Vol. 136: 133–146, 2019
https://doi.org/10.3354/dao03394

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published online October 17

Health assessment of the cleaner fish ballan wrasse *Labrus bergylta* from the British south-west coast

Jamie McMurtrie, David W. Verner-Jeffreys, Tamsin Cochrane-Dyet, Pete White, Ronny van Aerle, David Ryder, David Stone, Matthew Green, Stephen W. Feist, Irene Cano*

Cefas Weymouth Laboratory, Centre for Environment, Fisheries and Aquaculture Science, Weymouth DT4 8UB, UK

ABSTRACT: Wild-caught ballan wrasse *Labrus bergylta* are translocated en masse from the British south-west coast to Scotland for use as cleaner fish to tackle Atlantic salmon *Salmo salar* sea lice infestations; however, very little is known about the background health status of this species. This is the first health assessment of wild ballan wrasse from the British south-west. Wild-caught ballan wrasse (n = 75) from coastal populations of Dorset and Cornwall were subjected to a full health screen for viral, bacterial and parasitic infections and associated pathology. A range of metazoan and protozoan parasites were observed in histological sections, including copepods (sea lice *Caligus centrodonti*), nematodes, cestodes, digenean metacercariae, *Cryptocaryon*-like ciliates and an intestinal coccidian (*Eimeria* sp.) observed in 26.6% of the samples. The mycoplasma *Acholeplasma laidlawii* was associated with cytopathic effect in cell culture inoculated with tissue homogenates. The opportunistic pathogen *Photobacterium damselae damselae* was isolated from a single fish with a systemic infection. The isolate was confirmed to possess the virulence factors $hlyA_{ch}$ and plpV, previously associated with cell toxicity and pathogenicity to fish. There are no immediate concerns for the continued mass translation of ballan wrasse, however careful monitoring of the population is recommended.

KEY WORDS: Ballan wrasse · *Photobacterium damselae damselae* · Cleaner fish · *Eimeria* · Coccidian · *Caligus centrodonti* · Salmon aquaculture

1. INTRODUCTION

Atlantic salmon *Salmo salar* L. is an important and widely traded food source across the globe, with 70% of global production met by commercial farming. Supply of Atlantic salmon has increased by 384% since 1995, with a world production of 2248 t in 2016 (FAO 2018); however, production growth in recent years has dropped by 7% (Marine Harvest 2017). The largest concern to the industry has been parasitic sea lice, namely *Lepeophtherius salmonis* Krøyer and *Caligus* spp., estimated to cost the global industry £700 (~€950) million annually (Brooker et al. 2018b). Widely used chemicals for the removal of lice, such as the orally administered emamectin benzoate, marketed as SLICE®, and bath-administered hydrogen peroxide H_2O_2, are showing reduced efficacy (Jones et al. 2013, Helgesen et al. 2015), and in recent years attention has shifted to biological control using cleaner fish, in particular wrasse (Labridae spp.) and lumpfish *Cyclopetrus lumpus* L. (Brooker et al. 2018a).

Ballan wrasse *Labrus bergylta* (Ascanius, 1767) in the North Atlantic are currently regarded as the preferred species of cleaner fish, with some Scottish salmon farming companies achieving close to complete control of sea lice infestations with ballan wrasse without the need for chemotherapeutants (Hempleman 2017). Demand for ballan wrasse is largely met by wild-caught fish, although farming of cleaner fish has developed in the last 5 yr, with the

*Corresponding author: irene.canocejas@cefas.co.uk

Publisher: Inter-Research · www.int-res.com

production of 118 000 wrasse in the UK in 2016 (Munro & Wallace 2017). Due to long generation times, wild-caught broodstock have been used exclusively in wrasse culture to date (Brooker et al. 2018b), and as wrasse farming becomes more common, it is increasingly important to understand the health status of these wild populations which are either directly transferred for deployment on Scottish salmon farms or used as broodstock.

There is a risk of cleaner fish acting as vectors of disease, being asymptomatic carriers of bacterial, viral and parasitic disease agents (Rimstad et al. 2017). These threats may manifest following the stress of translocation, leading to mortality of cleaner fish stocks, but may also pose a risk through transmission to farmed Atlantic salmon that have not developed natural resistance via gradual exposure to these agents of disease. The risk of disease is not restricted to Atlantic salmon, as cleaner fish are known to escape from salmon farms and hybridise with local wrasse populations (Faust et al. 2018); thus, cleaner fish may also exert a disease threat to local species.

A number of diseases are known to be serious threats for both wrasse and salmon, but the level of risk they pose is often unclear. *Aeromonas salmonicida* is a bacterium that causes severe systemic disease globally in a range of fish hosts, with typical *A. salmonicida* causing furunculosis in salmonids. Although these typical strains have been isolated from wrasse cohabiting with infected salmon, disease in wrasse is dominated by atypical *A. salmonicida* strains, namely A-layer types V and VI. These remain genetically distinct from those that most commonly infect salmon (Gulla et al. 2016b).

Viral haemorrhagic septicaemia (VHS) is a serious notifiable disease agent (OIE 2018), with wrasse having suffered a mortality event associated with VHS virus (VHSV) genotype III (Munro et al. 2015). Atlantic salmon are susceptible to VHSV, with low pathogenicity experimentally observed with a range of European genotypes (I–III), and an increased pathogenicity when infected with genotype IVa (Lovy et al. 2013).

Amoebic gill disease (AGD) affects both ballan wrasse and Atlantic salmon (Karlsbakk et al. 2013) and is caused by the same aetiological agent, *Neoparamoeba perurans*. The parasite has a low host specificity and as such there is a high risk of transfer between different host species, a finding which has been confirmed experimentally between lumpfish and salmon (Haugland et al. 2017). However, the parasitic and microbial fauna of wild-caught ballan wrasse is poorly understood, and the transmission risk to both salmonid and cleaner fish is unclear. This is particularly the case in south-west England, where an estimated 1 million wrasse, of mixed species, are caught every year, with the majority of these landings known to be ballan wrasse destined for use as cleaner fish in Scottish Atlantic salmon farms (Riley et al. 2017). The aim of this study was to conduct the first health assessment of wild-caught ballan wrasse from south-west England, using a wide range of classical pathogen isolation techniques, molecular detection and metagenomic approaches.

