Experimental approaches to study the transmission and infection of *Cardiocephaloides longicollis* (Rudolphi, 1819) Dubois, 1982 (Trematoda, Strigeidae) in fish

Doctoral Thesis by

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CERTIFICAN que Dña. **Gabrielle Shira van Beest** ha realizado bajo su dirección, y con el mayor aprovechamiento, el trabajo de investigación recogido en esta memoria y que lleva por título "**Experimental approaches to study the transmission and infection of *Cardiocephaloides longicollis* (Rudolphi, 1819) Dubois, 1982 (Trematoda, Strigeidae) in fish**", para optar el grado de Doctora en Ciencias Biológicas.

Y para que así conste, en cumplimiento de la legislación vigente, se expide el presente certificado en **Valencia 7 de febrero de 2023**.

A.I. Born Torrijos F.E. Montero Royo J.A. Raga Esteve

A mi familia

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Summary

Summary

Trematodes (Platyhelminthes, Trematoda) are ubiquitous components of ecosystems, and in recent decades, researchers have shown increasing interest in them, especially in their transmission and infection strategies and mechanisms. Digenean trematodes form a large group, and usually have a complex life cycle with three hosts. Digenean first intermediate host is usually a mollusc, the second intermediate host can be an invertebrate or vertebrate host, and the definitive host is typically a vertebrate. In the definitive host, the parasite develops into an adult and reproduces sexually. The eggs of the parasite are then released to the environment where the miracidium, the first free-swimming larval stage of the life cycle, infects the first intermediate molluscan host. In the snail the miracidium then develops into a sporocyst or redia, in which the cercariae reproduce asexually. Numerous mature cercariae emerge from the snail to the water and quickly infect the second intermediate host. The cercariae penetrate the host and encyst as metacercariae, which undergo morphogenesis to become infective to the definitive host. In recent years, it has been suggested that some metacercariae may alter the host behaviour to increase the likelihood of being preyed by a suitable definitive host.

The strigeid *Cardiocephaloides longicollis* (Rudolphi, 1819) Dubois, 1982 (Trematoda, Digenea) is widely distributed along European coasts. Its life cycle has been replicated in the laboratory, allowing to have a complete description of the different stages of the life cycle. *Cardiocephaloides longicollis* cercariae develop in the sporocysts of infected nasariid snails (first intermediate host) and when mature, they emerge to the environment and infect the second intermediate host, fish, probably with help of sensory receptors. However, the cercariae penetration pattern into fish remains to be studied, as well as the mechanisms during their transmission. *Cardiocephaloides longicollis* infects primarily sparid fishes and encysts in their brain until the

metacercariae become infective and are trophically transmitted to the definitive host, mainly fish-eating larids, in which *C. longicollis* develops into an adult and reproduces sexually. The parasite eggs are then released into the water, where the miracidium infects the snail. Studying their structure offers a better understanding of their adaptations, but their mechanisms and transmission strategies are not yet well understood, which is critical given their importance in digenean life cycles.

The most important steps for the continuation of digenean life cycle are the transmission events from one host to the next. Trematodes have evolved adaptive strategies and behaviours to facilitate the host encounter and infection. For example, the adaptations and strategies of free-living stages are mostly associated with responses to cues from the environment and /or the host (chemo-attraction) and with mechanisms that rely mostly on larval morphology (e.g. furcocercariae exhibit a powerful and driven swim resulting from a well-developed bifurcated tail).

Parasites can deteriorate the host fitness and make them longer exposed to predators. For example, to reach the definitive host, some parasites may affect the behaviour of the second intermediate host to increase their likelihood of being preyed by the definitive host. Few studies have demonstrated that this behaviour is not due to a pathological side effect of the infection but is caused by the parasite itself. Therefore, to prove the existence of parasite-induced manipulation, theoretical studies must be combined with empirical work.

Laboratory models have proven useful to support parasitological studies, such as the model of *Toxoplasma gondii* infecting mice. *Sparus aurata* L. is an important fish in Mediterranean aquaculture, and is naturally infected with a variety of parasites, making it a widely used model in parasitological studies (e.g. the monogenean *Sparicotyle chrysophrii* and the nematode *Anisakis simplex*). Given the importance of fish used in experimental work, it is

important to follow the Guide to Animal Welfare and apply the 3Rs, which stand for “Replacement”, “Reduction” and “Refinement” to achieve high quality results. In addition, a tagging system is usually required to differentiate fish individually. However, tagging animals can have side effects and its success relies on many factors. Therefore, when establishing a line of research for specific fish species, preliminary trials to select an appropriate tagging system is recommended.

In this thesis, part of the life cycle of the digenean *C. longicollis* was reproduced in the laboratory, which provided the opportunity to conduct experimental assays. The main aim of this thesis was to better understand the transmission strategies of parasites with complex life cycles, to study in detail the behaviour of the cercariae after their emergence from the snail until the infection of the fish brain, and to optimize protocols for the study of potential effects of encysting metacercariae on fish behaviour.

In vivo fluorescent dyes can be useful in the study of larval stages such as trematode cercariae, but their impact needs to be tested beforehand to guarantee high quality results. Their use allowed to observe the penetration pattern of *C. longicollis* cercariae into *S. aurata* under laboratory conditions (**Chapter 4**). Cercariae were detected primarily in areas near the head, the dorsal fin and ventral side of the fish, indicating a preference for specific entry portals likely associated with areas close to the brain or with associated migration routes connected with it.

The versatile functionality of cercarial body structures allows them to display different behaviours according to their transmission stage (**Chapter 5**). In each stage of their transmission, cercariae use their structures in the most advantageous way, i.e. when crawling on the fish surface, the cercariae use the oral and ventral sucker, while when burrowing, they mostly perform peristaltic movements and use the surface spines and the oral sucker to open the path into

the fish tissue. *Cardiocephaloides longicollis* was detected migrating through connective tissue and muscle fibres toward the brain.

Visible Implant Elastomer tags (VIE) were found to be safe and reliable for long- and short-term tagging of a wide range of sizes of gilthead seabream under laboratory conditions (**Chapter 6**). Two injection sites were considered suitable; however, the caudal fin may be less reliable in long-term studies in growing fish. No severe immune response was observed, but migration to distant tissues of the main compound from VIE was detected, being this the first time, this has been reported in fish. Although no effects on fish health were detected, migration of VIE can likely be reduced by limiting the volume of silicone injected.

Preliminary results of behavioural experiments on individually infected fish showed that *C. longicollis* encysted in the brain of gilthead seabream did not appear to affect the perception of the light gradient, but it could affect the acuity of their responses, and probably alter other behaviours such as schooling and escape responses (**Chapter 7**). Therefore, further studies need to be conducted.

To conclude, *Cardiocephaloides longicollis* cercariae have been shown to have adaptive strategies to complete their life cycle. The use of *C. longicollis* as a model in the laboratory has provided a better understanding of the transmission strategies of parasites with a complex life cycle. The combination of different tools, such as *in vivo* dyes to label cercariae or tags to identify fish in experimental assays, have been shown to be necessary to gain empirical knowledge that supports theoretical research.

Resumen

Resumen

Introducción General

Los trematodos (Platelmintos) son parásitos cosmopolitas que se pueden encontrar en diferentes tipos de ecosistemas (Prietrock y Marcogliese 2003). Los trematodos más numerosos y estudiados son los digeneos. Estos parásitos tienen un ciclo de vida complejo que suele incluir tres hospedadores. Típicamente, un molusco es el primer hospedador intermediario, el segundo puede ser invertebrado o vertebrado y el definitivo suele ser un vertebrado (Cribb 2001, Gibson 2002). En este último hospedador, el parásito se desarrolla y se reproduce sexualmente. Posteriormente, los huevos, que contienen la larva miracidio en desarrollo, salen del hospedador definitivo y se liberan al agua donde infectan el primer hospedador intermediario, un molusco. En éste, el miracidio da lugar a la fase larvaria esporocisto o redia que, posteriormente, sale al exterior y se transforma en la cercaria, que es una larva nadadora. Éstas infectan al segundo hospedador intermediario, que puede ser un pez, donde se enquistan y posteriormente infectan al hospedador definitivo a través de la cadena trófica (Galaktionov y Dobrovolskij 2003). La transmisión a través de varios hospedadores permite una mayor dispersión del parásito y les conduce hasta el hospedador definitivo (Combes et al. 1994).

El estrigeido *Cardiocephaloides longicollis* (Rudolphi, 1819) Dubois, 1982 se encuentra en las costas europeas y su ciclo de vida fue descrito por primera vez por Prévot y Bartoli (1980). Estos autores consiguieron reproducir gran parte del ciclo de este parásito en el laboratorio; posteriormente las descripciones de Born-Torrijos et al. (2017) y Yoneva et al. (2022) han permitido conocer el desarrollo y morfología de *C. longicollis*. Sin embargo, las estrategias de

transmisión han sido muy poco estudiados y son clave para entender su comportamiento. *Cardiocephaloides longicollis* se reproduce asexualmente mediante esporocistos en el interior de caracoles nasáridos. Posteriormente, las cercarias maduras emergen al exterior, nadando en el agua hasta infectar al segundo hospedador intermediario, que suelen ser diferentes especies de peces teleósteo. Probablemente *C. longicollis* dependa de receptores sensoriales para encontrar el hospedador (Yoneva et al 2022). Una vez fijado al pez, probablemente penetre dentro del cuerpo y se desplace sobre y dentro del mismo con ayuda de la ventosa oral y ventral y las espinas de su superficie corporal (Yoneva et al. 2022). El estudio del patrón de penetración de la cercaria de *C. longicollis* resulta necesario para evaluar si hay zonas del cuerpo del pez más atractivas que otras como lugares de entrada (lo que se trata en el Capítulo 4 de la presente Tesis Doctoral), tal y como se ha revelado en otros digeneos (Karvonen et al. 2004). La natación de las cercarias de *C. longicollis* fue esbozada por Prévot y Bartoli (1980) como una vibración rápida ente dos puntos fijos del su cuerpo que les acerca a la superficie del agua, seguida de un descenso pasivo. Explorar en más detalle el comportamiento de esta fase larvaria desde el caracol (primer hospedador intermediario) al pez (segundo hospedador intermediario) es necesario para entender el éxito de su transmisión (Capítulo 5). El espectro de especies que actúan como segundos hospedadores intermediarios es más amplio que el de primeros hospedares intermediarios, ya que predominantemente infecta a peces espáridos, como es el caso de la dorada, *Sparus aurata* L. (Born-Torrijos et al. 2016). *Cardiocephaloides longicollis* se enquista en el cerebro del pez y con su ingesta se transmite a aves piscívoras, predominantemente de la familia Laridae. *Cardiocephaloides longicollis s*e desarrolla en el tracto digestivo de estas aves y, tras su reproducción sexual, los huevos de *C. longicollis* son liberados al agua completándose con ello el ciclo vital cuando el miracidio se desarrolla e infecta al molusco bentónico. Aunque la descripción de las estructuras ayuda a entender su biología, la comprensión

de los mecanismos últimos de transmisión es todavía parcial. Por ello, estudios de laboratorio pueden ayudar a obtener información empírica.

Como se ha comentado, los trematodos tienen un ciclo de vida realmente complejo en el que se transmiten entre diferentes hospedadores hasta alcanzar al hospedador definitivo. Uno de los pasos más importantes es la interacción de las fases larvarias, como el miracidio y la cercaria, con el hospedador intermediario. Ambas larvas están expuestas a las condiciones del medio acuático, y pueden afectar a su desarrollo o a su transmisión. Por ejemplo, el miracidio puede rastrear señales químicas del hospedador y la cercaria puede responder a cambios de pH, luz y temperatura para encontrar al hospedador o su hábitat (Sukhdeo y Sukhdeo 2004). Como es esperable, su transmisión también está asociada a su natación. Las cercarias con cola bifurcada, como las de *C. longicollis*, suelen tener la potencia de infectar hospedadores con movilidad activa como peces demersales que frecuentan en la columna de agua (Combes 1994, Haas 2003, Capítulo). Después del encuentro con el pez estas larvas presentan gran diversidad de respuestas que pueden ofrecer un gran rango de niveles de especificidad en la interacción entre larva y hospedador. desde el reconocimiento del hospedador, reconocimiento de la zona de penetración, a la ruta migratoria especifica al tejido final donde se ubica en el pez (Haas 2003, Hendrickson 1979). Después, los peces, y con ellos las larvas metacercarias del parásito, son consumidas por el hospedador definitivo (aves piscívoras). Para maximizar su transmisión, los trematodos pueden provocar cambios sutiles en el comportamiento del hospedador (Cézilly et al. 2013). Pero es complicado identificar si el cambio se debe a la manipulación directa del parásito o a un efecto secundario de la infección. En algunas especies se ha demostrado la existencia de manipulación del hospedador directamente por la acción de un parásito; el caso más conocido es *Euhaplorchis californiensis* que provoca un comportamiento errático en el pez y con ello una mayor exposición al depredador (Lafferty y Morris 1996).

Gran parte del conocimiento sobre los mecanismos y patrones de infección de los digeneos se ha estudiado utilizando peces como modelos experimentales. Desde que los peces se han convertido en una herramienta común en investigación, es importante aplicar los fundamentos de las 3Rs (Reemplazamiento, Reducción y Refinamiento), aparecidos en las guías del bienestar animal para los experimentos con animales vivos (Russell y Burch 1959, Fife-Cook y Franks 2019). Por eso es importante usar los métodos de marcaje (marcas) más adecuadas en los peces para el diseño de los estudios: desde los más básicos como los cortes en las aletas a marcas más sofisticadas como la telemetría (Sandford et al. 2020). Su fiabilidad depende de muchos factores, como el tipo de pez y su tamaño, ya que si se hace de forma incorrecta puede poner en riesgo los resultados de la investigación (Sandford et al. 2020). Es fundamental hacer pruebas preliminares para asegurar la eficacia y fiabilidad del método de estudio, en especial si se trata de investigación experimental. En esta Tesis Doctoral también se evalúa la aplicación de las marcas de Implantes Visibles de Elastómeros (VIE) en doradas en condiciones de laboratorio. Uno de las especies de peces más utilizadas en parasitología aplicada es la dorada, ya que es un hospedador natural de muchos parásitos del medio marino, se mantiene fácilmente en acuarios de experimentación y se produce industrialmente en piscicultura (López-Verdejo et al. 2022, Sitjà-Bobadilla et al. 2009, Villar-Torres et al. 2023). Para el desarrollo de esta tesis se ha utilizado el modelo *C. longicollis*-dorada (Capítulos 4 y 5) con el fin de ayudar a desvelar con detalle los mecanismos de transmisión de los digeneos. Para facilitar el estudio de ciclos de vida como éste, se utilizaron tinciones fluorescentes que han permitido visualizar con gran detalle los estados larvarios del parásito (Capítulo 4).

Objetivos

La finalidad de esta Tesis Doctoral ha sido conseguir un mejor entendimiento de la transmisión entre distintos hospedadores de digeneos parásitos de ambiente marino con un ciclo de vida complejo. Para ello, se ha utilizado como modelo el estrigeido *Cardiocephaloides longicollis* y se ha replicado parte de su ciclo de vida en el laboratorio. Los objetivos concretos de los estudios realizados han sido:

I. Investigar la validez de tinciones para el estudio de estados larvarios a corto y a largo plazo. Evaluar experimentalmente el efecto de tinciones fluorescente *in vivo,* Éster de Succinimidil-Carboxifluoresceína (CFSE) y "Hoechst 33342" (NB), sobre la supervivencia, actividad y capacidad infectiva de las cercarias. Identificar y describir el patrón de penetración de las cercarias de *C. longicollis* cuando entran el hospedador intermediario, usando *Sparus aurata* L. como modelo en el laboratorio, que a su vez es un hospedador natural de este parásito.

II. Investigar las funciones de las estructuras de la cercaria de *C. longicollis* en los mecanismos y locomoción de esta larva desde su emergencia del caracol hasta su enquistamiento en el cerebro de *S. aurata*. Analizar y describir la actividad de distintas estructuras del cuerpo de la cercaria durante diferentes fases de ciclo vital: búsqueda del hábitat del hospedador, anclaje al hospedador, reconocimiento, penetración e infección.

III. Evaluar experimentalmente la calidad de los estudios cuando se utilizan marcas para diferenciar individualmente los peces, usando marcas de Implantes Visibles de Elastómeros (VIE) en *S. aurata* en el laboratorio. Analizar la retención de los VIE y evaluar histológicamente los efectos de la marca considerando el tamaño del pez y su localización en éste. Con ello se pretende optimizar el uso de elastómeros en peces para la investigación aplicada a la parasitología.

IV. Mostrar la utilidad de las marcas VIE en un estudio experimental preliminar. Evaluar los cambios comportamentales provocados por el trematodo cerebral *C. longicollis* en *S. aurata*.

Materiales y Métodos Generales

El resumen de los materiales y métodos utilizados en los estudios que componen la tesis se explican en las secciones correspondientes en cada capítulo.

Capítulo 4

Estudio de cercaria teñida *in vivo* con tinción fluorescente revela la entrada de *Cardiocephaloides longicollis* (Rudolphi, 1819) Dubois, 1982 en la dorada, *Sparus aurata* L.

Introducción

El ciclo de vida de los trematodos se caracteriza por tener múltiples hospedadores y diferentes fases de desarrollo. La transmisión de las fases larvarias, como la cercaria, es crítica para el éxito de la infección del siguiente hospedador (Combes et al. 1994). Su transmisión es en general poco conocida y por ello actualmente es un importante foco de interés. La transmisión de cercarias puede parecer un gran desafío debido a su corta vida y limitación de su energía (Anderson y Whitfield 1975, Williams y Jones 1994), pero probablemente desarrollan estrategias que aseguren su encuentro con el siguiente hospedador, como es la emergencia en su mismo hábitat. En general, el proceso de penetración e infección de cercarias es poco conocido en la mayoría de las especies; por ejemplo, conocemos el punto de entrada de especies como *Diplostomum spathaceum* y *Centrocestus armatus* pero desconocemos el mecanismo de penetración (Karvonen et al. 2004, Paller et al. 2008). Tinciones

fluorescentes *in vivo*, como el CFSE, han sido aplicadas exitosamente en estudios de platelmintos y podrían ayudar a entender la estrategia de transmisión de las cercarias.

En este estudio se investiga la transmisión de la cercaria de *Cardiocephaloides longicollis* al segundo hospedador intermediario, concretamente su penetración en éste. Este digeneo infecta mayoritariamente peces demersales como la dorada (*Sparus aurata* L.) (Osset et al. 2005), uno de los peces más importantes en la acuicultura mediterránea. El objetivo de este estudio ha sido validar tinciones fluorescentes *in vivo* en cercarias de C. *longicollis*, para facilitar su seguimiento e identificar el patrón de penetración de las cercarias en el pez. A su vez, se han realizado pruebas para detectar posibles efectos negativos de la tinción sobre la supervivencia, actividad y capacidad de infección de las cercarias.

Materiales y Métodos

Se recogieron caracoles de la especie *Tritia reticulata* (L.) del Delta del Ebro (España) y fueron artificialmente infectados en el laboratorio con muestras fecales de gaviota recolectadas de la colonia de *Larus michahellis* en Benidorm (España) para asegurar la emergencia de cercarias. Tras la estimulación de su emergencia, las cercarias obtenidas fueron utilizadas en diferentes ensayos. Un total de 51 doradas juveniles procedentes de una piscifactoría de Burriana (Castellón, España) se mantuvieron en las instalaciones de la Planta de Acuarios de los Servicios Centrales de Apoyo a la Investigación Experimental (SCSIE) de la Universidad de Valencia (UV)) hasta el día del ensayo experimental

La supervivencia y actividad de las cercarias fueron evaluadas tras ser sometidas a tres concentraciones diferentes de dos tinciones fluorescentes *in vivo*: (i) Éster de Succinimidil-Carboxifluoresceína (CFSE, 20 µM, 50 µM, 100 µM) y (ii) “Hoechst 33342” (NB, 1- 3 gotas/ml). Las concentraciones se compararon con cercarias “control", no teñidas. Las cercarias fueron revisadas

cada dos horas bajo el estereoscopio durante 24 horas. En cada concentración se examinaron un total de 100 cercarias repartidas en cuatro réplicas. La actividad de la cercaria se evaluó en base a su actividad natatoria y la supervivencia hasta que la cercaria no mostrase ningún tipo de movimiento (incluyendo movimientos espasmódicos).

Para el estudio del patrón de penetración las cercarias fueron teñidas con CFSE (concentración de 50 μM). Un total de quince doradas fueron infectadas con 100 cercarias teñidas e inmediatamente fueron sacrificados con una sobredosis de MS222. Seguidamente se examinó el tegumento del pez bajo estereoscopio fluorescente. Las cercarias se localizaron por fluorescencia en un mapa del pez, siguiendo el sistema de coordenadas XY. La superficie del pez se dividió en diferentes zonas.

En el caso de la evaluación del efecto de las tinciones sobre la capacidad infectiva de las cercarias, 36 peces fueron infectados individualmente con una dosis de 50 cercarias durante 60 minutos. Los peces fueron sacrificados 20 días después de la infección y el contaje de metacercarias en el cerebro se comparó entre cercarias control, teñidas con CFSE y con NB.

El análisis del efecto de las tinciones en la supervivencia y actividad de las cercarias, se realizó comparando las diferencias entre las tinciones y concentraciones con un modelo mixto de regresión de riesgos proporcionales de *Cox* (MMCOxPH) y modelo de regresión de *Weibull (*RWM*)*. Para el examen del patrón de penetración en dorada, la distribución de las cercarias se analizó mediante modelos de procesos puntuales (PPM) con una simulación aleatoria espacial. Seguidamente, las diferencias de densidades de cercarias por zonas determinadas ((número de cercarias/número total de cercaria enganchadas al pez))/superficie de la zona (cm^2)) se analizaron con modelos mixtos lineales (LMM), usando las zonas como variables fijas y las réplicas como variables aleatorias. El análisis del posible efecto de la tinción sobre la capacidad de

infección de la cercarias, utilizó como variable respuesta el número de metacercarias detectadas en el cerebro en un modelo linear generalizado mixto (GLMM), usando el tipo de tinción como variable fija y las réplicas como variable aleatoria. Todos los análisis estadísticos se realizaron en el programa R (*R Development Core Team*, versión 3.0.1).

Resultados

En general, las diferentes concentraciones de cada tinción mostraron tener efectos en la supervivencia y en la actividad de las cercarias. Sin embargo, la concentración intermedia de la tinción CFSE no mostró ningún efecto sobre la supervivencia durante las primeras 5 horas después de la tinción (RWM, $P > 0,05$). Por tanto, el uso de CFSE a una concentración intermedia se consideró válido para estudiar el patrón de entrada de la cercaria de *C. longicollis* hasta cinco horas después de su tinción. El mapa de coordenadas de los peces mostró zonas frecuentadas por las cercarias como por ejemplo los ojos, las branquias, la aleta dorsal y la zona próxima a la región ventral (LMM, $P < 0,005$). También se observó que la tinción CFSE fue la única que tuvo un efecto significativo sobre la infección, con 1,62 veces menos metacercarias infectando el cerebro comparado con las cercarias control (GLMM, $P = 0,004$).

Discusión

En este estudio las tinciones fluorescentes *in vivo* han permitido seguir la distribución de cercarias de *C. longicollis* durante la penetración e infección de la dorada. En general, se ha detectado una mayor densidad de cercarias en el área de los ojos, branquias, aleta dorsal y cerca de la parte ventral. También se analizó el efecto de dos tinciones sobre la supervivencia, la actividad y la capacidad de infección de cercarias teñidas comparadas con cercarias sin teñir, observándose diferentes tipos de efectos según la tinción y la concentración aplicada. Es bien conocido que las cercarias pueden verse afectadas por factores

externos, como por ejemplo sustancias como herbicidas, pesticidas y varios metales pesados (Pietrock et al. 2001, Morley et al. 2002, 2003, Koprivnikar et al. 2006, Griggs y Belden 2008, Hua et al. 2016). La tinción NB incrementó la supervivencia y la actividad de las cercarias, probablemente debido a una mejor eficacia de consumo de glucógeno (Fried et al. 1982). La tinción CFSE mostró un incremento de la actividad de las cercarias a corto plazo, pero no afectó a su supervivencia. Sin embargo, es la única tinción que redujo el éxito de infección de *C. longicollis* en el cerebro de dorada. Esto podría ser debido a que CFSE tiñe las glándulas de penetración de la cercaria y compromete su penetración a través del tegumento del pez (Curwen et al. 2006, Paveley et al. 2009). Por tanto, usar CFSE como herramienta para estudiar parásitos transparentes es muy útil a corto plazo. Ambas tinciones a largo plazo requieren más pruebas para su uso.

Los detalles de la penetración de los trematodos en el hospedador son poco conocidos. Los resultados sugieren que *C. longicollis* es capaz de penetrar inespecíficamente áreas de la piel de la dorada, pero los resultados muestran una preferencia por ciertas zonas, probablemente asociado a su cercanía al cerebro o a una vía de transporte rápida como, por ejemplo, los nervios periféricos de los ojos, el sistema circulatorio al penetrar por la branquias (Höglund et al. 1991) e incluso a través del cordón nervioso central cuando penetran a través de la aleta dorsal (Höglund et al. 1991, Matisz et al. 2010).

Capítulo 5

La versatilidad de la simplicidad: Estructuras de *Cardiocephaloides longicollis* utilizados con diferentes propósitos durante la trasmisión de la cercaria.

Introducción

Los trematodos están caracterizados por tener un ciclo de vida complejo. La transmisión desde un molusco al siguiente hospedador intermediario depende de la fase larvaria, la cercaria. La cercaria nada libremente hasta encontrar el siguiente hospedador, invertebrado o vertebrado; teniendo en cuenta que su esperanza de vida suele ser corta, suele desarrollar estrategias que incrementan su dispersión para localizar el hospedador e infectarlo (Combes et al. 1994, Morley 2020). La mayoría de las especies son capaces de responder a estímulos externos como la gravedad, turbulencia, luz y sombras (Haas 1992, Fingerut et al. 2003, Haas 2003) y usarlo como guía para encontrar el hospedador, como por ejemplo su natación hacia la luz. La natación, en general, depende mayoritariamente de la estructura de la cola. Las cercarias pueden tener una cola simple o bifurcada. Las cercarias con cola bifurcada, llamadas furcocercarias, suelen tener una natación más dinámica e incluso más veloz que las cercarias sin cola bifurcada (Galaktionov y Dobrovolskij 2003, Morley 2020). Las furcocercarias suelen nadar con la cola por delante, típicamente llamado “tail-first” y, además, la bifurcación permite que la cercaria se mantenga flotando cuando necesite descansar, imitando un paracaídas al expandir las bifurcaciones a los lados minimizando con ello la decantación.

Cuando la cercaria interacciona con la superficie del hospedador, libera la cola y se arrastra sobre la piel hasta su punto de penetración donde excava su camino hacía el tejido de interés (Haas 2003, Horák et al. 2002). El tipo de locomoción en cada fase puede ayudar a entender la trasmisión de los parásitos entre

hospedadores, pero, desafortunadamente, no ha sido muy estudiado. Sin embargo, la ruta migratoria hacia el tejido de interés es un foco de investigación recurrente, ya que los trematodos infectan una gran variedad de órganos y, sorprendentemente, éstas no penetran habitualmente cerca del tejido de interés, si no que muestran rutas específicas adecuadas para avanzar, según cada especie hospedadora. Por ejemplo, la cercaria de *Diplostomum spathaceum* (Rudolphi, 1819) llega a los ojos de la trucha arco iris a través del sistema circulatorio (Betterton 1974, Höglund 1991), mientras que en el ciprínido *Pimephales promelas* llega a los ojos a través del tejido conectivo y del músculo (Ratanarat-Brockelman 1974).

El objetivo de este estudio es el análisis de los mecanismos utilizados por la furcocercaria desde su transmisión desde el caracol al pez, incluyendo su natación libre en el agua, búsqueda del hábitat del hospedador, desplazamiento sobre la piel del hospedador, mecanismo de penetración, identificación de la ruta migratoria hacía el tejido de interés y mecanismo de excavación. Para ello, se ha utilizado el estrigeido *Cardiocephaloides longicollis* (Rudolphi, 1819) Dubois, 1982, el cual tiene un ciclo de vida complejo (Prévot y Bartoli 1980, Osset et al. 2005) bien establecido experimentalmente en laboratorio, lo que permite estudiar en detalle el mecanismo de sus estructuras corporales en cada fase de su transmisión. Este digeneo se libera desde caracoles bentónicos y penetra en la piel de peces, como la dorada, y se enquista en su cerebro como metacercaria hasta que es consumido por el hospedador definitivo, un ave marina (Prévot y Bartoli 1980, Osset et al. 2005).

Materiales y Métodos

Las cercarias de *C. longicollis* se obtuvieron tras estimular su emergencia desde caracoles infectados (*Tritia reticulata* (L.)) recolectados de la laguna “Els Alsfacs” del Delta del Ebro (España). Un total de 30 doradas (*S. aurata* L.)

juveniles procedentes de una piscifactoría de Burriana (Castellón, España) se mantuvieron en las instalaciones del SCSIE hasta el día del ensayo experimental.

Las cercarias se tiñeron con rojo neutro y fueron grabadas en video con un estereoscopio o microscopio para su posterior análisis. Para el estudio de la natación, 16 cercarias fueron analizadas durante 300 segundos en una columna de agua, mientras que, para el estudio de su desplazamiento sobre la piel y la migración dentro del pez, 30 cercarias se colocaron artificialmente sobre un trozo de tejido de dorada fresco y fueron analizados durante 180 segundos. También se analizó estadísticamente la correlación entre las medidas de diferentes partes del cuerpo de la cercaria durante su fase de deslizamiento sobre la piel y excavación entre tejidos. Además, se calculó la media del éxito de anclaje de las cercarias, teniendo en cuenta la dosis de cercarias utilizada para el ensayo y tras confirmar estadísticamente que no hay diferencias significativas entre los peces con un modelo linear generalizado (GLM). Estos datos se obtuvieron parcialmente del estudio previo de van Beest et al. (2019). Todos los análisis estadísticos se realizaron con el programa R (*R Development Core Team*, versión 4.2.1) y los videos fueron editados con el programa Adobe Premier 2019 (versión 13.1.2, *Adobe Systems*).

La ultraestructura de la cercaria fue observada en detalle bajo el microscopio electrónico de barrido, enfocándonos en estructuras que intervienen en su transmisión y que no fueron estudiadas previamente por Yoneva et al. (2022), como es el caso de las espinas y la bifurcación de la cola.

Un total de 30 peces fueron infectados con una dosis de 100 cercarias cada pez siguiendo la metodología de van Beest et al. (2019). Estos fueron sacrificados secuencialmente a diferentes tiempos después de la infección, desde 30 minutos hasta 5 días. Los peces se dividieron en tres partes y se fijaron para su posterior análisis histológico.

Resultados

Tras el análisis de los videos grabados de cercarias teñidas con rojo neutro, se identificaron tres tipos de comportamiento durante su fase de natación libre: (1) posición de descanso (similar a un paracaídas), (2) natación "tail-first" y natación cuando el cuerpo de la cercaria apunta hacia delante, llamada "body-first". El 80% del tiempo analizado, las cercarias se encontraron en posición de descanso (lo que explicaría el posible desarrollo de fibras musculares en lado ventral cerca del punto de bifurcación de la cola) y un 19% nadando "tail-first", el cual parece desplazar la cercaria e incluso hacerle dar vueltas en U. Tras el análisis de los videos, se observó que la cola bifurcada actuaba como sistema propulsor, que consiste en dos secuencias: (1) brazada intensa de una de las bifurcaciones y (2) brazada de recuperación, cuando la bifurcación muestra un movimiento débil sin provocar un desplazamiento en el espacio de la cercaria. La combinación de ambas brazadas permite a la cercaria desplazarse en una dirección con "tail-first" o "body-first". Los resultados del anclaje mostraron una media de un 30% de cercarias ancladas.

Una vez se fija a la piel del pez, *C. longicollis* libera la cola y se desplaza siguiendo una locomoción similar a una sanguijuela, con pasos grandes hacia delante, usando las ventosas como puntos de anclaje. Una vez encuentra el punto de penetración, la ventosa ventral actuaría como ancla y la parte posterior junto con las espinas cefálicas empujarían y para perforar la piel. Para llegar al cerebro, se sugiere que *C. longicollis* se desplaza con movimientos peristálticos lentos con ayuda de sus espinas superficiales. La ultraestructura de las espinas mostró una morfología de tipo espina de rosal. Las correlaciones observadas entre las medidas del cuerpo de la cercaria confirmaron que las cercarias simulan el movimiento de la sanguijuela cuando se mueven sobre la piel y usan movimientos peristálticos cuando migran entre tejidos en dirección al cerebro.

Las observaciones previas y los análisis histológicos confirman que la *C. longicollis* se puede desplazar por células del tejido conectivo y fibras musculares hacia el cerebro sin provocar lesiones relevantes en el hospedador o una respuesta inmune.

