PATHOGENESIS AND TRANSMISSION OF LYMPHOCYSTIS DISEASE VIRUS (LCDV) IN GILTHEAD SEABREAM *(Sparus aurata* L.)

TESIS DOCTORAL

ESTEFANÍA JIMÉNEZ VALVERDE

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Programa de Doctorado en Biología Celular y Molecular
Departamento de Microbiología
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Memoria presentada por
Dª. **Estefanía Jiménez Valverde**
para optar al grado de
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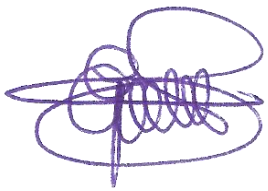
con mención internacional

Fdo. La doctoranda



FACULTAD DE CIENCIAS

PROGRAMA DE DOCTORADO EN BIOLOGÍA CELULAR Y MOLECULAR

D. **JUAN JOSÉ BORREGO GARCÍA** Catedrático del Departamento de Microbiología de la Universidad de Málaga, como **TUTOR** dentro del programa de doctorado "**BIOLOGIA CELULAR Y MOLECULAR**" y **DIRECTOR** del trabajo de tesis realizado por la doctoranda.

Dña. **Mª DOLORES CASTRO LÓPEZ**, Profesora Titular del Departamento de Microbiología de la Universidad de Málaga y **DIRECTORA** del trabajo de tesis realizado por la doctoranda.

INFORMAN:

Que la doctoranda **ESTEFANÍA JIMÉNEZ VALVERDE** ha realizado de forma satisfactoria y bajo nuestra supervisión las actividades de formación dentro del programa de doctorado arriba mencionado así como el trabajo investigador que se presenta y que lleva por título **"Pathogenesis and transmission of lymphocystis disease virus (LCDV) in gilthead seabream (*Sparus aurata* L.)"**. Este trabajo constituye su proyecto de Tesis para aspirar al Título de Doctora en Biología con mención internacional.

Y para que así conste, y tenga los efectos que correspondan, en cumplimiento de la legislación vigente, extendemos el presente informe en Málaga, a 14 de septiembre de 2016.

Fdo. El Tutor: Juan José Borrego García

Fdo. Los directores: Juan José Borrego García Mª Dolores Castro López

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RESUMEN

INTRODUCCIÓN

La fuerte demanda a nivel mundial de los productos derivados de la acuicultura ha abocado a que dicho sector haya experimentado un crecimiento exponencial durante las últimas décadas, constituyendo una alternativa suplementaria al sector de la extracción pesquera. En la actualidad es una de las actividades productivas con mayor interés económico, así como un instrumento adecuado para asegurar la sostenibilidad de los recursos naturales. La mayoría de las especies marinas cultivadas presentan alto valor comercial, a veces porque el volumen de las poblaciones salvajes es bajo o se encuentra en descenso. Este es el caso de la dorada (*Sparus aurata*), tradicionalmente cultivada en el norte de Italia y en el sur de España, que ha acabado convirtiéndose en una de las principales especies de peces cultivados, ocupando el tercer puesto en la Unión Europea y siendo el principal cultivo de peces en la acuicultura española.

Uno de los problemas más importantes a los que se enfrenta la acuicultura, y que puede limitar de forma significativa el cultivo de las especies piscícolas, es la aparición de patologías de etiología microbiana que ven favorecidas su propagación por las condiciones derivadas del cultivo intensivo. Dentro de las enfermedades infecciosas, las de origen vírico tienen una especial relevancia debido a diversos factores, como las altas mortalidades que provocan y la capacidad de inducir infecciones persistentes. Las infecciones víricas son, de hecho, un factor limitante para la expansión de la acuicultura debido a las pérdidas directas en la producción de peces, los costes derivados de la reducción de la productividad y la gestión de la enfermedad, sumados a la pérdida de mercados de exportación en relación con las restricciones comerciales. Además, hay que añadir a esta problemática las limitaciones impuestas por los métodos de diagnóstico y la escasez de tratamientos antivirales realmente efectivos. Por otra parte, existen numerosos virus capaces de desarrollar infecciones asintomáticas, con el consiguiente riesgo de liberación continua de partículas víricas al medio que puede pasar desapercibida. Esto podría implicar una rápida transmisión de la enfermedad en

el sistema de producción, pudiendo llegar a afectar a la totalidad de las fases del cultivo. Debido al riesgo de propagación, muchas de estas enfermedades víricas son de declaración obligatoria, apareciendo en las listas de la Organización Mundial de Sanidad Animal (OIE).

En los últimos años, el número de enfermedades de origen vírico descritas en las instalaciones de acuicultura se ha incrementado considerablemente, destacando aquellas que afectan a peces marinos cultivados. Las principales virosis que afectan a la piscicultura son las provocadas por los virus pertenecientes a las familias *Birnaviridae*, *Iridoviridae*, *Nodaviridae*, *Reoviridae* y *Rhabdoviridae*. Con respecto a los miembros de la familia *Iridoviridae*, estos pueden afectar a una gran diversidad de especies animales (vertebrados poiquilotermos y artrópodos) y tienen una amplia distribución geográfica. En el caso de peces cultivados, el virus de la enfermedad de linfocistis (LCDV), incluido en el género *Lymphocystivirus*, es el representante de la familia con mayor incidencia, afectando tanto a peces marinos como dulceacuícolas, siendo las familias *Centrarchidae*, *Percidae*, *Scienidae* y *Pleuronectidae* las que incluyen un mayor número de hospedadores susceptibles.

El Comité Internacional de Taxonomía Vírica (ICTV), basándose en diferentes características como el tamaño del virión, rango de hospedador, histopatología, perfiles proteicos y análisis de la secuencia del DNA, reconoce actualmente una única especie dentro del género *Lymphocystivirus*, *Lymphocystis disease virus 1* (LCDV-1), que corresponde a virus aislados de platija europea (*Platichthys flessus*) y de solla (*Pleuronectes platessa*). Además, se incluyen otros tres virus candidatos en el género: Lymphocystis disease virus 2 (LCDV-2), que corresponde a los aislados de lenguadina (*Limanda limanda*); Lymphocystis disease virus-China (LCDV-C), aislado de platija japonesa (*Paralichthys olivaceus*), y Lymphocystis disease rockfish virus (LCDV-RF), aislado a partir de pez roca coreano (*Sebastes schlegeli*). El genoma del LCDV-C se ha secuenciado recientemente y muestra considerables diferencias con la especie LCDV-1 en cuanto a tamaño, organización genómica e identidad de los productos génicos codificados, además del rango de hospedador. Por ello se ha propuesto que constituya una nueva especie del género *Lymphocystivirus*.

Una de las características distintivas de la familia *Iridoviridae* es la presencia de la proteína principal de la cápside (MCP), principal componente estructural de las partículas víricas, que representa hasta el 45% de los polipéptidos víricos totales. Los estudios filogenéticos basados en la secuencia del gen que codifica para la MCP han demostrado la existencia de variabilidad genética dentro del género *Lymphocystivirus*, estableciéndose 9 genotipos que difieren en múltiples propiedades biológicas, incluyendo rango de hospedador, características antigénicas, etc. La especie LCDV-1 constituiría el genotipo I; el genotipo II corresponde a los aislados de platija japonesa (LCDV-C); el genotipo III incluye los aislados del pez roca coreano (LCDV-RF); el genotipo IV, los aislados de cobia (*Rachycentron canadum*) y lubina japonesa (*Lateolabrax japonicus*) (LCDV-RC y LCDV-SB, respectivamente); el genotipo V incluye los aislados del pez tetra fantasía (*Parambassis baculis*) (LCDV-CB); el genotipo VI para los aislados de gourami (*Trichopodus leerii* y *T. Trichopterus*) (LCDV-TL); el genotipo VII incluye a los aislados de dorada y de lenguado senegalés (*Solea senegalensis*) (LCDV-SA y LCDV-SSE, respectivamente); el genotipo VIII corresponde a los aislados de perca americana (*Micropterus salmoides*) (cepa Leetown HNF); y el genotipo IX, constituido por los aislados de perca amarilla (*Perca flavescens*).

El LCDV es un virus icosaédrico de gran tamaño, que presenta partículas isométricas de 150 a 200 nm, con una cápside bilaminar y un núcleo central o "core" de apariencia filamentosa, separado de la cápside por un material amorfo de 10 a 20 nm de espesor. La estructura del virión está formada por una membrana interna lipoproteica, con un alto contenido en fosfolípidos, y por una cápside proteica constituida fundamentalmente por la MCP. Además, se ha observado la presencia de filamentos externos en la superficie del virión de aproximadamente 2,5 nm de longitud. El genoma del LCDV está constituido por una molécula lineal de DNA de doble cadena altamente metilado que presenta redundancia terminal, permutación circular, y un contenido G-C de aproximadamente el 30%. En cuanto al mecanismo de replicación del LCDV aún no ha sido completamente dilucidado, y sólo se ha podido establecer que la fase final de la morfogénesis se lleva a cabo en el citoplasma celular. No obstante, se ha propuesto como modelo de mecanismo de replicación el del

virus FV-3 (*Frog virus 3*, especie tipo del género *Ranavirus*), perteneciente también a la familia *Iridoviridae*.

El LCDV es el agente etiológico de la enfermedad de linfocistis (LCD), una de las primeras enfermedades víricas de peces descrita. Los primeros estudios se remontan a 1904, cuando Woodcock confundió los huevos del parásito *Lymphocystis johnstonei* con las células hipertrofiadas que son el síntoma característico de esta enfermedad. Años más tarde, Weissenberg determinó que el agente causal de la LCD era un virus, siendo confirmada su naturaleza vírica mediante microscopía electrónica por Walker y mediante el aislamiento del virus en la línea celular BF-2 por Wolf.

La LCD se ha descrito en más de 150 especies diferentes de peces teleósteos, tanto marinos como dulceacuícolas, y está ampliamente distribuida por todo el mundo, habiéndose detectado en los cinco continentes. En Europa, la enfermedad es endémica del Mar del Norte y del Mar Mediterráneo, donde afecta tanto a peces salvajes como cultivados. En España, la enfermedad se describió por primera vez en doradas cultivadas en 1988, y desde entonces se ha convertido en una de las patologías más frecuentemente registradas en las poblaciones de doradas cultivadas.

Esta enfermedad se caracteriza por la aparición de unos pequeños nódulos de color blanquecino a grisáceo localizados en la superficie del cuerpo y las aletas, que pueden aparecer aislados o más frecuentemente agrupados en racimos, pudiendo llegar a cubrir todo el cuerpo del animal. Estos nódulos también pueden aparecer en ojos, boca, branquias y, con menor frecuencia, en órganos internos como bazo, hígado, riñón, superficie intestinal, estómago y corazón, lo que implica un aumento de la mortalidad. Cada uno de estos nódulos corresponde a una célula fibroblástica hipertrofiada, denominada célula linfocística o linfociste, que presenta un diámetro de 0,1-2 mm y en cuyo citoplasma tiene lugar la replicación vírica. El aumento de tamaño de las células hipertrofiadas se debe a un incremento en el volumen del núcleo, nucleolos y citoplasma como resultado del desarrollo de uno o más cuerpos de inclusión citoplasmáticos, lo que origina la pérdida de la capacidad de división celular. Esto provoca la hipertrofia de la célula infectada que incrementa su volumen de 50.000 a 100.000 veces.

La LCD es una enfermedad auto-limitada que, dependiendo de la especie hospedadora y las condiciones ambientales, puede prolongarse por un tiempo variable. Así, por ejemplo, en doradas mantenidas a 20 °C las lesiones suelen remitir totalmente en unos 45 días. En las instalaciones de acuicultura la LCD suele presentar una elevada morbilidad, ocasionando graves pérdidas económicas relacionadas con una desfiguración de los animales que impide su comercialización. Además, los animales enfermos muestran un descenso del crecimiento y anemia, siendo más susceptibles a infecciones bacterianas secundarias, canibalismo y/o infestaciones parasitarias. Por otra parte, aunque normalmente no es una enfermedad fatal, se han descrito episodios de elevada mortalidad en piscifactorías, sobre todo en alevines de pequeño tamaño donde la afectación severa puede ocasionar muerte por asfixia o inanición. Cuando las lesiones remiten se aprecian cicatrices de color gris-azulado que acaban desapareciendo. En aquellos individuos en los que han desaparecido los síntomas se ha observado que el virus persiste en el hospedador, detectándose antígenos y genomas víricos en dermis, riñón, bazo e hígado. También se ha demostrado que es muy frecuente que las infecciones por LCDV cursen de forma asintomática.

Existen pocos estudios realizados sobre la patogénesis del LCDV, limitados en su mayoría a estudios histopatológicos de las lesiones localizadas en la piel y órganos internos. En el caso de las infecciones en dorada nuestro grupo de investigación ha establecido la distribución del virus en diversos tejidos mediante aislamiento de partículas infectivas en cultivos celulares, detección del genoma viral por PCR, así como por técnicas de inmunohistoquímica e hibridación *in situ* DNA-DNA. Los resultados de estos estudios sugieren que la infección por LCDV en alevines de dorada presenta un carácter sistémico, tanto cuando esta cursa de forma clínica como asintomática, y que la multiplicación del virus parece ocurrir en fibroblastos, hepatocitos y macrófagos. Sin embargo, no ha podido demostrarse si las partículas víricas infectivas procedían de una viremia o si se replicaban efectivamente en esos tejidos. Por otra parte, se comprobó que el virus persiste en el hospedador tras la desaparición de los síntomas, detectándose antígenos y genomas víricos en riñón, bazo, dermis e hígado. La persistencia de genoma viral también se ha demostrado en el caso del LCDV-C en platija japonesa, aunque limitada al tejido epidérmico.

Las rutas de transmisión del LCDV no se han dilucidado totalmente, si bien el contacto directo es la ruta más ampliamente aceptada, siendo las superficies externas, incluyendo las branquias, las principales vías de entrada del virus. En las instalaciones de acuicultura, la transmisión del LCDV se ve también favorecida por la alta densidad de población, así como por la eventual presencia de heridas en los animales. Por otra parte, aunque se ha propuesto que la acción de ectoparásitos y otros factores agresivos puede favorecer la transmisión del LCDV, su implicación no se ha demostrado empíricamente. Al igual que se ha establecido para otras infecciones víricas de peces con transmisión mediante vía hídrica, diversos autores han propuesto que el LCDV se transmite por vía alimentaria en las piscifactorías, lo que implicaría la contaminación vírica del alimento vivo (rotíferos y/o artemias). En cuanto a las vías de excreción del LCDV a partir de peces infectados, sólo se ha comprobado que se liberan al medio acuático al producirse la rotura de las células linfocísticas.

Desde 1996 nuestro grupo de investigación viene realizando estudios sobre la LCD en doradas cultivadas. A lo largo de estos años numerosas empresas del sector han solicitado nuestros servicios para el diagnóstico del LCDV en sus animales, lo que nos ha permitido constatar que la incidencia de la enfermedad en las piscifactorías es muy variable. Además, en muchas piscifactorías de la Comunidad Autónoma Andaluza y del resto de la cuenca mediterránea la situación se ha agravado en los últimos años, hasta convertirse en un problema crónico en muchas de las instalaciones de cultivo de dorada, sobre todo en "nurseries" y en la fase de pre-engorde. En algunas instalaciones el problema queda limitado a la imposibilidad de vender los animales con manifestaciones clínicas hasta que estas desaparezcan, lo que generalmente supone pérdidas económicas importantes, mientras que en otros casos se producen también importantes mortalidades, sobre todo cuando la enfermedad afecta a alevines de pequeño tamaño. Otros estudios realizados por nuestro grupo de investigación indican que el LCDV está presente de forma ubicua en muchas piscifactorías, siendo muy frecuentes las infecciones asintomáticas en alevines de dorada, comportándose estos animales como portadores del virus.

Al igual que ocurre en otras muchas infecciones víricas de peces, no existen medidas profilácticas adecuadas para el control de la LCD en doradas, sino que la prevención debe pasar por evitar la introducción del virus en las piscifactorías y por establecer medidas higiénico-sanitarias que permitan su eliminación. Para ello es esencial conocer cuáles son los reservorios del virus en las piscifactorías, así como su implicación en la transmisión vírica, para evitar la aparición de brotes y las pérdidas económicas que estos suponen.

Los principales objetivos planteados en la presente tesis doctoral fueron el estudio de la patogénesis del LCDV en dorada, y el establecimiento de las rutas de transmisión del mismo en larvas y alevines de esta especie. Para la consecución de estos objetivos se diseñaron y evaluaron técnicas moleculares para determinar los órganos diana del LCDV en el hospedador, tanto en infecciones agudas como en animales infectados de forma subclínica y en portadores recuperados de la enfermedad. Estas herramientas permitieron también la identificación de reservorios del virus en las instalaciones de cultivo larvario de dorada, y el estudio de su implicación en la transmisión vírica. Por último, se planteó establecer si la artemia es susceptible a la infección por LCDV.

CAPÍTULO 1: Métodos de diagnóstico para el virus de la enfermedad de linfocistis.

En este capítulo se recoge el diseño, evaluación y aplicación de métodos moleculares para la detección y/o cuantificación del LCDV genotipo VII en doradas cultivadas. Los métodos desarrollados se basan en la detección de genoma viral mediante PCR a tiempo real (qPCR) y LAMP (Loop-mediated isothermal amplification), así como un protocolo que permite la detección de transcritos virales en cultivos celulares (ICC-RT-PCR) para la detección y cuantificación de virus infectivos.

1.1. Detección y cuantificación del virus de la enfermedad de linfocistis mediante PCR a tiempo real.

La existencia de infecciones subclínicas producidas por el LCDV se ha puesto de manifiesto en repetidas ocasiones mediante el uso de métodos basados en la reacción en cadena de la polimerasa (PCR). Sin embargo, estos métodos no proporcionan resultados cuantitativos que pueden ser útiles en estudios epidemiológicos y patológicos. Por otra parte, los peces portadores del virus están involucrados en la transmisión viral, por lo que el uso de métodos de diagnóstico sensibles y específicos para detectar el LCDV es esencial para prevenir la enfermedad.

En el presente capítulo, se desarrolló un ensayo de qPCR para la detección y cuantificación del LCDV (genotipo VII). El ensayo se evaluó para el diagnóstico viral en estudios de vigilancia en diversas explotaciones de dorada, y también para identificar posibles reservorios del virus en las piscifactorías.

El protocolo de qPCR diseñado es específico para el LCDV, como se ha demostrado mediante el análisis de las curvas de disociación generadas para cada muestra. La sensibilidad analítica, determinada como el menor número de copias del plásmido recombinante utilizado en la curva estándar que puede ser detectado, fue de 2 copias de DNA por reacción. Por otra parte, el protocolo de qPCR mostró un amplio rango dinámico, extensible hasta concentraciones de 6 $\log_{10}$ de DNA plasmídico, y a un título infectivo desde 10^4 a 1 $TCID_{50}$. Además, la precisión del ensayo está avalada por la alta correlación de los coeficientes obtenidos para la curva estándar, y por la variación intra- e inter-ensayo de los valores de Ct. Utilizando el protocolo descrito en este capítulo se pueden detectar concentraciones tan bajas como 1 copia de DNA vírico por mg de tejido. Esta alta sensibilidad, combinada con su amplio rango dinámico, hace de este ensayo de qPCR una herramienta ideal para detectar bajas cargas virales en infecciones subclínicas por el LCDV, y, al mismo tiempo, cuantificar las variaciones de la carga viral durante el curso de la infección.

La aplicación del ensayo de qPCR a la detección del LCDV en piscifactorías ha demostrado que la determinación del estatus virológico de peces individuales, tanto enfermos como infectados subclínicamente, es posible

mediante el muestreo de una porción de aleta caudal. La prevalencia del LCDV en las poblaciones de dorada analizadas que no presentaban sintomatología varió del 30 al 100%, incluso en piscifactorías donde no se había detectado previamente la enfermedad. En estos peces, la carga viral estimada en aleta caudal fue de dos a cinco órdenes de magnitud menor que en peces enfermos. De hecho, la carga viral parece estar correlacionada con la manifestación de la enfermedad. Sin embargo, no ha podido determinarse si la baja carga viral detectada en infecciones subclínicas se asocia a una replicación del virus. Por todo ello, el protocolo de qPCR diseñado podría ser una herramienta valiosa para el estudio de la relación entre la multiplicación vírica y el inicio de los síntomas en infecciones experimentales con LCDV.

El análisis mediante qPCR de animales reproductores de una piscifactoría con historial previo de LCD también permitió identificar portadores del virus. En esta misma piscifactoría fue posible la detección y cuantificación por qPCR del LCDV en todas las muestras obtenidas de distintos puntos del sistema, como huevos fertilizados, larvas y alevines, así como en rotíferos y artemias utilizados para la alimentación de los mismos. El ensayo de qPCR mostró una alta sensibilidad clínica, pero con la ventaja de que se puede completar en unos 130 min, incluyendo la generación de la curva de disociación, lo que supone una considerable reducción de tiempo con respecto a otras técnicas con similar sensibilidad. Por otra parte, este ensayo permitió identificar la existencia de múltiples reservorios del LCDV en las instalaciones de acuicultura, y la importancia de la aplicación de tratamientos de desinfección eficaces para evitar la transmisión del virus a través de los huevos o del alimento vivo.

1.2. Ensayo LAMP (loop-mediated isothermal amplification) para la detección rápida del virus de la enfermedad de linfocistis.

El diagnóstico rápido del LCDV en animales infectados de forma asintomática es frecuentemente vital, por lo que hay que recurrir a métodos alternativos a los ensayos inmunológicos o a los basados en la PCR convencional, ya que estos requieren técnicas adicionales para incrementar su sensibilidad, como pueden ser la amplificación previa en cultivos celulares o la

hibridación en blot, que los hacen laboriosos y con tiempos de análisis prolongados. Estas dificultades metodológicas son subsanables mediante el uso de la qPCR, pero incluso en este caso podemos encontrar limitaciones debido al coste en reactivos o el equipamiento necesario para realizar dicha técnica.

El método LAMP desarrollado en este capítulo es una técnica sensible, específica, rápida y simple, que permite el diagnóstico del LCDV (genotipo VII) tanto en el laboratorio como en ensayos de campo, lo que supone una gran versatilidad, ya que el diagnóstico se puede realizar *in situ*. La sensibilidad del método se estimó en 10 copias de DNA viral, muy similar a la obtenida por qPCR y significativamente superior a la obtenida mediante PCR convencional. Además, el análisis de la temperatura de disociación de los productos obtenidos permite confirmar la amplificación específica del LCDV. La duración del ensayo, así como la monitorización de la amplificación, puede ser controlada a tiempo real de forma precisa. Así, este ensayo permitió la detección del LCDV en menos de 60 minutos en muestras de dorada y lenguado portadores del virus que cursaban la infección de forma subclínica, en los que la carga viral es extremadamente baja.

Por otra parte, la técnica ha sido diseñada utilizando una plataforma portátil (Genie® II), que permite realizar el diagnóstico en condiciones de campo, lo cual es especialmente interesante cuando ocurren brotes de infección en piscifactorías y el tiempo para determinar la presencia del virus, tanto cualitativamente como cuantitativamente, es limitado. La correlación observada entre la cantidad de DNA y los valores de tiempo de positividad (Tp) obtenidos indican que el protocolo LAMP diseñado puede aplicarse para la cuantificación del LCDV sin necesidad de otro equipamiento.

Uno de los puntos críticos en el diagnóstico molecular en muestras de campo es el requerimiento de muestras con DNA de alta calidad. Debido a que el método LAMP es menos sensible a sustancias inhibidoras o interferentes que la PCR, y a que su eficiencia no se ve afectada por la presencia de DNA no purificado, el ensayo LAMP diseñado ha podido utilizarse junto a un protocolo de extracción rápido de DNA que no requiere de reactivos tan costosos. Los resultados fueron similares a los obtenidos utilizando DNA purificado mediante un sistema de extracción comercial. Este método de extracción en crudo permite

ahorrar tiempo y costes, y es potencialmente adecuado para operadores menos experimentados.

1.3. Ensayo ICC RT-PCR (integrated cell culture RT-PCR) para la detección y cuantificación de LCDV infectivos.

El método estándar para el diagnóstico viral en peces está basado en el aislamiento del virus en cultivos celulares, seguido de la confirmación mediante técnicas moleculares o serológicas. Este procedimiento demuestra la infectividad del virus detectado, además de poder utilizarse para determinar el título vírico infectivo. Sin embargo, el LCDV es difícil de propagar en cultivos celulares y, en ocasiones, no produce efectos citopáticos (CPE) consistentes, especialmente en el caso de muestras obtenidas de peces infectados subclínicamente. Además, en dichas muestras, la aparición de CPE requiere al menos 14 días o incluso de un segundo pase ciego.

Por ello, en el presente capítulo, se desarrolló un ensayo en cultivos celulares seguido de la detección de transcritos virales mediante RT-PCR (ICC-RT-PCR), que se combinó con la hibridación en blot de los productos de RT-PCR, para la detección de partículas infectivas del LCDV. El método está basado en la detección de RNA mensajero vírico mediante RT-PCR a los cinco días post-inoculación en cultivos celulares. En el caso de virus DNA como el LCDV, la detección de transcritos en cultivos celulares es indicativo de la presencia de virus infectivos, permitiendo diferenciar estos de virus inactivados también presentes en la muestra. Además, la hibridación en blot permite aumentar considerablemente la sensibilidad en la detección de estos transcritos virales. Por último, este ensayo puede aplicarse para la determinación del título infectivo mediante el método del número más probable, expresándose como número más probable de unidades infectivas por unidad de volumen (MPNIU/ml).

El ensayo de ICC RT-PCR diseñado, que puede llevarse a cabo en sólo 7 días, mostró una alta sensibilidad, al menos 100 veces superior a otros métodos de diagnóstico viral basados en el desarrollo de CPE. Además, la ICC RT-PCR permitió la determinación del título infectivo en muestras con muy baja carga

vírica, incluyendo aquellas de animales portadores asintomáticos, en los que no fue posible obtener CPE después de 14 días de incubación en cultivos celulares. En estos animales asintomáticos, los títulos infectivos obtenidos mediante ICC RT-PCR fueron entre 2,2-2,7 logaritmos más bajos que la carga viral estimada mediante qPCR. Por tanto, aunque la qPCR es una técnica adecuada para el diagnóstico del LCDV, la cantidad de virus infectivos puede verse en ocasiones sobreestimada, al menos en peces infectados subclínicamente, lo que estaría relacionado con la detección de virus defectivos.

El ensayo de ICC RT-PCR diseñado permitió la detección y cuantificación de partículas infectivas del LCDV de forma rápida, específica y sensible, por lo que puede ser una herramienta de gran utilidad para el estudio de aspectos importantes de la infección por LCDV como la transmisión o la epizootiología.

CAPÍTULO 2: Patogénesis y transmisión del virus de la enfermedad de linfocistis.

Este capítulo aborda el estudio del tropismo del LCDV en diferentes órganos y tejidos de ejemplares juveniles de dorada, analizándose tanto animales enfermos como animales con infecciones subclínicas o recuperados de la enfermedad. Para ello se han utilizado técnicas de cuantificación vírica mediante qPCR y de detección de transcritos virales mediante RT-qPCR o hibridación *in situ* (ISH). Así mismo, se presenta el estudio histopatológico realizado en estos animales.

También se han estudiado las vías de transmisión del LCDV a larvas y alevines de dorada, estableciéndose la existencia de múltiples rutas para la transmisión horizontal del virus. Por último, se presentan los resultados obtenidos en infecciones experimentales realizadas con artemias con el fin de establecer el carácter de hospedador para el LCDV de este crustáceo.

2.1. Determinación de los órganos diana para la multiplicación del virus de la enfermedad de linfocistis en dorada.

El principal objetivo de este apartado fue el estudio de los mecanismos de patogénesis del LCDV en alevines de dorada, y en particular la determinación de los órganos diana para la multiplicación vírica. Para ello, se realizaron muestreos en poblaciones de dorada de piscifactorías, incluyendo ejemplares asintomáticos, ejemplares enfermos (es decir, que mostraban la sintomatología típica de la LCD), y ejemplares recuperados de la enfermedad. Los órganos que se analizaron fueron aleta caudal, intestino, hígado, bazo, riñón y cerebro. La carga viral en los distintos órganos de alevines de dorada se determinó mediante qPCR, mientras que la cuantificación relativa de la expresión del gen que codifica la MCP viral se realizó mediante RT-qPCR. Para determinar las células diana para la replicación del LCDV en los distintos órganos analizados, se procedió a la detección de RNA mensajero viral mediante ISH. Por otra parte, también se estudiaron las alteraciones histopatológicas asociadas a la LCD en los grupos de peces antes mencionados, empleándose dos técnicas tintoriales generales: hematoxilina-eosina y hematoxilina-V.O.F.

Los resultados obtenidos indican que la infección por LCDV en alevines de dorada presenta un carácter sistémico, tanto cuando estos muestran la sintomatología característica de la enfermedad como en el caso de infecciones subclínicas o asintomáticas. Estas infecciones subclínicas son además productivas, como lo demuestra el hecho de que fue posible detectar transcritos virales en todos los órganos analizados en ambos grupos de peces asintomáticos. Además, aunque la enfermedad es auto-limitada, el virus no se elimina después de que los animales se recuperen de la enfermedad, por lo que la infección es persistente, si bien con cargas virales sólo detectables mediante qPCR. Los análisis mediante ISH mostraron que el LCDV presenta un tropismo tisular amplio, replicándose al menos en fibroblastos de la dermis (donde la infección conlleva su transformación en linfocistes), hepatocitos, células del tejido hematopoyético de bazo y riñón, y en el cerebro.

En cuanto a los estudios histológicos realizados, en animales enfermos se observaron alteraciones histopatológicas de distinta consideración según el

órgano considerado, mientras que en peces recuperados la mayoría de los órganos presentaron características histológicas similares a los de los animales sanos. En peces recuperados sólo se detectaron cambios patológicos en intestino e hígado, aunque estos daños son menos severos que los observados en peces enfermos. Esto indicaría que dichos daños están asociados a una elevada carga vírica y que son reversibles, es decir, desaparecen cuando el animal se recupera de la enfermedad.

2.2. Transmisión del virus de la enfermedad de linfocistis a larvas y alevines de dorada.

Los análisis realizados en diversas piscifactorías españolas con historial de LCD demostraron que tanto larvas como alevines de dorada pueden ser portadores del virus, lo cual suele estar asociado con la transmisión vertical de los virus a partir de los reproductores. Los reproductores portadores de virus pueden excretarlos en sus fluidos reproductivos, infectando así a los óvulos en el momento de la fecundación o poco después. En el presente estudio, se analizaron muestras de sangre de reproductores de dorada utilizando PCR-hibridación para el diagnóstico del LCDV. El genoma viral se detectó en un 17,5% de los animales analizados, y los huevos fecundados obtenidos a partir de estos reproductores también resultaron positivos para el virus, así como la mayoría de las larvas nacidas de ellos, lo que sugiere una transmisión vertical del LCDV. Para abordar la cuestión de si el LCDV se transmite en la superficie del huevo o intra-óvulo, los huevos fertilizados se desinfectaron con yodo. Las larvas desarrolladas a partir de estos huevos desinfectados resultaron negativas tanto por PCR-hibridación como por ISH, lo que demostraría la localización superficial del virus en los huevos.

Las larvas obtenidas a partir de huevos LCDV-positivos presentaron DNA y antígenos víricos en la epidermis, lo que sugiere que los virus presentes en la superficie del huevo son capaces de infectar a dichas larvas. Sin embargo, cuando comienza la fase exotrófica, también se detectaron antígenos víricos en el tracto digestivo de algunas larvas, incluso antes de la introducción de

alimento vivo, lo que sugiere una transmisión a través del agua de virus excretados por larvas infectadas.

Los rotíferos y los nauplios de artemia constituyen el alimento vivo más frecuentemente utilizado en el cultivo larvario y post-larvario de dorada. Sin embargo, estos invertebrados se han considerado como posibles vectores para la introducción de diferentes patógenos microbianos en las instalaciones de acuicultura, entre ellos, algunos patógenos víricos. Las larvas LCDV-negativas alimentadas con rotíferos contaminados mostraron antígenos víricos en el tracto digestivo a los dos días de iniciarse la alimentación, detectándose también el virus en la epidermis unos días después. Por tanto, los rotíferos actúan como vector para la transmisión del LCDV a larvas de dorada. Además, se detectaron antígenos víricos en cerebro e hígado de algunos animales, lo que sugiere que en larvas de dorada la infección por LCDV puede ser también sistémica. La diseminación del virus hacia órganos internos desde la epidermis y/o el tracto digestivo podría producirse por vía sanguínea. Por otra parte, se analizó la carga y la expresión génica virales en alevines de dorada de 0,5-1 g a los que se alimentó con metanauplios de artemia contaminados experimentalmente con LCDV. Los resultados obtenidos demostraron que las artemias pueden participar también en la transmisión vía alimentaria del LCDV.

2.3. *Artemia* sp. como hospedador del virus de la enfermedad de linfocistis.

Artemia sp. es un crustáceo braquiópodo del orden Anostraca que constituye el alimento vivo más importante utilizado en acuicultura. Estudios previos realizados por nuestro grupo de investigación demostraron la presencia de partículas infectivas del LCDV en quistes comerciales de artemia, si bien pudo demostrarse que se trataba de una contaminación externa de los mismos, ya que no fue posible detectar el virus tras someterlos a un tratamiento de desinfección. Sin embargo, en los nauplios eclosionados a partir de estos quistes LCDV-positivos, al igual que los inoculados por baño con un aislado de LCDV procedente de dorada, fue posible la detección específica de genoma viral, localizándose este en el tracto digestivo de los nauplios. La detección del LCDV mediante ISH en nauplios de artemia infectadas por baño podría indicar que

estos actúan como bioacumuladores de patógenos desde el agua de cultivo. Además, como hemos indicado anteriormente, los metanauplios de artemia actúan como vector del LCDV, lo que implicaría la existencia de una transmisión horizontal del virus desde el agua a la cadena trófica. Sin embargo, estos resultados no permiten determinar si el artrópodo es sólo un vector mecánico o, por el contrario, es susceptible al LCDV. Por ello, en este apartado se realizaron infecciones experimentales en artemias (metanauplios, juveniles y adultos) con LCDV, analizándose en paralelo la carga y la expresión génica virales. Los resultados obtenidos sugieren que el LCDV es capaz de establecer una infección productiva en artemias, al menos en condiciones experimentales, si bien dicha infección cursa de forma subclínica. La carga viral (tanto en copias de DNA vírico como en título infectivo) en artemias es superior a la detectada en doradas asintomáticas infectadas por LCDV. Este es el primer caso descrito de un virus de peces que también infecta invertebrados, y amplía el rango de hospedador del LCDV a crustáceos.