2. MATERIALS AND METHODS

2.1. Sampling

Local fishermen provided 75 ballan wrasse fished from 3 sites on the south-west coast: Weymouth (n = 31) and Portland (n = 13) (Dorset population) and Falmouth (n = 31) (Cornwall population); the fish were delivered to the Cefas Weymouth Laboratory in September 2017. Fish were euthanised humanely according to UK Home Office procedures in compliance with the Animals (Scientific Procedures) Act 1986 (www.legislation.gov.uk/ukpga/1986/14/contents). Blood samples were collected and smears prepared. Gill arches were removed, and filaments were sampled for fresh microscopic examination and stained gill imprints. Gill samples were placed into saline for culture of eukaryotes. Kidney and spleen were swabbed for bacterial isolation. Gill arches and a pool of the kidney, spleen, liver, heart and brain were used for virology. Skin, muscle, intestine, kidney, spleen, liver, heart, gill and brain were fixed both in ethanol and 10% neutral-buffered formalin (NBF) for molecular and histological examinations. Eye and gonad tissue were taken solely for histological examination.

2.2. Histology

NBF-fixed tissue sections were transferred to 70% ethanol prior to processing and embedded in paraffin wax with the PELORIS II Premium Tissue Processing System (Leica Biosystems). Embedded blocks were sectioned at 3–4 µm thickness with a rotary microtome (Shandon Finesse) for haematoxylin and eosin (H&E) staining. Tissue sections were examined with a Nikon E800 light microscope and images captured using Lucia™ software. Following initial examinations, a selection of samples was re-cut for Giemsa, Gram and Ziehl–Neelsen stains for elucidation of protists and bacteria in tissues.

2.3. Bacteriology

Swabs were immediately streaked onto tryptic soy agar (TSA), sea water agar (SWA), defibrinated sheep blood (Oxoid) agar with 2% (w/vol) NaCl and thiosulfate–citrate–bile salts–sucrose agar (TCBS) (ThermoScientific). Plates were incubated at 15°C and observed daily for growth. Colonies of interest were subcultured onto SWA, for identification by cell morphology, Gram stain and TCBS growth screening. Selected isolates of interest where further characterised by 16S rRNA gene Sanger sequencing and enzymatic activities (API 20NE/20E kits, BioMérieux).

2.4. Virology

During sampling, tissues were directly placed in 1:10 transport media (Glasgow's MEM, supplemented with 10% foetal bovine serum, 200 IU ml^{-1} penicillin, 200 µg ml^{-1} steptomycin and 2 mM L-glutamine) and were homogenised and clarified by centrifugation for 15 min at 2500 × *g*. Homogenates were inoculated at 1:100 and 1:1000 dilution onto 48-well tissue culture plates (Falcon, BD Biosciences), with 3 different fish cell lines: epithelioma papulosum cyprini (EPC) (ATCC®: CRL-2872™), chinook salmon embryo (CHSE-214) (ATCC®: CRL-2872™), fathead minnow (FHM) (ATCC® CCL-42™), SSN-1 (ECACC 96082808) and bluegill fibroblast (BF-2) (ATCC®: CCL-91™). Inoculation of BF-2 was run in duplicate, with a supplementary infectious pancreatic necrosis virus (IPNV) neutralizing antisera (polyclonal goat anti-serotype Sp IPNV serum; Harlan Sera-lab). Samples were incubated at 15°C for 14 d and regularly examined for cytopathic effect (CPE). Monolayers were blind-passaged onto fresh cells for a further 14 d of incubation.

Samples showing CPE were filtered to 0.2 µm with a Ministart® NML syringe filter (Sartorius), and filtrate was reinoculated onto fresh cell monolayers. Viral nucleocapsid was extracted from the supernatant of the cells showing CPE as described below and subjected to further molecular analysis (reverse transcription [RT-]PCR).

2.5. Gill health examination

A gill arch from each fish was placed in 10 ml sterile sea water and then transferred to a sterile petri dish for incubation at 18°C, with regular observation for amoebas and other protists; selected samples were subsequently cultured on malt yeast agar (MYA). DNA was extracted from the cultures for amplification and Sanger sequencing of 18S small subunit (SSU) rRNA with primers ERIB1 and ERIB10 (Table 1).

2.6. DNA and viral nucleocapsid extraction

DNA extractions were performed using either the EZ1 extraction robot or the QIAamp® Investigator BioRobot® (Qiagen) following the manufacturer's instructions. Tissue samples were diluted to a 1:10 mass to volume ratio in digestion buffer G2 (when using the EZ1 robot) or ATL (when using the BioRobot) and homogenised with glass beads in a FastPrep®-24 Classic (MP Biomedicals), before 10 µl of Proteinase K were added and samples were incubated at 56°C for 3 h. DNA was eluted in 60 µl of elution buffer.

Following pathological screening, selected gill tissues had DNA extracted from formalin-fixed, paraffin-embedded tissue with the FFPE Tissue Kit (Qiagen), following the manufacturer's protocol, with DNA eluted in 30 µl ATE buffer.

An EZ1 Virus mini kit (Qiagen) was used for viral nucleocapsid extraction from tissues and inoculated cells, with RNA eluted in 60 µl of elution buffer.

2.7. Molecular detection of pathogens

RT of viral nucleocapsid was performed by incubation at 70°C for 2 min, before 200 units M-MLV reverse transcriptase (RTase)were added for an additional hour at 37°C. The total 20 µl reaction volume consisted of M-MLV RTase with M-MLV RTase 5× reaction buffer, 1 mM dNTP mix, 500 ng random primers, 25 units RNasin® Ribonuclease Inhibitor (Promega) and 4 µl of extracted viral nucleic acid.