Discusión

Los resultados sugieren que, aunque la cercaria muestre una estructura corporal muy básica, las estructuras se caracterizan por su versatilidad, proporcionando la capacidad de usarlas a través de las diferentes fases y hábitats que forman su ciclo de vida. En el caso de *C. longicollis*, el éxito de infectar el pez depende mayoritariamente de la capacidad de natación de la cercaria, ya que es liberada del caracol en la zona bentónica y debe nadar en contra de la gravedad para moverse en la columna de agua entre el fondo y la superficie del agua. Es posible que las cercarias aprovechen factores externos para su traslado, pero mayoritariamente parece depender de su cola. Este estudio sugiere que la cercaria de *C. longicollis* nada de forma diferente a lo anteriormente observado en otros casos (Prévot y Bartoli 1980, Combes et al. 1994). *Cardiocephaloides longicollis* propulsa el movimiento con la combinación de brazadas de la bifurcación de la cola, sin embargo, en otras especies se ha descrito las oscilaciones de lado a lado del tronco de la cola como propulsor del movimiento. La posición de descanso (efecto paracaídas) parece incrementar la fricción contra el agua, mediante el refuerzo de las fibras musculares en la parte ventral, cerca del punto de bifurcación y la presencia de espinas en la superficie de la cercaria. Además, se observó que la cercaria es capaz de cambiar el tipo de natación de “tail-first” a “body-first”, y viceversa, simplemente cambiando la fuerza aplicada en cada brazada de las bifurcaciones. Todas estas observaciones podrían explicar la baja especificidad de hospedadores intermediarios secundarios; el mecanismo de encontrar el hospedador parece inespecífico, sin embargo, la capacidad de engancharse y desengancharse de la piel de un

hospedador (resultados sin publicar) y la especificidad del patrón de penetración (van Beest et al. 2019) indican que, probablemente, *C. longicollis* utilice papilas sensoriales una vez fijado para identificar al hospedador (Yoneva et al. 2022). Seguidamente, la cercaria se arrastraría hasta el punto de entrada al pez, utilizando las ventosas que tienen una musculatura muy desarrollada (Yoneva et al 2022), lo que les permite una adhesión resistente para no desengancharse de la piel. Por contra, una vez dentro del pez, la cercaria se mueve lentamente con movimiento peristáltico, sin provocar lesiones tisulares. Sugerimos que este estrigeido llega al cerebro a través de una de las rutas más utilizadas por trematodos, el tejido conectivo y el músculo (Johnson 1971, Ratanarat-Brockelman 1974). Sin embargo, el número de cercarias detectadas, considerando que la tasa de infección es de un 30%, fue muy bajo. Es posible que el difícil procesado de tejidos de estructuras duras como el hueso, haya comprometido el seguimiento de la migración de las cercarias hacia el cerebro.

Capítulo 6

Implantes visibles de elastómero en dorada (*Sparus auratus* L.) para investigación experimental: localización de las marcas en pez para su optima retención y minimizar la aparición de lesiones histológicas

Introducción

El marcaje de peces es una herramienta que permite identificar y analizar peces individualmente, lo cual es muy útil en investigación aplicada. Existe una gran variedad de tipos de marcas (McFarlane et al. 1990) y, en general, se pueden diferenciar dos categorías: marcas naturales y artificiales. Las marcas naturales son características propias de los propios peces, fáciles de identificar, como la forma del cuerpo o marcas en la piel (Sandford et al. 2020). Sin embargo, aunque

se consideren seguras y no invasivas para el pez, no se suelen usar por su fácil alteración y por dar lugar a identificaciones incorrectas. Las marcas artificiales son comúnmente utilizadas para estudiar la biología del pez (McFarlane et al. 1990, Sandford et al. 2020). Para el diseño de las marcas es importante tener en cuenta que sean económicas, fáciles de manejar y reconocer sin comprometer la salud y el bienestar de los peces (aptitud física, crecimiento o comportamiento) (Macaulay et al. 2021, Sandford et al. 2020, Sneddon 2019). En general, las marcas artificiales más usadas en peces son las marcas externas, como por ejemplo marcas "Carlin", discos "Peterson", anclas "*T-bar*" o marcas "dardo" (Osbourn et al. 2011, Akins et al. 2014). Desafortunadamente, este tipo de marca puede provocar lesiones importantes, especialmente en espacios reducidos como son las granjas de peces o los centros de investigación, debido a su roce con otros peces o partes de las infraestructuras como paredes o redes (Mourning et al. 1994, Jepsen et al. 2015). Por tanto, en centros de investigación, el corte de aletas, marcaje de la piel con nitrógeno líquido y las marcas internas, como por ejemplo tatuajes y transmisores integrados pasivos (*Passive Integrated Transponder*, " PIT"), son de los métodos de marcaje más utilizados. El uso de marcas tipo tatuaje, con azul Alcian o elastómeros, generalmente muestran una óptima eficacia en condiciones de laboratorio, ya que no suelen depender del tamaño del pez (al contrario que, por ejemplo, las marcas PIT, ver Jepsen et al. 2005) y tienen una larga duración, (ej. al contrario de otras como el corte aletas o marcaje cutáneo Eriksen et al. 2011, Mortensen et al. 2013). Estas marcas son económicas, fáciles de aplicar y reconocer externamente (Cooke et al. 2013, Wilder et al. 2016), destacando los elastómeros, ya que son resinas biocompatibles que se pueden combinar con diferentes colores, ofreciendo muchos códigos de coloración. Éstos son utilizados en gran variedad de animales, desde invertebrados a vertebrados, y se considera que no afectan al bienestar del animal. Sin embargo, es aconsejable comprobar la eficiencia y los posibles efectos secundarios, ya que puede variar según diferentes factores como la especie y las condiciones en las que se encuentra el animal y la experiencia

del personal investigador que manipula a los animales. Este paso es importante, especialmente desde que se ha detectado la migración de la resina de los elastómeros a otros tejidos como el riñón en renacuajos. En algunos de estos casos se ha detectado una importante respuesta inmune (Cabot et al. 2021).

El objetivo principal de este estudio es evaluar y optimizar el marcaje de doradas (*Sparus aurata*) con elastómeros en condiciones de laboratorio. La dorada es uno de los peces más importantes en la acuicultura mediterránea y europea (FAO 2022) y es un animal recurrente en la investigación aplicada, como la parasitología marina (van Beest et al. 2019, López-Verdejo et al. 2022). Sin embargo, bajo condiciones de laboratorio, donde el espacio es limitado, se esperan posibles conflictos asociados a un comportamiento territorial en las doradas (McRobert y Bradner 1998, Pavlov y Kasumyan 2000). Por tanto, es importante utilizar marcas, como por ejemplo elastómeros, que no atraigan la atención de los miembros del grupo de peces y evitar un comportamiento agresivo (observaciones personales con otro tipo de marcas, resultados no publicados). A pesar de cumplir con muchos requisitos importantes para el adecuado marcaje de peces en condiciones de laboratorio, los elastómeros no son comúnmente utilizados en estudios con doradas (Astorga et al. 2005, Navarro et al. 2006, Montero et al. 2009). El objetivo de este estudio ha sido validar el uso de múltiples colores de elastómeros en doradas en condiciones de laboratorio, mediante el análisis de la retención de las marcas y la evaluación de la aparición de posibles lesiones tisulares u otros efectos secundarios en la salud, asi como el bienestar del pez a corto y a largo plazo. Para esto, se examinaron peces de diferentes tamaños marcados en dos zonas diferentes.

Materiales y Métodos

Las doradas fueron facilitadas por una piscifactoría en Burriana (Castellón, España) y se mantuvieron en tanques de experimentación de 3000 l en las instalaciones del SCSIE. Los peces se marcaron con elastómeros en dos zonas:

entre los radios de la cola y/o en entre radios y bases de los pterigióforos que dan base a la aleta dorsal. En el caso de los peces control, se inyectó tampón fosfato salino en vez del elastómero. La resina de los elastómeros se inyecta con una jeringa superficialmente, siguiendo las instrucciones del fabricante (*Northwest Marine Technology*).

Un total de 52 peces (32 peces marcados + 20 control) fueron monitoreados diariamente durante 85 días tras el marcaje (dtm) y su nivel de estrés fue cuantificado para detectar un posible deterioro del bienestar del pez en base a 3 niveles establecidos en un sistema aceptado por el Comité Ético de la UV (≤ 5 puntos, nivel de estrés muy leve; 6-10 puntos, nivel de estrés alto que hay que controlar; 11-16 puntos, el ensayo se debe cancelar y sacrificar los animales). En general, durante este estudio la puntuación obtenida ha sido 0, excepto dos semanas en las que la puntuación subió a 3 puntos tanto en los peces marcados como en los controles. En este caso, a pesar de que los síntomas mostrasen un nivel de estrés muy leve, se decidió pausar temporalmente el experimento de forma preventiva.

Para el estudio de la retención de las marcas, se midieron semanalmente 5 colores de elastómeros en peces de diferentes tamaños (20 peces con una longitud estándar de (LS, media ± DS), 20,7 ± 2,2 cm, y 12 peces de 28,8 ± 2,2 cm). Se realizaron un total de 10 muestreos a lo largo de 12 semanas (no se midieron en la semana 6 y 7, ya que peces control y marcados mostraron síntomas de nivel de estrés leve (3 puntos)). Al finalizar del experimento se sacrificaron los peces y los tejidos de alrededor de los elastómeros se fijaron para la evaluación histológica.

Un total de 228 doradas se fijaron para la evaluación histológica de los posibles efectos de los elastómeros en dos zonas diferentes (dorsal y caudal) a corto, medio y largo plazo, en diferentes tamaños de pez. A corto plazo (4, 8 y 20 dtm) se analizaron tejidos de 24 peces (12 peces de tamaño pequeño de 12,3 ± 5,0

cm; 12 peces de tamaño grande de 25,4 ± 2,6 cm), a medio plazo se analizaron 52 peces (85 dtm, longitud de los peces indicado previamente) y a largo plazo (180 dtm) se analizaron 152 peces (93 peces de tamaño pequeño 13,9 ± 3,5 cm; 56 peces de tamaño grande de 22,5 ± 3,8 cm).

Para confirmar la diferencia de tamaños entre peces, se analizaron las diferencias en cada ensayo con modelos lineales, usando la "longitud estándar" como respuesta variable y "tamaño de grupo" como variable explicativa. El posible efecto de los elastómeros en el crecimiento de las doradas (indicador importante del nivel de estrés en peces) se analizó con un modelo lineal usando la "diferencia de longitud entre día 85 tm y 1 tm" como respuesta variable y "grupo según la marca" como variable explicativa en cada grupo de tamaño. Para estudiar la retención de la marca, los coeficientes de variación de longitud de las marcas (calculado: CV_{tag} =DS/media × 100) se analizaron en modelos de regresión beta de cada grupo de tamaño por separado, usando "zona marcada" (dorsal y caudal), "color del elastómero" (naranja, verde, rosa, rojo y amarillo) y su interacción como variables explicativas. Todos los análisis estadísticos se realizaron con el progama R (*R Development Core Team*, versión 4.1.2) y la significatividad $\alpha = 0{,}050$.

Resultados

El 100% de las doradas estudiadas sobrevivieron a los experimentos y no mostraron efectos negativos en su salud o bienestar, en base a la puntuación obtenida según la tabla guía aceptada por el Comité Ético de la UV. El crecimiento de las doradas no mostró diferencias significativas entre peces marcados y control en ambos grupos de peces (LM, peces de tamaño pequeño y grandes $P \geq 0{,}050$). Más de un 85% de las marcas se identificaron correctamente hasta los 85 dtm tanto en peces de tamaño pequeño y como grande, siendo particularmente estable en la cola caudal, con más de un 90% de éxito de identificación en ambos grupos de peces. Las marcas en general podían mostrar

un (i) acortamiento, (ii) fragmentación o (iii) dispersión, pero las medidas semanales no mostraron un patrón evidente. Algunas marcas mostraron menor longitud a lo largo del tiempo cuando se inyectó en la dorsal de peces grandes. Sin embargo, algunas marcas en la aleta caudal mostraron un alargamiento de la longitud al inicio del experimento y que se redujo al final del experimento. Este hecho fue causado, probablemente, por una dispersión inicial provocada por su fragmentación, seguido por la desaparición de la parte fragmentada. Sin embargo, cuando se analizó esta variación (CV_{tag}) con modelos de regresión beta, los peces grandes mostraron marcas más estables a lo largo del tiempo, pero los peces de tamaño pequeño mostraron mayor variabilidad en las marcas inyectadas en la dorsal en comparación con la caudal (modelo de regresión beta, $P < 0{,}001$).

El análisis histológico a diferentes tiempos post marcaje (4, 8, 20, 85 y 180 dtm) confirmó la ausencia de efectos patológicos en la zona de inyección de la marca. Sin embargo, en muestras de 180 dtm se detectó, a simple vista, la migración de resina que compone los elastómeros a otros tejidos como el riñón y el bazo, predominantemente en peces de tamaño grande marcados en la aleta caudal (30% de peces marcados exclusivamente en la aleta caudal y 44,4% de peces marcados en ambas zonas de inyección). Bajo el microscopio, sorprendentemente, no se detectó una respuesta inmune importante, observándose solo algunos centros melanomacrófagicos (CMMs).

Discusión

Este estudio ofrece la última actualización sobre la aplicación, retención y posibles efectos secundarios de los elastómeros en doradas en condiciones de laboratorio. En general, vemos que los elastómeros cumplen con los requisitos de un marcaje eficaz para estudios a largo plazo, con una rápida recuperación del pez después del marcaje y no afecta negativamente a la salud y al bienestar

de las doradas, coincidiendo con resultados del estudio de Astorga et al. (2005) con doradas juveniles de granja.

El bienestar animal es una preocupación importante en la actualidad y por ello, es importante asegurar que la metodología aplicada no compromete el estado del animal. Por eso, hay tanta variedad de tipo de marcas que se adaptan a cada circunstancia y que cada vez son más sofisticados. Nuestros resultados confirman que cuando los elastómeros se marcan superficialmente en tejido trasparentes y son visibles y fiables en diferentes especies y zonas del cuerpo, sin afectar el bienestar del animal (Sandford et al. 2020). Al igual que en otras especies, su retención en dorada es alta, particularmente en la aleta caudal. La gran vascularización y porosidad del tejido dermal de la base de la aleta dorsal podría explicar la variabilidad que se ha detectado en estas marcas, especialmente en los peces que se encuentran en fase de crecimiento (~20 cm longitud estándar). Por tanto, la base de la aleta dorsal y la aleta caudal se consideran zonas de marcaje fiables, pero se aconseja evitar la zona dorsal en peces en crecimiento.

Los peces marcados no mostraron ningún signo de necrosis, degeneración, inflamación en las zonas de marcaje confirmando la biocompatibiliad de la resina con el tejido del pez. Sin embargo, se detectó una pequeña migración de este material al riñón y al bazo en muestras recolectadas 180 dtm. Ésta es la primera vez que se detecta este fenómeno en peces, pero se ha visto en otros animales como renacuajos y tritones. Tanto en anfibios como peces, el riñón y el bazo son los filtradores principales del sistema circulatorio. Por tanto, es posible que la resina se haya transportado a través del sistema circulatorio, especialmente en peces de un tamaño mayor de 22 cm, a través de una red vascular muy desarrollada que presentan en la aleta caudal (Xu et al. 2014). Como es de esperar, una resina biocompatible con características similares a otros poliésteres con aplicación medicinal no provoca una respuesta inmune importante cuando migra (Park et al. 2012). Aun así, en renacuajos marinos se

ha detectado una respuesta inmune severa a lo largo del tiempo y por tanto sería conveniente evitar su migración simplemente reduciendo el volumen de la resina inyectada en la aleta caudal. Por tanto, en todo caso sugerimos que se realicen pruebas preliminares para asegurar la eficacia y la fiabilidad de los elastómeros en peces; siendo además crucial familiarizarse con el proceso de marcaje y la identificación correcta de cada color utilizado.

Capítulo 7

Implantes visibles de elastómero en dorada (*Sparus auratus* L.) para investigación experimental: localización de las marcas en pez para su optima retención y minimizar la aparición de lesiones histológicas

Tras el estudio de la eficiencia de las marcas de implantes visibles de elastómeros (VIE) y sus posibles efectos secundarios, se concluyó que es un método de marcaje fiable y seguro para diferenciar individualmente doradas (*Sparus aurata* L.) en laboratorio (Capítulo 6). Por tanto, se marcaron doradas con marcas VIE para evaluar si el trematodo *Cardiocephaloides longicollis* afecta al comportamiento de los peces infectados. Este parásito penetra en su hospedador secundario, el pez, y migra hasta llegar al cerebro donde se enquista y se desarrolla en metacercaria (Prévot y Bartoli, 1980). Metacercarias han mostrado causar efectos en el hospedador que parecen incrementar las probabilidades de transmisión al hospedador definitivo (Lafferty y Morris 1996). Sin embargo, hasta ahora no se ha demostrado empíricamente si *C. longicollis* afecta de esta forma al hospedador. Por tanto, ensayos experimentales evalúan el comportamiento de doradas infectadas y sin infectar (control). Para ello se infectaron en el laboratorio catorce doradas con cercarias de *C. longicollis* obtenidas tras su emersión de caracoles infectados *Tritia reticulata* (L.). Se realizaron dos ensayos diferentes, primero se evaluó la

respuesta de los peces a un gradiente de luz provocado en una acuario tubular y en el segundo ensayo se evaluó la capacidad de huída cuando fueron capturado con un salabre. Los resultados fueron examinados estadísticamente con modelos mixtos generalizados en el programa R (*Development Core Team* (versión 4.2.1.)). Los resultados preliminares muestran una pequeña diferencia entre doradas control e infectadas en su posición en el gradiente de luz. Sin embargo, las doradas infectadas bajo la presión de captura parecen ser más fácil de capturar que las "control" cuando están mezcladas en un mismo banco de peces. Esto sugiere que la metacercaria de *C. longicollis* puede provocar cambios en el comportamiento de la dorada y facilite su captura. Pero necesita ser estudiado más a fondo.

Conclusiones

Los resultados de este proyecto han conducido a las siguientes conclusiones:

1) Los resultados de este proyecto, enfocado en las estrategias de transmisión del trematodo *Cardiocephaloides longicollis,* muestran que los trematodos son excelentes candidatos para estudiar las interacciones entre parásito y hospedador, a pesar de tener un ciclo de vida complejo, con diferentes hospedadores y diferentes estados larvales.

2) La tinción fluorescente *in vivo* CFSE (Éster de Succinimidil-Carboxifluoresceína) es una herramienta eficiente para el estudio de la transmisión a corto plazo de fases larvarias transparentes como las cercarias. Por otro lado, la tinción NB (Hoechst 33342/NucBlue) parece más fiable que CFSE a largo plazo, dado el hecho de que las cercarias teñidas con NB muestran valores de infección similares a las cercarias sin teñir. Es posible que la tinción de las glándulas de penetración con CFSE afecte a su actividad y por tanto a su capacidad de infección.

3) La cercaria de *C. longicollis* infecta al pez penetrando a través del tegumento y, aunque no muestre preferencia por un flanco concreto de la

dorada, la densidad es mayor en zonas específicas del cuerpo del pez. Parece que las zonas de penetración preferidas son la superficie de la zona cercana a los ojos, las branquias, la aleta caudal y cerca de la zona ventral. Por el contrario, la aleta caudal parece ser la zona menos atractiva como área de penetración para la cercaria. Esto sugiere que *C. longicollis* muestra especificidad en ciertas zonas para su penetración, probablemente debido a que son zonas cercanas al cerebro o a rutas específicas como el sistema circulatorio y sistema nervioso que ofrecen un paso de acceso eficiente al cerebro.

4) La cercaria de *C. longicollis* muestra una natación potente y dinámica, probablemente para alcanzar al segundo hospedador intermediario, el pez. La cercaria utiliza las ramas de la cola bifurcada como una hélice para propulsar y guiar la natación. Además, la cercaria utiliza la cola para mantener su posición en la columna de agua minimizando la decantación. Cuando la cercaria extiende las ramas de la cola, provoca un efecto paracaídas que reduce su hundimiento.

5) Las estructuras multifuncionales de la cercaria de *C. longicollis* son clave para completar la transmisión al pez. La ventosa ventral y oral participan cuando la cercaria se arrastra sobre el tegumento del pez, imitando a una sanguijuela hasta encontrar el portal de penetración. Durante la penetración, la ventosa ventral actúa como un ancla y la ventosa oral empuja en contra el tegumento para abrir camino. Los movimientos peristálticos y las espinas de rosal de la superficie corporal permiten que la cercaria excave a través de tejido conectivo y fibras musculares del pez de manera exitosa. Por tanto, estas estructuras adaptables muestran su importancia para que *C. longicollis* infecte eficazmente peces hospedadores.

6) Los análisis histológicos de la migración de *C. longicollis* en el pez revelan que este estrigeido llega al cerebro de *S. aurata* mediante la excavación de tejido conectivo y fibras musculares sin provocar lesiones o una respuesta inmune importante del pez hospedador. Sin embargo, se necesitan

hacer más estudios para excluir rutas migratoria alternativas que lleguen al cerebro.

7) Mediante la evaluación de los efectos de los implantes visibles de elastómeros (VIE) sobre la salud y el bienestar de los peces, se descartaron efectos negativos y se asegura la calidad de los resultados de la experimentación.

8) No se detectaron efectos negativos en el bienestar de los peces marcados con VIE, indicando su conveniencia en estudios visuales y su fiabilidad en un gran rango de tamaños de doradas marcadas en la aleta dorsal y/o caudal. Sin embargo, para un uso a largo plazo en peces en crecimiento, muestra menos permanencia en la aleta caudal.

9) Los análisis histológicos confirman la ausencia de una respuesta inmune severa asociada a las marcas VIE. Sin embargo, la silicona que compone los elastómeros muestra una migración al riñón y al bazo en un 10,5% de los peces pequeños y un 30,4% de los peces de gran tamaño. Aunque no se detecte una respuesta inmune importante, probablemente se pueda minimizar limitando el volumen de elastómero inyectado. Ésta es la primera vez que se detecta este fenómeno en peces.

10) Peces marcados con VIE han ayudado a identificar individualemten doradas infectadas con *C. longicollis* en ensayos comportamentales. Se ha descartado que las metacercarias enquistadas alteren la percepción de la luz. Sin embargo, probablemente afecte a la precisión del pez en otras actividades.

11) El uso de *C. longicollis* como modelo de laboratorio ha permitido entender mejor las estrategias de transmisión de los digeneos. Reproducir un ciclo de vida complejo en el laboratorio ayuda a apoyar el trabajo teórico con la experiencia empírica.

General Introduction

1. General Introduction

1.1 Trematode life cycle

Trematodes (Platyhelminthes, Trematoda) are globally distributed parasites, ubiquitous in very diverse ecosystems (Prietrock and Marcogliese 2003) probably due to using a broad range of animals as hosts, enhancing their transmission between drastically different habitats (Galaktionov and Dobrovolskij 2003, Prietrock and Marcogliese 2003). Currently, trematodes comprise two subclasses, Aspidogastrea and Digenea, the latter being of a greater interest as they form a much larger group of parasites comprising more than 2700 nominal genera and 18000 nominal species. Among digeneans, complex indirect life cycles are predominant, but there are also some direct life cycles in which parasites grow and become sexually mature in a single host before releasing propagules (eggs or larvae): e.g. the opecoelid *Plagioporus sinitsini* Mueller, 1934 or the two hemiurids *Bunocotyle progenetica* (Markowski, 1936) or *Parahemiurus bennettae* Jamieson, 1966 (see Poulin and Cribb 2002). Most common life cycles involve three hosts (Fig. 1.1). While the first intermediate host is usually a mollusc, the second intermediate host can be an invertebrate or vertebrate host, and the definitive host is a usually a vertebrate, ranging from teleosts, amphibians, reptiles, birds to mammals (Cribb 2001, Gibson 2002) (Fig. 1.1). After sexual reproduction of the adult stage of the parasite in the definitive host, the eggs of the parasite are released to the environment. The eggs contain a miracidium, which is the first larval stage in the digenean life cycle, and which hatches and infects the first intermediate molluscan host, usually by penetrating through the pallium, foot and tentacles. Miracidia then develop into sporocysts or rediae with sac-like morphology, but with a great diversity among them, e.g. in shape, size, and colour (Galaktionov and Dobrovolskij 2003). The sporocysts or rediae are packed with embryos in germinal sacs that develop into cercariae by asexual reproduction. When these swimming larvae mature, they emerge from the

molluscan host to infect the second intermediate host, invertebrates, or vertebrates (Fig. 1.1). The cercariae penetrate and migrate through the body of the second intermediate host to reach the target tissue where they encyst as metacercaria. Usually, cercariae undergo morphogenesis to become infective to the definitive host, which ingests the second intermediate host (Galaktionov and Dobrovolskij 2003). In general, encysting parasites do not cause tissue damage, however some species have been shown to provoke changes in neurotransmitter levels or to damage neural tissue (Shaw and Øverli 2012), which is likely to results in phenotypical alterations (Helland-Riise et al. 2020). Digenean parasites final development usually occurs in the definitive host tract, where they mature and are ready to reproduce sexually (Galaktionov and Dobrovolskij 2003). Although complex cycles involve higher chances to fail transmission, they allow for a greater dispersal dispersion of the parasite, leading to a suitable final host in a different habitat (Combes et al. 1994).

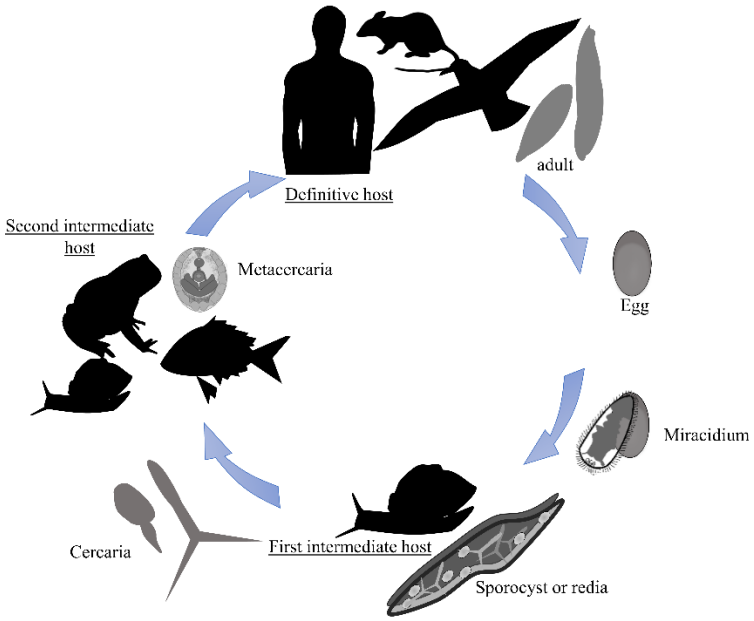


Figure 1.1. Representation of a general complex life cycle of a digenean trematode. The host spectrum is represented in black silhouettes, and the parasites in grey silhouettes.

1.2 Study parasite: *Cardiocephaloides longicollis* (Rudolphi, 1819) Dubois, 1982

The strigeid *Cardiocephaloides longicollis* (Rudolphi, 1819) Dubois, 1982 (Trematode, Digenea) is widespread along European coasts, especially in the Mediterranean, and its complex life cycle was first studied by Prévot and Bartoli (1980) (Fig. 1.2). After describing the occurrence of this species in the Brusc lagoon (Var, France) these authors accomplished to describe all stages except the miracidium by reproducing part of the life cycle under laboratory conditions. This description was recently extended using scanning and transmission electron microscopes to study the development of the two larval stages, the miracidium and the cercaria (Born-Torrijos et al. 2017, Yoneva et

al. 2022). However, some aspects of this parasite species are not yet well understood, in particular the transmission strategies used by the different larval stages to infect the next intermediate hosts. Therefore, nowadays, great efforts are being made to gain a holistic overview of the biology of this parasite.

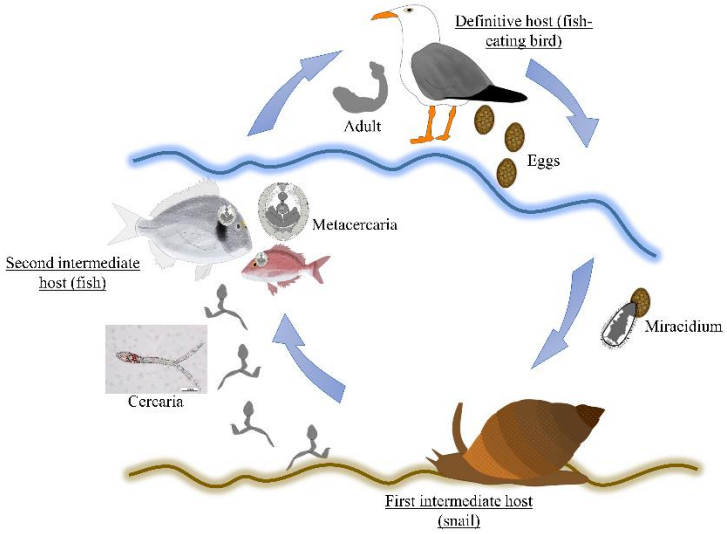


Figure 1.2. Representation of the complex life cycle of the strigeid *Cardiocephaloides longicollis,* with three hosts.

Cardiocephaloides longicollis cercariae develop in cylindrical-arcuate shape sporocysts infecting the digestive gland of nasariid snails (Prévot and Bartoli 1980, Born-Torrijos et al. 2016). To better understand the behaviour of *C. longicollis*, cercarial structures likely involved in parasite transmission have been described. For instance, the absence of eyespots and the four identified types of putative sensory structures indicate that *C. longicollis* cercariae probably rely on sensory receptors rather than light to infect the next host (Yoneva et al. 2022). The spine distribution on the cercarial body, i.e. over the entire body surface, on the oral sucker and nine transversal rings in the anterior body (Prévot and Bartoli 1980, Yoneva et al. 2022), indicates that these

cercariae use them for penetrating into fish and when moving on and within the host. Their penetration pattern into fish needs to be assessed to investigate whether cercariae show attraction to specific areas of the fish (Chapter 4), as has been observed in eye-fluke cercariae that reach the eyes of the fish by penetrating through the gills and the skin (*Diplostomum* spp.) (Karvonen et al. 2004). The swimming activity of *C. longicollis* cercariae has also been a focus of interest, as it is directly related to the transmission and infection strategy of the parasite. Their swimming has been described as a rapid lateral vibration between the two nodes of their body that leads them closer to the surface, followed by a passive descent using their bifucarted tail (furcocercariae type) as a parachute (Prévot and Bartoli 1980). Given the more detailed information available on the ultrastructural function of cercariae, further exploration of the swimming behaviour of *C. longicollis* in relation to their transmission behaviour from the snail to the brain of the fish host is necessary (see Chapter 5).

The host spectrum for the second intermediate host is wider than that of the first intermediate host, predominantly found infecting the brain of numerous sparid fish, including the important farmed fish *Sparus aurata* L. (Born-Torrijos et al. 2016). After occupying the target organ in fish, Prévot and Bartoli (1980) suggested that *C. longicollis* metacercariae become infective to the final host four months post-infection. Once this parasite is ingested along with the fish by piscivorous birds, mainly larid species (Born-Torrijos et al. 2016), they develop into an adult and remain in the anterior part of the host digestive tract (Prévot and Bartoli 1980) (Fig. 1.2). Following sexual reproduction of the adult, eggs of *C. longicollis* are released to the water where the miracidium develops and hatches (Fig. 1.2), likely depending on environmental parameters such as light or temperature (~20 days at 25 ºC, Born-Torrijos et al. 2017). Once miracidia have hatched, they must quickly infect the next host using an active host-finding strategy, i.e. swimming using

ciliary movements to locate and penetrate the snail tissue with help of the eyespots and well-developed nervous system (Born-Torrijos et al. 2017).

Although describing *C. longicollis* structures contributes to the understanding of its biology and transmission, the mechanisms are poorly understood. Given the important role of transmission strategies in completing digenean life cycles, even though they are difficult to investigate, it is crucial to increase our efforts toward a better knowledge of the broad range of strategies used by digenean parasites. The combination of different techniques, laboratory models and experimental assays, and sometimes direct observation and recording of larval behaviour (e.g. under the stereomicroscope and the fluorescent microscope, Chapter 4 and 5), helps to unravel the different mechanisms cercariae use when infecting the next intermediate host.

1.3 Transmission strategies of trematodes

As mentioned earlier, trematode complex life cycles are extremely diverse, however, most of them share common features, as parasites use at least one intermediate host and two free-living stages that inhabit different ecosystems and are commonly trophically transmitted from the second intermediate host to the definitive host. It is in these transmission events where parasites have evolved adaptive strategies and behaviours to maximize the host encounter and infection (Haas 2003, Brachs and Haas 2008, Haas et al. 2008).

One of the most important steps for the continuation of the trematode life cycle is the interaction of the larval stages, i.e. miracidia and cercariae, with the intermediate hosts. Usually, these are non-feeding stages whose short life span is limited at the expense of their energy reserves (Anderson and Whitfield 1975, Williams and Jones 1994). These larvae are free-living stages that swim in the water and are constantly exposed to environmental conditions that can act as cues for host- and/or habitat-recognition (Combes et al. 1994,

Marcogliese et al. 2001, Combes et al. 2002). Miracidia and cercariae have fundamental differences that result in agreater structural and behavioural variance in cercariae. This diversity may be related to the broad host range that cercariae infect (invertebrates and vertebrates), in contrast to the narrow host range of miracidia (molluscs) (Galaktionov and Dobrovolskij 2003).

