genes relacionados con la respuesta de estrés en hígado, intestino, músculo, branquias y cerebro de lenguado senegalés.
3. Determinar el efecto de la sustitución total de aceite de pescado por aceites vegetales en la dieta del lenguado senegalés sobre la expresión de los genes relacionados con la respuesta inmune intestinal. Estudiar el efecto del uso de aceites vegetales en la dieta sobre la expresión de los receptores de eicosanoides intestinales en *Solea senegalensis*.

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CAPÍTULO 2

MATERIAL Y MÉTODOS

2. Material y métodos

Para la consecución de los objetivos se realizaron dos experimentos:

I. *Experimento nutricional*. Se evaluaron los efectos de dos dietas constituidas a partir de aceites vegetales (VO) respecto a una dieta constituida con aceites de pescado (FO), sobre el rendimiento del pez, su composición bioquímica y la expresión relativa de varios genes relacionados con el estrés, el sistema inmunológico y las rutas metabólicas de los eicosanoides, tras ocasionarse un estrés por persecución con red durante 5 minutos.

II. *Experimento de respuesta fisiológica a cambios ambientales*. Se estudió la expresión relativa de los receptores de glucocorticoides (GRs) y las proteínas de shock térmico (HSPs) asociadas a estos, la hormona liberadora de corticotropina (CRH), su proteína de unión (CRHBP) y la propiomelanocortina (POMC), tras inducir un shock térmico de +5ºC, evaluándose la expresión de dichos genes a lo largo de 1 semana.

Los experimentos se desarrollaron en las antiguas instalaciones de cultivo del ICCM (Instituto Canario de Ciencias Marinas), actualmente denominado, Parque Marino Científico Tecnológico de la ULPGC (Universidad de las Palmas de Gran Canaria), situado en el puerto de Taliarte, en Gran Canaria, España.

Los ejemplares de lenguado senegalés, *Solea senegalensis,* utilizados en esta tesis procedían de la empresa local ADSA, pertenciente al grupo Tinamenor S.L., situado en Castillo del Romeral, Gran Canaria, España.

El análisis genético y bioquímico de las muestras se realizó en la División de Acuicultura y Genética Marina del IUSA (Instituto Universitario de Sanidad Animal y Seguridad Alimentaria), situado en Bañaderos, Gran Canaria, España.

Los análisis de cortisol se llevaron a cabo en el Departamento de Biología, Fisiología e Inmunología de la Universidad Autónoma de Barcelona, Bellaterra, España.

El Open Array se efectuó en el centro IFAPA (Instituto de Investigación y Formación Agraria y Pesquera), El Toruño, ubicado en el término municipal del Puerto de Santa María (Cádiz).

Las experiencias desarrolladas en esta tesis doctoral se englobaron dentro del ‘‘Plan Nacional de Bases para el Control de la Reproducción y Conocimiento del Sistema de Defensas Naturales del Lenguado’’ financiado por el JACUMAR.

2.1 Diseño experimental y toma de muestras

2.1.1 Efecto de la acción combinada del uso de aceites vegetales en la dieta y el estrés por manipulación, sobre la respuesta metabólica, inmunológica y de estrés en juveniles de Solea senegalensis

Los peces experimentales se seleccionaron dentro de un rango de 3,50 ± 0,24 g de peso. Desde el inicio del experimento se ubicaron en tanques de plástico de 45 l (Fig. 2.1). Cada tanque se abasteció con agua de mar filtrada a razón de 3 l/h, a una temperatura de entre 21,6-22,8°C, con un rango de oxígeno disuelto en agua de 6,20 ± 0,70 gl^{-1} y un fotoperiodo natural (12 horas de luz: 12 horas de oscuridad). Los peces se alimentaron manualmente *ad libitum* con dietas especialmente formuladas para esta experiencia, dos veces por día, 6 días por semana.

Se utilizaron tres tanques por dieta, con un total de 90 peces por tratamiento. El índice de ingesta se midió diariamente, pesando la cantidad de comida administrada y la desechada. Los parámetros de crecimiento se tomaron los días 0, 30, 60 y 90. Se determinó la tasa de

conversión alimenticia (FCR) y la tasa de crecimiento específico (SGR), utilizando las fórmulas detalladas a continuación:

$$FCR = \text{alimento consumido} / \text{ganancia de peso}$$

$$SGR = [(\ln W1 - \ln W0)]*100/t2-t1$$

Donde W0 y W1 se refieren respectivamente al peso seco de los peces al inicio y al final del experimento (por tanques), y t2-t1 se identifica como el intervalo de tiempo en días desde el comienzo y el final del experimento. El día 60 se realizó la toma de muestras por disección de 10 peces por tanque (30 peces por tratamiento). Se obtuvieron muestras de hígado, intestino y músculo para el análisis bioquímico y la determinación de ácidos grasos en cada uno de estos órganos. A otros 6 peces por tanque (18 peces por tratamiento) se les tomaron muestras de sangre a través de la vena caudal. Esta sangre se depositó dentro de tubos tipo Eppendorf heparinizados (Lithium heparine, Deltalab) que se centrifugaron a 800 *xg* durante 10 min para obtener el plasma que posteriormente se conservó a -80°C hasta realizarse el análisis de cortisol.

Al término del experimento, el día 90, se realizó un test de estrés por persecución con red. Antes de realizar el test de estrés, se sacrificaron 9 peces por dieta experimental de los que se obtuvieron las muestras control, y tras cinco minutos de persecución con red se sacrificaron otros 9 peces por dieta experimental. Los peces se diseccionaron con material autoclavado y sobre una superficie estéril, tomándose aproximadamente unos 60 mg de hígado, músculo e intestino de cada pez, y depositándose estas muestras en tubos Eppendorf junto con RNA Later (QIAGEN) a razón de 5 partes de este conservante por 1 de tejido y manteniéndolas en refrigeración durante 24 h. Tras este periodo de espera se extrajo el RNA Later de los tubos tipo Eppendorf y las muestras se conservaron a -80°C hasta su posterior análisis.

Todos los peces muestreados durante este experimento se sacrificaron utilizando una sobredosis anestésica de aceite de clavo en inmersión. Todo el material de disección y de almacenamiento manejado durante los muestreos fue debidamente autoclavado a 121 ˚C durante 30 min.

2.1.2 Efecto del choque térmico sobre la respuesta a estrés en juveniles de Solea senegalensis

En la segunda experiencia se utilizaron un total de 168 juveniles de lenguado senegalés con un peso medio de 62,3 ± 21,3 g. Los ejemplares se distribuyeron desde el principio en 24 tanques de plástico rectangulares de 45 l de capacidad (N= 7 animales/tanque) (Fig. 2.1), y se aclimataron a una temperatura de 18-19°C durante 30 días. Transcurrido este periodo, en la mitad de los tanques se ocasionó una subida gradual de temperatura durante 1 h hasta alcanzar los 23-24°C, manteniéndose así durante una semana. Cada tanque se abasteció con agua de mar filtrada a razón de 3 l/h, en un fotoperiodo natural (12 horas de luz: 12 horas de oscuridad). Con un rango de oxígeno disuelto en agua de 6,2 ± 0,7 gl^{-1}. Los peces se alimentaron manualmente *ad libitum* con una dieta comercial, dos veces por día, 6 días por semana.

Se tomaron muestras de un total de 96 peces (4 peces por tanque), repartidos en 4 puntos de muestreo tras el choque térmico: 1 h, 24 h, 3 d y 1 sem. Se recogieron muestras de 60 mg de hígado, músculo, branquias, cerebro e intestino, depositándose estas muestras en tubos tipo Eppendorf con RNA Later (QIAGEN) a razón de 5 partes de conservante por 1 de tejido, que se almacenaron en refrigeración durante 24 h. Tras este periodo de espera, se extrajo el RNA Later de los tubos tipo Eppendorf y las muestras se conservaron a -80°C, hasta su posterior análisis.

También se tomaron muestras de sangre de cuatro peces por tanque por medio de una punción en el seno caudal. Dichas muestras se depositaron en tubos tipo Eppendorf heparinizados (Lithium heparine, Deltalab) que se centrifugaron a 800 *xg* durante 10 min para obtener el plasma y que posteriormente se conservaron a -80°C hasta el análisis de cortisol.

Todos los peces se sacrificaron por medio de sobredosis anestésica de aceite de clavo en inmersión. El material de disección y de almacenamiento utilizado durante los muestreos fue debidamente autoclavado a 121 ˚C durante 30 min.

Figura 2.1: Tanques de 45 L con superficie de 60x40 cm para el cultivo de juveniles de *S.senegalensis.*

2.2 Formulación de dietas experimentales

Para la experiencia I se formularon tres dietas experimentales isonitrogenadas (56% de contenido proteico) e isoenergéticas, con un contenido de lípidos constante del 12%. La dieta control se formuló con aceite de pescado procedente de anchoa peruana como única fuente lipídica (100FO). Mientras para las otras dos dietas experimentales se sustituyó, en su totalidad, el FO como fuente lipídica por VO. Una de ellas se formuló con soja (100SO) y la otra con lino (100LO). Las dietas se prepararon en las antiguas instalaciones de investigación del ICCM utilizándose un mezclador (DANAMIX BM 330, Azpeitia, Gipuzcua, España) y una peletizadora (CPM, California Pellet Mill, EE.UU.).

2.3 Análisis bioquímico de dietas y tejidos

Todas las muestras se conservaron a -80 ˚C. Los análisis se realizaron por triplicado y para ello las muestras fueron correctamente homogenizadas.

2.3.1 Análisis proximal

Para el análisis proximal de las dietas formuladas en la experiencia I, se midieron los siguientes parámetros:

- Humedad

La humedad se determinó mediante el secado de las muestras a 105 ˚C hasta que se alcanzó un peso constante (AOAC, 1995).

- Contenido en cenizas

El contenido de cenizas se determinó mediante el secado de las muestras en un horno a una temperatura de 450˚C hasta que se alcanzó un peso constante (AOAC, 1995).

- Proteínas totales

Las proteínas totales se estimaron a partir del nitrógeno presente en el total de la muestra, utilizando la metodología de Kjeldhal (AOAC, 1995), después de la digestión de la muestra con ácido sulfúrico concentrado a una temperatura de 420 ˚C.

- Lípidos totales

Los lípidos totales se extrajeron de las dietas siguiéndose el método descrito por Folch y colaboradores (1957), donde se utiliza una mezcla de cloroformo / metanol (2:1, v / v) con un contenido del 0,01% en butil hidroxitolueno, E 321 (BHT). Después de la extracción lipídica, los disolventes se secaron bajo una atmósfera de nitrógeno para posteriormente determinar el peso de los lípidos de las muestras.

2.3.2 Análisis de ácidos grasos

Los esteres metílicos de los ácidos grasos (FAMEs), procedentes de las dietas experimentales y los órganos seleccionados, se obtuvieron por transmetilación de los lípidos totales a través del método detallado por Christie (1982). Los FAMEs se separaron, identificaron y cuantificaron por cromatografía líquida de gases bajo las condiciones descritas por Izquierdo y colaboradores (1992) (Tabla 2.1). Los ésteres de metilo individuales fueron identificados por comparación con estándares externos EPA 28, Nippai, Ltd, de Tokio, Japon.

Tabla 2.1: Condiciones del cromatógrafo para el análisis de los ácidos grasos

CROMATÓGRAFO	Shimadzu GC-14-A (división instrumental de Shimadzu), Kyoto, Japón
INTEGRADOR	Shimadzu C-R5A
COLUMNAS	Capilar de sílice fundida 30*0,32 mmj D.I (Supelco, Inc, Bellefonte, EEUU)
GAS DE TRANSPORTE	Helio
PRESIÓN DE GASES	He = 1; H_2 = 0,5; N_2= 0,5 y aire = 0,5 kg/cm^2
DETECTOR	FID a 250 ºC
Tª DE INYECCIÓN	250 ºC
HORNO	Tª inicial 180 ºC 10 min Incremento de 2,5 ºC por min Tª final de 215 ºC durante 12 min

2.4 Análisis del cortisol

La concentración de cortisol en plasma se determinó por radio inmuno ensayo (RIA) utilizando el método tripsina-antitripsina (Rotllant *et al*., 2001) en las instalaciones del Departamento de Biología Celular, Fisiología e Inmunología, de la Universitat Autonòma de Barcelona, Bellaterra, España.

2.5 Análisis genético

2.5.1 Extracción de RNA

Para la extracción del RNA se utilizó TRI Reagent (SIGMA-Aldrich, Sant Louis, Missouri) y un kit RNaesy Tissue Lipid de QIAGEN (Fig. 2.2), siguiéndose el protocolo descrito en el Apéndice 1 (Capítulo 9). Todas las muestras de tejido se homogenizaron mecánicamente en mezclas de 100 mg, utilizándose siempre material autoclavado y guantes sin talco. La calidad y cantidad del RNA resultante (Tabla 2.2) se evaluó por medio de un espectrofotómetro denominado NanoDrop 1000 (Thermo Scientific).

Figura 2.2: kit de extracción de RNA (RNaesy Tissue Lipid de QIAGEN), placa de frío, pipetas, tubos Eppendorfs autoclavados, guantes sin talco, columnas de filtrado y reactivos.

Tabla 2.2: Cuantificación y pureza del RNA

TEJIDO	EXP.	ng/µl			260/280			260/230		
MÚSCULO	1	278,32	±	15,97	2,05	±	0,04	1,89	±	0,35
HÍGADO	1	1.989,75	±	740,34	1,97	±	0,12	1,75	±	0,26
INTESTINO	1	1.443,14	±	304,60	2,04	±	0,02	1,97	±	0,05
HÍGADO	2	1.348,42	±	788,12	1,96	±	0,08	1,96	±	0,24
CEREBRO	2	540,88	±	38,22	2,12	±	0,03	1,96	±	0,40
BRANQUIA	2	366,71	±	18,76	2,11	±	0,02	1,49	±	0,61
INTESTINO	2	1.582,66	±	588,76	1,96	±	0,07	1,91	±	0,20
MÚSCULO	2	171,73	±	16,06	1,91	±	0,20	1,57	±	0,40

2.5.2 Síntesis cDNA

La reacción de transcripción inversa, para la síntesis de cDNA, se llevó a cabo en un volumen de 20 µl con un total de 1 µg de RNA siguiéndose el protocolo de síntesis del iScript ™ cDNA Synthesis Kit (Bio-Rad Hercules, California) (Tabla 2.3).

Tabla 2.3: Componentes de la reacción de síntesis de cDNA a partir de RNAm (izquierda) y el protocolo a seguir con el termociclador (derecha)

Componentes de la reacción		Protocolo de reacción
Mix de reacción iScript 5x	4 µl	5 minutos a 25 ˚C
Transcriptasa inversa iScript	1 µl	30 minutos a 42 ˚C
Agua libre de nucleasas	13 µl	5 minutos a 85 ˚C
RNA (1 µg total)	2 µl	4 ˚C ∞

2.5.3 RT-PCR

Todas las reacciones de RT-PCR, se realizaron en el iCycler thermocycler with optical module (Bio-Rad Hercules, California) utilizándose 12,5 µl de iQ™SYBRGreen Supermix (Bio-Rad), 1 µl de cDNA disuelto a 1/10 y 1,2 µl de oligos, con un volumen final de 25 µl.

Y teniéndose en cuenta las siguientes condiciones en el termociclador:

1. Desnaturalización del cDNA y activación enzimática: 95 ˚C durante 7 min
2. Unión de oligos: 40 ciclos de 70 ˚C o 60 ˚C durante 15 s
3. Extensión: 70 ˚C durante 30 s

A excepción de los análisis de RT-PCR pertenecientes a los genes de respuesta inmune innata que se realizaron a través de una plataforma de OpenArray ® (Life Technologies). Para cuyas reacciones se utilizaron 5 µl de TaqMan® OpenArray® Real-Time PCR Master Mix junto con el cDNA específico para cada una de las muestras. Tanto el cDNA como la amplicación Taqman se llevaron a cabo de acuerdo a los protocolos elaborados por el fabricante. Para la ejecución de los ensayos realizados con TaqMan se utilizó la OpenArray® Real-Time PCR Instrument (Life technologies), cargándose las muestras en placas de OpenArray con el Sistema OpenArray ® AccuFill ™ de acuerdo con los protocolos descritos por el fabricante.

Tras comprobarse que los genes amplificaban con una eficiencia próxima al 100%, se llevó acabo el análisis de expresión génica por el método comparativo CT ($2^{-\Delta\Delta CT}$) (Livak y Schmittgen, 2001) siguiéndose los pasos descritos a continuación:

1. Normalización del CT correspondiente al gen diana con el CT correspondiente al gen de referencia (ubiquitina o gapdh2), tanto para la muestra de ensayo (estresada) como para la muestra calibradora (control):

   CT (ensayo) = CT (diana, ensayo) - CT (de referencia, ensayo)

   CT (calibrador)= CT (diana, calibrador) - CT (de referencia, calibrador)

2. Normalización de la ΔCT de la muestra de ensayo con el ΔCT del calibrador: ΔΔCT = ΔCT(ensayo) - ΔCT(calibrador)

3. Cálculo del ratio $2^{-\Delta\Delta CT}$, que nos da el ratio de expresión normalizada que se traduce en el aumento o disminución de la expresión del gen diana en la muestra de ensayo (estresada) en comparación con el calibrador (control).

2.5.4 Oligos

De todos los oligos específicos para lenguado senegalés utilizados en RT-PCR (Tabla 2.4), una parte se extrajo de publicaciones científicas (Ubiquitina, HSP70, HSP90AA, HSP90AB, CRF, CRFBP, POMCA y POMCB); mientras que el resto (GR1, GR2, PLA2, LOX5, EP2, EP3y EP4) se diseñaron a partir de secuencias publicadas en el Genbank y la plataforma web de aquagenet (www.aquagenet.eu). Para el diseño de cada uno de ellos se utilizó el programa Oligo 7.0 Software (Medprobe) respetándose los siguientes parámetros:

- Eficiencia ≥ 500
- Dímeros en extremo 3´ con $\Delta G \geq -3$ kcal/mol
- Dímeros en todo el oligo con $\Delta G \geq -7$ kcal/mol
- Hardpin-loop con $\Delta G \geq -3$ kcal/mol
- Estabilidad en los extremos 3´ con $\Delta G \geq -6$ y -8
- Contenido de Adenina y Timina (A + T)de [50,0%]
- Contenido de Guanina + Citosina (G + C) de [50,0%]
- Diferencia de Temperatura de melting ™ entre oligos $\leq 1{,}0°C$
- Tamaño de oligo entre 17-25 pares de bases (pb)
- Tamaño de fragmento entre 100-200 pb

Los chips pertenecientes al OpenArray fueron diseñados en un formato de 56x48 muestras incluyéndose las sondas pertenecientes a los 56 genes relacionados con el sistema

inmune innato y la síntesis de prostaglandinas (Tabla 2.5. A, B, C, D, E, F, G y H). En el conjunto completo de genes evaluados se incluyeron 9 genes asociados a la defensa antiviral (ifnc, mx, irf1, irf2, irf3, irf7, irf8, irf9, irf10), 15 interleuquinas y sus receptores (il1b, il6, il8b, il8c, il10, il11a, il12b, il15, il17c, tnfa, il1rl1, cxcr2, il10rb, il15ra, il17ra), 8 relativos a las vías del complemento (c1ql4, c2, c3, c4a, c4b, c5, c9, cfh), 10 genes relacionados con los patrones moleculares asociados a patógenos o PAMP (pglyrp1, tlr1, tlr5s, tlr8b, tlr9, lyg1, lyg2, lyg3, lyc1, lyc2), 2 genes pertinentes a la síntesis de prostaglandinas (COX1A y COX2), 6 quimiocinas (cck3, ccl25, cxcl10, cxcl13, ccl19, ccl20) y 3 genes relacionados con la diferenciación antigénica (cd4, cd8a, cd8b), además de incluirse 3 genes de referencia (ub52, eef1a1 y gapdh2) (Infante *et al.,* 2008). Todas las secuencias se obtuvieron a partir del SoleaDB (http://www.juntadeandalucia.es/agriculturaypesca/ifapa /soleadb_ifapa/) y los oligos y sondas pertenecientes al OpenArray se perfilaron con la herramienta de diseño de Custom TaqMan ® (Life Technologies).

2.5.5 Clonación y amplificación de secuencias

Para la clonación de los genes del GR en lenguado senegalés, se diseñaron varios oligos sobre secuencias conservadas de estos genes en el falso halibut del Japón o *Paralichthys olivaceus* (Tabla 2.6), utilizándose el kit TOPO TA Cloning kit for Sequencing (Invitrogen ™) para realizar dicha clonación a través del protocolo detallado en el Apéndice 2 (Capítulo 9). Para amplificar la secuencia de los GRs se diseñaron los oligos detallados en la Tabla 2.7 y se siguieron los protocolos establecidos en el kit 3`RACE System for Rapid Amplification of cDNA Ends (Invitrogen ™) y 5´ RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Invitrogen ™), detallados en los Apéndices 3 y 4 (Capítulo 9) respectivamente.

Para el ensamblaje y comparación de las secuencias se utilizó el programa Mega 5.0.

Tabla 2.4: Oligos diseñados para RT-PCR

Gen		Secuencia 5´-3´	Pb	Acc no	Bibliografía
GR1	F	CCTGCCGCTTCCACAAGTGTCTGATG	130	AB614369	Benítez-Dorta *et al*., 2013
	R	TTCAACTGGTGGAGGTGGCGGTGT			
GR2	F	TCAGCGTGGAGTTCCCGGAGATG	92	AB614370	Benítez-Dorta *et al*., 2013
	R	GGTGGAACAGCAGCGGCTTGATG			
ubiquitina	F	AGCTGGCCCAGAAATATAACTGCGACA	93	AB291588	Infante *et al.,* 2008
	R	ACTTCTTCTTGCGGCAGTTGACAGCAC			
HSP 70	F	GCTATACCAGGGAGGGATGGAAGGAGGG	119	AB513855	Salas-Leiton *et al.,* 2010
	R	CGACCTCCTCAATATTTGGGCCAGCA			
HSP 90 AA	F	GACCAAGCCTATCTGGACCCGCAAC	105	AB367526	Manchado *et al.,* 2008
	R	TTGACAGCCAGGTGGTCCTCCCAGT			
HSP 90 AB	F	TCAGTTTGGTGTGGGTTTCTACTCGGCTTA	148	AB367527	Manchado *et al.,* 2008
	R	GCCAAGGGGCTCACCTGTGTCG			
CRH	F	CGGCGTCTATTACAAGGGAAAGTTGGGAAC	98	FR745427	Salas-Leiton *et al.,* 2012
	R	TCGGACCTCCTCCCCCTCTCCAT			
CRHBP	F	AGCTGCTGGGGGGCAATGGCATA	94	FR745428	Salas-Leiton *et al.,* 2012
	R	CCAACCTTCATCTGGGCGAGTCCTCT			
POMCA	F	CGGCCCATCACAGTCTACAGCTCCA	131	FR874846	Salas-Leiton *et al.,* 2012
	R	TACGCGCCGTCCTTTTTCTCGTG			
POMCB	F	GGATGCGGCAAAAGGGGGACA	111	FR874847	Salas-Leiton *et al.,* 2012
	R	CCCCATCTAAAGTGACCCATGCGGTA			
EP2	F	ATGGCGTTAGAGAGATGCTTTG	142	(www.aquagenet.eu)	-
	R	TACCAAACCCCACAAACGGAA			
EP3	F	GCTTTGCATTGGATCTCTGG	162	XM005459908.1	-
	R	CACAGGCCAAAAGTTGTCATGC			
EP4	F	ACCATCGCCACCTATGTGCAAG	120	NM001173955.1	-
	R	TATGGACATGGCACAGATAATGCT			
LOX5	F	AGTGATCCGAAAATGCACCAAA	151	(www.aquagenet.eu)	-
	R	ACTGATGCCGTCTAACACCT			
PLA2	F	CGGCCTTCTTGTCACATTCACA	113	FF285823	-
	R	CGACAACCCTTACACCGAGT			

Tabla 2.5A: Oligos de síntesis de prostaglandinas diseñados para Open Array

Síntesis de Prostaglandinas					
Nombre del Gen	**Abrev.**	**ACC NO**	**Secuencia del F**	**Secuencia del R**	**pb**
Ciclooxigenasa 1a	*COX1A*	AI39RPP	GTGATCTTAAGACTACGGCATCTGT	ATGAGACCCATTGAATTTACCAGTTCTT	96
Ciclooxigenasa 2	*COX2*	AI5IPVX	CCGACTTACAATGCGGATTATGGTT	TTGGGCAATCCTCTGGTACAG	100

Tabla 2.5B: Oligos de defensa antiviral diseñados para Open Array

Defensa antiviral					
Nombre del Gen	**Abrev.**	**ACC NO**	**Secuencia del F**	**Secuencia del R**	**pb**
Interferon C	*ifnc*	AI70L8D	GGAAACTCGATACCTTCATGAACCT	TGTGGACCAGTGGAGAGACA	73
Proteína Mx inducida por interferón	*mx*	AI89KEL	CATCCACCTGTCCCACACA	GCCCGCGATATTTGTCTTCATAGAT	83
Factor regulador de interferón 1	*irf1*	AIX00UK	GGGCGATTCGCTCTCAGA	GTGTGTACAGTGCTGTCGACT	60
Factor regulador de interferón 2	*irf2*	AIY9Y0S	GCTAATTTCCGCTGTGCTATGAA	TGATGCTTTTGTCCTTCACTTCCT	64
Factor regulador de interferón 3	*irf3*	AI0IW60	CGACCTCAAGAGATTCCCAAACTG	GCCAACATTCCTCCTATAAAGTCCTT	74
Factor regulador de interferón 7	*irf7*	AI1RVC8	CGAGATGGCGCAGATGGAA	GGCTGTTGTGTGACATCTGTAGAC	74
Factor regulador de interferón 8	*irf8*	AI20TJG	CCTCTACAGTTCGGACAGCAT	CTGGCGGTCGTACTCTATGAG	70
Factor regulador de interferón 9	*irf9*	AI39RPO	CCCAATACTGGCCAAAATCACAGAT	GGCCAGCATTGGGCCAATA	76
Factor regulador de interferón 10	*irf10*	AI5IPVW	CTGCAGGGACAGGTTCCT	GGCAACGACACCGAGTTC	95

Tabla 2.5C: Oligos de vías del complemento diseñados para Open Array

Vías del Complemento					
Nombre del Gen	**Abrev.**	**ACC NO**	**Secuencia del F**	**Secuencia del R**	**pb**
C1q-proteína 4	*c1ql4*	AIHSOLP	CTACGCTTCTAACAGTGTGATCCT	AGCTGCACACACACCTCATC	60
Complemento C2, factor B	*c2*	AII1MRX	AGTCAGCTCCAGGCTCTGT	CCGCCGGCTGATCTCAT	60
Complemento C3	*c3*	AIKAKX5	GACCAAACACAGTGTGTGAAATTCT	CTGCAGTTCTCTTCAGCACATG	104
Complemento C4 tipo 1	*c4a*	AILJI4D	GACTGACAAAGAATGATCGTTTGCA	CTGAGCACTTCGACAATGTATCCAT	79
Complemento C4 tipo 2	*c4b*	AIMSHAL	ACGAGTGCAAAGGACGGAATAATAA	CGTTTGCGTTGTCACCTTCAA	106
Complemento C5	*c5*	AIN1FGT	GGTGACAGCCTCCATGGT	CGGACGTACCTTCTCCATACAGA	71
Complemento C9	*c9*	AIPADM1	TCAAATTCAGCCCAAGTGAGGATAC	CTCAGACCGACATTCACATTCAC	64
Factor H	*cfh*	AIQJBS9	GCAGCAGACAAACAGCAACAG	GCTGACTGGTACAGTATGAATTCCT	101

Tabla 2.5D: Oligos de interleuquinas y receptores de interleuquinas diseñados para Open Array

Interleuquinas y sus receptores					
Nombre del Gen	**Abrev.**	**ACC NO**	**Secuencia del F**	**Secuencia del R**	**pb**
Interleuquina 1B	*il1b*	AI6RN14	CGCAGAAAGTGACATGTTGAGATTT	GGAAGCGGGCAGACATGA	83
Interleuquina 6	*Il6*	AI70L8C	ACAATTTCCTGCAGAGATAAAAGTAAGCT	CAAGCCCTCAGGCCTACAATATTAA	106
Interleuquina 8 tipo b	*Il8b*	AI89KEK	GAAGGTGGAAATAATTCCTGGAAGCT	TGTCTTTTTCAGCGTGGCAATG	70
Interleuquina 8 tipo c	*Il8c*	AIAAZKD	GCCGGCGTTACTGTCTGAA	GACTGAGCTCGTCTGACTGTAG	97
Interleuquina 10	*il10*	AIBJXQL	CCGTCTTTGTGTTATTTCTCCAACAG	TGGAGTTCAGCTTTGTGATGTCA	78
Interleuquina 11a	*Il11a*	AICSVWT	CTGCAGAGCCATGAGGATACAT	CTATCCACTACATTGCACTCTGACA	73
Interleuquina 12 beta	*il12b*	AID1T21	ACGCAGTACAGATCCAACGC	CCTGACAGTGAACCCCTGATC	106
Interleuquina 15	*il15*	AIFAR89	ATCTCTGTAAATAATTGCATTTGTTTATATTTTGT	AAGGACGGCAAGGTTTGTCA	84
Interleuquina 17 c	*il17c*	AIGJQFH	AGGCTCGCTGTCTTTGCT	GACTTTCCGTCGGAGGATTCC	63
Factor de necrosis tumoral alfa	*tnfa*	AI6RN15	CCATAGGCAGCAAAGTGTCTCT	CCTGTCCGTCTGTCAACGT	87
Receptor 1 de la interleuquina 1	*iIl1rl1*	AIRR9ZG	CACCACCAAAGATCATGAGGGTATC	CTGGAGCCTGATGAGTTATAAACCT	84
Receptores CXC R2	*cxcr2*	AIS075O	CGGTGCTGTATGCCTTTGTG	TCCATGAGGCCGATCTTCCT	76
Receptor beta de la interleuquina 10	*il10rb*	AIT96BW	GAGAGAACCACCAATGAGGAAGAAG	CAGAGAAGATCAGCGACACCAA	64
Receptor alfa de la interleuquina 15	*il15ra*	AIVI4H4	TGTCCGTGTTCAGAAATTCGTCAT	GCCTCAAATATCCGTCCACACAT	103
Receptor alfa de la interleuquina 17	*il17ra*	AIWR2OC	GCGGTGAACTGTCCTCCTT	TCATTGGGAATTTGAAGTGGTCCTT	74

Tabla 2.5E: Oligos de patrones moleculares de asociación a patógenos diseñados para Open Array

Patrones Moleculares de Asociación a Patógenos (PAMP)					
Nombre del Gen	**Abrev.**	**ACC NO**	**Secuencia del F**	**Secuencia del R**	**pb**
Proteína de reconocimiento de péptidoglicano 1	*pglyrp1*	AI20TJH	GCAGGCTCTGATGGACACAT	CCTATTGAGTTGTGTCCAAGAGTGT	60
Receptor de células Toll 1	*tlr1*	AIRR9ZH	CCCCATCCTCAATGAACTTCTCTTG	GGGCAGCTCTTCAGGTTGTT	75
Receptor de células Toll 5S	*tlr5s*	AIS075P	CAAAGTTCATCGTTCGGTCCAAATG	TGTGCCCTGTGACCAAACA	84
Receptor de células Toll 8B	*tlr8b*	AIT96BX	GGTATGTCAGATTTGGCAGCGAA	TCAACCTCTCCGGAAATGGATTTT	75
Receptor de células Toll 9	*tlr9*	AIVI4H5	AGTACCTCCAGCTGAGGAAGAG	CGTCCTCATCCTGTTCCAGAAAAG	98
Lisozima G 1	*lyg1*	AIWR2OD	CCAAATGGAGGTGGACACACT	CGCCTTGGCAGAGGTGTT	64
Lisozima G 2	*lyg2*	AIX00UL	GGACTCAAAAAGAGGAGCATATAACGG	GAGTGTGGTTACCTCCTTTTGGATT	77
Lisozima G 3	*lyg3*	AIY9Y0T	GCCCTTATTGCTGGCATCATCT	TCCCCCACCGTCAATAAGAGTAT	66
Lisozima C 1	*lyc1*	AI0IW61	CAGATCAACAGCCGCTATTGG	GCTGATTCCACATGCATTTGAAGTG	66
Lisozima C 2	*lyc2*	AI1RVC9	CCTGAGCCAGTGGGAGTCT	GATCTGGAGGATGCCGTAGTC	85

Tabla 2.5F: Oligos de quimioquinas diseñados para Open Array

Quimioquinas					
Nombre del Gen	**Abrev.**	**ACC NO**	**Secuencia del Fw**	**Secuencia del Rv**	**pb**
Quimioquina CC K3	*cck3*	AIAAZKE	GAGAGGACTTGGGTTTTTGAGAAGA	GTTGTAGTGGCAGGCGTAATC	80
Quimioquina CC 25	*ccl25*	AIBJXQM	CGGGACAAAGGAGAGCAACTT	AATCCATGTGGTCCAGCGT	69
Quimioquina CXC 10	*cxcl10*	AICSVWU	GGAGAAATGTGTGAACCCAGAGT	CGCGCTGCTCTTTTTCTCT	75
Quimioquina CXC 13	*cxcl13*	AID1T22	GTCAACGGGCTTCTCTCTGA	TGAGGCTTCATGAGTCCTGTTTATG	72
Quimioquina CC 19	*ccl19*	AIGJQFI	GCGGCAGGAACCTGTGT	CGTACGCTCAGCTGTTTGAC	59
Quimioquina CC 20	*ccl20*	AIFAR9A	ACACCGTCCAGACCATAAACAC	CTCGGACGGTGGAAGATGATG	61

Tabla 2.5G: Oligos de antígenos de diferenciación diseñados para Open Array

Antígeno de Diferenciación					
Nombre del Gen	**Abrev.**	**ACC NO**	**Secuencia del F**	**Secuencia del R**	**pb**
CD8 Alfa	*cd8a*	AIHSOLQ	GTGCCAGCATTAAAAGCAACG A	GCAGTCACAACTTCCGCTCTT T	82
CD8 Beta	*cd8b*	AII1MRY	GGTTTGGTCGGAGGATTCACT	GTCGTAAAGGACAAGTCCAA CAGA	80
CD4	*cd4*	AIKAKX6	GACCTCAGGCTGCAATGGT	TGAGCAGAGTGATGGACAGA CT	65

Tabla 2.5H: Oligos de genes de referencia diseñados para Open Array

Genes de Referencia					
Nombre del Gen	**Abrev.**	**ACC NO**	**Secuencia del F**	**Secuencia del R**	**pb**
Ubquitina UB52	*ubi*	AILJI4E	AGGCTAAGATTCAGGATAAGGA AGGA	TCCATCCTCCAGCTGTTTGC	80
Factor de elongación Eucariótico 1A1	*eef1a1*	AIMSHAM	CTGTTGGACGTGTCGAGACT	TCTCCACAGACTTGACCTCA GT	93
Gliceraldehído-3-fosfato deshidrogenasa 2	*gapdh2*	AIN1FGU	TCATTCCTGAGCTCAACGGTAA G	AGGTCAACCACTGACACATC AG	76

Tabla 2.6: Oligos diseñados para la clonación de secuencias

Gen		**Secuencia (5´a 3´)**	**pb**	**Programa termociclador**				
GRs Falso halibut del Japón (*Paralichthys olivaceus*) Acc No AB013444.1	F	TTC AGG ATG CCA CTA CGG	54	95 ˚C 4 ´	95 ˚C 30 ´´	55 ˚C 15 ´´	72 ˚C 30 ´´	72 ˚C 30 ´´
	R	ACA TCA GAA AGA GCC AGG AG	8	x1	x40			x1

Tabla 2.7: Oigos diseñados para amplificar fragmentos de DNA con los kits 3´y 5´ RACE

	GR1	**GR2**
F1	GTGGAAGGGCAGCATAATTACTTG	GGGGAGGAACGACTGCATCA
F2	GCCTGCCGCTTCCACAAG	CAACGGGATCATCAGTGC
F3	GGCAACCAGCCCAAGGTG	CTCGTGCCCACCATGCTGT
F4	CGCCACCTCCACCAGTTG	TCTGCCAGGCTTCCGTAACC
	AUAP	GGAACAGCATCGACTAGTAC
R1		TCGGGAGGACGGGAACC
R2	AGGCTTCAACTGGTGGAG	GGGAACCGGCATGATGTCAGG
R3	CGGGTGTCACCTTGGGCTGGTTG	GGCAGGCCGGACAGTTCTT
R4	GGTTTTGCGAGCTTCCAAGTT	CGTTCCTCCCCGCACAGAG
R5	CGGGCAGTTCTTTCTCCTGA	CACGAGCTGGGGCATCC
R6	TCACGCCTGGGGTACAGAGC	CCGGCATGATGTCAGGCACT
R7	TCAAGGCCGGGAGTGTCAA	ATTCCAGCTTGAAGACATTT
R8		CCTGCGGATCTTATCGATGA
	AAP	GGCCACGCGTCGACTATGAC

2.6 Análisis estadístico

Las medias y desviaciones estándar (SD) se calcularon para cada parámetro medido, excepto para los valores de cortisol, donde los valores se representaron como una media más el error estándar (SE). Para realizar los análisis estadísticos se siguieron las normas descritas por Sokal y Rohlf (1995).