PCR was performed in a 50 µl reaction volume consisting of 1× green GoTaq® flexi buffer, 2.5 mM $MgCl_2$, 1 mM dNTP mix, 1.25 units of GoTaq® G2 Hot Start Polymerase (Promega), 50 pmol of each forward and reverse primer (Table 1), 2.5 µl of either DNA or cDNA. After an initial denaturing step of 5 min at 95°C, samples were subjected to 35 cycles of 1 min at 95°C, 1 min at 55°C, 1 min at 76°C, followed by a final extension step of 10 min at 72°C in a PTC-225 Peltier thermal cycler (MJ Research). Amplified products were held at 4°C until being resolved on a 2% (w/v) agarose/TAE gel (40 mM Tris-acetate, pH 7.2, 1 mM EDTA, 1.0 µg ml^{-1} ethidium bromide)

Table 1. Primers used for PCR amplification. ISH: *in situ* hybridisation

PCR type	Primer/Probe	Sequence (5′–3′)	Reference
Virus — family Rhabdoviridae	Ves Gen 1 Ves Gen 2 Ves Gen 4	GGRMGDTTYTTYTCHYTRATGTC ATCAGRTCNGGYCTNCCRTTRTARTA ATNACKCKRAANACNGGNCCRTT	Ruane et al. (2014)
Virus — genus *Novirhabdovirus*	For EXT Rev EXT For INT	CACTSAARGTSAARGARATGG CARATNGTCCANACRTAYTG TTYCCDGARATMACCATGACC	This study
Virus — genus *Aquabirnavirus*	FEXT REXT Fint Rint	AAGACCMGDAACWHTGG TCDATYTTRAAGTTRATSCC TATGCNGACAACATMTACAT GRCTTCCCRCTGCCYTGVCC	Dixon et al. (2008)
Virus — family *Iridoviridae* (*Ranavirus*)	GenIridoFext2 GenIridorext2 GenIridoFint2 GenIridoRint2	TTCCCAGGTCGGGSGATTAC TCCACRACCATRTCCCTGAC CGCATAGGCTACKAYAACATG GTWRGCYTCSACGGTGTCG	This study
Virus — *family Iridoviridae* (*Megalocytivirus*)	GenIridoFext4 GenIridoRext4 GenIridoFint4 GenIridoRint4	GATGCGATGGAGACCCACTT TGCTCTGGCTGATGAGCAGG TTAATGTGTGGCTGCGTGTTA CATAGTCTGRCCGTTGGTGAT	This study
Bacteria — 16S rRNA gene	fD1 rP2	AGAGTTTGATCCTGGCTCAG ACGGCTACCTTGTTACGACTT	Weisburg et al. (1991)
Bacteria — ISH probe	S-D-Bact-0008-a-S-20 S-*-Univ-0536-a-A-18	AGAGTTTGATCCTGGCTCAG GWATTACCGCGGCKGCTG	Suau et al. (1999)
Aeromonas salmonicida qPCR	VapAF3 VapAR1 AS MB	ACTGTCTGTTACCCTGCCA GCTACTTCACCCTGATTGG 6-FAM-CGCGATCACATCAGCAGGC TTCAGAGTCACTGGATCGCG-MGB	Gulla et al. (2016a) Keeling et al. (2013)
Cultured ciliates 18S rRNA gene	ERIB1 ERIB10	ACCTGGTTGATCCTGCCAG CTTCCGCAGGTTCACCTACGG	Barta et al. (1997)
Neoparamoeba perurans qPCR 18S rRNA gene	Peru.F Peru.R Peru.P	GTTCTTTCGGGAGCTGGGAG GAACTATCGCCGGCACAAAAG 6-FAM-CAATGCCATTCTTTTCGGA-MGB	Fringuelli et al. (2012)
Coccidia — 18S rRNA gene	CoF CoR	CCCAATGAAAACAGTTTCGAGG CGGCATAGTTTATGGTAGAGATT	This study
Cryptocaryon — ITS	P1 NC2 S15	GTTCCCCTTGAACGAGGAATTC TTAGTTTCTTTTCCTCCGCT TGAGAGAATTAATCATAATTTATAT	Yoshinaga & Dickerson (1994) Sun et al. (2006) Chen et al. (2008)
Ciliate — 18S rRNA gene	384F 1147R	YTBGATGGTAGTGTATTGGA GACGGTATCTRATCGTCTTT	Dopheide et al. (2008)

at 120 V, 400 mA and visualised under UV irradiation using the Gel Dox Imager XR+ (Bio-Rad), with images captured by Quantity One 1-D Analysis Software (Bio-Rad).

Coccidian-specific primers (CoF and CoR; Table 1) were designed for the amplification of a 580–649 bp fragment of coccidian 18S SSU rRNA by multiple-sequence alignment of published coccidia sequences (Table S1 in Supplement 1 at www.int-res.com/articles/suppl/d136p133_supp1.xlsx). PCR amplification was performed on gut DNA extracts of 5 fish exhibiting pathology of intestinal coc-

cidiosis, followed by Sanger sequencing (see Section 2.9).

CoF and CoR primers were also used for generation of digoxigenin (DIG)-labelled probe (Roche) for *in situ* hybridisation (ISH). Tissue sections from a Weymouth and Falmouth fish were mounted on poly-lysine-treated slides (Sigma-Aldrich), with ISH performed as previously described (Cano et al. 2009). Briefly, permeabilisation of sections with Proteinase K (100 µg ml^{-1}, Promega) for 30 min at 37°C, the probe was denatured at 95°C for 5 min prior to overnight hybridisation at 42°C. Tissue sections were blocked with 6% (w v^{-1}) skimmed milk (Sigma), and NBT/BCIP (Roche) was used for detection of the hybridisation signal, prior to counterstaining of nuclei with Nuclear Fast Red (Sigma).