Several behavioural strategies that guarantee the transmission of miracidia have been described. To ensure the continuity of the cycle, it is crucial to avoid the miracidia to hatch within the definitive host tract before being released to the environment. Thereafter, the hatching process is likely to be triggered by environmental cues of the molluscan host habitat, such as light, osmotic pressure or temperature (Smyth and Halton 1983). For example, eggs of *Fasciola hepatica* L. (Fasciolidae) can hatch at 16–20°C under light conditions, overlapping with the preferred host conditions ((O. F. Müller, 1774) Lymnaeidae) (Rowan 1956). Similarly, miracidia usually respond to light and/or gravity to find the host habitat, such as the miracidia of *Schistosoma mansoni* Sambon, 1907 (Schistosomatidae) or *Echinostoma caproni* Richard, 1964 (Echinostomatidae), which have a strong negative geotaxis (swimming against gravity) dominated by a positive phototaxis (swimming toward light) that leads them to their host habitat (*Biomphalaria glabrata* (Say, 1818) Planorbidae) (Behrens and Nollen 1992). They are also able to maintain their position, as *S. mansoni* shows reduced speed and increased turning rate until they encounter the host by chemo-attraction (Mason and Fripp 1976). Chemo-attraction to the host has also been observed in parasites infecting snails as these are slowly moving targets (Haas 2003), however the exact mechanisms are not well understood (Haas 2003, Sukhdeo and Sukhdeo 2004). It can occur in miracidia a few hours after emerging (Campbell 1961, MacInnis 1965, Shiff and Kriel 1970) and it can have generic and specific responses to snail's signals-(Haberl et al. 2000).

Similarly, cercariae also use environmental conditions as cues to infect the next intermediate host (Haas 2003, Sukhdeo and Sukhdeo 2004). Given their limited energy and life span, quickly finding the next host is critical (Combes et al. 1994, Haas 1994). Some species emerge from the snail immediately overlapping time and space with the next host, likely stimulated by environmental conditions related to the host's presence in the water, such as pH levels, light and temperature (Bartoli and Combes 1986, Sukhdeo and Sukhdeo 2004, Koprivnikar et al. 2014). Cercariae tend to have slow motility and low swimming activity, whereas other cercariae have developed strategies to maximize the infection of the more distant hosts (Sukhdeo and Sukhdeo 2004). Many cercariae actively swim to the host habitat expecting to overlap in space, while others follow a different strategy that implies attracting the next host by simulating the behaviour of one of their natural preys (Combes et al. 1994, Haas 1994, Sukhdeo and Sukhdeo 2004, Santos et al. 2007, Prokofiev and Galaktionov 2009). Cercarial behaviours may also occur in response to environmental conditions such as light and/or gravity (Combes et al. 1994, Haas 1994, 2003). Cercariae of *C. longicollis* show photopositive and geonegative responses (swimming toward light and against gravity), locating themselves halfway up the water column where their intermediate fish hosts frequent the most (Bartoli and Combes, 1986).

Chemo-attraction mechanisms are not as commonly used by cercariae as by miracidia (e.g. *Hypoderaeum conoideum* (Bloch, 1782) (Echinostomatidae) (Galaktionov and Dobrovolskij 2003). Cues released into the environment by fast-moving hosts can be difficult for cercariae to track due to their active movement (Haas 2003), and therefore, cercariae usually maximise their little energy reserves trying to respond selectively to appropiate host stimuli. For example, the cercariae of several trematodes exhibit swimming bursts in response to shades that trigger their attachment to a surface (burst in response to shade that trigger the attachment of cercariae to a surface (Haas 2003, Sukhdeo and Sukhdeo 2004).

As expected, cercarial morphology is likely related to the transmission strategy to the next host (Santos et al. 2007, Morley 2020). For instance, the cercariae that emerge already in the habitat of the target host usually have a short tail that helps them crawl on the substrate and infect bottom-dwelling hosts, e.g. cercariae of *Lepocreadium pegorchis* (Stossich, 1901) (Lepocreadiidae) show low swimming activity and a geopositive response to infect bivalves (Combes et al. 1994). In contrast, the vast majority of cercariae that infect mobile hosts have a well-developed tail with a bifurcated stem forming two branches (furca tail), which usually offers a driven locomotion and longer duration floating in the water column (parachute-like) (Combes 1994, Haas 2003, Santos et al. 2007, Morley 2020, Chapter 5).

After encountering the host, the cercariae usually follow a behavioural pattern that Haas (2003) described in four phases, (1) host attachment, (2) enduring contact with the host, (3) creeping on surface and (4) penetration into host. The execution of these four phases can be species- and host-specific, providing a high diversity of strategies from low to high response specificity. Some species attach to a broad range of animals, however the low specificity in the attachment can be overcome in subsequent phases, e.g. the quick and non-specific attachment of *D. spathaceum*, stimulated by carbon dioxide, becomes fish-specific when enduring contact and penetration are stimulated by a specific substance in the mucus surrounding the fish (Haas et al. 2002). The penetration portals are not well known, overall cercariae enter unexpected areas as they tend to penetrate into areas distant from the target tissue, likely in search of faster connections to the target organ. For example, among diplostomids, *D. spathaceum* penetrates through the gills to infect the eyes of trout using the circulatory system (Höglund 1991), while *Posthodiplostomum ptychocheilus* (Faust, 1917) penetrates the dorsal and caudal fins to infect the brain via the central nervous cord (Hendrickson 1979). Species-specific routes are thus most likely chosen to penetrate and migrate to target tissues, and

therefore, these must be studied individually for the different species (Chapters 4 and 5).

Some parasites can be trophically transmitted to the definitive host and therefore, to maximise its consumption along with the second intermediate host, trematodes can provoke stealthy changes (Lafferty and Morris 1996, Moore 2002, Coats et al. 2010, Kortet et al. 2010, Kekäläinen et al. 2014) that usually affect multiple phenotypic alterations in host behaviour (Cézilly et al. 2013). For example, parasites can significantly reduce host fitness and alter their habitat by negatively affecting host's energy reserves, reproduction and competitive success (Kavaliers et al. 2000, Barber et al. 2003, Poulin 2007, Barber and Dingemanse 2010). This may lead infected hosts to expose themselves longer to predators due to inefficient food foraging (e.g. *D. spathaceum* reduces reaction distance to prey), erratic swimming behaviour or suppression of antipredator response (Barber et al. 2000, Barber and Dingemanse 2010). Getting to understand mechanisms of host manipulation is a focus of interest among researchers (Seppäla et al. 2005, Poulin et al. 2013,). However, this can be challenging because host behaviour alterations can be caused by direct manipulation of host behaviour (Poulin 2010, Cézilly et al. 2013, van Houte et al. 2013), but also by a pathological side effect of infection or by host defence response. Few studies have been able to prove host manipulation by a parasite species. One of the best-known examples was described by Lafferty and Morris (1996), who demonstrated that the flashing, surfacing, contorting, shimmying and jerking behaviours of California killifish (*Fundulus parvipinnis* Girard, 1854 (Fundulidae)) increased when *Euhaplorchis californiensis* Martin, 1950 (Heterophyidae) was found encysted in their brains, leading to an increase in their predation by a variety of piscivorous birds. Marcogliese et al. (2001) and Seppälä et al. (2004, 2005, 2011) have shown that cataracts induced by *Displostomum* spp. affect the fish host response by impairing their vision and thus its escape behaviour.

Overall, trematodes have evolved transmission strategies to ensure infection of the definitive host, which may be out of reach without the parasite-induced behaviours. One of the most challenging topics to investigate is the behavioural changes provoked by parasite manipulation, as experimental assays are required to test these changes. A preliminary experimental study on potential behavioural changes in gilthead seabream caused by *C. longicollis* was conducted as part of this thesis (Chapter 7), but further investigations are necessary to confirm this.

1.4 Experimental research

Fish have become a common tool in research, and it is important to apply the 3Rs fundaments of the globally recognized Guide to Animal Welfare, in which Russell and Burch (1959) suggested that the welfare of experimental animals is a prerequisite for successful research and a moral obligation. The application of the 3Rs, which stand for "Replacement", "Reduction" and "Refinement", is widely accepted to maximize the quality of studies without compromising animal welfare (Davies et al. 2018, Kirk 2018, Würbel 2017). Replacement refers to methods that avoid the use of animals or replace them with insentient material, e.g. animals with lower pain perception or computer models. Reduction refers to minimizing the number of animals used, while refinement refers to minimizing the pain and distress of animals by improving their welfare. Fulfilling the requirements of the 3Rs has improved scientific protocols and has encouraged researchers to handle experimental animals more carefully, to achieve reliable outcomes and minimize immune responses and undesirable effects of stress and infection (Sneddon 2017, Fife-Cook and Franks 2019).

The fish most used as experimental animals are zebrafish (*Danio rerio* Hamilton, 1822 (Cyprinidae)), three-spined stickleback (*Gasterosteus aculeatus* L. (Gasterosteidae)) and gilthead seabream (*S. aurata*). These fish

have become popular vertebrate models in a broad range of research fields, from medical and pharmaceutical applications to behavioural, ecological, and evolutionary research (stickleback (Huntingford et al. 2009, Barber and Scharsack 2010, Norton 2019), zebrafish (Chakraborty et al. 2009, Reed and Jennings 2010, Tavares and Lopes 2013, Powell et al. 2021, Tao et al. 2022,) and gilthead seabream (Aranguren et al. 2002, Rios-Fuster et al. 2021, López-Verdejo et al. 2022). Tagging methods, which can range from fin clipping to telemetry tags (Sandford et al. 2020, Rácz et al. 2021), are also commonly used in research on these species. Even though fish are commonly used in research, there is a lack of information on the potential side effects of tagging procedures on their welfare (Sandford et al. 2020, Macaulay et al. 2021, Rácz et al. 2021). Thus, the scientific outcome may be at risk if not conducted adequately, as their misuse may provoke severe side effects, such as reduced growth rate, injuries or behavioural changes (Deakin et al. 2019, Sandford et al. 2020). Therefore, when starting a research line on some fish species, as the present thesis, it is recommended that preliminary experiments be conducted at the beginning of a line of research on specific fish species, to test whether the chosen tagging protocol has potential side effects for the animal (see Chapter 6).

1.4.1 Applied parasitological studies

Getting to know parasites has been a focus of interest in research in recent decades. Parasites are present in a wide variety of ecosystems and in a broad range of organisms, and surprisingly, the more parasitism was understood, the more interesting it became. One of the obvious reasons for studying parasites is to mitigate the potential negative effects on infected hosts. One of the best-known cases is the infection of mice with *Toxoplasma gondii* (Nicolle and Manceaux, 1908) (Sarcocystidae) (Wang and Sibley 2020). Its research is very advanced and is deeply understood by the use of these hosts as parasite-host models. Parasite-host models are widely used in research as a tool to explore

different scenarios and to enrich knowledge on parasitism. These models are important to improve our understanding of parasite biology and transmission strategies.

The combination of theoretical and empirical research is key to study parasites, especially their transmission. Many authors consider that there is need of more of such host-parasite models to improve our understanding of parasite transmission (Seppälä and Jokela 2008, Poulin 2015). For investigating the host manipulation, there is a variety of models, such as *Diplostomum* sp., which infects salmonid rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) (Seppälä et al. 2004, 2005), *E. californiensis*, which infects California killifish, and a more recent model that infects zebrafish with *Pseudoloma neurophilia* Matthews, Brown, Larison, Bishop-Stewart, Rogers and Kent, 2001 (Glugeidae) (Midttun et al. 2020). Furthermore, other important mechanisms of parasite transmission have been investigated, as for instance the migration pathway of freshwater digenean parasites such as *Posthodiplostomum* sp. that infects fathead minnows (*Pimephales promelas* Rafinesque, 1820 (Cyprinidae)) (Matisz and Goater 2010, Matisz et al. 2010), or the infection process and establishment in the host, by the well-known nematode *Anisakis pegreffii* Campana-Rouget and Biocca, 1955 that infects European seabass (*Dicentrarchus labrax* (L.) (Moronidae)) and Sprague-Dawley rat (*Rattus norvegicus* (Berkenhout, 1759) (Muridae)) under laboratory conditions (Trumbić et al. 2021, Hrabar et al. 2022). One of the most used fish for marine parasitological studies is the gilthead seabream *S. aurata*. This fish is one of the most important species in Mediterranean aquaculture (~3.4% of global marine and coastal aquaculture production, FAO 2022) and is a natural host for a wide range of parasites in the wild and in aquaculture (e.g. from myxozoans to helminths, Pavlidis and Mylonas 2011). Therefore, a broad range of parasite models are used for gilthead seabream. One of the most problematic parasites in Mediterranean farms is *Sparicotyle*

chrysophrii (Van Beneden and Hesse, 1863) (Microcotylidae), which has been investigated by Sitjà-Bobadilla et al. (2009) and Villar-Torres et al. (2023) under laboratory conditions and its transmission was replicated in gilthead seabream. Similarly, the parasite model *Enterospora nucleophila* Palenzuela, Redondo, Cali, Takvorian, Alonso-Naveiro, Álvarez-Pellitero and Sitjà-Bobadilla, 2014 (Enterocytozoonidae) was used in gilthead seabream (Picard-Sánchez et al. 2021). The infection process and establishment of the nematode *Anisakis simplex* Davey, 1971 was also assessed by infecting gilthead seabream (López-Verdejo et al. 2022). Likewise, the model parasite of this thesis, *C. longicollis*, was used under laboratory conditions to infect this fish species as well (Chapters 4, 5 and 7). Establishing this host-parasite system in the laboratory provides an efficient tool to study its life cycle and offers a great opportunity to reveal the transmission mechanisms of marine digenean parasites. At the same time, the study of larval trematodes, is challenging, given the nature of some stages. Eggs, miracidia and cercariae are difficult to investigate given their size, and transparent nature, especially the hatched miracidia and cercariae (Galaktionov and Dobrovolskij 2003). Therefore, when examining these stages, specific techniques need to be involved in the protocols to make the larvae detectable and recognizable. One of these methods could be fluorescent dyes, which make the cercariae appear bright and shiny under the fluorescent microscope, as shown in Braun et al (2020) and LaFonte et al. (2015). Nevertheless, their use needs to be tested to ensure minimal impact on the survival or infection rate of the cercariae, which needs to be evaluated in advance.

To sum up the work carried in this thesis, **Chapter 4** evaluated the use of *in vivo* fluorescent dyes as a tool to facilitate the recognition and monitoring of larval stages such as cercariae, by analysing survival, activity and infection rate of cercariae. In addition, the penetration pattern of *C. longicollis* cercariae on fish skin was examined to determine whether cercariae have preferred areas to enter into fish. **Chapter 5** addressed the relationship between the body

mechanisms of *C. longicollis* cercariae and their transmission behaviour. Their mechanisms were analysed in different transmission phases: swimming in the water, crawling on and penetrating through the fish skin and burrowing within the fish. Both chapters have increased the knowledge on the transmission of parasites with a complex life cycle. **Chapter 6** tested and optimized the use of Visible Implant Elastomers tags on *S. aurata* under laboratory conditions. VIE were needed to individually identify the fish used in experimental research assessing whether *C. longicollis* has an impact on fish behaviour, which was preliminary evaluated in **Chapter 7**.

Aims and Objectives

2. Aims and Objectives

The main aim of the project is to achieve a better understanding of the transmission between hosts of marine digenean parasites with a complex life cycle. To this end, we used as a model organism the strigeid *Cardiocephaloides longicollis* (Rudolphi, 1819) Dubois, 1982, and replicated part of its life cycle under laboratory conditions, which allowed to study the behaviour of different parasite stages and, during the process, to optimize protocols for conducting experimental parasite infections in fish.

The specific objectives of this study are:

I. To investigate whether there is a valid and easy labelling system for the study of larval stages in short-term studies. To this end, to assess experimentally the effect of *in vivo* fluorescent dyes, i.e. carboxyfluorescein diacetate succinimidyl ester (CFSE) and Hoechst 33342 (NB), on cercarial survival, activity and infection rate. To identify and describe the penetration pattern of cercariae of *C. longicolis* into the intermediate host under laboratory conditions using the fish model *Sparus aurata* L., natural host for this parasite.

II. To investigate the role of cercarial structures on the mechanisms and locomotion of cercariae of *C. longicolis*, from their emergence from the snail to the encystment in the brain of *S. aurata*. To monitor and describe the activity of cercarial body structures during host-finding, -attachment, -recognition, -penetration and -infection behaviours.

III. To evaluate experimentally the quality of research when using individual marks to recognize fish, by using Visible Implant Elastomers (VIE) tags on *S. aurata* under laboratory conditions. To monitor the tag retention and assess histological effects depending on the tagging location and size of the fish. To optimize the use of VIE tags for applied parasitological research.

IV. To show the utility of VIE tagging in a preliminary experimental assay. To experimentally evaluate the potential behavioural changes provoked by the brain-encysting trematode *C. longicollis* infecting *S. aurata*.

General Materials and Methods

3. General Materials and Methods

This chapter provides an overview of the animals used in this study, the sampling sites and methods used to obtain them. General methods for studying larval stages such as the cercariae, as well as techniques such as microscopy, *in vivo* labelling and histology are also described. In addition, artificial infections of gilthead seabream and the procedure for tagging with Visible Implant Elastomers (VIE) are briefly described in this section. The setup and the design of the experimental behavioural trials and the general statistical analyses are also explained. A more detailed account of the materials and methods used in each study is provided in the relevant chapters (Chapters 4 to 7).

3.1 Animal collection and maintenance

3.1.1 Snail host

Snails *Tritia reticulata* (L. 1758) (Nassaridae) were manually collected in spring at a site on the "Beach of Eucalyptus" in the Els Alfacs lagoon (Ebro Delta, Spain) (40º37'35"N,0º44'31"E) (Fig. 3.1A, B) (Born-Torrijos et al. 2016). This lagoon is formed by sedimentation at the sides of the mouth of the Ebro River. The soft sediment and the dwarf eelgrass *Zostera noltei* Hornemann, 1832 (Zosteraceae) (Fig 3.1C) provide a habitat for a large number of snails. Because the lagoon experiences large daily fluctuations in temperature and water level, it is important to avoid extreme temperatures and low tides during snail collection (Fig. 3.1D, E), as snails typically excavate deeper into the mud and become difficult to find. Snails were transported to the laboratory in aerated seawater collected at the sampling site. After acclimating snails to artificial seawater (35 psu salinity) and temperature of 16ºC for one week, they were screened for infections (Fig. 3.1F, G) (details in 3.1.3 section). Snails were fed every other day with small-size feed fish pellets

(1,2–1,8 cm) and particulate feed for suspension-feeding of marine invertebrates.

3.1.2 Fish host

Juvenile gilthead seabream (*Sparus aurata* L.) were obtained from a hatchery in Burriana (Andromeda Group, Castellon, Spain). The fish were transported in oxygenated seawater to the SCSIE facility (Central Support Service for Experimental Research, University of Valencia). The fish were acclimated for one week and later kept in 3000 l tank, where they were daily exanimated by assessing their welfare, feeding habits and water quality parameters. Values between 7.9 and 8.4 for pH, 85 % for oxygen saturation (oximeter, Polaris-Oxyguard International A/S) and 35 psu salinity (Refractometer, Blau) were considered normal by the aquarium plant personnel. The fish were fed *ad livitum* with commercial fish pellets daily.

3.1.3 Parasite collection

To obtain freshly emerged cercariae, *C. longicollis* emission was stimulated by incubating *T. reticulata* snails individually in cylindrical wells (40 ml) (Fig. 3.1F, G). Snails were covered in seawater (35 psu salinity) for 24 hours at 25ºC, exposing them to a 12h:12h photoperiod, which triggered the emergence of mature cercariae to the water. The water was examined under stereomicroscope and cercariae were collected with a micropipette (20-200 µl, Eppendorf, Research plus), which allows their manipulation without damaging them.

Figure 3.1. Snail sampling site at "Els Alfacs" lagoon (Ebro Delta, Spain). (**A**) Map of the sampling site (Google), (**B**) image of the lagoon from above

and (**C**) underwater showing muddy sediment covered with dwarf eelgrass. (**D-E**) Image of manual sampling at the lagoon. (**F-G**) *Tritia reticulata* incubated in a well with seawater for infection screening.

3.2 Methodologies for the study of larval stages

3.2.1 Morphological and ultrastructural data

For morphological examination of *C. longicollis* cercariae, live cercariae were observed under a light microscope (Leica DMR, magnification up to 400X), identifying them according to the descriptions by Prévot and Bartoli (1980). For ultrastructural studies, the cercariae were examined by scanning and transmission electron microscopy (SEM, TEM). For this purpose, cercariae were fixed in 2.5% glutaraldehyde in phosphate-buffered saline (PBS, 0.1 mol L−1, pH 7.4) and preserved at 4 °C for 24 h. For SEM, cercariae were dehydrated in a graded series of acetone. The specimens were critical point dried with liquid CO2 (Fig. 3.2A, B) and mounted on stubs, sputter-coated with gold grids. For TEM, after their fixation with glutaraldehyde, specimens were washed three times in PBS (15 min each). Then, the samples were post-fixed in 2% osmium tetroxide for 2 h, washed three times in phosphate buffer (PB), followed by a gradual dehydration in a series of acetone. After this, specimens were infiltrated and embedded in Epon resin. First, semithin sections (400 nm) were cut using a glass knife, stained with toluidine blue, and observed using a light microscope. Then, ultrathin sections (60–90 nm) were cut through selected regions using a diamond knife on a Leica Ultracut UCT ultramicrotome. These were placed on copper grids, stained with uranyl acetate and lead citrate according to Reynolds (1963). The cercariae were observed under the Hitachi S4800 scanning electron microscope (Hitachi, Japan, accelerating voltage 1kV, SCSIE (Spain) and the JEOL JEM-1010 transmission electron microscope (JEOL, Tokyo, Japan, accelerating voltage 80 kV, equipped with a CCD digital camera Mega View III at the Laboratory

of Electron Microscopy, Institute of Parasitology, Biology Centre of the Czech Academy of Sciences (Czech Republic)).

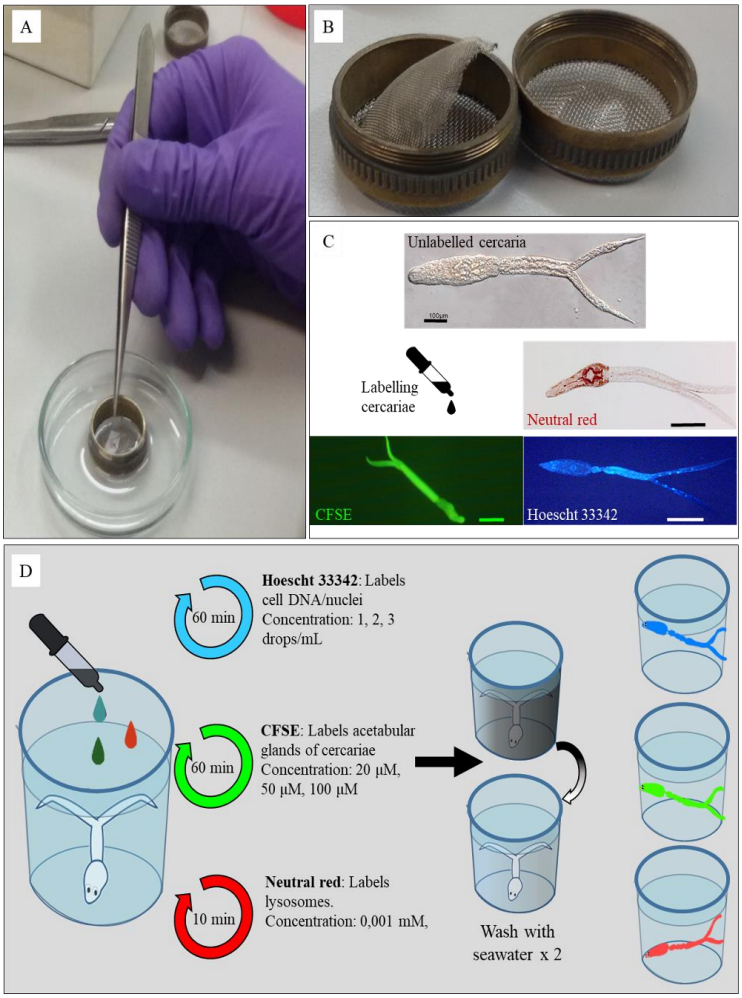


Figure 3.2. Summary of some methods used to identify cercariae larvae during the study. (**A-B**) Preparation of specimens for critical point drying with liquid CO2 for SEM analysis. (**C**) *In vivo* image of cercariae before and after labelling with CFSE, Hoescht 33342 and neutral red (sustituir fotos con las de cfse y nb). (**D**) Description of the cercariae-labelling process for each of the dyes used in this work. Scale bars C =100 µm. Authors: Ana Born Torrijos (C unlabelled cercaria)

3.2.2 *In vivo* labelling

Given the small size and transparent body of the cercariae, an *in vivo* labelling method is required to study them. In this PhD thesis, three different dyes have been used (Fig. 3.2C). First, two different fluorescent *in vivo* dyes, Carboxyfluorescein diacetate succinimidyl ester (CFSE) and Hoescht 33342 (NB) were tested, and then a non-fluorescent *in vivo* dye, neutral red, was tested (Fig. 3.2C, D). The toxicity of the dye, which could cause side effects in the survival and activity of cercariae, was tested in advance by applying different dye concentrations. To assure their efficiency and safety, the dyes were required to be powerful enough to facilitate cercarial visualization, but without affecting their performance. For this purpose, freshly emerged cercaria (< 6 h old) were placed in a well containing seawater (1 ml) together with the dye (see detail in Fig. 3.2D). To avoid dragging dye residues into the experimental assays, it was important to wash the cercariae twice before transfer (Fig. 3.2D).

3.2.3 Histological analyses

Samples were fixed in 4% formaldehyde up to two days for histological examination after being in formic acid for 12 h. Samples were dehydrated in graded alcohol and paraffin ((Shandon Histocentre 3, Thermo Electron Corporation), 24 h ethanol 70%, 2 × ethanol 90%, 3 × ethanol 96%, 50:50 ethanol 96%: xylol, 2 × xylol, 50:50 xylol:paraffin, 3× paraffin (all at 25°C for 0 min and 3× paraffin at 60°C for 60 min except the last one which takes place overnight)). The paraffin-soaked samples were then embedded in paraffin. The paraffin-embedded samples were cut in a midsagittal plane

using a microtome (Leica RM2255, Leica Biosystems, Germany). The obtained 7µm sections were stained with hematoxylin and eosin and mounted in DPX (2 × 3 min xylol, 2 x 3 min ethanol 96%, 3 min ethanol 70%, 3 min ethanol 50%, 5 min in tap water, 6 min hematoxylin, 10 min in tap water, 4 min alcoholic eosin, 5 min in tap water, 2 × 15 seconds (s) ethanol 96%, 2 × 3 min ethanol 100% and 2 × 3 min xylol). Histological sections were examined at magnifications between 100 and 400x (Leica DM 5000B, Jenoptik Laser Optik, system GmbH, Germany) and photographed (ProgRes C3, ProgRes CapturePro 2.7, Germany).

3.3 Experimental fish infection

To infect fish, these were placed individually in small cylindrical containers with sufficiently filtered seawater (35 psu salinity) to cover them. In order to reduce stressing factors for their infection, this process was designed considering the conditions of the facility where the fish are normally kept (water temperature, light and ambient noise). After 30 min acclimation, the fish were exposed to the cercariae for one hour, releasing them into the water (dose changed according to different assays: 50 – 150 cercariae). Then, the fish were placed in a larger container where they were closely monitored for the next 6 hours to inspect their well-being, and later transferred to 3000L tanks at the experimental aquarium plant from the Central Support Service for Experimental Research, University of Valencia.

3.4 Application of Visible Implant Elastomers on gilthead seabream

Gilthead seabreams were tagged with Visible Elastomer Implant tags (Northwest Marine Technology, NMT Inc, Shaw Island, WA) for visual-

experimental assays. The main components of this tag are silicone and colourant, which were prepared according to the manufacturer's instructions (10:1 dilution of coloured component and hardener) (Fig. 3.3A, B). Five different colours were used, which after mixing were loaded into an insulin syringe (BD 0.3 cc, with a 29 gauge needle) and superficially injected under the fish skin at the caudal fin and at the base of the dorsal fin (Fig. 3.3C, D, E). This required anesthetizing fish with MS222 (0.015% solution buffered in seawater of tricaine methanesulfonate, Sigma-Aldrich). When manipulating the fish, it was important to use soft materials and gloves to avoid damaging the skin of the fish (Fig. 3.3C), and to work quickly to avoid suffocating the fish during the process. After tagging the fish, residues from VIE tags were washed off and the fish were then isolated in seawater to slowly recover from the anaesthesia (Fig. 3.3F, G). The fish were monitored for the next six hours to assess their well-being after tagging. UV light was needed to see some colours, such as yellow, green and pink (Fig. 3.3D, E). Then, the fish were euthanized with an overdose of MS222 (0.030% solution buffered in seawater of tricaine methanesulfonate). Samples were collected for histological analyses to evaluate the reaction of VIE tags in the tissues. Therefore, these samples were prepared according to the histological techniques described in the methodological section for the study of larval stages (Section 3.2.3).

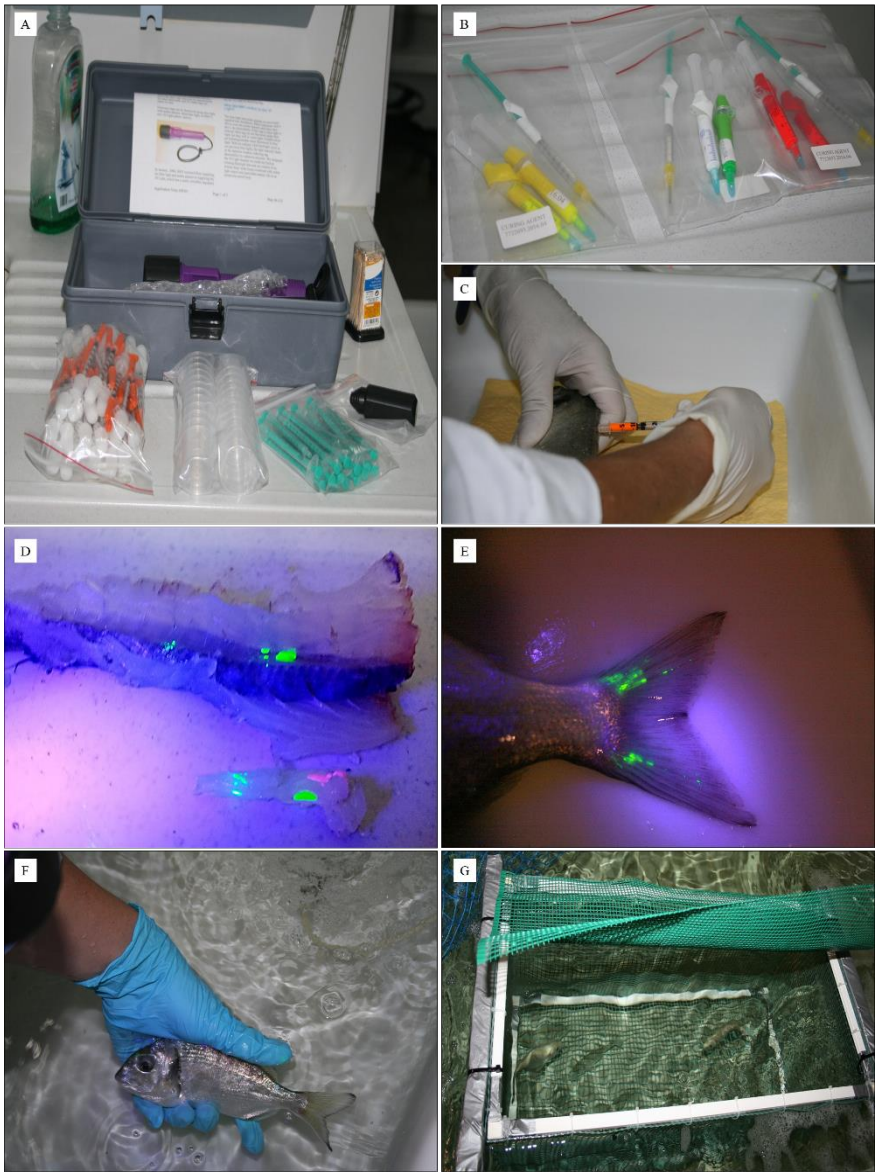


Figure 3.3. Images summarizing the process of tagging with Visible Implant Elastomers (VIE). (**A-B**) Material required for tagging the fish. (**C**) Orange VIE tag being superficially injected at the base of the dorsal fin of the fish. (**D**) Sample of two VIE tag colours superficially injected on the dorsal fin and (**E**)

green VIE tag injected into the caudal fin. (**F)** Tagged fish being washed to eliminate VIE residuals on the skin, and (**G**) isolated to monitor their recovery.