Los datos referidos al crecimiento del pez y la composición de ácidos grasos de la experiencia I fueron sometidos a un análisis de varianza (ANOVA) de una vía. Cuando los valores mostraban una F significativa las medidas individuales se compararon mediante un test múltiple de Duncan. Para las varianzas que no se distribuyeron normalmente se aplicó el test no paramétrico de Kruskall-Wallis sobre los datos.

Para el análisis estadístico de los datos obtenidos a partir de la RT-PCR realizada en el Capítulo 3 se utilizó un análisis de varianza (ANOVA) de dos vías, con la dieta y el estrés como factores fijos y un ANOVA de una vía, utilizándose la dieta como factor fijo, y los animales no estresados alimentados con 100FO como valor de referencia, además de un T-test para muestras independientes con el fín de encontrar diferencias significativas, entre animales estresados y no estresados en cada una de las dietas.

Mientras que, para el análisis estadístico de los datos obtenidos por medio de RT-PCR expuestos en el Capítulo 4 se realizó un ANOVA simple, utilizando como factor fijo la Tª.

Y por último para el análisis estadístico de los datos adquiridos a partir de RT-PCR en los Capítulos 5 y 6, se utilizó un análisis multivariante de varianza (MANOVA), para evaluar el efecto de la dieta, a la vez que un MANOVA de dos vías, para observar el efecto del estrés (utilizándose la dieta y el estrés como factores fijos), además de utilizarse una "t de student" para comparar los grupos de control y de estrés en cada una de las dietas.

En cada uno de los casos, el análisis estadístico se llevó a cabo por medio del programa SPSS para Windows versión 11.0, consideraronse significativos los valores de $P<0,05$ y

transformando los datos a logaritmo en caso de no cumplir con la normalidad y homogeneidad de varianza.

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CAPÍTULO 3

"DIETARY VEGETABLE OIL AND GLUCOCORTICOID RECEPTOR-RELATED GENES IN SENEGALESE SOLE"

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3. Total substitution of fish oil by vegetable oils in Senegalese sole (*Solea senegalensis*) diets: effects on fish performance, biochemical composition, and expression of some glucocorticoid receptor-related genes

3.1 Abstract

To study the substitution of fish oil by vegetable oils in fish diets, juveniles Senegalese sole (*Solea senegalensis*) were fed diets (56 % crude protein, 12 % crude lipid) containing either linseed (100LO) or soybean (100SO) oils in comparison with a 100 % fish oil-based diet (100FO) for 90 days. Samples of muscle, liver, and intestine were collected for biochemical analysis and for glucocorticoid receptor related genes, including GR1 and GR2, and the associated heat shock proteins HSP70, HSP90AA, and HSP90AB. Besides, basal levels of plasma cortisol were also determined. After the feeding period, a stress test, consisting on 5 min of net chasing, was applied to a selected population of each dietary group. Total replacement of fish oil by vegetable oils did not induced changes in fish growth and performance, but affected fatty acid profile of muscle, liver, and intestine, reflecting those tissues the characteristic fatty acids of each type of dietary oil. A tendency to conserve the ARA/EPA ratio could be observed in the different tissues, despite of the level of these fatty acids in diet. Chasing stress induced an increase of muscle GR1 and a reduction in intestinal GR2 relative expressions at any of the experimental diets assayed. In liver, chasing stress induced an increase in both GR1 and GR2 gene expression in fish fed fish oil diets. Similarly, chasing stress induced an increase of muscle HSP70 and decrease of HSP90AB in liver at any of the experimental diet assayed. Besides, vegetable oils decreased the expression of HSP70 in intestine, being the relative expression of liver HSP90AA increased by the inclusion of linseed oil in the diet, at any of the experimental conditions assayed.

3.2 Introduction

Fish oil (FO) in aquafeeds needs to be replaced by other types of oils, from marine or terrestrial origin, due to the limited availability of FO oil to cover the increased demand (Tacon and Metian, 2008). Thus, in the last years, there is an increasing effort to use sustainable alternative lipid sources, including different either single vegetable oils (VO) or their blends (Turchini *et al.,* 2009). Although fish growth and dietary utilization seems to be not substantially affected by 60–75 % replacement of FO with alternative lipid sources in almost finfish species studied (if essential fatty acid (EFA) requirements are covered), total substitution of FO by VO may induce a negative effect on growth of most marine fish species studied. Moreover, those negative effects are influenced by several factors such as water temperature, the type of oil used, and the amount of fish meal used in the diet or fish size (Sales and Glencross, 2011).

Vegetable oils are abundant in n-6 and n-9 C18 PUFAS, but are poor sources of long-chain polyunsaturated fatty acids (LC-PUFA).Although they have been used in diets for several marine fish species (Reviewed by Turchini, *et al.,* 2009; Sales and Glencross, 2011), themajor limitation to the use of vegetable oils as alternative lipid sources for aquafeeds is the risk of reduction of the health promoting effects provided by LC-PUFA (Turchini *et al.,* 2009). Moreover, the use of certain vegetable oils in diets could have a negative impact on fish health or welfare (Montero and Izquierdo, 2010). Indeed, the use of certain VO has been reported to reduce pathogen resistance and alter several immune system-related parameters (recently reviewed by Montero and Izquierdo, 2010; Montero *et al.,* 2010) including eicosanoid production (Bell *et al.,* 1995; Ganga *et al.,* 2005).Moreover, VO in fish diets have been found to affect stress resistance in different marine species (Montero *et al.,* 2003; Jutfelt *et al.,* 2007) by altering post-stress circulating levels of plasma cortisol (Ganga *et al.,* 2011a), the in vitro release of cortisol from inter-renal cells (Ganga *et al.,* 2011b). Finally, they may cause alterations in the morphology and physiology of target tissues (Caballero *et al.,* 2003, 2004,

2006), even affecting the maintenance of epithelial barrier functions and the regulation of the inflammatory response in the gastrointestinal tract (Oxley *et al.,* 2010).

During a stressful situation, cortisol, the main corticosteroid in fish, is released from interrenal cells into the circulatory system following an ACTH (Adrenocorticotropic hormone) induced response, entering cells cytosol by passive diffusion (Wendelaar-Bonga, 1997). Cortisol effects in the cell are mediated by the intracellular glucocorticoid receptors (GR), which are members of the nuclear receptor superfamily and act as steroid ligand dependent transcription factors, to control and regulate gene expression (Mommsen *et al.,* 1999). Within the cytosol, GR is included in a non-activated form within a multiprotein heterocomplex, together with several heat shock proteins (HSPs) such as HSP70 and HSP90, whose functions are the assembly, functionality, and transport of GR (Pratt and Toft, 1997). HSPs are associated with the GR until a hormone signal, such as cortisol, induces a conformation with lower affinity for HSPs. This action enables the GR to target sites of transcription activation.

Once GR is activated, dissociates from the HSPs dimers and the nuclear localization signals of GR are unmasked. Then, the receptor is capable to translocate into the nucleus and bind to a specific DNA region, the glucocorticoid response element (GRE), to regulate transcription of glucocorticoid responsive genes (Vijayan *et al.,* 2005; Aluru and Vijayan, 2009). On the other hand, activated HSP90 and HSP70 play a fundamental role on the folding and assembling of other cellular proteins, and they are involved in the regulation of kinetic partitioning between folding, translocation and aggregation, as well as in immune, apoptotic, and inflammatory processes (Roberts *et al.,* 2010). Besides, HSPs produced in response to stressful conditions help organisms to survive during conditions of stress, playing a critical role on the host defenses against neoplasia and chronic pathogens (Roberts *et al.,* 2010).

Although some evidences on the effect of certain nutrients on stress resistance of Senegalese sole (*Solea senegalensis*) have been recently reported (Alves-Martins *et al.,* 2011b), only few works focus on the role of dietary lipids on the regulation of stress response of this species. Those studies are centered on the modulatory role of essential fatty acids such as

eicosapentaenoic acid (EPA) (20:5n-3), docosahexaenoic acid (DHA) (22:6n-3), or arachidonic acid (ARA) (20:4n-6) on the whole post-larvae cortisol after stressful conditions (Alves-Martins *et al.,* 2011b). However, very little is known of the effect of dietary VO on juvenils response to stressful stimulus and on the expression of glucocorticoid- related genes. Thus, the objective of this study was to determine the effects of total FO substitution by VO in Senegalese sole juveniles in terms of fish performance, fatty acid composition, and certain welfare indicators such as circulating plasma cortisol concentration or expression of stress-related genes.

3.3 Materials and methods

3.3.1 Experimental diets

Three isonitrogenous (56 % crude protein), isolipidic (12 % total lipids), and isoenergetic diets were formulated: a control diet based on fish oil (100FO) (Peruvian anchovy oil) as the sole lipid source, and two vegetable oil diets where 100 %of the fish oil was replaced by either linseed oil (100LO) or soybean oil (100SO). Each diet was tested in triplicate. Ingredients and proximate composition and selected fatty acids contents are shown in Tables 3.1 and 3.2.

Table 3.1: Main ingredients and proximate composition from the experimental diets fed to Senegalese sole juveniles for 12 weeks

Diets/Ingredients	100FO	100LO	100SO
Fish meal	660	660	660
Wheat gluten	15	15	15
Starch	6.5	6.5	6.5
Fish Oil	8	-	-
Linseed oil	-	8	-
Soybean oil	-	-	8
Vitamin mix	2	2	2
Mineral mix	2	2	2
CMC	0.5	0.5	0.5
Biochemical composition (% D.W.)			
Crude protein	56.7 ± 2.2	56.8 ± 2.2	56.6 ± 2.2
Crude lipids	12.3 ± 2.2	13.0 ± 2.2	12.6 ± 2.2
Ash	10.1 ± 2.2	10.5 ± 2.2	10.3 ± 2.2

Table 3.2: Fatty acid composition of the experimental diets fed to Senegalese sole juveniles for 12 weeks (g $100g^{-1}$ total identified fatty acids)

	Diets		
Fatty acids	**100FO**	**100LO**	**100SO**
14:0	4.9	3.19	3.34
14:1n-7	0.0	0.0	0.0
15:0	0.3	0.21	0.3
16:0ISO	0.0	0.02	n.d.
16:0	19.9	15.7	15.9
16:1n-7	6.0	3.6	2.9
16:1n-5	0.2	0.2	0.2
16:2n-6	0.9	0.53	0.3
16:2n-4	0.0	0.61	0.41
17:0	0.9	0.04	0.42
16:3n-4	0.3	0.06	0.04
16:4n-3	1.3	1.00	0.65
18:0	4.1	4.96	3.01
18:1n-9	18.3	19.04	11.97
18:1n-7	3.0	2.1	1.4
18:1n-5	0.1	0.1	0.0
18:2n-6	6.1	9.6	36.8
18:3n-6	0.2	0.1	0.1
18:3n-4	0.2	0.0	0.0
18:3n-3	2.1	20.5	6.8
18:4n-3	1.0	0.6	0.5
18:4n-1	0.4	0.1	0.1
20:0	0.2	0.2	0.2
20:1n-9	1.4	0.5	0.3
20:2n-9	0.1	0.0	0.0
20:4n-6	0.8	0.5	0.6
20:3n-3	n.d.	0.1	0.0
20:4n-3	0.8	0.3	0.4
20:5n-3	12.0	8.8	5.6
22:1n-11	0.4	0.2	0.1
22:5n-3	3.1	1.3	2.6
22:6n-3	11.1	5.7	4.9
Total saturates	30.3	24.9	23.0
Total monoenes	29.5	25.8	17.4
∑n-3	31.4	38.3	21.5
∑n-6	7.3	10.2	37.5
∑n-9	19.8	19.6	12.4
∑n-3 LC-PUFA	27.0	16.2	13.6
ARA/EPA	0.1	0.1	0.1
EPA/DHA	1.1	1.5	1.1
n-3/n-6	4.3	3.8	0.6

n.d.non-detected

3.3.2 Experimental fish and samples collection

Two hundred and seventy Senegalese sole juveniles of 3.50 ± 0.24 g initial body weight were randomly distributed into 9 indoor fiberglass tanks (45 L of capacity and 60x40 cm of surface) of 30 fish per tank. Tanks were supplied with filtered seawater at a temperature of 21.6–22.8°C and natural photoperiod (around 12L:12D). Water dissolved oxygen values ranged 6.20 ± 0.70 g -1. Fish were manually fed until apparent satiation with the experimental diets for 12 weeks (twice daily, 6 days a week). Feed intake was daily determined and growth parameters were calculated at days 0, 30, 60, and 90. Food conversion ratio (FCR), defined as the amount of food ingested by the generated biomass, and specific growth rate (SGR) defined as [(ln final weight - ln initial weight)/number of days] 9 100 were also calculated.

At the end of the experimental period, ten fish per tank (30 per treatment) were killed by an overdose of anesthetic, and liver and muscle for biochemical and fatty acid determinations were obtained by dissection. Samples were kept at -80°C until analysis. Blood from 6 fish per tank (18 per dietary treatment) was collected by caudal sinus puncture with a 1-ml plastic syringe and was placed into a heparinized Eppendorf tube and was centrifuged at 800 g during 10 min to obtain plasma samples. Plasma samples were kept at -80°C until analysis. From the remaining fish, 9 fish per treatment were killed by overdose of anesthetic and were used to obtain samples of liver, muscle, and intestine for the determination of stress-related gene expression. These samples were quickly kept in RNA later and frozen at -80°C until gene expression analysis.

A chasing stress test was also conducted at the end of the experimental period, and 9 animals from each experimental diet were subjected to a 5 min net chasing. After this stressful situation, samples of liver, muscle, and intestine were obtained for determination of the relative expression of stress-related genes and were kept in RNA later and frozen at -80ºC until analysis.

3.3.3 Biochemical and fatty acid composition analysis

Biochemical and fatty acid composition analyses of diets and selected tissues were conducted following standard procedures (AOAC, 1995). Dry matter content was determined after drying the sample in an oven at 105°C to constant weight, ash by combustion in a muffle furnace at 600°C for 12 h, protein content (N x 6.25) was determined by Kjeldahl method, and crude lipid was extracted following the Folch method (Folch *et al.,* 1957). Fatty acids from total lipids were prepared by transmethylation (Christie, 1982) and separated by gas chromatography (Izquierdo *et al.,* 1992), being quantified by flame ionizator detector (FID) and identified by comparison with external standards (EPA 28, Nippai, Ltd. Tokyo, Japan). All analyses were conducted by triplicates.

3.3.4 Welfare indicators: plasma cortisol and expression of stress-related genes

Plasma cortisol concentration was determined by radio-immunoassay using the trypsin–antitrypsin method as previously described for marine fish species (Rotllant *et al.,* 2001).

Tissues sampled from each tank were pooled and total RNA was extracted from approximately 100 mg of those pools, using 1 ml TRI Reagent (SIGMA Aldrich, Saint Louis, MO, USA). Chloroform and isopropanol were used for separation and precipitation, respectively. The pellets were hydrated with 100 µl of Milli-Q sterile water, previously treated with 0.1 % DEPC and kept at -80°C until analysis. Total RNA concentration, purity, and quality were measured by NanoDrop 1000 Spectrophotometer (Thermo Scientific). The reverse transcription (RT) reactions were carried out in 20 µl volumes with iScript TM cDNA Synthesis Kit (Bio-Rad Hercules, CA, USA) containing 1 µg of total RNA. At the end of the RT reactions, all cDNA populations were kept at -20°C.

3.3.5 Real-time PCR (RT-PCR)

All PCRs were performed in i-cycler thermocycler with optical module (Bio-Rad Hercules, California) using 12.5 µl Brillant SYBR Green QPCR Master Mix (Bio-Rad Hercules, California), 1 µl of a 1:5 dilution of the cDNA, and the amount previously optimized of each primer (Table 3.3) in a final volume of 25 µl. Cycling conditions consisted of desnaturation and enzyme activation for 7 min at 95ºC, followed by 40 cycles at 95ºC for 15 s and 70ºC for 30 s. Within the oligo used, Heat Shock Protein (HSP) 90AA, 90AB, 70 and ubiquitin were previously described by Infante *et al.* (2008), Manchado *et al.* (2008), and Salas-Leiton *et al.* (2010). GR1 (Acc No AB614369.1) and GR2 (Acc No AB614370.1) sequences (Table 3.4) have been cloned and sequenced partially from larval cDNA of Solea senegalensis, using 3´RACE Rapid Amplification of System for cDNA Ends (Invitrogen TM) and 5´RACE System for Rapid Amplification of cDNA Ends (Invitrogen TM) cloning and subsequent sequencing.

Table 3.3: Primers of the different genes used

Target	Primer	Sequence 5´-3´	Amplicon (bp)
GR1	F	CCTGCCGCTTCCACAAGTGTCTGATG	130
	R	TTCAACTGGTGGAGGTGGCGGTGT	
GR2	F	TCAGCGTGGAGTTCCCGGAGATG	92
	R	GGTGGAACAGCAGCGGCTTGATG	
UBIQUITIN	F	AGCTGGCCCAGAAATATAACTGCGACA	93
	R	ACTTCTTCTTGCGGCAGTTGACAGCAC	
HSP 70	F	GCTATACCAGGGAGGGATGGAAGGAGGG	119
	R	CGACCTCCTCAATATTTGGGCCAGCA	
HSP 90 AA	F	GACCAAGCCTATCTGGACCCGCAAC	105
	R	TTGACAGCCAGGTGGTCCTCCCAGT	
HSP 90 AB	F	TCAGTTTGGTGTGGGTTTCTACTCGGCTTA	148
	R	GCCAAGGGGCTCACCTGTGTCG	

The primers were designed using Oligo v 6.89 program (Medprobe). Relative gene expression was estimated by the Δ–Δ method (Livak and Schmittgen, 2001) using ubiquitin

(Acc No AB291588.1) as housekeeping gene after check that the expression of this gene was not influenced by any of the experimental treatments used, as previously reported and proposed by several authors working on Senegalese sole (Infante *et al.,* 2008), unstressed fish fed 100FO diet considered as control or reference value.

Table 3.4: Glucocorticoid receptor (GR) 1 and 2 sequences

GR1

1 gtgaagaaag aaaaagatga tgactttatt cagctctgta ccccaggcgt gatcaaacag
61 gagaagacat ctgctggcca gattaattgt cgaataagtg gctcatcctc cacagttttg
121 cccaactcaa accccatttc aatctgtggt gtcagcacat caggaggacc gtcctaccac
181 tttggagtca acacaagtca aagtggtgaa gctcagcagc agaaggatca gaagccagtg
241 tccaaagtgt ttcttccagt gacaacaatc agtggaaatt ggagcagagg ccagggtgca
301 gttagcgcgg gtcttatgca gagagcaagt gactgcttct caagctcccc taccttctcc
361 accagctttg catgttctac ctccagacaa gaaggggcca ctgctacatc ctctggccag
421 gtaaagagtg caactcataa aatctgccaa gtgtgctccg atgaggcttc aggctgccac
481 tatggcgttc tcacgtgcgg cagctgtaag gtcttcttca agagagcggt ggaagggcag
541 cataattact tgtgtgctgg gaggaacgac tgcataatag acaagatcag gagaaagaac
601 tgcccggcct gccgcttcca caagtgtctg atggcaggca tgaacttgga agctcgcaaa
661 accaagaaga tgaaccgtta taagggcaac cagcagggca accagcccaa ggtgacaccg
721 ccacctccac cagttgaagc ctgcaccctg gttcccaagg gtatgcctca actggttccc
781 acaatgctgt ccctgctgaa ggccatcgag ccagagacca tctacgccgg ctacgacagc
841 accctgccca tcacctccac acgcctcatg acgaccctga accgattggg cggccgacag
901 gtcatctcag ctgtcaagtg ggccaaatct ctgccaggtt tcaggaacct gcacctggat
961 gaccagatga ccctgttgca gtgctcatgg ctcttcctca tgtctttcag tctgggctgg
1021 aggtcttatc aacagagcaa tggcaacatg ctctgttttg cacctgacct cgtcatcaat

GR2

1 atgccggttc ccgtcctccc gaggatgccc cagctcgtgc ccaccatgct gtctgtgctc
61 aaggccatcg agccagagat catctactcg ggctacgacg gcacgctgcc ggacaactcc
121 tcgcgcctca tgagcacgct caacaggctg gggggtcagc aggtcatctc tgcagtcaag
181 tgggccaagt ctctgccagg cttccgtaac ctgcacctgg acgaccagat gactctgctg
241 cagtgctcct ggctctttct gatgtcgttc ggtctgggat ggaggtcgta cgagcagtgc
301 aacggcagta tgctctgctt cgcccccgac ctcgtcatca acaaagagcg tatgaagctg
361 cccttcatga acgaccagtg tgagcaaatg ctgaaaatct gcaacgagtt tgtccgactg
421 caagtgtcct acgacgagta cctgtgtatg aaggtgctgc tgctgctcag tacagtaccc
481 aaagatggcc tgaagagcca gggggtgttt gacgagatca ggatgtcgta catcaaggag
541 ctcgggaaag ccatcgtcaa gagggaggag aacgccagtc agaactggca gcgcttctac
601 cagctaacta agctattgga ctccatgcag gagatggtgg aaggccttct acagatctgt
661 ttctacacct ttgtgaataa aaccctcagc gtggagttcc cggagatgct cgcagagatc
721 atcaccaacc agataccaaa attcaaagac gggagcatca agccgctgct gttccaccag
781 aaaggactgc cataaactgt gaagcaatga ctttaaaaaa aataaataaa tcctgcaatc
841 atgcaagagc ac

3.3.6 Statistical analysis

All data were tested for normality and homogeneity of variance. Means and standard deviations (SD) were calculated for each parameter measured except for cortisol values where values are represented as Mean plus standard error (SE). Statistical analyses followed methods outlined by Sokal and Rohlf (1995). Data of fish growth and tissue fatty acid composition were submitted to a one-way analysis of variance (ANOVA). When F values showed significance, individual means were compared using Duncan multiple comparison test. Significant differences were considered for $P<0.05$. If the variances were not normally distributed, the Kruskall–Wallis nonparametric test was applied to the data. Two way ANOVA was used for relative gene expression data, using diet and stress as fixed factors, and using unstressed 100FO as a reference value. Analyses were performed using SPSS software (SPSS for windows 11.0).

3.4 Results

At the end of the experimental period, the different diets fed did not induce significant differences in fish survival, being this parameter around 85 % for all the experimental groups (Table 3.5). However, the use of soybean oil as the main source of lipid induced a lower (P\0.10) fish growth in terms of final body weight (Table 3.5). No significant differences were found in SGR, which ranged from 1.51 for fish fed 100FO diet to 1.29 for fish fed 100SO diet. There were no significant differences among experimental groups in diet utilization (FCR ranging from 1.58 for 100FO fish to 1.66 for 100SO fish) or hepatosomatic index (around 0.60) (Table 3.5).

Inclusion of vegetable oils did not affect lipid content in liver (around 9 % fresh weight), muscle (around 2.5 % fresh weight), or intestine (around 3 % fresh weight) (Table 3.5). However, as expected, dietary oil determined the fatty acid profile of Senegalese sole. Thus, linoleic acid (18:2n-6) was accumulated in animals fed 100SO diet in all the tissues studied

(Tables 3.6, 3.7 and 3.8), whereas alpha-linolenic acid (18:3n-3) increased in tissues of fish fed 100LO-based diet (Tables 3.6, 3.7 and 3.8). Fish fed 100FO showed the highest values of saturated and LC-PUFA in the different tissues studied (Tables 3.6, 3.7 and 3.8). Among the different LC-PUFA, dietary vegetable oil significantly reduced the amount of EPA and ARA on the different tissues studied, but those values obtained for DHA were not significantly different among treatments for muscle and intestine, despite lowering its absolute value, in animals feeding with 100 % VO-based diets. Other fatty acids were affected by the type of diet, being 20:3n-3 significantly increased in the different tissues of animals fed on the vegetable oil-based diets when compared with fish oil-based diet.

Table 3.5: Senegalese sole growth, survival rate and plasma cortisol fed experimental diets. Mean ± SD, except for plasma cortisol values, represented as mean ± SE

Diets	100FO	100LO	100SO
Initial body weight (g)*	3.5 ± 0.2	3.5 ± 0.2	3.5 ± 0.3
Final body weight (g) **	13.7 ± 1.3[a]	13.2 ± 1.7[a]	11.0 ± 3.2[b]
SGR***	1.5 ± 0.2	1.5 ± 0.3	1.3 ± 0.2
FCR***	1.6 ± 0.3	1.6 ± 0.4	1.7 ± 0.5
Survival rate* (%)**	86.5 ± 2.6	85.3 ± 3.1	86.5 ± 3.0
HSI¥	0.6 ± 0.0	0.6 ± 0.1	0.6 ± 0.1
Lipid content (% W.W.)			
Muscle	2.3 ± 0.9	2.6 ± 0.4	2.5 ± 0.9
Intestine	2.8 ± 0.7	3.2 ± 0.6	3.0 ± 0.7
Liver	8.4 ± 1.3	8.9 ± 1.5	9.4 ± 1.1
Plasma cortisol (ng/ml)¥¥	8.4 ± 1.3	12.5 ± 1.5	10.0 ± 1.6

* n=30x3; ** n= 25x3; ***n=3; ¥ n=10x3; ¥¥ n=6x3. Different letters within a row denote significant differences at P<0.10

Dietary oils did not induce any significant change in basal levels of circulating plasma cortisol, although fish fed 100LO diet showed the highest (but not significantly different) value of circulating plasma cortisol concentration (ranging from 8.43 to 12.54 ng cortisol ml-1 plasma in fish fed 100FO or 100LO, respectively) (Table 3.5).

Regarding the expression of stress-related genes in muscle, relative expression of GR1 was significantly higher in stressed fish when compared with non-stressed animals, for any of the experimental diets assayed (Fig. 3.1 A). The combined effect of dietary soybean oil inclusion with the stress challenge showed a tendency to increase the relative expression of GR2 in comparison with non-stressed fish fed 100FO diet (Fig. 3.1 A).

In intestine, despite stressed fish tended to show lower relative values of GR1 expression than 100FO fed fish, no significant differences were found (Fig. 3.1 B). The relative expression of GR2 was significantly lower in intestine of stressed fish when compared with non stressed animals, at any of the experimental diets assayed with the 100FO as control diet, whereas no dietary effect was found on intestine GR2 relative expression (Fig. 3.1 B).

Dietary oils did not significantly affect the relative expression of GR1 and GR2 in liver (Fig. 3.1 C), although the relative expression of GR1 in fish fed 100SO diets showing the highest (but not significant) values. Chasing stress induced a significant increase in the relative expression of both GR1 and GR2 in fish fed 100FO (Fig. 3.1 C).

Muscle HSP70 relative expression (Fig. 3.2 A) was significantly higher in all stressed animals when compared with unstressed fish at any of the dietary treatments assayed. The relative expression of HSP90AB was significantly lower in muscle of all experimental conditions at experimental diets assayed when compared with fish fed 100FO diet except for 100SO diet in stressed fishes (Fig. 3.2 A). The relative expression of muscle HSP90AA showed a tendency to increase at any of the dietary treatments assayed subjected or not to a stress situation (Fig. 3.2 A).

In intestine, all dietary treatments showed significantly lower relative expression of HSP70 subjected or not to a stress situation when compared with non-stressed fish fed 100FO (Fig. 3.2 B). The use of vegetable oils and the stressful situation tended to down-regulate HSP90AA gene in intestine (Fig. 3.2 B).

In liver, there was not a significant effect of chasing stress on HSP90AA relative expression, but a significant increase in the relative expression of fish fed linseed oil-based diets either under stressful or non-stressful situations was detected (Fig. 3.2 C). Besides, no effect of diet was detected for liver HSP90AB relative expression, but the stressed animals showed a significant decrease when they were compared with non-stressed animals (Fig. 3.2 C).