ISH was also performed for confirmation of the bacterial infection identified by PCR and Sanger sequencing, using a DIG-labelled probe of partial 16S rRNA with the primers S-D-Bact-0008-a-S-20 and S-*-Univ-0536-a-A-18 (Table 1). The PCR reaction for the probe labelling was conducted as described above.

PCR amplifications of FFPE-extracted gill tissue were performed using the set of primers P1/NC2 and P1/S15 targeting the second internal transcribed spacer (ITS-2) of *Cryptocaryon*; the generic primers 18E/18G; and 384F/1157R, specific for ciliates, both assays targeting the 18S rRNA gene (Table 1).

2.8. AGD and *Aeromonas salmonicida* quantitative PCR (qPCR)

A qPCR assay targeting the 18S rRNA gene of *Neoparamoeba perurans*, which causes AGD, was run in duplicate with DNA extracted from 45 fish gill arches preserved in ethanol. Primers and probe (Table 1) were run with assay conditions as described previously (Fringuelli et al. 2012).

In addition, a qPCR assay targeting the A-layer (*vapA*) gene of all known strains of *A. salmonicida* was run in duplicate for a subset of the population with DNA extract of kidney and spleen tissue from 48 fish. qPCR reactions were run with assay conditions as described previously (Gulla et al. 2016a).

2.9. PCR Sanger sequencing

Following PCR amplification, gel fragments for sequencing were excised under a UV transilluminator and purified using spin modules (MP Biomedicals). Sequencing was completed with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) following the manufacturer's recommended procedure. Sequence analysis was performed with the ABI 3100 Avant Genetic analyser (Applied Biosystems) and Sequencher software (Gene Codes). Sequence similarity was determined by BLASTn (NCBI nt database March 2018; Altschul et al. 1990) and the Ribosomal Database Project (RDP Release 11, Update 5). The phylogenetic tree was generated from 100 bootstrap replications of the Tamura-Nei model based on partial deletions in MEGA v7.

2.10. Illumina MiSeq next-generation sequencing (NGS)

A bacterial isolate was selected for high-throughput sequencing. Sequencing libraries were prepared with Nextera XT kits (Illumina) and sequenced at Cefas by Illumina MiSeq using the 2× 300 bp paired-end protocol (following the manufacturer's recommended procedures).

2.11. Bioinformatics

Analysis of the sequenced bacterial culture was performed using a Linux virtual machine on the MRC CLIMB cloud computing infrastructure (Connor et al. 2016). Read quality was assessed using FastQC v0.11.7 (Andrews 2010), and Trimmomatic v0.36 (Bolger et al. 2014; default parameters) was used to remove adaptor sequences and low-quality bases. De novo assembly of paired and unpaired reads was performed with SPAdes v3.9.0 (Bankevich et al. 2012) and Unicycler v0.4.4 (Wick et al. 2017).

The genome was annotated using Prokka v1.13.3 (Seemann 2014). Kraken v1.0 was used to check for potential contaminants in the sequencing library (Wood & Salzberg 2014). BUSCO v3.0.2 was used to check the completeness of the genome (Waterhouse et al. 2018). The OrthoDB v9 (Waterhouse et al. 2013), customised for *Gammaproteobacteria* and available through the BUSCO website (https://busco.ezlab.org/), was used together with BUSCO v3.0.2 to assess the completeness of the genome.

Multilocus sequence typing (MLST) was carried out using a recently published scheme for *Photobacterium damselae* (Alba et al. 2016). Existing and novel alleles for each of the 6 housekeeping genes

were identified using mlst v2.10, which itself makes use of the PubMLST database (Jolley & Maiden 2010). Sequences from each gene were then concatenated, resulting in a total alignment of 2739 bp. All isolates whose details were published along with the original scheme were included in the concatenated alignment (Alba et al. 2016), as well as any genome on NCBI which included suitable metadata. A phylogenetic tree was constructed using FastTree v2.1.10 (Price et al. 2010).

3. RESULTS

3.1. Gross observations and histopathology

All fish observed during sampling were in a good, healthy condition with no clinical signs of disease (Table 2). Sea lice attached to the skin were observed on 21.3% of fish examined from both populations (Dorset and Cornwall), with the Cornwall population having the higher prevalence and infection intensity (38.7% in Cornwall versus 9% in Dorset); these were morphologically identified as *Caligus centrodonti* (Fig. 1A). The most prevalent parasite stages observed in tissue sections were eimerian sporocysts (Fig. 1B–D) infecting intestinal epithelial cells in 26.6% of all fish analysed. We also observed nematodes (Fig. 1E) and associated granulomas (Fig. 1F) in the liver and intestine in 16% of fish, and copepods on the gills (Fig. 1G) in 25.3% of fish. In addition, cestodes were found in the intestine in 4% of fish, and digenean metacercariae, *Trichodina* sp. and *Cryptocaryon*-like ciliates were also seen (Fig. 1H).

3.2. *Eimeria* sp. infection

No significant inflammatory response was associated with the presence of *coccidian* sporocysts in the intestine (Fig. 1B–D). PCR amplification and sequencing of 5 infected samples with coccidian primers (CoF, CoR) targeting the 18S SSU rRNA yielded a 596 bp consensus sequence. Nucleotide sequence similarity searches showed that this sequence is highly similar to sequences of *Eimera* spp. previously reported in fish (ranging from 93 to 98% similarity). Furthermore, phylogenetic analysis placed this species in a clade containing *Eimera* spp. (Fig. 2). Sequence data were submitted to GenBank under accession number MK397509.

ISH successfully labelled the parasite stages in the intestine (Fig. 1C,D) and confirmed the extent and intensity of infection, with sporocyst-filled oocysts observed in the intestine sections. A few oocysts were also observed in the intestinal lumen.