3.5 Infected fish used in behavioural experiments

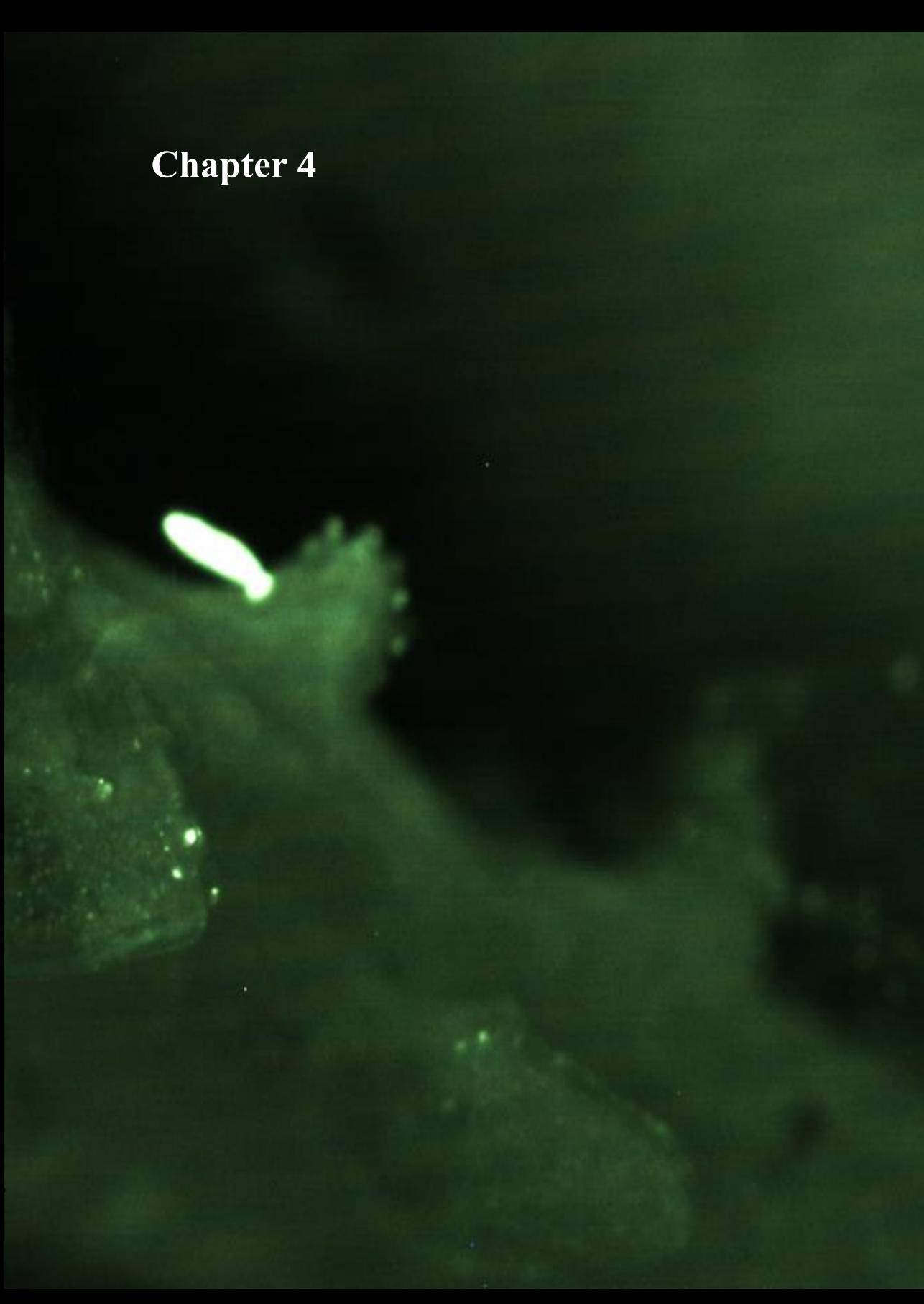
Fish with a mature infection were required for behavioural experimental studies. These were artificially infected as previously explained (Section 3.3) and their depth selection and escape behaviour were analysed. To this end, the behaviour of infected fish was compared to that of uninfected fish. Both groups, separate and mixed, were used in two different assays, (1) in a cylindrical and narrow aquarium to observe the effect of infection of fish behaviour in a light gradient, and (2) in a 300 cm diameter circular tank where fish were chased with a net to evaluate the infection effect on the escape response. In both assays, it was crucial to acclimate the fish to the new tank for 24 hours and to always feed them after the assay. To ensure their welfare, it was important to pay attention to whether these activities increased the stress level of the fish. Thus, if the aggression level raised or the fish showed injuries on the skin, the assay was immediately postponed. It was also important to measure the water parameters (pH, oxygen level, salinity) to guarantee the well-being of the fish.

3.6 Statistical analysis

All statistical analyses were performed using R (R Development Core Team, version 3.0.1; 4.1.2). To analyse the different data sets generated in the present PhD thesis, Generalized Linear Mixed Models (GLMMs), Generalized Linear Models (GLMs), Linear Mixed Models (LMMs) and Linear Models (LMs) were estimated using the *lme4* package (Bates et al. 2015, 2022). point process models (PPM, package spatstat, (Baddeley and Turner 2005), survival regression Weibull model (RWM) (package *survival,* (Therneau and Lumley 2015)) and mixed model Cox proportional hazards regression (MMCoxPH, package *coxme*, (Therneau 2018)) were used specifically in Chapter 4. Beta

regression models (*betareg* package (Zeileis et al. 2022)) were estimated specifically in Chapter 6. Statistical significance was set at $P < 0.05$, unless otherwise stated.

Chapter 4



4. Chapter 4

In vivo fluorescent cercariae reveal the entry portals of *Cardiocephaloides longicollis* (Rudolphi, 1819) Dubois, 1982 (Strigeidae) into the gilthead seabream *Sparus aurata* L.

Original paper published in: van Beest GS, Villar-Torres M, Raga JA, Montero FE, Born-Torrijos A. 2019. *In vivo* fluorescent cercariae reveal the entry portals of *Cardiocephaloides longicollis* (Rudolphi, 1819) Dubois, 1982 (Strigeidae) into the gilthead seabream *Sparus aurata* L. Parasites & Vectors 12:1–10. *https://doi.org/10.1186/s13071-019-3351-9*

4.1 Abstract

Despite their complex life cycles involving various types of hosts and free-living stages, digenean trematodes are becoming recurrent model systems. The infection and penetration strategy of the larval stages, i.e. cercariae, into the fish host is poorly understood and information regarding their entry portals is not well-known for most species. *Cardiocephaloides longicollis* (Rudolphi, 1819) Dubois, 1982 (Digenea, Strigeidae) uses the gilthead seabream (*Sparus aurata* L.), an important marine fish in Mediterranean aquaculture, as a second intermediate host, where they encyst in the brain as metacercariae. Labelling the cercariae with *in vivo* fluorescent dyes helped us to track their entry into the fish, revealing the penetration pattern that *C. longicollis* uses to infect *S. aurata*. Two different fluorescent dyes were used: Carboxyfluorescein diacetate succinimidyl ester (CFSE) and Hoechst 33342 (NB). Three ascending concentrations of each dye were tested to detect any effect on labelled cercarial performance, by recording their survival during the first 5 hours post-labelling (hpl) and 24 hpl, as well as their activity during 5 hpl. Labelled cercariae were used to track the penetration points into fish, and cercarial infectivity and later encystment were analysed by recording brain-encysted metacercariae in fish infected with labelled and control cercariae after 20 days post infection. Although the different dye concentrations showed diverse effects on both survival and activity, intermediate doses of CFSE did not show any short-term effect on survival, permitting a brighter and longer recognition of cercariae on the host body surface. Therefore, CFSE helped to determine the penetration points of *C. longicollis* into the fish, denoting their aggregation on the head, eye and gills region, as well as on the dorsal fin and the lower side. Only CFSE-labelled cercariae showed a decreased number of encysted metacercariae when compared to control. Overall, the study suggests that CFSE is an adequate labelling method for short-term *in vivo* studies, whereas NB would better suit *in vivo* studies on long-term performance.

Cardiocephaloides longicollis cercariae seem to be attracted to areas nearby the brain or probably connected to migration routes to neuronal canals.

Keywords: Cercarial penetration pattern, cercarial survival and activity, metacercarial encystment, Digenea.

4.2 Introduction

Host-parasite interactions represent a useful model for a better understanding of the evolution of animal life history traits and behaviours. Digeneans (Platyhelminthes, Trematoda) are particularly suitable for experimental studies; and, as they are highly diverse and spread all over the world, they are becoming recurrent model systems (Esch and Fernandez 1994, Esch et al. 2002, Poulin 2006, Keeney et al. 2008, Jephcott et al. 2016). Generally, their complex life cycles involve multiple developmental stages and transmission events (Galaktionov and Dobrovolskij 2003, Poulin 2007). In most life cycles, the first intermediate host, usually a gastropod, harbours larval stages (sporocysts or rediae), which mature so that fully developed cercariae are released into the water. In most cases, these larvae infect a second intermediate host within a certain period of time (hours to days), usually another mollusc or a fish, where they encyst as metacercariae before being consumed by definitive hosts, usually fish, birds or mammals, in which the parasites complete their life cycles by sexual reproduction (Combes et al. 1994).

Good timing of cercarial emergence in the target host habitat is crucial to contact their host, due to the limited life-span and energy reserves of cercariae needed until the infection of the second intermediate host (Anderson and Whitfield 1975, William 1994). Therefore, glycogen reserves need to be optimized (Haas et al. 1994, Haas and Roemer 1998). Although some species are passively transmitted to the second intermediate host, generally cercariae actively infect their next host, whether by chance, invading them anywhere along the entire body surface (Rohde and Rohde 2005), or by a specific infective strategy in particular areas. For example, *Centrocestus armatus* cercariae specifically infect the host through the gills (Paller and Uga 2008) and eye fluke cercariae specifically infect the fish eyes by penetrating the gills and the skin (e.g. *Diplostomum spathaceum,* Karvonen et al. 2004)). Once cercariae find their host, a complex process starts, with distinct phases of attachment and penetration. The acetabular glands of cercariae play an

important role in the penetration process into the host through the skin. Cercariae-host interaction activates their proteases and immunomodulatory activities, facilitating entrance into the host, as shown in *Schistosoma mansoni* (Curwen et al. 2006, Paveley et al. 2009). Nevertheless, there are no common attachment or penetration strategies among trematodes and, despite being essential to understand trematodes transmission, these processes remain unknown for most species, mostly because their study is highly difficult.

In vivo fluorescent dyes can be an adequate tool to study cercarial penetration patterns into fish. This method has been used with platyhelminths for several applications, including post-infection identification of trematode genetic clones (Keeney et al. 2008), lipid utilization in *S. mansoni* (Furlong et al. 1995), individual cestode tracking within intermediate host (Kurtz et al. 2002) and quantification of trematode larval infection success (LaFonte et al. 2015).The dyes suggested in this study have been previously employed to reveal, in particular, attachment, distribution and development of monogenean larvae on fish (CFSE, (Glennon et al. 2007, Trujillo-González et al. 2015)), to localize protein kinase C in cercariae (CFSE, (Ressurreição et al. 2015)) and to discriminate live and dead *S. mansoni* eggs (NB, (Sarvel et al. 2006)) and *S. mansoni* cercariae exposed to human serum (NB, (Da'dara and Krautz-Peterson 2014)).

For this study, cercariae of *Cardiocephaloides longicollis* (Rudolphi, 1819) Dubois, 1982 (Strigeidae) were labelled, allowing their tracking on fish surfaces and thus their entry portals into them. The cercariae of this digenean parasite penetrate the skin and migrate into the fish brain, where they encyst as metacercariae (Osset et al. 2005). The study of their transmission to the next intermediate host is particularly interesting as they infect fish farm species such as the gilthead seabream (*Sparus aurata* L.), which is one of the most important marine fish in the Mediterranean aquaculture, with up to 53.9% prevalences (Born-Torrijos et al. 2016). Therefore, the current work aimed to investigate

the penetration pattern of *C. longicollis* cercariae into fish. The use of fluorescent-labelling methods with cercariae is tested and evaluated by analyzing their effect on cercarial survival and activity after 24 hours (h) labelling as well as on their viability to successfully infect and encyst in a fish host.

4.3 Materials and methods

4.3.1 Parasite and host material

For obtaining *Cardiocephaloides longicollis* cercariae, the snail host *Tritia reticulata (L.)* was collected haphazardly during spring 2017 at the "Beach of the Eucalyptus" (40°37′35.0″N, 0º44′31.0″E) in *Els Alfacs* lagoon (Ebro Delta, Spain). After acclimatisation to laboratory conditions, snails were screened for infections by incubating them individually in cell well plates during 24 h at 25ºC, 12:12h light: dark cycle and 35 psu salinity, thereafter, being checked for cercarial emergence under the stereomicroscope. To guarantee enough emerged cercariae during experiments, uninfected snails were artificially infected: faecal samples were collected every two months during one year from an isolated, large colony of yellow-legged gull hosts *Larus michahellis* inhabiting an island (off Benidorm, Spain, Western Mediterranean, 38°30′07.6″N, 0º07′47.4″W). The faeces were checked under stereomicroscope and the eggs were morphologically identified according to Dubois (1938). Identification was confirmed molecularly in random samples as described in Born-Torrijos et al. (2017). Faeces containing *C. longicollis* eggs were dropped into snails' aquaria, and snails were periodically checked to detect infected specimens as previously described. Only newly emerged cercariae (all less than 2 h old), gathered from a pool of cercariae emerged from 12 different snail hosts, were used in the present study.

A total of 51 juvenile specific pathogen free (SPF) gilthead seabream (12.0 g and 6.7 cm standard length, on average), natural second intermediate hosts

of *C. longicollis,* were supplied by a hatchery from Burriana (Castellón, Spain), and maintained in 3,000L tanks at the installations of SCSIE (Central Support Service for Experimental Research, University of Valencia) until their use in different experimental assays. An ethic statement from the University of Valencia Animal Ethics Committee was granted (A1458654393594).

4.3.2 Experimental procedure

4.3.2.1. Effect of two *in vivo* fluorescent dyes on cercarial survival and activity

To assess the dye effect on cercarial survival and activity, cercariae were labelled by exposing them during 60 minutes (min) in dark conditions to three ascending concentrations (Fig. 1A), of two different dyes, diluted with filtered seawater: (i) Carboxyfluorescein diacetate succinimidyl ester (CFSE, low 20µM, intermediate 50µM, high 100µM; Sigma-Aldrich), which labels the acetabular glands of the cercariae, and (ii) Hoechst 33342 (NB, low 1 drop/mL, intermediate 2 drops/mL, high 3 drops/mL; Thermo Fisher Scientific), which labels the cell DNA/nuclei. All these concentrations were compared with control, unlabelled cercariae, which followed the same incubation process as labelled cercariae, but seawater was applied instead of the dye. A preliminary assay showed that parasite visualization was highest when cercariae were incubated for 60 min at dark conditions, in contrast to 10 and 30 min. After labelling, the dying solution was replaced with clean filtered seawater and cercariae were placed in individual 96-cell wells plates (Fig. 1A), where they were examined under the stereomicroscope every 2 h, starting at 1 h post-labelling (hpl) during 24 h. Each concentration was tested for a total of 100 cercariae, in 4 replicates of 25 cercariae each, at room temperature (approx. 25ºC), 12:12 h light: dark cycle and 35 psu salinity. Cercarial activity was classified by visual assessment as active (swimming cercariae) or inactive (cercariae barely swimming, showing erratic movements and spontaneous spasms), while cercarial longevity was recorded as the time until cercariae did not show spasmodic movements.

4.3.2.2. Penetration pattern on host surface

Fish were infected with labelled cercariae and analysed to determine the parasite penetration pattern on the host body surface (Fig. 1B). Fifteen *S. aurata* were infected with 100 cercariae each, which were previously labelled with an intermediate CFSE concentration under the same conditions previously described. After this process, cercariae were maximum between 3-6 h old. Additionally, unlabelled cercariae and fish tissue were examined under a fluorescence stereomicroscope (MZZ16F Leica) to discard any natural fluorescence that could interfere with the recorded data. Immediately following fish euthanasia by concussion to avoid the detachment of cercariae by chemicals, fish were placed into a black box with enough filtered seawater to cover them (90mm^3) and their body surface was observed under a fluorescence stereomicroscope coupled with a digital camera (MZZ16F Leica, DFC300 FX Leica). Both fish flanks, randomly alternating the first analysed one, were carefully examined for live parasites during an average time of 20 min/fish flank. Parasite location was recorded on an XY coordinate system based on a grid representing a fish flank, divided into different regions (Fig. 1B). Some zones, such as mouth, eyes and gills, were considered as particular zones as they may be common penetration areas for trematodes. Gills, oral folds, oral cavity, nasal chamber and ventral area were also examined. Scaled photographs were taken of each fish and this information was transferred into a digital XY coordinate system together with the location data in the free image-processing program ImageJ (1.47v, (Schneider et al. 2012)).

4.3.2.3. Dye effect on cercarial infectivity and metacercarial encystment in brain

To assess the effect of the fluorescent dyes on cercarial infectivity and later metacercarial encystment, control and NB or CFSE labelled cercariae were used to infect fish (Fig. 1C). After the acclimatisation of 36 fish in individual cylindrical containers with enough filtered seawater to cover them (approx. 200 cm^3), fish were exposed to 50 cercariae for 60 min (3 replicates of 4 fish

for each treatment, using intermediate dye concentrations). Thereafter, complete cercariae, tails and bodies that remained in the water after fish infection were counted to have an accurate initial infection value (3.5±0.3 complete cercariae and bodies were found after experiment in all treatments). The fish were measured, weighted and maintained in aquaria at room temperature, then euthanize them at 20 days post-infection (dpi) with MS222 (tricaine methanesulfonate, 0.03% solution buffered in seawater; Sigma-Aldrich). The number of metacercariae in the brain was then recorded after meticulous skull dissection (Fig. 1C).

4.3.3 Statistical analyses

To assess the variation in survival and activity rates of cercariae and investigate differences between concentrations in each treatment, data were tested by mixed model Cox proportional hazards regression (MMCoxPH, package *coxme*, (Therneau 2018)). If data did not show a constant proportional hazard between levels (validated at P>0.05 with function *cox.ph*, package *survival*), regression Weibull model (RWM) was used (package *survival,* (Therneau and Lumley 2015)) instead MMCoxPH. Survival analyses aim to model time to event data, usually considering death as the event. In this study, we used the death of a cercaria as the event to study their survival, and for studying cercarial activity, the inactivity of a cercaria was considered the event. First, to evaluate the effect of the concentration of each dye on the survival and activity of cercariae, survival analyses were used for each treatment separately (CFSE and NB), with concentrations as covariate (i.e. control, low, intermediate and high) in the RWM and in the MMCoxPH model (here adding the control treatment as the baseline with which the dye treatment were compared to, and replicates as random effect). Differences were evaluated by comparing the different concentrations of each fluorescent dye to the control treatment. Survival of cercariae was analysed after 5 hpl, i.e. the time during which the penetration pattern assay was run, and after 24

hpl, whereas the activity of cercariae was analysed after 5 hpl. To explore the survival and activity of labelled cercariae after 5 hpl, differences between intermediate concentrations of both dyes were analysed. For both survival and activity models, intermediate concentration of NB and CFSE was considered as a covariate and compared to control cercariae.

To study *C. longicollis* penetration pattern, data on both fish flanks were combined after discarding significant differences between the number of cercariae attached to the left and right body flanks by generalized linear model fitted with a quasibinomial structure (GLM, logit link function, package *lme4*, (Bates et al. 2015)), using the proportion of attached cercariae as response variable and the fish flank as fixed effect (Appendix 1: Table S1). In order to detect any pattern of the parasite distribution on the host´s body surface, the positions of cercariae were analysed by point process models (PPM, package *spatstat*, (Baddeley and Turner 2005)). For these two-dimensional parasite distribution analyses, parasites attached underneath the pectoral fins had to be excluded, resulting only in the omission of 2 cercariae and thus including the rest attached to both fish flanks. A complete spatial randomness simulation (CSR) was created based on Quadrat counting test and Kolmogorov-Smirnov test to confirm the assumption of uniformity of *C. longicollis* cercariae distribution on fish surface. A heat map was created to illustrate their position, using kernel density analysis (see Fig. 1B).

To detect the entry portals into the fish, the fish surface was first divided into 3 major regions (see Fig. 1B, head (H), body (B) and fins (F)) and afterwards into 11 regions (see Fig. 1B), subdividing the head into 3 sub-regions (eye (H1), mouth (H2), gills (H3)), the body into 3 sub-regions (upper (B1), middle (B2) and lower (B3) side), and the fins into 5 sub-regions (dorsal (F1), pectoral (F2), pelvic (F3), anal (F4) and caudal (F5) fins). To obtain the number of parasites for each region, the parasite counts were pooled from the XY coordinate grid data following the previously described regions (shown in Fig.

1B). Differences between regions were analysed by linear mixed models (LMM, package *lme4),* using Box-Cox transformed values (Fox and Weisberg 2011) of cercariae density (number of cercariae on region/total number of cercariae on fish)/ (region area (cm^2)/total fish area (cm^2)) as response variable, the established regions as fixed effect and the replicates as random effect.

To assess the dye effect on cercarial infectivity and metacercariae encystment in the fish´s brain, differences in the number of encysted metacercariae were analysed by generalized linear mixed model fitted with a binomial structure (GLMM, logit link function, package *lme4*), using the proportion of brain-encysted metacercariae as response variable, the dye type as fixed effect and replicates as random effect.

After GLM, LMM and GLMM, multiple pairwise comparisons were obtained using Tukey's all-pair comparisons test (package *multcomp*, (Hothorn et al. 2017)), adjusting p-values with Bonferroni correction. All statistical analyses were performed in R (R Development Core Team, version 3.0.1), with significance set at $\alpha = 0.050$.

4.4 Results

4.4.1 Survival and activity of labelled cercariae

Our analyses showed a significant decrease in the mortality risk, i.e. longer survival, of NB-labelled cercariae after 24 hpl in all three concentrations compared to control, i.e. 29.9%, 67.9% and 57.3% longer survival in low, intermediate and high concentration (RWM, low, P=0.015; intermediate and high, P<0.001, Appendix 1: Table S2i). After 5 hpl, i.e. the necessary time to run the penetration assay (see *Penetration pattern on host surface* in *Materials and Methods*), both intermediate and high concentration NB-labelled cercariae showed a significant increase of survival when compared to control, when mortality risk decreased by 71.5% and 70.1%, respectively (MMCoxPH, P=0.015 and P=0.019, respectively, Appendix 1: Table S2ii). At this time, cercarial activity significantly increased in all NB concentrations when compared to control cercariae, showing a reduction of the risk of inactivity (longer activity time) in low (57.7%), intermediate (55.7%) and high (62.7%) concentration (RWM, all P<0.001, Appendix 1: Table S3).

On the other hand, survival rate of CFSE-labelled cercariae showed a significant increase, showing a reduction in the mortality risk of 46.0% in low concentration compared to control after 24 hpl (RWM, P<0.001, Appendix 1: Table S4i), but did not show any significant differences after 5 hpl (MMCoxPH, Appendix 1: Table S4ii). Activity rate of CFSE-labelled cercariae after 5 hpl showed a significant increase as the probability of the risk of inactivity decreased by 66.2% in low concentration and 39.8% in intermediate concentration when compared to control (RWM, P<0.001, P=0.040, respectively, Appendix 1: Table S5).

The RWM analyses comparing survival and activity rates between intermediate concentration of NB and CFSE after 5 hpl showed that NB-labelled cercariae exhibit significantly higher cercarial survival, by reducing

the mortality risk by 78.4%, and the inactivity risk by 57.1% compared to control cercariae (Appendix 1: Table S6), while CFSE labelled cercariae did not show significant differences in survival rates ($P>0.05$, Appendix 1: Table S6i) and 39.8% significantly lower inactivity risk than control cercariae ($P=0.041$, Appendix 1: Table S6ii, Fig. 1A). This, together with our preliminary visual assessment, showed that the most adequate fluorescent dye and dose was the CFSE intermediate concentration, with longer permanence and powerful brightness that permits an easy recognition of labelled cercariae during 5 h. Thus, this concentration was chosen to label cercariae used in the following experiments.

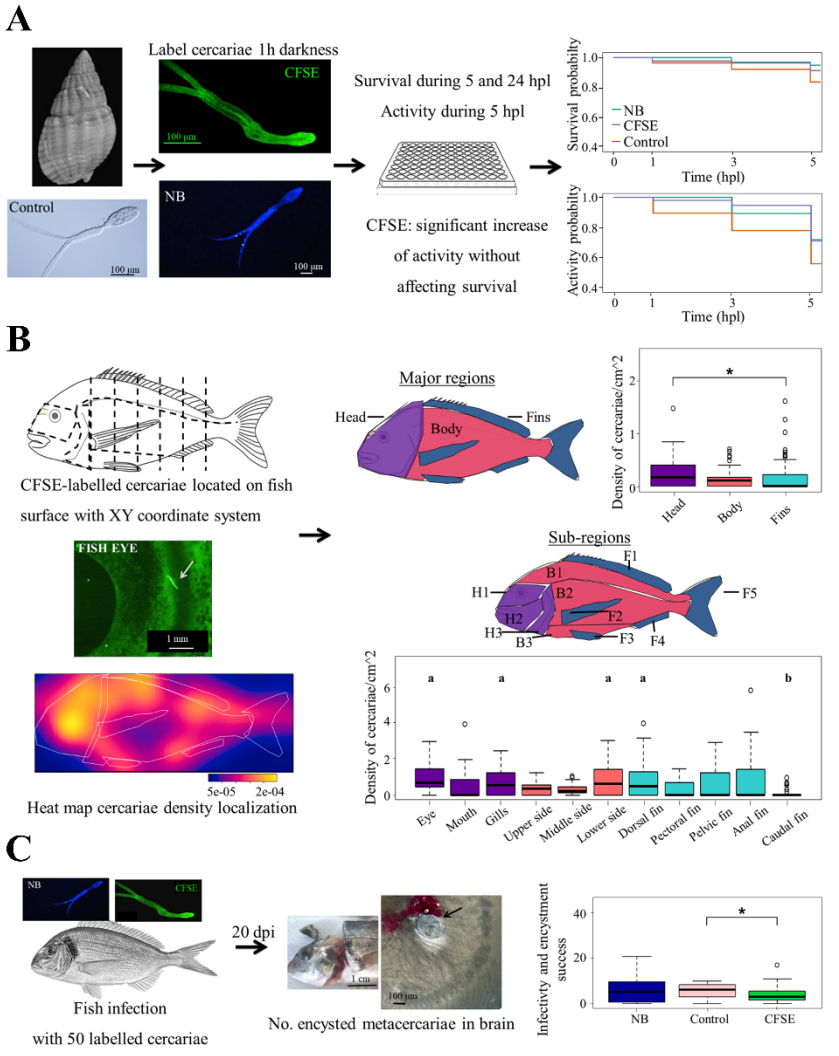


Figure 1. Summary of the main aims and results of our study. **(A)** Dyes effect on cercarial survival and activity, recorded during 24 hpl (hours post-labelling). Survival and activity curves representing the results of the regression Weibull model (RWM) for NB and CFSE labelled cercariae within 5hpl. **(B)** CFSE-labelled cercariae penetration pattern into fish observed and recorded in a XY coordinate system, divided into 3 major regions and 11 sub-

regions. Major region boxplot represents the LMM results, sub-region boxplot represents pairwise comparison results, where "a" indicates the sub-regions significantly different from "b" sub-region (i.e. caudal fin, showing the lowest cercarial density). **(C)** Dyes effect on cercarial infection and metacercarial encystment. The number of brain-encysted metacercariae was recorded 20 days post-infection (dpi). The boxplot shows the GLMM results. Horizontal bars represent median values, box edges indicate interquartile range, first and third quartile, whiskers indicate the minimum and maximum values. Significant differences are marked with an asterisk or with different letters.

4.4.2 Penetration pattern of *C. longicollis* into the fish host

Our results pointed to a non-uniform distribution of *C. longicollis* cercariae on the host´s body surface determined by CSR simulation created based on Quadrat counting test and Kolmogorov-Smirnov test (in both cases, $P<0.001$, Fig. 1B). The heat map illustrates the aggregation of attached fluorescent cercariae especially close to the head, eye and gills region, as well as to the dorsal area of the fish. Furthermore, analyses where the surface was divided into 3 major regions indicated a significant higher cercarial density on the head region compared to the fins, showing 1.10 times more cercariae (LMM, $P<0.001$, Appendix 1: Table S7i, Fig. 1B), this being supported by pairwise comparisons (Appendix 1: Table S7ii). When comparing cercarial density between sub-regions, our results showed that the density of cercariae was not significantly different between the eyes (H1), dorsal fin (F1), gills (H3) and lower side (B3) (LMM, all $P>0.05$), being these areas the most attractive ones. The first two showed significant differences with almost all other regions, while gills and lower side only showed significantly 1.56- and 1.53-times higher density than the caudal fin, respectively (LMM, both $P<0.001$). The eye region, which was the area showing the highest cercarial density, significantly differed from less attractive areas by a range from 1.37 and 1.84 times more (LMM, $P=0.025$ and $P<0.001$, respectively), with the caudal fin (F5) showing

the lowest density (P<0.001). The dorsal fin, the second most attractive area, showed a significant different density that ranged from 1.36 and 1.70 times more (LMM, P=0.021 and P<0.001, respectively), which included the mouth and the rest of fins, with the caudal fin being once again the one with the significantly lowest density (P<0.001). Pairwise comparisons only supported significant differences between the four most attractive areas (eye, dorsal fin, gills and lower side) with the caudal fin (F5) (pairwise comparisons, all P<0.010 except lower side, P=0.013, see Fig. 1B, Appendix 1: Table S8ii).

4.4.3 Dye effect on cercarial infectivity and metacercarial encystment

When looking at the differences in cercarial infectivity after labelling, our results showed that the number of encysted metacercariae after infection was not significantly different between control and NB-labelled cercariae (GLMM, P>0.05, Appendix 1: Table S9, Fig. 1C). However, CFSE-labelled cercariae showed 1.62 times significantly less infection and later metacercarial encystment than control cercariae (GLMM, P=0.004, Appendix 1: Table S9i, Fig. 1C). Pairwise comparisons were concordant with GLMM results (Appendix 1: Table S9ii).

4.5 Discussion

In this study, *in vivo* fluorescent labelling allowed the tracking of the distribution of *C. longicollis* cercariae during the host's penetration and infection, pointing to the eye region, gills, lower side and dorsal fin as the areas where cercariae attach most frequently. Furthermore, the investigation of the effects of two different fluorescent dyes on the survival and activity of cercariae, as well as on their viability to later infect and encyst in the target organ, has permitted the identification of the most adequate dose and substance to label trematode cercariae used in experiments. All cercariae labelled with

NB or CFSE dyes were detectable up to 24 h and probably longer although this was not further explored.

4.5.1 Short- and long-term effects of NB and CFSE fluorescent dyes

Ascending concentrations of both dyes were tested by observing labelled cercariae during 24 hpl, i.e. longer than needed for the fulfillment of the rest of experiments. Of particular interest are the first 5 h after cercarial emergence from the snail host and their labelling since, besides being the time needed to complete the penetration pattern experiment, it coincides with the highest infective period previously described for cercariae (3 to 6 h in *D. spathaceum,* (Karvonen et al. 2003); 1 to 9 h in *S. mansoni,* (Whitfield et al. 2003)). Thus, the study of specific effects of fluorescence during this period is crucial. Overall, NB showed a slight increase of cercarial survival in every concentration, as well as an increase of their activity after 5 hpl. The free-living cercarial stage is short-lived and non-feeding, dependent on glycogen storage for energy metabolism (Anderson and Whitfield 1975, Combes et al. 1994). Nevertheless, it is well known that external factors, including biotic and abiotic factors may affect trematodes´ life spans. Several publications have recently drawn our attention to toxic pollutant effects on trematode free-living stages, since they could affect cercarial survival and activity or infection success: for example, cadmium and zinc in different species, such as *D. spathaceum* (Morley et al. 2002, 2003), mercury in *Diplostomum sp.* (Pietrock et al. 2001); or herbicides and pesticides in several species (Koprivnikar et al. 2006, Griggs and Belden 2008, Hua et al. 2016). The precise mechanisms by which toxic compounds may affect cercarial behaviour remain unknown. In our study, NB showed to have a much larger effect on cercarial survival and activity, increasing both of them. An alteration of the neuromuscular stimulation that controls cercarial swimming (e.g. increased serotonin or decreased acetylcholine (Fried and Prior 1983)) or a more efficient consumption of

glycogen reserves affected by the labelling of the nucleic acid by NB could explain these effects (e.g. cytoxic and kinetic responses rely on the different cell types, even sometimes altering their viability and/or proliferative behaviour, as suggested by Fried et al. (1982)). Overall, CFSE seemed to have a smaller effect on cercarial behaviour, increasing their activity during the first 5 hpl but not their survival. Although mechanisms affected by external substances should be further studied, our current objective was to uncover which fluorescent dye could affect short-term cercarial performance, and it was demonstrated that intermediate CFSE concentration is an adequate method for this. On the other hand, CFSE was the only dye that affected cercariae in the long-term since it decreased fish infection, with 1.62 times less metacercarial encystment, showing that it affects cercariae infective capacity. CFSE labels the acetabular glands of cercariae, which have an important role during the penetration into the host´s skin due to their high protease activity (Curwen et al. 2006, Paveley et al. 2009). Hence, labelling the acetabular glands with CFSE could either inhibit their enzymes or lessen their stimulation thus affecting their capacity to recognize the host. Therefore, NB dye, which did not significantly affect *C. longicollis* cercarial infective capacity, would be a more adequate dye in *in vivo* studies on long-term larval performance.