Table 3.6: Muscle fatty acid composition of fish fed the different experimental diets (g $100g^{-1}$ fatty acids identified)

	Diets		
Fatty acids	**100FO**	**100LO**	**100SO**
14:0	3.3 ± 0.1	2.2 ± 0.3	2.1 ± 0.2
14:1n-7	0.1 ± 0.0	n.d.	n.d.
15:0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
16:0ISO	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0
16:0	19.0 ± 1.2	16.6 ± 1.7	16.5 ± 2.1
16:1n-7	4.3 ± 0.4	3.1 ± 0.4	3.0 ± 0.8
16:1n-5	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
16:2n-6	0.4 ± 0.1	0.3 ± 0.0	0.3 ± 0.0
16:2n-4	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
17:0	0.4 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
16:3n-4	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
16:4n-3	0.3 ± 0.0	0.2 ± 0.1	0.2 ± 0.0
18:0	3.4 ± 0.2	3.8 ± 0.2	3.2 ± 0.1
18:1n-9	28.1 ± 2.1^{a}	20.4 ± 2.4^{b}	29.2 ± 2.7^{a}
18:1n-7	5.7 ± 0.7^{a}	1.9 ± 0.5^{b}	2.5 ± 0.4^{b}
18:1n-5	0.3 ± 0.1	0.1 ± 0.0	n.d.
18:2n-6	6.6 ± 1.0^{a}	9.4 ± 1.0^{a}	22.3 ± 0.8^{b}
18:3n-6	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
18:3n-4	0.1 ± 0.0	0.0 ± 0.0	n.d.
18:3n-3	2.0 ± 0.3^{a}	20.4 ± 3.1^{b}	3.8 ± 0.2^{c}
18:4n-3	0.9 ± 0.1	0.8 ± 0.1	0.5 ± 0.1
18:4n-1	0.1 ± 0.0	0.1 ± 0.0	n.d.
20:0	0.2 ± 0.0^{a}	0.1 ± 0.0^{b}	0.2 ± 0.0^{a}
20:1n-9	3.5± 0.5	2.4 ± 0.7	2.6 ± 1.0
20:2n-9	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:4n-6	0.5 ± 0.0^{a}	0.4 ± 0.1^{ab}	0.3 ± 0.1^{b}
20:3n-3	0.1 ± 0.0^{a}	0.8 ± 0.0^{b}	0.4 ± 0.0^{c}
20:4n-3	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
20:5n-3	5.6 ± 0.7^{a}	4.2 ± 0.6^{ab}	3.1 ± 0.7^{b}
22:1n-11	2.4 ± 0.2	1.8 ± 0.3	1.8 ± 0.3
22:5n-3	0.9 ± 0.1	0.6 ± 0.1	0.5 ± 0.0
22:6n-3	11.0 ±1.9	8.7 ± 1.8	6.2 ± 2.8
∑saturates	26.5 ± 3.3	23.2 ± 3.8	22.4 ± 3.7
∑monoenes	44.4 ± 3.5^{a}	30.1 ± 3.9^{b}	39.3 ± 4.2^{ab}
∑n-3	20.9 ± 3.1^{a}	36.0 ± 4.1^{b}	15.1 ± 3.8^{a}
∑n-6	7.4 ± 1.1^{a}	10.0 ± 1.2^{a}	22.8 ± 2.3^{b}
∑n-9	31.8 ± 2.2^{a}	22.9 ± 1.9^{b}	31.9 ± 1.6^{a}
∑n-3 LC-PUFA	17.7 ± 2.5^{a}	14.5 ± 2.0^{ab}	10.5 ± 1.0^{b}
ARA/EPA	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
EPA/DHA	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
n-3/n-6	2.8 ± 0.1^{a}	3.6 ± 0.2^{b}	0.7 ± 0.0^{c}

Different letters within a row denote significant differences ($P<0.05$) n=10x3. n.d. non-detected

Table 3.7: Liver fatty acid composition of fish fed the different experimental diets (g $100g^{-1}$ identified fatty acids)

	Diets		
Fatty acids	**100FO**	**100LO**	**100SO**
14:0	4.7 ± 0.3^{a}	2.1 ± 0.3^{b}	1.8 ± 0.2^{b}
14:1n-7	0.0 ± 0.0	n.d.	n.d.
15:0	0.2 ± 0.1^{a}	0.1 ± 0.0^{b}	0.1 ± 0.0^{b}
16:0ISO	n.d.	n.d.	n.d.
16:0	19.0 ± 1.6^{a}	13.6 ± 1.7^{b}	15.5 ± 2.1^{ab}
16:1n-7	8.3 ± 1.2^{a}	3.0 ± 0.4^{b}	3.6 ± 0.8^{b}
16:1n-5	n.d.	n.d.	0.1 ± 0.0
16:2n-6	0.8 ± 0.1^{a}	0.2 ± 0.0^{b}	0.3 ± 0.0^{b}
16:2n-4	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
17:0	0.9 ± 0.1^{a}	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}
16:3n-4	n.d.	n.d.	n.d.
16:4n-3	1.0 ± 0.1^{a}	0.1 ± 0.1^{b}	0.1 ± 0.0^{b}
18:0	5.6 ± 0.4	4.8 ± 0.2	5.5 ± 0.1
18:1n-9	18.9 ± 3.4	22.2 ± 2.4	24.5 ± 2.7
18:1n-7	3.8 ± 1.0	3.0 ± 0.5	3.1 ± 0.4
18:1n-5	0.2 ± 0.1^{a}	0.1 ± 0.0^{b}	0.1 ± 0.0^{b}
18:2n-6	5.6 ± 1.5^{a}	10.2 ± 1.0^{b}	27.7 ± 0.8^{c}
18:3n-6	0.3 ± 0.0^{a}	0.2 ± 0.0^{a}	2.1 ± 0.0^{b}
18:3n-4	0.3 ± 0.0^{a}	0.1 ± 0.0^{b}	0.1 ± 0.0^{ab}
18:3n-3	1.0 ± 0.3^{a}	24.3 ± 3.1^{b}	2.5 ± 0.3^{c}
18:4n-3	1.6 ± 0.1^{a}	0.8 ± 0.1^{b}	0.5 ± 0.1^{b}
18:4n-1	0.5 ± 0.0^{a}	0.1 ± 0.0^{b}	0.1 ± 0.0^{b}
20:0	0.3 ± 0.0^{a}	0.1 ± 0.0^{b}	0.1 ± 0.0^{b}
20:1n-9	1.9 ± 0.5	1.4 ± 0.7	1.1 ± 1.0
20:2n-9	0.5 ± 0.2	0.2 ± 0.1	0.4 ± 0.1
20:4n-6	0.9 ± 0.1^{a}	0.2 ± 0.1^{b}	0.3 ± 0.1^{b}
20:3n-3	0.1 ± 0.0^{a}	1.2 ± 0.0^{b}	0.3 ± 0.0^{c}
20:4n-3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:5n-3	9.3 ± 0.7^{a}	1.9 ± 0.6^{b}	2.3 ± 0.7^{b}
22:1n-11	1.2 ± 0.2	1.2 ± 0.3	0.6 ± 0.3
22:5n-3	4.9 ± 0.1^{a}	1.4 ± 0.1^{b}	1.4 ± 0.0^{b}
22:6n-3	7.6 ± 0.9^{a}	6.1 ± 0.8^{ab}	5.1 ± 0.8^{b}
Total saturates	30.1 ± 3.3^{a}	23.4 ± 3.8^{b}	23.2 ± 3.7^{b}
Total monoenes	35.3 ± 3.5	30.4 ± 3.9	33.4 ± 4.2
∑n-3	25.5 ± 3.1^{a}	35.5 ± 4.1^{b}	12.4 ± 3.8^{c}
∑n-6	7.2 ± 1.1^{a}	10.1 ± 1.2^{a}	30.2 ± 2.3^{b}
∑n-9	21.4 ± 2.2	23.1 ± 1.9	26.0 ± 1.6
∑n-3 LC-PUFA	21.9 ± 2.5^{a}	13.9 ± 2.0^{b}	9.2 ± 1.0^{b}
ARA/EPA	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
EPA/DHA	1.2 ± 0.0^{a}	0.5 ± 0.0^{b}	0.5 ± 0.0^{b}
n-3/n-6	3.5 ± 0.1^{a}	3.5 ± 0.2^{a}	0.4 ± 0.0^{b}

Different letters within a row denote significant differences ($P<0.05$) n=10x3 n.d. non-detected

Table 3.8: Intestine fatty acid composition of fish fed the different experimental diets (g $100g^{-1}$ fatty acids identified)

	Diets		
Fatty acids	**100FO**	**100LO**	**100SO**
14:0	5.6 ± 0.1[a]	2.1 ± 0.3[b]	3.1 ± 0.4[b]
14:1n-7	0.1 ± 0.0	n.d.	n.d.
15:0	0.35 ± 0.0	0.2 ± 0.0	0.2 ± 0.1
16:0ISO	0.1 ± 0.0	0.1 ± 0.0	n.d.
16:0	22.4 ± 1.2[a]	16.6 ± 1.2[b]	16.5 ± 3.0[b]
16:1n-7	8.1 ± 0.4[a]	2.5 ± 0.4[b]	4.2 ± 1.3[b]
16:1n-5	0.2 ± 0.1	n.d.	n.d.
16:2n-6	0.9 ± 0.1[a]	0.3 ± 0.0[b]	0.5 ± 0.1[b]
16:2n-4	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.1
17:0	0.9 ± 0.0[a]	0.3 ± 0.1[b]	0.5 ± 0.2[b]
16:3n-4	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.1
16:4n-3	1.1 ± 0.2[a]	0.3 ± 0.1[b]	0.5 ± 0.1[b]
18:0	6.1 ± 0.6[ab]	8.7 ± 0.7[a]	4.8 ± 0.6[b]
18:1n-9	14.7 ± 3.0	18.4 ± 2.8	19.7 ± 3.0
18:1n-7	3.9 ± 0.7	2.4 ± 0.6	2.8 ± 0.6
18:1n-5	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0
18:2n-6	8.5 ± 1.0[a]	14.9 ±1.4[b]	31.1 ± 3.5[c]
18:3n-6	0.2 ± 0.0[a]	0.1 ± 0.0[b]	0.3 ± 0.0[c]
18:3n-4	0.3 ± 0.0[a]	0.1 ± 0.0[b]	0.1 ± 0.0[b]
18:3n-3	1.3 ± 0.3[a]	19.0 ±2.4[b]	2.8 ± 0.4[a]
18:4n-3	1.3 ± 0.1[a]	0.3 ± 0.1[b]	0.6 ± 0.1[a]
18:4n-1	0.5 ± 0.0[a]	0.1 ± 0.0[b]	0.2 ± 0.0[b]
20:0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
20:1n-9	1.8 ± 0.5	1.3 ± 0.4	1.6 ± 0.7
20:2n-9	0.3 ± 0.0[a]	0.1 ± 0.0[b]	0.2 ± 0.0[c]
20:4n-6	1.5 ± 0.1[a]	0.6 ± 0.1[b]	0.5 ± 0.1[b]
20:3n-3	n.d.	0.4 ± 0.2	0.2 ± 0.0
20:4n-3	0.6 ± 0.0[a]	0.3 ± 0.0[b]	0.3 ± 0.1[b]
20:5n-3	8.1 ± 0.7[a]	2.4 ± 1.0[b]	3.0 ± 0.8[b]
22:1n-11	1.3 ± 0.2	1.0 ± 0.2	1.1 ± 0.3
22:5n-3	2.3 ± 0.1[a]	0.6 ± 0.0[b]	0.7 ± 0.1[b]
22:6n-3	6.8 ± 0.9	5.8 ± 1.0	3.6 ± 0.7
Total saturates	35.2 ± 3.3[a]	28.3 ± 3.4[ab]	25.0 ± 3.7[b]
Total monoenes	31.1 ± 3.5	26.1 ± 3.7	30.2 ± 3.9
∑n-3	21.5 ± 3.1[ab]	29.1 ± 4.7[a]	11.7 ± 2.9[b]
∑n-6	10.5 ± 1.1[a]	15.7 ± 1.5[a]	32.1 ± 2.1[b]
∑n-9	16.7 ± 2.2[a]	19.9 ± 2.8[ab]	21.5 ± 2.0[b]
∑n-3 LC-PUFA	17.7 ± 2.5[a]	9.6 ± 1.0[b]	7.9 ± 1.2[b]
ARA/EPA	0.2 ± 0.0[a]	0.3 ± 0.0[b]	0.2 ± 0.0[a]
EPA/DHA	1.2 ± 0.0[a]	0.4 ± 0.0[b]	0.8 ± 0.0[c]
n-3/n-6	2.0 ± 0.1[a]	1.8 ± 0.3[a]	0.4 ± 0.1[c]

Different letters within a row denote significant differences ($P<0.05$) n=10x3 n.d. non-detected

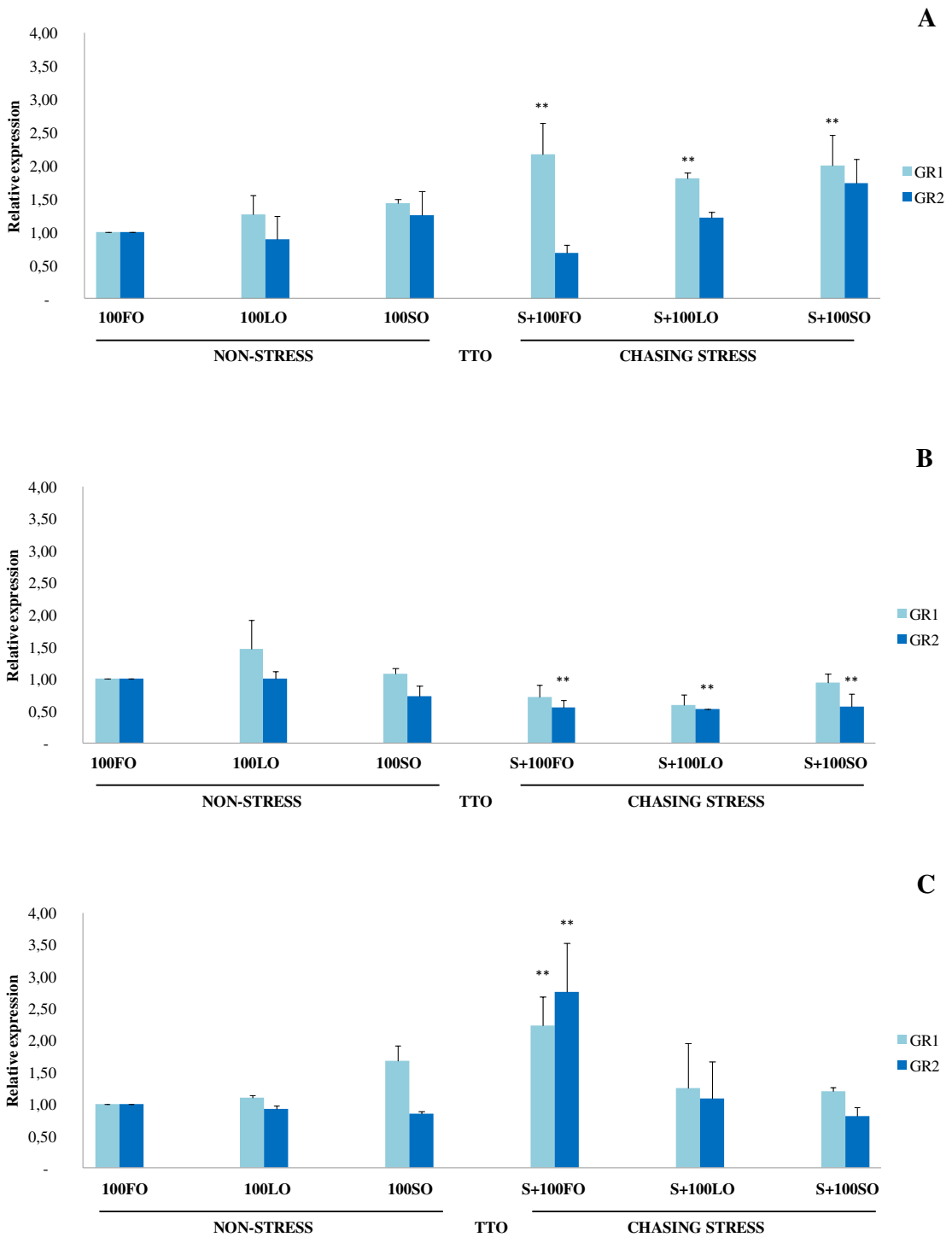

Figure 3.1: Relative expression of GR1 and GR2 genes in 1.A) Muscle, 1.B) Intestine and 1.C) Liver of Senegalese sole fed diets with total substitution of fish oil for either linseed or soybean oils, and subjected to chasing stress. Relative expression referred to the value of unstressed fish fed fish oil based diet. FO: fish oil; LO: Linseed oil, SO: Soybean oil, S: Stressed fish n=9. * denotes significant differences (P<0.05) with control fish (100FO) for a given gene. ** denotes significant differences between stressed and non-stressed fish in a specific diet.

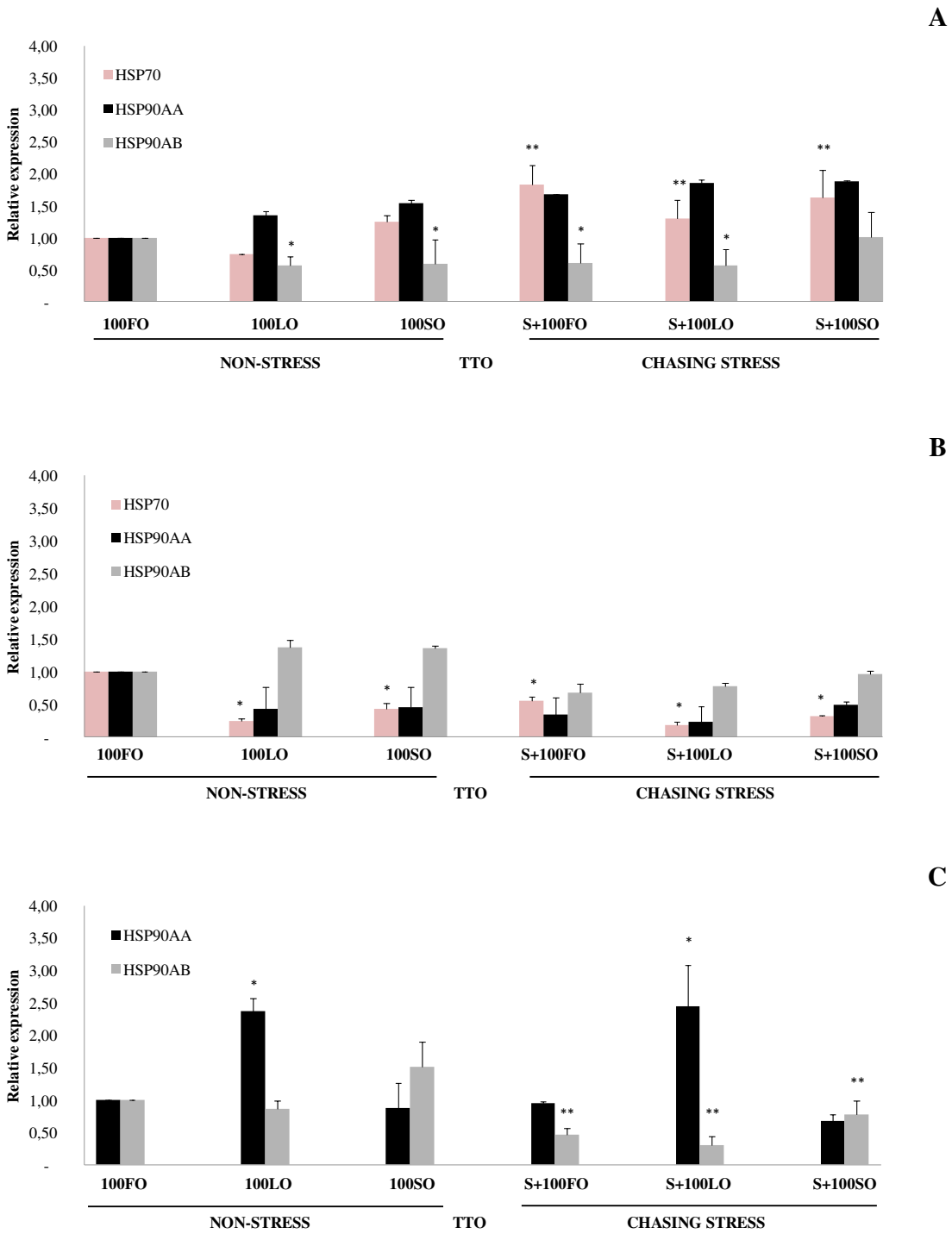

Figure 3.2: Relative expression of HSP70, HSP90AA and HSP90AB genes in 2.A) Muscle, 2.B) Intestine and 2.C) Liver of Senegalese sole fed diets with total substitution of fish oil for either linseed or soybean oils, and subjected to chasing stress. Relative expression referred to the value of unstressed fish fed fish oil based diet. FO: fish oil; LO: Linseed oil, SO: Soybean oil, S: Stressed fish n=9. * denotes significant differences (P<0.05) with control fish (100FO) for a given gene. ** denotes significant differences between stressed and non-stressed fish in a specific diet.

3.5 Discussion

Regardless of the lipid source assayed in the present study, Senegalese sole-specific growth rates were similar (Silva *et al.,* 2010) or even slightly higher (Borges *et al.,* 2009) than those formerly obtained for this species with diets containing 12 % crude lipids. Nevertheless, complete replacement of fish oil by soybean oil slightly reduced SGR leading to lower body weights at the end of the study. High inclusion levels of soybean oil in marine fish diets have been described to induce negative effects in fish (Sales and Glencross, 2011). For instance, lower growth is found in gilthead sea bream juveniles fed on diets with either 80 % (Izquierdo *et al.,* 2005) or complete fish oil replacement (Montero *et al.,* 2008) by soybean oil, as well as in other marine warm water species such as European sea bass (*Dicentrarchus labrax*) (Montero *et al.,* 2005) or grouper (*Epinephelus coioides*) (Lin *et al.,* 2007). Within the flatfish species, no negative effects of complete fish oil replacement by either linseed oil or soybean oil have been found on growth of turbot (Regost *et al.,* 2003) or of halibut fed a 70 % fish oil replacement by linseed oil (Alves-Martins *et al.,* 2011a). The expression of some genes involved in myogenesis, such as white muscle myogenic regulatory factor MyoD, may be down-regulated by the use of soybean in trout feeds (Alami-Durante *et al.,* 2010) and could be also contributing to the reduced growth found in Senegalese sole in the present study, since other myogenic genes (mylc2 and mrf4) are markedly affected by dietary lipids in this species (Campos *et al.,* 2010).

The type of dietary oil had a direct impact on the different tissues, as the fatty acid profile characteristic of the different dietary oils used was clearly reflected in the different tissues as described for other flatfish and other marine species (reviewed by Turchini *et al.,* 2009). Marine species have a limited capacity to elongate and desaturate C18 fatty acids into those considered as essential (EPA, DHA, and ARA) (Sargent *et al.,* 2002). The results of this experience could suggest some conservation of DHA in the different tissues of Senegalese sole, especially in muscle, since no significant differences in the relative amount of this fatty acid were found in fish tissues, as described for S. senegalensis in different dietary regimens (Morais *et al.,* 2004;

Alves-Martins *et al.,* 2011b), but the lower absolute value of this fatty acid in tissues indicates that a long-term feeding experiment is needed to check this crucial parameter within a complete on-growing period to elucidate how the use of dietary vegetable oils is affecting tissue DHA content up to commercial size. EPA and ARA decreased in the different tissues of fish fed vegetable oils, keeping a constant ARA/EPA ratio, as it occurs in other marine species such as gilthead sea bream (Montero *et al.,* 2010). ARA, docosapentaenoic acid (22:5n-3) (DPA), and variations in ratio ARA/EPA are preferentially retained in Senegalese sole Artemia-fed post-larvae under different dietary treatments (Alves-Martins *et al.,* 2011b), or soybean-enriched Artemia (Morais *et al.,* 2006). Although muscle and liver have been proposed to have a highly conservative fatty acid profile in Senegalese sole (Rueda-Jasso *et al.,* 2004), the levels of LA and LNA reflected those of the diet, with ratios ‘‘fatty acid in tissue/fatty acid in diet’’ close to 1. Nevertheless, increased 20:3n-3 contents, a direct product of 18:3n-3 elongation, in muscle and liver of fish fed vegetable oils, suggests an increased activity of the C18–20 elongase, as proposed for other flatfish as turbot (Regost *et al.,* 2003) or halibut (Alves-Martins *et al.,* 2011a), being this enzyme a non-limiting step for LC-PUFA synthesis as described for flatfish (Bell *et al.,* 1994; Regost *et al.,* 2003). Besides affecting intestine fatty acid profile, feeding complete replacement of dietary fish oil may also alter lipid absorption and transport. Particularly, an accumulation of supranuclear lipid droplets within enterocytes observed in soybean oil fed seabream was related to the higher re-acylation of the absorbed lipids and the subsequent increase on lipoprotein synthesis (Caballero *et al.,* 2003), resulting in an impaired transit capacity through the *lamina propria* (Caballero *et al.,* 2003, 2006), altering their fatty acid composition in gilthead sea bream and increasing plasma cholesterol and triacylglicerol concentrations (Caballero *et al.,* 2006).

Dietary lipids and particularly vegetable oils may cause also alterations in fish welfare (Montero *et al.,* 2008, 2010; Montero and Izquierdo, 2010). In agreement, basal plasma cortisol levels of Senegalese sole fed linseed oil were slightly higher than in fish fed the other diets. In gilthead seabream, feeding linseed oil raises post-stress plasma cortisol contents (Montero *et al.,*

2003; 2008; Ganga *et al.,* 2011a, b) and in vitro release of cortisol from interrenal cells, through cyclooxygenase and lipoxygenase metabolites (Ganga *et al.,* 2011b). Similarly, in Atlantic salmon smolts, feeding high n-3/n-6 diets increased post-stress plasma cortisol (Oxley *et al.,* 2010). The stress response of Senegalese sole under different stressful situations has been widely described, this species seeming to be especially susceptible to salinity changes (Arjona *et al.,* 2009), high stocking density (Costas *et al.,* 2008; Wunderink *et al.,* 2011), air exposure (Costas *et al.,* 2011a) or, in a lesser extend, handling (Aragão *et al.,* 2008; Costas *et al.,* 2011b). There is a high variability of plasma cortisol values in this species, as a result of individual differences in the stress coping styles (Silva *et al.,* 2010). Basal plasma cortisol of Senegalese sole in the present study was in the range reported in other studies (Aragão *et al.,* 2008; Costas *et al.,* 2008, 2011a; Arjona *et al.,* 2009). Prolonged feed deprivation has been shown to increase plasma cortisol in this species, denoting its functional role on energy mobilization (Costas *et al.,* 2011a) and gluconeogenesis activation.

However, the expression of glucocorticoid receptors has not been yet studied in Senegalese sole. There are multiple corticosteroid receptors in fish (Prunet *et al.,* 2006), and two different GR (GR1 and GR2) genes and one MR (mineralocorticoid receptor) gene are found in most of the teleostean fishes (Bury and Sturm, 2007), all of them with affinity for cortisol as a single ligand (Stolte *et al.,* 2008). GR gene duplicated copies (GR1 and GR2) that have been found in European sea bass (Terova *et al.,* 2005; Vizzini *et al.,* 2007) are in accordance with the GR gene duplication within the teleost lineage. Rainbow trout GR2 has a much greater transactivational sensitivity to cortisol (60-fold) than GR1 (Bury *et al.,* 2003), but similar transactivational sensitivity to cortisol for both GRs has been described for the Haplochromis burtoni (Greenwood *et al.,* 2003). In the present study, both GRs genes have been expressed in the three tissues studied (muscle, liver, and intestine), but only fish muscle showed a clear response to repetitive chasing stress, increasing the expression of these receptors. Although stress-associated increased plasma cortisol can modify the GR gene expression, contrasting results have been reported (Vijayan *et al.,* 2003; Terova *et al.,* 2005). In European sea bass, high

plasma cortisol levels due to a high rearing density stress decreased the liver GR2 expression (Terova *et al.,* 2005); although both the GR-mRNAs were significantly increased at 24 h, GR1 gene expression is the higher of the two genes. In addition, when (at 1 week) the expression of both GR genes decreased, the GR1 gene expression maintained a high level (Vazzana *et al.,* 2010). In agreement, Senegalese sole in the present study showed that GR1 expression after the chasing stress increased more than that of GR2 in muscle and intestine.

Despite feeding soybean oil has been found to affect glucocorticoid receptors in mice (Oarada *et al.,* 2007), in fish there is a lack of studies on the effect of dietary lipid sources on these receptors. The present study demonstrated a marked effect of dietary oils on the type of response to stress of glucocorticoid receptors gene expression on the different tissues of Senegalese sole. Liver of fish fed FO based diets increases the expression of both GRs after stress, an effect ameliorated by the use of dietary vegetable oils. In muscle, feeding vegetable oils, particularly soybean oil, caused an over expression of GR2 gene in response to the chasing stress. Feeding soybean oil also increased the expression of pro-inflammatory cytokines (Montero *et al.,* 2010) in other marine fish. In turn, the increased proinflammatory cytokines could regulate GRs gene expression as it occurs in higher vertebrates (Webster *et al.,* 2001). In these studies, the transcription factor NF-kB leads to the accumulation of the b-GR isoform that suppresses the transcriptional activity of a-GR isoform by interfering with the formation of active co activator complexes, in a proposed activity of self compensatory regulation of both isoforms of GRs (Charmandari *et al.,* 2005). This regulatory pathway has not been yet studied in fish, but the results of this and previous studies have demonstrated that dietary soybean oil increases pro-inflammatory cytokines and affect GRs gene expression post-stress.

In response to chasing stress, HSP70 gene expression increased in muscle of Senegalese sole, regardless the dietary lipid source. Increased circulating levels of extracellular HSP70 (eHSP70) could be envisaged as an immune-modulatory mechanism induced by exercise, besides other chemical messengers (e.g., cytokines) released during an exercise effort, which are able to binding a number of receptors in neural cells. Human studies suggest that increased

levels of eHSP70 in the plasma during exercise and the huge release of eHSP70 from lymphocytes during high-load exercise bouts may participate in the fatigue sensation, also acting as a danger signal from the immune system (Heck *et al.,* 2011). Feeding vegetable oils to non stressed sole reduced the gene expression of HSP90AB in muscle and the HSP70 expression in intestine. HSP70 gene expression is also regulated by other dietary factors such as starvation (Cara *et al.,* 2005), caloric restriction (Heydari *et al.,* 1993), or arginine supplementation (Wu *et al.,* 2010). Reduced HSP70 gene expression in intestine of Senegalese sole could be related to the lower ARA contents of this tissue in fish fed vegetable oils, since arachidonic acid is a potent modulator of HSPs in humans (Jurivich *et al.,* 1994). Nevertheless, intestinal HSPs may be also influenced by the intestinal bacterial flora (Kokura and Yoshikawa 2006), which in turn may be affected by dietary vegetable oils (Oarada *et al.,* 2007). Specifically, feeding linseed oil increased HSP90AA gene expression in liver of both stressed and non-stressed sole, in agreement with the higher basal and post-stress cortisol levels found in fish fed this lipid source in this and previous studies (Montero *et al.,* 2003; Montero and Izquierdo, 2010; Ganga *et al.,* 2011a). Increased gene expression of HSP90 has been also found in liver of rainbow trout fed alternative diets containing soybean meal (Sealey *et al.,* 2010). The higher HSP90 mRNA abundance corresponding to the elevation in this protein in fish reflects a role for glucocorticoids in the hepatic stress response process (Vijayan *et al.,* 2003). HSPs interact with the glucocorticoid receptor regulating its correct folding, activation, intracellular transport, transcriptional regulation, and decay (Grad and Picard, 2007). Several studies suggest that the intracellular ratio HSP90/GR can be a key regulator of steroid action, either positively or negatively (Kang *et al.,* 1999) suggesting that HSP90 can form the first line of defense against heat stress (Hori *et al.,* 2010). Further experiences should be conducted to elucidate the interaction between relative expression of HSPs and glucocorticoid receptors under different stressful conditions.

In summary, total replacement of fish oil by vegetable oils (either soybean or linseed oil) in diets for Senegalese sole altered the fatty acid profiles of muscle, liver, and intestine, which

reflected diet composition. However, in spite of reduction in LC-PUFA, the ratio ARA/EPA remains constant within tissues among the fish feeding different oil-based diets. Some evidences of fatty acid elongation processes can be also observed. There was a direct effect of handling stress on the expression of both GRs, increasing muscle GR1 and decreasing gut GR2 in stressed animals at any of the experimental diets assayed. Stressful conditions increased muscular HSP70 expression and decreased hepatic HSP90AB, independently of the diet; vegetable oils decreased the expression of HSP70 in intestine, being the relative expression of liver HSP90AA increased by the inclusion of linseed oil in the diet, at any of the experimental conditions assayed. Further studies are being conducted to better understand the effect of dietary lipids in stress response in this fish species and the physiological and molecular mechanisms implied.