3.3. *Cryptocaryon*-like gill infection

Histology revealed a fish infected by 4 ciliated parasites within 2 neighbouring gill lamellae (Fig. 1H). From their morphology, they were tentatively described as *Cryptocaryon*-like ciliates at the trophont stage of their life cycle. Ciliates were embedded within the gill tissue and were associated with localised cellular necrosis.

Attempts to sequence a PCR product from the ciliate using *Cryptocaryon*-specific, ciliate-specific and generic 18S primers were unsuccessful from either gill tissue preserved in ethanol or extracted from gill embedded in a paraffin wax block.

3.4. Isolation of *Photobacterium damselae damselae*

Bacterial culture plates were monitored daily for colony growth, with significant observations recorded. A total of 125 isolates were collected for characterisation, with 13 isolates identified by 16S sequen-

Table 2. Summary of the histopathology observations of ballan wrasse caught from the British south-west coast. Numbers represent the prevalence (%) of metazoans (nematodes, digeneans [metacercariae], cestodes), protistan parasites (*Trichodina* sp., *Cryptocaryon*-like and *Eimeria* sp.), copepods (unidentified copepod and *Caligus centrodonti*), and endosymbiont *Pseudocohnilembus persalinus*

Location	Number sampled	Nematodes	Digeneans	Cestodes	Copepods	*C. centrodonti*	*Trichodina* sp.	*Cryptocaryon*-like	*P. persalinus*	*Eimeria* sp.
Dorset	44	15.9	2.2	2.2	6.8	9.0	2.2	0	0	13.6
Cornwall	31	16.1	0	6.4	51.6	38.7	0	3.2	41.9	45.1
Total	75	16	1.3	4	25.3	21.3	1.3	1.3	17.3	26.6

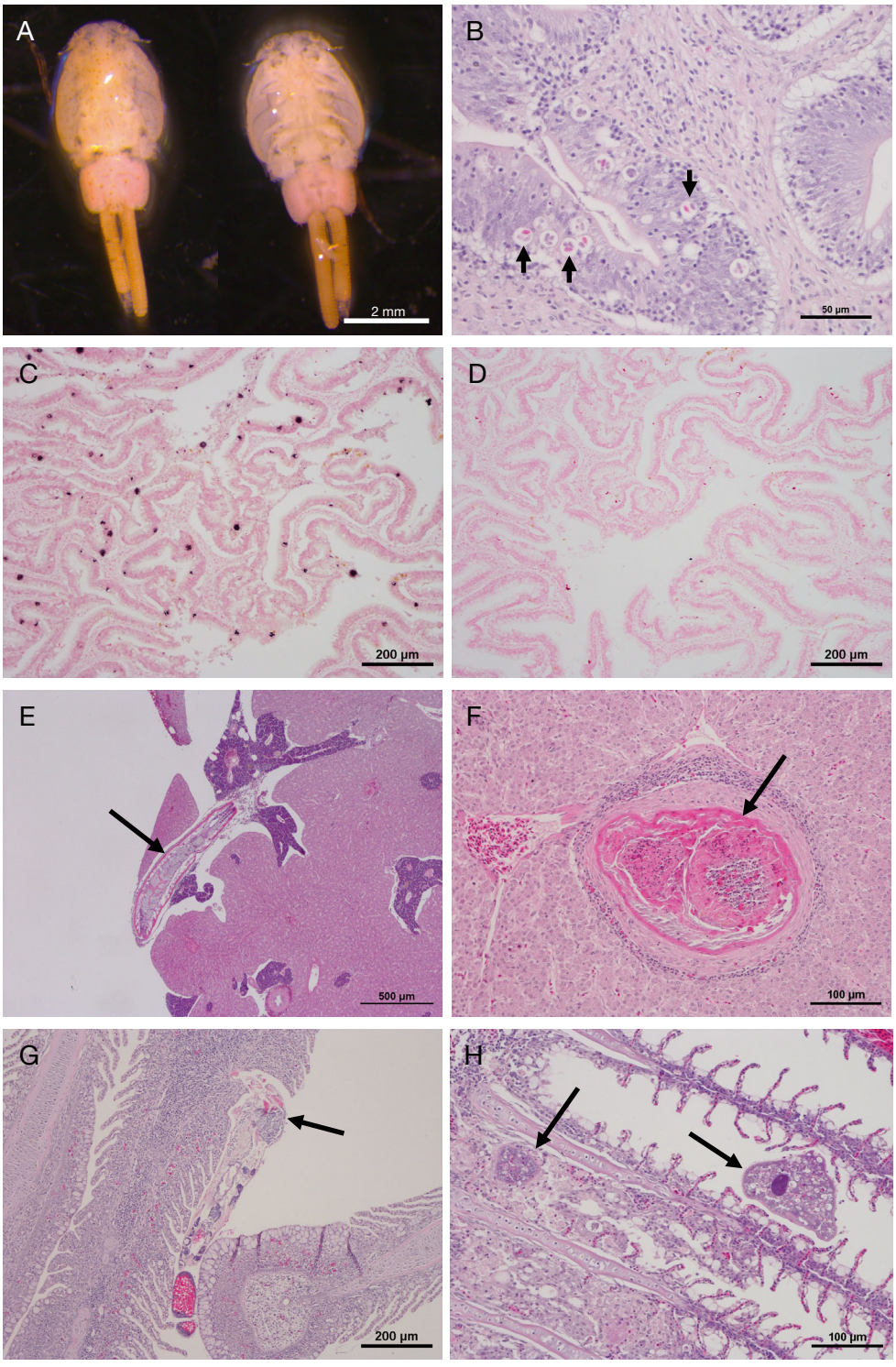

Fig. 1. Parasitology of ballan wrasse. (A) Sea lice *Caligus centrodonti* observed on the skin surface of wrasse, fixed in ethanol. (B) Focal infection of the intestinal lamina propria with multiple *Eimeria* sp. sporocysts (arrows); H&E stain. (C) *In situ* hybridisation (ISH) with strong binding to *Eimeria* sp. sporocysts with a DIG-labelled probe designed against an *Eimeria* sp.-specific region of the 18S rRNA gene. (D) ISH negative control with no specific probe binding; darker spots are artefacts. (E) Nematode (arrow) infection of the liver observed at initial attachment; H&E stain. (F) Liver granuloma (arrow) with necrotic centre associated with a nematode infection; H&E stain. (G) Copepod (arrow) attached and feeding at the gill lamella with minor inflammatory response; H&E stain. (H) *Cryptocaryon*-like ciliated parasites (arrows) attached between gill lamella with minimal pathogenic effect; H&E stain