Our study supports previous studies where CFSE labelling was proven as an appropriate method for a rapid and easy *in vivo* dye for small and translucent infective stages; e.g. myxosporean actinospores (Yokoyama and Urawa 1997); monogenean oncomiracidia (Trujillo-González et al. 2015); even for experiments on specific organs such as the study of the acetabular glands activation process in cercariae by Ressurreição et al. (2015). Although this method has been proven to affect long-term cercarial performance by decreasing infection success and metacercarial encystemt, it is adequate to study short-term survival and activity, allowing us to reveal the penetration pattern of *C. longicollis* cercariae into the fish host (see later). To our knowledge, NB has been previously used only for the discrimination between

live and dead *S. mansoni* eggs (Sarvel et al. 2006) and cercariae after exposure to human serum (Da'dara and Krautz-Peterson 2014), who used a less cell-permeant version of the dye. Thus, this is the first time that survival and activity rates in unlabelled and NB-labelled cercariae have been compared. We consider NB (Hoechst 33342) as a useful tool for differentiation between dead and live larval stages, but more studies on long-term survival of labelled cercariae are necessary before using this dye in experimental assays e.g. testing drug-resistance in human parasites.

4.5.2 Penetration pattern of *C. longicollis* larvae into fish host

Little is known about common attachment and penetration patterns of trematodes into their hosts and each trematode species probably develops a pattern with specific entry portals into the fish hosts. In this study, although *C. longicollis* cercariae showed a non-random distribution, they could be detected attached all over the fish's surfaces, but more frequently penetrating areas as close as possible to the brain, such as the eyes, gills, dorsal fin and lower side. The rest of the fins, the mouth area and the more muscular surfaces of the body, i.e. upper and middle body side, showed slightly smaller numbers of attached cercariae, although none so few as the caudal fin, which seemed to be much less attractive to be used as an entry point into the fish. Once the cercariae contact a possible host by chance, the specificity for fish invasion is achieved by contacting carbohydrates present in the mucus covering the hosts (see Haas et al. (2002) for *D. spathaceum* cercariae), such as happens in cercariae of *Opisthorchis viverrini*, where the attachment and penetration relies on the adequate identification of glycosaminoglycans, and proteins present on the fish surface (Haas et al. 1990). However, specific interactions between a parasite species and its host are the effective strategy to complete their life cycle. Cercariae species usually establish a suitable entry portal that can guarantee a higher infection success, such as the human skin for schistosomes (e.g. *S. mansoni* and *S. haematobium*, (Haas et al. 1994)) or the gills in different

trematodes (e.g. *C. armatus*, (Paller and Uga 2008)). Some cercariae, like those of the eye flukes, usually penetrate through the gills and skin to infect the eye (*D. spathaceum,* (Haas et al. 2002)). Fish mucus attractants could help cercariae to trace, by chemoreception, a favourable entry portal into the fish (Haas et al. 2002), but although host-recognition mechanisms seem to be crucial for their transmission, many species reach their target only after opportunistically penetrating the host, which can risk losing cercariae in a dead end host (Poulin 2007).

Many strigeoids migrate great distances within the host, from the cutaneous entry point to the target organ, as for example *Posthodiplostomum ptychocheilus*, whose metacercariae also establish in the brain of its intermediate fish host (Conn et al. 2008, Hendrickson 1979). However, and to the best of our knowledge, although the migrations routes are better known, the specific or preferred entry portals are still unclear for brain-encysting metacercariae. When comparing areas within the head, the eyes were highlighted as one of the most attractive areas, together with the gills, which accommodated more cercariae than the oral cavity (Appendix 1: Table S10). In any case, this study demonstrated that a high density of attached cercariae concentrate on areas which might connect and transport them faster to the brain. Therefore, for instance, the penetration of the head of the host and especially the gills may facilitate access to the blood in the circulatory system, as happens for example in *D. spathaceum* when infecting the eyes (Höglund 1991). On the other hand, the concentration of cercariae on the lower side might be related to the presence of the pelvic girdles, which are close to the head region in this fish species (see Fig. 1B), and cercariae penetrating the host by the dorsal fin could use peripheral nerves to access the central nerve cord or cranial nerves to directly reach the brain, as proposed in the studies with *P. ptychocheilus* cercariae of Hendrickson (1979) and Matisz et al. (2010). Hendrickson (1979) also suggested that the neuronal canal could be also easily accessed from the caudal fin, even more rapidly than cercariae penetrating in

areas much closer to the brain, but this route seems not to be the preferred by *C. longicollis* since the caudal fin did show lesser cercariae attached on its surface. Thus, overall, infection success of *C. longicollis* cercariae might result from the important attraction showed for areas related to the brain, either by their proximity or by the easy migration routes to reach it.

This study allowed us to have a better understanding of the cercarial transmission and penetration of *C. longicollis* into its fish host, by using optimized detection methods that facilitated our *in vivo* study on larval performance and offered a better visualization of cercariae on the fish surface. Our results validated CFSE as an adequate method to study short-term larval performance, while NB serves better for long-term studies, and demonstrated as well that *C. longicollis* cercariae show an attraction to areas nearby the brain or with easy access to migration routes. Future studies on possible migration routes to target organs within the fish will allow a better understanding of strigeoid life cycles.

4.6 References

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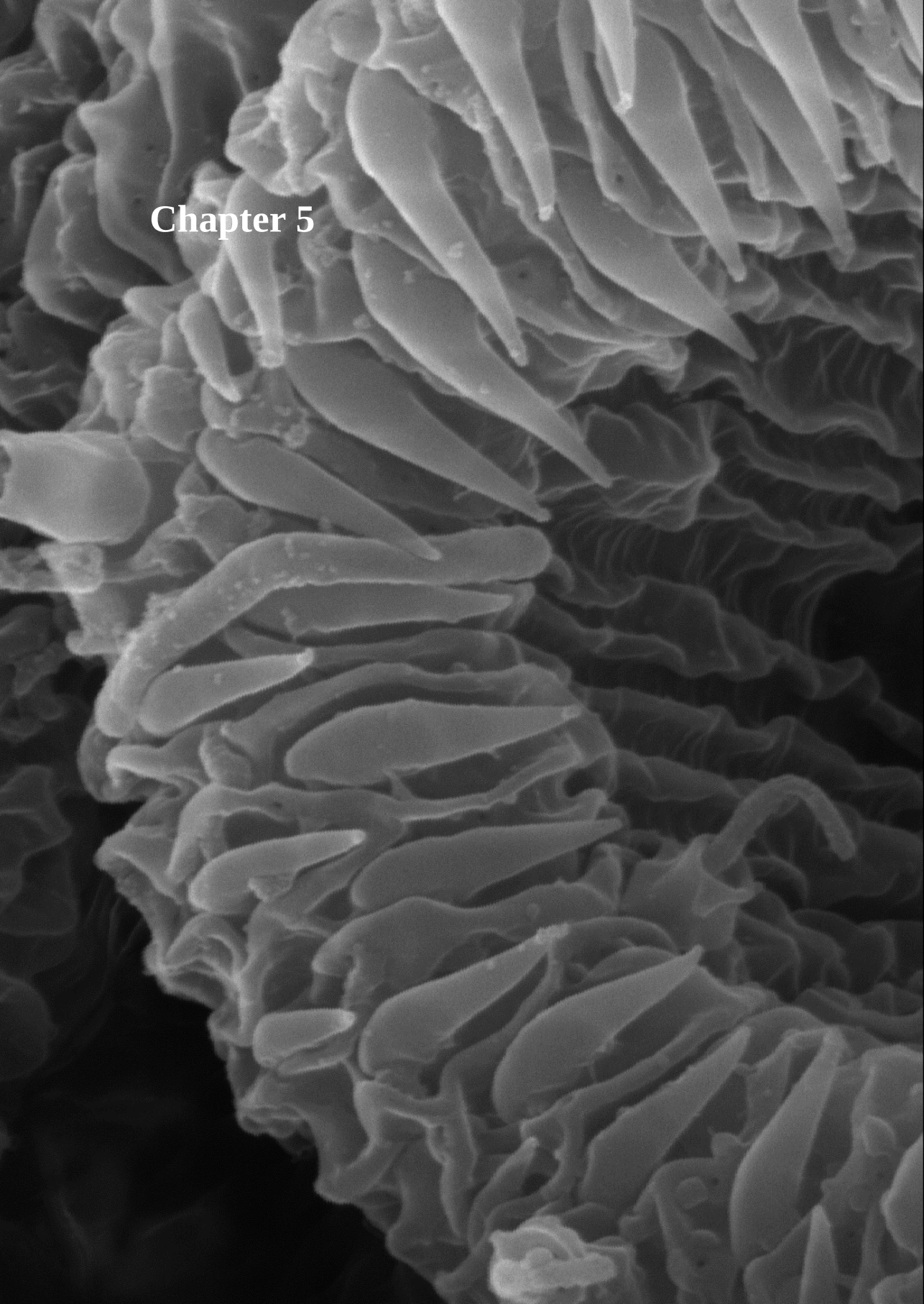

Chapter 5

5. Chapter 5

The versatility of simplicity: Structures of *Cardiocephaloides longicollis* used for different purposes during cercarial transmission

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5.1 Abstract

Transmission and infection strategies are critical for completing the life cycles of trematode parasites, which are characterized by complex life cycles involving multiple hosts and stages. Transmission between the first and second intermediate hosts typically relies on cercariae, a free-swimming larval stage that displays a series of behaviours to efficiently disperse, locate, attach to, and infect the next host. The aim of this study is to provide detailed information on behaviours used by furcocercariae (bifurcated tail) during its transmission from the snail to the fish host, using the laboratory-established model of *Cardiocephaloides longicollis* (Strigeidae). These cercariae are released from snails into seawater, where they swim, locate, penetrate the skin of fish, and encyst as metacercariae in their brain. In a series of *in vivo* assays, freshly-emerged cercariae were used to visually study their behaviour and locomotion. Histopathology of experimentally infected gilthead seabreams with *C. longicollis*, taken at sequential post-infections times, were analysed to localize the migrating cercariae to the fish brain. Our results show that cercariae make front to their challenging transmission by using their multifunctional structures for different purposes. While 80% of the behaviour was spend in a resting position, the most common swimming behaviour was with tail-first, which is commonly described in furcocercariae to reach the host microhabitat. However, *C. longicollis* relies more on the furcae of the tail by using them as a propeller providing thrust and guidance when they swim, instead of using the tail stem. After attaching to the fish skin, cercariae rapidly creep on it using the oral- and ventral-suckers simulating a leech-like movement until they find a suitable penetration site. To penetrate, cercariae press the cephalic structures against the skin, while the ventral sucker anchors the cercariae to it. After this, they switch their locomotion to a slow peristaltic movement, opening the path through tissues with the help of their cephalic structures and anchoring their body with their surface spines. This is

consistent with the post-penetration histological analyses, which suggested that *C. longicollis* cercariae move between the cells of the connective tissue and muscle fibres when migrating toward the fish's brain, without provoking relevant tissue damage or host responses. Understanding the versatility of cercarial structures to adapt to external conditions enriches our knowledge on parasites and their transmission ecology, opening the door to the design of avoidance methods in fish farm struggling with harmful parasites.

Keywords: parasite, life cycle, larval stage, cercariae behaviour, transmission strategies, multifunctional structures

5.2 Introduction

Trematode parasites are characterized by complex life cycles involving multiple hosts and stages and rely on their transmission from one host to the next (Galaktionov and Dobrovolskij 2003). Transmission from a molluscan intermediate host to the next invertebrate- or vertebrate-host, is typically performed by the free-swimming larval stages known as cercariae. The cercariae are released in the water and quickly find a suitable host by developing behaviour strategies to efficiently disperse, locate and infect the host (Bartoli and Combes 1986, Combes et al. 1994, Morley 2020). To ensure the continuity of the life cycle, it is important that the cercariae overlap in space and time with their next intermediate hosts. Most species respond to certain stimuli that allow them to locate themselves in the target microhabitat, such as gravity, turbulence, light and shade (Haas 1992, 2003, Fingerut et al. 2003). Therefore, depending on the characteristics of the next intermediate host, cercariae may for example swim upward to locate pelagic hosts or downward to locate bottom-habiting species (e.g., positive, or negative geotaxy, *Euhaplorchis* sp. and *Probolocoryphe lanceolate*, infecting fish and crabs, respectively, Fitzpatrick et al. 2016). Cercariae can alternate short and long swimming periods with a resting position (Graefe et al. 1967, Saladin 1980, Bartoli and Combes 1986, Galaktionov and Dobrovolskij 2003), which helps them to efficiently use the limited glycogen reserves concentrated mainly in their tails (Anderson and Whitfield 1975). The tail of cercariae is usually a long powerful locomotor structure of similar length to the body, with a well-developed muscle structure that propels the cercariae (Galaktionov and Dobrovolskij 2003), playing a critical activity during swimming. Some cercariae have a single stem tail, such as heterophids, and others have furcae, when the distal end of the stem organ is bifurcated, such as schistosomes (Galaktionov and Dobrovolskij 2003). These are known as furcocercariae and its morphology seems to improve their swimming since

their forked tail provides a more efficient driven locomotion, thus their swimming often being faster than mono-tailed ones (Morley 2020), and in addition allowing a longer floating duration. Furcocercariae use the furcae to swim with the posterior end pointing forward, usually known as swimming with the tail-first. This is produced by alternating contractions of the muscles of the right and left sides of the caudal stem, which provoke the oscillation of the cercariae during swimming, such as in *Schistosoma mansoni* cercariae for infecting humans (Graefe et al. 1967, Nuttman 1974, Krishnamurthy et al. 2017). When cercariae are in the resting position, they spread out the furcae, increasing the floating duration due to their surface gain, and acting parachute-like against the water resistance (Galaktionov and Dobrovolskij 2003).

Once attached to the surface of the host, the cercariae usually detach their tails and creep on the skin to penetrate and burrow into the host at a suitable site (Horák et al. 2002, Haas 2003). Cercarial behaviour and physiology during these phases are of particular interest (Haas 2003), as some species appear to be more sensitive to different stimuli, which can likely determine their transmission, e.g., *Trichobilharzia szidati* required less time to explore for a penetrating site than *S. mansoni* (Haas and Haeberlein 2009). Nevertheless, cercarial creeping-, penetrating- and burrowing-locomotion has been vaguely described except for a few examples (Kathi and Agarapu 2010, Horák et al. 2015). Furthermore, trematodes can be found in a wide variety of host organs, and although their penetration site could be expected to be close to the target organ to exploit their limited glycogen reserves and minimize the host's immune response (Ratanarat-Brockelman 1974), this is not a common approach (Höglund 1991). The most common routes to reach the target tissue are the circulatory system and connective tissue, but these can show a high level of trematode- and host-specificity. For example, the migration of diplosmatoid cercariae to the eyes has been shown to occur by different routes, depending on the parasite and the host

species. While *Diplostomum spathaceum* mainly follows the circulatory system to reach the eyes of trout (Betterton 1974, Höglund 1991), it migrates through muscle and connective tissue when infecting minnows (Ratanarat-Brockelman 1974). Moreover, cercariae can also take more convenient migration routes, e.g., following peripheral nerves to reach the central nervous system and efficiently enter the brain (e.g., *Posthodiplostomum ptychocheilus* infecting fish, Hendrickson 1979, Matisz et al. 2010), and even migrate through the brain to efficiently enter the nasal cavity (e.g., *Trichobilharzia regenti* infecting ducks, Blažová and Horák 2005).

The present study aims to understand the mechanisms used by a furcocercaria during its transmission from its snail host to the fish host, including its migration to the target organ, using the laboratory-established model of *Cardiocephaloides longicollis* (Rudolphi, 1819) Dubois, 1982 (Strigeidae). The cercariae of this digenean parasite are released into the water by bottom-dwelling snails and penetrate the skin of fish, migrating to the brain, where they encyst as metacercariae and are consumed by the definitive host, seabirds (Prévot and Bartoli 1980, Osset et al. 2005). Cercariae infect over 30 fish species from nine different families, including the gilthead seabream (*Sparus aurata* L.), an important farmed species in the Mediterranean (Born-Torrijos et al. 2016). This study provides detailed information on the different transmission phases of the cercariae of *C. longicollis*, whose versatile structures accomplish different goals (i.e. host-finding, -attachment, -recognition, -penetration, -infection) in different environments (i.e. water, host surface, within host body).

5.3 Materials and methods

5.3.1 Collection and maintenance of *Cardiocephaloides longicollis* hosts and cercariae release

Snails *Tritia reticulata* (L.) were manually collected during spring at the "Beach of the Eucalyptus" (40°37′35.0″N, 0°44′31.0″E) in Els Alfacs Lagoon (Ebro Delta, Spain), where snails have been described to have a high infection prevalence (Born-Torrijos et al. 2016). After acclimatizing to laboratory conditions, snails were incubated individually in a cylindrical well (40 ml) for 24 hour (h) at 25 °C, 12:12 h light: dark cycle and 35 psu salinity. Freshly emerged *C. longicollis* cercariae (< 2 h old) were pooled from six infected snails and used in the different assays.

A total of 30 juvenile pathogen-free gilthead seabream (*Sparus aurata*, mean±SD, 10.0 ± 5.3 g and 6.2 ± 2.1 cm standard length) were supplied by a hatchery from Burriana (Castellón, Spain). Fish were kept in 3000 l tanks at the facilities of SCSIE (Central Support Service for Experimental Research, University of Valencia) until used in experimental assays (A1390575271192).

5.3.2 Record of *in vivo* cercariae locomotion

The swimming behaviour of the cercariae was analysed visually by recording sixteen labelled cercariae swimming freely in a 3 mL seawater column for 300 s under a stereomicroscope. Attachment of the cercariae to the fish host was studied using an experimental assay conducted in a previous study with *C. longicollis* cercariae (van Beest et al. 2019). Briefly explained, fifteen fish were individually exposed to 100 cercariae (except two fish, which were exposed to 70 cercariae due to the lack of cercariae in that moment) for 30 min and their position on fish skin was recorded in a XY coordinate system. The average success of attachment of the cercariae to the fish skin was measured and to confirm no significant differences

between fish individuals, a generalized linear model was fitted (GLM with a logit link (binomial family), *lme4* package, Bates et al. 2022) 'attachment rate' (i.e. number of cercariae attached to a fish relative to the number of cercariae used for infection) as response variable and 'fish identity' as explanatory variable. Creeping and burrowing behaviour of thirty cercariae artificially placed on a freshly obtained dorsal fin of a gilthead seabream was recorded in a petri dish containing filtered seawater for 180 s under a microscope. The cercariae were measured (body length, fore- and hind-body, and the width of both suckers, i.e. oral-, ventral-sucker and the posterior) every 4.3 s for 180 s during their creeping and burrowing behaviour using Tracker 5.0 software. To investigate the relationship between these measurements and the above-mentioned areas, both factors were statistically correlated (package *GGally*, using *ggpairs*, Schloerke et al. 2021). All cercariae were labelled *in vivo* with neutral red (0.001 mM, 10 min) and recorded with a video camera (24 frames per second). All analyses were performed in R software (R Development Core Team, version 4.1.1) and videos were edited using Adobe Premier 2019 software.

5.3.3 Histopathology and ultrastructural data relevant to penetration and migration into fish

A total of 30 juvenile gilthead seabream fish were artificially individually infected with 100 cercariae each, following the infection method of van Beest et al. (2019). These were sacrificed sequentially at different time points post-infection, i.e. 30 min, 1, 2, 4, 8, 24, 36, 48, 72 h post-infection (hpi) and 5 days post-infection (dpi), by MS 222 overdose (Tricaine methanesulfonate, 0.030 % solution buffered in seawater; Sigma-Aldrich). Fish body was divided into three regions, (*a*) head (including operculum), (*b*) middle-body (including the organ cavity), and (*c*) posterior body. Samples were fixed in 10% buffered formalin for 2 days for histological examination after being in formic acid for 12 h. Samples were dehydrated

in graded alcohol and embedded in paraffin (Shandon Histocentre 3, Thermo Electron Corporation). Paraffin blocks were sectioned (5-7 µm, 20-40 sections per block), using a microtome (Leica HM330, Leica Biosystems, Germany). The sections were stained with hematoxylin and eosin and mounted in DPX. Histological sections were examined at magnification between 100 and 400x (Leica DM 5000B, Jenoptik Laser Optik, system GmbH, Germany) and photographed (ProgRes C3, ProgRes CapturePro 2.7, Germany).

While detailed information on the ultrastructure of *C. longicollis* cercariae has been described in a previous study (Yoneva et al. 2022), here we focus on those structures that were not included but are likely involved in the locomotion of cercariae during swimming and burrowing, i.e. the furcae and the rose-thorn spines on the fish body`s surface For this, we used the same procedure as described in Yoneva et al. (2022), using a Hitachi S4800 scanning electron microscope (Hitachi, Japan, accelerating voltage 1 kV, Central Service for Experimental Research of the University of Valencia (Spain)) and a JEOL JEM-1010 transmission electron microscope (JEOL, Tokyo, Japan, accelerating voltage 80 kV, equipped with a CCD digital camera Mega View III at the Laboratory of Electron Microscopy, Institute of Parasitology, Biology Centre of the Czech Academy of Sciences (Czech Republic)).

5.4 Results

5.4.1 Record of *in vivo* cercariae locomotion

Neutral red-labelled cercariae helped identify and register three different swimming behaviours, (1) resting position, i.e. body upside down with furcae spread out and acting parachute-like, which is reinforced by muscle fibres in the ventral side of the furcae near the splitting point (Fig. 1A), (2) swimming tail-first, i.e. swim with the tail directed forward (Fig. 1B), and (3) swimming body-first, i.e. swim with the body directed forward (Fig. 1C). The most common swimming behaviour observed was the cercariae in resting position, followed by swimming with tail-first and the less common was swimming with body-first, i.e. 80%, 19% and 1% of the time, respectively. During these latter two behaviours, the swimming and resting periods show variable durations, which resulted in the cercariae swimming being displaced in variable distances and even doing U-turns. Because *C. longicollis* has a bifurcated tail, the cercariae used the furcae as a swimming locomotion thrust system, consisting of two parts: the power stroke, i.e. one furca shows a powerful stroke, and the recovery stroke, i.e. the other furca shows a weak stroke (Fig. 1B, C and Appendix 2: Video S1). The power stroke was followed by a synchronous recovery stroke that caused the tail to oscillate between two fix points, namely the ventral sucker and the distal end of the tail stem (Fig. 1B and C). When the cercariae swam with the tail-first, the power stroke moved toward the head of the cercariae, while the recovery stroke spread out in opposite direction of the head of the cercariae (Fig. 1B). Repetition of this combination caused the cercariae to move with tail-first. In contrast, when swimming with body-first the power stroke spread out in opposite direction of the head of the cercariae while the recovery stroke weakly moved toward the head of the cercariae, and the repetition of this combination caused the cercariae to move with body-first (Fig. 1C).

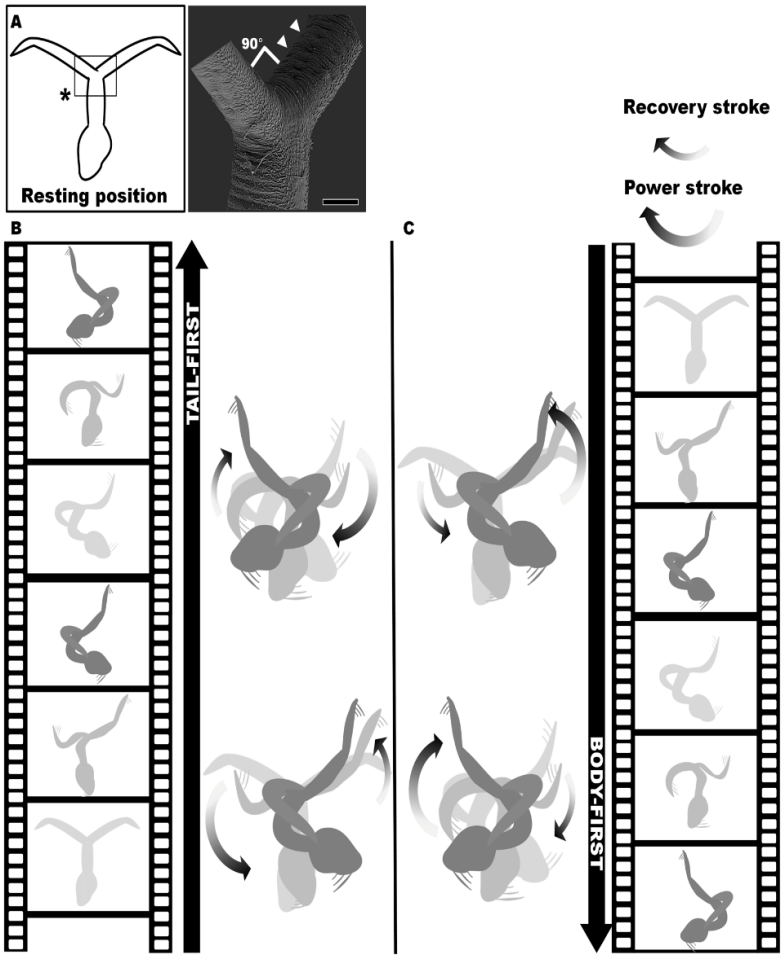

Figure 1. Cercarial swimming locomotion. **(A)** Resting position illustration and SEM picture of the ventral side of the furcae in the splitting point. Head-arrow indicate the development of muscle fibres. **(B)** Illustration of cercarial sequential locomotion display when swimming tail-first **(C)** and body-first. Two arrows with colour gradient, white to black, indicating the sequential display of the cercariae swimming; the large arrow indicates power stroke and the small one the recovery stroke. Scale bars A =10 μm.

The study of cercarial attachment showed an average of 30% success rate of cercariae attaching to the fish skin after 30 minutes (mean±SD, 30.05 ± 9.87), with no significant differences between fish individuals (GLM, estimate= -0.026, SE=0.131, z-value=-0.198, P=0.843). Creeping and burrowing locomotion of the cercariae was described based on the recorded behaviour, to facilitate that description the cercarial body was divided in four regions: (*a*) the head, containing the oral sucker, (*b*) the upper middle-body, containing the upper transversal rows of spines, (*c*) the middle-body, containing the ventral sucker and transverse rows of spines and (*d*) the posterior region (Figs. 2A and 4A). After the *C. longicollis* cercariae attached to the fish, they detached their tails and creeped on the host surface to find the penetration entry point (Fig. 2A and B). The cercariae simulated a leech-like movement with large onwards unidirectional steps to move forward, when the oral sucker detached from the fish skin and the region a and b stretched elongating forward until the oral sucker reattached to the skin (Fig. 2A Display 2). The ventral sucker, which probably served as an anchor while region a and b were elongating forward, later detached from the skin and regions b, c and d stretched and squeezed back and forward (Fig. 2A Display 3 - 4) until the ventral sucker attached to the skin (Fig. 2A Display 5 and Appendix 2: Video S2 and S3). Once the cercariae reached the entry portal into the host, the ventral sucker anchored to the skin and the fore-body moved back and forward (Fig. 3A and B, respectively), probably pushing their way in with the help of the cephalic spines (Fig. 3B ii). Observations of *C. longicollis* cercariae burrowing into fish tissue suggested that the extension and contraction of the body was not as evident as during creeping locomotion. The body moved onward peristaltically in small steps. The oral sucker and mouth structures (region a) might be strongly involved in opening the path (Fig. 4A Display 1 – 2), while the rose-thorn spines, which have a broad base and a single-curved pointed tip and are located on cercariae flanks surface (Fig. 4B), probably contributed

to anchoring the body to the surrounding tissue when it was squeezed, sticking out the spines perpendicularly to the cercarial tegument. They then transitioned to a flat and parallel position from tegument when the cercariae stretched their bodies, making possible their detachment from the tissue (Fig. 4A Display 1-2). To push the oral sucker forward, the region b stretched while the region c anchored the body (Fig. 4A Display 2 and C). After opening the path, region b anchored the body and regions c and d moved back and forward likely to detach the spines from region c, move forward and then anchor region c again (Fig.4A Display 4 and D and Appendix 2: Video S4 and S5).

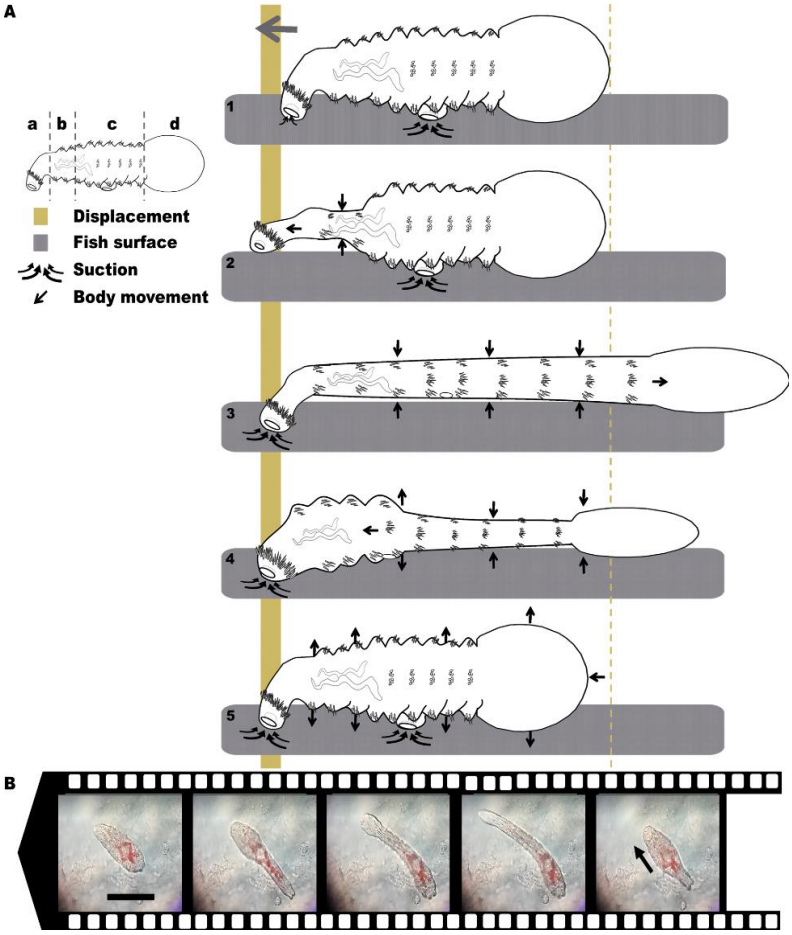

Figure 2. Proposed cercarial creeping locomotion. **(A)** Illustration of cercarial creeping locomotion (Display 1 to 5). The four regions of the body are detailed on the upper left corner ((*a*) the head, containing the oral sucker, (*b*) the upper middle-body, containing the upper transversal rows of spines, (*c*) the middle-body, containing the ventral sucker and transverse rows of spines and (*d*) the posterior region). **(B)** Living cercariae creeping on the surface of the fish´ dorsal fin. Black arrows indicate musculature motion and curved arrows indicate suction. Scale bars B =100 µm.

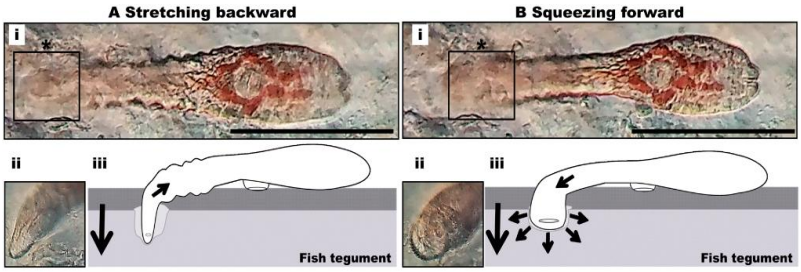

Figure 3. Proposed cercarial penetration locomotion. Picture of living cercariae (**i, ii**) and illustration (**iii**) of cercariae **(A)** squeezing forward and **(B)** stretching backward during penetration of the fish skin. Big arrows indicate direction of the cercariae and small arrows indicate the musculature motion. The asterisks in (**i**) show the location of the oral-sucker represented in (**ii**). Scale bars A, B = 100µm.