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CAPÍTULO 4

"EFFECTS OF THERMAL INCREASE ON THE SENEGALESE SOLE STRESS RESPONSE"

This manuscript has been submitted to *Fish Physiology and Biochemistry*

4. Effects of thermal stress on the expression of the glucocorticoid receptor complex linked genes in Senegalese sole (*Solea senegalensis*): Acute and adaptive responses

4.1 Abstract

The present work examined the short and long-term effects of a rise in temperature of from 18-19ºC to 23-24ºC, on the expression of genes related to the stress response regulation, in juveniles of Senegalese sole. The animals were exposed to heat shock of 5 degrees, after 1 month of acclimatization at 18-19ºC. After this process, samples of different tissues were taken from a total of 96 fish divided into four sampling points: 1 hour, 24 hours, 48 hours and 1 week. We quantified transcript levels of a wide set of genes involved in Hypothalamus pituitary Interrenal axis (glucocorticoid receptor 1 and 2, corticotrophin release hormone, corticotrophin release hormone binding proteins, proopiomelanocortin A and B), and cellular stress defense system (heat shock protein 70, 90AA and 90AB), along with blood samples to measure the plasma cortisol concentration. Our data show that the heat shock produced changes (either increase or decrease) in gene expression and plasma cortisol levels, and these responses showed marked differences between tissues. Taken together, these data indicate a good initial response to heat shock in Senegalese sole, suggesting that heat shock protein 90 may be a regulatory factor for the glucocorticoid receptor in the presence of steroid hormone, and the regulation of corticotrophin release hormone binding proteins transcription could represent a major mechanism to exert a negative feedback on adrenocorticotropic hormone release from the pituitary in sole.

4.2 Introduction

In fish as in other vertebrates, growth, reproduction and disease resistance are influenced by temperature, so changes of optimal temperature range could induce a thermal stress response compromising these processes (Cossins *et al.,* 1995). An allostatic state activates the cellular stress response (CSR) which involves prevention and repair of macromolecular damage incurred (McEwen and Wingfield, 2003). Besides, other mechanisms can be affected, including activation of molecular chaperones to refold proteins that have been denatured (Logan and Somero, 2011), initiation of proteolysis to remove proteins that cannot be rescued through activities of chaperones (Feder and Hofmann, 1999) or even apoptotic pathways if heat stress is severe (Kültz, 2005). The interaction between both CSR and apoptosis are complex, apoptosis being mediated in part by an increase of circulating cortisol levels (Bury *et al.,* 1998; Laing *et al.,* 2001), which would be enhanced by the glucocorticoid receptor (GR) (Van der Salm *et al.,* 2002). This mechanism is also regulated by the accumulation of Heat Shock Protein 70 (HSP70) that is related to a low GR protein content in cells (Boone *et al.,* 2002). This up-regulation of HSP 70 can block apoptosis through the inhibition of several caspase proteins (Beere, 2004) and naturalize damaged proteins before initializes the apoptotic process.

The variations of circulating cortisol regulate the Hypothalamus-pituitary-Interrenal (HPI) axis activation after stress, being the levels of cortisol differing among and within species (Pottinger *et al.,* 2010). In a first step, cortisol secretion can inhibit corticotrophin release hormone (CRH) transcription and also could modulate the synthesis of CRH receptors that mediate CRH actions (Westphal and Seasholtz, 2006). Besides, increases of cortisol can modulate the CRH binding proteins (CRHBP) that block CRH (Flik *et al.,* 2006). Cortisol is also involved in the synthesis and release of proopiomelanocortin (POMC) from the pituitary corticotrophs for adenocotricotropin hormone (ACTH) synthesis. The effect of stress on pituitary POMC mRNA levels varies according to the nature of the stressor stimulus (Aguilera, 1994).

At the cellular level, the effects of cortisol are mediated by intracellular glucocorticoid receptors (GR), members of the superfamily of nuclear receptors and act as ligand dependent transcription factors to control and regulate gene expression (Mommsen *et al.,* 1999). Most teleost have two glucocorticoid receptor genes (GR1 and GR2) that are expressed in several organs (Bury and Sturm, 2007; Stolte *et al.,* 2008). Depending on the teleost species, each GR requires a different concentration of cortisol to initiate transcription e.g., GR2 is 60-fold more sensitive than GR1 in rainbow trout (*Oncorhynchus mykiss*) (Prunet *et al.,* 2006). In the cytosol, GRs are in an inactive form within a multi protein complex along with several HSPs such as HSP70 and 90, whose functions include the assembly, functionality and transport of genetic resources (Pratt and Toft, 1997) and play an important role in the process of acquired thermo-tolerance (Kregel, 2002; Fangue *et al.,* 2006). In particular, HSP70 is essential in the assembly and maintenance of the glucocorticoid receptor heterocomplex (Hutchinson *et al.,* 1994; Pratt and Welsh, 1994). HSP90 has been suggested to stabilize the receptor heterocomplex against proteolytic degradation (Dundjerski *et al.,* 2000). Steroid receptors can bind hormones in the absence of HSPs, but there is considerable evidence that HSPs increase the binding capacity of the steroid receptor, facilitate nuclear translocation of the receptor complex, and increase the proteolytic half-life of the receptor complex (Pratt and Welsh, 1994; Czar *et al.,* 1997; Smith *et al.,* 1998). Analysis of hepatic tissue taken from hypercortisolemic rainbow trout, demonstrated that levels of free HSP70 decreased whereas the amount of HSP70 bound to the GR increased after exposure to heat shock (Basu *et al.,* 2003). Although HSPs have a relatively short half-life, their levels remain elevated in the whole organism long after the stressor is removed, which indicates their role in long-term adaptation and increased stress tolerance (Morimoto and Santoro, 1998), playing an important role in maintaining homeostasis (Iwama *et al.,* 1998).

The effects of temperature stress is of special importance in terms of predicting "adaptive" or "non-adaptive" responses in different fish species in the context of climate change (Mora and Maya, 2006; Somero, 2010). Senegalese sole (*Solea senegalensis*) is a marine teleost

that inhabits coastal and estuarine areas, which are subjected to wide changes in environmental salinity and temperature (Dinis *et al.,* 1999; Imsland *et al.,* 2003; Vinagre *et al.,* 2006). The effects of temperature changes on the Senegalese sole osmoregulatory systems and thyroid hormones has been previously determined (Arjona *et al.,* 2010) as well as the protein metabolic capacity of this species in response to rearing temperature (Campos *et al.,* 2013), but there is few information on the capacity of this species for adaptation to temperature changes in terms of response of the expression of stress-related genes. The objective of the present study was to analyze the stress-mediated response of Senegalese sole to a rearing temperature change, in terms of adaptive and non-adaptive response.

4.3 Materials and methods

4.3.1 Experimental fish and samples collection

One hundred and sixty eight Senegalese sole juveniles of 62.29 ± 21.26 g initial body weight from a local farm (ADSA, Castillo del Romeral, Gran Canaria, Spain) were randomly distributed into 24 indoor plastic tanks (45 l) of 7 fish per tank. Tanks were supplied with filtered sea water, at a temperature of 18-19 ˚C, and natural photoperiod (around 12L: 12D). Water dissolved oxygen values ranged 6.20 ± 0.70 g/l. Fish were manually fed until apparent satiation with a commercial diet for 5 weeks (twice daily, 6 days a week). After an acclimatization period of 30 days, a heat shock was applied to one half of the tanks (12 tanks) in experimentation. The experimental thermal shock consisted to an increase of 5ºC, immediate change from 18 to 24 ˚C in one hour, using with individual electronic heaters in each tank, in one hour.

Blood from 4 fish per tank was collected by caudal sinus puncture and stored into a tube previously treated with Lithium heparine (Deltalab). Blood was centrifuged at 800 *xg* during 10 min to obtain plasma samples. And samples were stored -80ºC until analysis.

All fish were sacrificed by anesthetic overdose of clove oil by immersion according to protocols approved by the animal welfare committee of the ULPGC.

Samples of 60 mg of intestine, liver, muscle, gills and brain were colleted from a total of 96 fish (four fish per tank, triplicate for each sampling point at either 18 or 24ºC, different triplicate tanks for each sampling point) divided into four sampling points after heat shock: 1 hour, 24 hours, 3 days and 1 week. Samples were placed in RNA Later (QIAGEN) at a ratio of 5 parts preservative to 1 part tissue.

4.3.2 Plasma cortisol and expression of stress-related genes

Plasma cortisol concentration was determined by radio-immunoassay using the trypsin–antitrypsin method as previously described for marine fish species (Rotllant *et al.,* 2001), in the Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, Bellaterra, Spain.

4.3.3 Stress-related genes

The expression of GR1, GR2, ubiquitin, HSP70, HSP90AA, HSP90AB, CRH, CRHBP, POMCA and POMCB genes was conducted using oligos previously described for this species (Infante *et al.,* 2008; Manchado *et al.,* 2008; Salas-Leiton *et al.,* 2010, 2012; Benítez-Dorta *et al.,* 2013), using a RT-PCR.

4.3.4 RNA extraction and cDNA synthesis

One hundred milligrams of tissue (equal amount from 4 fishes per tank, approximately 25 mg per fish) were pooled and total RNA was extracted using 1 ml TRI Reagent (SIGMA-Aldrich, Sant Louis, Missouri). Chloroform and isopropanol were used for separation and precipitation, respectively. The pellets were hydrated with 100 µl of Milli-Q sterile water, previously treated with 0.1% DEPC and kept at -80ºC until analysis. Total RNA concentration, purity and quality were measured by NanoDrop 1000 Spectrophotometer (Thermo Scientific). Considering as optimal, the values between 1.8-2.0 in the 260 nm / 280 nm wave length. The reverse transcription (RT) reactions were carried out in 20 µl volumes with iScriptTM cDNA Synthesis Kit (Bio-Rad Hercules, California) containing 1 µg of total RNA. At the end of the RT reactions, all cDNA populations were kept at -20ºC.

4.3.5 Real time (RT-PCR)

All PCR reactions were performed in i-cycler thermocycler with optical module (Bio-Rad Hercules, California) using 12.5 µl Brillant SYBR Green QPCR Master Mix (Bio-Rad Hercules, California), 1µl of a 1:5 dilution of the cDNA and the amount previously optimized of each primer (Table 4.1) in a final volume of 25 µl. Cycling conditions consisted of denaturation and enzyme activation for 7 min at 95°C, followed by 40 cycles at 95°C for 15 seconds and 70°C for 30 seconds. Each assay was done in duplicate.

In all cases, total RNA was treated twice with DNAse I using the RNAse-Free DNAse kit (Qiagen) for 30 min. RNA sample quality was checked on agarose gel, and quantification spectrophotometrically determined. Total RNA (1 mg) from each sample was reverse transcribed using the iScript_cDNA Synthesis kit (Bio-Rad). Lack of genomic DNA contamination was confirmed by PCR amplification of RNA samples in the absence of cDNA synthesis.

The relative gene expression was estimated by the Δ-Δ method (Livak and Schmittgen, 2001) using ubiquitin as housekeeping gene after check that the expression of this gene was not influenced by any of the experimental treatments used, as previously reported and proposed by others authors working on Senegalese sole (Infante *et al.,* 2008), being considered unstressed fish held at 18-19°C as control or reference value.

Table 4.1: Primers of the different genes used

Target	Primer	Sequence 5´-3´	Amplicon (bp)	Acc no	Bibliography
GR1	F	CCTGCCGCTTCCACAAGTGTCTGATG	130	AB614369	Benítez-Dorta *et al.,* 2013
	R	TTCAACTGGTGGAGGTGGCGGTGT			
GR2	F	TCAGCGTGGAGTTCCCGGAGATG	92	AB614370	Benítez-Dorta *et al.,* 2013
	R	GGTGGAACAGCAGCGGCTTGATG			
UBIQUITIN	F	AGCTGGCCCAGAAATATAACTGCGACA	93	AB291588	Infante *et al.,* 2008
	R	ACTTCTTCTTGCGGCAGTTGACAGCAC			
HSP 70	F	GCTATACCAGGGAGGGATGGAAGGAGGG	119	AB513855	Salas-Leiton *et al.,* 2010
	R	CGACCTCCTCAATATTTGGGCCAGCA			
HSP 90 AA	F	GACCAAGCCTATCTGGACCCGCAAC	105	AB367526	Manchado *et al.,* 2008
	R	TTGACAGCCAGGTGGTCCTCCCAGT			
HSP 90 AB	F	TCAGTTTGGTGTGGGTTTCTACTCGGCTTA	148	AB367527	Manchado *et al.,* 2008
	R	GCCAAGGGGCTCACCTGTGTCG			
CRH	F	CGGCGTCTATTACAAGGGAAAGTTGGGAAC	98	FR745427	Salas-Leiton *et al.*, 2012
	R	TCGGACCTCCTCCCCCTCTCCAT			
CRHBP	F	AGCTGCTGGGGGGCAATGGCATA	94	FR745428	Salas-Leiton *et al.*, 2012
	R	CCAACCTTCATCTGGGCGAGTCCTCT			
POMCA	F	CGGCCCATCACAGTCTACAGCTCCA	131	FR874846	Salas-Leiton *et al.*, 2012
	R	CCAACCTTCATCTGGGCGAGTCCTCT			
POMCB	F	GGATGCGGCAAAAGGGGGACA	111	FR874847	Salas-Leiton *et al.*, 2012
	R	CCCCATCTAAAGTGACCCATGCGGTA			

4.3.6 Statistical analysis

All data were tested for normality and homogeneity of variance. Means and standard deviations (SD) were calculated for each parameter measured except for cortisol values where

values are represented as mean plus standard error (SE). Statistical analyses followed methods outlined by Sokal and Rohlf (1995). When F values showed significance, individual means were compared using Duncan multiple comparison test. Significant differences were considered for $P<0.05$. If the variances were not normally distributed, the Kruskall–Wallis non-parametric test was applied to the data. Genetic data were calibrated to untreated control at 1h, 24 h or 3 days in the short-term exposure and to untreated control at 1 week in the mid-term exposure. Comparisons between groups were made by one-way analysis of variance followed by Tukey analysis, using the stress time exposure as fixed factor, and using stressed fish held at 23-24°C as a reference value. Analyses were performed using SPSS software (SPSS for windows 11.0).

4.4 Results

Acute and chronic changes in water temperature caused variations in cortisol levels and the expression of genes involved in stress response and behavior in the different tissues examined.

4.4.1 Plasma cortisol

The heat shock did not induced significant changes in levels of circulating plasma cortisol in any of sampling points, probably due to a high standard error (SE), although, one hour after the increase of 5°C the cortisol showed high level (18.6 ng/ml), slightly less than that produced seven days after the end of experiment (21.5 ng/ml). The values of the circulating cortisol in the rest of sample points ranged were between 4.5 and 8.3 ng/ml (Fig. 4.1).

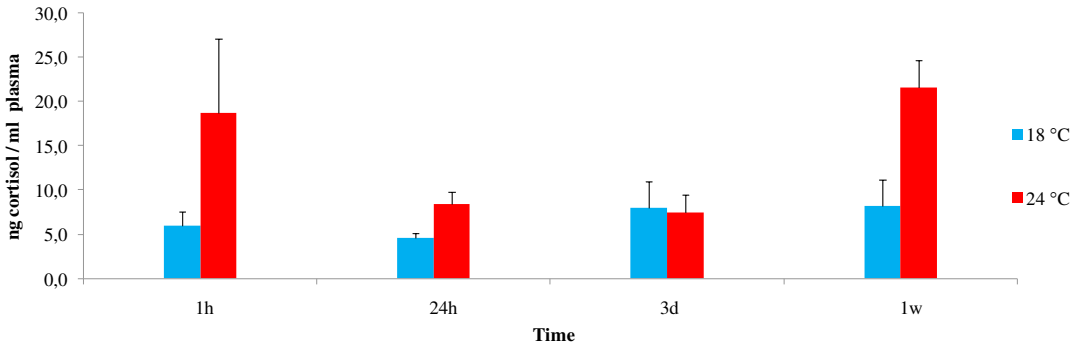

Figure 4.1: Circulating plasma cortisol levels after an increase of 5ºC in temperature

4.4.2 Expression of stress-related genes in liver

The relative expression of GR1 and GR2 was increased (P<0.05) 1 hour after the beginning of the heat shock (Fig. 4.2). The heat shock did not induce any change in the relative expression of HSP70 in liver (Fig. 4.2). However, the pattern of the relative expression of this gene presented a similar time course when compared to GR1, with a progressive decrease in values toward the end of the experimental period. HSP90AA gene (Fig. 4.2) increased (P<0.05) within the first 24 hours after initiation of thermal stress, then decreasing (P<0.05) until the end of the experimental period. For HSP90AB gene (Fig. 4.2) the highest (P<0.05) value of expression is observed at 24 hours post stress, then decreasing until the end of the experimental period as occurred with HSP90AA.

4.4.3 Expression of stress-related genes in muscle

The relative expression of GR2 was increased (P<0.05) on the third day post-stress (Fig. 4.3), HSP90AA was up regulated (P<0.05) one hour after heat shock (Fig. 4.3) and HSP90AB is

increased (P<0.05) at 1 week after heat shock (Fig. 4.3), while the increase of temperature had no effect on the relative expression of GR1 and HSP70 in the muscle (Fig. 4.3).

4.4.4 Expression of stress-related genes in intestine

Thermal stress induced a significant (P<0.05) increase of the relative expression of GR1, GR2 and HSP90AB after a week (Fig. 4.4). Besides, the increase of temperature had no significant effect on the relative expression of HSP70 and HSP90AA genes in the intestine (Fig. 4.4).

4.4.5 Expression of stress-related genes in gills

The relative expression of GR1 was significantly (P<0.05) increased 24 hours and one week after the start of the heat shock and (Fig. 4.5 A). GR2 expression was also significantly (P<0.05) increased after one week of thermal stress (Fig. 4.5 A). Heat shock stress caused a significant increase of HSP70 and HSP90AA genes expression after 24h (Fig. 4.5 B) whereas induced a significant increase (P<0.05) of HSP90AB after one week (Fig. 4.5 B).

4.4.6 Expression of stress-related genes in brain

The increase in temperature induced a significant (P<0.05) increase in relative expression of GR1 and GR2 at 24 hours, recovering the initial values after 3 days of acclimation at 24°C (Fig. 4.6 A). Thermal stress had no effect on the expression of HSP70 gene, although higher values were observed after 1 hour after the beginning of the heat shock (Fig. 4.6 B). However, the relative expression of HSP90AA gene reached a maximum value (P<0.05) after 1 hour of the start of heat shock (Fig. 4.6 B). Besides, the change of temperature induced a significant

increase in expression of HSP90AB during the first 24 hours from the start of heat shock being significantly higher at 1 and 24h after the beginning of the thermal stress (Fig. 4.6 B).

On the other hand, the increase of the temperature induced a significant (P<0.05) increase of the relative expression of CRHBP (Fig. 4.6 C) at 1 week (P<0.05) after the beginning the stress. There was no effect of temperature increase in the expression of POMCA and POMCB (Fig. 4.6 D) although higher values were observed after 1 week after beginning heat shock, while the relative expression of CRH remained stationary (Fig. 4.6 C).

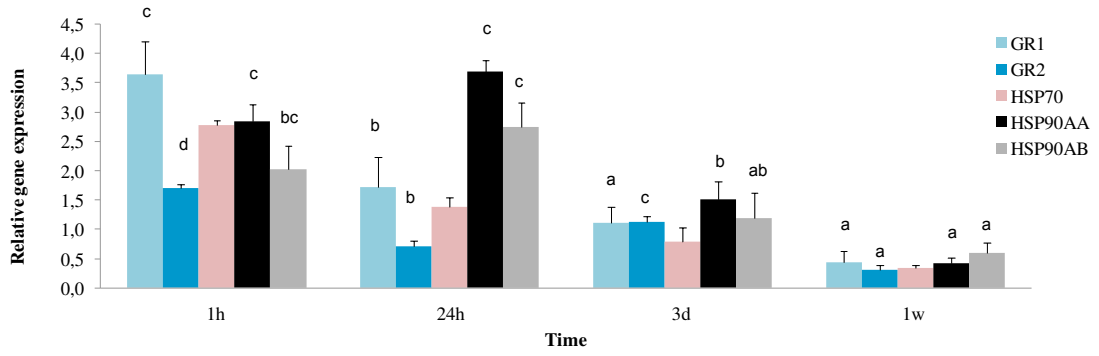

Figure 4.2: Relative expression of genes: GR1, GR2, HSP70, HSP90AA and HSP90AB in liver after the heat shock of +5ºC. Different letters denotes significant differences (P<0.05) among times after a temperature increase of 5ºC

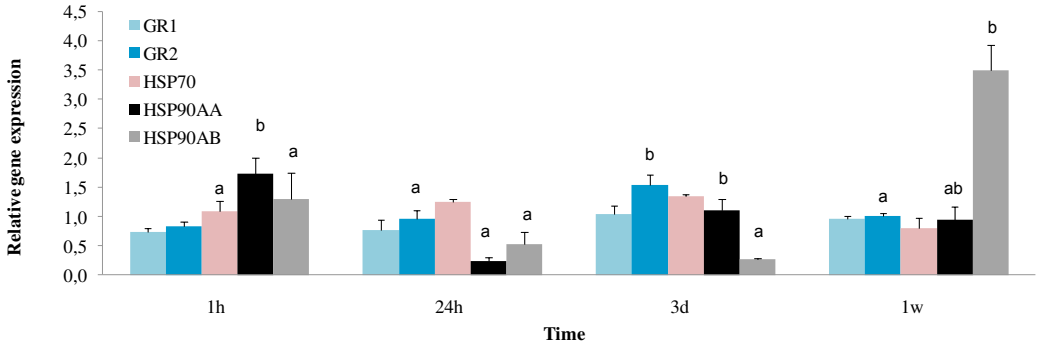

Figure 4.3: Relative expression of genes: GR1, GR2, HSP70, HSP90AA and HSP90AB in muscle after the heat shock of +5ºC. Different letters denotes significant differences (P<0.05) among times after a temperature increase of 5ºC

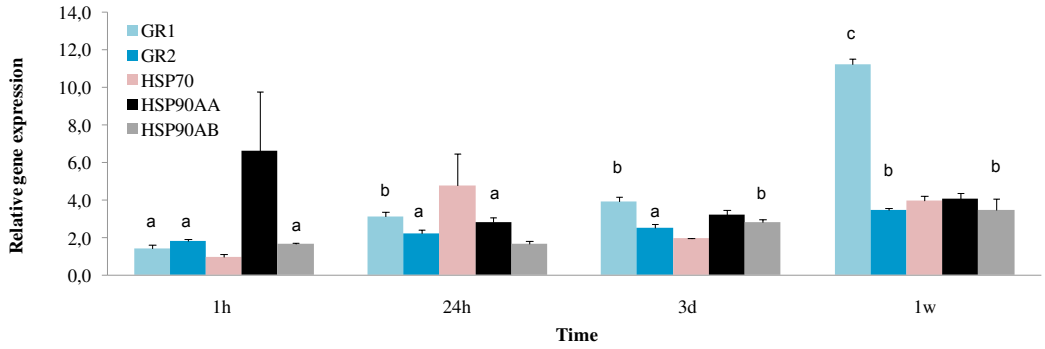

Figure 4.4: Relative expression of genes: GR1, GR2, HSP70, HSP90AA and HSP90AB in intestine after the heat shock of +5ºC. Different letters denotes significant differences (P<0.05) among times after a temperature increase of 5ºC

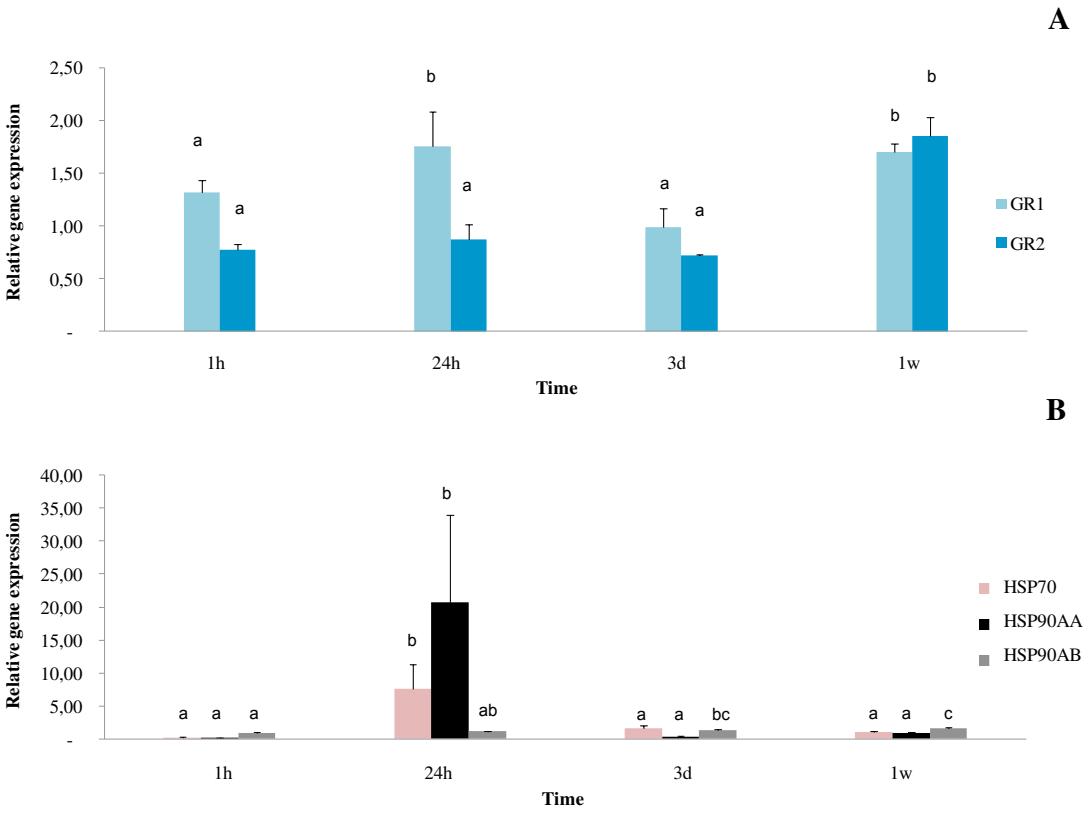

Figure 4.5: Relative expression of genes: (A) GR1 and GR2; (B) HSP70, HSP90AA and HSP90AB in gills after the heat shock of +5ºC. Different letters denotes significant differences (P<0.05) among times after a temperature increase of 5ºC

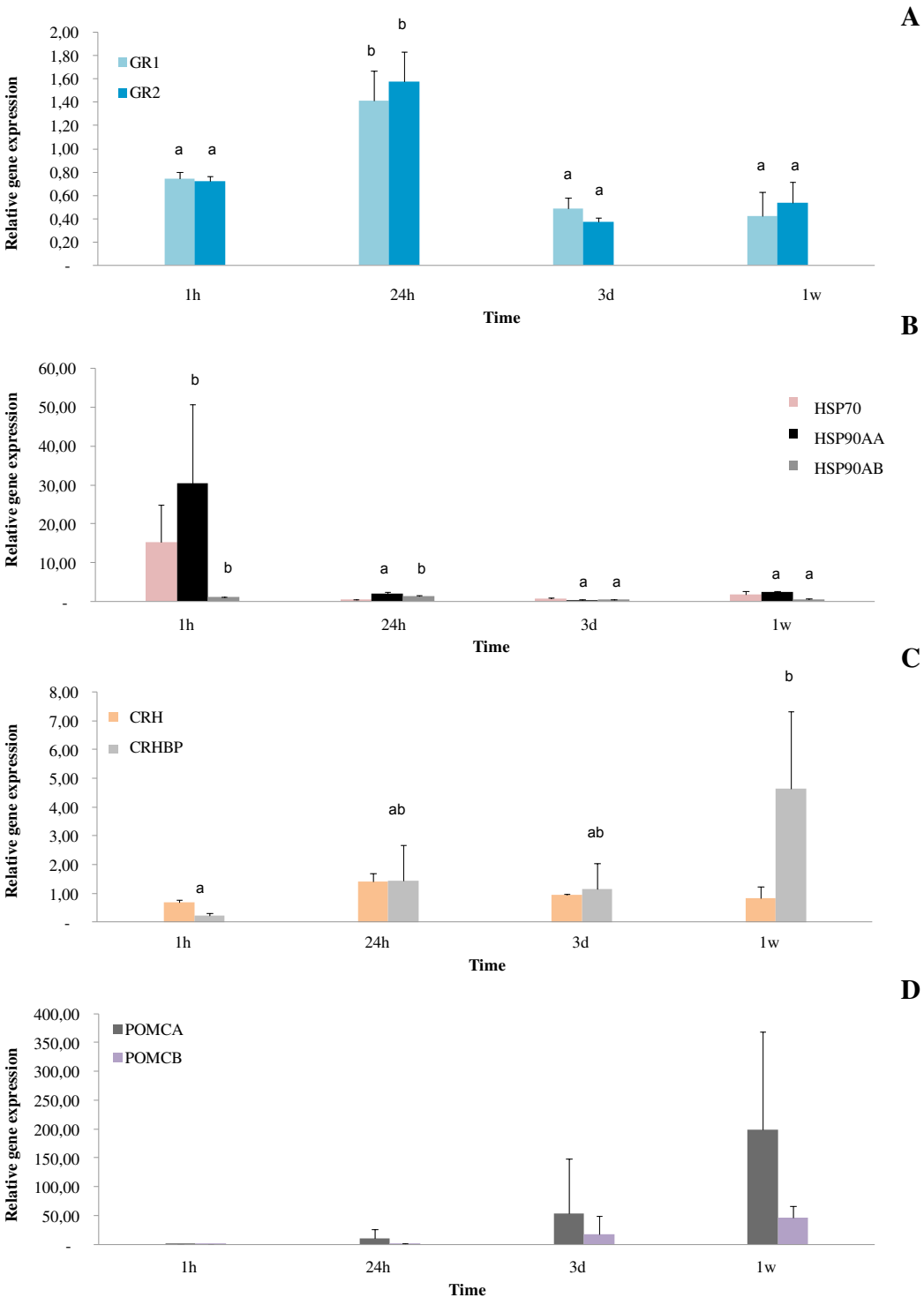

Figure 4.6: Relative expression of genes: (A) GR1 and GR2; (B) HSP70, HSP90AA and HSP90AB; (C) CRH and CRHBP; (D) POMCA and POMCB, in brain after the heat shock of +5ºC. Different letters denotes significant differences (P<0.05) among times after a temperature increase of 5ºC

4.5 Discussion

After a stress situation, increased circulating levels of cortisol as a short term response should provide alertness and energy for the animal to deal with the stressor and maintain homeostasis (Schreck, 1981; Mommsen *et al.,* 1999). At mid term, physiologic processes tend to adapt to compensate the stress with some limits (Schreck *et al.,* 2001). In the present study an increase of cortisol can be observed as a short term responses to the elevation of temperature, denoting the role of plasma cortisol as a sensitive indicator of thermal stress. This increase has been also observed in juvenile of Atlantic cod (*Gadus morhua* L.) exposed to an acute thermal challenge where the plasma cortisol showed an exponential increase with temperature (Pérez-Casanova *et al.,* 2008), and it is in agreement with several other studies on different fish species (Gamperl *et al.,* 1994; Wendelaar-Bonga, 1997; Mommsen *et al.,* 1999; Afonso *et al.,* 2008). However, after 1 week of thermal acclimation, a secondary peak of plasma cortisol was observed. Taking into account that this species usually lives in a wide range of temperature (Imsland *et al.,* 2003), this secondary elevation of cortisol could be denoting the attempted to scope an allostatic balance, as suggested by the results published by Costas *et al.* (2012) in Senegalese sole acclimated to 26ºC. However, a circumstance with a potentially negative impact on secondary stress factors in long term periods (McConnachie *et al.,* 2012) cannot be rejected and further experiences must be conducted with a long term acclimation time.

The cortisol acts as a transcription factor to modulate gene expression of the GR (Mommsen *et al.,* 1999), a key mediator of the vertebrate stress response (Wendelaar-Bonga, 1997; Charmandari *et al.,* 2004), leading to energy store mobilization to cope with stress (Mommsen *et al.,* 1999). A temperature changes has been described to trigger alterations of the GRs (Fernandino *et al.,* 2012) and HSPs (Roberts *et al.,* 2010) in fish. The presence of multiple corticosteroid receptors in teleosts may regulate a complex network of glucocorticoid signaling (Bury *et al.,* 2003; Schaff *et al.,* 2008), and the differential expression of these receptors may influence different pathways and coordinate responses during stress (Stolte *et al.,* 2008). Studies

by Vijayan *et al.* (2003) demonstrated that there is an autoregulation of GR mediated by cortisol during stress in fish. These mechanisms caused the absence of correlation between the abundance of GR mRNA and GR protein, suggesting that glucocorticoids may also affect the threshold of cellular stress, altering constitutive levels of HSP90 and HSP70 in the liver of trout (Sathiyaa *et al.,* 2001). The ability of cortisol or stress to promote the association of HSP70 with the GR could be an adaptive mechanism to enhance the functionality of the GR and ensure that glucocorticoid-mediated responses to stress occur efficiently (Basu *et al.,* 2003). HSPs elevated after a temperature shock is indicative of a stress (Roberts *et al.,* 2010), and are directly related to an increased thermo tolerance after a rise of cortisol (Basu *et al.,* 2002). These mechanisms had been described as adaptive processes in Atlantic salmon (*Salmo salar*) to cope with the thermal shock just before the smoltification (Pan *et al.,* 2000).