INTRODUCTION

1. Current state of aquaculture of gilthead seabream (*Sparus aurata* L.)

Global production of fish from aquaculture has grown substantially in the past five decades, being one of the fastest-growing animal-food-producing sectors and it currently represents more than 66% of global food fish consumption (FAO, 2014). Aquaculture is oriented not only to quantitative production but also to improve the quality of products, and its success is based on the control of species reproduction, technological innovation applied to facilities, and the development of specific foods.

Most cultured marine fish species are of relatively high commercial value, sometimes because wild stocks are small or declining. Currently, the aquaculture production of some fish species is substantially higher than fish produced by capture fisheries. This is the case of gilthead seabream (*Sparus aurata* L.), traditionally cultured in Northern Italy, as well as in Southern Spain, that has become one of the main products of the European aquaculture (FAO, 2014).

The gilthead seabream, belonging to the family *Sparidae*, is a common inhabitant in the Mediterranean Sea and the Eastern coastal areas of the Atlantic Ocean from the United Kingdom to the Canary Islands. It is a euryhaline fish present in both marine and brackish water environments, such as coastal lagoons and estuarine zones, in particular during the initial stages of its life cycle. This fish is gastronomically very appreciated and it is marketed both fresh and frozen.

Large-scale production of gilthead seabream juveniles was definitively achieved in 1988-1989 in Spain, Italy and Greece. This species has demonstrated a high adaptability to intensive rearing conditions, both in ponds and cages, and its annual production increases regularly every year, with an estimated global production of about 173,024 tonnes in 2014 (APROMAR, 2015). At present, aquaculture production of gilthead seabream is recorded in more than 20 countries, with Greece, Turkey and Spain being the major producers. Its farming is also carried out in Egypt, Tunisia, Italy, Cyprus, Croatia, Malta, Israel, France and

Portugal, and there is an incipient production in Albania, Algeria, United Arab Emirates and Bosnia-Herzegovina. Considering only food fish, gilthead seabream is the third species in production volume (109,030 tonnes) in the European Union aquaculture, and the second one in market value at first sale (565 million Euro) after Atlantic salmon (APROMAR, 2015). Regarding Spanish aquaculture, it is the leading fish species with a production volume of 16,230 tonnes in 2014 (APROMAR, 2015).

Although gilthead seabream is one of the most important fish species in the European aquaculture, more efforts should be made for the adoption of responsible and sustainable aquaculture practices that guarantees a more efficient production system while also promotes the welfare of farmed fish (FAO, 2014). Infectious diseases are one of the most significant constraints to sustainable aquaculture production (Subasinghe *et al.*, 2001). Major disease problems affecting gilthead seabream farms are bacterial infections such as pasteurellosis (caused by *Photobacterium damselae* subsp. *piscicida*) and vibriosis (*Vibrio alginolyticus*, *V. anguillarum* or *Photobacterium damselae* subsp. *damselae*), and the lymphocystis disease, the most frequently reported viral infection in farms in the South Atlantic and Mediterranean areas (Borrego *et al.*, 2001; Colorni & Padros, 2011). This fish species is also susceptible to several other viruses such as aquareovirus, betanodavirus and birnavirus, but they are usually asymptomatic infections (Bandin *et al.*, 1995; Rodger *et al.*, 1997; Castric *et al.*, 2001; Cherif *et al.*, 2009).

2. Major viral diseases affecting farmed fish

Large-scale aquaculture has been associated with environmental and microbial concerns worldwide as a consequence of its intensive culture and high-stocking density. Fish culture production mainly follows two procedures: (i) integrated management of coastal zones, performed mainly in marine and brackish waters using extensive and semi-intensive procedures, and (ii) the intensive farming of highly valuable fish and shellfish species, performed mostly in tanks and cages. In recent years, modern aquaculture has been characterized

by a strong increase in production output because of the application of innovative technologies in fish farms and the optimization of management strategies.

The potential of fish farming has been compromised by the emergence of infectious pathologies. Among the infectious diseases reported, those of viral aetiology are more difficult to control, due to the high susceptibility of aquatic animals at an early age, the lack of therapeutics, and limited knowledge of both the pathogenesis of viral infections and the natural resistance mechanisms in aquatic animals. Moreover, virus can produce subclinical infections, and the asymptomatic host may shed viral particles into the environment, which can lead to rapid transmission of the disease in the production system (Castric *et al.*, 2001; Terlizzi *et al.*, 2012). Major viral diseases associated with significant losses in aquaculture, and the host fish affected, are presented in Table 1.

Viruses are, therefore, a limiting factor for the expansion of aquaculture because of the direct losses of fish production, costs derived from reduced productivity and disease management, and loss of export markets related to trade restrictions (Whittington & Chong, 2007; Renault, 2009; Rigos & Katharios, 2010).

Table 1. Major viral diseases in farmed fish (according to Essbauer & Ahne, 2001; Walker & Winton, 2010; Kibenge *et al.*, 2012).

Virus	Genome	Family	Main fish species affected
Infectious pancreatic necrosis virus (IPNV)	dsRNA	*Birnaviridae*	Salmonid fish, sea bass, turbot, Senegalese sole, redbanded seabream
European eel virus (EVE)	dsRNA	*Birnaviridae*	Eel
Piscine reovirus (PRV)	dsRNA	*Reoviridae*	Atlantic salmon, Atlantic herring, Atlantic horse mackerel
Coho salmon virus (CSRV)	dsRNA	*Reoviridae*	Coho salmon, chum salmon
Piscine myocarditis virus (PMCV)	dsRNA	*Totiviridae*	Atlantic salmon
Infectious haematopoietic necrosis virus (IHNV)	(-) ssRNA	*Rhabdoviridae*	Salmonid fish, brown trout, rainbow trout
Viral haemorrhagic septicemia virus (VHSV)	(-) ssRNA	*Rhabdoviridae*	Salmonid fish, sea bass, turbot
Spring viraemia of carp virus (SVCV)	(-) ssRNA	*Rhabdoviridae*	Common carp, koi, grass carp, bighead carp , silver carp, crucian carp
European eel virus X (EVEX)	(-) ssRNA	*Rhabdoviridae*	Eel
Snakehead rhabdovirus (SHVR)	(-) ssRNA	*Rhabdoviridae*	Snakehead
Hirame rhabdovirus (HIRRV)	(-) ssRNA	*Rhabdoviridae*	Hirame, black seabream, grayling
Infectious salmon anaemia virus (ISAV)	(-) ssRNA	*Orthomyxoviridae*	Atlantic salmon
Viral nervous necrosis virus (VNNV)	(+) ssRNA	*Nodaviridae*	Grouper, sea bass, flatfish, Senegalese sole, gilthead seabream
Salmon pancreas disease virus (SAV1)	(+) ssRNA	*Togaviridae*	Atlantic salmon
Sleeping disease virus (SAV2)	(+) ssRNA	*Togaviridae*	Rainbow trout
Norwegian salmonid alphavirus (SAV3)	(+) ssRNA	*Togaviridae*	Atlantic salmon, rainbow trout
RV puffer fish (RV-PF)	(RT) ssRNA	*Retroviridae*	Puffer fish

Table 1. Continued.

Virus	Genome	Family	Main fish species affected
European sheatfish virus (ESV)	dsDNA	*Iridoviridae*	Catfish
Red sea bream iridovirus (RSIV)	dsDNA	*Iridoviridae*	Red sea bream, yellowtail, sea bass, Japanese parrotfish
Infectious spleen and kidney necrosis virus (ISKNV)	dsDNA	*Iridoviridae*	Ornamental freshwater fishes
Sea bass iridovirus (SBIV)	dsDNA	*Iridoviridae*	Sea bass
Rock bream iridovirus (RBIV)	dsDNA	*Iridoviridae*	Rock seabream
Orange-spotted grouper iridovirus (OSGIV)	dsDNA	*Iridoviridae*	Grouper, African lampeye, dwarf gourami
White sturgeon iridovirus (WSIV)	dsDNA	*Iridoviridae*	Sturgeon
Grouper iridovirus (GIV/TGIV/SGGIV)	dsDNA	*Iridoviridae*	Brown-spotted grouper, nursing grouper, yellow grouper
Channel catfish virus (CCV)	dsDNA	*Iridoviridae*	Channel catfish
Lymphocystis disease virus (LCDV)	dsDNA	*Iridoviridae*	Flounder, plaice, dab, gilthead seabream, Senegalese sole
Cyprinid herpesvirus-1 (CyHV-1)	dsDNA	*Herpesviridae*	Carp fish
Cyprinid herpesvirus-2 (CyHV-2)	dsDNA	*Herpesviridae*	Goldfish
Cyprinid herpesvirus-3 (CyHV-3/KHDV)	dsDNA	*Herpesviridae*	Koi, common, and ghost carps
Anguillid herpesvirus-1	dsDNA	*Herpesviridae*	Eel
Cutthroat trout virus	dsDNA	*Herpesviridae*	Cutthroat trout

3. Lymphocystis disease virus

3.1. Taxonomy and genetic diversity

Lymphocystis disease virus (LCDV) belongs to the family *Iridoviridae*. Members of this family have been described as causal agents, causing high mortalities in a wide range of invertebrate and lower vertebrate animals (Chinchar, 2002). According to the 9th Report of the International Committee on Taxonomy of Viruses, the family *Iridoviridae* is subdivided into five genera: *Iridovirus*, *Chloriridovirus*, *Ranavirus*, *Lymphocystivirus* and *Megalocytivirus* (Fig. 1) (Jancovich *et al.*, 2012).

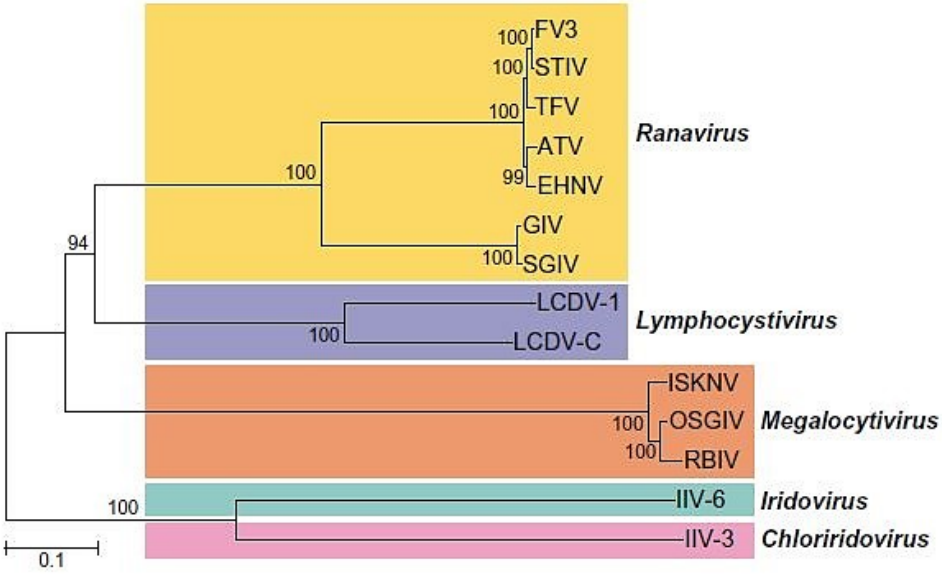


Figure 1. Concatenated phylogeny of 26 conserved ORFs sequences obtained from 14 full genome sequences of members of the family *Iridoviridae*. The neighbour-joining tree was obtained using MEGA4 (according to Jancovich *et al.*, 2012).

Three genera, *Lymphocystivirus*, *Megalocytivirus* and *Ranavirus*, have received attention due to the economic losses caused to the aquaculture industry (Chao *et al.*, 2002; Dong *et al.*, 2010; Whittington *et al.*, 2010; Kuttichantran *et al.*, 2012). Features that distinguish these genera are GC %

content, nucleotide sequence, cytopathological signs, the use of both host- and virus-encoded RNA polymerases and differences in the inferred amino acid sequence of key genes such as ATPase, methyltransferase or major capsid protein (MCP) (Sudthongkong *et al.*, 2002; Wang *et al.*, 2003; Do *et al.*, 2005b; Williams *et al.*, 2005; Kuttichantran *et al.*, 2012; Chinchar & Waltzek, 2014).

LCDV belongs to *Lymphocystivirus* genus, which includes one species *Lymphocystis disease virus 1* (LCDV-1) originally isolated from European flounder (*Platichthys flesus*) and European plaice (*Pleuronectes platessa*). In addition, three virus candidates are also included in this genus: LCDV-2, isolated from common dab (*Limanda limanda*); LCDV-C, isolated from Japanese flounder (*Paralichthys olivaceus*), and LCDV-RF, isolated from black rockfish (*Sebastes schlegelii*). LCDV-1 and related viruses are distinguished by host specificity, histopathology, viral protein profiles and DNA sequences (Jancovich *et al.*, 2012).

One of the distinctive features of the *Iridoviridae* family is the presence of a major capsid protein (MCP), which is the main structural component of the viral particles. MCP comprises 40-45% of the total viral polypeptides and has a molecular weight of approximately 50 kDa (Flügel, 1985). Phylogenetic inference of the iridoviral family is based on the relatively conserved MCP gene (Tidona *et al.*, 1998). Members of the same genus show more than 70% amino acid sequence identity within the MCP, whereas species from different genera show approximately 50% identity (Do *et al.*, 2005a; Williams *et al.*, 2005). On the basis of the MCP gene sequence, nine genotypes of *Lymphocystivirus* have been proposed to date (Fig. 2): LCDV-1 as genotype I; genotype II, consisting of Japanese flounder (LCDV-C) isolates; genotype III, which includes black rockfish (LCDV-RF) isolates; genotype IV, for cobia (*Rachycentron canadum*) and Japanese seabass (*Lateolabrax japonicus*) (LCDV-RC and LCDV-SB, respectively) isolates; genotype V includes painted glass fish (*Parambassis baculis*) (LCDV-CB) isolates; genotype VI for gourami (*Trichopodus leerii* and *T. trichopterus*) (LCDV-TL) isolates; genotype VII includes gilthead seabream and Senegalese sole (*Solea senegalensis*) (LCDV-SA and LCDV-SSE, respectively) isolates; genotype VIII for a largemouth bass (*Micropterus salmoides*) isolate (strain Leetown NFH); and genotype IX, including an American yellow perch (*Perca flavescens*) isolate (Kitamura *et al.*, 2006a;b; Hossain *et al.*, 2008; Kvitt *et al.*, 2008; Cano *et al.*, 2010;

Palmer *et al.*, 2012). The genetic diversity of LCDV has been related to the host fish species (Kitamura *et al.*, 2006a;b; Hossain *et al.*, 2008). However, when studying the evolutionary relationship of LCDV and its hosts, Yan *et al.* (2011) did not obtain significant evidence of co-speciation between LCDV genotypes and their host fish species.

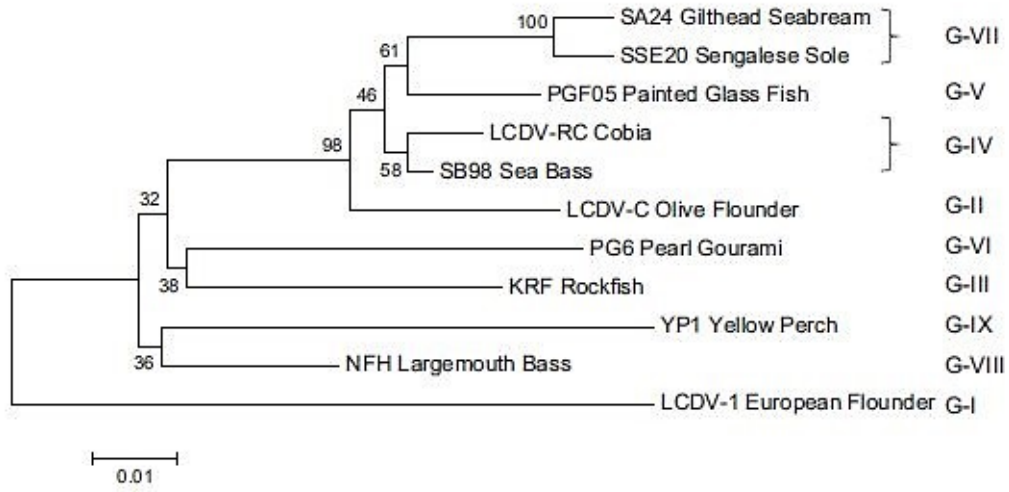


Figure 2. Phylogenetic tree showing the relationship between viral isolates belonging to the nine different LCDV genotypes. These relationships are based on the amino acid sequence of the major capsid protein (MCP) gene. The neighbour-joining tree was obtained using MEGA4 (modified from Cano *et al.*, 2010).

3.2. Virion structure

LCDV are large icosahedral viral particles that, depending on the host fish species, may vary in size from 120 to 340 nm in diameter (Tidona & Darai, 1999; Paperna *et al.*, 2001). The virus consists of a bilaminar capsid and a core that appears filamentous, displaying helicoidal symmetry (Madeley *et al.*, 1978; Samalecos, 1986; Heppell & Berthiaume, 1992) (Figs. 3 and 4). The core is surrounded by a membranous structure that is clearly demonstrated in decaying virus (Smail & Munro, 2001). Negative staining electron images of decaying viruses show that the outer electron-lucent layer of the capsid is composed of knobs, possibly attached to the inner capsid layer by a fringe of fibril-like external

protrusions of 2.5 nm in length (Jancovich *et al.*, 2012). The treatment of lymphocystis disease virions with papain before staining revealed a capsomer lattice structure, presumably because the papain removed the outer capsid (Samalecos, 1986).

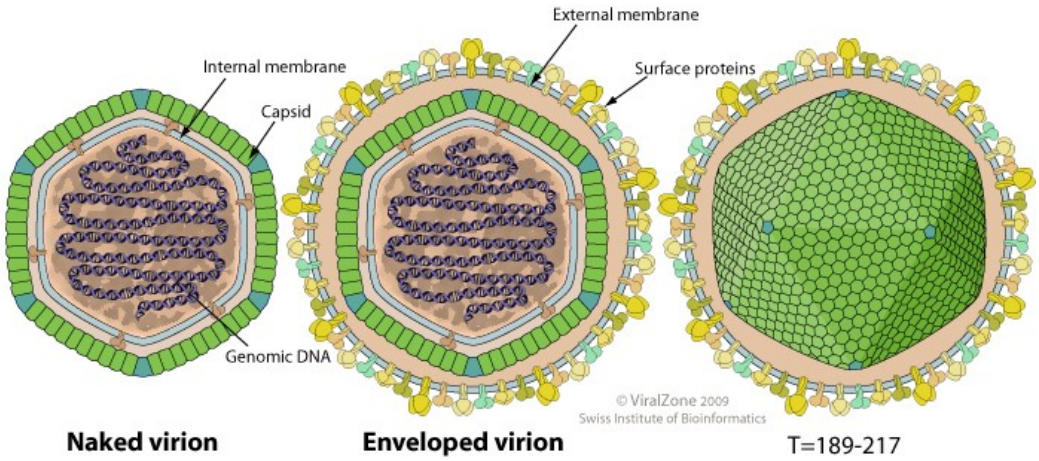


Figure 3. Diagram of the structure and organization of a virion of LCDV (obtained from ViralZone, Swiss Institute of Bioinformatics).

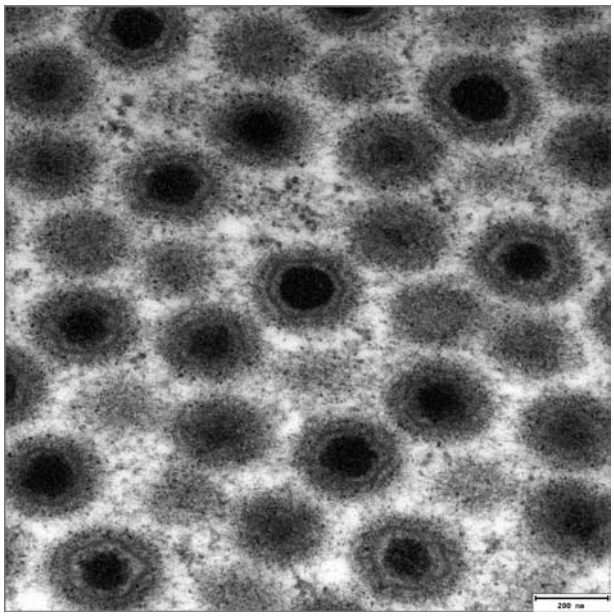


Figure 4. Transmission electron micrograph of LCDV particles in the cytoplasm of a lymphocystis cell in the caudal fin of *Sparus aurata*. Scale bar = 200 nm.

Virions are heat labile and can be inactivated by ether, glycerol, 5-iododeoxyuridine and UV-treatments (Wolf, 1988; Iwamoto *et al.*, 2002). Freezing-thawing cycles at -20°C may provoke a decrease in viral infectivity (Wolf, 1962). In contrast, the virions show stability to pH 6-9 and are resistant to ultrasonic treatment (Walker & Hill, 1980).

3.3. Chemical composition

Lymphocystis disease virions are composed of 42% proteins, 17% lipids and 1.6% nucleic acids, with sugars most likely representing a major portion of the remaining unidentified components (Robin *et al.*, 1983). SDS-PAGE analysis revealed the presence of 33 structural polypeptides, ranging from 4 to 220 kDa, in LCDV-1 virions isolated directly from fish tumours (Flügel *et al.*, 1982). However, purified virions obtained from other fish species showed a different electrophoretic pattern of 23 to 31 polypeptides ranging from 30 to 210 kDa (Robin *et al.*, 1984; Garcia-Rosado *et al.*, 2004). A common characteristic of all LCDV particles is the presence of a MCP of approximately 50 kDa composed of 459 amino acids, which represents up to 45% of the total protein content (Flügel *et al.*, 1982; Robin *et al.*, 1986; Heppell & Berthiaume, 1992). The MCP is one of the antigenic proteins identified in LCDV that immuno-reacted with Japanese flounder antisera from diseased fish and also from formalin-inactivated LCDV vaccinated fish (Jang *et al.*, 2011). The enzymatic activities associated with purified virions include a viral encoded ATP hydrolase, a protein kinase and a thymidine kinase (Flügel *et al.*, 1982; Darai *et al.*, 1983).

Several authors have reported the presence of carbohydrates in LCDV. Robin *et al.*, (1986) showed the presence of 10 glycoproteins in highly purified virus particles of an LCDV strain originally isolated from largemouth bass. In addition, Garcia-Rosado *et al.* (2004) reported the existence of 8 glycoproteins, with molecular weights ranging from 76 to 210 kDa, in viral particles isolated from gilthead seabream. Six of these glycoproteins presented a high content of mannose, and the other two contained a high proportion of sialic acid and N-acetylglucosamine, respectively.

Although LCDV is a non-enveloped particle, it may contain 5-17% lipids that are readily digested by a treatment with phospholipase that has been described for other iridoviruses (Robin *et al.*, 1983; Chinchar *et al.*, 2005). These phospholipids constitute an internal lipid membrane that lies between the DNA core and the viral capsid. The origin of the internal lipid membrane is unclear. The composition of the internal lipid membrane suggests that this membrane is not derived from host membranes but is rather produced *de novo*. However, it has been suggested that the internal lipid membrane is derived from fragments of the endoplasmic reticulum and plays a key role in virion assembly (Jancovich *et al.*, 2012).

The LCDV genome is a single lineal double-stranded DNA molecule of 102.6 kbp for LCDV-1 and 186.2 kbp for LCDV-C (Jancovich *et al.*, 2012). This genome is circularly permuted, terminally redundant, and heavily methylated (22%), with a GC content of 29.9% for LCDV-1 and 27.2% for LCDV-C (Darai *et al.*, 1983; Wagner *et al.*, 1985; Tidona & Darai, 1997a; Jancovich *et al.*, 2012). In addition, LCDV-1 DNA contains numerous short-direct, inverted and palindromic repetitive sequence elements (Schnitzler *et al.*, 1987; Schnitzler & Darai, 1989; Jancovich *et al.*, 2012).

3.4. Genome organization

Complete DNA sequences of LCDV-1 and LCDV-C have been determined. The former encoded 195 potential open reading frames (ORFs), whereas LCDV-C possesses 240 potential ORFs (Tidona & Darai, 1997b; Zhang *et al.*, 2004; Jancovich *et al.*, 2012). In LCDV-1, 108 largely non-overlapping ORFs are likely to represent viral genes, and 38 show significant homology to proteins related to virus replication and transcription, such as DNA polymerase (ORF 135R), DNA polymerase processing factor (ORF 003L), DNA-dependent RNA polymerases (ORF 016L, ORF 025L and ORF 171R), DNA methyltransferase (ORF 005L), methyl-sensitive restriction endonuclease with specificity for CCGG target sites (ORF 178L), structure-specific endonuclease (ORF 191R), DNA-dependent ATPase (ORF 054R), DNA puff protein homolog (ORF 108L), proteins homologous to an early transcription factor subunit (ORF 132L), late promoter transactivator

protein (ORF 032R), dsRNA-specific ribonuclease (ORF 137R), thymidine kinase (ORF 136R) and ribonucleoside-diphosphate reductases (ORF 027R and ORF 176L). In addition, other putative gene products showed significant homology to proteins involved in the virus-host interaction, including an insulin-like growth factor, a tumour necrosis factor receptor family, thioredoxin, cysteine proteinase, several protein kinases, a tissue differentiation factor, a collagen type IX homolog, b-hydroxy steroid dehydrogenase and ATPase, to name a few (ORFs 010L, 022R, 035L, 036R, 043R, 047L, 063L, 080R, 088R, 093R, 094R, 095L, 122R, 125R, 128L, 153L, 158L and 167L, respectively) (Flügel *et al.*, 1982; Koonin, 1993; Müller *et al.*, 1995; Tidona *et al.*, 1996; Tidona & Darai, 1997b; Sudthongkong *et al.*, 2002; Essbauer *et al.*, 2004; Kim & Lee, 2007; Pontejo *et al.*, 2013).

In the case of LCDV-C, Zhang *et al.* (2004) reported the presence of 240 potential ORFs and 176 non-overlapping putative viral genes. A search of the GenBank database using the 176 individual putative genes revealed 103 homologues to the corresponding ORFs of LCDV-1 and 73 potential genes that were not found in LCDV-1 or in other iridoviruses. Among these 73 genes, 8 genes contain coding sequences of conserved domains of cellular proteins, such as the caspase recruitment domain involved in apoptotic signalling (ORF 002L), thymidylate synthase (ORF 011L), the tumour necrosis factor receptor domain (ORF 016L), site-specific recombinase (ORF 047R), reverse transcriptase (ORF 051L), 7 transmembrane receptor (ORF 058L), the N-terminal domain of cell division protein 48 (ORF 209R) and collagen triple-helix repeat (ORF 216L). The remaining 67 novel genes do not show any significant homology with sequences in the public database.

Recently, López-Bueno *et al.* (2016), using 454/Roche GS-FLX Titanium sequencing system and Illumina assembled contigs, together with PCR and Sanger sequencing reactions, were able to obtain the full-length genome sequence of a LCDV isolate from gilthead seabream (LCDV-Sa). The genome is 208.5 kbp in length, significantly longer than that of the two LCDV previously sequenced, and it is therefore the longest known vertebrate iridovirus genome. The GC content of the LCDV-Sa genome (33%) is higher than those of LCDV-1 (29.1%) or LCDV-C (27.2%). Using PASC, these authors calculated that LCDV-Sa shared only 54.7% identity with LCDV-C or 38.9% identity with LCDV-1. In addition,

LCDV-Sa showed evidence of heavy genomic rearrangements as compared to LCDV-C, and an almost complete absence of co-linearity stretches with LCDV-1. Annotation of the full length LCDV-Sa genome indicated the presence of 183 putative ORFs encoding proteins larger than 30 amino acids. These included all 26 conserved iridovirus core genes (Eaton, 2007).

3.5. Viral multiplication

The replication mechanism of LCDV has not been investigated, but a model for frog virus 3 (FV-3), a member of the genus *Ranavirus*, has been proposed (Chinchar *et al.*, 2009; Jancovich *et al.*, 2012) (Fig. 5). The cellular receptor(s) for FV3 is unknown but viral entry is achieved by clathrin-mediated endocytosis. In the case of LCDV-C, a 27.8 kDa protein associated with beta-actin in the plasma membrane of flounder gill cells has been identified as the virus receptor (Wang *et al.*, 2011a). Following uncoating, viral cores enter the nucleus where first-stage DNA synthesis, and the synthesis of immediate early (IE) and delayed early (DE) viral transcripts, occurs. One or more virion-associated proteins act as transactivators and re-direct host RNA polymerase II to synthesize IE and DE viral mRNAs using the methylated viral genome as a template. The gene products encoded by the IE and DE viral transcripts include both regulatory and catalytic proteins. The viral DNA polymerase is involved in the first round of viral DNA synthesis.

The newly synthesized viral DNA may serve as the template for additional rounds of DNA replication and early transcription, or it may be transported to the cytoplasm where the second stage of viral DNA synthesis occurs. In the cytoplasm, viral DNA is replicated as large, branched concatemers that are processed to mature DNA during DNA packaging. Viral DNA methylation also occurs in the cytoplasm of the host cell; although its precise role is uncertain, it is hypothesised to protect viral DNA from endonucleolytic attack. The transcription of late (L) viral genes occurs in the cytoplasm, and full L gene transcription requires prior DNA synthesis. Homologues of the two largest subunits of RNA polymerase II are encoded by all iridoviruses. Whether this viral DNA-dependent RNA polymerase functions only in the cytoplasm to transcribe L viral genes or

whether it also plays a role in continued early transcription has not yet been determined.

Virion formation occurs in the cytoplasm within morphologically distinct areas named viral assembly sites. Within these assembly sites, concatemeric viral DNA is packaged into virions by a "headful" mechanism that results in the generation of circularly permuted- and terminally-redundant genomes, similar to those reported in the T-even Enterobacteria phages of the family *Myoviridae*. Following assembly, virions accumulate in the cytoplasm within large paracrystalline arrays or acquire an envelope by budding from the plasma membrane.

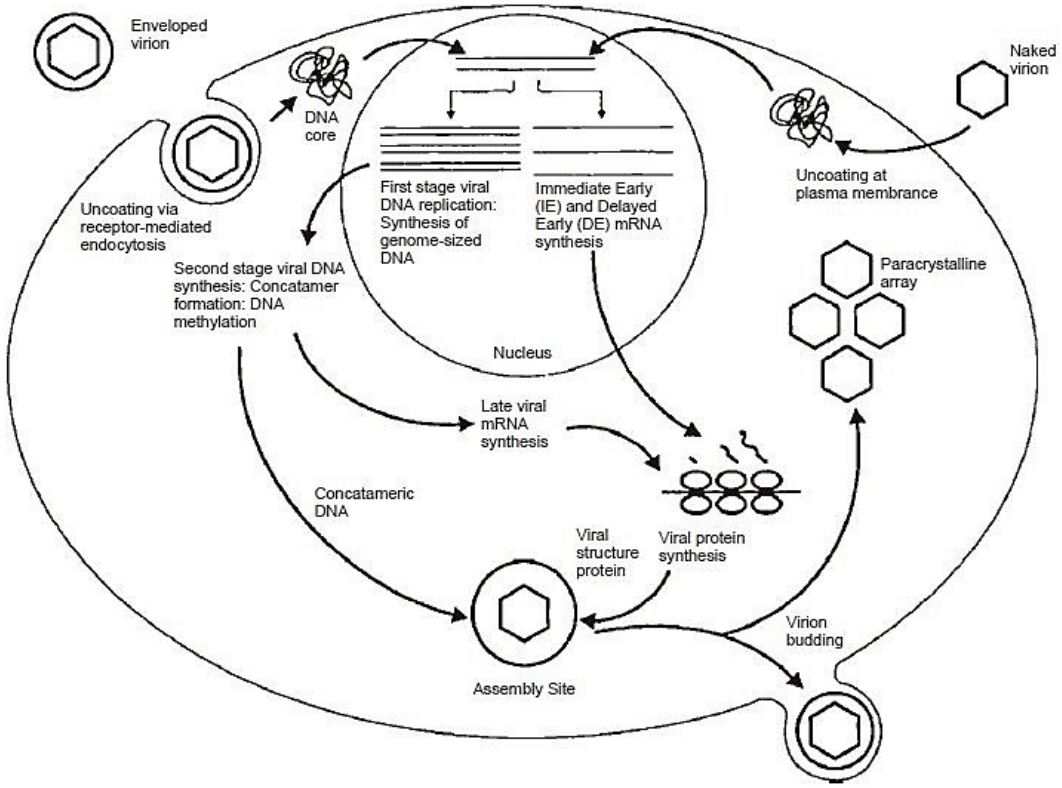


Figure 5. Iridovirus replication cycle. The multiplication cycle of frog virus 3 (FV3) is illustrated as proposed model for LCDV replication (based on Williams *et al.*, 2005).

4. Lymphocystis disease

Lymphocystis disease (LCD) is a well-known fish viral infection that is characterized by hypertrophy of fibroblastic cells in the dermis connective tissue of affected fish, occasionally proliferating as true epithelial tumours (Samalecos, 1986). This viral disease affects a wide variety of freshwater, brackish and marine fish species. LCD was one of the first fish viral diseases reported in the 19th century (Wolf, 1988), and its viral aetiology was demonstrated by electron microscopy by Walker (1962) and the subsequent virus isolation on BF-2 cell line by Wolf (1962). Although this disease is rarely fatal, fish showing the characteristic symptoms cannot be commercialized, causing important economic losses (Masoero *et al.*, 1986).