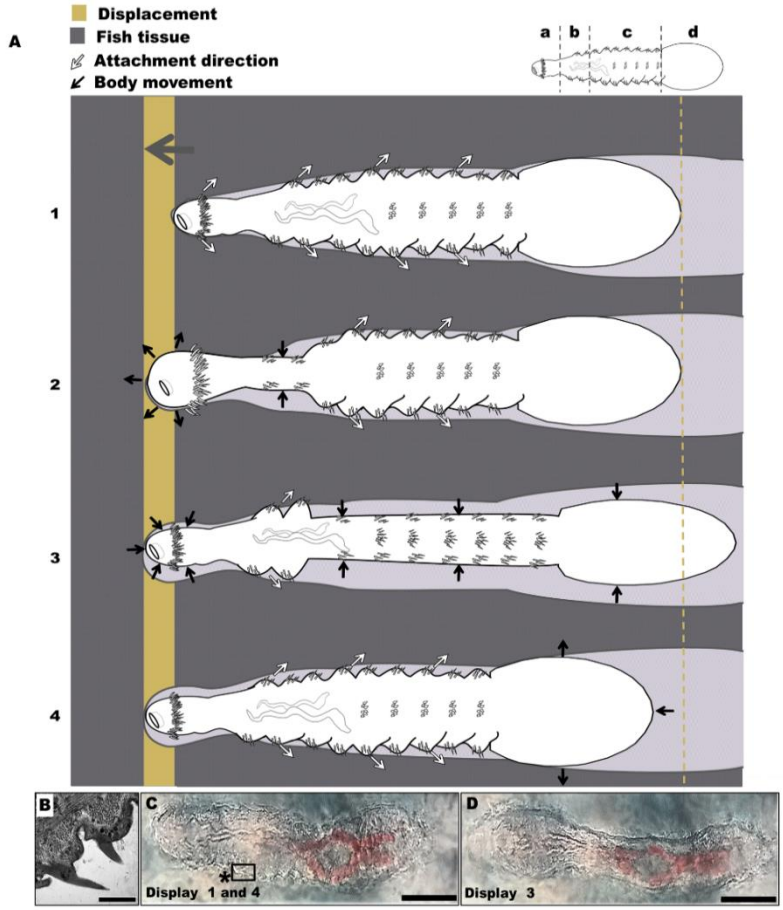

Figure 4. Proposed cercarial burrowing locomotion. **(A)** Illustration of cercarial burrowing locomotion (Display 1–4). Black arrows indicate musculature motion and white arrows indicate the direction of spine attachment to the tissue. The four regions of the body are detailed on the upper right corner (*a*) the head, containing the oral-sucker, (*b*) the upper middle-body, containing the upper transversal rows of spines, (*c*) the middle-body, containing the ventral-sucker and transverse rows of spines, and (*d*) the posterior region. **(B)** TEM picture of rose-thorn spines. **(C)** and **(D)** details of burrowing locomotion of live cercariae burrowing into fish's dorsal

fin. The asterisk in C shows the location of the spine represented in B. Scale bars B = 1µm; C, D = 20µm.

In addition to describing the cercariae observed in the videos, statistical analyses of their body measurements revealed the correlation between body regions during creeping and burrowing (Fig. 5A and B, respectively; measured regions indicated by lines). During creeping, the fore-body length and ventral sucker width were significantly correlated to the length of the cercariae, i.e. when the cercariae elongate, the fore-body elongates and the ventral sucker becomes narrower ($r=0.913$, $P<0.001$; and $r = -0.470$, $P=0.002$, respectively, Fig. 5C). Moreover, the width of the oral sucker showed a significant correlation between the length of the fore-body and the hind-body, i.e. the fore-body and oral sucker act as anchors when the hind body moves ($r = -0.422$, $P=0.005$; and $r = 0.460$, $P=0.002$, respectively). When *C. longicollis* cercariae burrowed, the fore- and hind-body length were significantly correlated with the body length ($r=0.641$, $r=0.573$ (both $P<0.001$), respectively). The ventral sucker was significantly correlated with the fore- and hind-body length, i.e. when the fore- or hind-body expands, the total length of the body increases, and consequently the width of the ventral region becomes narrower ($r=-0.385$, $r=-0.383$ (both $P=0.012$), respectively).

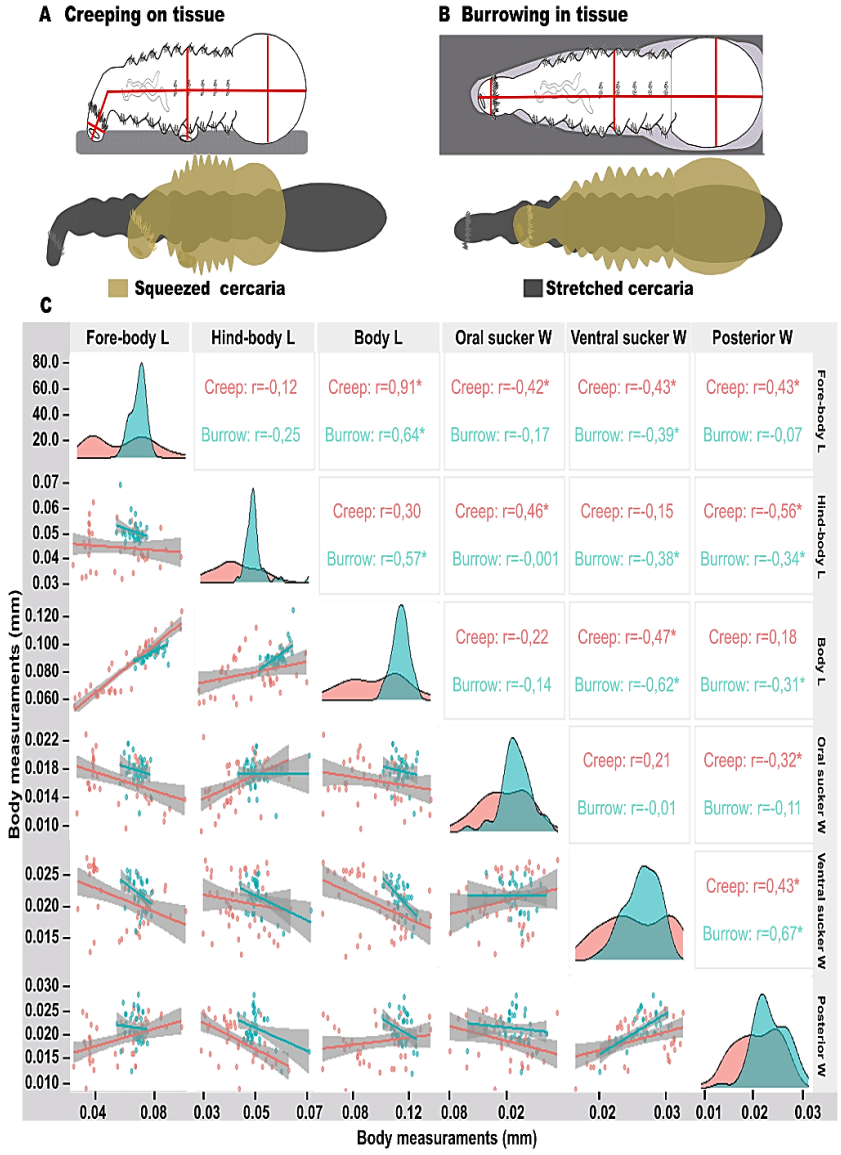

Figure 5. Body changes of cercariae during creeping and burrowing locomotion. Body changes of cercariae during creeping and burrowing locomotion. Illustration of **(A)** creeping and **(B)** burrowing cercariae. The lines indicate the measured regions that were compared (Length (L) and Width (W)). **(C)** Graphical and statistical results comparing measurements

between regions while creeping (red colour) and burrowing (blue colour). The regions are indicated on the right and on the top of the graph. Significant correlations are indicated with an asterisk.

5.4.2 Histopathology of cercariae migration into fish

Histological sections at different time points post-infection confirmed that *C. longicollis* cercariae mainly migrate through tissues containing connective tissue to infect the target organ, the brain of the fish. Cercariae of *C. longicollis* were detected in connective tissue 4 h post-infection (hpi) near the nostrils of the fish (Fig. 6A), in the connective tissue of the oral cavity, between muscle fibres near the peduncle and in the dermis near to the mandible. Most of the detected parasites displayed the characteristic histological features described in digenean cercariae, indicating that migrating cercariae were still viable. Unfortunately, cercariae detection was unexpectedly low, thereby, reporting relative counts of cercariae per tissue and time interval was not possible. In addition, cercariae with features in degeneration, with disaggregated-detached cells, were identified in sections of the fish dermis at 36 hpi (Fig. 6B), likely these being abortive cercariae. No relevant alterations or pathological features such as presence of necrotic material, detached or degenerated cells or inflammatory changes were detected associated to these cercariae during their burrowing and migration to the brain.

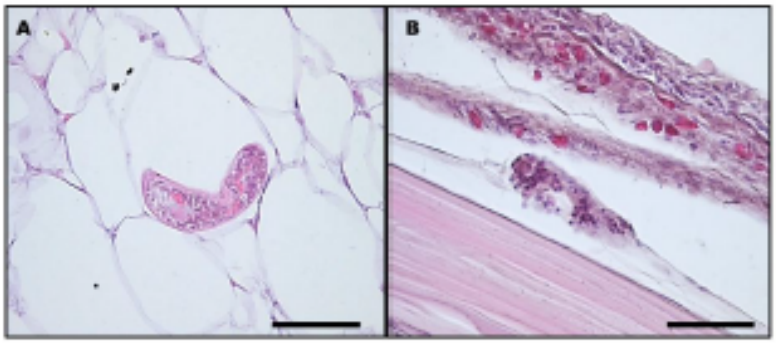

Figure 6. Histological sections of *Cardiocephaloides longicollis* cercariae infecting the fish. (A) Cercariae surrounded by adipose tissue below the orobranchial cavity 4 h post-infection (hpi) with no visual damage or alteration in the surrounding tissue and (B) cercaria found near the dermis of the fish head 36 hpi with disaggregated-detached cells. Scale bars A, B =50 µm.

5.5 Discussion

This study provides detailed information on the behaviour that *C. longicollis* furcocercariae use as a strategy to locate and infect their next intermediate host by swimming to the fish, creeping on its skin and burrowing into it. Our results suggest that despite the cercariae simplicity, these have shown a great versatility by using their organs for different purposes moving through different environments to ensure the continuity of their life cycle.

Cercariae exhibit strategical behaviours to successfully locate, attach and infect their next intermediate host (Combes et al. 1994). Once the cercariae emerge from their snail intermediate hosts, they need to place themselves close to their next intermediate fish hosts, mostly demersal fish in case of C. longicollis (Born-Torrijos et al. 2016). These cercariae use light and gravity for this (Bartoli and Combes 1986), suggesting that the likelihood of infecting these fish strongly relies on the cercarial performance to swim from the bottom dwelling to mid-water depth. However, even if some cercariae may benefit from external conditions such as water currents to facilitate their arrival to the suitable host-space (Morley 2020), the tail activity remains important for cercariae dispersal. Furcocercariae commonly swim with tail-first against gravity to reach the host-space (Combes et al. 1994, Krishnamurthy et al. 2017), which is consistent with our results. However, our results show a different swimming locomotion than that described previously for furcocercariae, including *C. longicollis* (Prévot and Bartoli 1980). While the swimming of the cercariae is triggered by the contraction of the longitudinal muscles of the tail stem, causing an oscillatory movement that moves the rest of the body from right to left (Bartoli and Combes 1986, Krishnamurthy et al. 2017), our study suggests that the thrust is not triggered by the muscular contraction of the stem of the tail, but by the stroke of the furcae against the water resistance, likely acting as a propeller guiding the cercariae. The combination of power strokes and synchronous recovery strokes of the furcae drives the cercariae to swim tail- or body-first. When C. longicollis cercariae are in a resting position, they spread out the furcae, covered by tegumental spines (Yoneva et al. 2022), to increase their

parachute-like effect against the waterresistance and maintain their position in the water column, which is consistent with the behaviour of most furcocercariae in a resting position (Horák et al. 2002, Galaktionov and Dobrovolskij 2003). Since our results suggest that *C. longicollis* cercariae remain in resting position most of the time, strong muscle fibres need to be present on the ventral side of the furcae splitting point, helping to reduce their sinking. Furthermore, the use of the furcae as a propeller and also as a parachute provides a versatile swimming behaviour, where they can switch from swimming with tail- to body-first by changing only the force they apply to the furca stroke. Even though some cercariae display specific behaviours near their potential hosts (Horák et al. 2002, Krishnamurthy et al. 2017), these two behaviours may indicate that *C. longicollis* responds quickly to nearby hosts, following a non-specific encounter mechanism that increases their attachment likelihood, thereby explaining the broad range of fish species from different families that *C. longicollis* cercariae can infect (Born-Torrijos et al. 2016). However, given the specific penetration pattern of *C. longicollis* cercariae (van Beest et al. 2019) and their ability to detach and reattach after an encounter (unpublished results, in laboratory conditions), the specificity in selecting the host appears to be acquired after attachment, using their four types of sensory papillae (Yoneva et al. 2022) or likely through a specific recognition mechanism as in other cercariae (e.g., Feiler and Haas 1988, Haas et al. 2002).

After attachment, the cercariae creep to the entry portals using the oral- and ventral-sucker leech-like movement, with the help of the well-developed musculature observed around the suckers (Yoneva et al. 2022), which allows a strong adhesion to the skin of the fish (Haas and van de Roemer 1998, Dorsey et al. 2002, Haas 2003). During this phase, the cercariae move rapidly and with wide movements, maintaining the attachment to the host surface with at least one of the suckers, probably reducing the risk of detachment as they are exposed to water resistance when the host moves. Nevertheless, it has been suggested that the water current does not affect creeping as much as expected, but rather an increased constriction due to limited space may stimulate the switch from a leech-like to a peristaltic movement, as has been observed in the adult stage of *S. mansoni* found in the venous system (Zhang et al. 2019). To penetrate the skin, *C. longicollis* cercariae probably open the path by pressing the mouth structures against the tissue, similar to *Trichobillarhzia* sp. during penetration (Horák et al. 2015). Peristaltic movements help them move slowly through tissues, while they mostly use the oral sucker and rose-thorn shaped spines, distributed over the entire body surface (Yoneva et al. 2022) to likely burrow into the body (Conn et al. 2008, Whitfield et al. 2003). The middle-body, with nine transverse rows of spines (Yoneva et al. 2022), might be strongly involved in the leech-like locomotion and peristaltic movement by stretching and squeezing the muscles. Diplostomatoid cercariae have been described migrating through the muscle, connective tissue, and the circulatory system to reach the target organ (e.g., the pericardium, the eyes (Ferguson 1943, Larson 1965, Johnson 1971, Betterton 1974, Ratanarat-Brockelman 1974, Höglund 1991Haas et al. 2007)). This is consistent with our results, as *C. longicollis*

cercariae migrate through connective tissue and between skeletal muscular fibres before reaching the brain. However, other brain-infecting cercariae migrate through more convenient tissues, i.e. using the peripheral nerves, which allow a rapid connection to the central nervous system (Matisz et al. 2010). This ability might be related to a response of the cercariae to cues in the host body that help orient themselves toward the target organ (Grabe and Haas 2004, Haas et al. 2007). This could also explain that the majority of cercariae migrate through the same path, but some cercariae may take a different route. For example, most *Ornithodiplostomum* sp. cercariae infect the pancreas or liver by migrating through muscle and connective tissue, however, a few migrate through the circulatory system (Matisz and Goater 2010). Our results also suggest that some of the cercariae that manage to penetrate the host, fail to reach the target tissue and become stuck in the dermis and in the skeletal muscle, as suggested by the disaggregated cercariae detected in few sections from the dermis above the fish scales, which could impair penetration into the host. Degenerated diplostomid cercariae have been described in the mesentery and liver when migrating toward the eyes of minnows (Ratanarat-Brockelman 1974). Although we analysed numerous sequential time points post- infection, the number of cercariae detected in the sections was unexpectedly low considering the infection rate of *C. longicollis* in experimental fish (~30% based on 12 fish, extracted from van Beest et al. 2019). This may be due to the relatively large size of the seabream juveniles used in this assay and the difficulties to process hard tissues such as bone, becoming a challenge to track day-to-day changes in the migration of cercariae in the fish. Therefore, although our results might indicate that *C. longicollis* larvae exclusively move through the connective tissue, we cannot rule out the possibility that larvae can be able to move through other specific tissues and paths, and that we may have missed important migration events between time intervals.

Nevertheless, the migrating cercariae of *C. longicollis* did not provoke any significant tissue damage or host responses, as commonly observed when cercariae move in spaces between bundles of cells rather than through them when migrating into muscle and connective tissue (Johnson 1971, Ratanarat-Brockelman 1974). This negligible detrimental effect of the cercariae on the host during their route may be considered as a minimal invasion strategy to not affect the host survival during the parasite's invasion. The combination of the burrowing movements and cercarial spines likely allows the cercariae to migrate between the muscular skeletal fibres, burrowing into a particular collagen abundant extracellular matrix, which avoids the damage on cellular structures and facilitates the tissue healing (Johnson 1971, Padrós et al. 2018). Since the connective tissue is a common fish body tissue, it allows the cercariae multiple options for their migration to reach their target organs (Johnson 1971, Ratanarat-Brockelman 1974). Given the great variety and different levels of specificity in the migration pathways of trematodes within their hosts, the use of this parasites as laboratory models might help to elucidate the mechanisms underlying the use of certain migration routes over others.

Overall, *C. longicollis* cercariae have shown a great versatility in adapting to external conditions by using their structures for different functions, e.g., the oral sucker can help to hold the body while creeping on the surface of the host, but it can also open the path while penetrating and burrowing into the fish. Linking the description of cercariae versatile organs to their behaviour helps to understand trematode transmission strategies and therefore enriches our knowledge on parasites and their ecology. This may be especially helpful for fish farms struggling with harmful parasites, as methods targeting the biological functions of cercariae could be used to avoid infecting fish, e.g., by using water currents targeting attachment mechanisms.

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Chapter 6

6. Chapter 6

Visible Implant Elastomers in gilthead seabream (*Sparus aurata* L.) for experimental research: preferred injection sites to optimize tag retention and minimize histological effects

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6.1 Abstract

Fish tagging is an important tool for individual or group identification and is commonly used for biological, ecological and fisheries management studies. The key for ensuring the quality of these studies lies primarily in the tag design, which should be economically affordable, easy to handle and provide long-term, efficient detection, without compromising fish health and welfare. A great variety of tags exists (e.g., natural marks, T- anchor tags, fin-clipping, passive integrated transponders), but all have their limitations. Visible Implant Elastomers (VIE) tags are often used in experimental studies, where small fish are often used due to space limitations. VIE tags are coloured, biocompatible silicone marks that are injected under the skin. The colour is externally visible, which allows for efficient tag identification and reduces handling of the fish. In this study, we aim to validate VIE tags on gilthead seabream, an important model in marine parasitology research. Fish of different sizes were tagged on the dorsal and/or caudal fin and monitored weekly for up to 180 days. Overall, VIE tags were visible and no indicators suggested an impaired welfare of the tagged fish, e.g., no mortality or reduced growth rate of the fish, no morphological abnormalities, or injuries, nor any conspicuous swimming or feeding activity, similar to untagged fish. Tag retention analysis (Beta regression model) demonstrated that injection site and fish size were critical for tag optimization. Histological analysis revealed no severe tissue response when the silicone was in connective tissue, however, the silicone sometimes migrated to organs such as the kidney and spleen. Although this phenomenon is known in tadpoles, it had not yet been observed in fish. It was particularly frequent when tagging the caudal fin, likely related to their well-developed vascularization, where the silicone probably reaches the circulatory system. This migration had no effect on fish health, as melanomacrophage centers involved in phagocytosis were the only response in the kidney and spleen when this occurred, and this could probably be avoided by reducing the volume of VIE material injected into the

caudal fin. Considering the low toxicity of VIE tags, and despite the migration of the material in the fish, we recommend these tags as a safe option for use under laboratory conditions at two tagging sites in a wide range of sizes of gilthead seabream. In addition to providing the most up-to-date information on the use, retention, and effects of VIE tags on fish for research purposes, we also suggest testing VIE tags prior to experimentation to avoid compromising the robustness of the study.

Keywords: fluorescent coloured VIE tag, optimization, experimental fish testing, tag migration

6.2 Introduction

Studies that investigate different aspects of fish biology from physiological, behavioural, ecological and even evolutionary perspectives typically use fish tags, which are particularly useful in monitoring fish in applied research and aquaculture. There are a variety of tags with different capabilities and limitations that can be divided into two groups, i.e. natural *versus* artificial tags (McFarlane et al. 1990, Fig. 1). Natural tags use natural marks or specific characteristics to distinguish populations, stocks or even individual fish. Some of these natural tags can be used to externally identify fish, such as by body shape, size, potential body malformations, skin marks or scars resulting from previous skin lesions, and mottling patterns in some fish species (Sandford et al. 2020). Other natural tags, such as circulus pattern of scales, genetic markers or even parasites, may also serve as references (Mosquera et al. 2003). Although natural tags are considered a non-intrusive method, they are not commonly used in some research areas because they can be eventually altered by internal or external factors, limiting their use in some fish species and presence of stable phenotypical traits (e.g., Yoshizaki et al. 2009, Narejo et al. 2008, Merz et al. 2012, Castillo et al. 2018). Artificial tagging methods are frequently used to help observers distinguish one fish from another. There is a wide variety from fin clipping and branding to more sophisticated external and internal tags such as archival tags or tags associated with acoustic and radiotelemetry satellite position tags (McFarlane et al. 1990, Parker et al. 1990, Sandford et al. 2020). These are important for studying populations or individual movements and migrations, age and growth, survival, and behaviour of fish (McFarlane et al. 1990, Sandford et al. 2020). Therefore, a successful artificial tag design should be economically affordable, easy to manage, and provide efficient and long-term identification without compromising fish health and welfare by maintaining natural fish growth, behaviour and fitness. Ideally, this combination will help identify potential harm to fish so that it is possible to intervene and maintain fish

welfare (McFarlane et al. 1990, Huntingford et al. 2006, Sneddon 2019, Sandford et al. 2020, Macaulay et al. 2021).

Tagging success is highly dependent on the type of tag, its location, and the tagging process itself, which includes capture, anaesthesia, handling tag application and fish recovery (Jepsen et al. 2015). The most common artificial tags used in observational studies that do not require fish handling are external tags. These are attached to the body of the fish by an incision or anchor that penetrates the fins, opercula, skin, or muscle (McFarlane et al. 1990, Bergman et al. 1992, Osbourn et al. 2011, Akins et al. 2014). In fisheries, tags associated with a colour and identification code containing information about the individual, stock or study are commonly used, such as Carlin tags, Peterson discs, opercula and jaw tags, spaghetti, T-bar anchors or dart tags (Fig. 1). They are economic, easy to apply, especially for large fish, and easy to detect. Unfortunately, in some cases these tags can cause injuries that can affect growth, swimming behaviour and even fish health and survival (Bergman et al. 1992, Hühn et al. 2014). This problem can be particularly severe when space is limited, as in fish farms and research facilities, and can result in fish scraping other specimens, walls, structures, or nets (Mourning et al. 1994, Jepsen et al. 2015) to the point that tagged fish no longer represent the group under study (Macaulay et al. 2021). This problem can be avoided by using internal tags that are inserted and implanted intramuscularly or intraperitoneally into the fish body (Sandford et al. 2020). Nowadays, the most common internal tags are passive integrated transponder tags (PIT tags), which are activated by a tag reading device and code wire tags (CTW), which require a special detector to be read (Fig. 1). Internal tags can be read without having to catch or handle the fish and their small size allows for tagging a wide range of fish species and sizes (Sandford et al. 2020). However, it is important to remember that these tags require a longer handling time when injected, which causes more surgical stress and increases the risk of infection after release (Boitani and Fuller, 2000). techniques continue to be developed that address society and fish farmer

concerns, such as farm welfare and environmental sustainability (Noble et al. 2018, Bovenkerk and Meijboom, 2020).

6.2.1 Artificial tagging in fish research

Fish tagging in research allows to minimize the number of animals and obtain more information from the same animals used, which is known as "Reduction", one of the three Rs in animal research (Russell and Burch, 1959). Furthermore, their use is required when fish cannot be kept in separate tanks or aquaria because of the experimental design or setup (Fig. 1, tags used in experiments are indicated with an asterisk). Because there is no perfect tagging method, tag design can be challenging as it depends on the fish species, size, and life stage as well as the duration and aim of the study. Small fish are often used in this type of work, and therefore external tags are generally not used to avoid tag dragging that could affect fish welfare (Jepsen et al. 2015). Body tagging, such as fin clipping and freeze-branding (Fig. 1, indicated with an asterisk), has been used for several years as an artificial tagging method under laboratory conditions because it is easy, inexpensive, and quick to perform. However, the number of tag combinations is limited (Pine et al. 2012, Mortensen et al. 2013) and their fast regeneration can be problematic for individual recognition in long-term studies, as the shape of the cut or mark on the skin changes and fades as the fish grows (Eriksen et al. 2011, Mortensen et al. 2013). The small PIT on small fish is usually used to identify fish individually during experiments (Sandford et al. 2020), however, it is size-dependent and may be associated with fitness losses (Jepsen et al. 2005). Dye injections, which are easy to apply, to recognize and economical (Cooke et al. 2013, Wilder et al. 2016), are an interesting alternative for visual identification of fish. The use of Visible Implant Elastomers (VIE) in fish has increased (Croft et al. 2004, Jungwirth et al. 2015a, 2015b, Kozłowski et al. 2017, Booth and Beretta 2021, Rácz et al. 2021), as it meets the requirements for an efficient fish tagging technique and has no obvious negative effects on fish welfare (Sandford et al. 2020). VIE tags consist of a

biocompatible type of silicone, mainly composed by siloxane with different colours that are injected as a viscous liquid that hardens into a solid mass. When these VIE tags are injected under the skin or into transparent structures such as fins, the colours are externally visible.

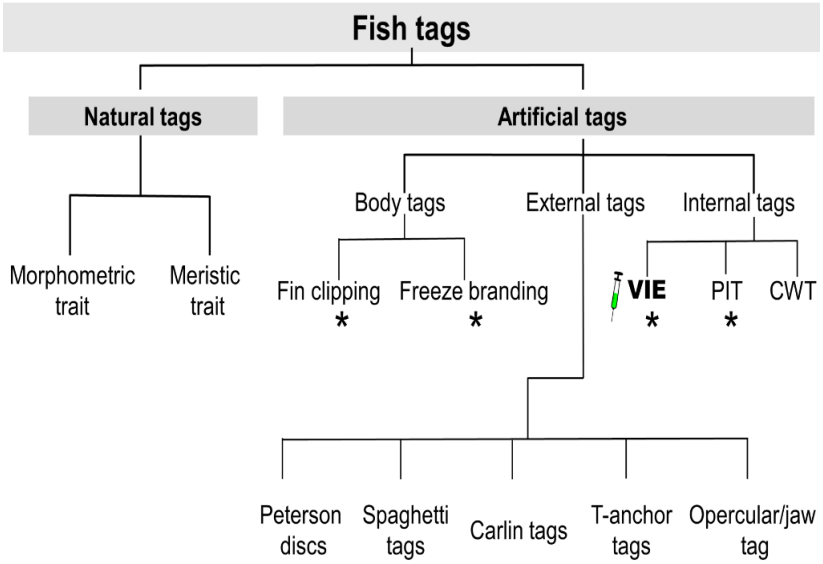

Figure 1. Overview of the most frequently tag types used for fish studies. Those tags commonly used in experimental research are indicated with an asterisk. VIE tags used in the present study are indicated in bold.

6.2.2 Visible Implant Elastomers in fish tagging

VIE tags are typically used in long-term studies of a great variety of animals, from invertebrates to vertebrates (e.g. blowflies, Moffatt 2013, lobsters, Neenan et al. 2015, frogs, Sapsford et al. 2015, fish, Kozłowski et al. 2017). Nevertheless, VIE tags also have certain limitations, such as tag loss, misidentification of individuals after a long period of time after tagging (FitzGerald et al. 2004, Sapsford et al. 2015) and limited colour combinations. Several studies have evaluated and optimized the use and retention of VIE tags in different fish species, usually finding high retention rates and infrequent tag losses, such as observed in bluegills (Dewey and Zigler, 1996), salmonids (Hughes et al. 2000, Olsen and Vøllestad, 2001), European bullhead and *Cottus gobio* (Bruyndoncx et al. 2002). Although previous studies have shown that VIE tags do not appear to cause pathological damage or severe reactions in immunocompetent tissues in fish (Petrie-Hanson 2013, Henrich et al. 2014, Featherstone et al. 2016, Hohn and Nastoll et al. 2016, Rácz et al. 2021), it is critical to conduct complementary assessments using different methods to guarantee their safety. This is particularly important as VIE material has been shown to migrate from the injection site to distant tissues, e.g., the mesentery, colon, and coelomic cavity in marine toads, which showed a severe immune response in the kidney over time (Cabot et al. 2021).

The present study aims to evaluate and optimize the use of VIE tags in gilthead seabream (*Sparus aurata* L.), as this fish is one of the most important species in Mediterranean and European aquaculture (FAO 2022) and is used in basic and applied research, especially as a model in marine parasitology research (van Beest et al. 2019, Lopez-Verdejo et al. 2022). It has been suggested that VIE tags do not negatively affect the growth and survival of gilthead seabream tagged on the dorsal skin of juvenile fish (<18 g) (Astorga et al. 2005), and since then, only few studies have been conducted using VIE tags in this species (e.g., behavioural and PIT tagging evaluation, Navarro et al. 2006, Montero et al.

2009). However, it is expected that under laboratory conditions, where space is limited and fish shoals are small, this species, which relies on phenotypic homogeneity in the fish group (McRobert and Bradner, 1998; Pavlov and Kasumyan, 2000), becomes highly territorial and responds aggressively to fish with visible external tags or even new skin marks (observed with different external tags previously used, unpublished results). Thereby, we aimed to validate the use of multicoloured VIE tags in gilthead seabream research by monitoring tag retention and, evaluating potential tissue damage and side effects on fish health and welfare that VIE tag might induce. This was tested in a short- to long-term study using a broad range of fish sizes and two injection sites.

6.3 Materials and Methods

6.3.1 Animal maintenance and monitoring fish and VIEs tags

Gilthead seabreams (*Sparus aurata* L. ~8.2 g average weight) were supplied by a hatchery in Burriana (Castellón, Spain), and maintained in 3000 l tanks at the SCSIE facilities (Central Support Service for Experimental Research, University of Valencia) for acclimation until their use in experimental assays Fish were anaesthetized using MS222 (0.015% solution buffered in seawater of tricaine methanesulfonate, Sigma-Aldrich) and immediately tagged with VIEs according to the manufacturer's instructions (Northwest Marine Technology, NMT Inc, Shaw Island, WA), by injecting the liquid VIE solution (10:1 dilution of coloured component and hardener) under the skin with an insulin syringe (BD 0.3 cc, with a 29 gauge needle). Intradermic tags were injected into the base of the dorsal fin, applied superficially in the space between the radials and the basals of the pterygiophores that support the dorsal fin (as close to the skin as possible to be visible) or/and into the radials of the caudal fin, except in the control/untagged fish, in which phosphate-buffered saline (pH=7.2) was injected instead. To evaluate the impact of VIE tagging on fish health, a total of 52 fish (20 control and 32 tagged) were monitored using welfare indicators commonly used in fish management and accepted by the Ethics Committee of the University

of Valencia (Spain) (see Table 1). These indicators are routinely checked in the aquarium facilities where the fish of this study are kept in order to quantify the stress level of the fish during experimental assays, helping to detect any symptoms of welfare impairment in the fish. In addition to daily observation of fish survival, feeding activity, ventilation rate, skin colour and swimming pattern (Huntingford et al. 2006, Sneddon 2019), they are weekly supervised when anesthetized prior to the VIE tag measurement (see below). For this, four categories are checked, each with four levels that quantify changes with a score from 0 to 3, monitoring fish growth rate, conspicuous behaviours and presence of morphological abnormalities or injuries. If the score is between 6 and 10, fish are considered to be under stress conditions, and if higher than 11, the assay must be stopped and the fish must be euthanized (Table 1). In this study, the obtained score was 0, except in two weeks when the score was 3 (see below), and for precautionary reasons the fish were not handled for that period.

To investigate tag retention (fading of tags), five tag colours (orange, green, pink, red or yellow) were tested in different combinations by tagging 20 small fish (standard length (SL, mean ± SD), 20.7 ± 2.2 cm, and weight (mean ± SD), 282.8 ± 52.8 g) and 12 large fish (28.8 ± 2.2 cm, 347.2 ± 36.6 g). Ten additional small and large fish were monitored as untagged control fish in each experiment. During weekly monitoring, the length of the VIE tag was measured using a Vernier calliper (±0.03mm) under the VI-Light (black light: Deep violet ~405 nm) after the fish were anaesthetized. A total of ten tag measurements were taken over twelve weeks, as the fish showed symptoms of stress in weeks six and seven (score 3 out of 16, Table 1) and could not be handled. These included irritated skin in the abdomen and in the side flanks likely caused by manipulations, even when gently performed under anaesthesia for approximately 30 seconds, as they affected both tagged and untagged fish that recovered after two weeks without manipulations. At the end of the experiment, fish were euthanized by an

overdose of the anaesthetic MS222 (0.03% solution buffered in seawater), and tissue and VIE tag samples were collected for histological evaluation.

Table 1. Fish welfare evaluation assessment table used as a reference for ethical procedures approved by the University of Valencia, Spain. Variables typically used in fish management to quantify and evaluate fish stress and welfare impairment under experimental conditions. Note: If an animal scores 3 in more than one variable, all 3 scores become a score of 4.