However, in Antarctic species like the Emerald Rock Cod (*Trematomus bernacchii*), the mRNA levels of proteins associated with apoptosis and DNA damage increased after heat stress, whereas HSP70 mRNA does not change, suggesting a potential failure regulation of heat stress in this species (Buckley and Somero, 2009). An up-regulation in HSP expression has been described as adaptive mechanism of eurythermal fish to temperature increase (Podrabsky and Somero, 2004; Buckley *et al.,* 2006; Buckley and Somero, 2009), failing this mechanisms when there is a lack of adaptive processes to heat stress in Antarctic fish (Hofmann *et al.,* 2000; Buckley and Somero, 2009). The response of HSPs to thermal stress seems to be tissue dependent (Palmisano *et al.,* 2000). These authors described a higher HSP90 expression in muscle, brain and gills of Chinook salmon following heat shock, when compared to liver, kidney and tail fin tissues (Palmisano *et al.,* 2000). This is in agreement with the results at the present study, where brain HSP expression is 7-fold higher than liver or even muscle, denoting the availability of this species to cope with thermal increases (Dyer *et al.,* 1991). Besides, Logan and Somero (2011) described a initial increase of HSP70 and HSP90AA expression in gills of Longjaw mudsucker (*Gillichthys mirabilis*) under a thermal shock of +9°C. This set of events

has been proposed as an ability to respond to heat stress in fish (Buckley and Somero, 2009; Hofmann *et al.,* 2000).

The stressful conditions have been shown to mobilize glucocorticoid receptors in fish (Prunet *et al.,* 2006). This activation depends on the intensity of the stress, cortisol lacking to bind to GR1 in non- or mild stressful conditions whereas both GR1 and GR2 may be mobilized in high stressful conditions (Bury *et al.,* 2003; Prunet *et al.,* 2006). The mRNA levels of GRs have been described to be up regulated in tilapia (*Oreochromis mossambicus*) after sea water acclimation. Among the different stressors, temperature have been described to induce serious alterations of the GR-systems, both in mammals (Matić *et al.,* 1998) and fish (Fernandino *et al.,* 2012). Fernandino *et al.* (2012) described an increased expression of GRs of pejerrey (*Odontesthes bonariensis*) larvae held at different Tª. Those larvae held at 29ºC showed significant increased GR1 expression when compared to larvae held at 17ºC. This is in agreement with the results obtained in the present experiment, when an increase of GR expression happens after a Tª increase. Specifically, GR1 expression was increased in liver and brain in the first 24 h after heat stress whereas other tissues as intestine GR1 increased after 1w of thermal stress, with no effect on muscle. The response of GR1 and GR2 seemed to be tissue specific in Senegalese sole. The differential GR tissue response against stress has been suggested for rainbow trout treated with slow release cortisol implants (Teles *et al.,* 2013). Differential tissue distribution of GR has been also described for the cichlid fish *Haplochromis burtoni* by Greenwood *et al.* (2003). Those authors described that GR2 was more highly expressed than GR1 in most of the tissues, at basal levels, at described also for rainbow trout (Ducouret *et al.,* 1995). However, in the present experiment, GR1 is more expressed than GR2 in liver, intestine and gills, after stressful conditions in agreement with those results found for Tilapia gills after handling stress (Aruna *et al.,* 2012). The apparent discrepancy among studies could be due to the type and intensity of the stress (Prunet *et al.,* 2006), and for different species studied since higher GR1 expression has been described for other fish species (Tagawa *et al.,* 1997).

The activation of GR depends not only on the expression of GR gene, but also on the intracellular HSP90/GR, either positively or negatively (Kang *et al.,* 1999). The binding of HSP90 allow GR to be competent for ligand binding (Segnitz and Gehring, 1997). The retention of GR either cytoplasma or nuclear is dependent of the ratio HSP90/GR and the intracellular localization of GR, being the nuclear retention of GR attenuated by the over expression of HSP90 (Tago *et al.,* 2004). Increase of intracellular HSP90 levels results in an increased HSP90/GR ratio, mainly in the nucleus, that inhibits GR binding to its DNA response element (Kang *et al.,* 1999). The positive modulation of the amplitude of response to steroids is the result of an optimal HSP90/GR ratio, whereas abnormally low or high ratios will negatively interfere with the response of GR (Qian *et al.,* 2001). An increase of HSP90/GR ratio has been proposed in rainbow trout hepatocytes treated with cortisol and heat shock as a modulator of the GR-dependent promoter activity (Sathiyaa *et al.,* 2001). These changes are favouring tissue responsiveness to glucocorticoid and could be mechanisms to increase tissue responsiveness to glucocorticoid stimulation (Vijayan *et al.,* 2003), offsetting the physiological consequences of GR protein down-regulated evident with the hyper-cortisolemia observed in fish (Pottinger *et al.,* 1994; Shrimpton and McCormick, 1999). However, this mechanism is dependent not only on the amount of cortisol after stress but also on heat shock (Sathiyaa *et al.,* 2001). This is in agreement with the results obtained in the present experiment, when an increased HSP90/GR ratio can be found after 24h of thermal stress, corresponding with a peak of plasma cortisol. How this elevation of HSP90/GR ratio is an adaptive mechanism remains unclear, but a preventive role on proteasomal degradation of GR has been proposed both in mammals (Segnitz and Gehring, 1997) and in fish (Aluru and Vijayan, 2007).

The response of GR complex to cortisol leads to different effects depending not only on the type of tissue, but also on the type of stress and the evolution of the response to stress, these tissue specific mechanisms needed to be elucidated (Le *et al.,* 2005; Vegiopoulos and Herzig, 2007; Aruna *et al.,* 2012). A specific GR response for each tissue throughout the time after heat shock has been observed in the present study in terms of relative quantification. For instance,

the brain showed an over-expression of GR gene 24h after thermal stress. Upon binding of cortisol, the glucocorticoid receptor (GR) in brain regulates the transcription of specific target genes, including those that encode the stress hormones corticotropin-releasing hormone (CRH) and ACTH, to auto-regulated the stress response in Zebra fish (Ziv *et al.,* 2013). These authors established that disruption of GR genomic activity results in a hyper activated HPI axis. In that sense, GR activity appears to be protective of the brain by dialing down the stress response (Ziv *et al.,* 2013). This over-expression has been also described for tilapia during the course of seawater acclimation and handing stress (Aruna *et al.,* 2012), or for hybrid striped bass (*M. chrysops x M. saxatilis*) under stress by infection with *Photobacterium damselae sp. Piscicida* (Acerete *et al.,* 2009). In the present experiment, 24 h after the onset of heat shock, the expression of CRH tended to be higher along with GRs in the brain, suggesting a possible role of GR to control the effect of the stress response through CRH in the brain. Further experiments are needed to clarify this brain GR response against other type of stress in Senegalese sole. Another tissue involved directly in the adaptation of teleost to environmental stress is the gills (Mommsen, 1984; McCormick, 1995, 2001; McCormick *et al.,* 2008). The aerobic cost for protein synthesis in the gills is high, consuming up to 70% oxygen during stressful situations (Houlihan *et al.,* 1995; Lyndon and Houlihan, 1998), including the effect of temperature (Pörtner and Knust, 2007). However, no changes in the GRs expression in gills were observed in the present study as a short term response to thermal shock, suggesting a possible mechanism to decrease tissue responsiveness to high cortisol levels (Teles *et al.,* 2013). These results are in agreement with previous studies in salmonids, where the acute elevation of cortisol, decrease the maximum binding capacity of gill GRs leading to a diminished sensitivity to circulating cortisol (Maule and Schreck, 1991; Shrimpton and Randall, 1994). However, the expression of GR1 appear up-regulated after 24h from the heat shock, maybe for the faster capable to respond to stress than GR2, as describe Aruna *et al.* (2012) in gills under handling stress. Further experiences are required to elucidate the short-term response of GR genes to different stressful situations such as salinity changes.

As a short-term response to thermal stress, the liver GR expression increased during the first hours, corresponding to the maximum of plasma cortisol found at the present study. Cortisol-mediated molecular changes in the gluconeogenic and protein catabolic pathways are GR-activated in trout hepatocytes, suggesting a key role for GR-specific signaling in this adaptive response (Aluru and Vijayan, 2007). Thus, stimulated glucose production by the high level of cortisol is mainly translated into a greater capacity for hepatic gluconeogenesis (Vijayan *et al.,* 1994). The short-term response in liver of Senegalese sole could be suggesting the increase of liver metabolic activity to cope with the heat stress, because the animals need to increase their metabolism and energy appropriately dosed (Mora and Maya, 2006). One of the prominent features of liver metabolism is the ability for de novo glucose synthesis, gluconeogenesis, in order to provide glucose for extra-hepatic tissues, including brain and gills during periods of stress (Mommsen *et al.,* 1999). GR-mediated corticosteroid signaling significantly increased glucose production in hepatocytes of trout (Aluru and Vijayan, 2007). This mechanism has been observed in bluegill sunfish (*Lepomis macrochirus*) with intraperitoneal implant of cortisol, during short term, but not during the long-term (McConnachie *et al.,* 2012). Increases in plasma glucose during a glucocorticoid stress response is the result of mobilization of carbohydrate energy stores, and these resources are finite (Mommsen *et al.,* 1999; Barton, 2002). In the present experiment the expression of both GRs in the liver after 1 week after thermal stress remained unchanged, while intestinal GR expression were increased, corresponding to a new increase of plasma cortisol. The observed results in intestine are in agreement with previous results in other species such as Mozambique tilapia (*Oreochromis mossambicus*) subjected to salinity changes or cortisol implantation (Takahashi *et al.,* 2006). Those authors observed increases in intestinal GR under chronic treatments, suggesting the importance of the GR up-regulation as a mechanism to adapt to a stressful situation in the intestinal tissue through regulation of tissue differentiation, development and metabolism. Besides, the induction of apoptosis in intestine appears to play an important role by increasing the intestinal epithelial permeability during stressful situations such as adaptation of

euryhaline fish to seawater, cortisol playing an important role by stimulating the intestinal apoptosis (Takahashi *et al.,* 2006).

On the other hand, HSP90AB has been mainly associated to long-term cell adaptation (Sreedhar *et al.,* 2004). In the present experiment, as a mid-term response to thermal stress, HSP90AB increased significantly in intestine, and also in muscle in agreement with results reported for Chinook salmon (Palmisano *et al.,* 2000). HSP90 has been proposed to play a tissue reorganization role in temperature acclimation through its action on proteolytic destruction of denatured enzyme isoforms or protein phosphorilation (Imamura *et al.,* 1998). Further experiments are required to elucidate the role of those genes in long-term temperature acclimation in Senegalese sole, as this is a species subjected to a wide range of temperature fluctuations when exploded semi-extensively and extensively (Arjona *et al.,* 2010; Castro *et al.,* 2012).

Interestingly, the thermal stress induced some changes in brain at mid-term. After one week, thermal-induced elevation of POMC and CRHBP expression was found in Senegalese sole. In this species, the decrease of POMCA mRNA has been considered an adaptive response of the fish to farm stocking density conditions (Wunderink *et al.,* 2011). The POMCB mRNA down regulation after short or long dexamethasone exposure has been proposed as mechanism to modulate the ACTH-mediated synthesis of cortisol (Salas-Leiton *et al.,* 2012). Besides, the CRHBP has been also reported as an inhibitor of the CRH-mediated ACTH release in pituitary mammal cells (Potter *et al.,* 1991). Both stress and glucocorticoids can up-regulate CRHBP mRNA expression, who in turns exerts a negative feedback on CRH actions (Huising *et al.,* 2004; Westphal and Seasholtz, 2006). The thermal-induced increase of CRHBP found in the present study could be indicating the activation of a negative feedback on the ACTH release from the pituitary in sole (Salas-Leiton *et al.,* 2012) after one week of thermal stress. However, certain POMC elevation after one week (although not significant) together with an increase of plasma cortisol at this point found in the present experiment could also indicate a non-well adaption of Senegalese sole to the new thermal conditions. Thus, further experiments are needed

to elucidate how Senegalese sole respond to longer acclimation periods in response to thermal changes.

In summary, thermal stress by increasing heat stress induced an elevation of plasma cortisol of juvenile Senegalese sole after 1 hour as a short-term response, and a consecutive increase after one week, as a mid-term response. Senegalese sole seems to respond positively in terms of adaptive mechanism, with a rapid over-expression of the relative expression of GRs and HSPs in the liver and brain tissue, significantly higher one hour post stress, denoting the fast and acute response of those tissues to a rapid change on temperature. The ratio HSP90/GR also increased 24 h after thermal shock, ratio proposed to be an adaptive mechanism to prevent proteasomal degradation of GR. As a mid-term response, the elevation of brain CRHBP mRNA expression one week after thermal shock could be an adaptive mechanism of negative feedback on HPI axis. Further experiences are required to elucidate how Senegalese sole responds to longer periods of acclimation to thermal increases.

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CAPÍTULO 5

"DIETARY VEGETABLE OIL AND IMMUNE-RELATED GENES IN SENEGALESE SOLE INTESTINE"

5. Dietary vegetable oils: effects on the expression of immune-related genes in Senegalese sole (*Solea senegalensis*) intestine

5.1 Abstract

The decreased availability of fish oil, traditionally used as oil source in marine aquafeeds, has lead to the search for alternatives oils. Vegetable oils (VO) are being extensively used as lipid sources in marine fish diets, inducing an imbalance on certain dietary fatty acids. Alteration on the dietary ratio of n-6/n-3 has been described to have detrimental effects on fish immunity. Senegalese sole has high susceptibility to stress and diseases, and little is known on the effects of dietary VO on its immunity.

Senegalese sole were fed diets (56% crude protein, 12% crude lipid) containing linseed (LO), soybean (SO) or fish (FO) oils as unique oil source. After 90 days of feeding, a stress test, (5 min of net chasing) was applied. Fish intestine were collected for biochemical analysis and expression of immune-related genes using Open-array RT PCR.

The use of VO did not induced changes in fish growth, but affected fatty acid profile of intestine and expression of immune-related genes. The use of SO (rich in n-6 fatty acids) induced an over-expression of those genes related to complement pathway, recognizing pathogen associated to molecular patterns, defensive response against bacteria, defensive response against viruses, antigen differentiation, cytokines and their receptors.

This general over-expression could indicate a chronic activation of inflammatory processes in fish gut. However, when a stress was applied, most of genes do not respond equal to those from fish fed n-3 oil based diet, maybe indicating the deleterious effects of a chronic

inflammation induced by n-6 dietary fatty acids and the subsequent low immune potential to they react when stressful conditions appear.

5.2 Introduction

In the last two decades, an intense global research effort has focused on studies on alternatives to fish oil and meals for aquaculture feeds, focusing specially on salmonids and Mediterranean marine fish (reviewed by Sales and Glencross, 2011). Vegetable oils have been proved to be the best candidates for the replacement of fish oil in aquafeeds, due to their low price and availability. However, in contrast to fish oil, which is rich in long chain polyunsaturated fatty acids (LC-PUFA), the main fatty acids of vegetable oils are fundamentally C18 PUFA, with n-6 C18 PUFA, such as linoleic acid (LA, 18:2n-6) as the main fatty acid presented in those oils (Sargent *et al.,* 2002). Thus, the use of vegetable oils such as SO or sunflower oils leads to a high inclusion of dietary LA, that has been described to negatively affect growth performance (Turchini *et al.,* 2009; Sales and Glencross, 2011) and specifically nonspecific immunity (reviewed by Montero and Izquierdo, 2010), due to unbalances of the ratio n-3/n-6 in the diet.

To avoid impacts on fish health, a well-balanced n-3/n-6 fatty acid ratio is required to maintain good health conditions (Simopoulos, 2008), since immune cells structure and functioning, tissue integrity, cell signaling and eicosanoids, depend directly on the dietary balance of fatty acids (Calder, 2006; Yaqoob and Calder, 2007). In fish nutrition, the use of VO rich in n-6 fatty acid has been described to alter several parameters of the immune system in different aquaculture species, depending on the type of vegetable oil used, the species studied and the level and period of substitution (reviewed by Montero and Izquierdo, 2010). Unbalances of n-3/n-6 ratio due to the use of dietary vegetable oils can affect piscine immunity at different levels: i) effects on immune cells functionality (i.e., phagocytosis, macrophage respiratory burst activity) (Sheldon and Blazer, 1991, Montero *et al.,* 2003), ii) immune cell fatty acid

composition (Waagbo *et al.,* 1995; Farndale *et al.,* 1999; Montero *et al.,* 2003), iii) humoral immunological processes such as serum lysozyme activity or alternative complement activity (Montero *et al.,* 2003; Lin *et al.,* 2007), iv) eicosanoid production (Gjøen *et al.,* 2004; Ganga *et al.,* 2005) and v) alterations of expression of immune-related genes (Montero *et al.,* 2008, 2010; Calduch-Giner *et al.,* 2012). Those dietary induced alterations of immune system can compromise fish resistance to diseases, although the susceptibility to infection depends on complex interactions between environment, pathogen and fish (Lodemel *et al.,* 2001; Brandsen *et al.,* 2003; Montero *et al.,* 2010, Kiron *et al.,* 2011). Gilthead sea bream (*Sparus aurata*) fed with a diet rich in VO exhibited a worse disease outcome when challenged with the intestinal parasite *Enteromyxum leei*, with some immunological parameters such as haematocrit, complement and lysozyme activity decreased when compared to infected fish fed a FO diet (Estensoro *et al.,* 2012). However, Calduch-Giner and co-authors (2012) showed that this replacement of 66% of fish oil by a blend of VO in gilthead sea bream diets did not modified the intestinal transcriptome in a basal state, but there were significant effects when those fish were exposed to a intestinal parasite challenge, being those changes a consequence of the different progression of the disease under different dietary treatments. In this sense, Seierstad *et al.* (2009) did not found effects of vegetable oils on the expression of pro-inflammatory cytokines of lipopolysaccharide (LPS)-stimulated head kidney from Atlantic salmon (*Salmo salar*) *ex vivo* incubated in plasma from fish fed on different lipid sources.

However, other authors have been described effects on expression of genes implied in selected immune pathways. The complete substitution of fish oil by either single VO (SO or LO) or blends of them (50/50) induced a chronic hepatic expression of *mx* protein (protein of resistance against mixovirus) gene in non-infected gilthead seabream (Montero *et al.,* 2008). VO replacement levels (50-60%) increased the cumulative mortality in gilthead sea bream challenged with *Vibrio alginolyticus* or increased the intestine expression of tumor necrosis factor alpha (*tnfa*) in *Photobacterium damselae* subsp. piscicida infected animals (Montero and Izquierdo, 2010; Montero *et al.,* 2010). Studies on effects of FO substitution in intestinal

transcriptome are very recent (Morais *et al.,* 2012a, b; Moldal *et al.,* 2014) and traditionally has been focused on the effects of replacement of fish meal by vegetable proteins, particularly soybean meal (Baeverfjord and Krogdahl, 1996), due to its potential to cause enteritis in particular in salmonids. Dietary VO induces histological changes in fish enterocytes, mainly due to supranuclear lipid droplet formation, altered reacylation mechanisms and decreased phospholipid synthesis (Caballero *et al.,* 2002; 2003).

The effects of dietary FO replacement by VO on fish intestinal transcriptome suggested morphological and structural changes to the intestinal muscle layer (Morais *et al.,* 2012b), expression of genes related to cell proliferation and apoptosis (Olsvik *et al.,* 2007), even affecting the maintenance of epithelial barrier functions and the regulation of the inflammatory response in the gastrointestinal tract (Oxley *et al.,* 2010), denoting the impact of the dietary lipids in intestine health and integrity.

Senegalese sole has been described to be affected by dietary vegetable oils in its fatty acid metabolism (Navarro-Guillén *et al.,* 2014), performance and response to stressful situations (Benitez-Dorta *et al.,* 2013). Although there are some studies on the effect of diet variations in Senegalese sole gut functionality and health, such as the use of pro-biotics (Saenz de Rodrigañez *et al.,* 2009) or feeding ratios (Salas-Leiton *et al.,* 2010), little is known on the effect of dietary oils on intestine immune-related genes. Thus, the objective of this study was to elucidate the effect of replacing dietary fish oil by vegetable oils on Senegalese sole on gut immune-related genes before and after an acute stress.

5.3 Material and methods

5.3.1 Experimental diets

Three isonitrogenous (56% crude protein), isolipidic (12% total lipids) and isoenergetic diets were formulated: a control diet based on fish oil (100FO) (Peruvian anchovy oil) as the sole lipid source, and two vegetable oils diets where 100% of the fish oil was replaced by either linseed oil (100LO) or soybean oil (100SO). Each diet was tested in triplicate. Ingredients and proximate composition and selected fatty acids contents are shown in Tables 5.1 and 5.2.

Table 5.1: Main ingredients and proximate composition from the experimental diets fed to Senegalese sole juveniles for 12 weeks

Diets /Ingredients	100FO	100LO	100SO
Fish meal	660	660	660
Wheat gluten	15	15	15
Starch	6.5	6.5	6.5
Fish Oil	8	-	-
Linseed oil	-	8	-
Soybean oil	-	-	8
Vitamin mix	2	2	2
Mineral mix	2	2	2
CMC	0.5	0.5	0.5
Biochemical composition (% D.W.)			
Crude protein	56.7 ± 2.2	56.8 ± 2.2	56.6 ± 2.2
Crude lipids	12.3 ± 2.2	13.0 ± 2.2	12.6 ± 2.2
Ash	10.1 ± 2.2	10.5 ± 2.2	10.3 ± 2.2

Table 5.2: Fatty acid composition of the experimental diets fed to Senegalese sole juveniles for 12 weeks (g $100g^{-1}$ total identified fatty acids)

	Diets		
Fatty acids	**100FO**	**100LO**	**100SO**
14:0	4.9	3.19	3.34
14:1n-7	0.0	0.0	0.0
15:0	0.3	0.21	0.3
16:0ISO	0.0	0.02	n.d.
16:0	19.9	15.7	15.9
16:1n-7	6.0	3.6	2.9
16:1n-5	0.2	0.2	0.2
16:2n-6	0.9	0.53	0.3
16:2n-4	0.0	0.61	0.41
17:0	0.9	0.04	0.42
16:3n-4	0.3	0.06	0.04
16:4n-3	1.3	1.00	0.65
18:0	4.1	4.96	3.01
18:1n-9	18.3	19.04	11.97
18:1n-7	3.0	2.1	1.4
18:1n-5	0.1	0.1	0.0
18:2n-6	6.1	9.6	36.8
18:3n-6	0.2	0.1	0.1
18:3n-4	0.2	0.0	0.0
18:3n-3	2.1	20.5	6.8
18:4n-3	1.0	0.6	0.5
18:4n-1	0.4	0.1	0.1
20:0	0.2	0.2	0.2
20:1n-9	1.4	0.5	0.3
20:2n-9	0.1	0.0	0.0
20:4n-6	0.8	0.5	0.6
20:3n-3	n.d.	0.1	0.0
20:4n-3	0.8	0.3	0.4
20:5n-3	12.0	8.8	5.6
22:1n-11	0.4	0.2	0.1
22:5n-3	3.1	1.3	2.6
22:6n-3	11.1	5.7	4.9
Total saturates	30.3	24.9	23.0
Total monoenes	29.5	25.8	17.4
∑n-3	31.4	38.3	21.5
∑n-6	7.3	10.2	37.5
∑n-9	19.8	19.6	12.4
∑n-3 LC-PUFA	27.0	16.2	13.6
ARA/EPA	0.1	0.1	0.1
EPA/DHA	1.1	1.5	1.1
n-3/n-6	4.3	3.8	0.6

5.3.2 Experimental fish and samples collection

Two hundred and seventy Senegalese sole juveniles with 3.50 ± 0.24 g of initial body weight were randomly distributed into 9 indoor PVC tanks (45L of capacity and 60x40 cm of surface) of 30 fish per tank. Tanks were supplied with filtered seawater at a temperature of 21.6-22.8ºC, and natural photoperiod (around 12 L: 12 D). Water dissolved oxygen values ranged 6.20 ± 0.70 g l^{-1}. Fish were manually fed until apparent satiation with the experimental diets for 12 weeks (twice daily, 6 days a week). Feed intake was daily determined and growth parameters were calculated at days 0, 30, 60, and 90. Food conversion ratio (FCR), defined as the amount of food ingested by the generated biomass, and specific growth rate (SGR) defined as [(ln final weight - ln initial weight)/number of days] x 100 were also calculated.

At the end of the experimental period, ten fish per tank (30 per treatment) were sacrificed by an overdose of anesthetic, and intestine for biochemical and fatty acid determinations were obtained by dissection. Samples were kept at -80ºC until analysis. Blood from 6 fish per tank (18 per dietary treatment) was collected by caudal sinus puncture with a 1 ml plastic syringe, and was placed into a heparinized Eppendorf tube and was centrifuged at 800 *xg* during 10 min to obtain plasma samples. Plasma samples were kept at -80°C until analysis. From the remaining fish, 9 fish per treatment were sacrificed by overdose of anesthetic, and were used to obtain samples of intestine for determination of stress-related gene expression. These samples were quickly kept in RNA later and frozen at -80ºC until gene expression analysis.

A chasing stress test was also conducted at the end of the experimental period and 9 animals from each experimental diet were subjected to a 5 min net chasing. After this stressful situation, samples of intestine were obtained for determination of the relative expression of stress-related genes and were kept in RNA later and frozen at -80ºC until analysis.

5.3.3 Biochemical and fatty acid composition analysis

Biochemical and fatty acid composition of diets and selected tissues were conducted following standard procedures (AOAC, 1995). Dry matter content was determined after drying the sample in an oven at 105ºC to constant weight, ash by combustion in a muffle furnace at 600ºC for 12 h, protein content (N x 6.25) was determined by Kjeldahl method and crude lipid was extracted following the Folch method (Folch *et al.,* 1957). Fatty acids from total lipids were prepared by transmethylation (Christie, 1982) and separated by gas chromatography (Izquierdo *et al.,* 1992), being quantified by flame ionizator detector (FID) and identified by comparison to external standards (EPA 28, Nippai, Ltd. Tokyo, Japan). All analyses were conducted by triplicates.

5.3.4 Plasma cortisol

Plasma cortisol concentration was determined by radio-immunoassay using the trypsin–antitrypsin method as previously described for marine fish species (Rotllant *et al.,* 2001).

5.3.5 RNA isolation and gene expression analysis

For RNA isolation, intestines sampled from each tank were pooled (n = 3 independent pools/ condition) and approximately 100 mg were used for total RNA using 1 ml TRI Reagent (SIGMA-Aldrich, Sant Louis, Missouri). Homogenization of intestine as carried out in the Tissue Lyser II instrument (Qiagen) using sing Matrix D (Q-BioGene) for 2 min at speed of 30Hz. In all cases, total RNA was treated once with DNase I using the RNase-Free DNase kit (Qiagen) for 30 min. Chloroform and isopropanol were used for separation and precipitation, respectively. The pellets were hydrated with 100 µl of Milli-Q sterile water, previously treated with 0.1% DEPC and kept at -80ºC until analysis. RNA sample quality was checked in agarose

gels and quantification was performed with a NanoDrop 1000 spectrophotometer (Thermo Scientific).

RT-PCR analysis for innate immune genes (Tables 5.3A, B, C, D, E, F and G) was performed using the OpenArray® Real Time PCR platform (Life Technologies). Total RNA (2 µg) from each sample was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies) and qPCR reactions were done using the TaqMan® OpenArray® Real Time PCR Master Mix. All cDNA and Taqman amplification procedures were carried out in accordance with the manufacturer's protocol. To run the Taqman assays, we used an OpenArray® Real-Time PCR Instrument (Life technologies). Samples were loaded into OpenArray plates with the OpenArray® AccuFill™ System according to the manufacturer's protocols. The openarray chips were designed in the format 56x48 containing probes for 56 genes related with innate immune system and prostaglandin synthesis including. The complete set of genes evaluated included 9 genes associated to the antiviral defense (*ifnc*, *mx*, *irf1*, *irf2*, *irf3*, *irf7*, *irf8*, *irf9*, *irf10*), 15 interleukins and their receptors (*il1b, il6, il8b*, *il8**c**, il10, Il11a*, *il12b*, *il15*, *il17c*, *tnfa*, *Il1rl1*, *cxcr2*, *il10rb*, *il15ra*, *il17ra*), 8 complement factors (*c1ql4*, *c2*, *c3*, *c4a*, *c4b*, *c5*, *c9*, *cfh*), 5 related to pathogen-associated molecular patterns (PAMPs) recognition (*pglyrp1*, *tlr1*, *tlr5s, tlr8b, tlr9*), 5 lysozymes (*lyg1*, *lyg2*, *lyg3*, *lyc1*, *lyc2*), 3 differentiation antigens (*cd4, cd8a, cd8b*) and 6 chemokines (*cck3, ccl25, cxcl10, cxcl13, ccl19, ccl20*). Also, 3 reference genes (*ub52*, *eef1a1* and *gapdh2*) were included (Infante *et al.,* 2008). Full name, as well as primers and amplicon size are indicated in Tables 5.3A, B, C, D, E, F and G. Each subarray was loaded with 5.0 µl of master mix containing specific cDNA and PCR mixture. All sequences were obtained from *SoleaDB* (http:// www.juntadeandalucia.es /agriculturaypesca /ifapa /soleadb_ifapa /). Primers and probes were designed using the Custom TaqMan® Assay Design Tool (Life Technologies). For analysis, raw data were imported into the Datassistv 3.01 software and Ct values exported and analysed using the $2^{(-\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001). Relative gene expression was calculated by using the $2^{(-\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001) using *gapdh2* as reference gene for normalization (Infante *et al.,* 2008). The

FO diet was used as calibrator to compare the effect of diets whereas the unstressed group for each dietary treatment was used to analyze the effect of chasing stress in each diet.

PermutMatrix was used to conduct cluster analysis for different diets using log 2 of fold-change with parameters set as following: Dissimilarity: Euclidean distance, Hierarchical: McQuitty's criteria Method, Seriation: Multiple-fragment heuristic (MF).