Lymphocystis disease (LCD) has been described in more than 150 species of fish from both marine and freshwater environments (Anders, 1989; Marcogliese *et al.*, 2001; Paperna *et al.*, 2001; Bunkley-Williams *et al.*, 2002; Sheng *et al.*, 2007a; Hossain *et al.*, 2008; Xu *et al.*, 2014; Huang *et al.*, 2015). The affected species belong to evolutionarily advanced orders of bony fish (teleosts), mainly including the families *Cichlidae*, *Osphronemidae*, *Centrarchidae*, *Gobiidae*, *Chaetodontidae*, *Pomacentridae*, *Sciaenidae*, *Serranidae* and *Pleuronectidae*. To date, LCD has not been reported in less-advanced fish orders, such as siluriformes, cyprinids and salmonids.

The disease is cosmopolitan, being widely distributed in all continents (Plumb, 1993). In Europe, LCD is an endemic disease in the North Sea and Mediterranean zones, affecting both wild and cultured fish species, such as European flounder, common dab, European plaice, grey gurnard (*Eutrigla gurnardus*), gilthead seabream, blackspot seabream (*Pagellus bogaraveo*) and Senegalese sole (Paperna *et al.*, 1982; Anders, 1989; Basurco *et al.*, 1990; Moate *et al.*, 1992; Garcia-Rosado *et al.*, 1999; Dethlefsen *et al.*, 2000; Alonso *et al.*, 2005). LCD is also a common fish disease in Asian aquaculture, particularly affecting Japanese flounder, black rockfish, cobia, Japanese seabass, Japanese amberjack (*Seriola quinqueradiata*) groupers (orange-spotted grouper, *Epinephelus coioides*, brown-marbled grouper, *E. fuscoguttatus*, and giant

grouper, *E. lanceolatus*), and red seabream (*Pagrus major*), as well as ornamental aquarium fish species (Matsusato, 1975; Tanaka *et al.*, 1984; Chen, 1996; Park & Sohn, 1996; Muroga, 1997; Chun, 1998; Xu *et al.*, 2000; Zhang, 2002; Xing *et al.*, 2006; Hossain *et al.*, 2008; Xu *et al.*, 2014; Huang *et al.*, 2015).

4.1. Disease features

The main characteristic of LCD is the appearance of small cream-coloured nodular lesions on the fish skin and fins (Colorni & Diamant, 1995; Sarasquete *et al.*, 1998) (Fig. 6). Each nodule consists of an LCDV-infected cell, named lymphocyst or lymphocystis cell, of up to 1 mm in diameter (Paperna *et al.*, 1982). These hypertrophied cells may occur singly or grouped in raspberry-like clusters of tumour appearance. These cellular aggregates are usually whitish in colour, but when they cover epithelial tissue that is rich in chromatophores, the chromatophores may render them greyish or darker (Wolf, 1988; Smail & Munro, 2001). In heavily affected fish, lymphocysts may cover the entire body, spreading from the gills to the fins (Paperna *et al.*, 1982; Flügel, 1985; Le Deuff & Renault, 1993; Xing *et al.*, 2006). Less frequently, they have also been described on eyes, causing exophthalmia, and internally over the mesenteries, peritoneum and several internal organs (Huizinga & Cosgrove, 1973; Russell, 1974; Dukes & Lawler, 1975; Howse *et al.*, 1977; Wolf, 1988; Colorni & Diamant, 1995; Xing *et al.*, 2006).

Diseased fish show low growth rates, which may be caused by the anaemia generally associated with this disease (Nishida *et al.*, 1998; Iwamoto *et al.*, 2002). Mortalities are typically limited to those individuals whose swimming, breathing, or feeding is severely impaired by particularly large and cumbersome growths of infected cells (Colorni & Padros, 2011). In fish farms, LCD outbreaks may favour secondary bacterial infections, cannibalism and/or parasitic infestations, factors that may increase mortality rates (Williams *et al.*, 2005; Colorni & Padros, 2011; Dezfuli *et al.*, 2012; Haddad-Boubaker *et al.*, 2013).

LCD is a chronic and self-limiting disease that, depending on the host fish species and environmental conditions, may persist for a variable period of time (Williams, 1996). Thus, the LCD-associated lesions may be evident for 1 year in

cold-water fish, whereas they disappear after several weeks in warm-water species (Paperna *et al.*, 1982; Gonzalez de Canales *et al.*, 1996).

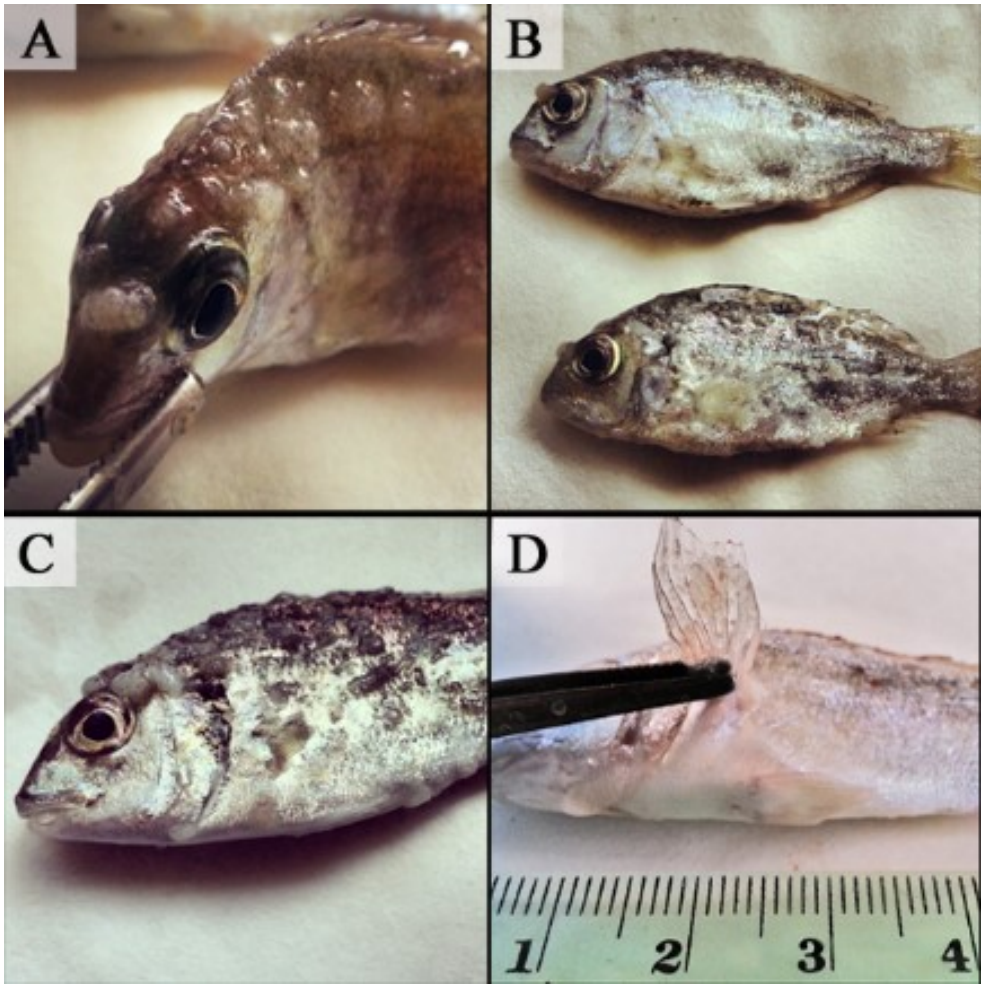


Figure 6. Specimens of juvenile gilthead seabream showing lymphocystis nodules grossly visible on fins a body.

LCDV infection has been described in bluegill (*Lepomis macrochirus*) (Dunbar & Wolf, 1966) and European plaice (Roberts, 1976). Although the time course for the development and regression of lymphocysts is quite different in both fish species (28 days at 25°C in bluegill compared to 3 months at 10°C in plaice), certain definitive stages can be recognized:

(i) Infected fibroblast-like cells cease dividing and increase their size. These hypertrophied cells show basophilic cytoplasms and develop prominent nuclei and nucleoli (Flügel, 1985).

(ii) As the cell enlarges, cytoplasmic inclusions surrounded by halo-like clear areas become evident. Electron microscopy studies revealed that these areas are viral factories (Spitzer *et al.*, 1982).

(iii) During maturation, a hyaline capsule becomes clearly demonstrable by haematoxylin-eosin staining (Peters & Schmidt, 1995). In gilthead seabream the hyaline capsule is composed of sulphate- and carboxyl-glycoproteins (Gonzalez de Canales *et al.*, 1996; Sarasquete *et al.*, 1998).

(iv) Finally, lymphocysts reach a degenerative phase in which nuclei and nucleoli appear condensed and poorly defined (Smail & Munro, 2001). Inclusions remain near the periphery, and the hyaline capsule degenerates. Macrophages and phagocytic cells appear around the degenerated lymphocysts and may invade them. Lymphocysts collapse may cause de novo infection of adjacent fibroblasts. Viral shedding appears to occur only after lysis of the lymphocysts.

4.2. Viral pathogenesis and host immunity

Data about LCDV pathogenesis are limited and are mainly restricted to histopathological studies of skin lesions (Gonzalez de Canales *et al.*, 1996; Sheng & Zhan, 2004; Sheng *et al.*, 2007b). More recently, immunohistochemistry (IHC), immunofluorescence (IF) or *in situ* hybridization (ISH) techniques have been used to study the course of the viral infection, as well as the LCDV tropism (Xing *et al.*, 2006; Sheng *et al.*, 2007b; Cano *et al.*, 2009a). These latter authors applied the IHC and ISH techniques for LCDV detection in gilthead seabream specimens from the same population. LCDV was detected in all tested organs (skin/fin, gills, intestine, liver, spleen and kidney), both in diseased and recovered fish. Fibroblasts, hepatocytes and macrophages appear to be target cells for virus replication. These results indicate that the virus establishes a systemic infection in gilthead seabream and persists in fish for an unknown period after the disappearance of clinical signs (Cano *et al.*, 2009a).

The DNA microarray technology is a useful tool for studying viral gene expression and allows the analysis of complex transcriptional profiles of large DNA viruses. In addition, this technique enables the characterization of host gene expression in response to viral infections, providing a better understanding of the underlying pathogenic mechanisms. The process of lymphocyst formation has been investigated in experimentally infected Japanese flounder, determining in parallel the viral genome amount and the changes in fish gene expression (Iwakiri *et al.*, 2014). The LCDV genome was first detected from the dorsal fins at 14 days post-infection (dpi), and the amount of viral genomes gradually increased in synchrony with lymphocyst development. The number of host genes that change their expression levels increased dramatically between 28 and 42 dpi. The results of the microarray analysis suggested that apoptosis inhibition, cell cycle arrest and alterations of collagen fibres may be implicated in lymphocyst formation in Japanese flounder fin cells.

The viral factors involved in lymphocyst formation are currently unknown. However, at least two viral gene products encoded by LCDV-C, a G protein-coupled receptor homolog (GPCR) and the thymidylate synthase (TS), can mediate cellular transformation *in vitro* when expressed in fish cell lines. GPCR inhibits cellular apoptosis in transient transfected fathead minnow (FHN) and epithelioma papulosum cyprini (EPC) cells (Huang *et al.*, 2007), whereas FHN cells constitutively expressing TS showed a transformed phenotype (Zhao *et al.*, 2008).

Little is known about immunity to LCDV, but there is the suggestion that by replicating primarily in the skin, LCDV is shielded from an antiviral response until late in infection (Chinchar *et al.*, 2009). The proliferation of macrophages and epithelioid cells around lymphocysts in the dermis has been described as an immune response in several fish species (Roberts, 1976; Colorni & Diamant, 1995; Sheng *et al.*, 2007b). Recently, Dezfuli *et al.* (2012) demonstrated that piscidin 3-expressing acidophilic granulocytes, but not mast cells, are recruited and activated in the dermis of gilthead seabream in response to LCDV infection. In addition, an enhanced phagocytic capability was observed in head kidney cells from American plaice (*Hippoglossoides platessoides*) suffering LCD (Marcogliese *et al.*, 2001).

In the Japanese flounder, the humoral immune response was monitored by antibody titration in sera from apparently healthy, lymphocystis diseased and recovered fish from an aquaculture facility, and the recovered animals showed the highest ELISA absorbance values (Nishida *et al.*, 1998). Lorenzen & Dixon (1991) reported an increase in seroprevalence and antibody titres against LCDV in wild European flounder populations. These findings suggest that fish can recover from LCD and develop acquired immunity.

There are also an increasing number of studies on the *in vivo* modulation of innate immunogenes expression after LCDV infection. These include the IRF-3 (interferon regulatory factor 3), IRF-7, IRF-8 and IRF-9 in Japanese flounder (Hu *et al.*, 2010; 2011; 2013; 2014), and the STAT2 (signal transducer and activator of transcription2) and STAT3 in turbot (*Scophthalmus maximus*) (Wang *et al.*, 2011b; 2013). All of them are up-regulated shortly after infection, which demonstrates their role in the antiviral response of the host. The anti-LCDV activity of two of the gilthead seabream Mx proteins (SauMx1 and SauMx2) also has been demonstrated *in vitro* by using three clonal populations of transfected CHSE-214, which stably express each of the SauMx proteins (Fernandez-Trujillo *et al.*, 2013).

The recent identification of a single major genetic locus controlling susceptibility to LCDV infection in Japanese flounder opens the way to selective breeding programs designed to develop flounder populations that are highly resistant to LCD (Fuji *et al.*, 2006; 2007). More recently, Hwang *et al.* (2011) demonstrated that TLR-2 (Toll-like receptor 2) mapped with the previously reported microsatellite marker that is associated with LCDV resistance, suggesting a relationship between host immune response and disease resistance.

4.3. Diagnostic methods

Although LCDV infections are generally benign and self-limiting, there are commercial concerns due to market rejection caused by the warty appearance of infected animals. For this reason, the development of rapid and sensitive diagnostic tools is very important for controlling the spread of this viral disease. Rapid diagnostic methods are required during the course of an outbreak,

whereas highly sensitive methods are required to detect subclinical viral infections in carrier fish (Sanz & Coll, 1992).

Classically, the diagnosis of LCDV has been based on the observation of disease symptoms. However, the development of rapid and specific diagnostic tools to control the viral dissemination in fish farms is highly advised because neither effective treatments nor commercially available vaccines currently exist. At present, the only feasible measures for disease prevention in aquaculture systems are general prophylactic practices, such as the exhaustive control of the fish to be introduced into the aquaculture system, the use of effective decontamination methods to prevent viral transmission from asymptomatic brood stock to larvae, and the supply of virus free-live food (Anders, 1989; Yoshimizu, 2009).

4.3.1. Virus isolation in cell cultures

The official method for diagnosis of fish viruses, established by the World Organisation for Animal Health (OIE), is based on virus isolation using susceptible cell lines and further confirmation by serological or molecular techniques (OIE, 2014). Although this procedure is widely accepted and currently used to validate other diagnostic methods, it is time consuming, and its effectiveness largely depends on the cell line used.

LCDV is not readily propagated in cell culture and showed a narrow host cell range; therefore, virus isolation usually requires homologous cell lines. The first report of LCDV isolation was from bluegill using a fry cell line (BF-2), although these viruses can also propagate onto a largemouth bass cell line (LBF-1) (Wolf *et al.*, 1966). Bejar *et al.* (1997) developed a fin-derived cell line from gilthead seabream (SAF-1) that supports the replication of LCDV isolated from this fish species (Perez-Prieto *et al.*, 1999), as well as from European flounder, common dab and European plaice (Alonso *et al.*, 2007). Some LCDV gilthead seabream isolates also replicate to a lesser extent on BF-2 cells (Garcia-Rosado *et al.*, 1999; Alonso *et al.*, 2007). The cytopathic effects (CPE) caused by LCDV on SAF-1 cells are similar to those reported on BF-2 cells, consisting in cellular rounding and the enlargement and presence of cytoplasmatic inclusions that become evident

after 3-10 days of incubation (Garcia-Rosado *et al.*, 1999). Iwamoto *et al.* (2002) tested 39 fish cell lines to propagate LCDV isolated from Japanese flounder, and only a homologous cell line (HINAE) exhibited CPE after 6-9 days of incubation. Similarly, Zhang *et al.* (2003) tested 13 fish cell lines for isolation of LCDV, recording viral replication only on two of them (GCO and GCK cell lines) derived from the grass carp (*Ctenopharyngodon idella*). Nevertheless, CPE characteristics induced by LCDV-C in these cell lines are markedly different from those previously described. LCDV-C can also be propagated onto the FG-9307 cell line, derived from Japanese flounder gill tissue, but in this case, the virus induced apoptotic cell death (Hu *et al.*, 2004).

In the years since 2010, several studies have been performed to obtain effective cell lines for LCDV *in vitro* propagation and vaccine development. Researchers at the Chinese Academy of Fisheries Sciences (Qingdao, China) have established at least seven cell lines that have proven to be susceptible to LCDV-C and turbot reddish body iridovirus (TRBIV). The cell lines derived from different fish species, such as turbot, half smooth tongue sole (*Cynoglossus semilaevis*), brown-marbled grouper and stone flounder (*Kareius bicoloratus*) (Wei *et al.*, 2009; Sha *et al.*, 2010; Wang *et al.*, 2010; Wei *et al.*, 2010; Xu *et al.*, 2011a; Zhang *et al.*, 2011; Zheng *et al.*, 2012).

4.3.2. Serological techniques

Serological techniques are usually performed for viral identification after virus isolation on cell culture or, alternatively, they can be used as diagnostic tools for direct viral detection in fish tissues (Sanz & Coll, 1992). These techniques can also be applied to determine the presence of antibodies against a particular pathogen in the serum of fish (Hattenberger-Baudouy *et al.*, 1995; LaPatra, 1996).

Several serological techniques, such as indirect immunofluorescence, flow cytometry and immunoblot, have been used for LCDV detection in cell culture (Garcia-Rosado *et al.*, 2002; Cano *et al.*, 2006). Among these, the immunoblot assay using an antiserum against a 60-kDa viral protein showed the highest sensitivity (32 ng/ml of purified LCDV), allowing the detection of viral antigens in

SAF-1 cells inoculated with 10 $TCID_{50}$/ml at 5 days post-inoculation (pi). Virus detection by CPE development in the same cells (recorded at 14 dpi) was only possible with inoculation titres above 10^2 $TCID_{50}$/ml. This immunoblot assay also proved to be effective for LCDV diagnosis in gilthead seabream tissue homogenates, from both diseased and asymptomatic fish, although a previous amplification step in cell culture was required for asymptomatic samples (Cano *et al.*, 2006).

Cheng *et al.* (2006) developed a panel of five monoclonal antibodies (MAb) to LCDV that have been applied for LCDV diagnosis in Japanese flounder using enzyme-linked immunosorbent assay (ELISA) and immunoblot (Xing *et al.*, 2006). More recently, these MAbs have been used for the development of two rapid detection tools: a gold immunochromatographic test strip (Sheng *et al.*, 2012a) and an antibody microarray (Sheng *et al.*, 2013). The sensitivity of both methods (1 and 0.55 µg/ml of purified LCDV, respectively) makes them suitable for detecting LCDV antigens in asymptomatic fish, as has been demonstrated by these authors. Moreover, the test strip allows on-site detection of LCDV without requiring specialized equipment or personnel.

To date, the detection of fish antibodies against a virus has not been accepted as a routine screening method for assessing the viral status of fish populations because of gaps in the knowledge on the serological response of fish to virus infections (LaPatra, 1996). However, antibody detection can be used at the population level as an indicator of previous exposure to the virus (Hattenberger-Baudouy *et al.*, 1995). The ELISA technique has been used to determine both the seroprevalence of the LCDV, as well as the determination of specific antibody titre, in several wild European flounder populations (Lorenzen & Dixon, 1991; Dixon *et al.*, 1996) and in cultured Japanese flounder (Nishida *et al.*, 1998).

4.3.3. PCR-based techniques

New sanitary regulations for fish have been implemented, which include sampling for the detection of asymptomatic LCDV carriers; therefore, it is necessary to develop molecular tools for diagnostic purposes (OIE, 2014).

Polymerase chain reaction (PCR) is a rapid, sensitive and highly specific technique for detecting iridoviral infections (Mao *et al.*, 1997; Grizzle *et al.*, 2003). In the case of LCDV, several PCR techniques based on the sequences of MCP coding genes have been developed in recent years. Using this technique, Cano *et al.* (2007) successfully detected LCDV from different marine fish species (European flounder, common dab, European plaice and gilthead seabream) collected from both Northern and Southern Europe. PCR combined with blot hybridization demonstrated to be adequate for virus detection in tissue homogenates of asymptomatic gilthead seabream carriers (Cano *et al.*, 2007; 2009a). Similar PCR-based assays have been developed by other authors to detect LCDV in other fish species, such as the Japanese flounder, black rockfish, turbot, redwing sea robin (*Lepidotrigla microptera*) and white-spotted puffer (*Arothrom hispidus*) (Xing *et al.*, 2006; Hossain *et al.*, 2007; Sheng *et al.*, 2007a; Zhan *et al.*, 2010).

The aforementioned studies have demonstrated the applicability of the PCR-based methods to detect LCDV in asymptomatic carriers, but they do not provide quantitative results that can be useful in epidemiological and pathological studies. Based on competitive PCR technology, Zan *et al.* (2007) established a semi-quantitative method for LCDV detection in Japanese flounder tissues. Real-time PCR is a powerful technique that has been used for the detection and quantification of several viral fish pathogens, including different iridoviruses (Wang *et al.*, 2006; Pallister *et al.*, 2007; Gias *et al.*, 2011), showing better sensitivity than conventional PCR. Regarding LCDV, Palmer *et al.* (2012) developed a real-time PCR assay using fluorogenic primers, which proved to be reliable in the detection and quantification of subclinical infected yellow perch. More recently, a new real-time PCR assay has been developed and applied for viral quantification in diseased and asymptomatic gilthead seabream (Ciulli *et al.*, 2015).

4.3.4. Loop-mediated isothermal amplification (LAMP)

LAMP is a technique in which DNA is quickly amplified under isothermal conditions with high specificity and sensitivity (Notomi *et al.* 2000). LAMP-

mediated diagnosis has been successfully used for the detection of viral pathogens in the aquaculture industry, including several iridoviruses (Caipang *et al.*, 2004; Mao *et al.*, 2008; Zhang *et al.*, 2009; Ding *et al.*, 2010; Sung *et al.*, 2010; Min *et al.*, 2013). Li *et al.* (2010) developed and evaluated a LAMP assay for the rapid detection of LCDV from both diseased and apparently healthy Japanese founders. The assay was found to be very specific because no cross-reactivity was obtained using other iridoviruses, and its detection limit was similar to that of real-time quantitative PCR. Due to LAMP amplifies under isothermal conditions (between 63 and 65 ºC) a thermal cycler is not required. In addition, LAMP products can be detected visually using several fluorescent dyes that bind to dsDNA, such as SYBR Green, calcein or ethidium bromide, or by the formation of a white precipitate, magnesium pyrophosphate, as a by-product of the amplification reaction. Therefore, LAMP can be widely used for viral diagnosis, particularly in resource-limited settings.

4.4. Lymphocistis disease virus transmission

Only a few studies have been conducted on the fate of LCDV outside the host and whether it is able to remain viable for an extended period of time in water or sediments. However, it is classically assumed that viral transmission occurs through the skin and gills of fish by direct contact or by waterborne exposure (Wolf, 1988; Bowser *et al.*, 1999; Kvitt *et al.*, 2008). Trauma of the skin via handling or netting, mating, parasitism and aggressive behaviour favour viral transmission among fish (Wolf, 1988; Plumb, 1993; Smail & Munro, 2001). Sheng *et al.* (2007b) and Cano *et al.* (2009a) reported the possible transmission of LCDV by feeding in aquaculture facilities.

Artemia nauplii have been considered as possible vector for the introduction of bacteria, viruses and protozoa into aquaculture facilities for decades, mainly because of its ability to filter and accumulate particles from aquatic environment, making this crustacean a potential candidate for the transmission of several pathogenic microorganisms. According to some authors, *Artemia* could act as reservoir or mechanical vector of pathogenic bacteria, such as *Bacillus*, *Erwinia*, *Micrococcus*, *Staphylococcus* and *Vibrio* (Austin *et al.*,

1982; Tatani *et al.*, 1985; Muroga *et al.*, 1987; Nicolas *et al.*, 1989). Mortensen *et al.* (1993) highlighted the potential role of *Artemia* as a vector for infectious pancreatic necrosis virus (IPNV) in larvae of turbot, while Skliris *et al.* (1998) concluded that both rotifers and *Artemia* could act as mechanical vector for viral nervous necrosis virus (VNNV). Previous studies by Cano *et al.* (2009b) showed the presence of infectious LCDV in commercial *Artemia* cysts, although it was an external contamination, since the virus could not be detected after a decapsulation treatment. Viral genome and antigens were detected in the digestive tract of nauplii hatched from contaminated cysts, but not at the umbrella and instar I stages. This might indicate that nauplii can accumulate virus from water contaminated with LCDV particles derived from breaking cyst. Moreover, nauplii also became LCDV-contaminated after bath challenge. These results suggest that *Artemia* should be consider a key factor in the introduction of LCDV in fish hatcheries, and therefore, it is critical to perform further studies to establish its role in the transmission of LCDV to cultured fish.

In aquaculture facilities, a high percentage of the fish population could be infected by LCDV, likely reflecting the ease of horizontal transmission, and viral infection incidences up to 70% have been described (Paperna *et al.*, 1982; Sano, 1988; Matsuoka, 1995; Xing *et al.*, 2006). The prevalence of LCD is affected by fish density, human manipulation, low salinity, water temperature, reduced oxygen conditions, nutritional deficiencies and chemical and biological water pollution (Paperna *et al.*, 1982; Bowser *et al.*, 1988; Berthiaume *et al.*, 1993; Sindermann, 1996; Vethaak & Jol, 1996; Mellergaard & Nielsen, 1997; Austin, 1999; Bowser *et al.*, 1999; Grygiel, 1999; Kitamura *et al.*, 2007). Hossain *et al.* (2009) demonstrated the importance of temperature on the persistence of LCDV in Japanese flounder epidermal tissues. These authors found that lymphocystis cells appeared on the skin and fins at 35 days post-challenge at 20°C, but no clinical signs were observed in the fish reared at 10° and 30°C, although LCDV could be detected by PCR. They concluded that at low temperatures, LCDV is able to persist over a long period of time in the fish epidermis, producing a subclinical infection.

4.5. Disease control and prevention

Viral disease prevention and control rely on the application of specific prophylactic measures (i.e., vaccination), or, alternatively, on the use of general control strategies, such as improved husbandry and water quality, better nutrition and lower stocking densities (OIE, 2014).

Control of LCDV in intensive culture operations would demand scrupulous disinfection procedures at all stages of production, screening and quarantine of each fish lot to be introduced, and treatment of raw seawater used in the fish facility (Bowden *et al.*, 1995). However, only a few studies have been performed on physical and chemical treatments against LCDV. Havikrishnan *et al.* (2010c) used a bath treatment with formalin, hydrogen peroxide and Jenoclean for LCDV-infected Japanese flounder. The authors concluded that these chemical agents enhanced the fish innate immune response and increased the fish resistance to the disease. However, these treatments cannot be systematically applied in aquaculture practice. For this reason, these authors evaluated the effect of herbal extracts and probiotics added to the fish diet in the course of LCDV infection in Japanese flounder, concluding that they act as immunostimulants that reduce the incidence of LCD (Havikrishnan *et al.*, 2010a;b).

Although there is no commercial vaccine available for LCDV infection, both inactivated and genetically engineered vaccines targeting LCDV have been designed and evaluated in recent years. Formalin- and heat-inactivated LCDV were used as vaccines, and proved to have a protective effect in Japanese flounder (Yoshimizu & Iwamoto, 2001; Xu *et al.*, 2011b). Nevertheless, its use is hampered by the necessity to obtain large amounts of purified virus particles directly from diseased fish lesions.

DNA vaccination is based on the administration of plasmid DNA (pDNA) encoding a protective antigen, rather than the antigen itself. The subsequent expression of the antigen by cells in the vaccinated hosts triggers the host immune response. A single intramuscular injection of low amounts of DNA induces rapid and long protection in fish against economically important viruses affecting aquaculture production (Lorenzen & LaPatra, 2005). Zheng *et al.* (2006)

designed a DNA vaccine against LCDV composed of a plasmid containing a 0.6-kbp fragment of the MCP gene of LCDV-C. The expression of several immune-related genes significantly increased after vaccination, and specific anti-LCDV immunoglobulins were also detected in the sera of vaccinated fish (Zheng *et al.*, 2010). In addition, this vaccine induced effective protection against LCD in Japanese flounder after intramuscular injection (Zheng *et al.*, 2011).

Oral DNA-based immunotherapy is a new strategy for fish immunization in intensive culture. However, the rate of degradation of DNA vaccines by nucleases and acidic conditions in the fish gastrointestinal tract may reduce vaccine efficiency. To avoid this, the vaccine DNA can be delivered encapsulated in micro- or nanoparticles that prevent its degradation. Microspheres of alginate, chitosan, and poly/DL-lactide-co-glycolide (PLGA) were tested by Tian *et al.* (2008a;b;c) for oral delivery of the LCDV pDNA vaccine cited above. Following immunization, the authors detected transgene expression in several organs from fish vaccinated with encapsulated pDNA. The encapsulated vaccine also induced higher levels of antibodies compared to control fish vaccinated with naked pDNA. Later, Tian & Yu (2011) demonstrated a significant increase in resistance to LCDV infection after oral administration of the pDNA vaccine encapsulated into PLGA nanoparticles.

5. *Artemia* as live food in aquaculture

Fish larval rearing is generally carried out under controlled hatchery conditions and requires specific culture techniques. The incomplete development of the larval digestive system during the early first-feeding has become one of the major bottlenecks preventing the successful production of many farmed fish. Usually, formulated feeds do not fulfil all the nutritional requirements and, therefore, result in poor growth and low survival of fish larvae. However, live food organisms seem to provide essential nutrient during larviculture, such as fatty acids, free amino acids, vitamin C and carotenoids. In addition, live food has a triggering effect by their continuous movement, allowing an enhanced perception, while the swimming activity assures a good

distribution of food items in the water column, facilitating more frequent encounters with the developing larvae which in most cases have a low mobility (Lavens & Sorgeloos, 1996).

Brine shrimps of the genera *Artemia* (Crustacea, Branchiopoda, Anostraca) constitute the most important live food for aquaculture (Sorgeloos *et al.*, 1986). Easy management, development characteristics, small size and high nutritional value, makes them suitable for both larvae and juvenile fish culture (Tizol, 1994). At present, it is the best suitable live food and, in many cases, the only one appropriate for many aquatic species in their early stages of life. They are usually supplied as *Artemia* nauplii or metanauplii, depending on the fish size at feeding.

5.1. Morphology and life cycle

In its natural environment, under determined stressful conditions, *Artemia* produces cysts that are metabolically inactive and do not further develop as long as they are kept dry. Artemia cyst consisted mainly of three external structures (Fig. 7) that enclosed the resting embryo (Morris & Afzelius, 1967; FAO, 1986): (i) Corium or alveolar layer: the external envelope of the cyst composed of lipoproteins impregnated with chitin and haematin. The haematin concentration determines the colour of the shell. Its main function is to provide protection for the embryo against mechanical disruption and UV radiation. (ii) Outer cuticular membrane: multilayer membrane that acts as a permeability barrier and protects the embryo from penetration by molecules larger than the CO_2 molecule. (iii) Embryonic cuticle: a transparent and highly elastic layer separated from the embryo by the inner cuticular membrane (this becomes the hatching membrane during hatching incubation).

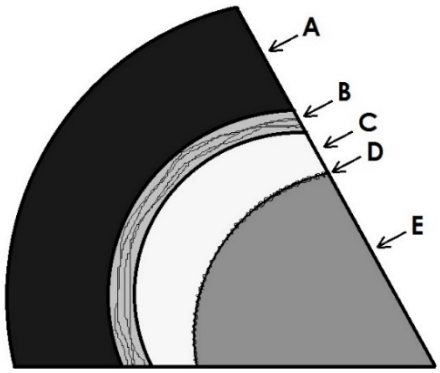


Figure 7. Diagram showing the structure of *Artemia* cyst. (A) Corium or alveolar layer; (B) outer cuticular membrane; (C) embryonic cuticle; (D) inner cuticular membrane; (E) embryo.

Upon immersion in seawater, the biconcave-shaped cysts hydrate and become spherical (Fig. 8), and inside the shell, the embryo resumes its interrupted metabolism. Approximately 20 h later, the outer membrane bursts (this is called "breaking" stage) and the embryo appears, surrounded by the hatching membrane. While the embryo hangs underneath the empty shell ("umbrella" stage), the development of the nauplius is completed and within a short period of time the hatching membrane is ruptured ("hatching") and the free-swimming nauplius is released (FAO, 1986).

Figure 8. *Artemia* cysts dehydrated (A), hydrated (B), and decapsulated (C).

The first naupliar stage (also named instar I) has an average size of 400 to 500 µm in length, is orange-brown (for accumulation of yolk reserves) and has three pairs of appendages: two pairs of antennae and jaws. In this first stage, the digestive system is not yet functional, since the mouth and anus still remain closed. About 24 h later, a second moult occurs and the nauplius goes to a second larval stage, named instar II, which has a fully functional digestive system and is now able to eat small food particles between 1 and 40 µm. Brine shrimps grow and develop by moulting, going through a series of 14 to 17 different stages (nauplii, metanauplii and juveniles) in which major changes, both morphological and functional, occur.

Adult *Artemia* (10-12 mm in length) has an elongated body with two stalked complex eyes, a linear digestive tract, sensorial antennulae and 11 pairs of functional thoracopods. The male has a paired penis in the posterior part of the trunk region and two hooked graspers in the head. The female can easily be recognized by the brood sac or uterus situated just behind the 11th pair of thoracopods (Fig. 9).