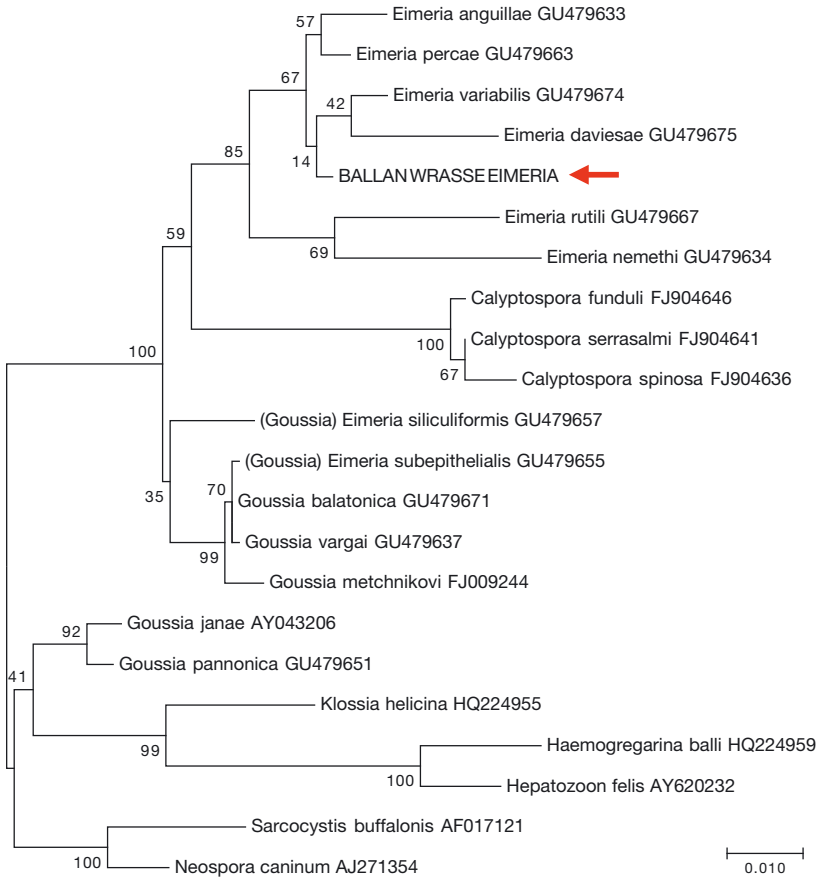

Fig. 2. Maximum-likelihood tree showing phylogenetic relationship of the 18S rRNA gene between the ballan wrasse intestinal *Eimeria* sp. coccidian (GenBank accession no. MK397509) and a selection of closely related coccidian species.

cing belonging to the genera *Vibrio* (3), *Allivibrio* (4), *Pseudoalteromonas* (2), *Flavobacterium* (1) and *Photobacterium* (3). *Aeromonas salmonicida* was not isolated from any culture plates. A modified qPCR assay for *A. salmonicida* was tested with typical and atypical strains (5b2, 5b4, 6) as positive controls. The assay was negative for 48 selected samples of pooled kidney and spleen.

A single fish revealed a systemic infection (Fig. 3), with non-acid-fast, Gram-negative bacilli (Fig. 3B). Pathological changes associated with the bacterial infection included lymphocytic infiltration in the intestine, splenic ellipsoids and interfollicular inflammation of the ovary. Relatively low numbers of Gram-negative bacilli were associated with significant inflammation and necrosis. Connective tissue associated with the kidney and adipose tissue of the intestine both revealed an active infection, with large masses of bacteria bordered by necrotic cells and a large inflammatory response involving degranulation of granulocytes (Fig. 3A). Blood smears revealed numerous bacteria of the same morphology, and vascular accumulation of bacteria in the intestine was observed.

Heavy bacterial growth of a single prominent colony type was observed on all bacteria culture plates from kidney and spleen swabs from this single fish. Three colonies were selected from SWA for further testing, revealing a Gram-negative bacillus with an API 20NE profile (1340204) with 99.6% identity to *Photobacterium damselae*. Following 16S rRNA amplification, sequencing and assembly was performed to generate a 1432 bp consensus sequence (GenBank accession number MK064503). BLASTn searches and RDP analysis confirmed that this sequence was highly similar to *P. damselae* (99% similarity) (Fig. S1 in Supplement 2 at www.int-res.com/articles/suppl/d136p133_supp2.pdf). A tree based on concatenated sequences from the glycerol uptake facilitator protein (*glpF*), DNA gyrase subunit B (*gyrB*), methionine-tRNA ligase (*metG*), NAD(P) transhydrogenase subunit alpha (*pntA*), dihydroorotase (*pyrC*) and cholera toxin transcriptional activator (*toxR*) genes was constructed, which included all isolates from the *damselae* subspecies, as well as being rooted by 3 isolates from the *piscicida* subspecies (phylogeny tree in Fig. 4, metadata in Table. S2 in Supplement 1). The isolate from ballan wrasse grouped with other isolates from the *damselae* subspecies, with subspecies identification confirmed by positive results for the biochemical tests API20NE (Essam et al. 2016): gas production from glucose, nitrate reduction to nitrite, urease production and observed green colony growth on TCBS agar and weak haemolysis on blood agar.