Table 5.3A: Primers of interleukins and receptors

Interleukins and Receptors					
Gene Name	**Abbrev.**	**ACC NO**	**Forward Primer Seq.**	**Reverse Primer Seq.**	**pb**
Interleukin 1B	*il1b*	AI6RN14	CGCAGAAAGTGACATGTTGAGATTT	GGAAGCGGGCAGACATGA	83
Interleukin 6	*Il6*	AI70L8C	ACAATTTCCTGCAGAGATAAAAGTAAGCT	CAAGCCCTCAGGCCTACAATATTAA	106
Interleukin 8 type b	*Il8b*	AI89KEK	GAAGGTGGAAATAATTCCTGGAAGCT	TGTCTTTTTCAGCGTGGCAATG	70
Interleukin 8 type c	*Il8c*	AIAAZKD	GCCGGCGTTACTGTCTGAA	GACTGAGCTCGTCTGACTGTAG	97
Interleukin 10	*il10*	AIBJXQL	CCGTCTTTGTGTTATTTCTCCAACAG	TGGAGTTCAGCTTTGTGATGTCA	78
Interleukin 11a	*Il11a*	AICSVWT	CTGCAGAGCCATGAGGATACAT	CTATCCACTACATTGCACTCTGACA	73
Interleukin 12 beta	*il12b*	AID1T21	ACGCAGTACAGATCCAACGC	CCTGACAGTGAACCCCTGATC	106
Interleukin 15	*il15*	AIFAR89	ATCTCTGTAAATAATTGCATTTGTTTATATTTTGT	AAGGACGGCAAGGTTTGTCA	84
Interleukin 17 c	*il17c*	AIGJQFH	AGGCTCGCTGTCTTTGCT	GACTTTCCGTCGGAGGATTCC	63
Tumor necrosis factor alpha	*tnfa*	AI6RN15	CCATAGGCAGCAAAGTGTCTCT	CCTGTCCGTCTGTCAACGT	87
interleukin 1 receptor type I	*Il1rl1*	AIRR9ZG	CACCACCAAAGATCATGAGGGTATC	CTGGAGCCTGATGAGTTATAAACCT	84
CXCR2 receptors	*cxcr2*	AIS075O	CGGTGCTGTATGCCTTTGTG	TCCATGAGGCCGATCTTCCT	76
Interleukin-10 Receptor Subunit beta	*il10rb*	AIT96BW	GAGAGAACCACCAATGAGGAAGAAG	CAGAGAAGATCAGCGACACCAA	64
Interleukin-15 Receptor Subunit alpha	*il15ra*	AIVI4H4	TGTCCGTGTTCAGAAATTCGTCAT	GCCTCAAATATCCGTCCACACAT	103
Interleukin 17 receptor A	*il17ra*	AIWR2OC	GCGGTGAACTGTCCTCCTT	TCATTGGGAATTTGAAGTGGTCCTT	74

Table 5.3B: Primers of pathogen-associated molecular patterns (PAMP)

Pathogen-Associated Molecular Patterns (PAMP)					
Gene Name	**Abbrev.**	**ACC NO**	**Forward Primer Seq.**	**Reverse Primer Seq.**	**pb**
Peptidoglycan recognition protein 1	*pglyrp1*	AI20TJH	GCAGGCTCTGATGGACACAT	CCTATTGAGTTGTGTCCAAGAGTGT	60
Toll-Like Receptor 1	*tlr1*	AIRR9ZH	CCCCATCCTCAATGAACTTCTCTTG	GGGCAGCTCTTCAGGTTGTT	75
Toll-Like Receptor 5S	*tlr5s*	AIS075P	CAAAGTTCATCGTTCGGTCCAAATG	TGTGCCCTGTGACCAAACA	84
Toll-Like Receptor 8 B	*tlr8b*	AIT96BX	GGTATGTCAGATTTGGCAGCGAA	TCAACCTCTCCGGAAATGGATTTT	75
Toll-Like Receptor 9	*tlr9*	AIVI4H5	AGTACCTCCAGCTGAGGAAGAG	CGTCCTCATCCTGTTCCAGAAAAG	98
g-type lysozyme 1	*lyg1*	AIWR2OD	CCAAATGGAGGTGGACACACT	CGCCTTGGCAGAGGTGTT	64
g-type lysozyme 2	*lyg2*	AIX00UL	GGACTCAAAAAGAGGAGCATATAACGG	GAGTGTGGTTACCTCCTTTTGGATT	77
g-type lysozyme 3	*lyg3*	AIY9Y0T	GCCCTTATTGCTGGCATCATCT	TCCCCCACCGTCAATAAGAGTAT	66
c-type lysozyme 1	*lyc1*	AI0IW61	CAGATCAACAGCCGCTATTGG	GCTGATTCCACATGCATTTGAAGTG	66
c-type lysozyme 2	*lyc2*	AI1RVC9	CCTGAGCCAGTGGGAGTCT	GATCTGGAGGATGCCGTAGTC	85

Table 5.3C: Primers of chemokines

Chemokines					
Gene Name	**Abbrev.**	**ACC NO**	**Forward Primer Seq.**	**Reverse Primer Seq.**	**pb**
C-C chemokine CK3	*cck3*	AIAAZKE	GAGAGGACTTGGGTTTTTGAGAAGA	GTTGTAGTGGCAGGCGTAATC	80
C-C chemokine 25	*ccl25*	AIBJXQM	CGGGACAAAGGAGAGCAACTT	AATCCATGTGGTCCAGCGT	69
CXC chemokine 10-like	*cxcl10*	AICSVWU	GGAGAAATGTGTGAACCCAGAGT	CGCGCTGCTCTTTTTCTCT	75
C-X-C chemokine 13	*cxcl13*	AID1T22	GTCAACGGGCTTCTCTCTGA	TGAGGCTTCATGAGTCCTGTTTATG	72
C-C chemokine 19	*ccl19*	AIGJQFI	GCGGCAGGAACCTGTGT	CGTACGCTCAGCTGTTTGAC	59
C-C chemokine 20	*ccl20*	AIFAR9A	ACACCGTCCAGACCATAAACAC	CTCGGACGGTGGAAGATGATG	61

Table 5.3D: Primers of complement pathways

Complement Pathways					
Gene Name	**Abbrev.**	**ACC NO**	**Forward Primer Seq.**	**Reverse Primer Seq.**	**pb**
C1q-like protein 4	*c1ql4*	AIHSOLP	CTACGCTTCTAACAGTGTGATCCT	AGCTGCACACACACCTCATC	60
Complement C2 factor B	*c2*	AII1MRX	AGTCAGCTCCAGGCTCTGT	CCGCCGGCTGATCTCAT	60
Complement C3	*c3*	AIKAKX5	GACCAAACACAGTGTGTGAAATTCT	CTGCAGTTCTCTTCAGCACATG	104
Complement C4-1	*c4a*	AILJI4D	GACTGACAAAGAATGATCGTTTGCA	CTGAGCACTTCGACAATGTATCCAT	79
Complement C4-2	*c4b*	AIMSHAL	ACGAGTGCAAAGGACGGAATAATAA	CGTTTGCGTTGTCACCTTCAA	106
Complement C5	*c5*	AIN1FGT	GGTGACAGCCTCCATGGT	CGGACGTACCTTCTCCATACAGA	71
Complement C9	*c9*	AIPADM1	TCAAATTCAGCCCAAGTGAGGATAC	CTCAGACCGACATTCACATTCAC	64
Factor H	*cfh*	AIQJBS9	GCAGCAGACAAACAGCAACAG	GCTGACTGGTACAGTATGAATTCCT	101

Table 5.3E: Primers of antiviral defense

Antiviral Defense					
Gene Name	**Abbrev.**	**ACC NO**	**Forward Primer Seq.**	**Reverse Primer Seq.**	**pb**
Interferon C	*ifnc*	AI70L8D	GGAAACTCGATACCTTCATGAACCT	TGTGGACCAGTGGAGAGACA	73
Interferon-induced GTP-binding protein M	*mx1*	AI89KEL	CATCCACCTGTCCCACACA	GCCCGCGATATTTGTCTTCATAGAT	83
Interferon regulatory factor 1	*irf1*	AIX00UK	GGGCGATTCGCTCTCAGA	GTGTGTACAGTGCTGTCGACT	60
Interferon regulatory factor 2	*irf2*	AIY9Y0S	GCTAATTTCCGCTGTGCTATGAA	TGATGCTTTTGTCCTTCACTTCCT	64
Interferon regulatory factor 3	*irf3*	AI0IW60	CGACCTCAAGAGATTCCCAAACTG	GCCAACATTCCTCCTATAAAGTCCTT	74
Interferon regulatory factor 7	*irf7*	AI1RVC8	CGAGATGGCGCAGATGGAA	GGCTGTTGTGTGACATCTGTAGAC	74
Interferon regulatory factor 8	*irf8*	AI20TJG	CCTCTACAGTTCGGACAGCAT	CTGGCGGTCGTACTCTATGAG	70
Interferon regulatory factor 9	*irf9*	AI39RPO	CCCAATACTGGCCAAAATCACAGAT	GGCCAGCATTGGGCCAATA	76
Interferon regulatory factor 10	*irf10*	AI5IPVW	CTGCAGGGACAGGTTCCT	GGCAACGACACCGAGTTC	95

Table 5.3F: Primers of differentiation antigen

Differentiation Antigen					
Gene Name	**Abbrev.**	**ACC NO**	**Forward Primer Seq.**	**Reverse Primer Seq.**	**pb**
CD8 ALPHA	*cd8a*	AIHSOLQ	GTGCCAGCATTAAAAGCAACGA	GCAGTCACAACTTCCGCTCTTT	82
CD8 BETA	*cd8b*	AII1MRY	GGTTTGGTCGGAGGATTCACT	GTCGTAAAGGACAAGTCCAACAGA	80
CD4	*cd4*	AIKAKX6	GACCTCAGGCTGCAATGGT	TGAGCAGAGTGATGGACAGACT	65

Table 5.3G: Primers of housekeeping genes

Housekeeping Genes					
Gene Name	**Abbrev.**	**ACC NO**	**Forward Primer Seq.**	**Reverse Primer Seq.**	**pb**
Ubiquitin UB52	*ubi*	AILJI4E	AGGCTAAGATTCAGGATAAGGAAGGA	TCCATCCTCCAGCTGTTTGC	80
Eukaryotiic elongation factor 1A1	*eef1a1*	AIMSHAM	CTGTTGGACGTGTCGAGACT	TCTCCACAGACTTGACCTCAGT	93
Glyceraldhyde 3 phosphate dehydrogenase 2	*gapdh2*	AIN1FGU	TCATTCCTGAGCTCAACGGTAAG	AGGTCAACCACTGACACATCAG	76

5.3.6 Statistical analysis

All data were tested for normality and homogeneity of variance. Means and standard deviations (SD) were calculated for each parameter measured except for cortisol values where values are represented as Mean plus standard error (SE). Statistical analyses followed methods outlined by Sokal and Rohlf (1995). Data of fish growth and tissue fatty acid composition were submitted to a one-way analysis of variance (ANOVA). When F values showed significance, individual means were compared using Tukey multiple comparison test. Significant differences were considered for $P<0.05$. If the variances were not normally distributed, the Kruskall–Wallis non-parametric test was applied to the data.

RT-PCR datas were log-transformed in order to comply with normality and homogeneity of variance. One-way multivariate analysis of variance (MANOVA) approach was used to

compare the effect of the diet. Moreover, a two-way MANOVA was used to test the effect of stress using the diet and stress as fixed factors. Moreover, t-student was carried to compare control and stress groups for each diet. Analyses were performed using SPSS software (SPSS for windows 11.0).

5.4 Results

At the end of the experimental period, the different diets fed did not induce significant differences in fish survival (around 85% for all the experimental groups), FCR ranging (from 1.58 for 100FO fish to 1.66 for 100SO fish), SGR (ranged from 1.51 for fish fed 100FO diet to 1.29 for fish fed 100SO diet) or hepatosomatic index (around 0.60) (Table 5.4). However, the use of soybean oil as the main source of lipid induced a lower ($P<0.10$) fish growth in terms of final body weight (Table 5.4).

Inclusion of vegetable oils increased linoleic and linolenic acids in intestine of fish fed diets with soybean (100SO) or linseed (100LO) oil, respectively (Table 5.5). But it did not affected lipid content in intestine (around 3% fresh weight) (Table 5.4). There were no significant differences in the docosahexanoic acid (DHA; 22:6n-3) content of intestine between diets, but the fish fed with vegetable oil showed the lowering values. As occurs with the amount of eicosapentaenoic acid (EPA; 20:5n-3) and arachidonic acid (ARA; 20:4n-6), significantly reduced in fish fed with vegetal oil dietary (Table 5.5).

The fish fed 100LO diet showed the highest, but not significant, cortisol value at basal state. With a ranging from 8.43 to 12.54 ng cortisol/ml plasma in fish fed 100FO or 100LO respectively (Table 5.4).

Table 5.4: Senegalese sole growth, survival rate and plasma cortisol fed experimental diets. Mean ± SD, except for plasma cortisol values, represented as mean ± SE

Diets	100FO	100LO	100SO
Initial body weight (g)*	3.5 ± 0.2	3.5 ± 0.2	3.5 ± 0.3
Final body weight (g) **	13.7 ± 1.3[a]	13.2 ± 1.7[a]	11.0 ± 3.2[b]
SGR***	1.5 ± 0.2	1.5 ± 0.3	1.3 ± 0.2
FCR***	1.6 ± 0.3	1.6 ± 0.4	1.7 ± 0.5
Survival rate* (%)**	86.5 ± 2.6	85.3 ± 3.1	86.5 ± 3.0
HSI¥	0.6 ± 0.0	0.6 ± 0.1	0.6 ± 0.1
Intestine lipid content (% D.W.)	2.8 ± 0.7	3.2 ± 0.6	3.0 ± 0.7
Plasma cortisol (ng/ml)¥¥	8.4 ± 1.3	12.5 ± 1.5	10.0 ± 1.6

* n=30x3; ** n= 25x3; ***n=3; ¥ n=10x3; ¥¥ n=6x3. Different letters within a row denote significant differences at P<0.10

Table 5.5: Intestine fatty acid composition of fish fed the different experimental diets (g $100g^{-1}$ fatty acids identified)

	Diets		
Fatty acids	**100FO**	**100LO**	**100SO**
14:0	5.6 ± 0.1[a]	2.1 ± 0.3[b]	3.1 ± 0.4[b]
14:1n-7	0.1 ± 0.0	n.d.	n.d.
15:0	0.35 ± 0.0	0.2 ± 0.0	0.2 ± 0.1
16:0ISO	0.1 ± 0.0	0.1 ± 0.0	n.d.
16:0	22.4 ± 1.2[a]	16.6 ± 1.2[b]	16.5 ± 3.0[b]
16:1n-7	8.1 ± 0.4[a]	2.5 ± 0.4[b]	4.2 ± 1.3[b]
16:1n-5	0.2 ± 0.1	n.d.	n.d.
16:2n-6	0.9 ± 0.1[a]	0.3 ± 0.0[b]	0.5 ± 0.1[b]
16:2n-4	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.1
17:0	0.9 ± 0.0[a]	0.3 ± 0.1[b]	0.5 ± 0.2[b]
16:3n-4	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.1
16:4n-3	1.1 ± 0.2[a]	0.3 ± 0.1[b]	0.5 ± 0.1[b]
18:0	6.1 ± 0.6[ab]	8.7 ± 0.7[a]	4.8 ± 0.6[b]
18:1n-9	14.7 ± 3.0	18.4 ± 2.8	19.7 ± 3.0
18:1n-7	3.9 ± 0.7	2.4 ± 0.6	2.8 ± 0.6
18:1n-5	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0
18:2n-6	8.5 ± 1.0[a]	14.9 ±1.4[b]	31.1 ± 3.5[c]
18:3n-6	0.2 ± 0.0[a]	0.1 ± 0.0[b]	0.3 ± 0.0[c]
18:3n-4	0.3 ± 0.0[a]	0.1 ± 0.0[b]	0.1 ± 0.0[b]
18:3n-3	1.3 ± 0.3[a]	19.0 ±2.4[b]	2.8 ± 0.4[a]
18:4n-3	1.3 ± 0.1[a]	0.3 ± 0.1[b]	0.6 ± 0.1[a]
18:4n-1	0.5 ± 0.0[a]	0.1 ± 0.0[b]	0.2 ± 0.0[b]
20:0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
20:1n-9	1.8 ± 0.5	1.3 ± 0.4	1.6 ± 0.7
20:2n-9	0.3 ± 0.0[a]	0.1 ± 0.0[b]	0.2 ± 0.0[c]
20:4n-6	1.5 ± 0.1[a]	0.6 ± 0.1[b]	0.5 ± 0.1[b]
20:3n-3	n.d.	0.4 ± 0.2	0.2 ± 0.0
20:4n-3	0.6 ± 0.0[a]	0.3 ± 0.0[b]	0.3 ± 0.1[b]
20:5n-3	8.1 ± 0.7[a]	2.4 ± 1.0[b]	3.0 ± 0.8[b]
22:1n-11	1.3 ± 0.2	1.0 ± 0.2	1.1 ± 0.3
22:5n-3	2.3 ± 0.1[a]	0.6 ± 0.0[b]	0.7 ± 0.1[b]
22:6n-3	6.8 ± 0.9	5.8 ± 1.0	3.6 ± 0.7
Total saturates	35.2 ± 3.3[a]	28.3 ± 3.4[ab]	25.0 ± 3.7[b]
Total monoenes	31.1 ± 3.5	26.1 ± 3.7	30.2 ± 3.9
∑n-3	21.5 ± 3.1[ab]	29.1 ± 4.7[a]	11.7 ± 2.9[b]
∑n-6	10.5 ± 1.1[a]	15.7 ± 1.5[a]	32.1 ± 2.1[b]
∑n-9	16.7 ± 2.2[a]	19.9 ± 2.8[ab]	21.5 ± 2.0[b]
∑n-3 LC-PUFA	17.7 ± 2.5[a]	9.6 ± 1.0[b]	7.9 ± 1.2[b]
ARA/EPA	0.2 ± 0.0[a]	0.3 ± 0.0[b]	0.2 ± 0.0[a]
EPA/DHA	1.2 ± 0.0[a]	0.4 ± 0.0[b]	0.8 ± 0.0[c]
n-3/n-6	2.0 ± 0.1[a]	1.8 ± 0.3[a]	0.4 ± 0.1[c]

Different letters within a row denote significant differences (P<0.05) n=10x3 n.d. non-detected

5.4.1 Effect of diets on gene expression

To evaluate the effect of the three experimental diets on the innate immune system, we quantified the mRNA levels of a gene panel involved in the antiviral defense (9), interleukins and receptors (15), complement pathway (8), pathogen-associated molecular patterns (PAMPs) recognition (5), lysozymes (5), chemokines (6) and differentiation Antigens (3) in gut of animals fed for 90 days. Four RT-PCR assays (genes putative encoding *ifnc*, *ccl25*, *cxcl13* and *tlr5s*) did not amplify in any of the samples analyzed. Hierarchical clustering analysis (Fig. 5.1) based on the 50 innate immune-related genes analyzed showed expression signatures that set the 100SO diet apart and clearly differentiated of 100FO and 100LO diets. Also, most of the significant genes related with complement pathway, interleukins and c-type lysozymes clustered together and exhibiting similar expression profiles in the samples analyzed.

Statistical analysis identified 19 genes with significantly higher mRNA abundance in animals fed 100SO (Fig. 5.2 A, B, C and D). This set of genes included six complement genes from via classica, the *c2* (without significant result) and *c4a* (3.9 and 5.7 -fold higher in 100SO fed animals than 100FO, respectively), three from the common way, the *c3*, *c5* and *c9* (55.1-, 30.5- and 52.9-fold, respectively higher in 100SO fed animals than 100FO), and the complement regulatory gene *cfh* (58.4-fold higher in 100SO fed animals than 100FO). Also the interleukins *il1b*, *il10*, *il11a* and *il12b* and the interleukin receptors *il1rl1* and *cxcr2* also showed higher mRNA levels in fish fed 100SO than 100FO and 100LO (6.8-, 6.5-, 6.4-, 3.0-,- 2.4-, and 2.4-fold higher in 100SO fed animals than 100FO, respectively). The remaining genes activated by the 100SO diet included two c-type lysozymes, *lyzc1* and *lyzc2*, two interferon related factors, *irf2* and *irf7*, the toll-like receptor *tlr9*, and the differentiation antigen *cd4*. Moreover, we identified two genes downregulated in fish fed 100LO diet, the interleukin 17c (*il17c)* and and toll-like receptor 1 (*tlr1*) (0.3- and 0.4-fold lower in 100LO fed animals than 100FO respectively).

5.4.2 Effect of a chasing stress on gene expression

To evaluate the effect of an acute stress on gene expression and its association with the diet, a chasing stress for 5 min was performed. A two-way MANOVA analysis using diet and stress as fixed factors identified statistically differences associated to stress for *cd8a*, *cd8b*, *irf8*, *mx*, *il8c*, *il12b*, *il15*, *il1rl* and *lyzg3* (Fig. 5.3 A, B, C, D and E). *Cd8a*, *cd8b*, *mx*, *il8*, *il15c*, *lyzg3* mRNA levels decreased in soles fed 100FO group, *irf8* in both 100FO and 100SO groups and *il12b* in 100SO. In most of these genes, soles fed 100LO did not vary mRNA levels. A separate analysis of gene expression by diet also identified a reduction of mRNA levels in *irf9*, *cck3*, *il15ra*, *tlr1* in 100FO and an increase of *il1b* in 100FO and *il17c* in 100LO. Complement factors *c3*, *c5*, *c9* increased mRNA although not significantly except *cfh* transcripts that increased in soles fed 100LO.

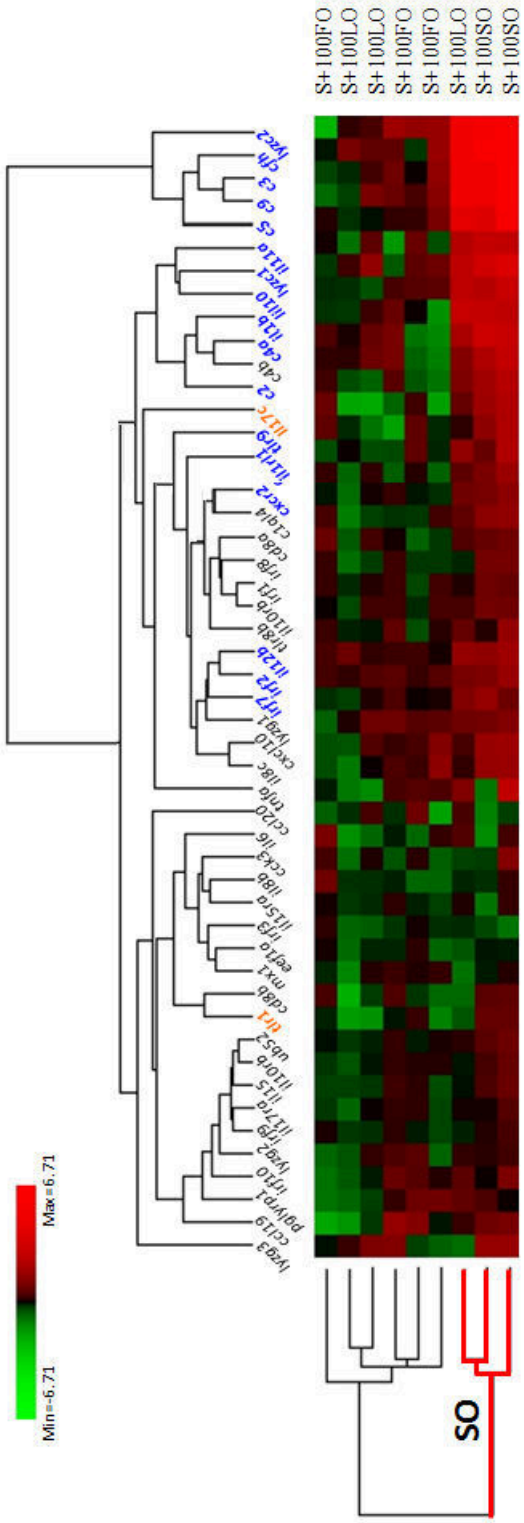

Figure 5.1: Genes in overrepresented functional annotation clusters from DAVID analysis were clustered according to their relative gene expression values, using PermutMatrix hierachical clustering software. Each gene is represented by a single row of coloured boxes; each phenotype is represented by a single column. As shown on the lengend, up-regulated genes are indicated with red of increasing intensity and down regulated genes indicated by green of increasing intensity

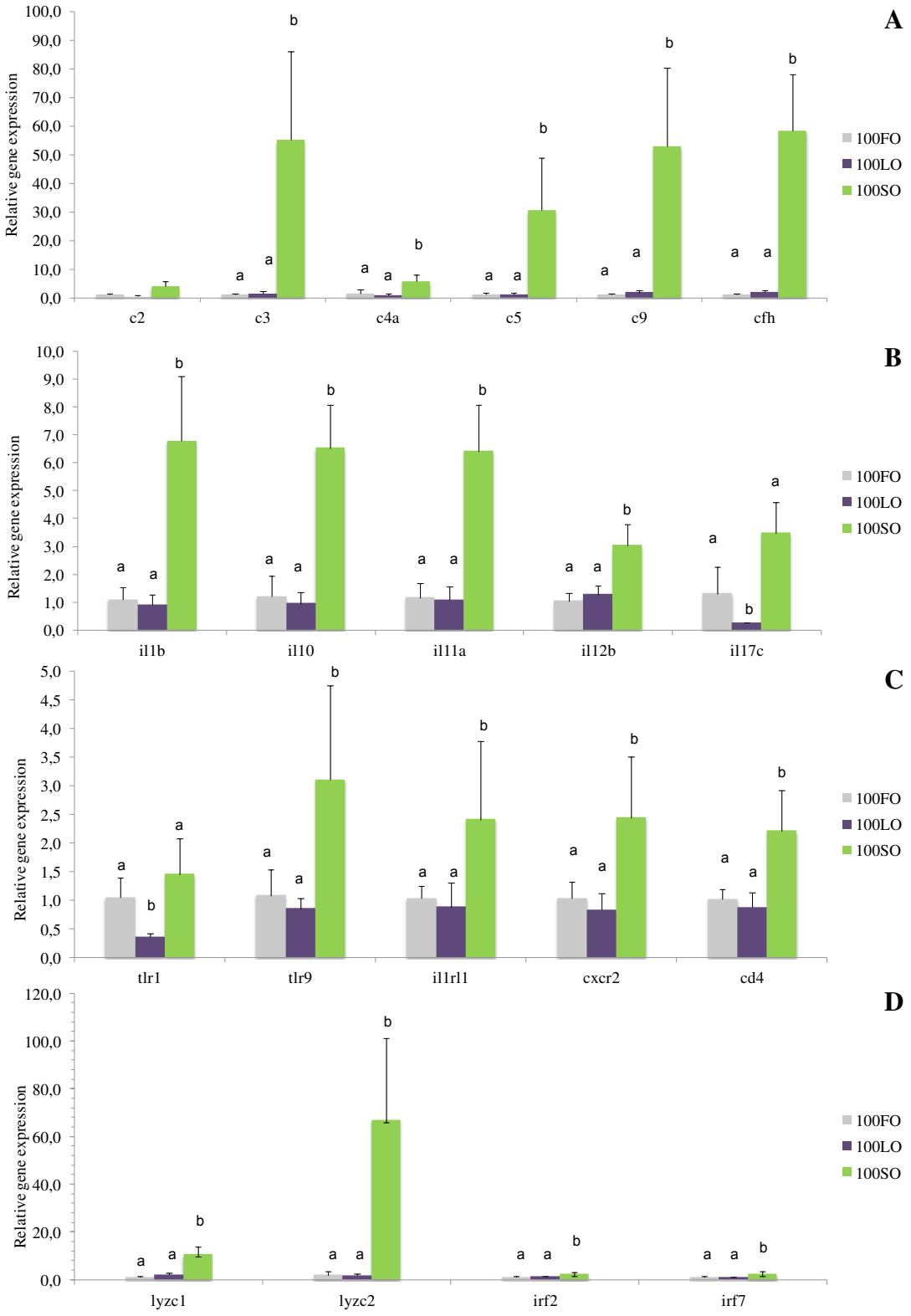

Figure 5.2: Relative expression of (A) alternative complement pathway genes (c2, c3, c4a, c5, c9 and cfh); (B) interleukins (il1b, il10, il11a, il12b and il17c); (C) Toll-like receptors (tlr1, tlr9), interleukin receptors (il1rl1 and cxcr2) and cluster of differentiation 4 (cd4); Lysozymes c (lyzc1 and lyzc2); (D) Interferon regulatory factor (irf2 and irf7) in intestine of Senegalese sole fed diets with total substitution of fish oil (100FO) for either linseed (100LO) or soybean oils (100SO), at basal state. Different letters denotes significant differences ($P<0.05$) among diets.

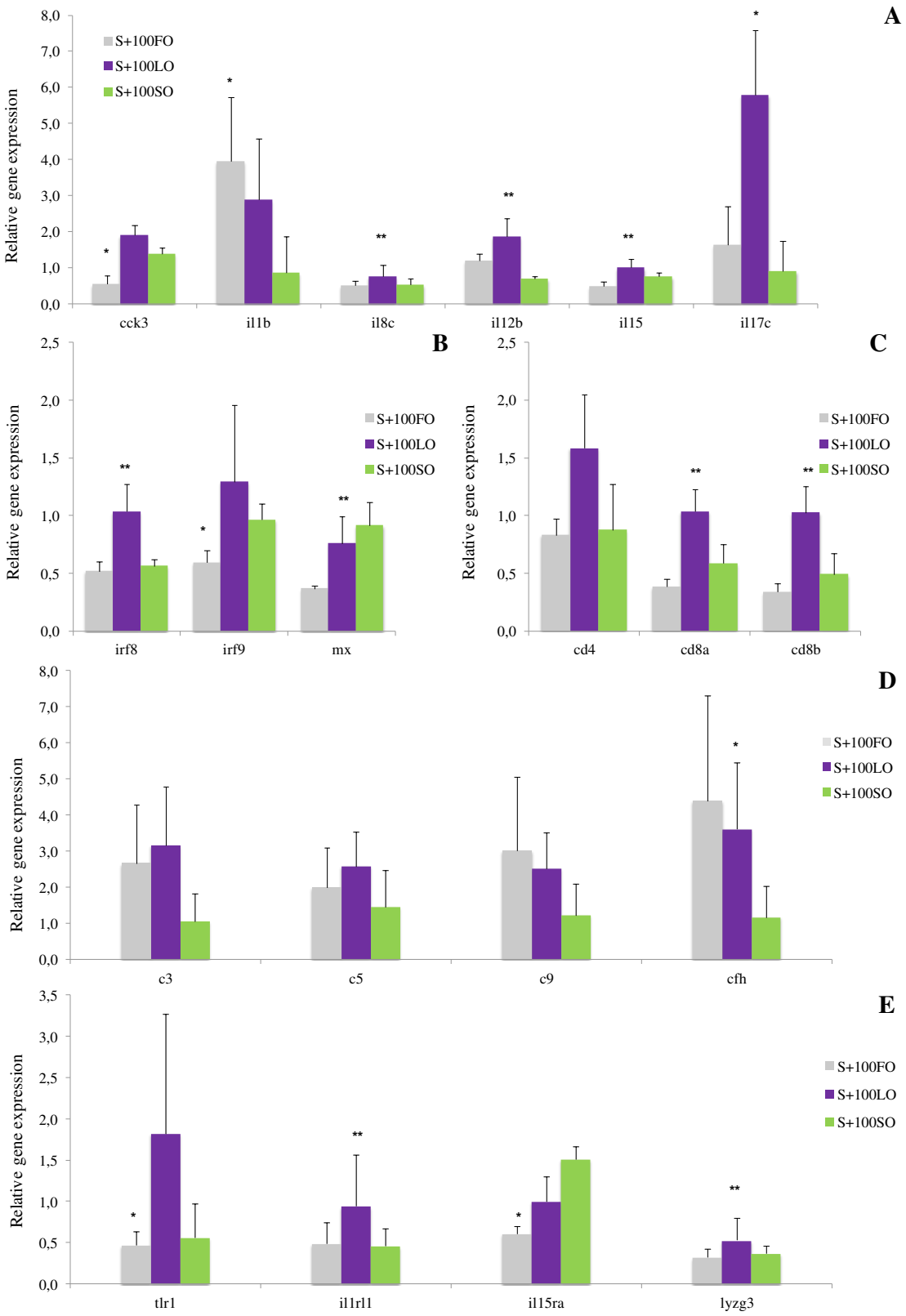

Figure 5.3: Relative expression of (A) Cholecystokinin 3 (cck3) and interleukins (il1b,il8c, il12b, il15 and iil17c); (B) Interferon regulatory factor (irf8 and irf9) and Interferon-induced protein mx (mx); (C) cluster of differentiation (cd4, cd8a, cd8b); (D) alternative complement pathway genes (c3, c5, c9 and cfh); (E) Toll-like receptors 1 (tlr1), interleukin receptors (il1rl1 and il15ra) and cd4; Lysozymes g 3 (lyzg3), in intestine of Senegalese sole fed diets with total substitution of fish oil (S+100FO) for either linseed (S+100LO) or soybean oils (S+100SO), and subjected to chasing stress. * denotes significant differences (P<0.05) for a gene between stressed and non-stressed fish in a specific diet. ** denotes significant differences (P<0.05) for a gene among diets in stressed fish.

5.5 Discussion

Complete replacement of fish oil by soybean oil reduced SGR leading to lower body weights at the end of the study, being the fish performance and fatty acid composition under these experimental diets previously discussed for this species (Benitez-Dorta *et al.,* 2013). Those results are in agreement with other studies in flatfish, such as that describing the effect of complete fish oil replacement by linseed oil or soybean oil on turbot (Regost *et al.,* 2003). In this sense, halibut fed a 70 % fish oil replacement by linseed oil (Alves-Martins *et al.,* 2011) was not also affected by the type of dietary oil. This relatively low impact of the total substitution of fish oil by vegetable oils in the Senegalese sole could be related with the relatively low LC-PUFA requirements observed in post-larval of this species (Morais *et al.,* 2004). A potential synthesis of DHA from EPA has been recently demonstrated in this species at molecular level by the functional characterization of fatty acyl desaturase with delta-4 activity (delta-4fad) and fatty acyl elongase (elovl5) (Morais *et al.,* 2012c) and even from α-linolenic acid (ALA, 18:3 n-3) (Navarro-Guillén *et al.,* 2014). In a very recent study, Navarro-Guillén and co-authors (2014) determined the effects of Artemia enrichment emulsions including either olive oil or soybean oil on DHA metabolism (absorption and catabolic oxidation) of post-larvae of this species. Those authors provided evidences that DHA is synthesized from EPA at physiological significant rates through a mechanism involving the transcriptional up-regulation of delta-4fad when dietary DHA is limiting. Benitez-Dorta and co-authors (2013) found similar levels of DHA in different tissues of Senegalese sole fed either fish oil based diets or vegetable oil based diets, that could be suggesting a selective retention of DHA or either a *de novo* synthesis of DHA under very low-DHA dietary levels through the routes proposed by Navarro-Guillén and co-authors (2014). This could be in agreement with the results obtained in the present experiment, where the ratio EPA/DHA of fish fed vegetable oils, and specially that from animals fed linseed oil based diet, were significantly lower than that from animals fed the fish oil based diet. Besides, post-larvae fed soybean oil treatment did not reflected the fatty acid

profile of the diet, showing higher than expected levels of docosapentaenoic acid (DPA, 22-5n-3), EPA, DHA, ARA and n-6 docosapentaenoic acid (DPA n-6, 22:5n-6) and much lower than expected levels of ALA. The utilization of ALA for elongation was also suggested in juveniles of Senegalese sole (Benitez-Dorta *et al.,* 2013) due to the increased amount of 20:3n-3 in different tissues of animals fed a linseed oil based diet, as previously described for other flatfish such as turbot (Regost *et al.,* 2003) or halibut (Alves-Martins *et al.,* 2011) suggesting a increased activity of C18-20 elongase. Further experiences must be conducted in juvenile Senegalese sole to elucidate the presence of those metabolic pathways, since this effect has been also observed in the present study Senegalese sole intestine, that clearly reflected the fatty acid profile of the diet with higher amount of ALA in fish fed linseed oil based diet and higher amount of LA in those fish fed soybean oil based diet, but with no significant differences of DHA among treatments.