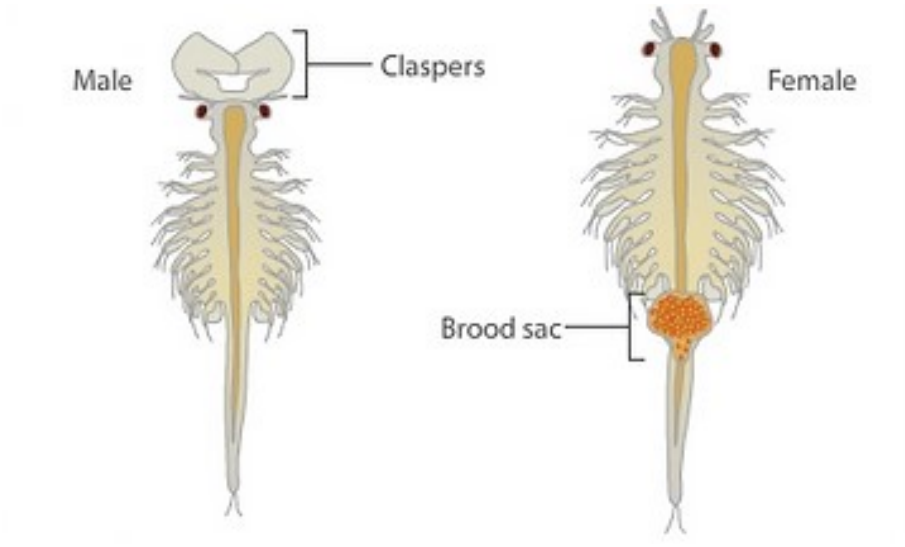


Figure 9. Schematic representation of adult brine shrimps. Female are slightly larger than males and their brood sac are easy visible to the naked eye (adapted from http://learn.genetics.utah.edu).

Eggs develop in two tubular ovaries in the abdomen and migrate via two oviducts into the uterus. Fertilized eggs usually develop into free-swimming nauplii (ovoviviparous reproduction) that are released by the mother. In extreme conditions (e.g., high salinity, low oxygen levels), the embryos only develop up to the gastrula stage. At this moment, they get surrounded by a thick shell (secreted by the brown shell glands located in the uterus), enter a state of metabolic cessation or dormancy (diapause), and are then released by the female as cysts (oviparous reproduction). The cysts usually float in the high salinity waters and are blown ashore where they accumulate and dry. Cysts are in a state of quiescence and can resume their further embryonic development when hydrated in optimal hatching conditions (FAO, 1986; Polanco, 2000).

OBJETIVOS

Los principales objetivos de la presente Tesis doctoral han sido el estudio de la patogénesis del virus de la enfermedad de linfocistis (LCDV) en dorada (*Sparus aurata* L.) y el establecimiento de las rutas de transmisión de este virus en larvas y alevines de esta especie. Con el fin de alcanzar estos objetivos, se diseñaron y evaluaron técnicas moleculares para determinar los órganos diana del LCDV en el hospedador. Estas herramientas se aplicaron también a la identificación de reservorios del virus en las instalaciones de cultivo larvario de dorada, y al estudio de su implicación en la transmisión vírica. Por último, se planteó establecer si la artemia es susceptible a la infección por LCDV.

Por lo tanto, los objetivos parciales de este estudio son:

1. Diseño, evaluación y aplicación de técnicas moleculares para la detección y cuantificación del LCDV.

2. Determinación de los órganos diana para la multiplicación del LCDV en alevines de dorada y estudio histopatológico de los animales infectados.

3. Estudio de las vías de transmisión del LCDV a larvas y alevines de dorada.

4. Determinación de la susceptibilidad de *Artemia* frente a la infección por LCDV.

AIMS

The main aims of this PhD Thesis include studying the pathogenesis of lymphocystis disease virus (LCDV) in gilthead seabream (*Sparus aurata* L.), and determining its transmission routes to larvae and fingerlings of this fish species. In order to achieve these aims, molecular techniques will be designed and evaluated to identify target organs for viral replication in gilthead seabream. These tools will also be used to identify viral reservoirs in farm facilities, and to study their role in viral transmission. Finally, the question of whether the brine shrimp *Artemia* is a susceptible host for LCDV infection will be investigated.

Therefore, the objectives of this thesis are to:

1. Design, evaluate and apply of molecular techniques for the detection and quantification of LCDV.

2. Identify the target organs for LCDV replication in gilthead seabream and perform histopathological studies on infected fish.

3. Evaluate routes of LCDV transmission in cultured gilthead seabream larvae and fingerlings.

4. Determine the susceptibility of *Artemia* to LCDV infection.

CHAPTER 1:

DIAGNOSTIC METHODS FOR LYMPHOCYSTIS DISEASE VIRUS

1. Real-time PCR assay for lymphocystis disease virus genotype VII detection and quantification

1.1. INTRODUCTION

The only adequate measure for LCD prevention in the aquaculture systems is the use of general prophylactic practices, such as good husbandry practices, reduced stocking density and the virological control of fish to be introduced in the farm facilities in order to detect carrier fish (Anders, 1989). These animals may pose a risk for the introduction of LCDV in fish farms, as direct contact between fish specimens is considered the main route of LCDV spreading (Wolf, 1988). Moreover, asymptomatic carrier breeders may also be involved in LCDV transmission to fish larvae (Chapter 2, section 2). The detection of subclinical viral infections in carrier fish requires the use of sensitive diagnostic methods (Sanz *et al.*, 1992). In this context, PCR-based methods have proved to be adequate for LCDV detection in apparently healthy fish (Cano *et al.*, 2007; Zan *et al.*, 2007; Kvitt *et al.*, 2008; Hossain *et al.*, 2009; Haddad-Boubaker *et al.*, 2013). The PCR-hybridization assay developed by Cano *et al.* (2007) not only allowed the detection of LCDV in carrier gilthead seabream, but also in rotifer and artemia used as live food for larval stages, which makes it a valuable tool for the detection of other potential LCDV foci in fish farms (Cano *et al.*, 2009). Nevertheless, this assay is relatively time-consuming, not readily applied to screening large sample numbers, and does not provide quantitative results, that can be useful in epidemiological and pathological studies of LCDV.

Real-time PCR (qPCR) has been used for the detection and quantification of numerous viral fish pathogens, including LCDV in yellow perch (Palmer *et al.*, 2012), and has been proved to be useful to overcome the disadvantages of conventional PCR above mentioned (Pallister *et al.*, 2007; Pepin *et al.*, 2008; Cutrín *et al.*, 2009). Recently, a qPCR assay has been developed and applied for the detection and quantification of LCDV in a low number of samples of diseased and recovered gilthead seabream. Nevertheless, the use of the assay

for LCDV monitoring in different stages of the fish production cycle was not investigated (Ciulli *et al.*, 2015).

The objective of this study was to establish the applicability of a new qPCR assay for LCDV genotype VII diagnosis in surveillance studies. In addition, this assay has been evaluated using samples from a gilthead seabream hatchery, in order to demonstrate its utility to detect several sources of LCDV in the fish farm.

1.2. MATERIALS & METHODS

1.2.1. Sample collection and DNA extraction

Juvenile gilthead seabream specimens were sampled at four aquaculture farms. In two of these farms (farms A and B), a total of 11 diseased and 25 asymptomatic (i.e., without signs of LCD) fish were collected during an outbreak of LCD, whereas in the other two (farms C and D), only asymptomatic fish were observed, and 24 of them were collected. Juvenile fish were euthanized by anaesthetic overdose (MS-222) (Sigma-Aldrich) before sampling. Samples of caudal fin (approximately 1 cm^2 in size) were aseptically cut off, frozen immediately at -20 °C in dry ice and sent to the lab. In addition, 9 gilthead seabream samples were collected at the hatchery in another farm suffering LCD outbreaks over several years (farm E). Samples consisted of fertilized eggs (one sample of 100 mg), larvae (4 pools of 10–15 animals), and fingerlings (up to 2 g of weight) (4 pools of 5–10 animals). No LCD clinical signs were observed in any of the sampled fingerlings. Larvae and fingerlings were also euthanized by anaesthetic overdose.

One sample of each rotifers (100 mg), commercial artemia cysts (100 mg), decapsulated artemia cysts (100 mg) and artemia metanauplii (100 mg) used as live food for larvae were also collected. These samples were washed with sterile seawater, gently dried and fresh frozen for shipment to the lab. In this farm, 50 broodstock were also analysed. These animals neither showed symptoms of LCD nor had LCD history, as stated by the farm records. For sampling purposes, these specimens were anaesthetized with MS-222 in seawater at a final concentration

of 30 mg/ml. For each specimen, samples of caudal fin and blood were obtained. Blood samples were collected from the branchial arches using S-Monovette 4.5 ml LH (Sarstedt), chilled to 4 °C and sent to the lab for analysis within 24 h, whereas samples of caudal fin were obtained and stored as described above.

In each aquaculture farm, specialized personnel carried out the sampling procedures described. Fish used in this study have been treated in compliance with the Spanish legislation (RD 53/2013, BOE no. 34).

Samples were homogenized in Leibovitz's L-15 medium (Gibco) (10%, w/v) as described previously (Alonso *et al.*, 2005), except samples of eggs, rotifers and artemia, which were ground in liquid nitrogen. Total DNA was extracted from 200 µl of tissue homogenates or 50 mg of tissue powder using the QIAamp DNA Minikit (Qiagen) according to the manufacturer's instructions. Finally, DNA was extracted from 200 µl of heparinised blood using the ReliaPrep Blood gDNA Miniprep System (Promega). In all cases, DNA was eluted in a final volume of 100 µl and stored at -20 °C until use as template for PCR-hybridization and qPCR. Prior to PCR assays, purified DNA was quantified spectrophotometrically using a NanoDrop 1000 spectrophotometer (Thermo Scientific), and DNA was diluted to achieve a final concentration of 20 ng/µl.

1.2.2. PCR-hybridization

LCDV genome was detected using the PCR-hybridization assay described by Cano *et al.* (2007). Briefly, a 270-bp fragment of the MCP gene of LCDV was amplified by PCR. PCR products were denatured and blotted onto a Hybond-N nylon membrane (GE Healthcare), and hybridization was carried out using an LCDV-specific digoxigenin (DIG)-labelled probe and the chemiluminescent substrate CSPD (Roche).

1.2.3. Cloning a fragment of MCP gene

LCDV isolate SA9 was used as source of viral DNA (Cano *et al.*, 2006). A fragment of the viral MCP gene was amplified by PCR using the primers LCDVs-F and LCDVs-R described by Kitamura *et al.* (2006b). PCR was performed in a 50-µl reaction volume containing 10 µl of 5X Colorless GoTaq Flexi Buffer (Promega), 3 mM MgCl2 (Promega), 5 µl of 0.2 mM dNTP (Roche), 1.25 U of GoTaq DNA polymerase (Promega), and 2 µl of each primer at 15 pmol/µl. DNA was amplified by the use of one denaturation step at 95 °C for 5 min, followed by 35 cycles of denaturation (95 °C for 1 min), annealing (50 °C for 30 s), and extension (72 °C for 1 min), and a final extension step at 72 °C for 10 min. PCR products were run in 2% agarose gels, purified using the High-Pure PCR Product Purification kit (Roche), and cloned into the pCR4-TOPO vector (TOPO TA cloning kit) (Invitrogen) for subsequent transformation in *Escherichia coli* (One Shot competent cells, Invitrogen) following manufacturer's instructions. Recombinant plasmid DNA was purified from *E. coli* cells with a commercial kit (High Pure Plasmid Isolation kit, Roche), and insert size was verified by PCR and sequencing, using the M13 primers provided in the cloning kit. The cloned MCP gene fragment was 609 bp in length, corresponding with nucleotide positions 99 to 707 of the LCDV SA9 MCP gene (GenBank accession no. GU320728).

1.2.4. Real-time PCR assay

Primers for qPCR (RT-LCDV-F: 5′-ACGTTTCTCGAGGCGGAGAT-3′, and RT-LCDV-R: 5′-CGGACGTTTGCTTGACCAA-3′) were designed to target the MCP gene of LCDV genotype VII, using Primer Express Software v3.0 (Applied Biosystems). This primer set generates a 150-bp amplicon within the 609-bp cloned fragment of MCP gene (nucleotide positions 173 to 322 of the LCDV SA9 MCP gene).

Real-time PCR reactions were carried out in 96-well plates (Applied Biosystems), in a final volume of 50 µl containing 25 µl of 2x Power SYBR Green PCR Master Mix (Applied Biosystems), 3 µl of each primer at 15 pmol/µl, and 10 µl of DNA. PCR amplifications were performed in a 7500 Real-Time PCR System

(Applied Biosystems). The thermal profile was: 50 °C for 2 min; 95 °C for 10 min, and 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Finally, dissociation curve analysis was carried out automatically in order to allow detection of non-specific amplification products.

1.2.5. Standard curve for LCDV quantification

To quantify the amount of viral DNA in different samples, a standard curve was generated using the recombinant plasmid described above. The concentration of the purified plasmid was determined by spectrophotometry as described above, and the plasmid stock was diluted to serve as template for qPCR (10-fold serial dilutions ranging from 10^6 to 10^2 copies, and then two-fold dilutions from 50 to 25 copies, and from 16 copies to 1 copy).

The sensitivity of the qPCR assay was also determined in terms of infectious viral particles. LCDV SA9 stock was titrated in SAF-1 cells, using the 50% cell culture infectious dose ($TCID_{50}$) endpoint dilution assay as described previously (Alonso *et al.*, 2005). An aliquot of 1 ml of the viral stock with a titre adjusted to 1 x 10^6 $TCID_{50}$/ml was subjected to a 10-fold serial dilution in Leibovitz's L-15 medium. The DNA of each dilution was extracted as previously specified, and used for qPCR. The amount of infective virus analysed per reaction was 1 x 10^5 to 1 x 10^0 $TCID_{50}$.

Milli-Q water and DNA from one LCDV-negative gilthead seabream sample (Cano *et al.*, 2007) were included within each qPCR run as no-template and negative controls, respectively. In each 96-well, plasmid dilutions for the standard curve were run along with the samples and controls, using three technical replicates. The number of copies of LCDV DNA in each well was calculated from its cycle threshold (Ct) value by interpolation in the standard curve (C_t versus log copy number), using the SDS Software v1.3 (Applied Biosystem). The amplification efficiency (E) was calculated from standard curves using the formula $E = (10^{-1/S} - 1) \times 100$ (S being the slope of the linear fit). Viral loads in samples were calculated as the mean of the three replicates, and expressed as viral DNA copies per milligram of tissue (per µl in blood samples).

1.2.6. Assay repeatability and reproducibility

To evaluate the precision of the qPCR, the intra- and inter-assay variability was determined using the recombinant plasmid. To assess intra-assay variation, four plasmid DNA dilution series (from 10^5 to 2 copies per reaction) were prepared and tested simultaneously in the same plate. Four separate PCR runs were carried out to assess inter-assay variation, using also seven dilutions of plasmid DNA. The mean, standard deviation (SD) and coefficient of variation (CV) were calculated independently for each DNA dilution.

1.3. RESULTS

1.3.1. Evaluation of the real-time PCR assay

Specificity of the qPCR was determined by analysis of the dissociation curves generated in each experiment. Standard and positive samples gave a single PCR product with a melting temperature of 77.7 ± 0.5 °C, which correspond with that deduced from the sequence of the expected fragment. The size of amplicons was monitored by agarose gel electrophoresis, and bands were observed at the expected size (150 bp).

The linear dynamic range, efficiency and precision of the qPCR assay were evaluated using a recombinant plasmid containing a 609-bp fragment of the LCDV MCP gene. Standard curves generated from four independent assays demonstrated a linear relationship between the amount of plasmid DNA and C_t values over a wide range of concentration, from 10^6 copies (C_t = 16.25 ± 0.48) to 2 copies (C_t = 34.09 ± 1.14) per reaction (Fig. 10A). The regression analysis yielded a correlation coefficient (r) ≥ 0.994 and an amplification efficiency of 101.89 ± 5.11%. The mean intra-assay variation was 1.38 ± 0.87% when analysing four replicates of plasmid dilutions, whilst the mean inter-assay variation among four experiments was 2.63 ± 0.48% (Table 2). These CV values were considered acceptable to validate the repeatability and reproducibility of the assay.

A linear relationship between the infective titre of a viral suspension and Ct values was also observed for viral amounts ranging from 1 x 10^4 to 1 x 10^0 $TCID_{50}$ per reaction (r = 0.999) (Fig. 10B), with 1 x 10^0 $TCID_{50}$ yielding a mean viral DNA copy number of 1.2 x 10^2.

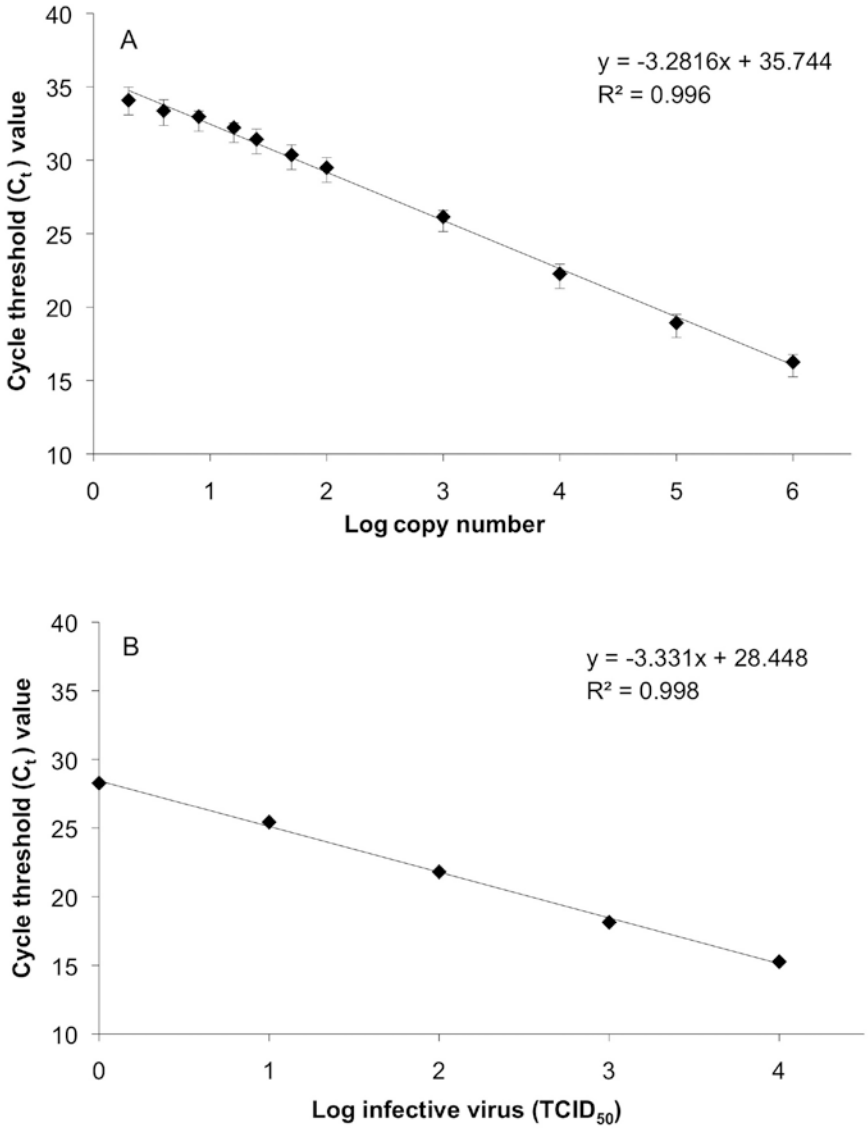


Figure 10. Dynamic range and sensitivity of the real-time PCR assay for LCDV detection. (**A**) Standard curve obtained using dilutions of plasmid DNA ranging from 10^6 to 2 copies per reaction. Linear regression was performed on mean data from four separate runs. The logarithm to base 10 (log) of the plasmid copy number versus the cycle threshold (C_t) value is represented. (**B**) Standard curve showing a linear relationship between the log of the amount of infective virus (expressed in $TCID_{50}$) per reaction and their corresponding C_t values.

Table 2. Intra- and inter-assay variability of the real-time PCR.

	Intra-assay variation[a]		Inter-assay variation[b]	
Copies/reaction	Cycle threshold (C_t)[c]	CV (%)	Cycle threshold (C_t)[c]	CV (%)
1 x 10^5	19.92 ± 0.16	0.78	18.92 ± 0.54	2.83
1 x 10^4	23.34 ± 0.17	0.73	22.27 ± 0.60	2.69
1 x 10^3	26.87 ± 0.18	0.66	26.14 ± 0.46	1.75
1 x 10^2	30.22 ± 0.27	0.89	29.49 ± 0.71	2.39
1.6 x 10^1	32.15 ± 0.47	1.45	32.22 ± 0.87	2.72
4 x 10^0	34.15 ± 0.80	2.34	33.37 ± 0.89	2.68
2 x 10^0	35.23 ± 0.99	2.80	34.09 ± 1.14	3.35
Overall CV[c] (%)		1.38 ± 0.87		2.63 ± 0.48

[a] Intra-assay variation was determined on four replicates of recombinant plasmid dilutions analysed in the same PCR run.
[b] Inter-assay variation was calculated on values obtained in four separate PCR runs.
[c] Mean ± standard deviation (SD).
CV: coefficient of variation.

1.3.2. Surveillance study of LCDV in gilthead seabream farms

In the present study, individual gilthead seabreams were collected from four farms with different background regarding to LCD. Farm C had no records of LCD, either before or after this study, whereas farm D suffered LCD outbreaks in last years. In farms A and B an outbreak of LCD was going on at the time of sampling, affecting several tanks. In these latter farms, fish from two different cycles of production were sampled, one suffering and other unaffected by LCD.

A total of 60 juvenile fish, both asymptomatic and diseased, were analysed by the qPCR assay developed in this study. LCDV was detected in all farms, 30 to 100% of fish being identified as LCDV-infected (Table 3). In parallel, asymptomatic fish were also analysed by PCR-hybridization, corroborating in all cases their infection status. In asymptomatic fish, viral loads in caudal fin ranged between 1 copy and 3.3 x 10^2 copies of viral DNA per mg of tissue, whereas in

animals with signs of the disease, viral loads were between 2.9 x 10^4 and 6.6 x 10^5 copies of viral DNA/mg.

Table 3. LCDV detection and estimated viral load determined by real-time PCR in caudal fin from diseased and asymptomatic gilthead seabream juveniles.

Fish population[a]	LCD signs[b]	LCDV detection[c]	Viral load[d]	
			Load range	Mean ± SD
A	-	73.3 (15)	1 - 15	(4 ± 3.99) x 10^0
A	+	100 (6)	1.2 x 10^5 - 6.6 x 10^5	(3.53 ± 2.77) x 10^5
B	-	30.0 (10)	1 - 330	(1.1 ± 1.89) x 10^2
B	+	100 (5)	2.9 x 10^4 - 6.9 x 10^4	(5 ± 1.75) x 10^4
C	-	87.5 (16)	1.7 - 54	(1.6 ± 1.56) x 10^1
D	-	100 (8)	12 - 140	(5.3 ± 5.88) x 10^1

[a] A, B, C and D are four gilthead seabream farms located in the Mediterranean area. Only farm C had no previous reports of lymphocystis disease (LCD).
[b] Typical lymphocystis disease signs: +, presence; -, absence.
[c] Percentage of LCDV-positive fish (total number of fish analysed).
[d] Copies of viral DNA per mg of tissue.
SD: standard deviation.

Samples were also collected at the hatchery in another farm with previous LCD records (farm E). Rotifer and artemia metanauplius cultures, and artemia cysts were positive for LCDV. Viral loads estimated for these samples were about 10^2 copies of viral DNA per mg (Table 4). In addition, one sample of decapsulated artemia cysts was analysed, and found to be LCDV-positive (1.2 x 10^1 copies of viral DNA/mg). LCDV was also detected by qPCR in non-disinfected gilthead seabream fertilized eggs, in animals collected at different tanks in the larval rearing unit (1- to 26-d-old larvae), and in fingerlings collected in the weaning area. Viral loads in these gilthead seabream samples ranged from 1.3 x

10^0 to 2.2 x 10^1 copies of viral DNA/mg. All the samples collected at this hatchery were also found to be LCDV-positive by PCR-hybridization.

Finally, broodstock from farm E were analysed using the qPCR assay (Table 5). LCDV was detected in 22 out of the 50 caudal fin samples analysed, with estimated viral loads ranging from 8.7 to 61 copies of viral DNA/mg. When blood samples were considered, only 9 of the fish were identified as LCDV-positive (1.3 ± 1.37 copies of viral DNA/ µl).

Table 4. Estimated viral load determined by real-time PCR in samples from a gilthead seabream hatchery.

Sample[a]	Viral load[b]
Rotifer culture	1.5 x 10^2
Artemia cysts	1.9 x 10^2
Decapsulated artemia cysts	1.2 x 10^1
Artemia metanauplii	1.5 x 10^2
Fertilized eggs	4.1 x 10^0
Larvae (1-d-old)	3.4 x 10^0
Larvae (6-d-old)	2.5 x 10^0
Larvae (8-d-old)	3.6 x 10^0
Larvae (26-d-old)	5.1 x 10^0
Fingerlings (0.3 g)	1.1 x 10^1
Fingerlings (0.9 g)	1.3 x 10^0
Fingerlings (1.6 g)	2.2 x 10^1
Fingerlings (2 g)	9.7 x 10^0

[a] Samples consisted of pooled animals.
[b] Copies of viral DNA per mg of tissue.

Table 5. LCDV detection and estimated viral load determined by real-time PCR in caudal fin and blood from asymptomatic gilthead seabream breeders.

		Viral load[b]	
Sample	**LCDV detection[a]**	**Load range**	**Mean ± SD**
Caudal fin	44 (50)	8.7 - 61	$(2.7 \pm 1.40) \times 10^{1}$
Blood	18 (50)	0.2 - 4.4	$(1.3 \pm 1.37) \times 10^{0}$

[a] Percentage of LCDV-positive fish (total number of fish analysed).
[b] Copies of viral DNA per mg of tissue (caudal fin samples) or per µl (blood samples).
SD: standard deviation.

2. Rapid and sensitive detection of lymphocystis disease virus genotype VII by loop-mediated isothermal amplification

2.1. INTRODUCTION

Loop-mediated isothermal amplification (LAMP) is a powerful technique designed to amplify nucleic acids with high specificity, sensitivity, and rapidity under isothermal conditions (Notomi *et al.*, 2000). The assay uses DNA polymerase and a set of specific primers that recognize six different sequences on the target DNA, to achieve high selective nucleic acid amplification. Other advantages of this technique, compared to PCR-based methods, are that the repeated denaturation of dsDNA to ssDNA is not required, which potentially reduces time to amplification, and that it is relatively easier to use and does not require expensive reagents and equipment.

In recent years, several LAMP assays have been designed and applied for the detection of different fish viruses, including several iridoviruses (Caipang *et al.*, 2004; Mao *et al.*, 2008; Zhang *et al.*, 2009; Ding *et al.*, 2010; Min *et al.*, 2013; Hwang *et al.*, 2015). In the case of LCDV, a LAMP assay was developed for the diagnosis of LCDV, but only could detect isolates obtained from the Japanese flounder, which belong to genotype II (Li *et al.*, 2010).

The objective of this study was to design a LAMP assay for the rapid and sensitive detection of LCDV genotype VII using an instrument for real-time fluorescence measurement of amplification. In addition, the assay has been evaluated to assess its applicability using samples of lymphocystis (LC)-diseased and asymptomatic gilthead seabream and Senegalese sole cultured in fish farms in Southern Spain.

2.2. MATERIALS & METHODS

2.2.1. Virus isolates and fish samples

LCDV SA25, belonging to the genotype VII isolated from gilthead seabream, was used as reference strain in this study. Strains of four iridoviruses: epizootic haematopoietic necrosis virus (EHNV), European sheatfish virus (ESV), doctor fish virus (DFV) and frog virus 3 (FV3), and one aquatic herpesvirus, cyprinid herpesvirus 3 (CyHV3), were used to evaluate the specificity of the LAMP assay.

Thirteen juvenile gilthead seabream specimens were collected at two aquaculture farms located in the South Atlantic coast of Spain. Seven of these fish showed signs of LCD, whereas the other six were asymptomatic (i.e. without typical signs of the disease). In addition, three specimens of Senegalese sole without signs of LCD were collected from another fish farm in the same area. Fish were euthanized by anaesthetic overdose and samples of their caudal fins were aseptically cut off and frozen immediately at -20 ºC (section 1.2.1).

2.2.2. LAMP primer design

In the first approach, we intended to design a LAMP assay valid for all LCDV genotypes described so far. To achieve this, a ClustalW pairwise alignment was carried out using MegAlign v7.0.0 (Lasergene, DNASTAR). The LCDV MCP gene sequences used, available from GenBank, were L63545.1 (genotype I), AB212997.1 (genotype II), AB213004.1 (genotype III), AB247938.1 (genotype IV), AB299163.1 (genotype V), AB299164.1 (genotype VI), GU320735.1 (genotype VII), GU290550.1 (genotype VIII), and GU939626.2 (genotype IX). Based on this alignment, it was impossible to identify a consensus sequence adequate for the design of specific LAMP primers. For this reason, primers were designed based on the sequence of the MCP gene of a LCDV genotype VII isolate (GenBank accession no. GU320735.1), using the Lamp Designer 1.10 program (Premier Biosoft International, Palo Alto, CA, USA). Primer set consisted of two outer primers (F3 and B3), two inner primers (FIP and BIP), and two loop primers (Loop-F and

Loop-B). Sequences and location of the primers within the MCP gene are shown in Table 6 and Fig. 11, respectively.

Table 6. Sequences of primers designed for the LCDV LAMP assay.

Primers	Sequences (5' – 3')
F3	GTTCTGGATCGCCCAATT
B3	TATCATGTTGTCGTAACCTACG
FIP (F1c + F2)	AACTTAACAGCGGGTATACGCATCTCGAGGCGGAGATTAC
BIP (B1c + B2)	CGGCACGATTCGATGGTGTAATGAGCCACCAAATCGTTAA
Loop-F	CCGTCATCCAGGCATTGA
Loop-B	CAAGCAAACGTCCGTTCAAT

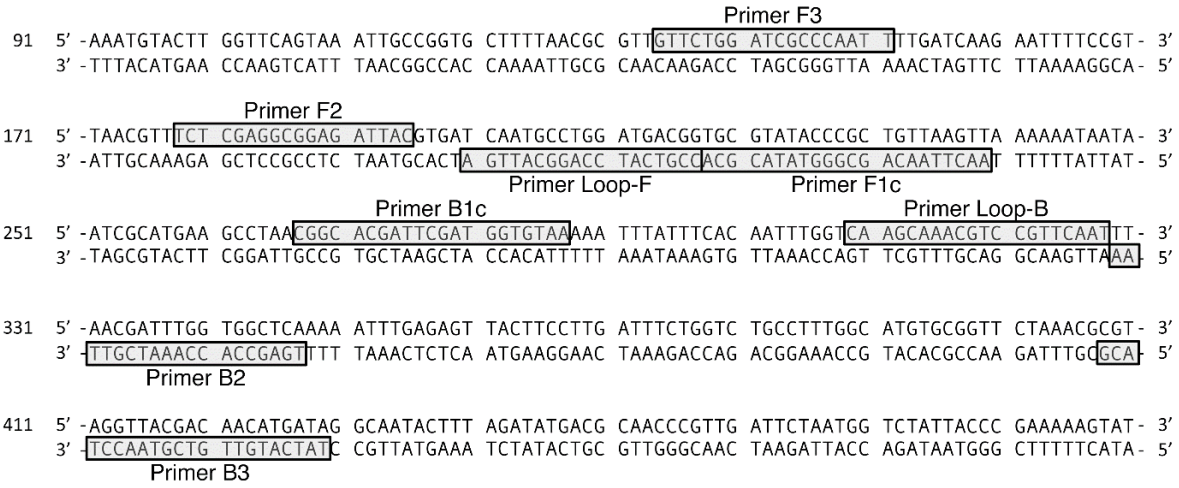


Figure 11. Primers for the detection of LCDV by LAMP. Partial DNA sequence of the MCP gene of LCDV (accession no. GU320735.1) showing target region of the primers.

The suitability of the LAMP primers for LCDV genotype VII detection was tested *in silico* through ClustalW alignment analysis. The analysis was performed using all the MCP sequences belonging to genotype VII (from isolates of both gilthead seabream and Senegalese sole), available at GenBank (accession nos.

GU320724.1 to GU320739.1, EF184306.1, HE650105.1, and HE650106.1). The specificity of the outer primers was also analysed by means of a virtual PCR using Serial Cloner v2.6.1 (http://serialbasics.free.fr/Serial_Cloner.html).

2.2.3. Construction of recombinant plasmid

A fragment of 1356 bp of the viral MCP gene of LCDV SA25 was amplified by PCR using the primers LC1-F and LC1-R described by Kitamura et al. (2006a), and cloned into the pCR4-TOPO vector as previously specified (section 1.2.3). The concentration of the recombinant plasmid was spectrophotometrically determined, and the plasmid stock was diluted to serve as template for LAMP reaction.

2.2.4. Optimization of LAMP reaction temperature

LAMP reactions were carried out in a final volume of 25 µl containing 15 µl of Isothermal Master Mix (OptiGene), 1 µM each of FIP, BIP, F3 and B3 primers, and 0.5 µM each of Loop-B and Loop-F primers. Aliquots of 5 µl of plasmid DNA (equivalent to 10^5 copies) were added as template. Isothermal amplifications were performed in a Genie® II system (OptiGene) that allows real-time monitoring of LAMP reactions. The reaction temperature was optimized using a block gradient from 62-69 ºC. Reactions were incubated at chosen temperature for 40 min, and then subjected to a slow annealing step (0.05 ºC/s) from 95 ºC to 75 ºC to identify specific amplification. The amplification ratio (change in fluorescence over time), annealing/melting temperature (Ta) of the product, and time of positivity (Tp) were obtained from the Genie® II software (OptiGene).

2.2.5. Specificity and sensitivity of the LAMP assay

The specificity of the developed LCDV LAMP assay was evaluated by testing the six fish viruses mentioned above. DNA from LCDV SA25 and SAF-1 cells were used as positive and negative controls, respectively. Viral and cellular DNAs

were extracted using the EZ1 Virus Mini Kit (Qiagen), according to the manufacturer's instructions.