The full genome sequence was 4.40 Mb in length and was predicted to contain 3801 protein-coding sequences. The assembled sequence data were uploaded to NCBI's Whole Genome Sequence database and published under accession GCA_004794735.1, whilst the raw data were uploaded to the Single Read Archive under accession SRR8447033. BUSCO was

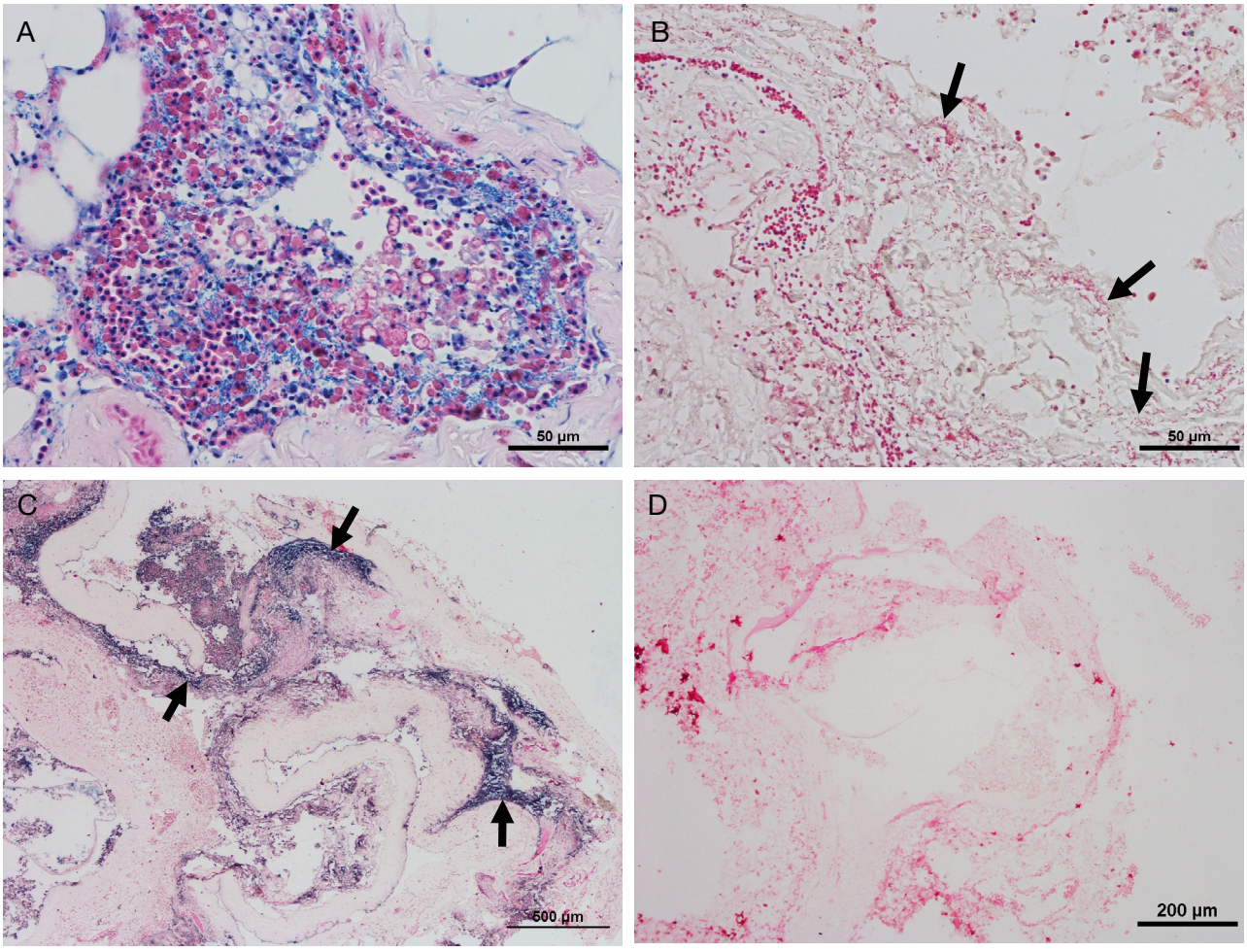

Fig. 3. Histopathology of ballan wrasse with systemic bacterial infection of *Photobacterium damselae damselae*. (A) Lesion of the kidney serosa with large numbers of bacteria, inflammatory response, lymphocyte infiltration and necrotic tissue; Giemsa stain. (B) Numerous Gram-negative bacilli bacteria (arrows) observed in intestinal adipose tissue; Gram stain. (C) *In situ* hybridisation (ISH) of intestinal adipose tissue suffering severe necrosis, with strong binding (arrows) of DIG-labelled probe designed against a portion of the *P. damselae damselae* 16s rRNA gene. (D) ISH negative control with no probe binding

used to confirm that 98.9% of the core housekeeping genes associated with *Gammaproteobacteria* were present within the genome (447 complete BUSCO groups out of a total of 452 included within the database). The isolate was not in possession of the highly virulent pPHDD1 plasmid; however, genes for the chromosomally encoded toxins haemolysin ($hlyA_{ch}$) and phospholipase-hemolysin (*plpV*) (Rivas et al. 2011) were identified.

ISH performed on tissue sections from this fish confirmed the presence of the isolated *P. damselae* showing strong hybridisation signal in the necrotic regions of kidney serosa and intestinal adipose tissue (Fig. 3C,D). ISH also confirmed the presence of low numbers of bacteria in the spleen, ovary and heart.