From the fatty acid profile point of view, intestine of Senegalese sole fed soybean oil based diet showed a significant lower n-3/n-6 ratio, whereas linseed oil based diet showed similar n3/n6 ratio that fish fed fish oil diet, with similar ARA/EPA ratio among treatments. However, as expected, the levels of ALA and the subsequent ratio ALA/LA in intestine of fish fed linseed oil based diet was higher than that from the other experimental groups. Increases of n-6 fatty acids have been widely described to affect immune system in an extended way in higher vertebrates (Calder, 1996, 2002, 2006; Yaqoob, 2004; Yaqoob and Calder, 2007). Dietary n-6 fatty acids have an important role in inflammation processes, stimulating production of pro-inflammatory cytokines such as *tnfa*, *il1*, and *il6*, and increasing vasodilatation or vascular permeability (Calder, 2002). In contrast, ALA and n-3 PUFA inhibit lymphocyte proliferation and natural killer cells activity, as well as pro-inflammatory cytokines by lymphocytes (Yaqoob and Calder, 1995, 2007; Wallace *et al.,* 2001). Within the present study, a wide range of immune-related genes was analyzed under the different dietary regimes: eight genes from the complement system, 15 from the inflammatory response (interleukins and receptors), 6 quemokines, 5 lysozymes, 9 genes involved in the viral response (interferon and

mx proteins) and 4 toll-like receptors. The results showed a clear increase of inflammatory response in intestine of those animals fed SO based diet, including significant increases of some proinflammatory interleukins (*il1b, il11a, il12b* and *il17c*). This is in agreement with results previously reported for other marine fish such as gilthead sea bream, in which an increase of basal levels of *tnfa* in head kidney of fish fed SO based diet was recorded (Montero *et al.,* 2010). In that study, dietary oils rich in n-6 fatty acids induced also an over-expression of intestine pro-inflammatory cytokines *tnfa* and *il1b* after pathogen challenge (Montero *et al.,* 2010). This effect has been widely described in mammals, with described pro-inflammatory effects of oral administration of LA rich oils such as sunflower oil (Masi *et al.,* 2012). Magdalon *et al.* (2012) described an accelerated release of *il1b* in LPS stimulated macrophages of rats fed LA. Kirpich and co-authors (2013) found that LA enriched oil is a significant contributing factor to ethanol mediated intestinal inflammatory response and mucus layer alterations in rodents, by increasing *tnfa* and decreasing the antimicrobial factor CRAMP. A low dietary n-3/n-6 ratio induces the activation of *il1*, up-regulating inflammatory gene (*NF-kB*) expression (Toboreck *et al.,* 2002) which leads to increases indices of inflammation.

Not only the inflammatory cytokines are affected by dietary high inclusion levels of LA, but also other immune parameters. Expression of some genes involved in the complement pathway was over-expressed in the SO based diet group. High substitutions of FO by SO (80%) in diet affected serum alternative complement pathway activity in gilthead seabream juveniles (Montero *et al.,* 2003). SO emulsions have been described to have a selective and reversible effect on the *c2* component of the complement cascade in human mononuclear phagocytes (Kolski and Stunk, 1981). C-type lysozymes gene expression is also affected by the substitution of fish oil by SO, the up-regulation of both *lyzc1* and *lyzc2* in the 100SO group being similar to that found after LPS sensibilization or bacterial infection (Fernández-Trujillo *et al.,* 2008). Other immune-related genes, such as Toll-Like receptors (*tlrs*) are up-regulated in fish fed 100SO diet, in agreement with those results obtained by Lee and co-authors (2003) who described the differential modulation of *tlrs* by fatty acids, being those receptors preferentially

inhibited by n-3 PUFA in dendritic cells (Weatherill *et al.,* 2005). The *TLRs* play an essential role in the detection of pathogens, recruiting *il1r*-associated kinase via adaptor myeloid differentiation factor 88 (MyD88) (Vollmer, 2006), subsequently inducing activation of nuclear factor-kappa B (*NF-kB*) and mytogen activated protein kinases (Akira and Hoshino, 2003). The activation of this pathway induces the proliferation of *cd4*+T cells (Lee *et al.,* 2003). In the present experiment, fish fed SO based diet showed an increased *cd4* gene expression, in agreement with the subsequent increased *tlrs* gene expressions. Similar results were described by Chen *et al.* (2012) for broiler chickens, who described increased mRNA expression of cluster of both differentiation antigens *cd4*+ receptor and *tlr3* in thymus by increasing n-6/n-3 ratio in the diet. Moldal *et al.* (2014) found a moderately higher transcript levels of *cd3* in the pyloric caeca of SO fed Atlantic salmon, indicating a higher number of T lymphocytes as *cd3* is part of the T cell receptor complex and is expressed in T lymphocytes (Liu *et al.,* 2008).

Interestingly, the some genes involved in the interferon response, the *irf2* and *irf7*, were also up-regulated in those Senegalese sole fed SO based diet. This is in agreement with previous results obtained for gilthead sea bream fed a diet with total substitution of FO by SO inducing an increase of the basal constitutive expression of liver *mx* transcript (Montero *et al.,* 2008), since the *mx* proteins are induced by type I interferon (alpha and beta) (Tafalla *et al.,* 2004). On the other hand, a clear cross-talk between interferon-gamma and the unsaturated fatty acid (and specifically LA) turnover in phospholipids has been described (Darmani *et al.,* 1993). Besides, interferon has been shown to be an inflammatory inducer with the activation of *NF-kB* (Tsubota *et al.,* 1999).

As shown in the overrepresented functional annotation clusters from DAVID analysis obtained in the present study, immune system of Senegalese sole fed SO based diet were clearly over-expressed not only in terms of inflammatory response but also other immune pathways such as the viral response or the *tlrs*, giving a general inflammatory response in the intestine of Senegalese sole fed a diet with low n-3/n-6 ratio in tissue. This is in agreement with the results obtained in soybean meal-induced inflammation in Atlantic salmon, transcription levels of

transforming growth factor β (*TGF-β*) that is produced by T lymphocytes, were reported to be up-regulated by 7-folds combined with 20-folds up-regulation of *il1b* (Marjara *et al.,* 2012). Similarly, although in a lesser extends, *TGF-β* were 2-fold up-regulated in pyloric caeca of Atlantic salmon fed SO based diet (Moldal *et al.,* 2014), denoting effects of this oil on the inflammatory processes. Linoleic acid has been described as a potent activator of *NF-kB* through the phosphorilation of IkappaB (inhibitor of kappa B) proteins (Toborek *et al.,* 1996) promoting general inflammatory processes, but further experiments are required to elucidate the specific role of dietary LA in activating *NF-kB* and, subsequently, the general inflammatory response. However, inflammation due to other effects, such as alterations on the gut-associated microbiota and the subsequent changes in transcellular transport on endotoxins, cannot be discarted since fatty acids have been proved to differentially regulate intestinal epithelial endotoxin transport through the regulation of the lipid draft mediated permeability, being this transport decreased in diets rich on n-3 fatty acids (Mani *et al.,* 2013).

However, other studies conducted in marine fishes failed to find effects of FO substitution by VO in gut-associated immune parameters. Calduch-Giner *et al.* (2012) did not found modifications of the basal intestine transcriptome of gilthead sea bream fed a 66% VO in the diet. The differences found among studies could be due to the different species studied, the type of VO (or blend of VO) used or different experimental conditions (i.e. time of feeding indirect effects as microbiota disbiosis, temperature, water quality or size of fish). It is interesting to point out, however, that Calduch-Giner and co-authors (2012) found a significant effect of the dietary VO on the intestinal transcriptome when the fish where exposed to a parasite challenge with the myxosporean *Enteromyxum leei*, including a strong down-regulation of some genes related to complement pathway. Similarly, Estensoro and co-authors (2012) found that 66% of FO substitution by VO in gilthead sea bream diets had no effect on the number of IgM- immuno reactive cells or on IgM expression in non-infected fish, whereas the infection with *E. leei* had a strong effect on the intestine of VO based diets fed fish, denoting a combined challenge-diet effect. This type of combined effects was also observed for gilthead

sea bream fed total substitution of FO by VO and subjected to a bacterial challenge test (Montero *et al.,* 2010), being the expression of pro-inflammatory cytokines strongly up-regulated in infected fish fed SO based diet. Oxley and co-authors (2010) also found a combined effect of high inclusion of plant ingredients and a challenge test on the intestinal inflammatory response of Atlantic salmon. Those authors postulated that a high replacement with plant-derived ingredients enhanced the cyclooxygenase-2 induction and synthesis of pro-inflammatory eicosanoids in the intestine of Atlantic salmon in response to acute physiological stress by net chasing. Indeed it seems that a combined effect of use of plant-derived ingredients and some stressful situations, such as an infection or physical stress, is producing a magnification of the effects of the stressful situation. This effect has been described for mammal (Fukurawa *et al.,* 1999) and is in agreement with the results obtained in the present experiment, where some of the immune-related genes from fish fed SO based diet are down-regulated or unchanted after a physical stressful situation (net chasing), compared to their basal levels.

However, in contrast to those findings, ne interesting point found in this study is the up-regulation of *il1b* and *il17c* after stress in fish fed 100FO or 100 LO diets (high n-3/n-6 dietary ratio), whereas fish fed 100 SO did not respond properly, suggesting an exhaustion of the immune response to stressful situation in 100SO fed fish. Changes in the number and proportion of circulating lymphocytes coupled with a rise in systemic inflammatory markers (i.e. cytokines and acute phase proteins) illustrates that the immune system actively responds to acute stressor exposure (Speaker and Fleshner, 2012), including immediate increases of *il1b* and *il6* after severe stress (Steptoe *et al.,* 2007). Besides, *il17c* is reported to stimulate the release of *il1b* from the monocytic cell line in mice (Yamaguchi *et al.,* 2007) and its function is also to promote the formation of tight junctions (Reynolds *et al.,* 2012). The increase of *il17c* expression in 100LO could be also related to the increase of *cd4* found in this experimental group immediately after stress, since *cd4* T cells are involved in the expression of *il17* family members, especially *il17a*, *il17c* and *il17f* (Yamaguchi *et al.,* 2007).

Those immediate responses of interleukins to acute stress in fish fed high n-3/n-6 dietary ratios could be producing the observed increases of other gut immune-related genes in those animals. The *il1b* has been reported to increase the production of complement component *c3* in enterocytes (Moon *et al.,* 1999), and it is regulated by hyperthermia or endotoxin-induced stress (Moon *et al.,* 1997). Complement cascade has been also described to be altered by other stressful situations, such as the post-traumatic stress disorder in humans that induced a hyper-activation of the complement classical pathway, whereas hypo-activates the alternative pathway (Hovhannisyan *et al.,* 2010).

The alterations of immune-related genes found as immediate response to chasing stress in Senegalese sole vary with the type of dietary oil used, fish fed SO based diet showing no response to stressful situation, whereas fish fed LO based diet, with the higher ALA/LA ratio in diet and tissue, showed similar response (or even more up-regulation) that the FO group. Alpha linolenic acid has been proved to reduce significantly the endoplasmic reticulum stress associated with lipotoxicity produced by saturated fats accumulation in primary rat hepatocytes (Zhang *et al.,* 2011), whereas linoleic acid has been demonstrated to induce endoplasmic reticulum (ER) stress (Zhang *et al.,* 2012). There is a relationship between ER stress and inflammation, some signaling pathways connecting both through various mechanisms, including the production of reactive oxygen species (ROS), the release of calcium from the ER, the activation of the transcription factor NF-kB or the mitogen-activated protein kinase (MAPK) (Zhang and Kaufmann, 2008). We have no evidences of these mechanisms in Senegalese sole, although ALA has been proved to modulate stress response in marine fish (Ganga *et al.,* 2011) and ameliorate deleterious effects of induced infection in gilthead sea bream (Montero *et al.,* 2010). Further experiments are required to elucidate the specific role of 18C fatty acids from plant origin in the immune system of this Senegalese sole in combination with stressful situations.

In summary, total substitution of FO by SO induces an up-regulation of different intestine immune-related genes, including some interleukins, components of the complement, the viral

response and the toll-like receptors response, among others, suggesting a chronic-like inflammatory situation in Senegalese sole intestine. When a stressful situation appears, such as a severe exercise after chasing stress, fish fed SO based diets do not respond equal than those fed FO, suggesting an exhaustion of the immune system that is not able to respond to the demand induced by the stress. However, fish fed LO based diet, with the higher ALA/LA ratio, showed an opposite response, increasing expression of some immune genes and suggesting a positive role of n-3 rich oils under stressful situations. Further experiments are required with different types of stress to which Senegalese sole has been proved to be very sensitive.

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CAPÍTULO 6

"DIETARY VEGETABLE OIL AND EICOSANOID-RELATED GENES IN SENEGALESE SOLE INTESTINE"

6. Dietary vegetable oils: effects on the expression of immune-related genes in Senegalese sole (*Solea senegalensis*) intestine: Eicosanoid receptors

6.1 Abstract

For aquaculture of marine species to continue to expand, dietary fish oil (FO) must be replaced with more sustainable vegetable oil (VO) alternatives. VO in fish diets has a modulating role on fish stress response and the Senegalese sole is a promising species for the Mediterranean aquaculture. The nature of dietary lipids and their concentration in essential fatty acids have a direct effect on eicosanoid metabolism and immune response. To study those effects, juvenile Senegalese sole were fed for 90 days diets (56% crude protein, 12% crude lipid) containing either linseed (100LO) or soybean (100SO) oils in comparison to a 100% fish oil based diet (100FO).

Samples of intestine were collected for biochemical analysis and for eicosanoid cascade gen expression, including phospholipase A2 (PLA2), cyclooxygenase type 1A (COX1A) and 2 (COX2), 5-lipoxygenase (LOX5), and eicosanoid receptors type 2 (EP2), 3 (EP3) and 4 (EP4). After the feeding period, a stress test, consisting on 5 minutes of net chasing, was applied to a selected population of each dietary group in order to check the combined effect of stress and diet on the genes expression.

The relative expressions of genes involved in eicosanoids cascade were affected in a different manner. No change was found in the LOX5 gene expression, under any of the experimental conditions, but a significantly higher expression values for COX2 gene expression could be observed in fish fed 100SO in comparison with those fed 100FO and 100LO, at

unstressed conditions. After chasing stress, there was a significantly lower expression of COX2 in fish fed with 100FO and an increase of COX1A expression.

Fish fed VO based diets showed a reduced intestine EP4 gene expression in unstressed conditions, but a higher ($P<0.05$) expression of EP4 gene and significantly lower expression of EP2 and PLA2 genes in fish fed 100LO after chasing stress.

In summary, including LO and/or SO as substitutes to FO in Senegalese sole, may alter the expression of genes related with eicosanoid cascade, such as COXs and EPs in the intestine and consequently modulate their response to stressful conditions.

6.2 Introduction

As explained in previous Chapters, the utilization of vegetable oils in fish diets not only could affect the ratio n-3/n-6 fatty acids, but also can produce a reduction of the long chain polyunsaturated fatty acid (LC-PUFA) (Turchini *et al.,* 2009; Montero and Izquierdo, 2010). The ability to convert α-linolenic acid (ALA, 18:3 n-3) to the long chain n−3 highly unsaturated fatty acids (eicosapentaenoic acid; EPA, 20:5n-3 and docosahexaenoic acid; DHA, 22:6n-3) depends greatly on the enzymatic capacity of fatty acid elongases and desaturases *in vivo*, which in turn seems to be linked to the evolutionary history of the species and its relation with the habitat-specific food web structures (Castro *et al.,* 2012). The dietary substitution of FO is a challenging process in marine species because they have a low ability to bioconvert linolenic acid (LA; 18:2 n-6) and ALA into long chain PUFA, resulting in a dietary requirement for , arachidonic acid (ARA; 20:4n-6), EPA and DHA (Tocher, 2003, 2010) which are essential to promote high growth performance and feed efficiency. DHA and EPA are essential fatty acids playing several biological roles, acting as important elements for the fluidity of cytoplasmic membranes and as precursors of eicosanoids, which are involved in inflammatory response (Von Schacky, 2006). Eicosanoids are oxygenated derivatives of polyunsaturated fatty acids

produced from membrane phospholipids (mainly ARA and EPA) by the action of phospholipases, cyclooxygenases and lipoxygenases (Rowley *et al.,* 1995). Two cyclooxygenase isoforms have been identified and are referred to as COX1 and COX2. Under many circumstances the COX1 enzyme is produced constitutively (i.e., gastric mucosa) whereas COX2 is inducible (Dubois *et al.,* 1998). It is generally consider that the eicosanoids derived from ARA have potent proinflammatory effects (Secombes, 1996) and the elevation of prostaglandin E2 (PGE2) (arachidonate metabolite) induces the production and release of inflammatory cytokines (Bagga *et al.,* 2003) and it can be more potent inhibitor than prostaglandin E3 (PGE3) (derived from EPA) (Secombes *et al.,* 1994). Leukotriene B4 (LTB4) increases vascular permeability, has chemotactic properties for leukocytes, induces lysosomal enzymes, and enhances generation of reactive oxygen species and production of proinflammatory cytokines (Calder, 2006), and its effects over the proliferation of leukocytes is more sensitive that the effects of leukotriene B_5 (LTB5) (Secombes *et al.,* 1994). In higher vertebrates, LTB5 and PGE3, among other EPA-derived eicosanoids, are frequently less potent than their equivalent derivatives from ARA, and have important antiinflammatory effects through mediators termed "E-series resolvins" (Serhan, 2006), although in marine fish, the production of EPA-derived eicosanoids could have important significance in fish metabolism (Ganga *et al.,* 2005), despite their short life *in vivo* (Rowley *et al.,* 1995).

The diverse actions of prostanoids are mediated by membrane bound receptors on neighboring cells. Exiting some types and subtypes of prostanoid receptors, including four types of EP receptors: EP1, EP2, EP3, EP4 for PGE_2 (Woodward *et al.,* 2011). EP1 induces Ca^{2+} moblizitation whereas EP2 and EP4 mediate the increase of cAMP, and EP3 mediates decrease in cAMP (Hirataand and Naruyima, 2012). EP1 and EP2 bind PGE_2 with lower affinity than EP3 and EP4 (Ricciotty and Fitzgerald, 2011)

Whithin the intestine, EP receptors are involved in modulating gastrointestinal mucosal integrity (Takeuchi *et al.*, 2010). Very briefly, in mammals, the actions of PGE_2 are related to inhibition of stomach contraction (EP1), stimulation of duodenal HCO_3- secretion (EP3/EP4),

inhibition of small intestinal contraction (EP4), and stimulation of mucus secretion (EP3/EP4) or down-regulation of cytokine secretion in the colon (EP4). PGE_2 also showed a healing-promoting effect on gastric ulcers and intestinal lesions through the activation of EP4 receptors (Takeuchi *et al.,* 2010)

However, very little is known on the eicosanoid receptors in fish intestine and physiological functions associated to this tissue, being eicosanoid recently molecularly characterized for zebrafish (Tsuge *et al.,* 2013). Thus, the objective of this study is to clarify the effect of dietary vegetable oils in intestinal eicosanoid EP receptors in *Solea senegalensis*.

6.3 Materials and methods

Samples used in the present study were obtained from the experiment described in Chapter 5 (see details there). Methodology used is also described in Chapter 5.

6.3.1 RNA extraction and cDNA synthesis

Tissues sampled from each tank were pooled and Total RNA was extracted from approximately 100 mg of those pools, using 1 ml TRI Reagent (SIGMA-Aldrich, Sant Louis, Missouri). Chloroform and isopropanol were used for separation and precipitation, respectively. The pellets were hydrated with 100 µl of Milli-Q sterile water, previously treated with 0.1% DEPC and kept at -80ºC until analysis. Total RNA concentration, purity and quality were measured by NanoDrop 1000 Spectrophotometer (Thermo Scientific). Total RNA (2 µg) from each sample was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). Each subarray was loaded with 5.0 µl of master mix containing specific cDNA and PCR mixture.

6.3.2 *Real time PCR (RT-PCR)*

Within the oligo used, ubiquitin were previously described by Infante *et al.* (2008). The primers EP2, EP3, EP4, PLA2 and LOX5 (Table 6.1) were designed using Oligo 7.0 program (Medprobe). The RT-PCR reactions for these genes were performed in *i-cycler* thermocycler with optical module (Bio-Rad Hercules, California) using 12.5µl Brillant SYBR Green PCR Master Mix (Bio-Rad Hercules, California), 1 µl of a 1:5 dilution of the cDNA and the amount previously optimized of each primer in a final volume of 25 µl. Cycling conditions consisted on desnaturation and enzyme activation for 7 min at 95°C, followed by 40 cycles at 95°C for 15 seconds and 60°C for 30 seconds.

COX1A and COX2 primers (Table 6.1) and probes were designed using the Custom TaqMan® Assay Design Tool (Life Technologies). RT-PCR analysis for these genes was performed using the OpenArray® Real-Time PCR platform (Life Technologies). RT-PCR reactions were done using the TaqMan® OpenArray® Real-Time PCR Master Mix. All cDNA and Taqman amplification procedures were carried out in accordance with the manufacturer's protocol. To run the Taqman assays, we used an OpenArray® Real-Time PCR Instrument (Life technologies). Samples were loaded into OpenArray plates with the OpenArray® AccuFill™ System according to the manufacturer's protocols.

The sequences of EP2, LOX5, COX1A and COX2 were obtained from *SoleaDB* (http://www.juntadeandalucia.es/agriculturaypesca/ifapa/soleadb_ifapa/), while the rest of sequences for the EP3 (XM005459908.1), EP4 (NM001173955.1) and PLA2 (FF285823) were extracted from (www.ncbi.nlm.nih.gov/pubmed).

Relative gene expression was estimated by the Δ-Δ method (Livak and Schmittgen, 2001) using ubiquitin (Acc No AB291588.1) as housekeeping gene (Infante *et al.,* 2008) for the primers EP2, EP3, EP4, PLA2 and LOX5, and using gapdh2 as housekeeping gene (Infante *et al.,* 2008) for the primers COX1A and COX2. The FO diet was used as calibrator to compare

the effect of diets whereas the unstressed group for each dietary treatment was used to analyze the effect of chasing stress in each diet.

Table 6.1: Different primers used

Target		Primers 5´-3´	Fragment size (bp)
PLA2	F R	CGGCCTTCTTGTCACATTCACA CGACAACCCTTACACCGAGT	113
EP2	F R	ATGGCGTTAGAGAGATGCTTTG TACCAAACCCCACAAACGGAA	142
EP3	F R	GCTTTGCATTGGATCTCTGG CACAGGCCAAAAGTTGTCATGC	162
EP4	F R	ACCATCGCCACCTATGTGCAAG TATGGACATGGCACAGATAATGCT	120
gapdh2	F R	TCATTCCTGAGCTCAACGGTAAG AGGTCAACCACTGACACATCAG	76
ubiquitin	F R	AGCTGGCCCAGAAATATAACTGCGACA ACTTCTTCTTGCGGCAGTTGACAGCAC	93
LOX5	F R	AGTGATCCGAAAATGCACCAAA ACTGATGCCGTCTAACACCT	151
COX1A	F R	GTGATCTTAAGACTACGGCATCTGT ATGAGACCCATTGAATTTACCAGTTCTT	96
COX2	F R	CCGACTTACAATGCGGATTATGGTT TTGGGCAATCCTCTGGTACAG	100

6.3.3 Statistical analysis

All data were tested for normality and homogeneity of variance. Means and standard deviations (SD) were calculated for each parameter measured except for cortisol values where values are represented as Mean plus standard error (SE). Statistical analyses followed methods outlined by Sokal and Rohlf (1995). Data of fish growth and tissue fatty acid composition were submitted to a one-way analysis of variance (ANOVA). When F values showed significance, individual means were compared using Tukey multiple comparison test. Significant differences were considered for $P<0.05$. If the variances were not normally distributed, the Kruskall–Wallis non-parametric test was applied to the data.

RT-PCR datas were log-transformed in order to comply with normality and homogeneity of variance. One-way multivariate analysis of variance (MANOVA) approach was used to compare the effect of the diet. Moreover, a two-way MANOVA was used to test the effect of stress using the diet and stress as fixed factors. Moreover, t-student was carried to compare

control and stress groups for each diet. Analyses were performed using SPSS software (SPSS for windows 11.0).

6.4 Results

6.4.1 Prostaglandin synthesis

The utilization of soybean oil as unique oil source in the diet induced a significantly higher ($P<0.05$) expression values for COX2 gene in comparison with those fed 100FO and 100LO, under baseline conditions (Fig 6.1A), being expression of COX1A also increased 2.5 fold in this experimental group. No change was found in the PLA2 or LOX5 gene expression, under any of the experimental conditions (Fig 6.1A).

The utilization of vegetable oils in the diet did not induced changes in the expression of EP2 and EP3 of unstressed fish, whereas expression of EP4 gene was significantly reduced in those fish fed any vegetable oil-based diet (Fig. 6.1 B).

After chasing stress, there was a significantly lower expression of COX2 in fish fed with 100FO diet (Fig 6.2 A). Besides, COX1A was over-expressed in FO based diet, showing 1.5 fold increases when compared with unstressed fish. Chasing stress induced a significantly higher ($P<.0.05$) expression of EP4 gene and a significantly ($P<0.05$) lower expression of EP2 and PLA2 genes in fish fed 100LO (Fig 6.2 A and B). No effect of chasing stress was observed in expression of EP3 gene at any of the different dietary treatments assayed.

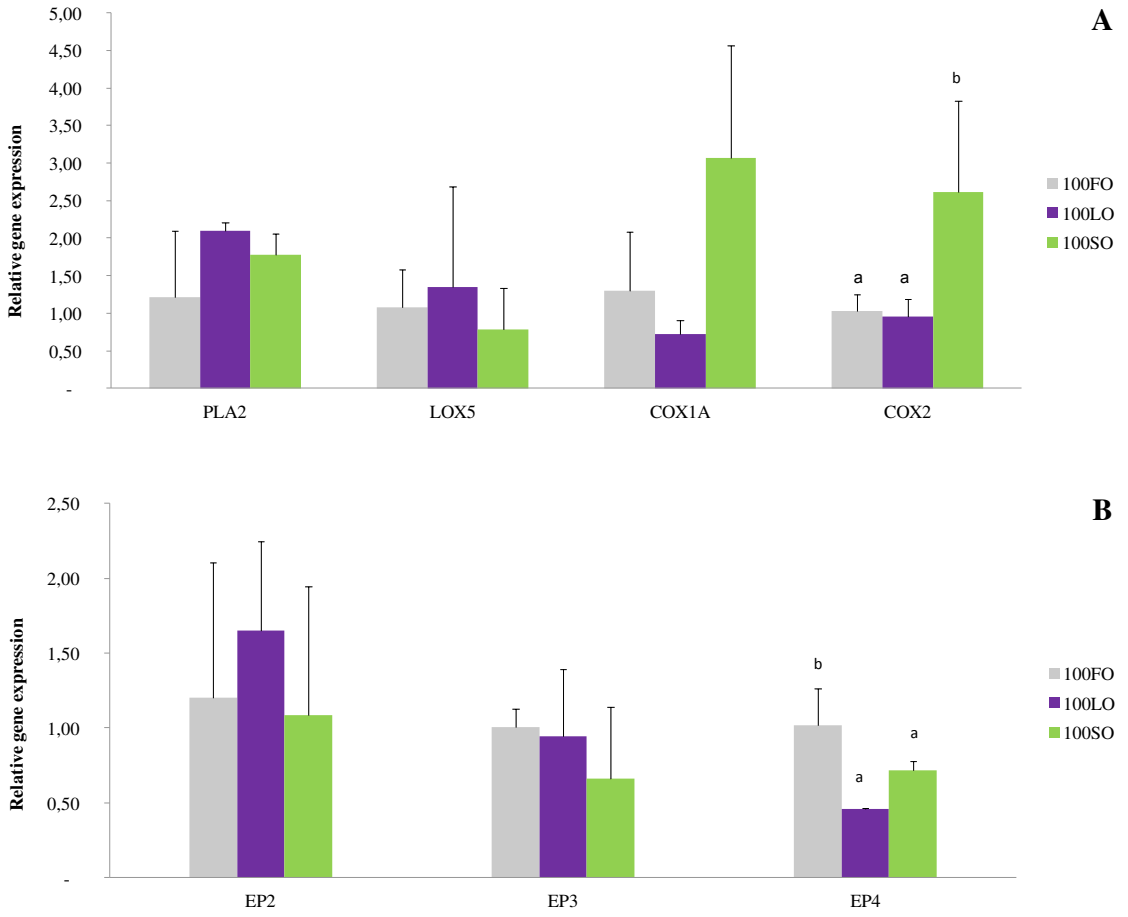

Figure 6.1: Relative expression of genes: (A) PLA2, LOX5, COX1A and COX2; (B) EP2, EP3 and EP4 in intestine, at baseline and over stress situation in fish fed with 100FO (FO), 100LO (LO) and 100SO (SO). Different letters denotes significant differences (P<0.05)

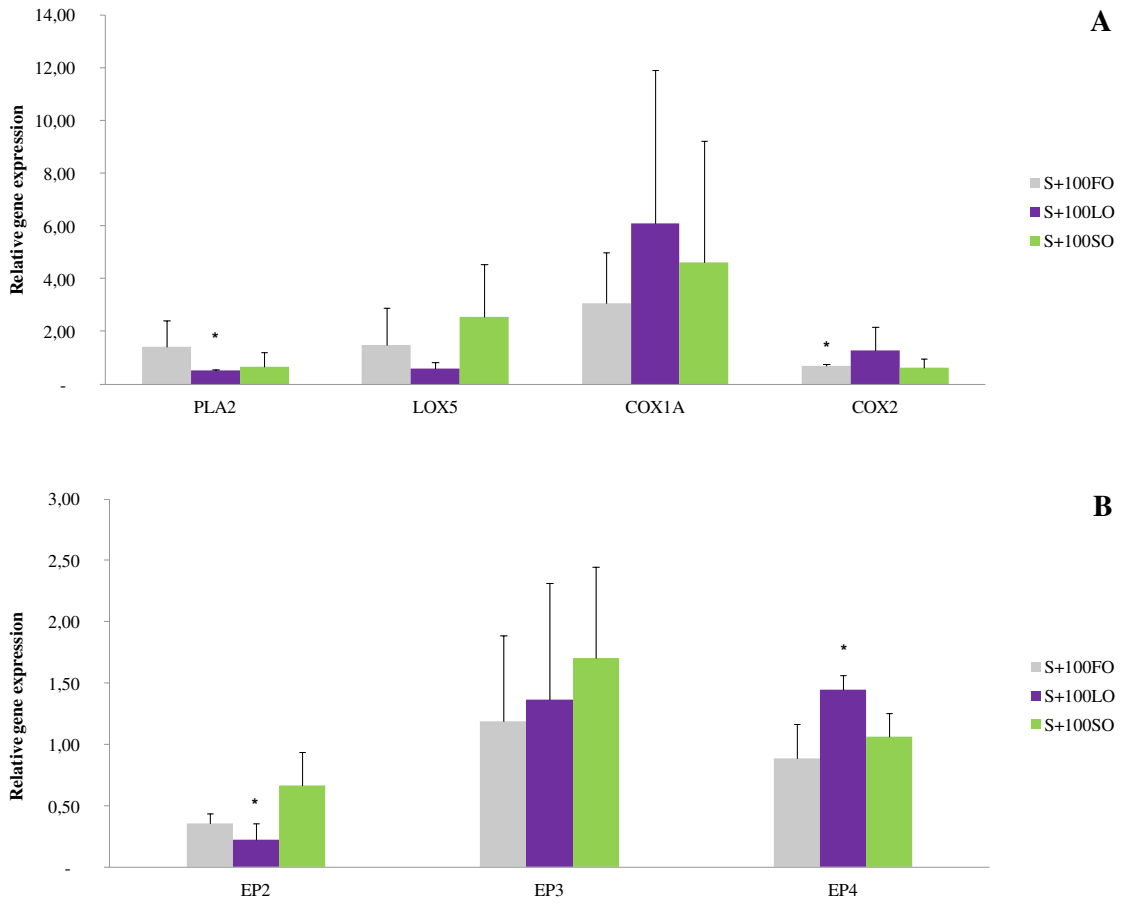

Figure 6.2: Relative expression of genes: (A) PLA2, LOX5, COX1A and COX2; (B) EP2, EP3 and EP4 in intestine, over stress situation in fish fed with 100FO (FOE), 100LO (LOE) and 100SO (SOE). * denotes significant differences (P<0.05)

6.5 Discussion

The importance of dietary ARA/EPA ratios is generally recognized due to the known competition between these two fatty acids for both inclusion into cellular membrane phospholipids by fatty acyl tranferases and subsequent eicosanoid formation and action (Sargent *et al.,* 1999). Within the present experiment, the ratio ARA/EPA of intestine was equal among different dietary treatments, although the total amount of both fatty acids was reduced in intestine of fish fed any of the VO based diet. However, although similar ARA/EPA ratios were observed in fish intestine among dietary groups, some effects on the eicosanoid cascade related genes could be observed in the present experiment.