To determine the sensitivity of the assay, ten-fold serial dilutions (10^6 to 10^0 copies) of the recombinant plasmid were used as template for LAMP. Plasmid DNA was diluted in Milli-Q water supplemented with DNA extracted from SAF-1 cells (100 ng per reaction). The optimized LAMP protocol was performed for three independent assays. Standard curves were generated by plotting the number of copies of plasmid DNA against Tp for each particular concentration.

2.2.6. Application of the LAMP assay for LCDV detection in fish samples

The suitability of the LAMP assay for the detection of LCDV was evaluated by testing samples from both LC-diseased and asymptomatic juvenile fish. Total DNA was extracted from 30 mg of caudal fin by using the E.Z.N.A. Tissue DNA Kit (Omega Bio-tek), following the protocol provided in the kit. DNA was eluted in a final volume of 50 µl and stored at -20 ºC until used as template for LAMP and qPCR. The LAMP assay was performed as specified above, but the amplification was monitored for up to 50 min. The qPCR analysis was carried out using the methodology described in section 1.2.

Finally, a modified Hot-SHOT protocol (Meeker *et al.*, 2007) was evaluated as a quick DNA extraction method, using caudal fin samples obtained from one LC-diseased gilthead seabream specimen. Briefly, a sample of 100 mg of caudal fin was ground using a sterile mortar and pestle, and transferred to a microcentrifuge tube containing 200 µl of 50 mM NaOH. After brief vortexing, the mixture was incubated at 95 °C for 20 min and then cooled to 4 °C, followed by the addition of 1/10 volume of 1 M Tris-HCl (pH 8.0) to neutralize the alkaline solution. Three µl of the supernatant obtained was immediately used for the LAMP assay. In parallel, DNA from another 100 mg of the same sample was extracted by using the EZ1 Virus Mini Kit, and 3 µl was used as template for LAMP. As positive control, 2 µl of LCDV SA25 DNA was combined with 3 µl of DNA from the Hot-SHOT extraction method to assess possible inhibition of the LAMP assay by the Hot-SHOT supernatant. DNA from SAF-1 cells was used as negative control.

2.3. RESULTS

2.3.1. Optimization of LAMP reaction temperature

The LAMP assay was evaluated at 62-69 ºC using as template 10^5 copies of the recombinant plasmid containing a fragment of the LCDV MCP gene. Products were amplified across this range of temperature with varying rapidity, with Tp values lower than 18 min. A temperature of 64 ºC was chosen as the optimal amplification temperature (Tp = 12 min, 57 s), and was applied in the subsequent analysis.

2.3.2. Specificity and sensitivity of the LCDV LAMP assay

No amplification was observed in LAMP reactions performed with DNA from SAF-1 cells (negative control), neither with DFV, FV3 or CyHV3 DNAs (Fig. 12A). When DNA from EHNV and ESV were used as template, some fluorescence could be observed but only after fluorescence normalization (Fig. 12A). With non-normalized data, fluorescence signal was lower compared to that showed by negative control (data not shown). Nevertheless, the annealing curves of the amplified products showed a single peak in the range of 84-86 ºC, only for LCDV DNA (Fig. 12B). These results indicate that the LAMP primers were specific to isolates of LCDV, and no cross-reactions have been observed with the other fish DNA viruses analysed.

Pairwise comparisons among MCP gene sequences belonging to LCDV genotype VII showed nucleotide identities ≥ 98%, and no mismatches were recorded in the regions corresponding to LAMP primers, except for two sequences (GU320724.1 and GU320734.1, with 100% identity) that showed two transitions at nucleotides 4th and 16th in primer Loop-B region. No amplifications were obtained while running *in silico* PCR with F3/B3 primers, using as template MCP sequences belonging to different LCDV genotypes except for genotype VII.

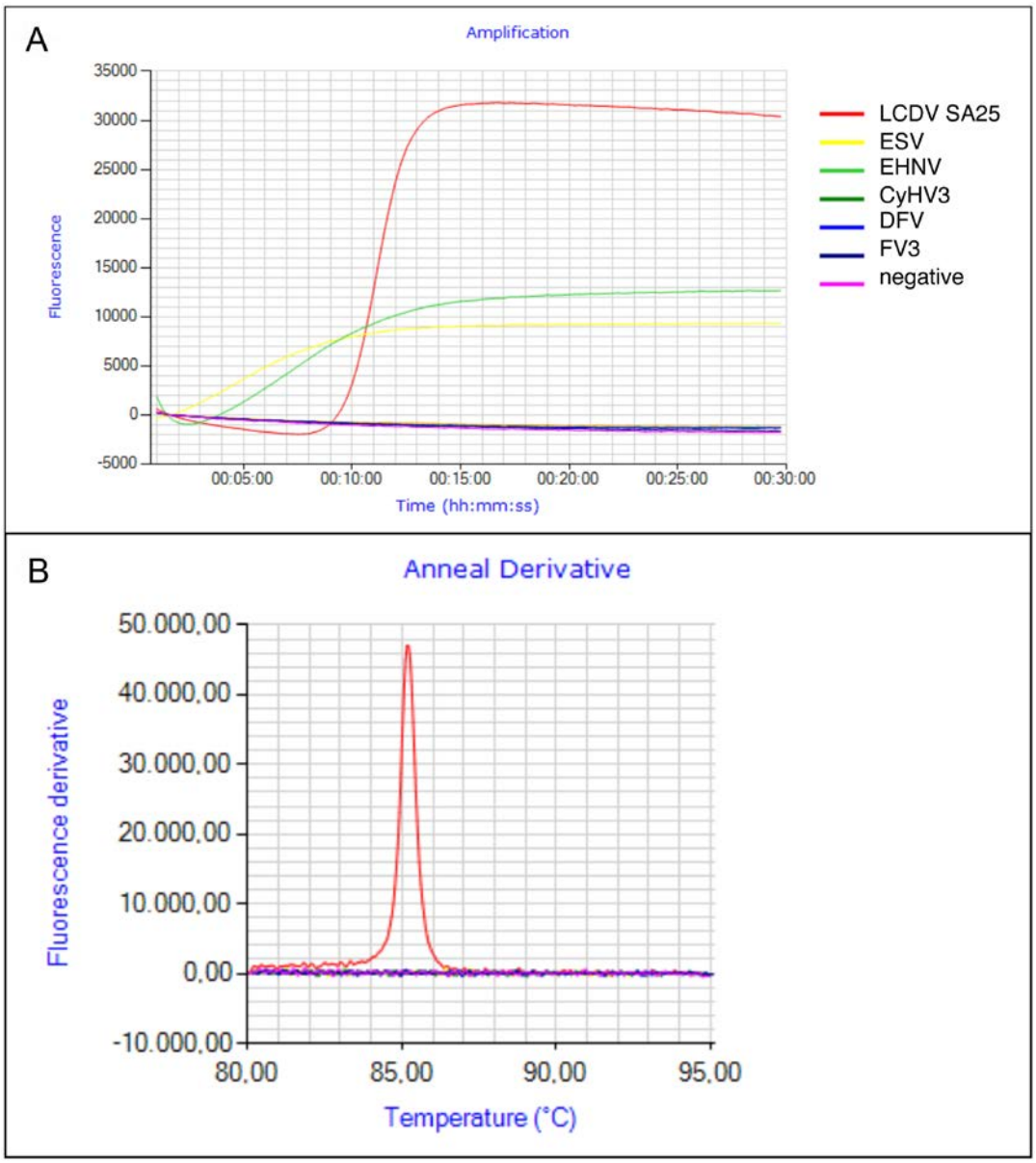


Figure 12. Specificity of the LCDV LAMP assay. (**A**) Genie® II software screenshot showing real-time isothermal amplification of DNA isolated from different viruses. Fluorescence was normalized to the background for 0-180 s. (**B**) Anneal derivative of isothermal amplified products. Negative control: DNA from SAF-1 cells.

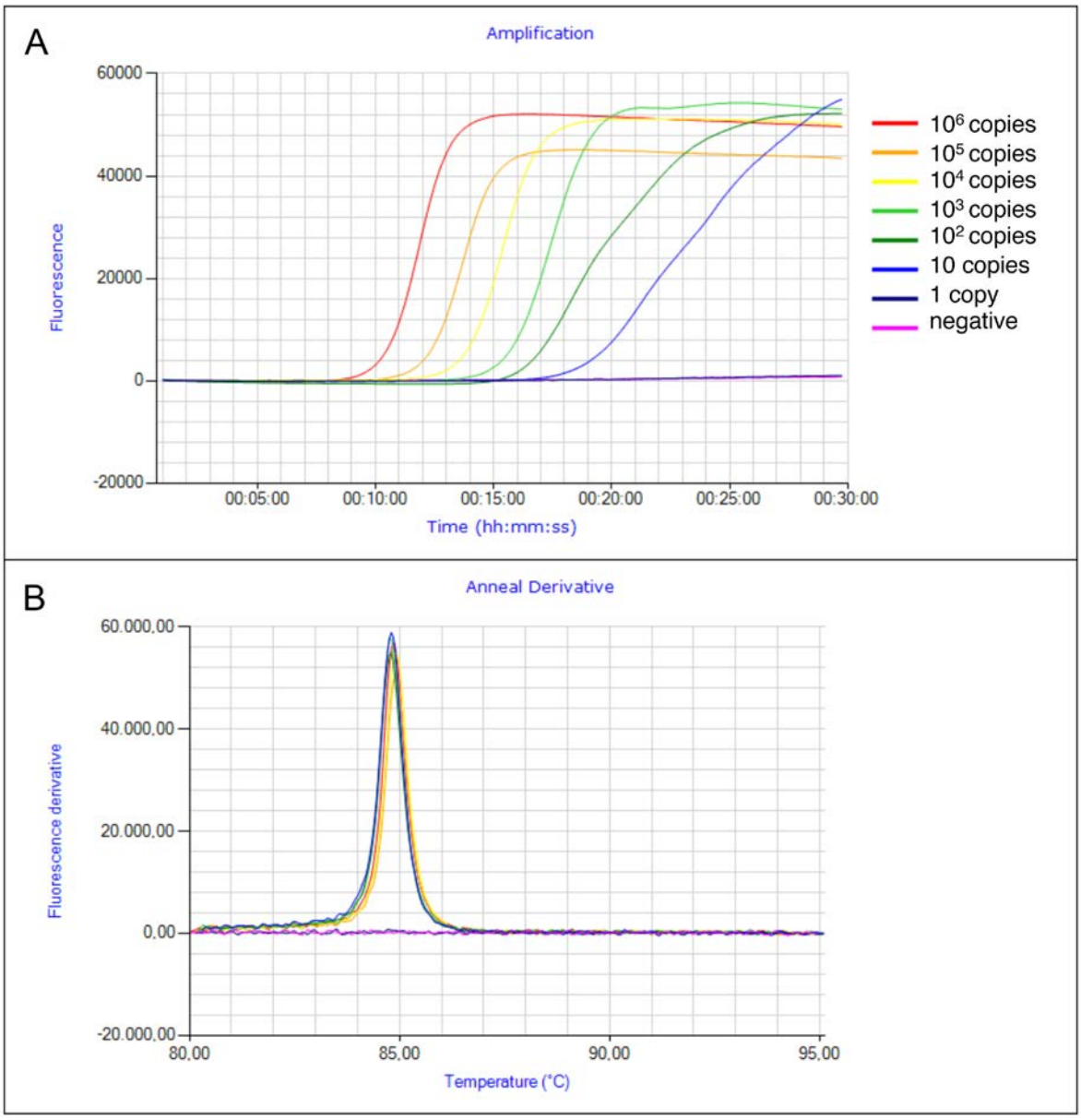


Figure 13. Sensitivity of the LAMP assay for LCDV detection. (**A**) Genie® II software screenshot showing real-time isothermal amplification of ten-fold serial dilutions of plasmid DNA ranging from 10^6 to 1 copies per reaction. Fluorescence was normalized to the background for 0-180 s. (**B**) Anneal derivative of isothermal amplified products. Negative control: DNA from SAF-1 cells.

Ten-fold serial dilutions of the recombinant plasmid ranging from 10^6 to 1 copies were used as template for the LAMP assay. The analytical sensitivity of the assay was estimated to be 10 copies per reaction (Fig. 13). Data analysis of three

independent assays revealed a significant correlation between the number of DNA copies and the Tp values ($r \geq 0.983$) (Fig. 14).

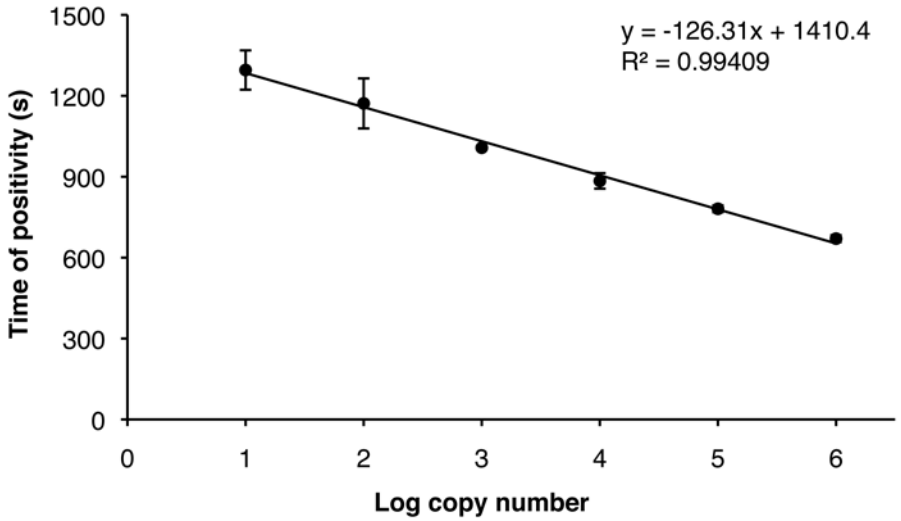


Figure 14. Correlation between the logarithm to base 10 (log) of the plasmid copy number and the time of positivity (Tp) value performed on mean data from three independent assays.

2.3.3. LCDV diagnosis by LAMP

The applicability of the LAMP assay for detection of LCDV in fish farms was evaluated by comparing its diagnostic capability with that of the qPCR assay (Table 7). LCDV was detected by LAMP in all samples from diseased gilthead seabream analysed, and even in those samples collected from asymptomatic carrier fish, with qPCR-estimated viral loads ranged between 1.9 and 2.7 x 10^1 copies of viral DNA per mg of tissue. Furthermore, no isothermal amplification was observed in samples where no LCDV genome was detected by qPCR. Tp values depend on the disease status, ranging from 3 min, 34 s and 11 min, 25 s for LC-diseased fish, and between 17 min, 32 s and 44 min, 26 s for asymptomatic carrier fish (Table 7). A correlation between the viral load estimated by qPCR and the Tp value ($r \geq 0.963$) was obtained for viral loads above the analytical sensitivity of the assay (using the protocol as described, this correspond to > 3.3 copies of viral DNA/mg).

Table 7. Evaluation of the LCDV LAMP assay using caudal fin samples from juvenile fish.

Sample	LCD signs[a]	Tp (LAMP assay)	Viral load[b] (qPCR assay)
Gilthead seabream			
No. 1	+	9 min, 59 s	1.1×10^5
No. 2	+	9 min, 32 s	1.3×10^5
No. 3	+	3 min, 34 s	1.2×10^6
No. 4	+	3 min, 42 s	1.1×10^6
No. 5	+	7 min, 26 s	3.5×10^5
No. 6	+	11 min, 5 s	2.8×10^4
No. 7	+	9 min, 27 s	1.9×10^5
No. 8	-	17 min, 32 s	2.7×10^1
No. 9	-	-[c]	<1
No. 10	-	-	<1
No. 11	-	26 min, 59 s	8.5×10^0
No. 12	-	29 min, 31 s	7.2×10^0
No. 13	-	-	<1
Senegalese sole			
No. 1	-	34 min, 45 s	2.6×10^0
No. 2	-	23 min, 46 s	9.4×10^0
No. 3	-	44 min, 26 s	1.9×10^0

[a] Typical lymphocystis disease signs: +, presence; -, absence.
[b] Copies of viral DNA per mg of tissue.
[c] Absence of isothermal amplification.
Tp: Time of positivity.

Finally, an analysis was performed to determine if the modified Hot-SHOT DNA extraction protocol chosen as an easy field protocol could negatively affect or inhibit the isothermal polymerase. The LAMP assay detected LCDV genome in DNA extracted using the Hot-SHOT method with a relatively high fluorescence signal (Fig. 15) and a shorter Tp value (7 min, 43 s *vs* 12 min, 41 s) compared to DNA extracted using the commercial kit from the same sample.

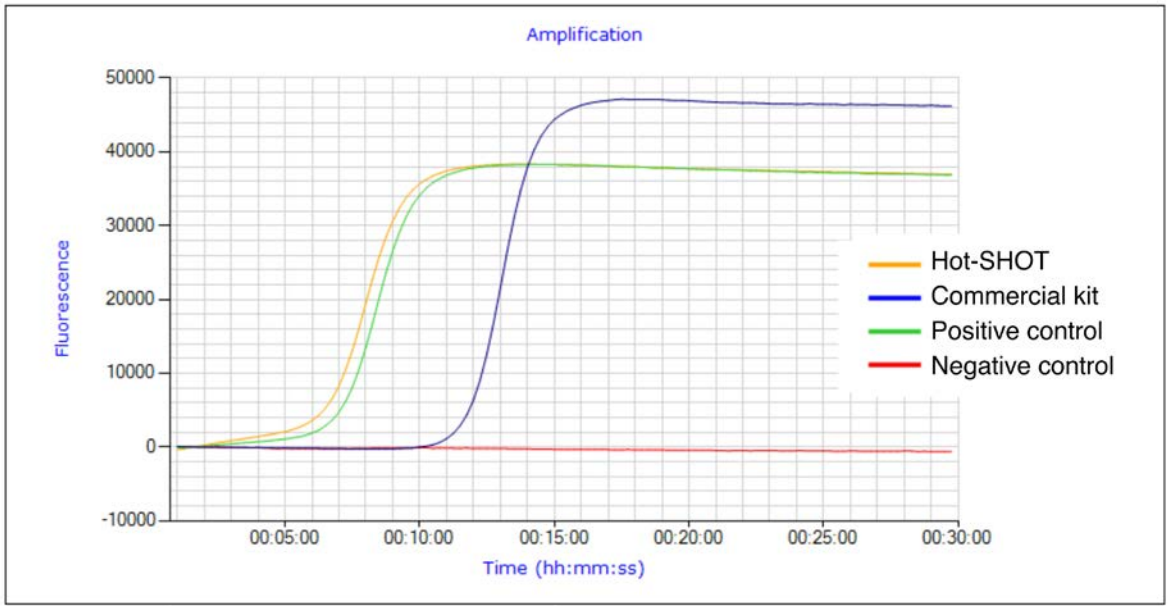


Figure 15. Comparison of DNA extraction method on LCDV detection by LAMP. DNA extracted from LC-diseased gilthead seabream by a modified Hot-SHOT extraction method and a commercial extraction kit. Fluorescence was normalized to the background for 0-180 s. Positive control: DNA from LCDV SA25 combined with DNA from the modified Hot-SHOT extraction method. Negative control: DNA from SAF-1 cells.

3. Evaluation of an integrated cell culture RT-PCR assay to detect and quantify infectious lymphocystis disease virus

3.1. INTRODUCTION

Viral disease prevention requires the use of rapid and sensitive diagnostic tools to avoid the spread of viruses in fish farms. In the case of LCDV, PCR-based methods have proved to be adequate for viral detection and quantification in several samples, including carrier fish and live food (Cano *et al.*, 2007; Ciulli *et al.*, 2015; section 1). However, these molecular methods do not provide any information on infectivity, which it is essential to establish the actual risk of viral transmission.

The standard method for diagnosis of fish viruses is based on virus isolation in cell culture and further confirmation by serological or molecular techniques (OIE, 2015). This procedure demonstrated virus infectivity and can also be used to determine viral infectious titres. However, LCDV is not easily propagated in cell culture, and virus isolation usually requires homologous cell lines (Iwamoto *et al.*, 2002). LCDV isolated from gilthead seabream can be cultured in the SAF-1 cell line, but often does not provoke clear and consistent cytopathic effects (CPE). This is particularly true when analysing samples with low viral loads, such as those collected from subclinically infected animals (Cano *et al.*, 2006). In addition, in these samples, CPE expression requires at least 14 d or even a blind passage step (Cano *et al.*, 2007).

The integrated cell culture (ICC)-PCR method has been applied for the detection of human viruses in environmental samples and has proved to be faster and more sensitive than methods based on CPE expression, minimizing the occurrence of false negatives when the viral load is low or with viruses that do not produce apparent CPE (Reynolds *et al.*, 2001; Lee *et al.*, 2005; Dong *et al.*, 2010; Li *et al.*, 2010). However, PCR sensitivity may lead to the detection of nucleic acids from inactivated viruses present in the samples. In the case of DNA viruses such as LCDV, the detection of viral mRNA by reverse transcription (RT)-

PCR during cell culture can be used to detect exclusively infectious virus (Ko *et al.*, 2003).

In the present study, we have developed an ICC-RT-PCR assay for the detection of infectious LCDV in samples with low viral loads. The suitability of the assay for viral titre determination using the most probable number (MPN) method was also evaluated.

3.2. MATERIALS & METHODS

3.2.1. Virus and cell lines

Three LCDV isolates from gilthead seabream, designated SA9, SA18 and SA25, were used in the present study (Table 8). Isolates were obtained from diseased fish as described previously (Garcia-Rosado *et al.*, 1999), maintained at -80 ºC, and used directly without further cell passage unless otherwise stated. LCDV strain Leetown NFH (ATCC VR-342) was used as reference strain of LCDV. To determine the specificity of the assay, an isolate of the iridovirus ESV, kindly provided by K. Way (CEFAS Laboratory, Weymouth, United Kingdom), was also used.

LCDV SA isolates were cultured in the SAF-1 cell line, whereas VR-342 and ESV were propagated in BF-2 cells. Both cell lines were grown in 24-well plates at 25 °C in Leibovitz's L-15 medium supplemented with 2% L-glutamine (Sigma-Aldrich), 1% penicillin-streptomycin (Sigma-Aldrich) and 10% foetal bovine serum (FBS) (Gibco). Semi-confluent cell monolayers were inoculated with 200 µl per well of the appropriate viral suspensions dilutions. After 2-h adsorption at 20 ºC, the inoculum was removed, and the cells were washed twice with phosphate buffered saline (PBS) (0.01 M, pH 7.4), prior to the addition of maintenance medium (L-15 medium with 2% FBS). Inoculated cells were incubated at 20 ºC and processed at 3, 5, and 7 d post-inoculation (pi) for ICC-RT-PCR, or maintained up to 14 dpi to observe CPE.

Table 8. Lymphocystis disease virus isolates from gilthead seabream used in this study.

Isolate	Isolation location (year)	Reference
SA9	Southwestern Spain (2001)	Cano *et al.*, 2006
SA18	Southwestern Portugal (2008)	Cano *et al.*, 2010
SA25	Northeastern Italy (2011)	This study

3.2.2. ICC-RT-PCR-hybridization assay

For ICC-RT-PCR, inoculated cells were harvested at the time points specified above. After washing twice with PBS, cells were suspended in PBS and pelleted by centrifugation. Total RNA was extracted using the E.Z.N.A. Total RNA Kit I (Omega Bio-tek). This RNA was treated with RNase-free DNase I (Roche) for 30 min at 37 °C, and stored at -80 ºC until used as a template for RT-PCR. In order to confirm the absence of residual contaminating viral DNA, total RNA was used as template for PCR following the protocol specified for RT-PCR but omitting the cDNA synthesis step and using GoTaq DNA Polymerase (Promega).

RT-PCR was performed using primers LCDVs-F and LCDVs-R (Table 9), and the PrimeScript One Step RT-PCR Kit Ver.2 (Takara) according to the manufacturer's instructions. The amplification conditions included an initial cDNA synthesis step at 50 ºC for 30 min, followed by denaturation at 94 ºC for 2 min, 35 cycles at 94 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s, and a final elongation step at 72 °C for 10 min. The amplified DNA (609 bp) was visualized by electrophoresis on a 2% agarose gel with ethidium bromide staining or was subjected to dot-blot hybridization using a DIG-labelled probe internal to the region amplified by RT-PCR. The probe was generated using the PCR DIG Probe Synthesis Kit (Roche), with a set of primers (RT-LCDV-F and RT-LCDV-R2) (Table 9) designed to amplify a fragment of 238 bp within the MCP gene of LCDV. The amplification profile for probe labelling was: 2 min at 95 ºC, and 35 cycles of 1 min at 95 ºC, 1 min at 53 ºC and 10 min at 72 ºC, with a final step of 10 min at 70 ºC and 5 min at 25 ºC. RT-PCR products (10 µl) were denatured and blotted onto

a Hybond-N nylon membrane, and hybridization was carried out using the chemiluminescent substrate CSPD, following previously described procedures (Cano *et al.*, 2007).

Table 9. Nucleotide sequences of forward and reverse primers used for RT-PCR and probe labelling.

Primer	Sequence (5' - 3')	Position[a]	Reference
LCDVs-F	YTGGTTCAGTAAATTACCRG	99-118	Kitamura *et al.*, 2006b
LCDVs-R	GTAATCCATACTTGHACRTC	688-707	Kitamura *et al.*, 2006b
RT-LCDV-F	ACGTTTCTCGAGGCGGAGAT	173-192	This study
RT-LCDV-R2	ACGCGTTTAGAACCGCACAT	391-410	This study

[a] Genomic position of the primers in the LCDV SA9 major capsid protein (MCP) gene (GenBank accession no. GU320728).

3.2.3. Sensitivity of the ICC-RT-PCR assay

To evaluate the sensitivity of the ICC-RT-PCR assay, SAF-1 cells were inoculated in triplicate with LCDV isolate SA25 at different infectious titres. Non-inoculated cells were used as negative control, and cells inoculated with the same viral isolate inactivated by UV light were used as non-infective control. The viral suspension (10^2 $TCID_{50}$/ml) was inactivated by a 60-min exposure to a UV germicidal lamp (G15T8, Sankyo Denki Co.).

3.2.4. ICC-RT-PCR assay for viral quantification

The ICC-RT-PCR assay was applied to determine the infectious titre of several viral stocks, both obtained directly from diseased gilthead seabream or after a first passage on SAF-1 cells, using the MPN method. SAF-1 cells were

inoculated in triplicate with tenfold serial dilutions of virus. Viral titre, expressed as MPN of infectious units per unit volume (MPNIU/ml), was estimated using a statistical MPN table for 3 replicates and 3 dilutions with a confidence level of 95% (APHA, AWWA, WPCF, 1999). The reproducibility of ICC-RT-PCR quantal assay was evaluated by titrating the virus stocks in duplicate or triplicate. Viral stocks were also titrated in parallel using the $TCID_{50}$ endpoint dilution assay (Reed & Muench, 1938).

Finally, the assay was used to quantify infectious LCDV in samples from five asymptomatic gilthead seabream juveniles. Samples consisted of caudal fin homogenates (20%, w/v), obtained as specified in section 1.2.1. Viral loads were estimated in parallel using qPCR (section 1.2).

3.3. RESULTS

3.3.1. Specificity and sensitivity of the ICC-RT-PCR assay

The specificity of the ICC-RT-PCR assay for LCDV detection was determined by analysing different viral isolates. A fragment of *ca.* 600 bp was amplified by RT-PCR, using as a template total RNA extracted from cells inoculated with LCDV, but not ESV. Nevertheless, minor amplicons were observed in agarose gels that also appeared when RNA extracted from non-inoculated SAF-1 or BF-2 cells were used (data not shown). These bands can mask the positive amplicon, especially in samples with low viral titres. Therefore, a dot-blot hybridization of the RT-PCR products was performed, which permits the identification of LCDV specific amplification products (Fig. 16).

ICC-RT-PCR allowed specific detection of viral mRNA in SAF-1 cells inoculated with LCDV SA25 at different viral titres and different times as shown in Fig. 17. The detection limit was 1 $TCID_{50}$/ml at 3 dpi, and 0.1 $TCID_{50}$/ml at 5 and 7 dpi. Based on these results, a 5-d incubation period was used for further analysis. No viral mRNA was detected from cells inoculated with UV-inactivated virus, although viral DNA was detected at all times points analysed.

In the case of CPE development on SAF-1 cells, the detection limit was 10^1-10^2 $TCID_{50}$/ml at 14 dpi (Table 10), and no CPE were observed at 5 dpi even for the highest inoculation titres.

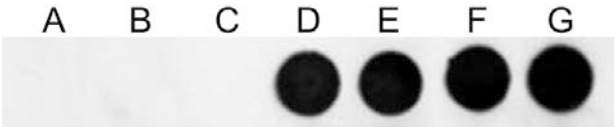


Figure 16. Specific detection of LCDV mRNA using RT-PCR followed by dot-blot hybridization. The assay was performed on total RNA extracted from SAF-1 cells inoculated with LCDV isolates (D) SA9, (E) SA18 and (F) SA25, or from BF-2 cells inoculated with (G) LCDV strain VR-342 or (C) ESV. Cells were inoculated with 10^3 $TCID_{50}$/ml, and processed at 5 d post-inoculation. Negative controls: RNA extracted from non-inoculated SAF-1 (A) or BF-2 (B) cells.

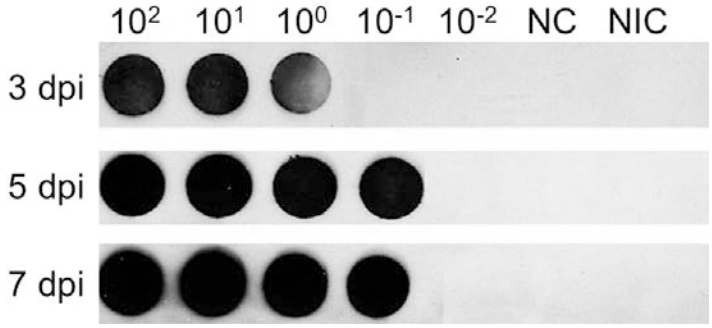


Figure 17. Viral mRNA detection by ICC-RT-PCR in SAF-1 cells inoculated with different titres of LCDV SA25 at different times post-inoculation. Titres expressed in $TCID_{50}$/ml. NC (negative control): non-inoculated SAF-1 cells. NIC (no infectivity control): SAF-1 cells inoculated with UV-inactivated virus (10^2 $TCID_{50}$/ml).

Table 10. LCDV detection by cytopathic effect observation in SAF-1 cells inoculated with different viral titres at 14 d post-inoculation.

	Viral titre ($TCID_{50}$/ml)				
Isolate	**10^3**	**10^2**	**10^1**	**10^0**	**10^{-1}**
SA9	+	+	-	-	-
SA18	+	+	-	-	-
SA25	+	+	+	-	-

3.3.2. Viral quantification by ICC-RT-PCR

The ICC-RT-PCR assay was also applied to determine the infectious titre of several viral isolates and found to be reproducible (Table 11).

Table 11. Comparison of viral infectious titres estimated by the ICC-RT-PCR assay and the development of cytopathic effects (CPE).

Viral stock[a]	ICC-RT-PCR assay[b]	CPE assay[c]
LCDV SA18	2.2×10^7 *	2.8×10^6
LCDV SA25	1.2×10^8 *	2.8×10^7
LCDV SA9 (1P)	1.2×10^3 **	No CPE
LCDV SA18 (1P)	$(3.9 \pm 1.4) \times 10^3$ **	No CPE
LCDV SA25 (1P)	$(5.6 \pm 1.6) \times 10^3$ **	No CPE

[a] Viral stocks were obtained directly from fish homogenates or after one passage on SAF-1 cells (1P).

[b] The ICC-RT-PCR assay was carried out in duplicate (*) or triplicate (**). Titres (media ± standard deviation) expressed as MPNIU/ml.

[c] Titres expressed as $TCID_{50}$/ml.

No CPE: CPE were not observed in the inoculated cells maintained up to 14 dpi.

In viral stocks obtained directly from diseased fish, the infectious titres estimated by the ICC-RT-PCR assay were nearly one order of magnitude higher than those obtained by the $TCID_{50}$ method. Furthermore, after a first passage on SAF-1 cells, viral titre dropped below the detection limit of the CPE assay, but it was still possible to titrate those viral stocks using ICC-RT-PCR (Table 11).

Finally, the detection and quantification of infectious LCDV was possible in all the gilthead seabream carriers analysed, although no CPE could be observed in cell cultures inoculated in parallel with those homogenates and maintained up to 14 dpi. Infectious titres estimated by the ICC-RT-PCR assay were 2.2-2.7 log_{10} lower than viral loads obtained by qPCR (Table 12).

Table 12. LCDV quantification in samples from asymptomatic gilthead seabream juveniles using ICC-RT-PCR and qPCR assays.

Fish sample[a]	ICC-RT-PCR assay[b]	qPCR assay[c]
1	1.8×10^1	3.1×10^3
2	3.7×10^1	2.0×10^4
3	1.8×10^1	8.6×10^3
4	3.7×10^1	1.9×10^4
5	3.7×10^1	1.5×10^4

[a] Samples consisted of caudal fin homogenates.
[b] Viral infectious titres expressed as MPNIU/ml.
[c] Viral loads expressed as copies of viral DNA/ml.

CHAPTER 2:

PATHOGENESIS AND TRANSMISSION OF LYMPHOCYSTIS DISEASE VIRUS

1. Target organs for lymphocystis disease virus replication in gilthead seabream

1.1. INTRODUCTION

LCD is a self-limiting disease characterized by the hypertrophy of fibroblastic cells in the connective tissue of fish (Samalecos, 1986). These hypertrophied cells, named lymphocysts or lymphocystis cells, are usually observed on the skin and fins, although they have also been described in several internal organs (such as the stomach, spleen, liver, kidney and heart) (Rusell, 1974; Dukes & Lawler, 1975; Howse *et al.*, 1977; Wolf, 1988; Sindermann, 1990; Colorni & Diamant, 1995). In gilthead seabream, LCD-associated lesions have been described only in the fish skin and fins, and usually disappear after 20-45 days depending on water temperature (Paperna *et al.*, 1982; Gonzalez de Canales *et al.*, 1996; Kvitt *et al.*, 2008).