Variables		Score
Fish growth	Normal growth	0
	Mild effects: Weight loss < 10%	1
	Moderate effects: Weight loss 10 – 20 %	2
	Severe effects: Animals do not feed. Weight loss > 20%.	3
Fish behaviour and fitness	Normal group swimming	0
	Mild effects: Change in skin colour and heavy presence of surface mucus	1
	Moderate effects: Mild injury on fish surface (cephalic, dorsal or abdominal area or fins)	2
	Severe effects: Conspicuous swimming, isolation from the group, inactivity and/or injury on > 20% of the fish surface	3
Stress response to routine management procedures	Normal	0
	Mild effects: Sudden and rapid escape behaviour	1
	Moderate effects: Lower and/or erratic activity	2
	Severe effects: Inactivity, accelerated ventilation, injury caused by escape response	3
Clinical parameters	Normal	0
	Mild effects: Isolated injuries on fish surface (cephalic, dorsal or abdominal area or fins)	1
	Moderate effects: Mild haemorrhage or ulceration on fish surface	2

Severe effects: Severe ulceration or extensive injury on fish surface	3

6.3.2 Histopathology of intradermic VIE tags in fish tissue

To evaluate the VIE tag effects on fish tissues on a short- and long-term period, samples were collected from a total of 176 gilthead seabream for histological analyses (Fig. 2). For the short-term study, 24 fish (12 small fish, 12.3 ± 5.0 cm, 59.9 ± 3.5 g; 12 large fish, 25.4 ± 2.6 cm, 245.9 ± 8.8 g) were tagged according to the previously described protocol, with untagged sites as control tissue, and euthanized at different time points, i.e. 4, 8 and 20 days post-tagging (dpt). For the long-term study, 152 fish (10 untagged and 86 tagged small fish (13.9 ± 3.5 cm, 68.4 ± 26.1 g); 10 untagged and 46 tagged large fish (22.5 ± 3.8 cm, 207.6 ± 31.8 g)) were used and euthanized at 180 dpt. For histological analyses, 2 cm^2 tissue samples of skin and fins containing the VIE tag were collected. Samples from liver, kidney and spleen were also collected from 180 dpt fish for the evaluation and monitoring of possible migration of VIE material. All samples for histological examination were immediately fixed in 10% buffered formalin for 48 hours. Samples containing hard tissues (bones, fin rays) were decalcified in 10% formic acid for 12 h. Samples were then dehydrated in graded alcohol series and embedded in paraffin (Shandon Histocentre 3, Thermo Electron Corporation). Paraffin blocks were sectioned (4-7 µm, 20-40 sections per block) using a microtome (Leica HM330, Leica Biosystems, Germany). The sections were stained with haematoxylin and eosin, mounted in DPX, and examined under the microscope Leica DM 5000B with a coupled camera (ProgRes C3, ProgRes CapturePro 2.7, Germany) and the microscope Leica CTR6000 with ultraviolet (UV) light source and with a coupled camera (Leica DFC 480, Germany).

6.3.3 Statistical analysis

To confirm the size difference between the two groups of fish (small and large gilthead seabream) in each assay (Fig. 2), different linear models were fitted (LM; package *lme4*, Bates et al. 2022), using 'standard length' of fish as response variable and the 'size group' (small *vs* large) as explanatory variable. Because the size difference was confirmed in all assays (LM, $P<0.001$, Appendix 3: Table S1), the following models were run separately for small and large fish. To analyse the effects of the tags on fish growth up to 85 dpt (Fig. 2, middle-term), linear models were fitted for small and large fish separately, using the 'length difference' between the first and last measurements (SL at 85 dpt – SL at 1 dpt (cm)) as the response variable (centered variable) and the 'tag group' of fish (tagged *vs* untagged/control) as explanatory variable. To investigate the retention rate of VIEs tags over time in fish tissues, the coefficient of variation of the tag length (calculated as CV_{tag} =SD/mean × 100) was analysed in beta regression models, for each size group of fish separately. The CV_{tag} indicates whether the variance of the tag is low, meaning that the tag shows an optimal retention in the fish after 85 dpt, or whether on the contrary, it fades or splits into several smaller tags, as observed in some cases, suggesting that the use of tags is not recommended in this case. Since the data did not meet the requirements to fit in a linear mixed model, the beta regression model allowed to analyse the CV_{tag} by applying a logit link (package *betareg*, Zeileis et al. 2022), using 'injection site' (dorsal *vs* causal), 'tag colour' (orange *vs* green *vs* pink *vs* red *vs* yellow) and their interaction as explanatory variables. For the large fish group, the model estimated a bias correction (type= "BC") (as opposed to the default value as for the small fish group, i.e. type= "ML") to correct for the low number of large fish in the middle-term assay (n=12). All statistical analyses were performed in R (R Development Core Team, version 4.1.2) with significance set at $\alpha = 0.050$.

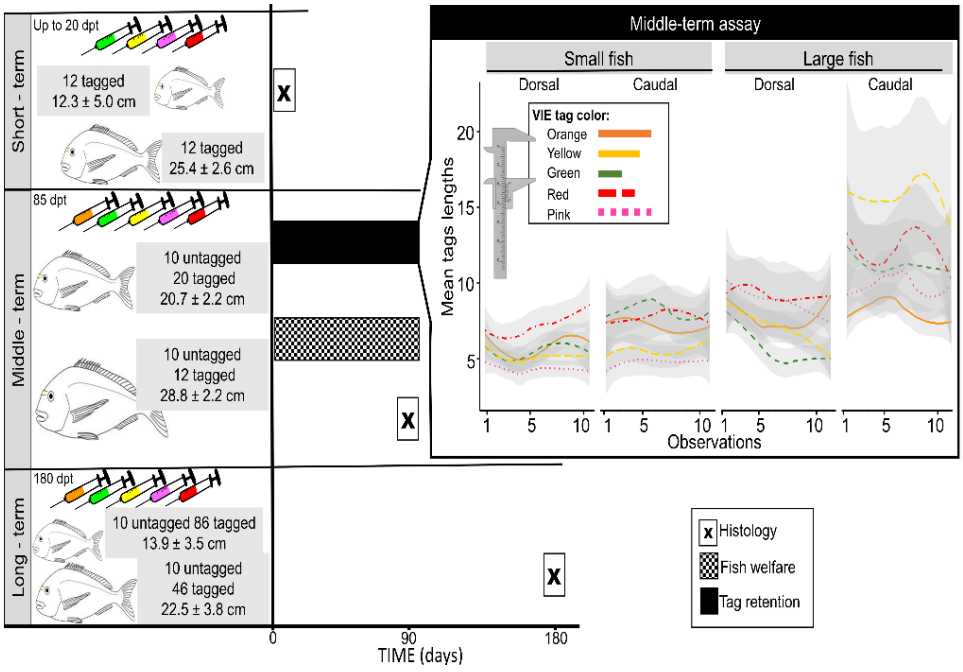

Figure 2. Experimental design of three different assays conducted in this study, e.g., short-, middle- and long-term (dpt, days post-tagging) (on the left). Mean ± SD of fish standard length is presented for each fish group. Graphical results of the middle-term assay representing the variation in VIE tag length. These were monitored weekly for 12 weeks, producing 10 observations (fish could not be handled in weeks six and seven due to stress symptoms) on five different tag colours (right part). The lines represent the mean variation, while the shaded area represents the 95% confidence intervals. Abbreviations: ch, chromatophore; cn, connective tissue; ep, epithelial cells; m, muscle. Scale bars: A, B = 1 cm; C, E=100 µm; D, F=200 µm.

6.4 Results

6.4.1 Evaluation of the effects of VIE tags on fish health and monitoring VIE tag retention

There was a 100% of survival of all fish, and no negative health effects (Table 1) were observed in both small and large fish under experimental conditions, as tagged and untagged fish showed no changes in their skin colour (stress conditions cause darker colouration in this species, Rotllant et al. 2000a, 2000b, Arends et al. 1999, Tort et al. 2011) or ventilation rate (no faster opercula beats and good gills irrigation). In addition, inspection of fish under anaesthesia did not reveal any morphological abnormalities or injuries, except at weeks 6 and 7 when fish could not be handled due to slight stress symptoms (see details in section 6.3.1 and Table 1). Feeding and swimming patterns of tagged and untagged fish were also similar, and no territorial or aggressive responses were observed toward the marked fish regardless of VIE colour. Furthermore, tags showed no effect on fish growth, as no significant differences were detected between tagged and untagged fish up to 85 days post-tagging (dpt) (Fig. 2, middle-term assay: LM; small fish, estimate ± SE = -0.259 ± 0.205, P=0.218; large fish, estimate ± SE = -0.168 ± 0.104, P=0.121, Appendix 3: Table S2).

Up to 85.6% of the tags were successfully identified by day 85 post- tagging, particularly for small fish the tag-detection rate was 86.6% (129/149) (average initial-length of missing-tags was 4.1 ± 1.8 mm (mean ± SD)), with 81.9% (86/105) of tags on the dorsal fin and 97.7% (43/44) of tags on the caudal fin detected 85 day post- tagging. Similarly, for large fish the tag-detection rate was 83.7 % (87/104) (average initial-length missing-tags was 7.7 ± 2.0 mm), with 80.8% (59/73) of tags on the dorsal fin and 90.3% (28/31) of tags on the caudal fin detected day 85 post- tagging. In addition, it was possible to visually identify three typical patterns in which the tags changed during the experimental work, affecting their size, (i) shortening, (ii) fragmentation or (iii) dissemination/scattering (Fig. 2, middle-term assay, on right side of the figure).

Tag length did not show an obvious standard pattern, as certain colours appeared to become shorter over time, e.g., pink, yellow and green tags showed decreased length when injected into the dorsal fin of large fish (Fig. 2). In others, however, tag length increased during the sampling period and decreased during the last weeks of the experiment, e.g., in the caudal fin, orange, yellow and green in small fish and all colours in large fish followed this pattern (Fig. 2). This could likely be due to fragmentation and dispersal of material resulting in an elongated appearance of the mark, followed by its disappearance, usually after the seventh observation (~nine weeks post-tagging). Nevertheless, when this variation was analysed in both fish size groups, different results were obtained. In the large fish group, tag retention (CV_{tag}) did not differ significantly between injection sites or colour, suggesting that tags are retained similarly over time (beta regression; $P > 0.05$, Table 2 (ii), Figs. 2 and 3A, B). However, in the small fish group, CV_{tag} showed higher variability when injected into the dorsal fin base compared to the caudal fin (beta regression; estimate ± SE = -0.94 ± 0.28, $P < 0.001$, Table 2 (i)), whereas this variation was comparable between different colours (beta regression; $P > 0.05$, Table 2).

Table 2. Results of Beta Regression model analysing the difference of coefficient of variation (CV_{tag}) between injection sites and tags colours, in the (i) small and (ii) large fish size groups (CV_{tag} (mm) ~ injection site (dorsal *vs* caudal site) × tag colour (orange, green, pink, red, yellow)). The categorical variables 'injection site' and 'tag colour' was assessed in comparison with the reference injection site 'dorsal' and 'orange' tag. R^2 represents the variability explained by fixed effects. Statistically significant results (at $\alpha = 0.050$) are indicated in bold.

Beta regression	Estimate	SE	*z-value*	P-value
(i) Small fish (R^2=0.08)				
Intercept	-0.673	0.210	-3.201	**0.001**
Caudal injection site	-0.945	0.279	-3.389	**<0.001**
Green tag	-0.044	0.285	-0.153	0.878
Pink tag	0.288	0.319	0.901	0.368
Red tag	0.251	0.328	0.764	0.445
Yellow tag	0.007	0.298	0.022	0.983
Caudal fin: Green tag	0.149	0.375	0.396	0.692
Caudal fin: Pink tag	-0.159	0.451	-0.352	0.725
Caudal fin: Red tag	0.290	0.416	0.698	0.485
Caudal fin: Yellow tag	0.406	0.397	1.021	0.307
(ii) Large fish (R^2=0.09)				
Intercept	-0.417	0.214	-1.946	0.052
Caudal injection site	-0.970	0.602	-1.611	0.107
Green tag	-0.336	0.395	-0.850	0.396
Pink tag	0.223	0.648	0.345	0.730
Red tag	-0.234	0.307	-0.763	0.445
Yellow tag	-0.031	0.273	-0.115	0.908
Caudal fin: Green tag	0.516	0.796	0.648	0.517
Caudal fin: Pink tag	-0.143	1.475	-0.097	0.923
Caudal fin: Red tag	1.112	0.684	1.625	0.104
Caudal fin: Yellow tag	0.737	0.694	1.062	0.288

6.4.2 Histopathology of the intradermic VIE tags on fish

Histological sections at different post-tagging times, i.e. 4, 8, 20, 85 and 180 days, confirmed that the VIE tags did not induce any relevant pathology at the injection site. While green, yellow and red tags had a granular/crystalline texture (Fig. 3C, D, F), pink and orange ones appeared as a fragile mesh (Fig. 3E). These were surrounded by only a single or double layer of fibroblasts, with visually no encapsulation or coordinated recruitment of inflammatory cells around the aggregated material. No relevant necrotic, degenerative, or inflammatory responses were observed in association with the presence of VIE aggregated material (Fig. 3C-F). Only in a few sections, a very slight displacement of the surrounding tissue (dermis, muscle) was observed. However, at 180 dpt (long-term assay) VIE tag material was unexpectedly detected in other areas, mainly in kidney and spleen (Fig. 4), after macroscopic visual inspection of the fish with VI light. In tagged small fish (~13.9 cm SL), VIE tag migration was detected in 10.5% (9/86), with 16.3% (7/43) migration occurring when fish were tagged at both injection sites, 10.1% (2/18) when exclusively injected at the caudal site, and no migration if only injected at the dorsal site (0/25). In tagged large fish (~22.5 cm SL), VIE tag migration was detected in 30.4% (14/46), with 44.4% (9/30) migration occurring when fish were injected at both sites, 30% (4/9) when injected exclusively at the caudal site and 14.3% (1/7) when injected at the dorsal site only. In the histological sections of these organs, clusters of VIE material were found aggregated in spleen and kidney tissues, surrounded by a very thin layer of connective tissue, similar to those observed in the skin and fins (Figs. 3 and 4). Except for the presence of melanomacrophage centers (MMCs) surrounding or containing some VIE tag aggregations (Fig. 4), no necrotic, degenerative, or inflammatory response was observed in any of the sections. When UV light was applied, the VIE aggregates and even single small-size particles were easily detected (Fig. 4D–F).

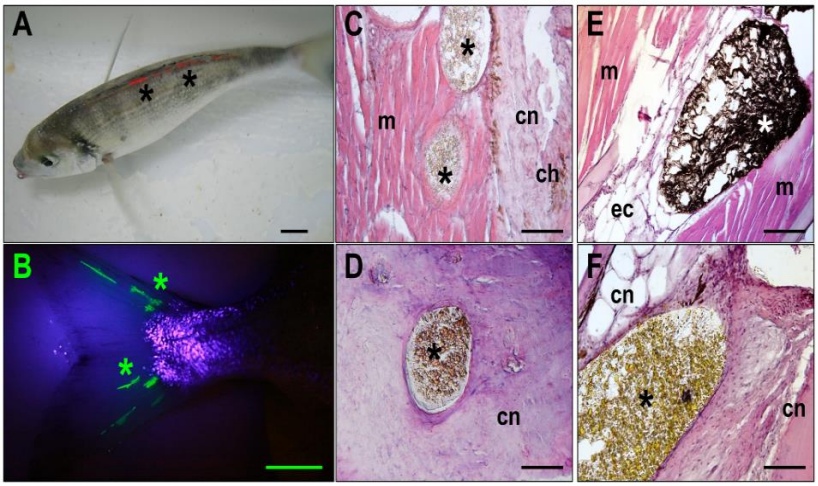

Figure 3. **(A)** Tagged-fish with red VIE tag, **(B)** tagged caudal fin with green VIE tag under VI-light. **(C-F)** Histological sections of multicolour VIE tags material in proximal tissue to the injection site. **(C-D)** red VIE tag, **(E)** pink VIE tag and **(F)** yellow VIE tag. Tags are indicated with an asterisk.

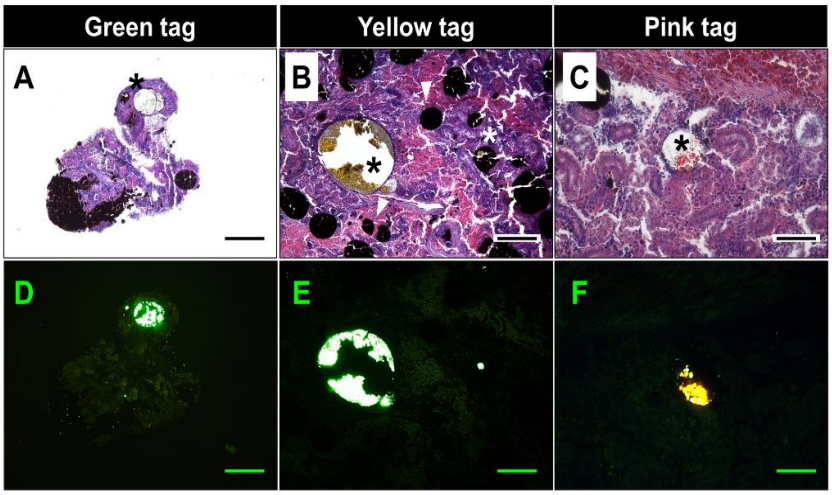

Figure 4. Histological sections of VIE tag material that migrated into the kidney of the fish **(A-C)** under bright field and **(D-F)** under UV light. The presence of melanomacrophage centers is indicated by head arrows and tag location by an asterisk. Scale bars: A,B, D, E= 200 µm; C, F = 50 µm.

6.5 Discussion

This study provides the most up-to-date information on the application, retention and potential side effects of VIE tags on gilthead seabream used for research purposes. VIE tags meet the requirements for an efficient tagging system for long-term studies, with rapid recovery of fish after tagging, no negative effects on the health or welfare of gilthead seabream, therefore being consistent with the results of Astorga et al. (2005). No aggressive reactions of other fish toward the colours of the VIE tags were observed, which is of particular importance as externally visible tags can be a problem for territorial fish under laboratory conditions (McRobert and Bradner, 1998, Pavlov and Kasumyan, 2000, unpublished results). VIE tags are therefore safe for tagging gilthead seabream under laboratory conditions for research purposes that rely on visual assessments. However, our results suggest that before applying VIE tags on gilthead seabream, the tags need to be tested in advance, especially

considering two main factors that have been shown to be key for a successful design for tagging this species under laboratory conditions, i.e. fish size and tag injection site. Moreover, the combination of fish welfare monitoring and histological analysis helps to evaluate the effects of VIE tags and ensures the reliability of the results obtained.

Currently, animal welfare is the focus of interest as it guarantees the quality of research results; hence the application of tagging methods should be tested to safeguard animal welfare standards (e.g., Jepsen et al. 2015, Macaulay et al. 2021). Our results are consistent with previous studies, in which intradermic tags, including VIE, have been shown to be reliable for visual assessments and safe to use on multiple species and body sites (Sandford et al. 2020). Furthermore, VIE have shown high retention when injected into specific areas, such as the rostrum of sturgeon (Kozłowski et al. 2017), the lower operculum of crappies (Pomoxis sp. Fryda et al. 2007), the ventral surface of European silver eels (Anguilla anguilla, Simon 2007), and also in the dorsal and caudal peduncle of bluegills for up to about seven months (Lepomis macrochirus, Dewey and Zigler, 1996). Similarly, in Astorga et al. (2005), juvenile gilthead seabream (<18 g) were tagged under the skin of the dorsal site. Our results showed that VIE tags in gilthead seabream from a wide range of fish sizes (18 −34 cm) were optimally preserved for up to 180 days without compromising their welfare. During weekly monitoring of the smaller fish (~20.7 cm SL), tags injected into the caudal fin showed less variability, suggesting a more stable retention compared to the dorsal injection site. This greater variability in the permanence of the tag at the dorsal site may be related to the sponginess and vascularization of the dermal tissue in this area, which favours the displacement of the VIE material into adjacent tissues. Given that the tag was injected into the connective tissue at the base of the dorsal fin, between the radials and basal pterygiophores, the VIE tag material likely spread and filled nearby pores beyond the needle hole, whereas the tag in the caudal fin injected into the radials, was probably better retained because the connective tissue surrounding the radials is richer in

collagenous fibres and not as spongy as at the base of the dorsal fin (Brennan et al. 2005). The fact that this variability in the dorsal fin was detected in the smaller fish is probably associated to the fragmentation, dissemination and/or reduction of the tags caused by the newly developing scales in a growing body (Simon and Dörner, 2011). Our results suggest that injection of VIE tags into the dorsal fin base and caudal fin radials is safe and efficient, however, for optimal preservation we recommend that the initial length of the tag in the dorsal fin should be at least four mm long when tagging small fish (~20.7 cm SL) in order to avoid porous tissue in growing fish, guaranteeing the visualization of the tags in a long-term study.

None of the VIE-tagged fish showed signs of necrosis, degeneration, inflammation or even a severe foreign-body reaction at the injection site, reinforcing the assumption of good biocompatibility of this material. However, partial migration of the VIE material to other tissues was observed 180 days post-tagging of the fish, with the kidney and spleen being the main organs where this material was found. This phenomenon has also been observed in other aquatic animals such as toads and smooth newts, with no apparent damage (Brannelly et al. 2013, Kopecký 2020). Similarly, no signs of inflammation or relevant alterations were detected in our study either. Kidney and spleen are the two main organs involved in filtration of the circulatory system in fish and amphibians, which could be related to the material accumulation in these tissues. However, accumulation of VIE tags in marine toads in the vasa recta led to progressive distention and rupture of the vessels, secondary granuloma formation, and engulfment of the tag material by macrophages (Cabot et al. 2021), which was not observed in the present study. Therefore, regardless the important role of the kidney and spleen as immunocompetent tissues, being the major lymphomyeloid tissue in teleosts (Bjørgen and Koppang 2021), no obvious cellular alterations occurred when VIE material migrated there, nor did they cause changes in nodal regulatory activity, as growth of tagged and untagged fish was similar. Nevertheless, although the silicone of the VIE tag

acted as an inert material in the tissues, the appearance of MMCs likely indicates that fish recognize the VIE tag as exogenous inert material. When biomaterial is implanted into other vertebrates, macrophages and giant cells respond similarly to it (Sheikh et al. 2015). This is not an unexpected finding as fish macrophages, such as the MMC, are involved in phagocytosis and in the accumulation of non-metabolizable waste materials, acting as "metabolic waste dumps" (Steinel and Bolnick, 2017; Carreras-Colom et al., 2022). Polyesters with similar properties to VIE tags components, e.g., aliphatic polyesters (AP), are commonly used in medicine because they are biocompatible polyesters with low toxicity (Park et al. 2012, Diaz et al. 2014, Arif et al. 2019). Thus, they are usually considered safe, even when injected directly into the organism or migrating to other tissues.

The migration of VIE material to the kidney and spleen occurred more frequently in larger fish (~22.5 cm), especially when fish were tagged in the caudal fin, which has well-developed vascularization due to its large locomotor activity (Xu et al. 2014). This, likely increases the chances of injecting the VIE material directly into a vessel when the material is injected into the caudal radials, enhancing the migration of the tag through the circulatory system before solidifying. This phenomenon could be avoided by reducing the volume of VIE material injected into the caudal fin, but further studies are needed to verify this. Similarly, we consider that a preliminary test is needed before using VIE tags, not only to evaluate the efficiency and safety of VIE tags on fish, but also to acquire skills in injecting VIE material into fish. Moreover, to avoid misidentification, it is also crucial to test the observer's skills in recognizing tag colours, especially distinguishing between pink and red tags, and green and yellow tags, which could be confused (similar observations in Astorga et al. (2005)). Regarding risks to the environment, although our results suggest that VIE tag material can migrate to distant tissues in tagged fish and potentially be released into the environment, we consider that VIE-tagged fish do not pose a risk to the environment as this type of silicone is not considered toxic and the volume of material used to tag the fish is despicable, making it a safe option for

wild fish studies (e.g., lemon damselfish in lagoons (Booth and Beretta 2021), intertidal fish in Portugal (Compaire et al. 2022), wild cichlids (Jungwirth et al. 2015a, 2015b)).

Based on our results VIE tags need to be tested in advance when starting new research lines, by monitoring of fish welfare and VIE tag retention, as well as histological analysis. We suggest two tagging sites on gilthead seabream that have been shown to be reliable for short- and long-term studies, preferably tagging the dorsal base when fish are larger than ~20 cm, and limiting the volume of VIE material when tagging the caudal fin to reduce migration of material to other tissues. We believe this information can help researchers striving to use the best possible evidence to make informed decisions about tagging fish.

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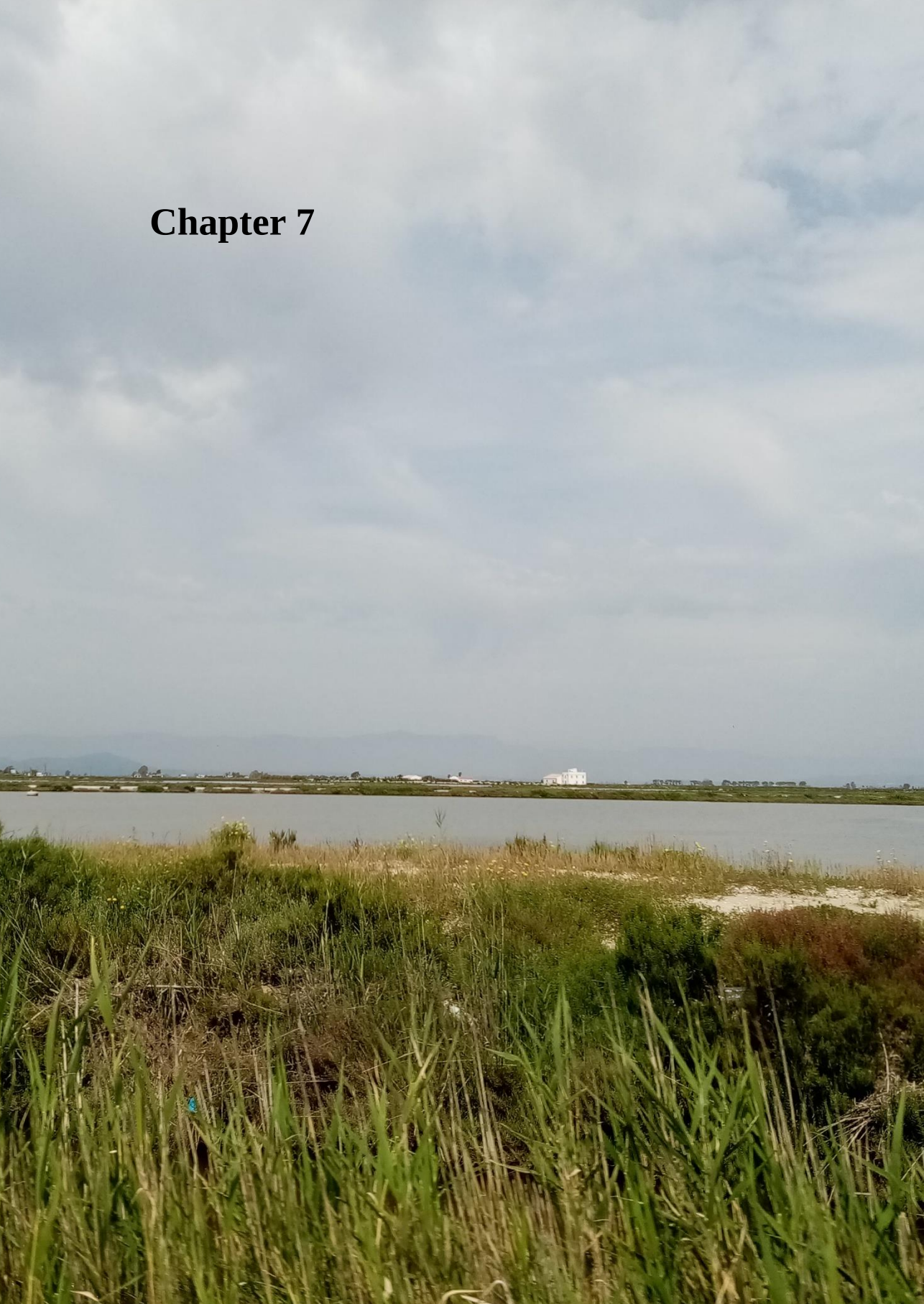
Chapter 7



7. Chapter 7

Use of Visible Implant Elastomers (VIE) in experimental assays: VIE tagging gilthead seabreams for experimental evaluation of behavioural changes provoked by brain-encysting trematode *Cardiocephaloides longicollis* (Strigeidae)

In this chapter, preliminary results are presented as an example of the use of VIE tags in experimental studies of fish parasites. This work has been presented at conferences of the British Society for Parasitology Spring and the Annual Meeting of the American Society of Parasitologists, and is currently being analyzed. A manuscript including these results will be prepared and submitted in 2023.

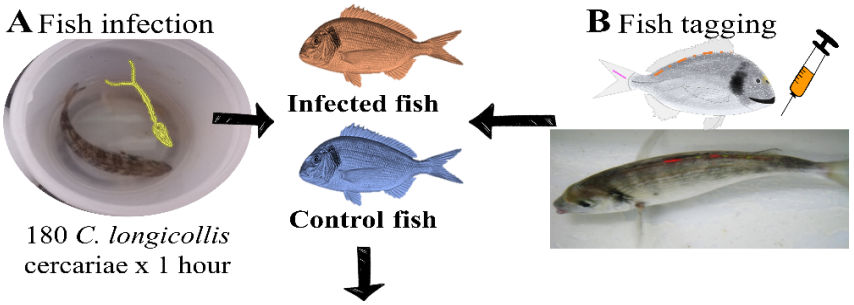
van Beest GS, Montero FE, Raga JA, Born-Torrijos A. Experimental evaluation of behavioural changes in gilthead seabream infected by brain-encysted *Cardiocephaloides longicollis* (Trematoda, Strigeidae) metacercariae. British Society for Parasitology Spring Meeting. 8th-11th April 2018, Aberystwyth, Gales, UK. Conference session

van Beest GS, Montero FE, Raga JA, Born-Torrijos A. Experimental evaluation of behavioural changes in gilthead seabream infected by brain-encysted *Cardiocephaloides longicollis* (Trematoda, Strigeidae) metacercariae. 93rd Annual Meeting of the American Society of Parasitologists. 21th-24 th June 2018 Cancun, Mexico. Poster session.

Use of Visible Implant Elastomers (VIE) in experimental assays: VIE tagging gilthead seabreams for experimental evaluation of behavioural changes provoked by brain-encysting trematode *Cardiocephaloides longicollis* (Strigeidae)

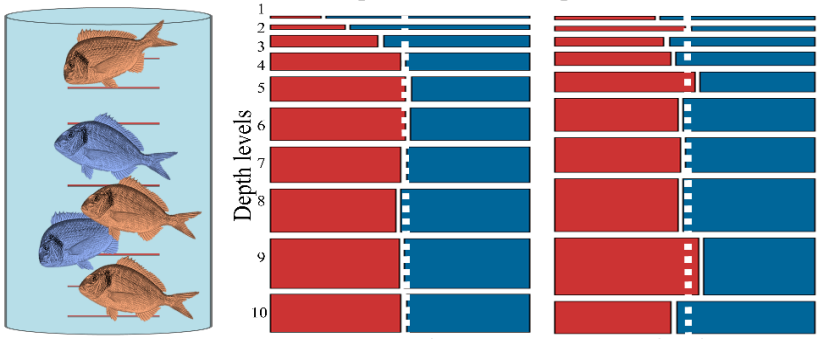
After investigating the potential effects of using VIE tags on *Sparus aurata* L., we concluded that this method is a safe option for individuall tagging of fish without altering their overall health and welfare under laboratory conditions (Chapter 6). Based on this, VIE tags were applied in an experimental assay to test whether the trematode *Cardiocephaloides longicollis* (Rudolphi, 1819) Dubois, 1982 affects the behaviour of infected fish. The cercariae of this parasite penetrate the skin and migrate into the brain, where they encyst as metacercariae (Prévot and Bartoli, 1980). It is commonly believed that the cysts can cause significant alterations in fish behaviour increasing their transmission to the definitive host, as has been evidenced in other brain-infecting trematodes (e.g. *Euhaplorchis californiensis* Martin, 1950, Lafferty and Morris 1996). However, this behavioural pathology suggested to be provoked by *C. longicollis*, has never been studied experimentally (Osset at al. 2005, Born-Torrijos et al. 2016). In the behavioural experiments, where a total of fourteen fish were experimentally infected with 180 *C. longicollis* cercariae each, emerged from *Tritia reticulata* (L.), five different colours of VIE tags were applied. Additionally, fourteen fish were subjected to the same infection procedure without the addition of the cercariae, thus serving as uninfected control fish (Fig. 1A, B). Behavioural experiments were performed six months post-infection to guarantee that the metacercariae were infective to the definitive host (Prévot and Bartoli, 1980). Two different assays were conducted: (1) Ten fish were placed in a plexiglass tube (200 cm height, 30 cm diameter) with an effective light-dark gradient. The water column was divided vertically

into 20 cm sections and the position of each fish was recorded every two hours for 30 minutes at 1-min intervals for three days in three different assays, i.e. control, infected and mixed fish group (Fig. 1C); (2) Fourteen fish were placed in a circular tank (mimicking aquaculture installations, 120 cm height, 300 cm diameter) and fish were captured everytwo hours for six minutes, six times per day for the same three assays (Fig. 1D). All recorded data were analyzed using generalized linear mixed models in R software (R Development Core Team (version 4.2.1)). Preliminary results showed little differences in the light gradient behaviour response between control (uninfected) and infected fish, as both were swimming at similar depths and light-dark gradient in the three assays conducted, i.e. control and infected fish separately and mixed (Fig. 1C). Similarly, infected fish showed no significant differences on the capture rate compared to control fish when fish were separated. However, when the fish were mixed in the same tank, the infected fish were caught more frequently than the control fish (Fig. 1D). This may indicate that encysted metacercariae induce a behavioural alteration in the infected fish that is related to neuronal disorder, but likely not to light perception. Although most metacercariae were infecting the optic lobes, the enhancement of their consumption by predators might not be a consequence of a decrease of light perception, but to other features of fish vision, such as visual acuity. This could lead tostealthy changes in fish behaviour during schooling or escaping from potential predators, which needs further investigation.