First of all, the PLA2 gene expression remained unaffected in basal levels, but a reduction of PLA2 gene expression could be observed in fish fed 100LO diet after stress. A reduction of cytosolic PLA2 has been observed when the in culture medium of canine mastocytoma cell line c2 was supplemented with ALA (Gück and Fuhrmann, 2005). The PLA2 activity has been also shown to be inhibited indirectly by high levels of ALA in the diet (Ballou and Cheung, 1985). High ALA dietary content has been showed to increase secretion of plasma cortisol in marine fish (Ganga *et al.*, 2011). A relationship between expression of PLA2 and whole post-larvae cortisol in Senegalese sole after 3 hours of stress was proposed (Alves-Martins *et al.,* 2013). The secretory phospholipase A2 (sPLA2) has been described to be activated under stressful conditions through the activation of G protein after ACTH stimulation (Stocco *et al.,* 2005). However, a clear relationship between expression of PLA2 and stress cannot be assumed due to the fast of sPLA2-induced release of ARA after trophic hormone stimulation (less than one minute) (Stocco *et al.,* 2005) and most of the previous studies evaluate PLA2 after several minutes or even hours. Further experiments need to be conducted to elucidate the relationship between changes in PLA2 after stress in fish fed ALA-rich diets.

The use of SO in Senegalese sole diets induced an increased expression of COX2 at basal stages, effect that is directly linked with the pro-inflammatory induction by this oil in intestine of Senegalese sole described in Chapter 5**.** N-6 fatty acids in the VO dietary group may increase, or at least not depress, COX2 expression induced by lipopolysaccharide (LPS) (Holen *et al.,* 2011). These observations are supported by the finding of high levels of PGE2 in plasma of SO fed salmon after injection of LPS (Gil-Martens *et al.,* 2010), and that COX2 expression in a mammalian macrophage cell line could be induced by n-6 but not n-3 unsaturated fatty acids (Lee *et al.,* 2001). Delayed PG biosynthesis, which proceeds gradually over a long term period after a pro-inflammatory stimulus, is accompanied by de novo induction of COX2, which is an absolute requirement irrespective of the coexistence of COX1 (Murakami *et al.,* 2000). However, in contrast to the traditional view that COX2 is induced in response to pathophysiological reactions and COX1 serves as a housekeeping enzyme for maintenance of

mucosal integrity (Vane *et al.,* 1998), both isoenzymes can act either alone or in concert towards mucosal defense (Dubois *et al.,* 1998; Wallace *et al.,* 2000).

The preference of COX2 over COX1 is related to the ability of COX2 to metabolize lower concentration of ARA to eicosanoids than those required for COX1, denoting that the amount of ARA supplied by cytosolic PLA2 critically influences which COX isozymes are utilized (Kulmacz and Wang 1995; Shitashige *et al.,* 1998; Murakami *et al.,* 1999). Murakami *et al.* (2000) suggested that the two COX isozymes are functionally coupled with the two distinct downstream PGE_2 synthase (PGES) enzymes. Under high demand of metabolites such as an immediate response to stressful situation, glutathione-dependent cytosolic PGES is predominantly linked with COX1 (Tanioka *et al.,* 2000). The over-expression of COX1 obtained in the present experiment after stress in fish fed FO based diet, which had the higher amount of ARA in the tissue, could be in agreement with that but further experiments (i.e. *in vitro* studies) are required to elucidate the immediate response of the enzymes related to eicosanoid synthesis to a stressful situation.

The effect of dietary VO on PG production depends on the fatty acid content in the dietary oils and the degree of FO substitution, as well as the ability of the fish species to produce LC-PUFA. ALA has been shown to increase EPA in leukocyte PLs (Bell *et al.,* 1993) and reduced the production of ARA-derived PGE2 and thromboxane B2 (TXB2), resulting in increased anti-inflammatory activity (Bell *et al.,* 2003). The PGE2, the most bioactive eicosanoid in terms of immune system can regulates cellular immune responses through distinct EP receptors on different immune cell populations (Akaogi *et al.,* 2004). In that sense, in absence of EP2, the T-cells would be resistance to PGE2 (Nataraj *et al.,* 2001). This tendency of reduced LPS-stimulated PGE2 production in blood culture has been described to be accompanied by an increased production of cytokines (Faber *et al.,* 2011). It is possible that the deletion of the EP2 receptor may have uncovered stimulatory actions of other EP receptors, such as EP3, that might contribute to the apparent resistance of EP2-deficient cells to PGE2 (Nataraj *et al.,* 2001).

At unstressed stage, fish fed any of the VO based diets showed a depleted expression of EP4 receptor. This is of special importance since the activation of EP4 receptor by PGE2 increase the mucus production and decreases the intestine contraction, reducing the translocation of bacterial through intestinal physical barrier (Takeuchi, 2010). PGE_2-induced mucus secretion is mainly mediated through EP4 receptors in a cAMP/PKA-dependent manner (Dey *et al.,* 2006). Activation of both EP2 and EP4 receptors offer cytoprotection of intestinal cells by increased mucus secretion, and through inhibition of hypermotility medieted by EP4 alone (Kunikata *et al.,* 2002). Mucosal EP4 receptor expression has been described to be upregulated in T-lymphocytes, increasing the expression of this gene in *lamina propia* T lymphocytes in ulcerative colitis (Takafuji *et al.,* 2000). Signaling via EP4 receptors plays a critical role in maintaining normal mucosa integrity (Kabashima *et al.,* 2002).

In conclusions, SO is increasing the expression of COX1A and 2 as results of the inflammatory-like situation described in Chapter 5**.** However, as occurred with the rest of the genes studied previously, those fish fed SO based diet failed to respond properly under a stressful situation that demands activation of the different mechanisms. The low level in the EP4 expression found in the present study could be indicating that the intestine of those fish fed VO oil based diets are less protected to cell damage or injury than those animals fed FO based diets. It is interesting to point out that the immediate response to chasing stress induce different responses depending on the type of dietary oil, increasing significantly the expression of EP4 gene in the gut from fish fed LO diet, suggesting that those animals fed on n-3 fatty acid rich diets are better prepared to cope with situations that potentially could affect the intestine integrity by increasing the EP4 gene expression whereas those animals fed on n-6 fatty acid rich diet showed a reduced EP4 gene expression with the subsequent reduced protection to intestine damage.

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CAPÍTULO 7

CONCLUSIONES

7. Conclusiones generales

1. La utilización de aceites vegetales (ya sea de soja o de lino) en la dieta del lenguado senegalés, modifica los perfiles de ácidos grasos del músculo, hígado e intestino, reflejando estos tejidos el perfil característico de cada aceite dietético utilizado.

2. La utilización de aceites vegetales en la dieta induce una reducción de ácidos grasos poliinsaturados de cadena larga (LC-PUFA) en los distintos tejidos de lenguados senegalés. Sin embargo, la relación de ARA/EPA y los niveles de DHA se mantienen constantes en los tejidos, lo que sugiere la existencia de mecanismos de elongación y desaturación de ácidos grasos de cadena corta hacia LC-PUFAs.

3. Se secuencian por primera vez los receptores de glucocorticoides, GR1 y GR2, para el lenguado senegalés.

4. El uso de aceites vegetales en la dieta disminuye la expresión relativa de la HSP70 en intestino de lenguado senegalés y la sustitución total de aceite de pescado por aceite de lino aumenta la expresión relativa de la HSP90AA en hígado.

5. El estrés por persecución induce un incremento en la expresión relativa del GR1 en el músculo y una disminución en la expresión relativa del GR2 en el intestino. Además, induce un incremento de la expresión relativa de la HSP70 en músculo y disminuye la expresión relativa de la HSP90AB en hígado. Todas estas respuestas son independientes del tipo de aceite dietético utilizado.

6. El incremento gradual de temperatura ambiental de 5ºC en una hora induce un aumento de la expresión de los GRs y las HSPs en tejido hepático y cerebro de

lenguado senegalés durante las primeras 24 horas, indicando la respuesta rápida de estos tejidos a un cambio de temperatura ambiental.

7. El incremento gradual de temperatura ambiental de 5ºC en una hora induce un aumento de la relación HSP90/GR en hígado y branquias, sugiriendo un mecanismo de protección frente a la degradación proteosómica del GR.

8. El incremento gradual de temperatura ambiental de 5ºC en una hora induce la elevación de la expresión del gen CRHBP en el cerebro de lenguado senegalés una semana después del estrés térmico, sugiriendo un mecanismo adaptativo de retroalimentación negativa sobre el eje HPI.

9. La sustitución total de aceite de pescado por aceite de soja en dietas para el lenguado senegalés induce un incremento de la expresión intestinal de genes relacionados con el sistema inmune, incluyendo interleuquinas, componentes de la vía clásica del complemento y de la respuesta viral, ciclooxigenasas y receptores "toll-like", sugiriendo una respuesta semejante a un proceso inflamatorio intestinal.

10. Cuando se somete al lenguado senegalés a un estrés agudo por persecución, la sustitución total de aceite de pescado por aceite de soja en dieta induce una falta de respuesta generalizada en términos de cambios en la expresión de genes inmunes comparado con la respuesta de los peces alimentados con la dieta basada en aceite de pescado. Este resultado podría sugerir un sistema inmune exhausto en estos peces alimentados con soja.

11. El efecto combinado del uso de aceite de lino con un estrés agudo por persecución en el lenguado senegalés, induce un incremento de la expresión intestinal de genes relacionados con el sistema inmune, en comparación con la respuesta de los animales alimentados con la dieta basada en aceite de pescado. Estos resultados podrían sugerir la idoneidad del uso de aceites ricos en n-3 frente

al uso de aceites ricos en n-6 para mejorar el potencial de respuesta inmune intestinal.

12. La sustitución total de aceite de pescado por aceites vegetales en dietas para el lenguado senegalés induce una disminución basal de la expresión intestinal del gen receptor de protaglandinas EP4. Debido al papel de este receptor en la producción de mucus intestinal, este resultado sugiere una posible disminución del potencial de protección de la mucosa intestinal.

13. Cuando se somete al lenguado senegalés a un estrés agudo por persecución, la sustitución total de aceite de pescado por aceite de lino en dieta, induce un incremento de la expresión intestinal del gen EP4 en comparación con la respuesta de los peces alimentados con la dieta basada en aceite de soja. Este resultado podría sugerir la idoneidad del uso de aceites ricos en n-3 frente al uso de aceites ricos en n-6 para potenciar la protección de la mucosa intestinal frente a situaciones de estrés.

CAPÍTULO 8

RESUMEN DE LOS CAPÍTULOS

8. Resumen de los capítulos

8.1 Sustitución total de aceite de pescado por aceites vegetales en dietas para lenguado senegalés (*Solea senegalensis*): efectos sobre el rendimiento del pez, su composición bioquímica y la expresión de algunos genes relacionados con los receptores de glucocorticoides

Con la finalidad de determinar el efecto del uso de aceites vegetales en la dieta del lenguado senegalés (*Solea senegalensis*) se sustituyó el 100% del aceite de pescado (FO) de la dieta control por aceite de lino (LO) o soja (SO) durante 90 días, realizándose posteriormente un test de estrés por persecución con red durante 5 minutos. A continuación, se realizarón análisis sanguíneos, bioquímicos y de expresión génica sobre muestras de músculo, hígado e intestino. En dichos análisis se desveló que el crecimiento y rendimiento del pez no se vieron afectados por el uso de aceites vegetales en la dieta, reflejándose dichos efectos en el perfil de ácidos grasos del músculo, hígado e intestino. A su vez, también se observó una tendencia de conservación en los niveles de ácido araquidónico/ácido eicosapentanoido (ARA/EPA) de los diferentes tejidos analizados, a pesar del nivel de estos ácidos grasos en las diferentes dietas utilizadas. Al ocasionarse un estrés agudo por persecución, se produjo un aumento en la expresión del receptor de glucocorticoides 1 (GR1) muscular y una reducción en la del receptor de glucocorticoides 2 (GR2) intestinal independientemente del tipo de aceite utilizado en la dieta. En el hígado se indujo un aumento en la expresión del GR1 y GR2 en peces alimentados con dietas formuladas con FO tras ocasionarse el estrés. Del mismo modo, se ocasionó un aumento en la expresión muscular de las proteínas de shock térmico 70 (HSP70) y una

disminución de las proteínas de shock térmico 90AB (HSP90AB) hepáticas, con independencia de la dieta utilizada. Además, con el uso de aceites vegetales (VO) en la alimentación del lenguado, se disminuyó la expresión del HSP70 en el intestino, aumentándose la expresión de las proteínas de shock térmico 90AA (HSP90AA) en el hígado por el uso de LO en la dieta, para cualquiera de las condiciones experimentales ensayadas.

8.2 Efectos del estrés térmico sobre la expresión de genes relacionados con el complejo receptor de glucocorticoides en lenguado senegalés *(Solea senegalensis)*: respuesta aguda y adaptativa

El objetivo del presente estudio se basó en examinar los efectos de la temperatura a corto y medio plazo sobre los genes que regulan la respuesta a estrés en juveniles de lenguado senegalés. Para ello, los peces se sometieron a un estrés de +5ºC, después de un mes de aclimatación a 18-19ºC. Tras este proceso, se obtuvieron muestras de hígado, cerebro, branquias, músculo e intestino de un total de 96 peces en cuatro periodos de tiempo diferente (1h, 24h, 3d y 1 sem) una vez se alcanzaron los 23-24ºC de temperatura en los tanques sometidos a estrés. Se analizó la expresión de genes involucrados en el eje hipotálamo-pituitaria-interrenal (GR1, GR2, CRH, CRHBP, POMCAA y POMCAB) y en el sistema de defensa celular frente a estrés (HSP70, HSP90AA y HSP90AB), además de los niveles de cortisol en plasma sanguíneo. Como resultado se obtuvieron fluctuaciones en los niveles de cortisol plasmático y en la expresión de los genes anteriormente mencionados difiriendo estos últimos entre los diferentes tejidos muestreados. Del conjunto de los datos analizados en este experimento se concluyó que el lenguado senegalés es capaz de manifestar una buena respuesta inicial ante un shock térmico de +5ºC. También se observó la posible actuación de la HSP90 como mecanismo regulador del GR en presencia de cortisol, y de la CRHBP como el mayor

instrumento para ejercer una retroalimentación negativa sobre la ACTH en la pituitaria del lenguado.

8.3 Aceites vegetales en dieta: efectos sobre la expresión de genes relativos al sistema inmune en intestino de lenguado senegalés (*Solea senegalensis*)

En este artículo se evidenciaron los efectos del uso de VO en la dieta sobre el balance en n-6/n-3 del intestino de juveniles de lenguado senegalés y cómo estos aceites repercuten en la respuesta inmune de los peces sometidos a un estrés agudo de persecución con red de 5 min de duración. Para ello se utilizaron las muestras de intestino recopiladas en el Capítulo 3 y se estudió la expresión de varios genes relacionados con la respuesta inmune a través de un Open-Array. Como ya se expuso en el Capítulo 3, el uso de aceites vegetales en la dieta no afectó al crecimiento de los peces, pero sí al perfil de ácidos grasos del intestino. En relación a los nuevos resultados derivados de los genes de respuesta inmunitaria se observó que el uso de SO (rico en ácidos grasos n-6) indujo la sobre expresión de interleuquinas, componentes de la vía clásica del complemento, genes de respuesta viral y receptores "toll-like", en estado basal. Con el tratamiento de todos estos datos en conjunto se visualizó lo que podría ser una activación crónica de los procesos inflamatorios en el intestino de lenguados alimentados con dietas ricas en SO, mientras que tras ocasionarse el estrés la mayoría de los genes se regularon a la baja, quizás a consecuencia de una inflamación crónica ocasionada por el abuso de ácidos grasos n-6 en la dieta. A su vez, en los lenguados alimentados con LO se observó lo contrario, atenuándose los efectos derivados de una respuesta inmune frente a una situación de estrés puntual por persecución.

8.4 Aceites vegetales en dieta: efectos sobre la expresión de genes relativos al sistema inmune en intestino de lenguado senegalés (*Solea senegalensis*): receptores de eicosanoides

El objetivo de este estudio se focalizó en clarificar los efectos de una dieta constituida al 100% con VO sobre la expresión génica de los receptores de eicosanoides (EPs) y las ciclooxigenasas (COXs) en el intestino de juveniles de lenguado senegalés, tanto en estado basal como tras ocasionarse un estrés por persecución de 5 min. Para ello se utilizaron las muestras de intestino mencionadas en el Capítulo 3 y se estudió la expresión de varios genes relacionados con la respuesta inflamatoria a través de una RT-PCR y un Open-Array. Como ya se desveló en los Capítulos 3 y 5, el crecimiento de los peces no se vió afectado por el uso de VO en la dieta, al contrario que el perfil de ácidos grasos analizado en el intestino. En relación a los resultados derivados de la expresión de los genes relativos al metabolismo lipídico, en estado basal se observó que el uso de dietas formuladas con SO como único recurso lipídico indujeron unos valores significativamente elevados para el gen COX2 en comparación con el resto de dietas, observándose lo mismo para el gen COX1A aunque sin resultados significativos, y en referencia a los EPs sólo se redujo de manera significativa la expresión del EP4 en aquellos lenguados alimentados con VO en comparación con los alimentados con FO. Tras ocasionarse el estrés por persecución, se observó una bajada en la expresión de la COX2 para aquellos peces alimentados con FO, mientras que en aquellos lenguados alimentados con LO, aumentó la expresión del EP4 y disminuyó la del EP2 y la PLA2. El conjunto de resultados obtenidos sugirió que el uso de SO en la dieta de juveniles de lenguado senegalés aumentó la expresión de la COX1A y la COX2 en estado basal como resultado de la acción inflamatoria descrita en el Capítulo 5. Mientras que, el estrés por persecución indujo diferentes respuestas dependiendo del tipo de aceite utilizado en la dieta, observándose un aumento significativo en la expresión del gen EP4 intestinal de aquellos peces alimentados con la dieta constituida a base de LO, lo que

sugiere que los animales alimentados con dietas ricas en ácidos grasos n-3 están mejor preparados para hacer frente a situaciones que podrían afectar a la integridad del intestino frente a aquellos alimentados con una dietas ricas en ácidos grasos n-6.

CAPÍTULO 9

APÉNDICES

9. Apéndices

9.1 Apéndice 1

9.1.1 Protocolo de extracción de RNA a partir de muestras tisulares, utilizando TRI Reagent (SIGMA-Aldrich, Sant Louis, Missouri) y un kit RNaesy Tissue Lipid de QIAGEN:

1. Se añade 1ml de Tri Reagent por cada 50-100 mg de tejido y se homogenizan en un tubo tipo Eppendorf de 2ml con 4 bolas de cristal, el cual se coloca en el Tissue Lyser II de QIAGEN (Fig. 9.1) durante 2 min a máxima frecuencia (30 Hz) para conseguir el homogenizado mecánico del tejido.

Figura 9.1: Tissue Lysser II de Qiagen

2. Se deposita el homogenizado en un tubo Eppendorf limpio y se mantiene a Tª ambiente (15–25°C) durante 5 min. Con lo que se ayuda a disolver los complejos núcleo-proteicos.
3. Se añaden 200 µl de cloroformo al tubo, se agita durante 15 s y se deja a Tª ambiente de 2 a 3 min.
4. Se centrifuga el tubo a 12.000 *xg* durante 15 min a 4ºC. De esta manera se consigue una solución trifásica:
   - Fase orgánica de color rojo = proteínas
   - Fase intermedia de color blanco = DNA
   - Fase superior incolora = RNA
5. El sobrenadante se transfiere a un tubo limpio, se añade 1 volumen de etanol al 70% por cada volumen de RNA (normalmente 600 µl) y se mezcla bien.
6. Esta mezcla se deposita en una columna de filtrado dentro de un tubo Eppendorf de 2 ml que viene incluida en el kit y se centrifuga a Tª ambiente (15–25°C) durante 15 s a 8000 *xg* (10.000 rpm), tras lo cual se descarta el sobrenadante.
7. Se añaden 350 µl de Tampón RW1 sobre la columna de filtrado y se centrifuga durante 15 s a 8.000 *xg* (10.000 rpm) para limpiar la membrana, descartándose de nuevo el sobrenadante.
8. Se realiza un tratamiento con DNAsa añadiendo 80 µl de una mezcla formada por 70 µl de RDD + 10 µl de DNAsa directamente a la columna de filtado, y dejándose en reposo durante 30 min a Tª ambiente.
9. Se añaden 350 µl de Tampón RW1 sobre la columna de filtrado y se centrifuga durante 1 min a 8.000 *xg* (10.000 rpm) para limpiar la membrana, descartándose de nuevo el sobrenadante.
10. Se añaden 500 µl de Tampón RPE sobre la columna de filtrado y se centrifuga durante 15 s a 8.000 *xg* (10.000 rpm) para limpiar la membrana, descartándose por tercera vez el sobrenadante.

11. Se añaden 500 µl de Tampón RPE sobre la columna de filtrado y se centrifuga durante 2 min a 8.000 *xg* (10.000 rpm) para limpiar la membrana, descartándose por cuarta y última vez el sobrenadante.
12. Por último se deposita la columna de filtrado sobre un tubo limpio de 1,5 ml, se añaden de 30 a 80 µl de agua libre de nucleasas y se centrifuga durante 1 min a 8.000 *xg* (10.000 rpm) para limpiar la membrana, guardándose el sobrenadante donde se encuentra el RNA extraído.

9.2 Apéndice 2

9.2.1 Protocolo de clonación con TOPO TA Cloning kit for Sequencing (Invitrogen ™):

1. Se realiza un mezcla con los reactivos detallados en Tabla 9.1, donde se incluyen los productos de PCR que se van a clonar, y se mantienen a Tª ambiente durante 30 min.

Tabla 9.1: Reactivos que se unen al vector de clonación

Producto de PCR	0,5 - 4 µl
Sal	1 µl
H2O	Hasta 5 µl
Total	6 µl

2. Se descongela la bacteria One Shot ® TOP10 y se añaden 2 µl de la mezcla configurada en el paso 1 para transformar la bacteria en una célula competente.
3. Se incuba el tubo con la bacteria en hielo durante 30 min.
4. Se provoca un shock térmico de 42˚C durante 30 s y se vuelve a depositar el tubo en hielo.
5. En un tubo Falconer se añaden la bacteria junto a 250 µl de medio tipo S.O.C. Después se inclina el tubo, se abre ligeramente y se incuba durante una hora a 37˚C.

6. Se siembra la ligación en una placa de agar LB ultrapuro y se incuba a 37˚C durante 24 h.

Protocolo y composición del agar LB ultrapuro para 20 placas de petri de 90 mm de diámetro (Tabla 9.2):

Se disuelven 17,5 gr de agar LB ultrapuro en 500 ml de H_2O MilliQ y se autoclava a 120 ˚C durante 30 min. Una vez disminuya la Tª se añaden los reactivos detallados en la Tabla 9.2.

Tabla 9.2: Composición del agar ultra puro

COMPONENTES DEL GEL	
Ampicilina	500 µl [50 mg/ml]
Kanamicina	500 µl [50mg/ml]
X-Gal	500 µl [50 mg/ml]
IPTG	500 µl [50 mg/ml]

7. Se seleccionan las colonias de color blanco y se resiembran en una placa nueva, donde se incuban denuevo a 37˚C durante 24h.

Se recogen las colonias con palillos, previamente autoclavados a 120ºC durante 30 min, y se diluyen en 25 µl de H_2O MilliQ. Para lisar la bacteria y obtener el fragmento de DNA clonado se aplica a los tubos una temperatura de 94ºC durante 4 min y después se centrifuga a 12.000 *xg* durante 2 min. La bacteria lisada se queda en el fondo del tubo y el sobrenadante de DNA se secuencia utilizando el Kit BIG DYE (Tabla 9.3) tras precipitar la muestra.

Protocolo para precipitación de muestras:

1. Se colocan en un tubo tipo Eppendorf de 1,5 ml los siguientes reactivos:
   - 14,843 µl de EtOH absoluto
   - 4,4 µl de H_2O
   - 0,75 µl de acetato sódico 3 M pH 5
   - 5 µl de muestra
2. Se agita la mezcla depositada en el Eppendorf, permaneciendo 15 min a Tª ambiente.

3. Se centrifuga el Eppendorf con la reacción a máxima velocidad durante 25 min.

4. Se retira el sobrenadante del tubo y se añaden 62,5 µl de EtOH al 70 %.

5. Se centrifuga nuevamente el tubo a máxima velocidad entre 5 y 10 min y se retira el sobrenadante del tubo.

6. Y por último, se deposita el sobrenadante en un tubo limpio a Tª ambiente durante al menos 10 min.

Tabla 9.3: Protocolo de secuenciación para BIG DYE kit

COMPONENTES DE REACCIÓN DE SECUENCIACIÓN			PROGRAMA TERMOCICLADOR		
BIG DYE	0,5 µl		94 ºC	3 min	x 1 ciclo
Tampón 5x	1,5 µl		96 ºC	10 s	
Oligos (10 pmoles/ µl)	1 µl		55 ºC	5 s	x 25 ciclos
Muestra	2 µl		60 ºC	4 min	
Total	5µl		4 ºC	∞	x 1 ciclo

9.3 Apéndice 3

9.3.1 Protocolo de amplificación de secuencia con el kit 3`RACE System for Rapid Amplification of cDNA Ends (Invitrogen ™) y sus componentes (Tabla 9.4):

Tabla 9.4: Componentes del kit 3`RACE System for Rapid Amplification of cDNA Ends (Invitrogen™)

COMPONENTES	
Tampón 10X PCR	[200 mM Tris-HCl (pH 8,4), 500mM KCl]
Mix dNTP 10 mM	(10 mM each dATP, dCTP, dGTP, dTTP0, 1M DTT)
$MgCl_2$ 25 mM	
SuperScript™ II transcriptasa	(RT, 200 unidades/µl)
Oligo adaptador	(AP, 10 µM)
Oligo universal	(UAP, 10 µM)
Oligo universal	(AUAP, 10 µM)
RNase H E. coli	(2 unidades/µl)
Agua DEPC	
RNA	(50 ng/µl)
Oligos específicos de RNA	(GSP, 10 µM)

1. Se centrifugan todos los componentes especificados en la Tabla 9.4.
2. Se depositan 5 µg de RNA procedente de lenguado senegalés en un tubo Eppendorf junto con 11 µl de H_2O DEPC.
3. Se añade 1 µl de oligo AP al tubo, se calienta a 70ºC durante 10 min y se deposita en hielo durante 1 min.
4. Se añaden al tubo 7 µl de la mezcla de productos especificados a continuación:

Tampón PCR 10X	2 µl
MgCl2 25 mM	2 µl
Mezcla dNTP 10 mM	1 µl
DTT 0,1 M	2 µl

5. Tras agitar la mezcla, se calienta el tubo a 42ºC durante 5 min.
6. Se añade 1 µl of SuperScript™ II RT en el tubo y se incuba a 42°C durante 50 min.
7. Se termina la reacción incubando el tubo a 70ºC durante 15 min.
8. Tras enfriar el tubo, se centrifuga brevemente y se añade 1 µl de RNase H.
9. Tras mezclar bien todos los componentes del tubo, se incuba a 37ºC durante 20 min.
10. Por último se procede a conservar esta reacción a –20ºC.

Posteriormente se chequean los productos de PCR mediante un gel de electroforesis junto con un marcador de tamaño molecular para constatar los pesos moleculares de las secuencias amplificadas.

9.4 Apéndice 4

9.4.1 Protocolo de amplificación de secuencia con el kit 5´ RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Invitrogen ™) y sus componentes (Tabla 9.5):

Tabla 9.5: Componentes del kit 5´ RACE System for Rapid Amplification of cDNA Ends, version 2.0 (Invitrogen ™)

COMPONENTES	Vol.	Tª
Tampón PCR 10X [200 mM Tris-HCl (pH 8,4), 500 mM KCl]	500 µl	–20°C
25 mM $MgCl_2$	500 µl	–20°C
Mix 10 mM dNTP [10 mM each dATP, dCTP, dGTP, dTTP]	100µl	–20°C
0,1 M DTT	100 µl	–20°C
Transcriptasa inversa SuperScriptII (200 units/µl)	10 µl	–20°C
Mix Rnase	10 µl	–20°C
5X tailing buffer [50 mM Tris-HCl (pH 8,4), 125 mM KCl, 7,5 mM $MgCl_2$]	500 µl	–20°C
2 mM dCTP	50 µl	–20°C
Transferrasa terminal deoxynucleotidyl	15 µl	–20°C
Oligo 5' RACE (AAP, 10 µM)	80 µl	–20°C
Oligo universal de amplificación (UAP, 10 µM)	40 µl	–20°C
Oligo universal de amplificación (AUAP, 10 µM)	40 µl	–20°C
Agua tratada con DEPC	1,25 ml	–20°C
Columnas S.N.A.P.	10	4°C
Tubos colectores	10	4°C
Solución de unión (6M Yoduro de sodio)	30 ml	4°C
Tampón de lavado	1 ml	4°C

- **Síntesis de cDNA**

  1. Se reúnen en un tubo Eppendorf de 1,5ml los componentes detallados en la Tabla 9.6 utilizándose RNA procedente de diversos tejidos de juveniles de lenguado senegalés.

Tabla 9.6: Componentes para la síntesis de cDNA

COMPONENTES	
GSP1	2,5 pmoles (~10 to 25 ng)
RNA	1-5 µg
Agua tratada con DEPC	hasta un volume final de 15,5 µl

2. Se incuba el tubo durante 10 min a 70°C para desnaturalizar el RNA. Después se mantiene en hielo durante 1 min. Se centrifuga levemente y se añaden por orden los componentes especificados a continuación:

Tampón PCR 10X	2,5 µl
$MgCl_2$ 25 mM	2,5 µl
Mix dNTP 10 mM	1 µl
0,1M DTT	2,5 µl
Volume final	8,5 µl

3. Se mezclan con cuidado en el tubo y se centrifugan durante unos segundos, seguidamente se incuba la reacción del tubo a 42ºC durante 1 min.
4. Se añade 1µl of SuperScript™ II RT al tubo. Se mezcla cuidadosamente y se incuba durante 50 min a 42°C.

Note: 30 min de incubación suelen ser suficientes para un RNA mensajero de cadena corta (<4 kb), para transcripciones más largas se requieren al menos 50 min.

5. Se incuba el tubo a 70ºC durante 15 min para terminar la reacción.
6. Denuevo se centrifuga el tubo de 10 a 20 s y se mantiene a 37°C.
7. Se añade 1 µl de la mezcla de RNasa al tubo, y se incuba durante 30 min a 37ºC.
8. Se centrifuga el tubo durante unos segundos y se deposita en hielo.

A partir de aquí se puede conservar la reacción a -20°C.

- **Purificación de cDNA por columna S.N.A.P.**

1. Se atempera la solución de unión a Tª ambiente.
2. Se atempera el agua milliQ que vayamos a utilizar a 65°C.
3. Se añaden 120 µl de solución de unión (NaI 6M) al cDNA en un tubo Eppendorf.
4. Se transfiere la solución cDNA/NaI a una columna S.N.A.P. y se centrifuga a 13.000 *xg* durante 20 s.
5. Se añaden 0,4 ml de tampón de lavado 1x a 4°C dentro de la columna.

6. Se centrifuga la columna a 13.000 *xg* durante 20 s, se descarta el sobrenadante y se repite este paso 3 veces más.
7. Se lava la columna con 400 µl de etanol al 70 % frío (4ºC) y se centrifuga a 13.000 *xg* durante 20 s, tirando el sobrenadante y repitiendo este paso una vez más.
8. Se centrifuga de nuevo la columna a 13.000 *xg* pero esta vez durante 1 min.
9. Por último, se transfiere la columna a un tubo limpio, se añaden 50 µl de agua MilliQ (previamente calentada a 65°C) y se centrifuga a 13.000 *xg* durante 20 s para diluir el cDNA.

- **TdT Tailing del cDNA**

1. Se añaden los siguientes componentes a un tubo tipo Eppendorf de 1,5 ml y se mezclan cuidadosamente:

DEPC-treated water	6,5 µl
5X tailing buffer	5,0 µl
2 mM dCTP	2,5 µl
cDNA purificado	10,0 µl
Volume final	24,0 µl

2. Se incuba la mezcla en el tubo durante 2 o 3 min a 94°C. Se deposita el tubo en hielo durante 1 min, se centrifuga unos segundos y se vuelve a depositar en hielo.
3. Se añade 1 µl de TdT al tubo, se mezcla cuidadosamente y se incuba durante 10 min a 37°C.
4. Para inactivar el TdT, se mantiene el tubo con la reacción a una Tª de 65°C durante 10 min, se centrifuga el tubo durante unos segundos y se deposita la reacción en hielo.

Posteriormente se chequean los productos de PCR mediante un gel de electroforesis utilizando un marcador adecuado al tamaño molecular de la secuencia amplificada.