Data on lymphocystis pathogenesis are very scarce and generally limited to histopathological studies of skin lesions (Gonzalez de Canales *et al.*, 1996; Sheng & Zhan, 2004; Sheng *et al.*, 2007b). However, several studies have shown that viral antigens can be detected in a number of organs of infected fish, not only in lymphocystis lesions (Xing *et al.*, 2006; Sheng *et al.*, 2007b; Cano *et al.*, 2009a). In gilthead seabream, DNA-DNA hybridization and immunohistochemistry were used to detect LCDV in diseased and recovered juveniles. Viral genomes and antigens were detected in the different organs analysed, including the caudal fin, gills, intestine, liver, spleen and kidney, suggesting that LCDV establishes a systemic and persistent infection in this fish species (Cano *et al.*, 2009a). In addition, LCDV is frequently detected by PCR-based methods in apparently healthy seabream (Cano *et al.*, 2007; Ciulli *et al.*, 2015; Chapter 1, section 1), which indicates that they may be subclinically infected. However, further studies are necessary to confirm these results and to establish if these infections are productive.

Thus, the objective of the present study was to determine the target organs and cells that support LCDV replication in gilthead seabream juveniles,

both LC-diseased and subclinically infected. In addition, a histopathological study of LCD was also conducted.

1.2. MATERIALS & METHODS

1.2.1. Fish samples

Gilthead seabream specimens were obtained from two fish farms located in southwestern Spain. In the first farm, fish without signs of LCD (6-10 g in weight) were collected, and constituted the group named "asymptomatic". This farm had no record of an LCD outbreak in more than 15 years. In the second farm, diseased individuals (6-10 g) showing typical external signs of LCD were collected, and two months after disease signs disappeared in the fish population, another group of fish (15-20 g) was sampled. These fish constituted the "diseased" and the "recovered" groups, respectively. Fish used in this study were treated according to the Spanish directive (RD 53/2013, BOE no. 34), and were euthanized by anaesthetic overdose (Chapter 1, section 1.2.1).

Samples of the caudal fin, intestine, liver, spleen, kidney and brain of nine individuals from each experimental group were aseptically collected and individually processed for subsequent homogenization and nucleic acid extraction. In addition, the same organs were collected from three fish in each group for *in situ* hybridization (ISH) and histological examination. Fish samples were fixed in 4% paraformaldehyde (Sigma-Aldrich) in DEPC-treated PBS (pH 7.2) for 24 h at 4 °C, and embedded in paraffin using standard histological procedures. Fixed caudal fin samples were decalcified with a solution containing 10% EDTA (Sigma-Aldrich) and 4% paraformaldehyde in DEPC-treated water at pH 7.0 for 10-15 days at 4 °C. Tissue sections (5-7 µm) were mounted on TESPA (3-triethoxysilylpropylamine)-treated slides.

1.2.2. DNA and RNA extraction and cDNA synthesis

Total DNA and RNA were extracted using the E.Z.N.A. Tissue DNA Kit and the E.Z.N.A. Total RNA Kit I (Omega Bio-tek), respectively, following the manufacturer's instructions. Total RNA was treated with RNase-free DNase I (Roche) for 30 min at 37 °C. RNA purity and quantity was determined using NanoDrop 1000 (Thermo Scientific). After DNase treatment, total RNA was used in the qPCR reaction in order to control for the absence of viral genomic DNA. First-strand DNA synthesis was carried out with 1 µg of total RNA and random hexamer primers using the Transcriptor First Strand cDNA Synthesis Kit (Roche). DNA and cDNA were stored at -20 °C until used as template for qPCR.

1.2.3. LCDV DNA quantification and gene expression

The qPCR protocol described in Chapter 1 (section 1.2.) was used to quantify the amount of viral DNA in the samples. The number of copies of LCDV DNA was calculated by interpolation in a standard curve, and viral load expressed as viral DNA copies per milligram of tissue.

Relative quantification of MCP gene expression was carried out by RT-qPCR, following the protocol mentioned above but using 20-µl final volume reactions and 2 µl of cDNA (at a 1/30 dilution). Normalized relative MCP expression levels were calculated for each sample applying the formula: $F = \log_{10} [(E + 1)^{40-Ct}/N]$ (Segarra *et al.*, 2014), where E is the amplification efficiency of the qPCR, Ct (threshold cycle) corresponds to the PCR cycle number, N is the maximal number of viral DNA copies/mg of tissue detected minus the number of viral DNA copies/mg of tissue determined by absolute qPCR for the sample, and Ct of 40 arbitrarily corresponds to "no Ct" by qPCR.

Results obtained for viral DNA quantification and relative gene expression were statistically analysed using a Mann-Whitney U-test followed by a Holm-Bonferroni correction for multiple comparisons.

1.2.4. RNA *in situ* hybridization and histopathology

DIG-labelled RNA probes were synthesized by *in vitro* transcription with the DIG RNA Labelling Kit (Roche) using a 150-bp fragment of the viral MCP gene (nucleotide positions 173 to 322 of the LCDV SA9 MCP gene, GenBank accession no. GU320728) cloned into the pCRII Dual Promoter vector (Invitrogen) as the template. The RNA probes were produced from 1 µg of linearized plasmid using T7 (antisense) or SP6 (sense) polymerases.

Deparaffinised and rehydrated tissue sections were permeabilized for 30 min with 10 µg/ml proteinase K in a buffer containing Tris-HCl 0.05 M pH 7.6 (40%, v/v) and $CaCl_2$ 1 M (4%, v/v) in DEPC-treated water at 37 °C. Sections were pre-hybridized with formamide (50%, v/v), 20x SSC (25%, v/v), torula yeast RNA (50 mg/ml), heparin sodium salt (5 mg/ml), Denhardt's solution (2%, v/v), CHAPS (2%, w/v) and Tween 20 (0.5%, v/v) in DEPC-treated water for 3 h in a 2x SSC saturated atmosphere at 55 °C. Sense and antisense probes were denatured at 85 °C for 5 min, and hybridization was performed overnight at 60 °C. After hybridization, sections were washed as previously described (Ortiz-Delgado *et al.*, 2006) and treated with 1% blocking reagent (Roche) in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) for 1 h at room temperature. Then, the slides were incubated with anti-digoxigenin-AP (Roche) overnight at 4 °C, and the hybridization signals were detected using NBT/BCIP (Roche) according to the manufacturer's instructions. All reagents were supplied by Sigma-Aldrich unless otherwise stated. Finally, sections were dehydrated and mounted in Eukitt® quick-hardening mounting medium (Sigma-Aldrich).

In parallel, tissue sections were stained with haematoxylin-eosin (HE) and haematoxylin-V.O.F. (Sarasquete & Gutierrez, 2005) for histological examination.

1.3. RESULTS

1.3.1. Viral load and gene expression

LCDV was detected by qPCR in all the samples analysed. Viral load in different organs from the three experimental groups are shown in Fig. 18. In diseased fish the highest viral loads were detected in the caudal fin (3.5 ± 2.4 x 10^5 copies of viral DNA/mg of tissue), followed by the kidney (1.2 ± 0.2 x 10^4 copies of viral DNA/mg) and brain (2.4 ± 0.9 x 10^3 copies of viral DNA/mg). In fish from the asymptomatic and recovered groups, low-titre infections were observed, with estimated viral loads between 0.4 and 27.5 copies of viral DNA per mg. No significant differences ($p < 0.05$) were observed between the asymptomatic and the recovered groups, except for liver samples. In fish from the asymptomatic group, the number of LCDV DNA copies in the brain was significantly higher than in the caudal fin ($p < 0.01$).

MCP gene expression was analysed as an indicator of viral productive infection. In diseased fish, relative viral gene expression values were similar to viral loads in the different organs analysed (Fig. 19). Thus, the highest relative expression values were detected in the caudal fin, followed by those in the kidney and brain. F values were significantly higher ($p < 0.01$) in these organs than in the other internal organs analysed. Viral gene expression was observed in all organs collected from fish from the asymptomatic and recovered groups, with relative values significantly lower ($p < 0.01$) than those obtained in samples from diseased fish. In asymptomatic fish, F values in the caudal fin were significantly higher ($p < 0.01$) than those in other tested organs, with the exception of the liver. No significant differences ($p < 0.01$) were observed between both experimental groups except for the caudal fin and brain samples, with F values significantly higher in the asymptomatic and recovered groups, respectively.

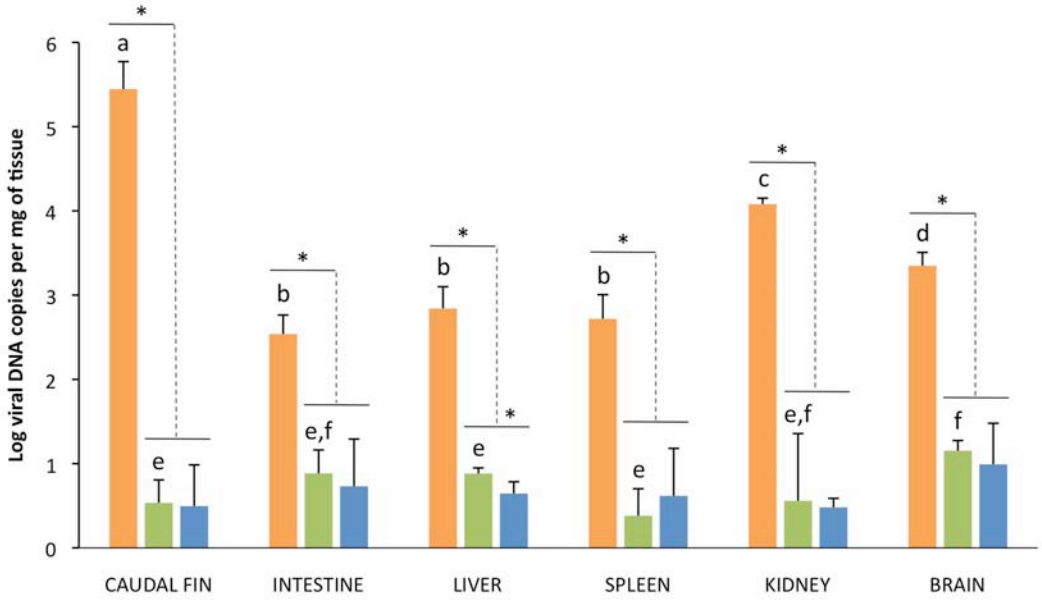


Figure 18. Viral loads in samples from different organs of gilthead seabream determined by qPCR. Experimental groups: diseased, orange; asymptomatic, green; recovered, blue. Different letters indicate significant differences between organs in the same experimental group. *Significant differences between groups. Significant level $p < 0.01$ (Mann-Whitney U-test, Holm-Bonferroni correction). Error bars represent ± standard deviation (n = 9).

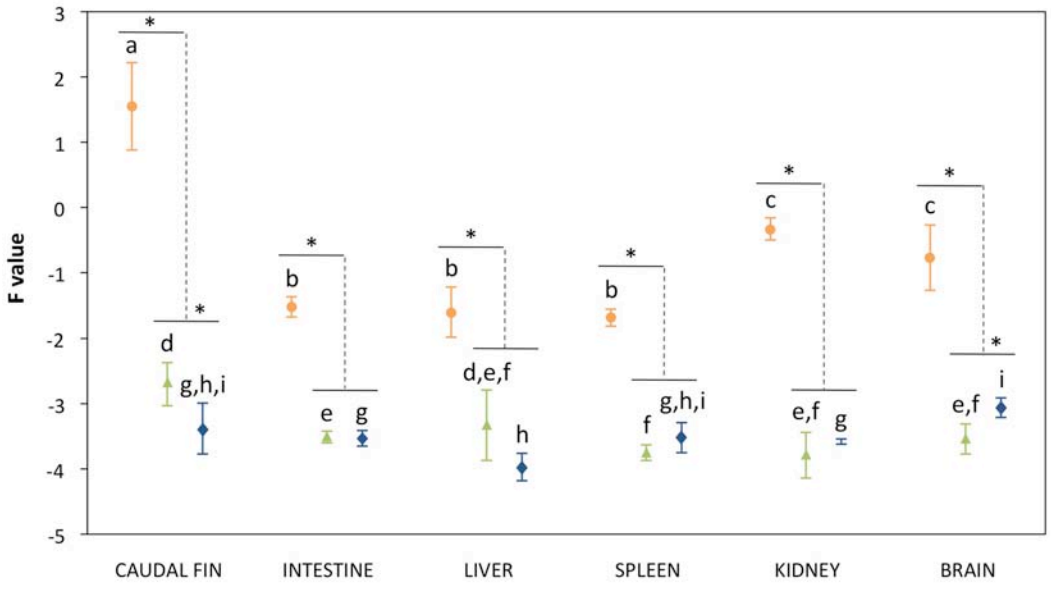


Figure 19. Relative MCP gene expression values in samples of gilthead seabream juveniles. Experimental groups: diseased, orange; asymptomatic, green; recovered, blue. Different letters indicate significant differences between organs in the same experimental group. *Significant differences between groups. Significant level $p < 0.01$ (Mann-Whitney U-test, Holm-Bonferroni correction). Error bars represent ± standard deviation (n = 9).

1.3.2. RNA *in situ* hybridization

Viral transcripts were detected by ISH in all organs from the diseased fish, whereas no signal was observed in the sections from fish belonging to the asymptomatic and recovered groups (results not shown). No labelling was observed in the negative controls using sense probe for ISH.

In sections of the caudal fin, the hybridization signal was strong, and labelling was observed as cytoplasmic inclusions in the lymphocysts (Fig. 20A) and in some cells in the surrounding connective tissue. In liver sections, numerous hepatocytes showed marked labelling in their cytoplasm (Fig. 20B). The hybridization signal was widely distributed in the splenic pulp, although in some areas the signal appeared concentrated around melanomacrophage centres (MMC) and ellipsoids (Fig. 20C). In the kidney, the hybridization signal was mostly confined to the haematopoietic tissue (Fig. 20D). In sections from the brain, ISH labelling was observed in the cytoplasm of cells in the granular layer (Fig. 20E). Finally, tissue sections from the intestine were also ISH-positives, but the tissue was so damaged by the ISH protocol that it was not possible to distinguish the localization of the labelled cells.

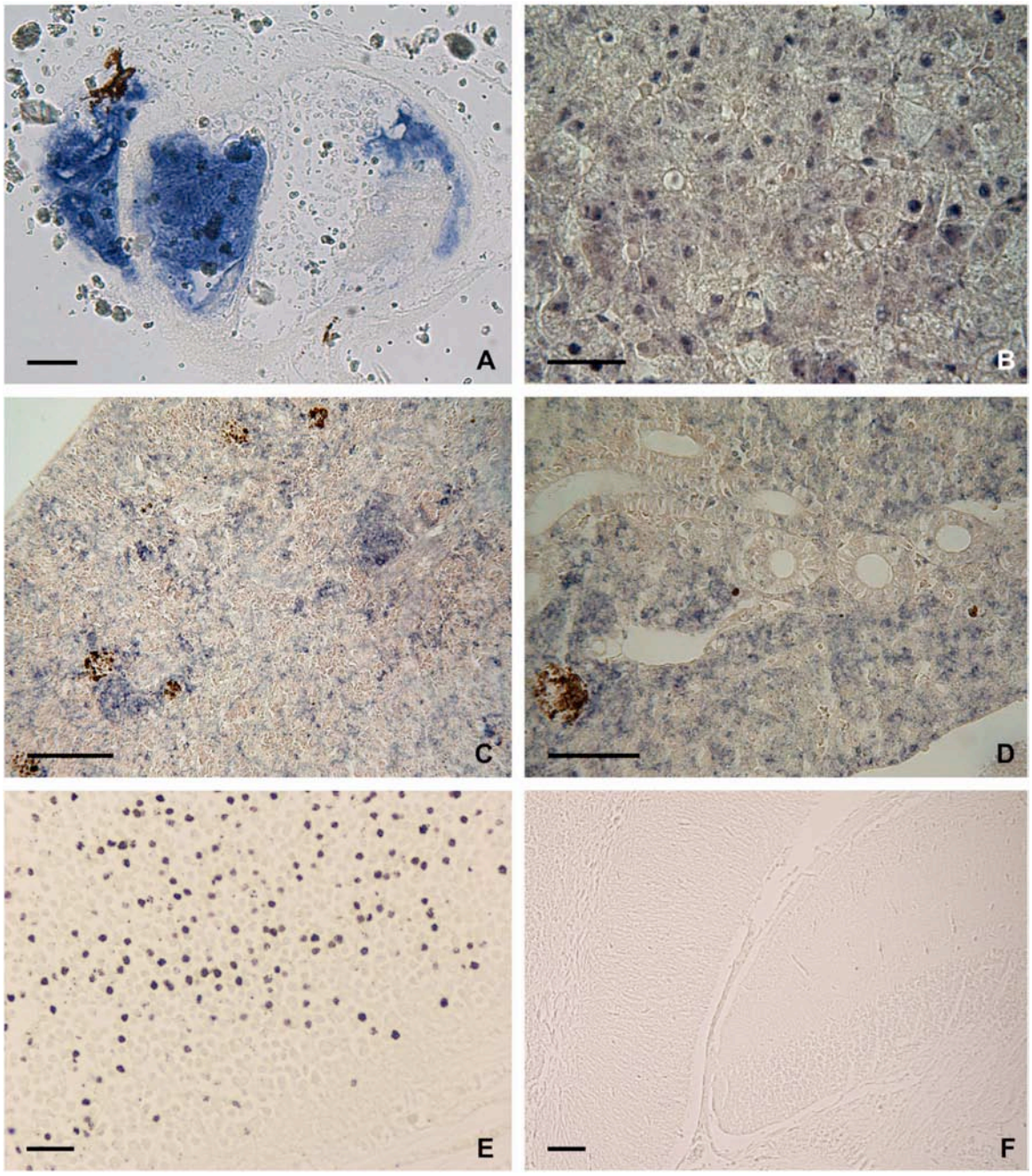


Figure 20. Lymphocystis disease virus (LCDV) detection in tissues from gilthead seabream demonstrated by *in situ* hybridization (ISH). The signal is observed microscopically as dark blue staining. (A) Lymphocyst in caudal fin showing viral mRNA in its cytoplasm. (B) ISH-positive hepatocytes in the liver of diseased fish. (C,D) Hybridization signal in the splenic pulp and in kidney interstitial cells, respectively, form diseased fish. (E): Viral transcripts in brain section from diseased fish. (F) ISH-negative brain section from recovered fish. Scale bars: (A, B, D) 50 µm; (C, F) 100 µm; (E) 20 µm.

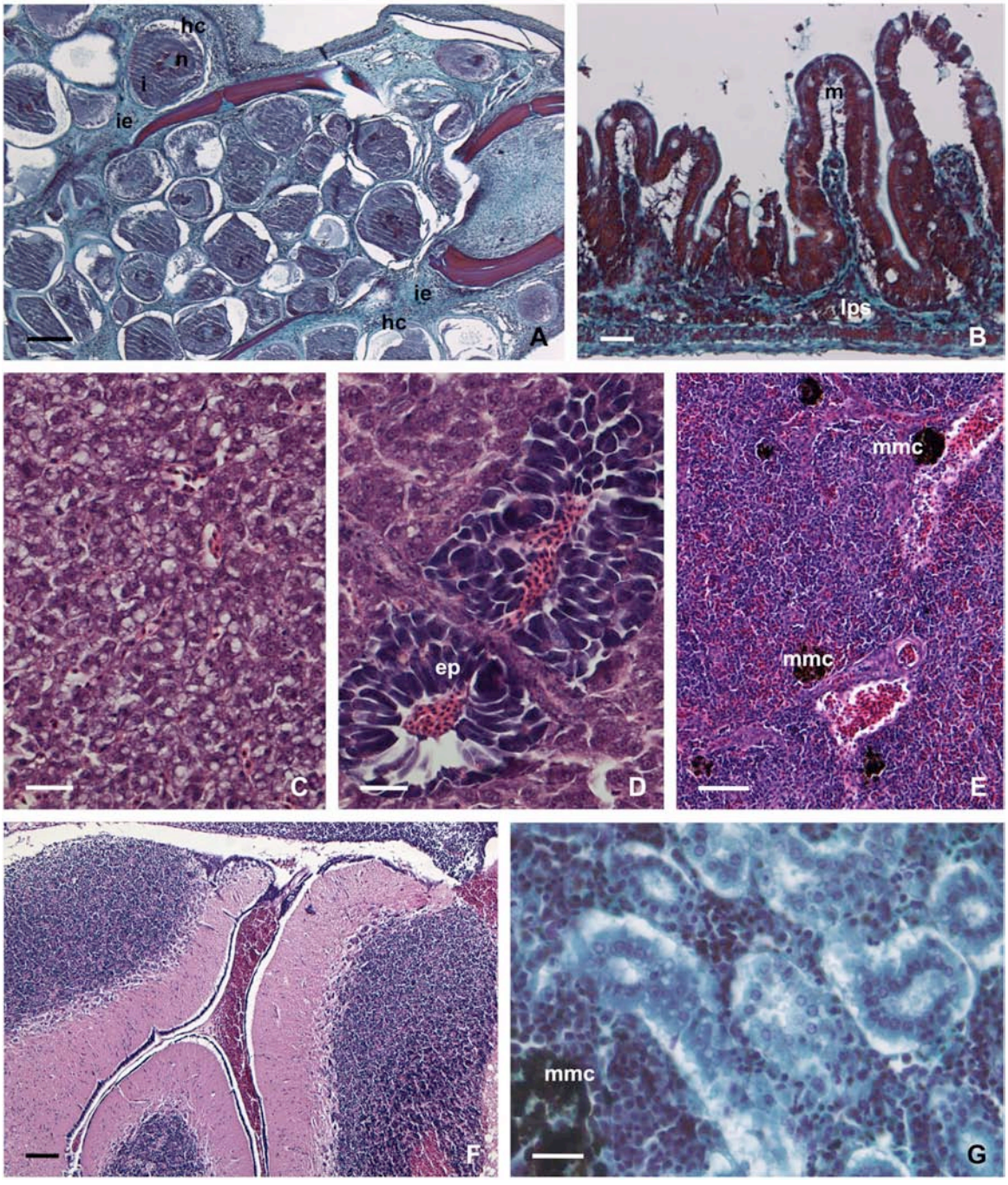
1.3.3. Histopathological study

In all the lymphocystis-diseased fish analysed, clusters of typical hypertrophied fibroblasts (lymphocysts) enclosed by a hyaline capsule were observed in the dermis of the caudal fin where they were surrounded by an abundance of inflammatory epithelioid cells exhibiting dark inclusions within the cytoplasm (Fig. 21A). Regarding internal organs, no lymphocysts were detected, but different types of histological alterations were observed depending on the organ analysed. The intestinal villi appeared dilated, and oedematous separation of the mucosa and inflammation of the submucosa layer were evident (Fig. 21B). Hepatocytes showed altered shape, signs of strong vacuolization and increased cytoplasmic basophilia (Fig. 21C), with some areas of hyaline necrosis (pyknotic nuclei) in the hepatic parenchyma and noticeable MMC. Several exocrine pancreatic cells showed some retraction and disruption of typical acinar structure (Fig. 21D). An increase in the number of MMC was also observed in the splenic parenchyma (Fig. 21E). Interestingly, brain ventricles appeared haemorrhagic in diseased specimens (Fig. 21F). Finally, in the proximal kidney, renal tubules were occluded and their epithelial cells showed strong hyaline degeneration, disorganization and noticeable vacuolization, as well as nuclear changes observed in pyknotic cells. Moreover, as in the liver and spleen, MMC increased in the kidney of diseased fish (Fig. 21G).

When gilthead seabream specimens had recovered from lymphocystis disease, most organs and tissues showed a normal structure and cellular pattern (Fig. 22), resembling those observed in fish from the asymptomatic group (Fig. 23) or in healthy gilthead seabream specimens from similar studies. Indeed, the caudal fin recovered its normal structure without a trace of lymphocysts (Fig. 22A). Oedematous signs in the intestinal mucosa disappeared, although some dilatation of the intestinal brush border was still visible (see Figs. 22B *vs* 23A). Hepatocytes showed their characteristic polygonal shape, although their cytoplasm appeared slightly basophilic with small signs of steatosis (see Figs. 22C *vs* 23B). In fish from both the recovered and the asymptomatic groups, a normal architecture of the exocrine pancreas was observed (Figs. 22D and 23B), and brain ventricles appeared without haemorrhagic focus (Figs. 22E and 23C). In the spleen, the number of MMC in the recovered fish was reduced in comparison to

diseased fish, and similar to that observed in asymptomatic animals (Figs. 22F and 23D). Additionally, in the recovered fish, renal tissue showed signs of recovery and a small number of MMC was seem and the necrosis focus disappeared.

Figure 21. Histopathology of lymphocystis disease in gilthead seabream juveniles. (A) Caudal fin section with lymphocysts in several developmental stages (haematoxylin-V.O.F.); hyaline capsule (hc), nucleus (n); cytoplasmic inclusion (i); inflammatory epithelioid cells (ie). (B) Cross-section of the intestinal villi showing oedema in the mucosa (m) and inflammatory reaction in the lamina propria/submucosa (lps). The mucosal epithelium appears hyperchromatic (haematoxylin-V.O.F.). (C) Histological section of liver showing hepatocytes with cytoplasmic vacuolization and loss of polygonal shape (HE). (D) Pancreatic acinar cells (ep) exhibiting retraction and disruption of their structure (HE). (E) Spleen section showing numerous melanomacrophage centres (mmc) (HE). (F) Histological section of brain showing haemorrhagic ventricles (HE). (G) Histological section of kidney showing hyaline necrosis and vacuolization in the epithelial cells of renal tubules (haematoxylin-V.O.F.). Scale bars: (A) 200 µm; (B, C, D) 50 µm; (E, F) 100 µm; (G) 20 µm.



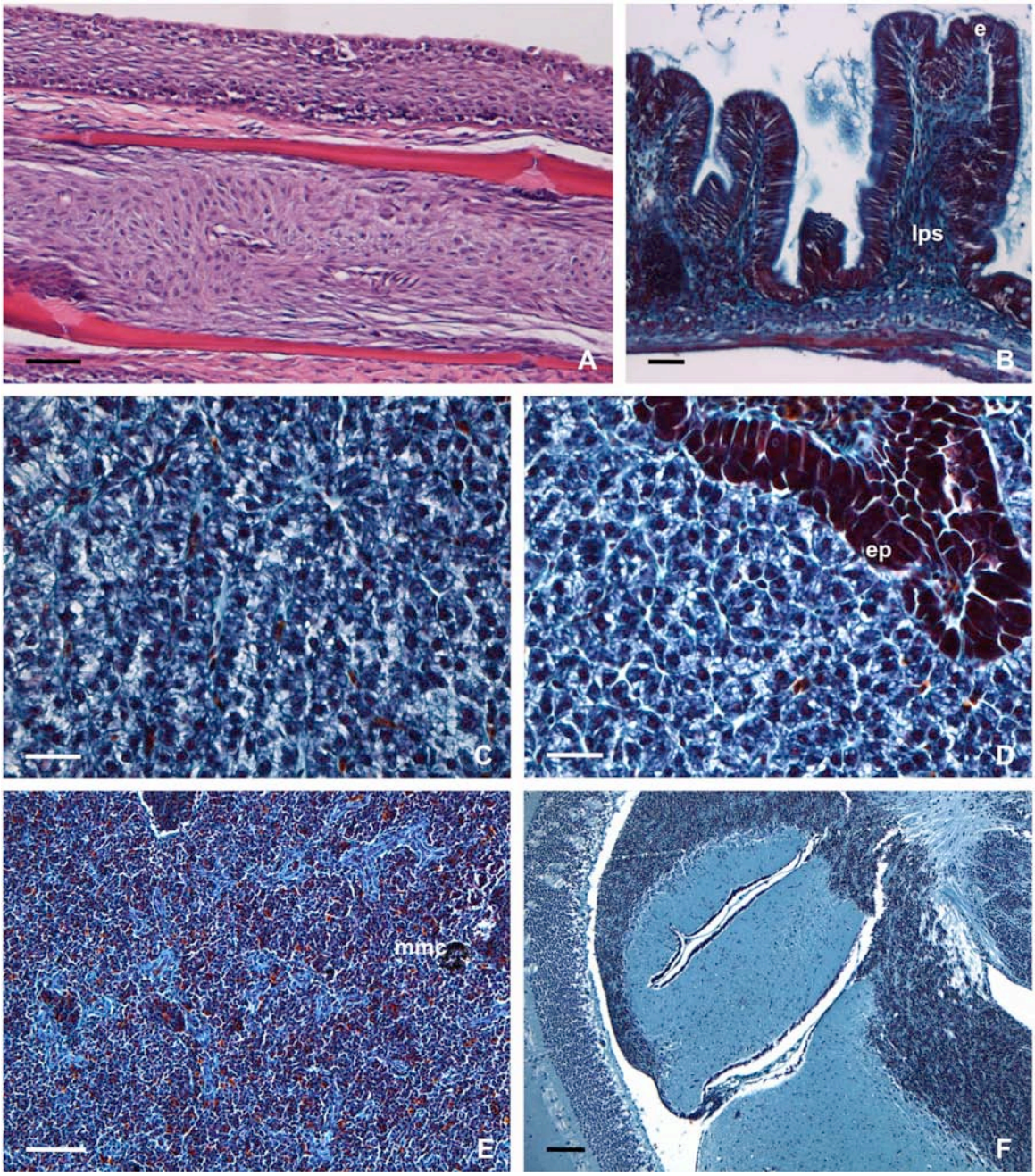


Figure 22. Histopathology of gilthead seabream recovered from lymphocystis disease. (A) Caudal fin section showing normal structure (HE). (B) Cross-section of the intestinal villi showing hyperchromatic epithelium (e) and inflammation in lamina propria/submucosa (lps) (haematoxylin-V.O.F.). (C) Basophilic polygonal-shaped hepatocytes in liver section (haematoxylin-V.O.F.). (D) Portion of exocrine pancreas (ep) showing basophilic pancreatic acini (haematoxylin-V.O.F.). (E) Spleen section showing a few melanomacrophage centres (mmc) in the parenchyma (haematoxylin-V.O.F.). (F) Histological section of brain with empty cerebral ventricles (HE). Scale bars: (A, B, C, D) 50 µm; (E, F) 100 µm.

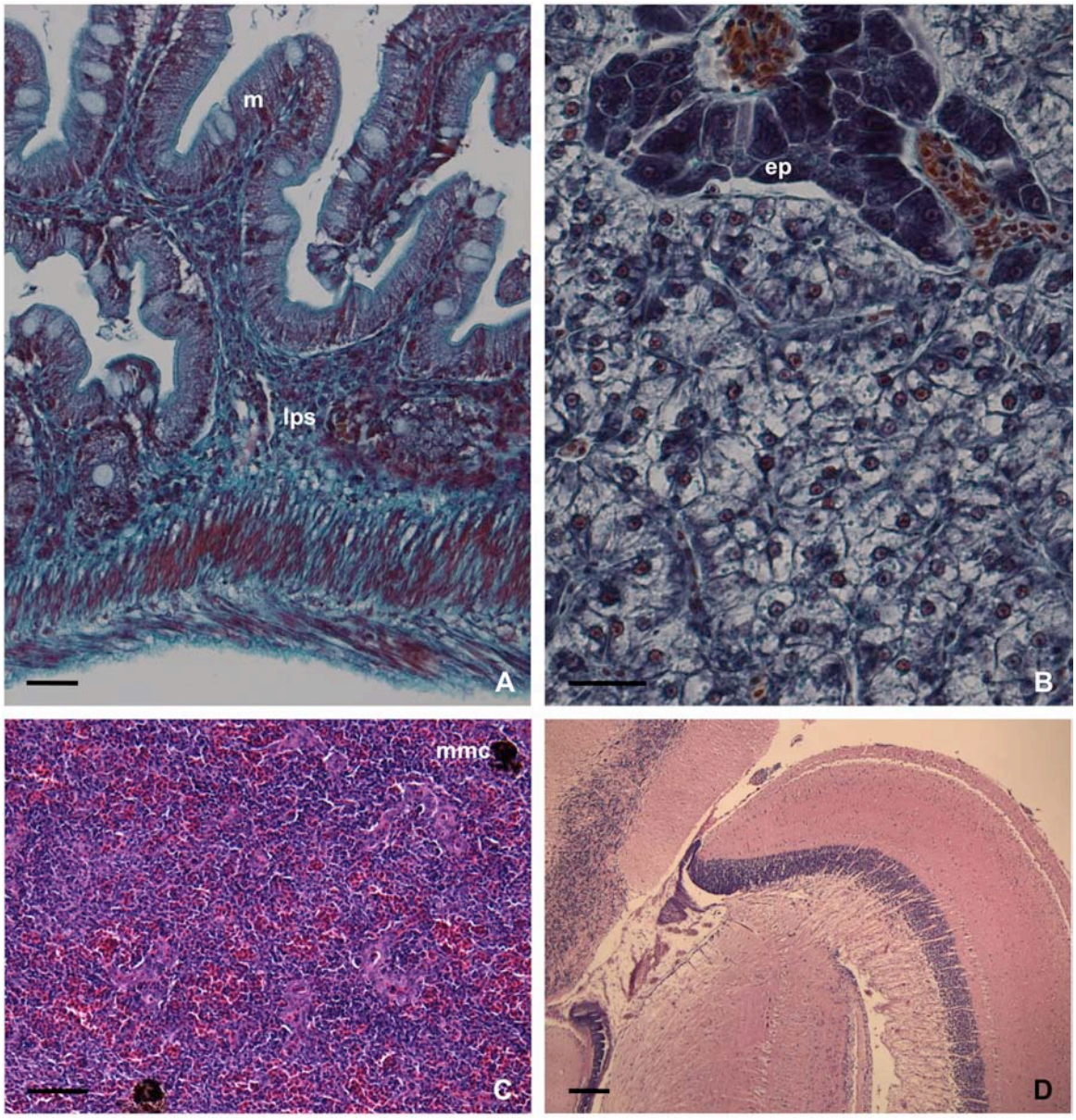


Figure 23. Histology of asymptomatic gilthead seabream. (A) Intestine section showing normal structure of the mucosa (m) and the lamina propria/submucosa (lps) (HE). (B) Histological section of kidney showing hepatocytes with polygonal shape, clear cytoplasm and nucleus in the periphery. Portion of exocrine pancreas (ep) with basophilic pancreocites distributed in acini (haematoxylin-V.O.F.). (C) Histological section of spleen with a few melanomacrophage centres (mmc) (HE). (D) Histological section of brain (HE). Scale bars: (A, B) 50 µm; (C) 100 µm; (D) 200 µm.