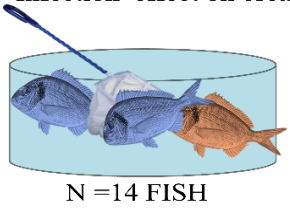
Results of behavioral experiments

C Infection effect on swimming behavior and depth



Small differences in use of space in all assays

D Infection effect on escape behaviour



3.5 more experimental fish were captured in the mixed group, showing:
-Elapsed reaction
-Decreased schooling

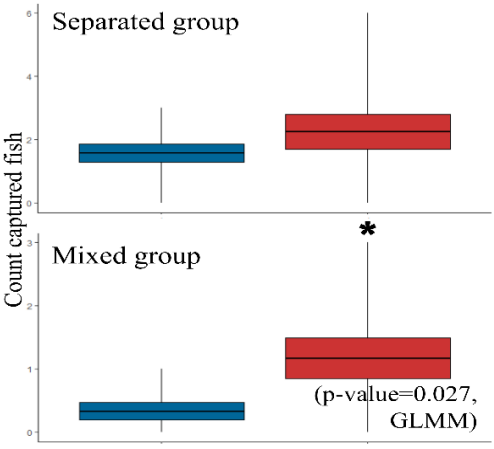


Figure 1. Design of the experimental study analyzing behavioural changes potentially induced by *Cardiocephaloides longicollis*. (**A**) Infection process of gilthead seabream (*Sparus aurata*) with *C. longicollis* and (**B**) tagging process of gilthead seabream with Visible Implant Elastomer tags. Preliminary results of two behavioural assays of infected and control fish: (**C**) depth swimming location of fish, according to their perception of the light gradient in the tubular tank and (**D**) rate of capturing fish with a net.

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Conclusions

8. Conclusions

The results of this PhD thesis have led to the following conclusions:

1) This project, which focused on the transmission strategies of the trematode *Cardiocephaloides longicollis*, shows that trematode parasites are good candidates for the study of parasite-host interactions despite their complex life cycle, the different hosts and different parasite and free-living stages.

2) The *in vivo* fluorescent dye CFSE (carboxyfluorescein diacetate succinimidyl ester) is an efficient tool to study the transmission of transparent larvae, such as cercariae, in short-term assays. On the other hand, the dye NB (NucBlue) seems to be more reliable than CFSE in long-term studies, given the similar infection rate of NB-labelled and unlabeled cercariae. The labeling of the penetration glands by CFSE likely impairs the cercariae penetration performance and hence their ability to infect the fish brain.

3) *Cardiocephaloides longicollis* cercariae infect fish by penetrating their skin and even though cercariae show no preference for a specific fish flank, their density is higher in specific areas of the body of the gilthead seabream. The surface of the area close to the eyes, gills, dorsal fin and areas near the ventral side of the fish appear to be preferred entry portal into the fish. In contrast, the caudal fin is the least attractive surface for cercariae to penetrate through. This suggests that *C. longicollis* specifically favors their penetration through specific areas that may be attractive due to their short distance to the brain or to specific pathways such as the circulatory or nervous systems, providing efficient access to the brain.

4) *Cardiocephaloides longicollis* cercariae show strong and driven swimming activity, likely to reach the second intermediate host, the fish. The cercariae use the branches of the forked tail as a propellers to thrust and guide their swimming activity. Furthermore, the powerful tail is also important in

maintaining their position in the water column by opening the forked tail to create a parachute-like effect that reduces their sinking.

5) The multifunctional structures of cercariae of *C. longicollis* are key to complete their transmission to the fish: The ventral sucker and the oral sucker are strongly involved inthe caterpillar-like crawling toward the penetration portals, whereas during penetration, the ventral sucker acts as an anchor while the oral sucker pushes against the skin to open the path. The peristaltic movements, along with the cephalic and surface rose-thorn spines, allow the cercariae to successfully burrow into the connective tissue and muscle fibers. Well-adapted structures have been thus shown to be imperative for effective infection of *C. longicollis* fish hosts.

6) Histological analyses of the migration of *C. longicollis* in fish reveal that this strigeid reaches the brain of the gilthead seabream by burrowing through connective tissue and muscular fibers without provoking any damage or significant host immune response. However, further studies are needed to exclude alternative migration paths to the brain.

7) The testing of Visible Implant Elastomers (VIE) tags on the health and welfare of gilthead seabream excludes any effects on the fish and ensures the quality of the experimental results.

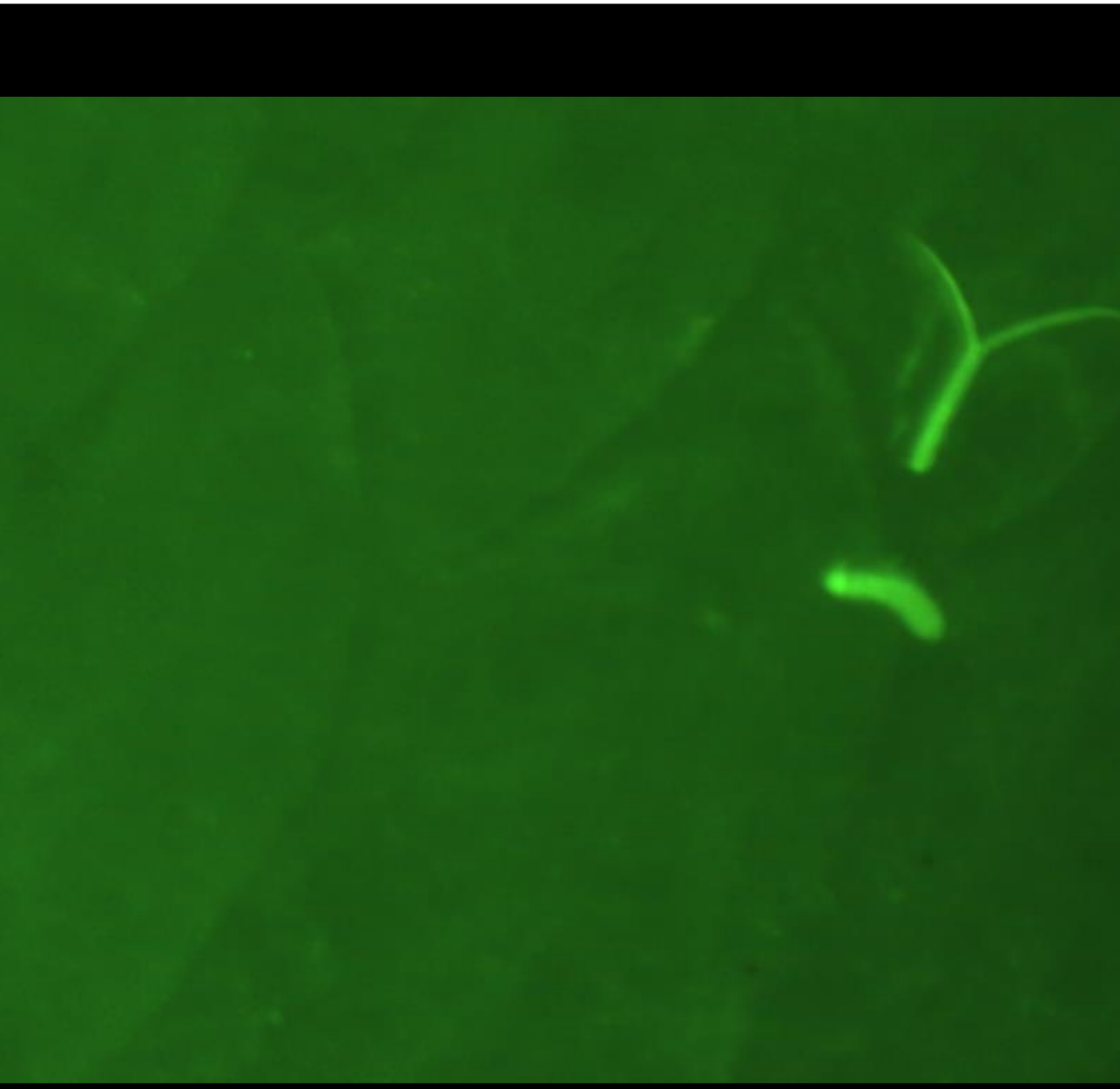
8) No negative effects on VIE-tagged fish welfare have been observed, pointing to the convenience in their use in visual studies and their reliability on a broad size range of gilthead seabream when tagged on the dorsal and/or caudal fin. Nevertheless, their use on growing fish in long-term studies shows less permanence in the caudal fin.

9) Histological analyses confirm the absence of a severe tissue response associated with the VIE tags. However, the silicone component migrates to the kidney and spleen in 10.5% of small fish and 30.4% of large fish. Although no severe immune reaction is observed, this could be easily minimized by limiting the injected volume of the tag. This phenomenon is reported for the first time in fish.

10) VIE tags help to individually indentify gilthead seabream infected with *C. longicollis* in behavioural assays. It could be excluded that encysting metacercariae alter ligth perception of fish. However. they probably affect the precision of the fish in other activities.

11) The use of *C. longicollis* as a laboratory model has improved our understanding of digenean transmission strategies. Reproducing a complex life cycle under laboratory conditions helps to support theoretical information with empirical experience.

Appendix 1



9. Appendix 1

Appendix 1. Supplementary material to Chapter 4

Appendix 1: Table S1. Evaluation of effect of fish's flank on cercarial attachment success.

	Estimate	**SE**	***z-value***	**P-value**
(i) GLM				
Intercept (=Left flank)	-3.076	0.116	-6.597	**<0.001**
Right flank	0.069	0.161	0.419	0.675
(ii) Pairwise comparison				
Right- Left	0.069	0.161	0.419	0.675(0.675)

Results evaluating with (i) generalized linear model (GLM) (Proportion of attached cercariae ~ Fish flank) and (ii) pairwise comparison. The intercept value in the GLM stands for the mean number of cercariae successfully attached to the left fish flank on the logit scale, to which the right flank is compared. The estimate of the right flank is added to the intercept value. Statistically significant results (at α=0.050) are indicated in bold, with the corresponding P-value obtained after Bonferroni correction given in parentheses.

Appendix 1: Table S2. Evaluation of effect of different NB concentrations on cercarial survival.

	Estimate	**SE**	***z-value***	**P-value**
(i) RWM				
Survival 24 hpl				
Intercept (=Control)	2.345	0.033	70.94	**<0.001**
Low Concentration	0.110	0.045	2.45	**0.015**
Intermediate Concentration	0.352	0.045	7.87	**<0.001**
High Concentration	0.263	0.045	5.82	**<0.001**
Log(scale)	-1.176	0.040	-29.60	**<0.001**
	$exp^{(\beta)}$	**se(β)**	***z-value***	**P-value**
(ii)MMCoxPH				
Survival 5 hpl				
Low Concentration	0.471	0.438	-1.720	0.086
Intermediate Concentration	0.285	0.517	-2.430	**0.015**
High Concentration	0.299	0.517	-2.340	**0.019**

Results evaluating (i) the effect of different concentrations of NB on cercarial survival after 24 hpl with Weibull regression model (RWM) (alive cercariae ~ NB concentration) and (ii) after 5 hpl with mixed model Cox Proportional Hazards regression (MMCoxPH) (alive cercariae ~ NB concentrations + replicates (random)). The intercept in (i) stands for the survival rate of the control cercariae, to which the other three levels are compared, i.e. low, intermediate and high NB concentration. The hazard rate ($exp^{(\beta)}$) of control cercariae in (ii) is 1, to which the other levels are compared. If $exp^{(\beta)}<1$, mortality risk is reduced. Statistically significant results (at $\alpha=0.050$) are indicated in bold. We also provide the scale parameter which indicates with log(scale) the Weibull distribution estimation.

Appendix 1: Table S3. Evaluation of effect of NB concentration treatment on cercarial activity.

	Estimate	SE	*z-value*	P-value
RWM				
Activity 5 hpl				
Intercept (=Control)	2.524	0.144	17.660	**<0.001**
Low Concentration	0.753	0.230	3.280	**<0.001**
Intermediate Concentration	0.714	0.215	3.320	**<0.001**
High Concentration	0.863	0.237	3.640	**<0.001**
Log(scale)	-0.132	0.085	-1.560	0.119

Results of regression Weibull model (RWM) evaluating the effect of NB concentration treatment on cercariae activity rate after 5 hpl (active cercariae ~ NB concentration). The intercept value stands for the activity rate of the control, to which the other three levels are compared, i.e. low, intermediate and high NB concentration. Statistically significant results (at α=0.050) are indicated in bold. We also provide the scale parameter which indicates with log(scale) the Weibull distribution estimation.

Appendix 1: Table S4. Evaluation of effect of CFSE different concentrations on cercarial survival.

	Estimate	SE	*z-value*	P-value
(i) RWM				
Survival 24 hpl				
Intercept (=Control)	0.346	0.033	71.076	**<0.001**
Low Concentration	0.189	0.045	4.166	**<0.001**
Intermediate Concentration	0.065	0.045	1.423	0.155
High Concentration	0.003	0.047	0.060	0.953
Log(scale)	-1.180	0.041	-28.492	**<0.001**
	$exp^{(\beta)}$	**se(β)**	***z-value***	**P-value**
(ii)MMCoxPH				
Survival 5 hpl				
Low Concentration	0.433	0.458	-1.830	0.068
Intermediate Concentration	0.503	0.438	-1.570	0.120
High Concentration	0.728	0.408	-0.780	0.440

Results evaluating (i) the effect of CFSE different concentrations on cercarial survival after 24 hpl with regression Weibull model (RWM) (alive cercariae ~ CFSE concentration) and (ii) after 5 hpl with mixed model Cox Proportional Hazards regression (MMCoxPH) (alive cercariae ~ CFSE concentrations + replicates (random)). The hazard rate ($exp^{(\beta)}$) of control cercariae in (ii) is 1, to which the other levels are compared. If $exp^{(\beta)}<1$, mortality risk is reduced. Statistically significant results (at $\alpha=0.050$) are indicated in bold. We also provide the scale parameter which indicates with log(scale) the Weibull distribution estimation.

Appendix 1: Table S5. Evaluation of effect of CFSE concentration treatment on cercarial activity.

	Estimate	SE	*z-value*	P-value
RWM				
Activity 5 hpl				
Intercept (=Control)	2.479	0.130	19.060	**<0.001**
Low Concentration	0.871	0.230	3.790	**<0.001**
Intermediate Concentration	0.408	0.199	2.050	**0.040**
High Concentration	0.112	0.187	0.600	0.549
Log(scale)	-0.220	0.083	-2.670	**0.008**

Results of regression Weibull model (RWM) evaluating the effect of CFSE concentration treatment on cercariae activity rate at 5 hpl (active cercariae ~ CFSE concentration). The intercept value stands for the activity rate of the control, to which the other three levels are compared, i.e. low, intermediate and high CFSE concentration. Statistically significant results (at α=0.050) are indicated in bold. We also provide the scale parameter which indicates with log(scale) the Weibull distribution estimation.

Appendix 1: Table S6. Evaluation of effect of dyes on cercarial survival and activity.

	Estimate	**SE**	***z-value***	**P-value**
RWM				
(i) Survival				
Intercept (=Control)	4.318	0.482	8.958	**<0.001**
NB	1.810	0.662	2.735	**0.006**
CFSE	0.902	0.536	1.682	0.093
Log(scale)	0.166	0.181	0.918	0.359
(ii) Activity				
Intercept (=Control)	2.473	0.130	19.040	**<0.001**
NB	0.671	0.195	3.440	**<0.001**
CFSE	0.403	0.197	2.040	**0.041**
Log(scale)	-0.232	0.092	-2.530	**0.012**

Results of regression Weibull model (RWM) evaluating the effect of NB and CFSE intermediate concentration on (i) cercarial survival (alive cercariae ~ treatment) and (ii) activity rates (active cercariae ~ treatment) after 5 hpl. The intercept value in (i) stands for the survival rate and in (ii) for the activity rate, of the control cercariae, which the other two levels are compared, i.e. NB and CFSE both with intermediate concentration. Statistically significant results (at α=0.050) are indicated in bold. We also provide the scale parameter which indicates with log(scale) the Weibull distribution estimation.

Appendix 1: Table S7. Evaluation of major regions of fish's surface effect on cercarial density.

	Estimate	**SE**	***t-value***	**P-value**
(i) LMM				
Intercept (=Head)	2.838	0.158	18.000	<0.001
Body	0.447	0.223	2.008	0.050
Fin	0.690	0.200	3.458	<0.001
	Estimate	**SE**	***z-value***	**P-value**
(ii) Pairwise comparison				
Body – Head	0.448	0.223	2.008	0.110(0.134)
Fin – Head	0.690	0.200	3.458	0.002(0.002)
Fin-Body	0.242	0.200	1.213	0.444(0.675)

Results of (i) linear mixed model (LMM) (Attached cercariae density ~ major region + replicates (random)) and (ii) pairwise comparison evaluating the effect of major regions on cercarial density, calculated as number of cercariae/area cm^2 (Box-Cox transformed values). The intercept value in the LMM stands for the mean density of cercariae attached to the head region, to which the other two regions are compared, i.e. body and fins. The estimate of each variable is added to the intercept value. Statistically significant results (at α=0.050) are indicated in bold, with the corresponding P-value obtained after Bonferroni correction given in parentheses. We also provide: random effect 'replicatse', variance<0.001.

Appendix 1: Table S8. Evaluation of effect of sub-regions of fish's surface on cercarial density.

	Estimate	**SE**	***t-value***	**P-value**
(i) LMM				
Intercept (=H1, eye)	0.969	0.092	10.484	**<0.001**
H2, mouth	0.364	0.128	2.836	**0.005**
H3, gills	0.144	0.128	1.122	0.263
B1, upper side	0.307	0.128	2.392	**0.017**
B2, middle side	0.289	0.128	2.252	**0.025**
B3, lower side	0.161	0.130	1.240	0.215
F1 dorsal fin	0.067	0.128	0.522	0.602
F2, pectoral fin	0.376	0.128	2.930	**0.003**
F3, pelvic fin	0.391	0.128	3.045	**0.002**
F4, anal fin	0.367	0.128	2.859	**0.004**
F5, caudal fin	0.631	0.128	4.913	**<0.001**
	Estimate	**SE**	***z-value***	**P-value**
(ii) Pairwise comparisor				
Mouth-Eye	0.364	0.128	2.836	0.144(0.251)
Gills-Eye	0.144	0.128	1.122	0.990(1.000)
Upper side-Eye	0.307	0.128	2.392	0.372(0.921)
Middle side-Eye	0.289	0.128	2.252	0.467(1.000)
Lower side-Eye	0.161	0.130	1.240	0.978(1.000)
Dorsal fin-Eye	0.067	0.522	0.128	1.000(1.000)
Pectoral fin-Eye	0.376	2.930	0.128	0.113(0.187)
Pelvic fin-Eye	0.391	3.045	0.128	0.083(0.128)
Anal fin-Eye	0.367	0.128	2.859	0.136(0.234)
Caudal fin-Eye	0.631	0.128	4.913	**<0.010(<0.010)**
Gills-Mouth	-0.220	0.128	-1.714	0.829(1.000)
Upper side-Mouth	-0.057	0.128	-0.444	1.000(1.000)
Middle side-Mouth	-0.075	0.128	-0.584	1.000(1.000)
Lower side-Mouth	-0.204	0.130	-1.571	0.895(1.000)
Dorsal fin-Mouth	-0.297	0.128	-2.314	0.424(1.000)
Pectoral fin-Mouth	0.012	0.128	0.094	1.000(1.000)

Pelvic fin-Mouth	0.027	0.128	0.210	1.000(1.000)
Anal fin-Mouth	0.003	0.128	0.023	1.000(1.000)
Caudal fin-Mouth	0.267	0.128	2.077	0.594(1.000)
Upper side-Gills	0.163	0.128	1.270	0.974(1.000)
Middle side-Gills	0.145	0.128	1.130	0.989(1.000)
Lower side-Gills	0.017	0.130	0.128	1.000(1.000)
Dorsal fin-Gills	-0.077	0.128	-0.600	1.000(1.000)
Pectoral fin-Gills	0.232	0.128	1.808	0.776(1.000)
Pelvic fin-Gills	0.247	0.128	1.923	0.702(1.000)
Anal fin-Gills	0.223	0.128	1.737	0.816(1.000)
Caudal fin-Gills	0.487	0.128	3.791	**<0.010(<0.010)**
Middle side-Upper side	-0.018	0.128	-0.140	1.000(1.000)
Lower side-Upper side	-0.147	0.130	-1.131	0.989(1.000)
Dorsal fin-Upper side	-0.240	0.128	-1.870	0.737(1.000)
Pectoral fin-Upper side	0.069	0.128	0.538	1.000(1.000)
Pelvic fin-Upper side	0.084	0.128	0.653	0.999(1.000)
Anal fin-Upper side	0.060	0.128	0.467	1.000(1.000)
Caudal fin-Upper side	0.324	0.128	2.521	0.292(0.643)
Lower side-Middle side	-0.129	0.130	-0.992	0.996(1.000)
Dorsal fin-Middle side	-0.222	0.128	-1.730	0.820(1.000)
Pectoral fin-Middle sid	0.087	0.128	0.678	0.999(1.000)
Pelvic fin-Middle side	0.102	0.128	0.794	0.999(1.000)
Anal fin-Middle side	0.078	0.128	0.607	0.999(1.000)
Caudal fin-Middle side	0.342	0.128	2.661	0.218(0.428)
Dorsal fin-Lower side	-0.094	0.130	-0.723	0.999(1.000)
Pectoral fin-Lower side	0.216	0.130	1.665	0.854(1.000)
Pelvic fin-Lower side	0.230	0.130	1.779	0.793(1.000)
Anal fin-Lower side	0.207	0.130	1.594	0.885(1.000)
Caudal fin-Lower side	0.470	0.130	3.631	**0.013(0.016)**
Pectoral fin-Dorsal fin	0.309	0.128	2.408	0.361(0.882)
Pelvic fin-Dorsal fin	0.324	0.128	2.524	0.290(0.639)
Anal fin-Dorsal fin	0.300	0.128	2.337	0.407(1.000)
Caudal fin-Dorsal fin	0.564	0.128	4.392	**<0.010(<0.010)**

Pelvic fin-Pectoral fin	0.015	0.128	0.116	1.000(1.000)
Anal fin-Pectoral fin	-0.009	0.128	-0.071	1.000(1.000)
Caudal fin-Pectoral fin	0.255	0.128	1.983	0.660(1.000)
Anal fin-Pelvic fin	-0.024	0.128	-0.187	1.000(1.000)
Caudal fin-Pelvic fin	0.240	0.128	1.868	0.739(1.000)
Caudal fin-Anal fin	0.264	0.128	2.055	0.610(1.000)

Results of (i) linear mixed model (LMM) (Attached cercariae density ~ sub-region + replicates (random))) and (ii) pairwise comparison evaluating the effect of sub-regions on cercarial density, calculated as number of cercariae/area cm^2 (Box-Cox transformed values). Fish surface is divided in 11 regions, representing the original fish division and so, subdividing the head into 3 sub-regions (eye (H1), mouth (H2), gills (H3)), the body into 3 sub-regions (upper (B1), middle (B2) and lower (B3) side), and the fins into 5 sub-regions (dorsal (F1) pectoral (F2), pelvic (F3), anal (F4) and caudal (F5) fins). The intercept value in the LMM stands for the mean density of cercariae attached to the fish eye sub-region (H1), to which the other sub-regions are compared. The estimate of a variable is added to the intercept value. Statistically significant results (at α=0.050) are indicated in bold, with the corresponding P-value obtained after Bonferroni correction given in parentheses. We also provide: random effect 'replicates', variance =0.004.

Appendix 1: Table S9. Evaluation of dyes effect on cercarial infectivity and metacercariae encystment success.

	Estimate	SE	*z-value*	P-value
(i)GLMM				
Intercept (=Control)	-1.880	0.471	-3.993	**<0.001**
NB	-0.224	0.174	-1.285	0.199
CFSE	-0.536	0.186	-2.891	**0.004**
(ii) Pairwise comparison				
Control-NB	0.224	0.174	2.285	0.403(0.596)
CFSE-NB	-0.312	0.191	-1.631	0.232(0.308)
CFSE-Control	-0.536	0.186	-2.891	**0.011(0.012)**

Results of (i) generalized linear mixed model (GLMM) (Proportion of brain-encysted metacercariae ~ dye type + replicates (random)) and (ii) pairwise comparison evaluating the effect of NB and CFSE on cercarial infectivity and metacercariae encystment success. The intercept value in the GLMM stands for the mean number of cercariae control, encysted as metacercariae in the fish's brain on the logit scale, to which the other treatments are compared. The estimate of a variable is added or subtracted to the intercept value. Statistically significant results (at α=0.050) are indicated in bold, with the corresponding P-value obtained after Bonferroni correction given in parentheses. We also provide standard error (SE). Random effect 'replicates', variance<0.616.

Appendix 1: Table S10. Evaluation of effect of fish's head sub-regions on cercarial density.

	Estimate	**SE**	***t-value***	**P-value**
(i) LMM				
Intercept (=H1, Eye)	0.968	0.009	105.899	**<0.001**
H2, mouth	0.048	0.013	3.699	**<0.001**
H3, gills	0.016	0.013	1.228	0.223
	Estimate	**SE**	***z-value***	**P-value**
(ii) Pairwise comparison				
Mouth-Eye	0.048	0.013	3.699	**<0.001(<0.001)**
Gills-Eye	0.016	0.013	1.228	0.437(0.437)
Gills-Mouth	0.032	0.013	2.470	**0.036(0.036)**

Results of (i) linear mixed model (LMM) (Attached cercariae density of only fish head surface ~only head sub-regions + replicates (random)) and (ii) pairwise comparison evaluating the effect of head sub-regions on cercarial density, calculated as number of cercariae/area cm^2 (Box-Cox transformed values). The fish head surface is divided into 3 sub-regions (eye (H1), mouth (H2), gills (H3)). The intercept value in the LMM stands for the mean density of cercariae attached to the fish eye sub-region (H1), to which the other head sub-regions are compared. The estimate of a variable is added to the intercept value. Statistically significant results (at α=0.050) are indicated in bold, with the corresponding P-value obtained after Bonferroni correction given in parentheses. We also provide standard error (SE). Random effect 'replicates', variance <0.001.

Appendix 2

10. Appendix 2

Appendix 2. Supplementary material to Chapter 5

Videos available online at the Comparative & Integrative Biology webpage (https://academic.oup.com/icb/article/62/3/461/6620838), as “Supplementary data”.

Appendix 2: Video S1. Animated image of a cercaria swimming with the tail-first. (file “icb-2022-0119-File014.mpg")

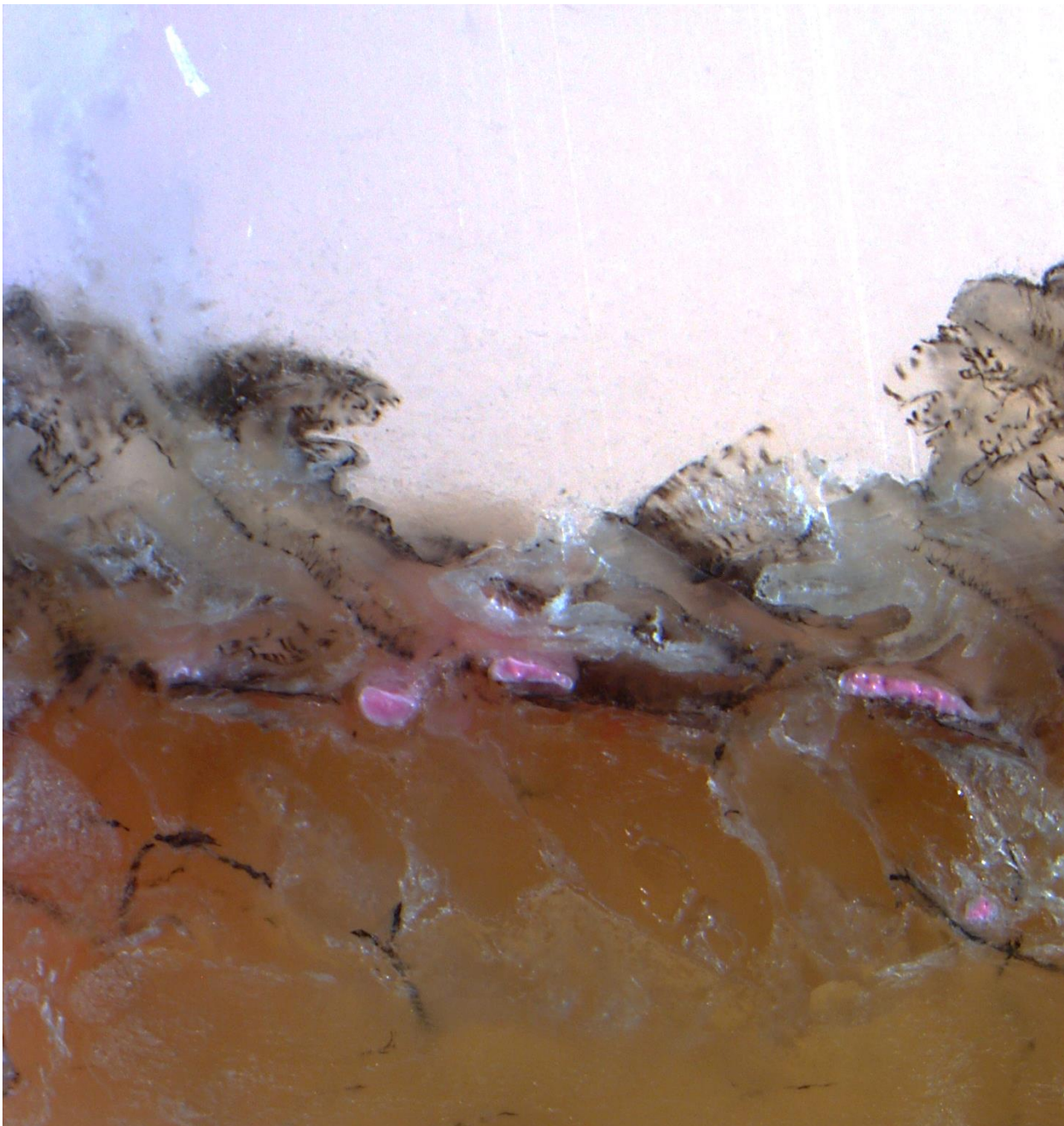
Appendix 2: Video S2. Animated image of a cercaria creeping on the fish surface. (file “icb-2022-0119-File015.mpg")

Appendix 2: Video S3. Video of a cercaria creeping on the fish surface. (file “icb-2022-0119-File016.mp4”)

Appendix 2: Video S4. Animated image of a cercaria burrowing within the fish tissue. (file “icb-2022-0119-File017.mpg")

Appendix 2: Video S5. Video of a cercaria burrowing within the fish tissue. (file “icb-2022-0119-File018.mp4")

Appendix 3



11. Appendix 3

Appendix 3. Supplementary material to Chapter 6

Appendix 3: Table S1. Results of linear models (LM) analysing the difference in size between two fish group sizes in the (i) short-, (ii) middle- and (iii) long-term studies (fish standard length (cm) ~ size group (small *vs* large fish). The categorical variable 'size group' was assessed in comparison with the reference size group 'small'. R^2 represents the variability explained by fixed effects. Statistically significant results (at $\alpha = 0.050$) are indicated in bold.

LM	Estimate	SE	*t-value*	P-value
(i) Short-term assay (R^2=0.95)				
Intercept	10.120	0.398	25.400	**<0.001**
Large group	10.830	0.565	19.220	**<0.001**
(ii) Middle-term assay (R^2=0.77)				
Intercept	21.227	0.375	56.660	**<0.001**
Large group	7.491	0.570	13.130	**<0.001**
(iii) Long-term assay (R^2=0.57)				
Intercept	13.910	0.370	37.580	**<0.001**
Large group	18.577	0.610	14.060	**<0.001**

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Appendix 3: Table S2. Results of linear model (LM) analyzing the difference in growth between two tag groups of fish after 85 days post-tagging (middle-term study) in the (i) small and (ii) large fish size group (fish length difference in 85 days (cm) ~ tag group (tagged *vs* untagged fish). The categorical variable 'tag group' was assessed in comparison with the reference tag group 'tagged fish'. R^2 represents the variability explained by fixed effects. Statistically significant results (at $\alpha = 0.050$) are indicated in bold.

LM	Estimate	SE	*t-value*	P-value
(i) Small fish (R^2=0.02)				
Intercept	0.089	0.120	0.741	0.465
Untagged group	-0.259	0.205	-1.263	0.218
(ii) Large fish (R^2=0.07)				
Intercept	0.076	0.070	1.092	0.288
Untagged group	-0.168	0.104	-1.619	0.121

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ALEXANDER PP VI