2. Transmission of lymphocystis disease virus to gilthead seabream larvae and fingerlings

2.1. INTRODUCTION

Transmission of LCDV has not yet been completely elucidated but direct contact is the route classically accepted for the spreading of LCDV infection, the skin and gills being the main portals of entry (Wolf, 1988; Bowser *et al.*, 1999). Viral transmission by cohabitation or by contaminated water has been demonstrated for several fish viruses, such as iridoviruses (Drennan *et al.*, 2006; Kurobe *et al.*, 2011), rhabdoviruses (Traxler *et al.*, 1993; Muroga *et al.*, 2004), aquabirnaviruses (Rodriguez Saint-Jean *et al.*, 2003), betanodaviruses (Johansen *et al.*, 2003) and orthomyxoviruses (Totland *et al.*, 1996). Some studies suggest the transmission of LCDV via the alimentary canal (Sheng *et al.*, 2007b; Cano *et al.*, 2009a), similar to that established for other waterborne viral fish diseases (Wolf, 1988; Peducasse *et al.*, 1999; Woodland *et al.*, 2002; Kurobe *et al.*, 2011).

There are no previous data supporting vertical transmission of LCDV (i.e. from broodstock to progeny via the eggs) as has been described for other fish viral pathogens, such as infectious pancreatic necrosis virus (IPNV), infectious haematopoietic necrosis virus (IHNV), viral nervous necrosis virus (VNNV), infectious salmon anaemia virus (ISAV) or white sturgeon iridovirus (WSIV) (Mulcahy & Pascho, 1985; Bootland *et al.*, 1991; MacAllister *et al.*, 1993; Mushiake *et al.*, 1994; Georgiadis *et al.*, 2001; Breuil *et al.*, 2002; Nylund *et al.*, 2007). With the exception of IPNV, which is transmitted intra-ovum, fish viruses are generally transmitted on the egg surface (Brock & Bullis, 2001). Consequently, disinfection of the egg surface can be used as a control strategy to prevent viral transmission, reducing disease outbreaks caused by these infectious agents (Grotmol & Totland, 2000; Buchan *et al.*, 2006).

Routine virological analyses carried out in gilthead seabream hatcheries from farms suffering recurrent LCD outbreaks in juveniles have demonstrated that larvae, post-larvae and fingerlings were usually infected by LCDV, and also

rotifers and *Artemia* naupliar/metanaupliar stages used as live food (Chapter 1, section 1; unpublished results).

Based on these results, the aim of the present study was to establish the possible source of LCDV infection in gilthead seabream larvae and fingerlings in an effort to shed light on LCDV transmission routes. In addition, iodine-based disinfection has been tested on eggs as a preventive treatment for LCDV infection.

2.2. MATERIALS & METHODS

2.2.1. Transmission studies with gilthead seabream larvae

Gilthead seabream eggs were obtained by natural spawning from a broodstock held in a farm with previous reports of LCD. The broodstock were composed of 42 fish, with a sex ratio of two males per female (Moretti *et al.*, 1999). Before stocking in the spawning tank, heparinised blood samples were collected from the fish (Chapter 1, section 1.2.1), sent to the laboratory within 24 h and analysed for LCDV detection by PCR-hybridization (see below).

Fertilized eggs were collected from the spawning tank via an overflow egg collector and placed in a 20-l bucket with clean, running, seawater. Eggs were divided into two batches (50,000 eggs each). Three samples of eggs (500 µl) from each batch were randomly collected and analysed for LCDV detection using both classical virological techniques and PCR-hybridization. Afterwards, one egg batch was stocked at a density of approximately 550 eggs per litre in a 120-l cylindro-conical tank at 19 ± 0.5 °C and 34 g/l salinity. In the other batch, eggs were disinfected by dipping in an active iodine (50 mg/l) solution for 10 min (Moretti *et al.*, 1999). Briefly, eggs were taken out of the water using a filter and quickly placed in a bucket containing 300 ml of aerated seawater supplemented with the disinfectant. After 10 min, eggs were rinsed with clean seawater, sampled for LCDV detection and stocked as described above. Both egg batches were incubated directly into the larval rearing tanks. After hatching,

rearing tanks operated in a flow through seawater system (Moretti *et al.*, 1999). Temperature and salinity were kept as indicated above.

From the fourth day post-hatch (dph), the larvae were fed with rotifers, *Brachionus plicatilis* (Bs and S-1 strains), at a concentration of 10 rotifers/ml. Rotifers were cultured in the hatchery, harvested as described elsewhere (Moretti *et al.*, 1999) and tested for LCDV before being fed to the fish. Three samples of harvested and rinsed rotifers (100 µl each), collected before feeding fish at 4, 6 and 8 dph, were analysed.

2.2.2. Brine shrimp LCDV inoculation

Brine shrimp *Artemia* cysts (AF, INVE) were decapsulated using a mixture of sodium hypochlorite (0.5 g active chlorine per gram of cysts) and sodium hydroxide (0.15 g/g cysts), following the procedure specified by Moretti *et al.* (1999). Residual hypochlorite was neutralized with sodium thiosulfate (0.1%, w/v, 5 min). Decapsulated cysts were hatched in sterile seawater (33 g/l salinity) at 26 ºC (Lavens & Sorgeloos, 1996). After a 48-h incubation, hatched instar II nauplii were separated from the unhatched and empty cysts and transferred to aquaria with fresh sterile seawater. Nauplii were reared to naupliar stage (4 d post hatching) at 26 ºC, with continuous aeration and a 24-h photoperiod, and fed with Mikrozell (Hobby).

Nauplii were inoculated by immersion with LCDV isolate SA25 (10^2 $TCID_{50}$/ml) (Cano *et al.*, 2009b), whereas animals inoculated with Leibovitz's L-15 medium (Gibco) were used as control group. After 24 h, nauplii were filtered through a synthetic net, washed and transferred to aquaria with fresh sterile seawater, and maintained as specified above. Eight days later, metanauplii from both experimental groups were filtered, washed and used to feed gilthead seabream fingerlings. The presence of LCDV on brine shrimp (two samples of 100 mg per experimental group) was determined by PCR-hybridization.

2.2.3. Gilthead seabream oral challenge

Gilthead seabream fingerlings (0.5-1 g) were obtained from a research marine aquaculture facility with no record of LCD. Five fish were randomly collected and analysed by PCR-hybridization to ensure that they were LCVD-free. Fish were divided into two groups (50 individuals per group) and stocked at a density of 2 g/l in aquaria with filtered seawater. Fish were maintained at 20-22 ºC and a 12-h photoperiod, and fed an artificial diet (500-800 µm) at a feeding rate of approximately 5% fish body weight per day.

For oral challenge, fingerlings were fed once with *Artemia* metanauplii inoculated with LCDV or L-15 medium (challenged and control groups, respectively) at a concentration of 0.2 g/l. The following day, an artificial diet was resumed, and fish were maintained at the conditions indicated above for 30 d.

2.2.4. LCDV detection by PCR-hybridization

DNA was extracted from fertilized egg samples, larvae 2 and 10 dph (two pools of 30 animals), fingerling samples, rotifer samples and artemia samples using DNAzol (Invitrogen), according to the manufacturer's instructions. Larvae and fingerlings were euthanized by anaesthetic overdose as previously specified (section 1.2.1). In the case of gilthead seabream fingerlings, samples consisted of the caudal part of the body (approximately the posterior one third of the fish body). In addition, DNA was extracted from heparinised blood samples collected from the broodstock using the QIAamp DNA blood kit (Qiagen).

A specific PCR combined with dot-blot hybridization was used to detect the LCDV genome as described in Chapter 1 (section 1.2.2).

2.2.5. Virological analyses

In parallel to viral DNA detection, eggs, pooled larvae (2 and 10 dph) and rotifer samples were homogenized (20%, w/v) in Leibovitz's L-15 medium supplemented with 2% L-glutamine, 1% penicillin-streptomycin and 2% FBS.

Homogenates were centrifuged at 5,000 x g for 10 min at 4 °C, filtered (0.45-µm pore-size filter) and used to inoculate SAF-1 cells. Cell cultures were maintained at 20 °C until the appearance of CPE (up to 15 dpi).

2.2.6. LCDV DNA quantification and gene expression

Seven gilthead seabream fingerlings from both the oral challenged and control groups (i.e. fed on metanauplii inoculated with LCDV and L-15 medium, respectively) were randomly sampled at 7, 12 and 24 dpi. Samples, obtained as specified in section 2.2.4, were homogenized (10%, w/v), and DNA and RNA were extracted from 200 µl of each homogenate using the Illustra triplePrep Kit (GE Healthcare), following the manufacturer's instructions. DNase treatment and cDNA synthesis were carried out as specified in section 1.2.2.

Viral DNA quantification and MCP gene expression were achieved by qPCR and RT-qPCR, respectively, following the protocols described in section 1.2.3.

2.2.7. Whole-mount *in situ* hybridization

Larvae were processed for whole-mount ISH following the protocol described by Cano *et al.* (2009b). Briefly, 10 animals were randomly sampled at 2 and 3 dph, and fixed with 4% neutral buffered formalin (NBF; Merck) at 4 °C overnight. After washing with PBS supplemented with Tween-80, the animals were rehydrated and permeated by sonication and proteinase K treatment. Hybridization was carried out at 42 °C overnight using the DIG-labelled probe mentioned in Chapter 1 (section 1.2.2). The endogenous phosphatase activity was blocked with 1 mM levamisole (Sigma-Aldrich), and the hybridization signal was detected using NBT/BCIP (Roche) for 1–2 h at room temperature. Animals were mounted using Aquatex (Merck).

2.2.8. Immunohistochemistry

NBF-fixed larvae sampled from 4 to 9 dph (10 animals per sample point) were dehydrated and embedded into paraffin following standard histological protocols, and sections (6 µm) were mounted on silane-treated slides (Sigma-Aldrich).

Deparaffinised and rehydrated sections were analysed by immunohistochemistry (IHC) according to previously reported procedures (Cano *et al.*, 2009a;b). After Triton X-100 permeabilization, sections were treated with levamisole or H_2O_2 (Merck) for endogenous phosphatase or peroxidase blocking, respectively. An anti-LCDV serum immunoabsorbed onto a monolayer of SAF-1 cells was used as primary antibody (Garcia-Rosado *et al.*, 2002), and pre-immunized rabbit serum as a negative control. Anti-rabbit IgG conjugated with alkaline phosphatase or with peroxidase (Sigma-Aldrich) was employed as secondary antibody.

Alkaline phosphatase activity was developed with NBT/BCIP, whilst a solution of diaminobenzidine (Sigma-Aldrich) and H_2O_2 was used for peroxidase detection. Tissue sections were mounted with a coverslip using Entellan (Merck) or Aquatex, respectively. In parallel, some sections were stained with HE for histological studies.

2.3. RESULTS

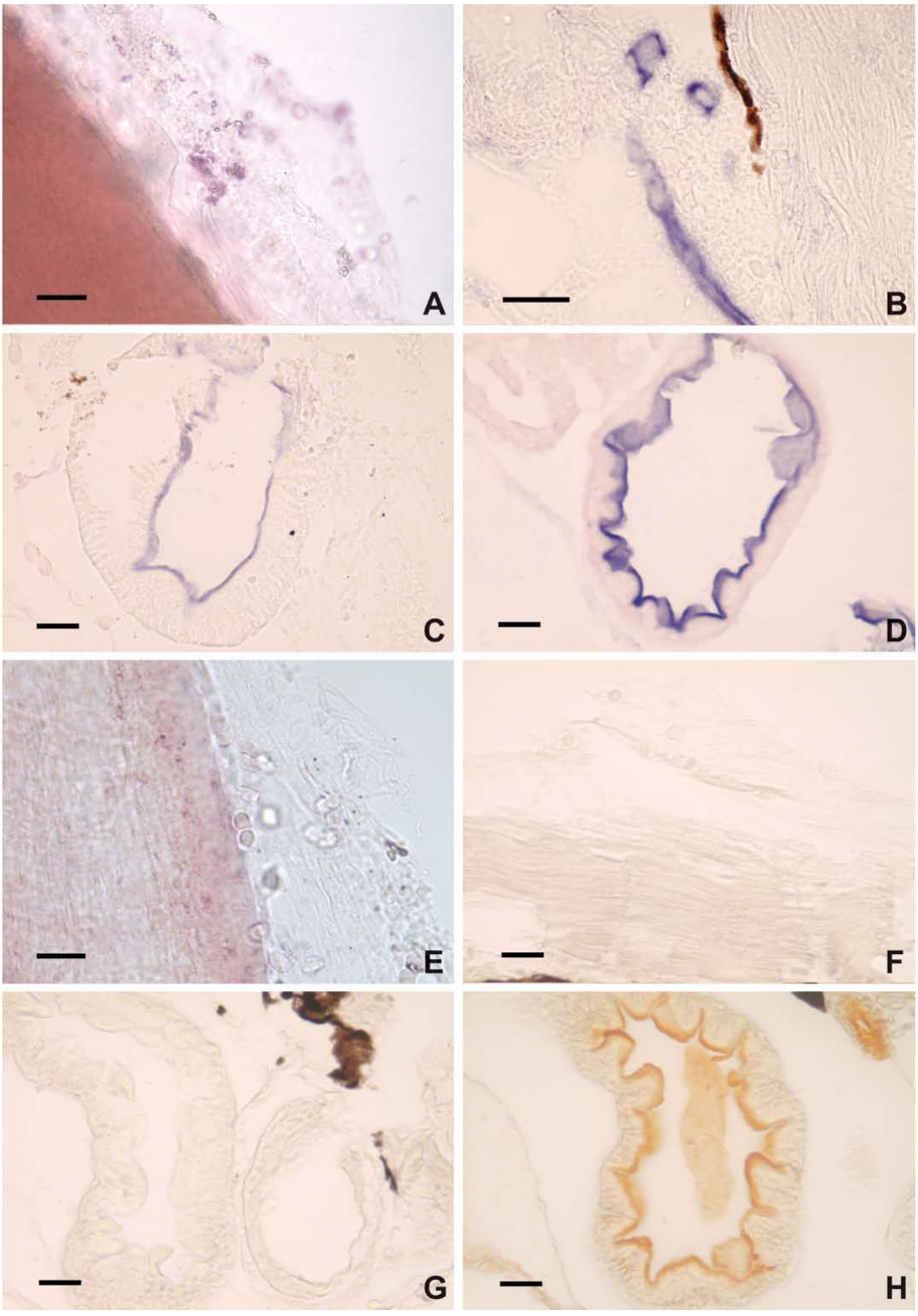
2.3.1. LCDV transmission to gilthead seabream larvae

LCDV genome was detected by PCR-hybridization in the blood from 7 out of 40 gilthead seabream specimens analysed. Using the same methodology, eggs spawned by these animals were also found to be LCDV positive, as well as 2-day-old larvae hatched from them (in both cases, 100% of the analysed samples were positive). Conversely, all the analyses performed to detect viral DNA from iodine-treated eggs, or from their larvae, generated negative results. Larvae analysed at 10 dph (from both experimental groups, i.e. non-disinfected

and disinfected eggs) showed positive results for LCDV detection by using PCR-hybridization.

The inoculation of SAF-1 cells with homogenates from PCR-hybridization positive eggs and larvae resulted in the appearance of LCDV-typical CPE (i.e. rounded and enlarged cells showing cytoplasmic inclusions) on the SAF-1 cell line. No CPE were observed on cells inoculated with homogenates from disinfected eggs or from their newly hatched larvae.

Figure 24. Lymphocystis disease virus (LCDV) detection in gilthead seabream larvae hatched from LCDV-positive (A-D) and negative (i.e. iodine-disinfected) (E-H) eggs, demonstrated by *in situ* hybridization (ISH) and immunohistochemistry. Hybridization signal is observed microscopically as dark blue staining (A, E), whereas immunolabelling appears either as dark blue (B-D) or brown (F-H) in colour, depending on secondary antibody used (conjugated with alkaline phosphatase or peroxidase, respectively). (A) Viral genome detection by whole-mount ISH in a 2-day-old larva. The hybridization signal is located in the epidermis. (B) Immunopositive primordial fin of a larva 4 dph. (C) 4-day-old larva showing a weak immunostaining in the digestive border. (D,H) LCDV antigens in the digestive tract of 6-day-old larvae. (E) ISH-negative epidermis in a larva 3 dph. (F,G) Immunonegative epidermis and digestive tract, respectively, of a larva analysed at 5 dph. Scale bars: 20 µm.



The whole-mount ISH technique showed the presence of viral DNA in the epidermis of larvae (2 and 3 dph) hatched from LCDV-positive eggs (Fig. 24A); 92.9% of the larvae examined were positive. In addition, IHC analyses also showed the presence of viral antigens in these animals. In all the larvae analysed at 4 dph, immunostaining was observed in the epidermis of body skin and primordial fins (Fig. 24B). In some sections (33.3% of the observed larvae), the digestive borders appeared weakly positive (Fig. 24C). No immunolabelling was observed when preimmune serum was used as primary antibody. Two- to five-day-old larvae hatched from disinfected eggs did not exhibit LCDV specific signal when they were analysed either by whole-mount ISH or IHC (Fig. 24E–G).

Four days after hatching, larvae deriving from both groups of eggs (disinfected and non-disinfected) were fed rotifers daily. The rotifer cultures were LCDV positive, as demonstrated by PCR-hybridization and CPE development on SAF-1 cells. In larvae hatched from LCDV-positive eggs, an increase in immunolabelling in the skin, digestive tract and digestive content was observed after feeding began (Fig. 24D). More than 97% of these animals showed viral antigens in the skin, and 88.9% of them also showed immunopositive digestive tracts. In addition, more than 92% of larvae from disinfected eggs analysed by IHC showed LCDV antigens in the epidermis and digestive tract from 6 dph (Fig. 24H). Microscopic examination of IHC processed larvae was systematically focused on the skin and digestive tract, although in some sections of larvae 6 dph onwards (from both experimental groups), immunolabelling was observed in brain and liver (no data on prevalence of viral antigens were recorded).

Microscopic examination of HE-stained tissue sections of larvae did not reveal histological alterations in LCDV-positive animals compared with negative ones.

2.3.2. LCDV transmission to gilthead seabream fingerlings

Artemia metanauplii were effectively contaminated by LCDV, as demonstrated by PCR-hybridization, whereas metanauplii in the control group remained LCDV-negative.

In fingerlings fed on the LCDV-positive metanauplii (challenged group) LCDV was detected by qPCR in all fish and at all time points analysed. At 7 dpi, the estimated viral load ranged between 10.6 and 26.8 copies of viral DNA per mg of tissue. Five days later, viral loads were significantly higher ($p < 0.01$), and they remained at similar values at 24 dpi (Fig. 25A). No LCD symptoms were observed in these fish at the end of the experiment (30 dpi). MCP gene expression was also detected in all fish analysed, with the highest F values observed at 12 dpi (Fig. 25B). Neither LCDV genomes nor mRNA were detected in fish from the control group (i.e. fed on metanauplii inoculated with L-15 medium).

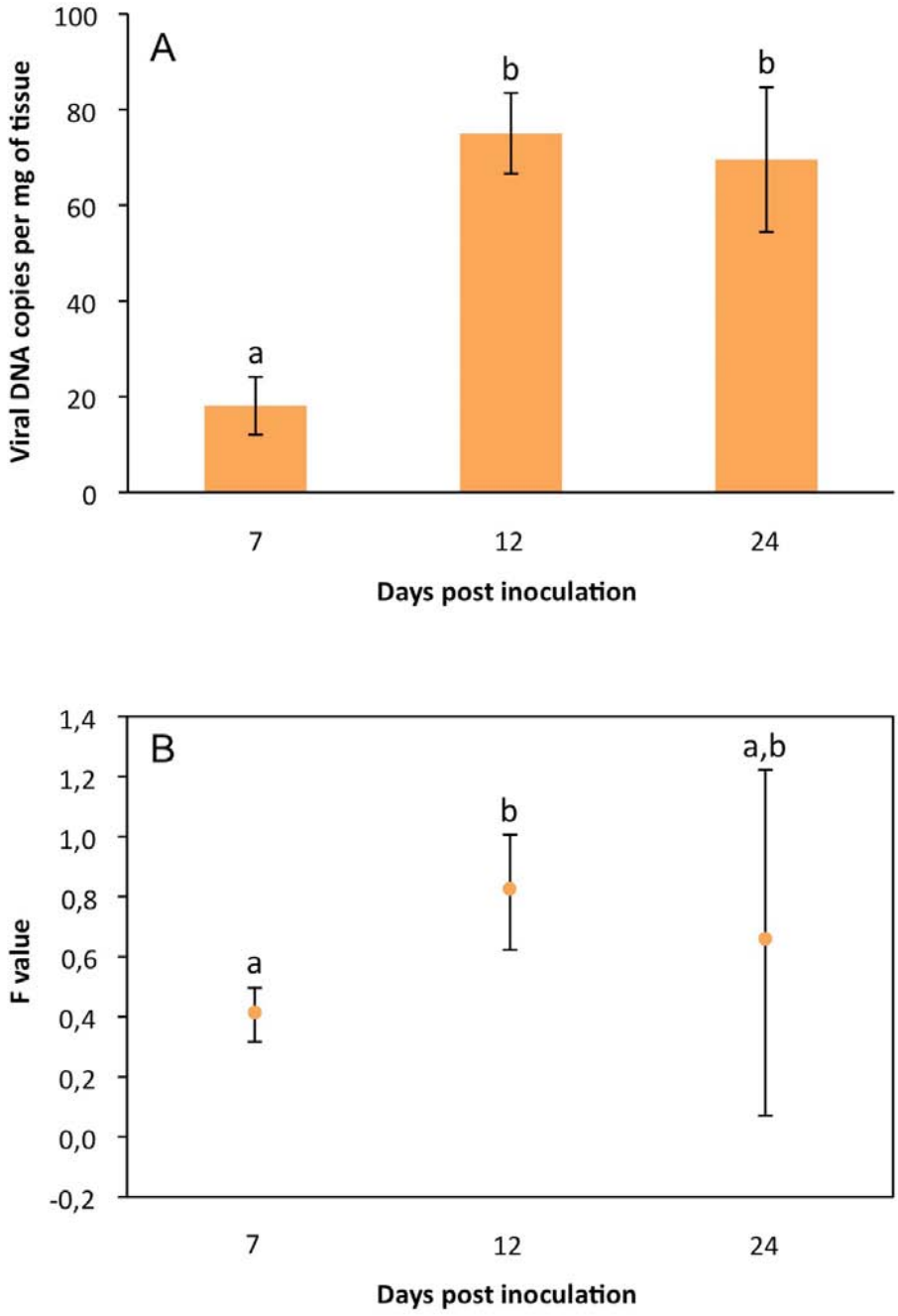


Figure 25. Viral loads (A) and relative major capsid protein gene expression values (B) in gilthead seabream fingerlings orally challenged with LCDV-positive *Artemia* metanauplii (mean ± standard deviation; n = 7). Different letters indicate significant differences ($p < 0.01$) (Mann-Whitney U-test, Holm-Bonferroni correction).

3. *Artemia* sp., a susceptible host for lymphocystis disease virus

3.1. INTRODUCTION

The brine shrimp *Artemia* (Crustacea, Branchiopoda, Anostraca) is an aquatic crustacean frequently used for feeding of fish in larval stages in aquaculture practice (Lavens & Sorgeloos, 1996; Sorgeloos *et al.*, 2001). Several authors have considered *Artemia* nauplii as a possible source for the introduction of microorganisms into the rearing systems, including bacteria, viruses and protozoa (Austin & Allen, 1982; Muroga *et al.*, 1987; Nicolas *et al.*, 1989; Mortensen *et al.*, 1993; Skliris & Richards, 1998; Mendez-Hermida *et al.*, 2006), and in most studies, a mechanical carrier stage has been proposed (Overstreet *et al.*, 1988; Mortensen *et al.*, 1993; Skliris & Richards, 1998).

LCDV has been detected by PCR-based methods in *Artemia* cysts and nauplii collected in gilthead seabream hatcheries (Cano *et al.*, 2009b; Chapter 1, section 1). The studies have demonstrated that *Artemia* nauplii become easily contaminated with LCDV by immersion challenge, and they can act as a vector for LCDV transmission to gilthead seabream (Cano *et al.*, 2009b; section 2.3.2). Furthermore, infectious LCDV persists along the *Artemia* life cycle, as viral genome and antigens and CPE on SAF-1 cells have been observed (Cano *et al.*, 2009b). These findings suggest that *Artemia* might act as a reservoir of LCDV and can support viral replication to some extend.

On the basis of these results, experimental infections with LCDV using different developmental stages of *Artemia* we carried out to determine if this crustacean is a susceptible host for LCDV.

3.2. MATERIALS & METHODS

3.2.1. Experimental infections

Artemia cysts were decapsulated and hatched, and nauplii were reared to the adult stage as previously described (section 2.2.2). Three different developmental stages (metanauplii, juveniles and adults) were taken from this stock at 8, 14 and 21 dph, respectively.

The infectivity of LCDV at different developmental stages of *Artemia* was tested by immersion challenge, using an infectious dose of 10^2 $TCID_{50}$/ml during 24 h as specified in section 2.2.2. Two LCDV isolates were used for the challenges, LCDV SA25, from gilthead seabream (belonging to genotype VII), and LCDV strain Leetown NFH (ATCC VR-342) (genotype VIII). Brine shrimp inoculated with Leibovitz's L-15 medium were used as the control group.

Samples of brine shrimp, approximately 100 mg in weight, were collected at several times post-inoculation (1, 3, 5, 8, 12, 15 and 23 d). The animals were washed with sterile seawater, gently dried and frozen in liquid nitrogen. Samples were ground in liquid nitrogen using a Mixer Mill MM400 (Retsch), and subsequently used for both nucleic acid extraction and virological analysis.

3.2.2. LCDV DNA quantification and gene expression

Total DNA and RNA were extracted from 20 mg of tissue powder using the Illustra triplePrep Kit. DNase treatment and cDNA synthesis were carried out as specified in section 1.2.2.

Viral DNA quantification and MCP gene expression were carried out by qPCR and RT-qPCR, respectively, following the protocols described in section 1.2.3, except that relative viral gene expression values (Rv) were calculated using the comparative delta-Ct method with *Artemia* actin expression used for normalization. No significant differences in Ct values were observed for this housekeeping gene between different experimental groups during the course of the infection (CV = 1.02%, Kruskal-Wallis test H = 2.27, $p > 0.13$). The calibrator

used for each experimental infection was a sample of brine shrimp for the control group collected at 8 dpi. Primers for *Artemia* actin gene detection by qPCR (Art-actin-F: 5'-GGTCGTGACTTGACGGACTATCT-3', and Art-actin-R: 5'-AGCGGTTGCATTTCTTGTT-3') were designed using Primer Express Software v3.0 (Applied Biosystems) based on the sequence obtained from GenBank (accession no. X52602.1).

3.2.3. Virological analysis

A total of 50 mg of tissue powder was suspended in 1 ml of Leibovitz's L-15 medium supplemented with 2% L-glutamine, 1% penicillin-streptomycin and 2% FBS, and clarified by centrifugation (10,000 x g for 5 min at 4 °C). These homogenates were used for viral analysis as specified in section 2.2.5 or were kept at -20 °C until used for virus titration. Infectious titres were determined using the ICC-RT-PCR protocol described in Chapter 1 (section 3.2.2).

3.3. RESULTS

To establish if LCDV replicates in *Artemia* cells, experimental infections were carried out using LCDV SA25 and three developmental stages of *Artemia*. The time course of the experimental infection was studied by analysing viral load and MCP gene expression in parallel. In challenged metanauplii, viral load increased by more than 2 orders of magnitude from the first to the eighth day post-infection (from 7.6×10^0 to 1.7×10^3 copies of viral DNA/mg of tissue), keeping above 10^2 copies of viral DNA/mg during the entire sampling period (Fig. 26A). In *Artemia* juveniles and adults, the time course of the infection was similar to that obtained for metanauplii, reaching the maximal value at 8 dpi (6.7×10^2 and 7×10^2 copies of viral DNA/mg of tissue, respectively) (Fig. 26A). Relative expression of viral MCP transcripts showed a similar temporal evolution for the three experimental groups analysed, reaching the highest value at 8 dpi (Fig. 26B). Neither LCDV genomes nor mRNA were detected in brine shrimp inoculated with L-15 medium.

No CPE could be observed in cell cultures inoculated with LCDV-infected *Artemia* homogenates and maintained up to 14 dpi. Nevertheless, by using the ICC-RT-PCR assay, viral infectious titre determination was carried out at 8 dpi. The estimated viral titres were 9.3 x 10^1 MPNIU/mg for metanauplii and juveniles, and 2.3 x 10^2 MPNIU/mg for infected adults.

Viral load and MCP gene expression were also investigated in *Artemia* metanauplii challenged with LCDV ATCC VR-342. Viral load reached the maximal value at 12 dpi (1.7 x 10^2 copies of viral DNA/mg of tissue), and the same was observed for relative viral gene expression (Fig. 27). In this case, the viral titre at 8 dpi was 7.5 MPNIU/mg, one order of magnitude lower than obtained in metanauplii infected by LCDV SA25.

In any of the experimental groups, clinical signs or mortality were not observed in the *Artemia* cultures.

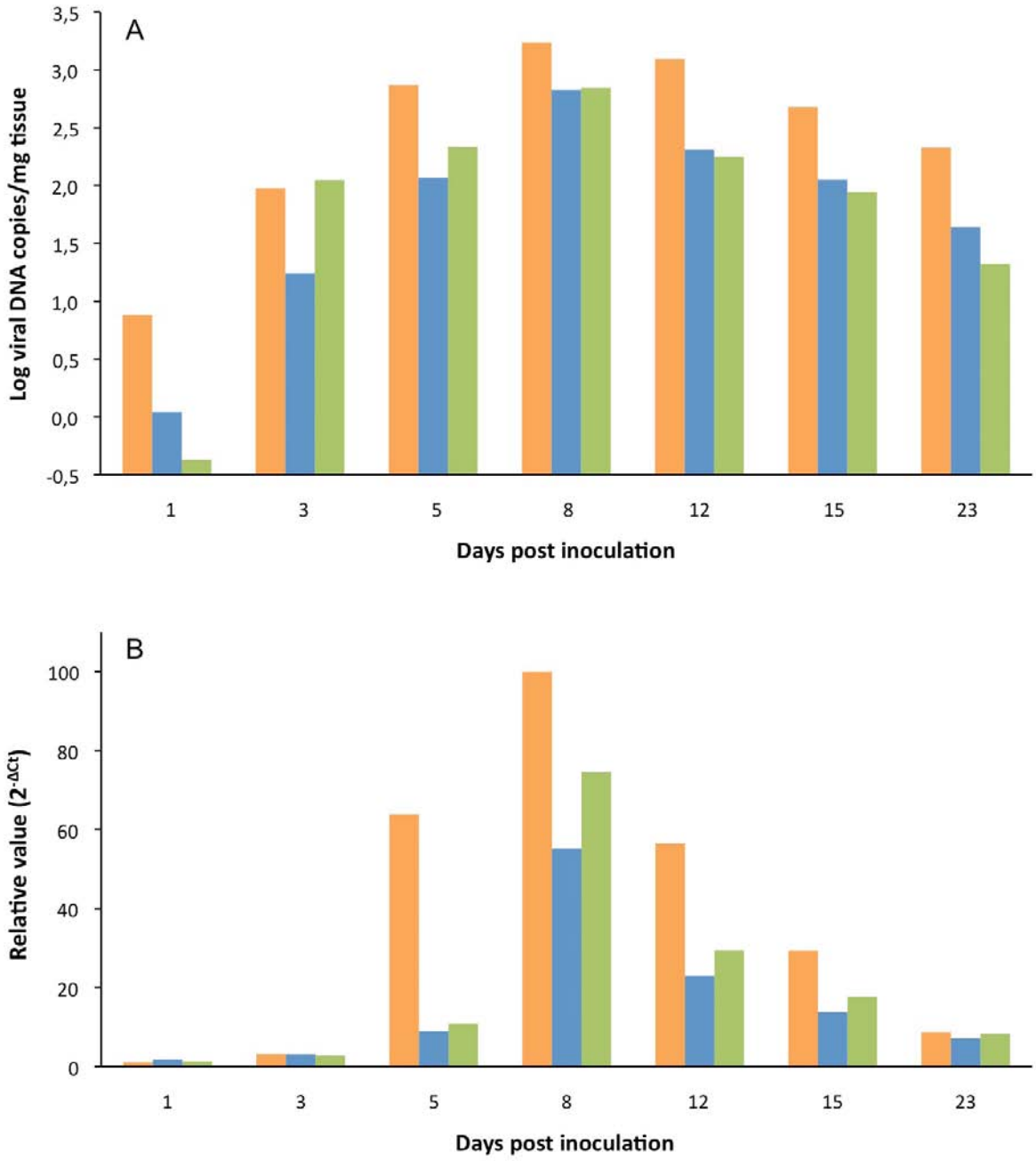


Figure 26. Temporal evolution of viral loads (A) and relative MCP gene expression values (B) in different developmental stages of *Artemia* inoculated with LCDV SA25. Orange: metanauplii (8 d post-hatching); blue: juveniles (14 d post-hatching) (blue); green: adults (21 d post-hatching).

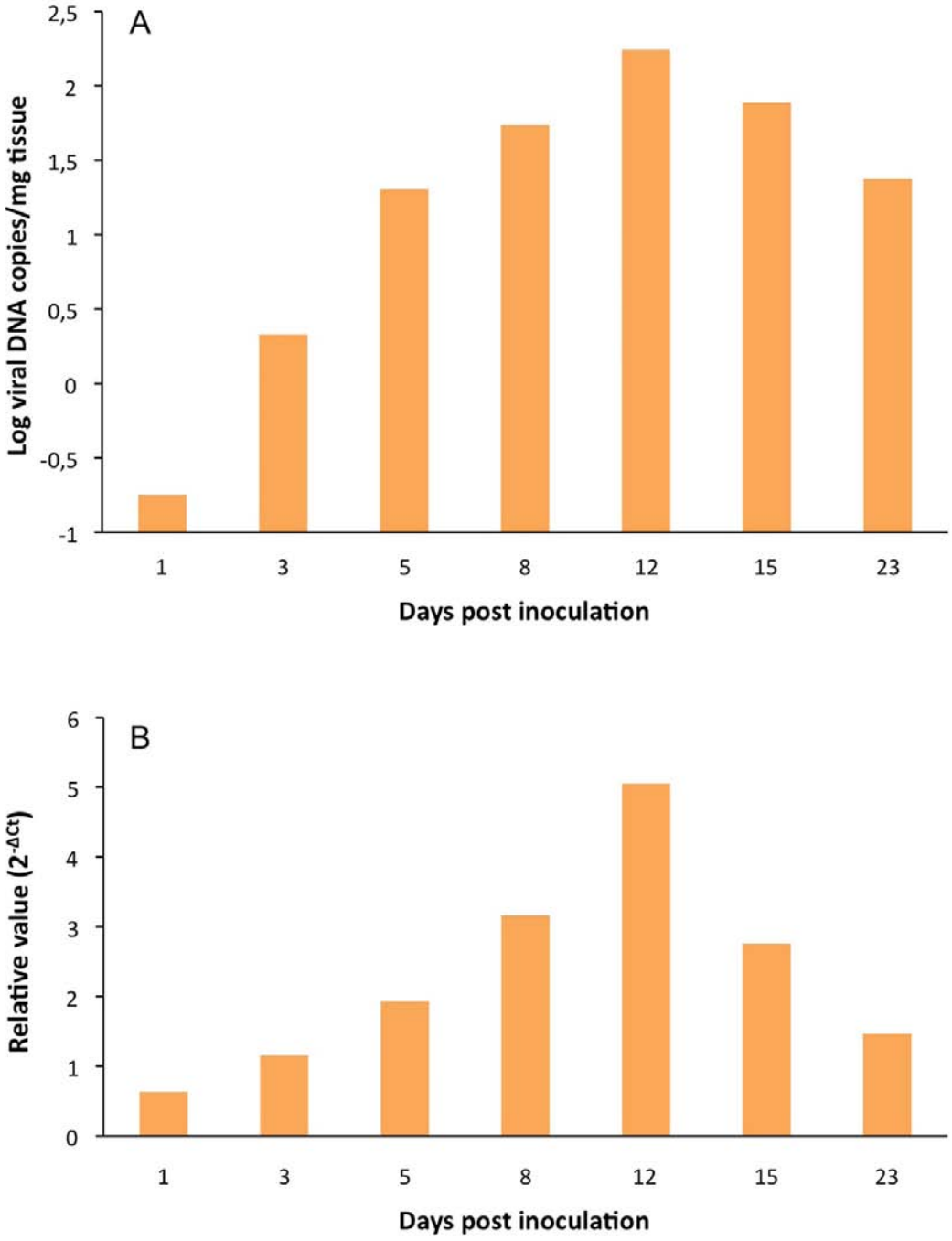


Figure 27. Temporal evolution of viral loads (A) and relative MCP gene expression values (B) in *Artemia* metanauplii (8 d post-hatching) inoculated with LCDV ATCC VR-342.

DISCUSSION

1. DIAGNOSTIC METHODS FOR LYMPHOCYSTIS DISEASE VIRUS

LCD outbreaks are frequently observed in the Mediterranean gilthead seabream aquaculture (Borrego *et al.*, 2001). Although it is usually described as a self-limiting disease, there are several reports on mortalities up to 45 % in juvenile fish, which may be related to secondary bacterial infections or with particularly large growth of lymphocysts, which severely impaired fish breathing or feeding (Colorni & Padros, 2011; Dezfuli *et al.*, 2012; Haddad-Boubaker *et al.*, 2013). As no effective treatments or commercially available vaccines currently exist, LCD prevention in hatcheries must rely on the selection of LCDV-free broodstock, the use of effective decontamination methods to prevent viral transmission from asymptomatic broodstock to larvae, and the supply of virus-free live food (Cano *et al.*, 2009b; Yoshimizu, 2009). During the growing period, the selection of non-infected fish is also advisable, as LCDV-positive juveniles may become symptomatic under stress conditions, such as transport to on-growing facilities. Moreover, little information is available on a number of epidemiological questions concerning LCDV infections, as the number of virus particles required to induce the disease, the number of genome copies present in asymptomatic and diseased fish, and the kinetics of viral replication.

Previous studies have demonstrated the applicability of a PCR-based method to detect LCDV in asymptomatic gilthead seabream carriers as well as in *Artemia* cultures (Cano *et al.*, 2007; Cano *et al.*, 2009b). Nevertheless, this method is relatively time-consuming, as an additional step of blot-hybridization of the PCR products is required to detect LCDV-positive samples. Furthermore, this assay is not quantitative and applicable for routine diagnosis.

In the present study, a qPCR assay has been developed and applied to detect and quantify LCDV in different samples. The assay was specific for LCDV, as demonstrated by analysis of the melting curves generated from each sample. Its analytical sensitivity, determined as the smallest copy number of the plasmid standard reliably detected, was 2 copies of DNA per reaction. The qPCR assay also showed a wide linear dynamic range, extending to 6 $\log_{10}$ concentrations of plasmid DNA, and infectious titres from 10^4 to 1 $TCID_{50}$. In addition, the precision

of the assay was supported by the high correlation coefficients obtained for the standard curves, and the intra- and inter-assay variation of Ct values.

Using the protocol as described, the qPCR assay allows the detection of the virus at levels as low as 1 copy of viral DNA per mg of fish tissue. This high sensitivity, combined with its wide dynamic range, makes the qPCR assay suitable to detect low viral loads in subclinical LCDV infections, and, at the same time, to quantify variable viral loads in the course of infection. In addition, it could be useful in searching for potential LCDV reservoirs.

The application of the qPCR assay to LCDV surveillance in fish farms has shown that monitoring the infection in individual fish, both diseased and subclinically infected, is possible by sampling caudal fin as reported previously (Cano *et al.*, 2007; Kvitt *et al.*, 2008). The prevalence of LCDV infection in the asymptomatic gilthead seabream populations analysed varied from 30 to 100 %, even in one farm with no previous LCD records. In these fish, estimated viral load in caudal fin was two to five orders of magnitude lower than in diseased fish. Thus viral load seems to correlate with disease manifestation. The low viral loads detected in subclinical infections may represent a status associated with viral replication as it has been established for juvenile fish during the studies on viral pathogenesis carried out in the present Thesis. In addition, the qPCR assay developed could be a valuable tool to study the correlation between viral multiplication and the onset of symptoms in experimental LCDV infections.

Palmer *et al.* (2012) developed a real-time PCR method using fluorogenic primers, specific for LCDV genotype IX sequences, which proved to be reliable in the detection of subclinically infected yellow perch, although its sensitivity was 5 x 10^2 copies of DNA per mg/l. LCDV quantification by qPCR has also been carried out in a reduced number of samples from diseased and recovered gilthead seabream (Ciulli *et al.*, 2015). The analytical sensitivity of the former assay was 5.2 copies of DNA per reaction that is more than twice the value reported in the present study. Furthermore, although the authors reported some quantitative data, viral loads were not expressed in terms of viral DNA copies per amount of tissue, which prevents further comparisons (Palmer *et al.*, 2012; Ciulli *et al.*, 2015).

Carrier fish were also identified in the broodstock from a farm with LCD records by analysing caudal fin samples by qPCR. The assay was applied in parallel to blood samples, and although LCDV could be detected, estimated viral load, and also clinical sensitivity, was lower than that obtained in caudal fin analysis. In this farm, the q-PCR assay allowed the quantitative detection of LCDV in all samples collected in the hatchery, including fertilized eggs, larvae and fingerlings, and also rotifer cultures, and *Artemia* metanauplii and cysts used for larval rearing. In these samples, as well as in caudal fin samples from asymptomatic juvenile fish, the qPCR assay showed the same clinical sensitivity than the PCR-hybridization protocol described by Cano *et al.* (2007), but is completed in 130 min, including melting curve generation, which considerably reduces the time required for LCDV diagnosis. In addition, the results of this study support the existence of multiple reservoirs of LCDV in the farm facilities, and the importance of proper application of effective disinfection treatments, as those recommended by the FAO (Moretti *et al.*, 1999), to avoid viral transmission through fish eggs or live food.

The qPCR assay developed in this study has proved to be a rapid, sensitive and reliable method for LCDV diagnosis in surveillance studies. Nevertheless, this technique has limitations as a result of the expensive reagents and equipment required that could prevent its routine use in fish farms. The LAMP method, due to its sensitivity, specificity, efficiency, rapidity and simplicity is a promising molecular technique for identifying some infectious diseases, and can be developed in the laboratory for subsequent deployment in the field, thus making it a suitable method for rapid diagnosis (Boonham *et al.*, 2014). LAMP assays have been applied to detect several fish viruses of high economic relevance (Savan *et al.*, 2005), with a sensitivity 10- to 100-times higher compared to conventional PCR (Caipang *et al.*, 2004; Mao *et al.*, 2008; Zhang *et al.*, 2009; Min *et al.*, 2013), and comparable to that of qPCR assays (Li *et al.*, 2010; He *et al.*, 2013).

In this study, a LAMP assay was designed for the detection of LCDV genotype VII. The assay has proved to be specific for this LCDV genotype, with no cross-reactions observed with the other iridoviruses tested. The analytical sensitivity of the LAMP assay, determined using ten-fold serial dilutions of the plasmid standard, was 10 copies of viral DNA, which is similar to that obtained by

qPCR, and significantly higher compared to conventional PCR (Cano *et al.*, 2007). When used on fish samples, the LAMP assay showed the same clinical sensitivity as qPCR, allowing the detection of LCDV in subclinically infected gilthead seabream and Senegalese sole. In addition, the apparatus used to perform the LAMP reactions provides information on the annealing/melting temperature of the products, which confirms the specific amplification of LCDV genotype VII in the samples. The LAMP assay is completed in less than 60 min (the duration of the assay can be precisely controlled as the amplification is monitored in real-time), even in samples from asymptomatic carriers where the viral load can be extremely low, which reduces the time required for LCDV diagnosis in comparison to the qPCR assay.

Since the Genie® II apparatus allows isothermal amplification on a low power portable platform, the LAMP protocol developed could be used in field conditions for the diagnosis of LCDV infection. In viral outbreaks occurring on fish farms, it is important to determine the presence of the pathogenic fish virus not only qualitatively, but also quantitatively. The correlation observed between the amount of template DNA, obtained from both recombinant plasmid or fish tissues, and the corresponding Tp values indicates that the LAMP assay might be useful for at least semi-quantifying LCDV without the need of expensive equipment.

One critical point of the application of diagnostic molecular methods to field samples is the requirement of high-quality DNA as template. Some authors have pointed out that LAMP is less prone to interference or inhibition by biological substances than PCR (Kaneko *et al.*, 2007; Francois *et al.*, 2011), and its efficiency does not seem to be affected by the presence of non-target genomic DNA in the reaction mixture (Notomi *et al.*, 2000). In the present study, a modified Hot-SHOT protocol has been used for crude DNA extraction, and resulted in an isothermal amplification similar to that obtained using DNA purified with a commercial extraction system. This protocol omits complex DNA purification processing; saves time, labour, and cost in the assay, and is potentially suitable for less experienced operators.

Molecular methods such as qPCR above discussed are adequate for viral detection and quantification, but they cannot be used if infectious viruses need

to be detected and/or quantified, which requires virus isolation on cell culture. LCDV is difficult to propagate in cell culture, and does not produce clear and consistent CPE, especially in samples collected from subclinically infected fish. In the present study, an ICC-RT-PCR assay, followed by dot-blot hybridization of the RT-PCR products, was developed to improve the detection of infectious LCDV.

The sensitivity of the ICC-RT-PCR assay was at least 100-fold higher than viral diagnosis obtained by CPE development. This could be partially due to the hybridization step that certainly increased the sensitivity of RT-PCR compared to agarose gel detection, as reported by other authors (Phromjai *et al.*, 2002; Cano *et al.*, 2007). In addition, the ICC RT-PCR assay could be completed in 7 d, including the dot-blot hybridization step, which considerably reduces the time required for LCDV titration when compared to the $TCID_{50}$ method (between 14 and 21 d) (Walker & Hill, 1980; Garcia-Rosado *et al.*, 1999; Cano *et al.*, 2007).

The sensitivity of the developed assay enabled the quantification of infectious LCDV in samples with low viral loads, including those from asymptomatic carrier fish, in which no CPE was recorded after a 14-d incubation period. The results obtained for viral stocks after a first passage on SAF-1 cells indicate that gilthead seabream LCDV isolates do not replicate efficiently *in vitro*, as had been suggested previously (Walker & Hill, 1980; Cuilli *et al.*, 2015).

In gilthead seabream carriers, infectious titres were more than 2.2 log_{10} lower than viral loads obtained by qPCR. Thus, although qPCR is a useful technique for routine LCDV diagnosis, the actual amount of infectious virus may be overestimated when used for viral quantification, at least in subclinically infected fish. This low viral productivity in fish tissues may be related to partial replication or defective virus assembly (Walker & Hill, 1980; Peters & Schmidt, 1995).

2. PATHOGENESIS OF LCDV IN GILTHEAD SEABREAM

The pathognomonic signs of LCD are the appearance of small pearl-like nodules on the skin and fins. The nodules are usually grouped in clusters, are papillomatous in appearance and can cover the entire body surface of the fish (Wolf, 1988). These nodules consist of LCDV-infected hypertrophied dermal fibroblasts (up to 1 mm in diameter), named lymphocysts or lymphocystis cells (Paperna *et al.*, 1982; 1987; Bowden *et al.*, 1995).

LCDV is considered a dermatropic virus (Wolf, 1988); however, in some fish species, lymphocysts have also been observed in the mesenteries, peritoneum, and several internal organs, which could indicate that the infection can become systemic under certain conditions (Howse *et al.*, 1977; Sinderman, 1990; Colorni & Diamant, 1995; Smail & Munro, 2001). Moreover, using sensitive immunological and molecular diagnostic methods, LCDV has been detected in different organs of fish without internal lesions (Sun *et al.*, 2003; Xing *et al.*, 2006; Sheng *et al.*, 2007b; Kvitt *et al.*, 2008; Ciulli *et al.*, 2015). These findings suggest a systemic condition for LCD although it has not been shown if viruses detected in different organs proceed from productive infections or are actually the result of an underlying viraemia.

In the present study, lymphocystis cells were exclusively observed in the dermis of the skin and fins of diseased juvenile gilthead seabream. Nevertheless, viral genomes were detected by qPCR in all of the organs analysed. Viral gene expression was also detected in all the samples, with the highest relative expression values recorded in the caudal fin, followed by those in the kidney and brain. Accordingly, the highest viral loads were detected in the fins, and the amount of viral genomes in the kidney and brain were significantly ($p < 0.01$) higher than in other internal organs analysed. These results support that LCDV establishes a systemic infection in gilthead seabream, similar to infections reported for other iridoviruses, such as ranaviruses and megalocytiviruses (Weber *et al.*, 2009; Whittington *et al.*, 2010).

Recent studies carried out in Japanese flounder and turbot have shown that LCDV genome copy numbers increased in all organs analysed during the

course of experimental infections, and that the extensive range of viral target tissues is, at least partially, the result of the wide distribution of the LCDV-C receptor (Sheng *et al.*, 2015; Wu *et al.*, 2015). Whether this receptor, a membrane protein of 27.8 kDa first identify in FG cells (Wang *et al.*, 2011a; Sheng *et al.*, 2012b), is present in gilthead seabream cells, and also a receptor for LCDV-Sa attachment, needs to be investigated.

Viral MCP transcripts were detected by ISH in order to identify susceptible cells supporting LCDV productive infection. As expected, LCDV expression was observed on lymphocysts located on the caudal fin but also in some cells in the surrounding connective tissue. Viral transcripts were also detected in hepatocytes, and in cells of the splenic pulp, the kidney interstitium, and the brain granular layer. This distribution of viral mRNA is similar to results of previous work that detected viral genomes and antigens in several organs of juvenile gilthead seabream (Cano *et al.*, 2009a). In the present study, it was not possible to determine which cell type contained viral transcripts in the intestine. Nevertheless, Cano *et al*. (2009a) detected LCDV-positive cells in the connective tissue of the lamina propia. Furthermore, other authors also described the detection of LCDV genomes and/or antigens in the gill lamella of LC-diseased Japanese flounder, black rockfish, and gilthead seabream (Xing *et al.*, 2006; Sheng *et al.*, 2007; Cano *et al*., 2009a). Together, these results support a broad range tissue tropism for LCDV, similar to that established for megalocytiviruses, which were described to be mesotheliotropic (Gibson-Kueh *et al.*, 2003; Marcos-Lopez *et al.*, 2011).

On the basis of the results obtained, the permissive cells for LCDV replication seem to be fibroblasts, hepatocytes, and cells of the mononuclear phagocyte system, as previously suggested (Garcia-Rosado *et al.*, 2002; Cano *et al.*, 2009a). The LCDV-C receptor has been detected in the membrane of a small portion of turbot peripheral leucocytes, which could indicate that they are susceptible to LCDV infection, resulting in LCDV spreading to different host tissues via the bloodstream (Sheng *et al*., 2015). In the gilthead seabream brain, viral transcripts were detected in cells of the granular layer, which suggests that microglial cells or infiltrating macrophages may be susceptible to LCDV, although neurons cannot be ruled out as a susceptible cell type. Further

immunocytochemical studies should be carried out to identify LCDV-infected cells in brain, using, for example, OX-42 or FL.1 antibodies that recognize monocyte-derived cells in fish (Cuoghi & Mola, 2007).

LCDV was detected at low levels in all of the organs analysed from asymptomatic and recovered fish. In addition, these organs support viral gene expression, indicating that the fish are subclinically infected by LCDV, and that this infection is also systemic. In asymptomatic fish, the highest relative viral expression value was recorded in the caudal fin, whereas the brain seems to be the main organ that supports viral expression in the recovered fish. As previously suggested by other authors (Kvitt *et al.*, 2008; Cano *et al.*, 2009a), LCDV establishes a systemic and persistent infection in gilthead seabream juveniles, which may extend for at least two months after disappearance of clinical signs.

Subclinically infected fish may be essential for LCD epizootiology. Thus, asymptomatic fish have been considered responsible for LCD outbreaks that appear in aquaculture facilities under stressful rearing conditions (Sindermann, 1996; Mellergaard & Nielsen, 1997; Austin, 1999; Cano *et al.*, 2007). These conditions might stimulate virus replication and the consequent development of symptoms (Iwakiri *et al.*, 2014). Moreover, it is assumed that fish can recover from LCD and develop acquired immunity (Nishida *et al.*, 1998; Smail & Munro, 2001). Nevertheless, recovered fish are persistently infected and, consequently, may be LCDV-carriers that could transmit the virus to naïve fish.

Histopathological studies carried out in LC-diseased fish have been focused on the description of lymphocystis cells, with few reports dealing with histological observations of internal organs, except when lymphocysts were also present (Paperna *et al.*, 1982; Colorni & Diamant, 1995; Gonzalez de Canales *et al.*, 1996; Sheng & Zhan, 2004).

In the present study, LC-diseased gilthead seabream specimens showed lymphocystis cells only in the dermis of the skin and fins, with histological characteristics resembling those previously described in this fish species (Gonzalez de Canales *et al.*, 1996). Histological alterations of different severities were also observed in all of the organs analysed, including necrotic changes in the liver and kidney, inflammatory response in the intestine submucosa, and

intraventricular haemorrhage. Necrotic changes in the epithelium of the proximal renal tubules were the only histological alterations described so far in gilthead seabream juveniles affected by LCD (Le Deuff & Renault, 1993; Cano *et al.*, 2009a). Nevertheless, histological damages similar to those observed in the present study in the liver, kidney or intestine, have also been described in LC-diseased snakeskin gourami (*Trichogaster pectoralis*) and kelp bass (*Epinephelus moara*) (Paperna *et al.*, 1987; Yongjia *et al.*, 1996). These histopathological changes can be directly related to viral replication, as in the case of the liver, where hepatocytes are actually infected by LCDV (as demonstrated by ISH in the present study or by observation of viral particles by TEM in kelp bass), where in other cases, indirect relation could be proposed. Thus, epithelial necrosis of renal tubules has been associated with substances produced by infected cells in the interstitial tissue, or, alternatively, with severe alterations in osmoregulation resulting from multiple skin lesions (LeDeuff & Renault, 1993; Cano *et al.*, 2009a). In addition, an increase in MMC was observed in the liver, spleen, and kidney, which could be associated with a cellular response to viral infection (Agius & Roberts, 2003). The proliferation of epithelioid cells around lymphocysts has also been described as an immune response against LCDV (Roberts, 1976; Colorni & Diamant, 1995; Sheng & Zhan, 2004). Finally, in recovered fish, most organs and tissues showed normal histological features, indicating that histopathological alterations associated with LCD are reversible.

3. TRANSMISSION ROUTES FOR LCDV IN GILTHEAD SEABREAM

In most Mediterranean gilthead seabream farms outbreaks of LCD affecting juvenile specimens (0.5-5 g) occurred sporadically (Garcia-Rosado *et al.*, 1999; Kvitt *et al.*, 2008; unpublished data). The analyses carried out in several Spanish farms with a history of LCD have shown that larvae through to juvenile fish are infected with LCDV, as demonstrated by viral genome detection using PCR-base methods (Chapter 1, section 1; unpublished data).

In general, viral diseases affecting larvae and juveniles are characteristically associated with vertical transmission of the virus by asymptomatic broodstock (Bootland *et al.*, 1991, Watanabe *et al.*, 2000). These carrier spawners shed the virus in their reproductive fluids, infecting ova at the time of fertilization or thereafter (Mushiake *et al.*, 1994; Nguyen *et al.*, 1997; Arkush *et al.*, 2004; Smail & Munro, 2008).

In the present study, blood samples from seabream broodstock were analyzed by PCR-hybridization (Cano *et al.*, 2007). Viral genome was detected in 17.5% of the tested animals, which were considered to be asymptomatic carriers. Seabream eggs spawned from these broodstock were also positive for LCDV, as well as most larvae hatched from them. These results suggest a vertical transmission of LCDV.

To address the question of whether LCDV is transmitted on the egg-surface or intra-ovum, fertilized eggs were disinfected with iodine. The treatment with active iodine resulted in the elimination of viral DNA from eggs, showing the superficial location of this viral contamination. Moreover, larvae developed from disinfected eggs produced negative results by both PCR-hybridization and whole-mount ISH. Therefore, the iodine treatment used in this study, which is recommended by the FAO for the culture of this fish species (Moretti *et al.* 1999), has proven to remove egg-surface LCDV contamination. This treatment has also proven to be effective in the elimination of WSIV on sturgeon eggs (Drennan *et al.*, 2006).

Although some seabream broodstock were LCDV-carriers, viral detection in ovary or reproductive fluids must be carried out to confirm vertical transmission. In experimentally LCDV-infected Japanese flounder and turbot viral load increased in the ovary over time, suggesting vertical transmission of the virus (Sheng *et al.*, 2015; Wu *et al.*, 2015). Alternatively, other routes for viral shedding could be considered (for example, faeces, urine or skin mucus) (LaPatra *et al.*, 1989; Wolf, 1988). Virus excreted by asymptomatic breeders could contaminate seawater in egg collectors, contributing to horizontal transmission to the eggs (Mazelet *et al.*, 2011).

The skin is identified as one of the key portals of viral entry accepted for LCDV (Wolf, 1988). In the present study, larvae hatched from LCDV-positive eggs exhibited viral DNA and antigens in the epidermis, which suggests that virus present on the egg surface at the time of hatching, can result in infection of the epidermis of newly hatched larvae. At 4 dph, which corresponds to the beginning of the exotrophic phase, viral antigens were also detected in the digestive tract of some larvae, even before the introduction of live food, suggesting a waterborne transmission of virus shed by infected larvae.

Larvae hatched from disinfected eggs remain LCDV-negative during the endotrophic phase. After feeding on LCDV-positive rotifers, immunolabelling was observed in the digestive tract, which suggests that viral entry can also be achieved via the alimentary canal, as has been previously proposed by Cano *et al.* (2009a). In these animals, the epidermis became immunopositive 2 d later. Whether the virus spreads from the digestive tract to the epidermis or directly from rotifer-sourced-LCDV contaminated seawater remains unknown. These results indicated that rotifers could serve as a vector in LCDV transmission to gilthead seabream larvae. Previous studies have established that *B. plicatilis* can act as reservoir for several fish and crustacean viral pathogens (Skliris & Richards 1998; Yan *et al.*, 2004). In addition, the transmission of white spot syndrome virus (WSSV) from infected *B. plicatilis* to shrimp, *Fenneropenaeus chinensis*, and crayfish, *Procambarus clarkii*, larvae has been previously reported, demonstrating its role as viral vector (Zhang *et al.*, 2006; Yan *et al.*, 2007).

As previously demonstrated for LCDV-infected gilthead seabream juveniles, LCDV infection may also be a systemic condition in larvae, as viral

antigens have been detected in brain and liver in some larvae. Viral spreading to internal organs could be achieved from the epidermis and/or the digestive tract via the bloodstream, as previously proposed (Sheng *et al.*, 2015; Wu *et al.*, 2015).

Several authors have considered *Artemia* nauplii as a possible source for the introduction of microorganisms into rearing systems, including bacteria, viruses, and protozoa (Nicolas *et al.*, 1989; Mortensen *et al.*, 1993; Sahul Hameed *et al.*, 2002; Sudhakaran *et al.*, 2006; Sivakumar *et al.*, 2009). However, only a few studies have demonstrated the role of *Artemia* as vectors involved in the transmission of microbial pathogens to fish (Olson, 1976; Grisez *et al.*, 1996; Sitja-Bobadilla *et al.*, 2005; Mendez-Hermida *et al.*, 2007). In the present study, gilthead seabream fingerlings fed on LCDV-contaminated metanauplii become infected, as demonstrated by the increase in LCDV genome copies during the experimental period, and the detection of viral transcripts in these animals. These results indicate that *Artemia* metanauplii could be a vector for LCDV transmission to gilthead seabream.

Lymphocystis disease control is based on preventive husbandry practices, such as the removal of affected animals and other potential viral sources, and a reduction in stressful conditions (Anders, 1989; Paperna *et al.*, 1982). Knowledge of transmission patterns for LCDV can aid in disease control. The results obtained strongly suggest that LCDV is egg-surface transmitted, and can be removed by iodine-based disinfection, which can be considered as an adequate preventive measure for application in gilthead seabream hatcheries. The supply of virus-free live food appears also important, thus decontamination methods that can be safely applied to rotifer and brine shrimp cultures have to be evaluated.

4. LYMPHOCYSTIS DISEASE VIRUS INFECTION IN BRINE SHRIMP

A number of studies have confirmed the role of *Artemia* nauplii as vectors for several crustacean viruses, such as *Macrobrachium rosenbergii* nodavirus (MrNV), hepatopancreatic parvo-like virus (HPV), white spot syndrome virus (WSSV), and infectious myonecrosis virus (IMNV) (Sudhakaran *et al.*, 2006; Sivakumar *et al.*, 2009; Zhang *et al.*, 2010; Da Silva *et al.*, 2015). In addition, *Artemia* appear to be susceptible to some of these viruses, including WSSV and MrNV, with the infection being asymptomatic (Li *et al.*, 2003; Sudhakaran *et al.*, 2007). Regarding fish pathogens, *Artemia* nauplii have proven to be a mechanical vector only in the case of microsporidia and *Vibrio anguillarum* (Olson, 1976; Grisez *et al.*, 1996), although some studies have shown that they could also accumulate viral pathogens and protozoa (Mortensen *et al.*, 1993; Skliris & Richards, 1998; Mendez-Hermida *et al.*, 2007).

In the present thesis, it has been demonstrated that *Artemia* metanauplii can act as a vector for LCDV, participating in viral transmission to gilthead seabream via the alimentary route. Previous studies demonstrated that infectious virus could be detected in *Artemia* nauplii inoculated with LCDV by immersion, and the virus persisted to the adult stage, and from adults to reproductive cysts (Cano *et al.*, 2009b). These results led us to consider the hypothesis that *Artemia* could be susceptible to LCDV infection, acting as reservoir and biological vector for LCDV.

The results obtained in the experimental infections demonstrated that *Artemia* could be infected by LCDV at different developmental stages, since viral loads increased during the course of the experiments. In addition, viral transcripts were also detected, showing similar temporal evolution. Thus, *Artemia* seems to be a susceptible host for LCDV, at least in experimental conditions, with the resulting infection being asymptomatic. This is the first description of a fish virus that also infects invertebrates. Viral loads and infectious titres estimated in LCDV-infected *Artemia* were higher than those obtained in the present thesis for subclinically infected gilthead seabream fingerlings or juveniles.

It is notable that brine shrimp doubled or tripled in size during the course of the experimental infections, particularly in those performed with metanauplii and juveniles. Thus, viral loads expressed per mg of tissue are difficult to interpret, and do not reflect actual viral loads per individual. Taking this into account, the number of genome copies probably increased in each brine shrimp during the experiments.

Viral replication kinetics were similar in the four experimental infections carried out. Nevertheless, relative viral gene expression values were higher in metanauplii compared to juveniles or adults infected by LCDV SA25, although this difference was not reflected in infectious titres. Differences in relative viral expression values were observed in metanauplii infected by both viral isolates, which might indicate that viral infectivity is variable among LCDV genotypes.

CONCLUSIONES

1. El ensayo de qPCR desarrollado en esta tesis doctoral constituye un método sensible, específico y fiable para la detección y cuantificación del genotipo VII del LCDV en doradas. Además, el método es rápido y apropiado para estudios de vigilancia epidemiológica, donde es esencial la detección de infecciones subclínicas en peces portadores. Su empleo es también útil para la identificación de reservorios del virus o para estudios de replicación vírica en doradas.

2. El ensayo de LAMP diseñado permite la detección rápida y específica del LCDV (genotipo VII) con una alta sensibilidad, por lo que presenta algunas ventajas comparado con otras técnicas moleculares para su aplicación en programas de vigilancia víricos en instalaciones de acuicultura.

3. El ensayo ICC-TR-PCR desarrollado en este estudio es una técnica rápida, específica y sensible para la detección y cuantificación de LCDV infectivos. Esta técnica puede ser una herramienta valiosa para estudios epizootiológicos y de transmisión, donde es necesario determinar tanto la presencia como la cantidad de virus infectivos.

4. La infección del LCDV en dorada es sistémica, incluso en el caso de infecciones subclínicas, identificándose diferentes órganos como dianas primarias o secundarias para la replicación del virus. El curso de la infección por LCDV puede ser crónico, presentando los peces una infección persistente durante un periodo de tiempo indeterminado.

5. El LCDV presenta un tropismo muy amplio. Además de los fibroblastos de la dermis, que se convierten en linfocistes como consecuencia de la infección por LCDV, existen células en hígado, bazo, riñón, intestino y cerebro que son capaces de soportar una infección vírica productiva. Las células permisivas para la replicación del LCDV parecen ser fibroblastos, hepatocitos y células del sistema fagocítico mononuclear.

6. Los cambios histopatológicos asociados con la enfermedad de linfocistis aparecen en diferentes órganos de alevines de dorada, y no siempre están relacionados directamente con la replicación del virus. Dichos cambios histopatológicos revierten cuando los peces se recuperan de la enfermedad.

7. Los reproductores asintomáticos de doradas son portadores del LCDV, y podrían liberar partículas víricas en sus fluidos reproductivos y/o excreciones, provocando la transmisión del virus a las larvas. Además, los rotíferos constituyen un vector de infección para el LCDV, tanto por el consumo de alimento contaminado por el virus, como por el agua de cultivo.

8. El LCDV provoca una infección productiva en *Artemia*, al menos en las condiciones experimentales utilizadas, lo cual extiende el rango de hospedador del LCDV a crustáceos. Además, los metanauplios de *Artemia* pueden actuar como vector para la transmisión del LCDV a doradas.

CONCLUSIONS

1. The qPCR assay developed in this PhD thesis is a sensitive, specific and reliable method for the detection and quantification of LCDV genotype VII in gilthead seabream. The method is rapid and appropriate for viral surveys for which the detection of subclinical LCDV infections in carrier fish is essential. The assay is also valuable to identify reservoirs or to study viral replication in fish.

2. The LAMP assay was able to successfully detect LCDV genotype VII in a short period of time. The speed, specificity and sensitivity of this technique give it an advantage over other molecular methods for field use in surveillance programmes.

3. The ICC-RT-PCR assay developed in this study is a rapid, specific and sensitive technique for the detection and quantification of infectious LCDV. This technique will be a valuable tool in epizootiological and transmission studies for which both the presence and the amount of infectious virus needs to be determined.

4. LCDV infection is a systemic condition in gilthead seabream, even for subclinical infections, where several organs seem to be primary or secondary targets for virus replication. The course of LCDV infection can be chronic, with fish persistently infected for an undetermined period of time.

5. LCDV has a broad range tissue tropism. In addition to dermis fibroblast that become lymphocysts after LCDV infection, cells from the liver, spleen, kidney, intestine, and brain could support a productive viral infection. The permissive cells for LCDV replication seem to be fibroblasts, hepatocytes and cells of the mononuclear phagocyte system.

6. Histopathological changes associated with LCD appear in different organs in juvenile gilthead seabream, and are not always directly related to viral replication. In recovered animals, these histopathological changes are reverted.

7. Asymptomatic LCDV-carrier gilthead seabream broodstock may shed viral particles in their reproductive fluids and/or other excretions, which may, in turn, be transmitted to their larvae. In addition, rotifers constitute a key route for LCDV infection, both by consumption of contaminated food or as a source for virus in the rearing seawater.

8. LCDV establishes a productive infection in *Artemia*, at least under the experimental conditions examined here, which extend the host range of LCDV to crustaceans. Furthermore, *Artemia* metanauplii can act as a vector for LCDV transmission to gilthead seabream.

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