3.5. Virology results and identification of mycoplasma *Acholeplasma laidlawii*

No viral pathogens were isolated by cell culture from any sample, although CPE was observed in the second blind passage of EPC cells from 28 samples (18%). CPE was sporadically observed across both the Cornwall and Dorset populations, with no discernible trend. RT-PCR analysis of these samples yielded no amplification with generic primers for species in the genera *Novirhabdovirus* and *Aquabirnavirus*, while generic primers for *Iridovirus* and *Vesiculovirus* produced multiple bands of varying molecular weight for each sample. Bands of similar molecular weight to expectations were sequenced, and all were identified as a mixture of cyprinid host

Fig. 4. Phylogenetic tree of the concatenated sequences of glycerol uptake facilitator protein (*glpF*), DNA gyrase subunit B (*gyrB*), methionine-tRNA ligase (*metG*), NAD(P) transhydrogenase subunit alpha (*pntA*), dihydroorotase (*pyrC*) and transmembrane regulatory protein (*toxR*) genes of *Photobacterium damselae* subspecies. Red arrow highlights the ballan wrasse *P. damselae damselae* isolate. Associated metadata can be found in Table S2 in Supplement 1

DNA and *Acholeplasma laidlawii*, a bacterial mycoplasma. RT-PCR confirmed that VHSV was not present in the sampled population subset.

Original sample homogenates were filtered at 0.2 µm to remove mycoplasma and reinoculated on EPC cells. No CPE was observed after blind passage for any sample.

3.6. *In vitro* culture of gill ciliates

Fresh gill imprints taken and examined during sampling did not reveal any amoebas in the population, and isolation of amoebas was also unsuccessful. A qPCR assay was not able to detect the presence of *N. perurans* in gill tissue from a random selection of half of the population samples (n = 45). During attempted amoeba isolations, large numbers of fast-moving ciliates with matching characteristics and an ellipsoid morphology of approximately 30 × 14 µm, were observed in 13 fish from the Cornwall population (42%). PCR amplification and sequencing of the 18S SSU rRNA from 2 samples yielded a 681 bp consensus sequence with 100% sequence identity, according to BLAST, with *Pseudocohnilembus persalinus*. Histology gill sections of affected fish did not reveal the presence of any ciliates with matching morphology, and no significant pathological changes to the gills were noted.

4. DISCUSSION

The wild capture of ballan wrasse from the UK is an emerging fishery fuelled by the requirement for cleaner fish, with total reported landings of wrasse estimated to have increased by 368% over the last 6 yr (Riley et al. 2017). However, data on wrasse capture is minimal, and many landings go unreported or under generic 'demersal fish' labels. The health status of these wild populations is relatively

unknown, and as such the transfer of possible salmonid and labrid pathogens and parasites is currently unchecked.

We identified multiple parasites, including nematodes and cestodes in the digestive tract, nematodes and digenean metacercariae in the liver and ciliates and copepods in the gill. None of these parasites appeared to pose a significant health threat, since only minimal pathological changes were evident. However, heavy infections of ectoparasites under culture conditions might compromise osmoregulation (Feist & Longshaw 2008).

The sea louse *Caligus centrodonti* was visually identified on the surface of 20% of the sampled ballan wrasse. This is a known wrasse specialist, and transfer to salmon does not occur (Bron & Treasurer 1992). However, as a parasite of all 5 wrasse species that occupy the British Isles (Treasurer 1997), the continued unintended introduction of *C. centrodonti* to Scottish waters may increase transfer to wild wrasse populations. There is also a potential risk of transferring *C. centrodonti* from wild-caught wrasse broodstock to hatchery-reared juveniles. Close monitoring and treating incoming broodstock is advised (Powellet et al. 2018).

The ciliated *Cryptocaryon irritans* is the causative agent of marine whitespot for a range of marine fish species, invading the skin, gills and eyes, resulting in epithelial hyperplasia and respiratory difficulties (Colorni & Burgess 1997). None of the reported gross clinical signs associated with marine white spot were noted in the fish in this study, which presented low numbers of *Cryptocaryon*-like ciliated parasites.

Generally, *Cryptocaryon* does not cause heavy infections in the wild due to low host density but is recognised as a significant problem in aquaria, where infection spreads quickly (Colorni & Burgess 1997). Therefore, in an intensive hatchery rearing environment, more susceptible juveniles could be at a high risk of infection where theronts can quickly spread to new hosts.

Eimeria sp. coccidians are considered ubiquitous parasites of fish gut epithelium (Molnár et al. 2012). Histopathological screening revealed focal infections of intestinal epithelial cells with coccidian parasites in 20% of sampled fish. Coccidian parasites have previously been reported in the family Labridae, but as far as we are aware not in ballan wrasse. *E. banyulensis* was isolated from goldsinny wrasse *Ctenolabrus rupestris* on the French Mediterranean coast (Daoudi 1987), and a heavy unidentified coccidian infection of the intestinal epithelium was identified in a single cuckoo wrasse *Labrus mixtus*, originating from the Scottish west coast (Gibson 1995). Little tissue disruption was observed in this cuckoo wrasse, with only minor changes to the immediate surrounding area of cysts. This mirrors the current findings in ballan wrasse, with no significant pathological changes noted. However, intestinal tissue sections from all sampled fish had high numbers of eosinophils, which are associated with responses to parasitic infections (Alvarez-Pellitero 2008). Phylogenetic analysis of the 18S SSU rRNA consensus sequence reveals that this coccidian sits firmly within the *Eimeria* genus, although it is apparent that this sequence remains distinct from previously reported *Eimeria* species. Due to divergence from known coccidian sequences, lack of previous reports in ballan wrasse and the known host-specific nature of coccidia, we consider this coccidian to be a likely new species of the genus *Eimeria*. ISH confirmed that the identity of observed coccidia matched the obtained 18S sequence in fish from both the Dorset and Cornwall populations. These protists show a high degree of host specificity and have a monoxenous life cycle (Wiedmer et al. 2017); therefore, the risk of infecting cocultured salmon is very low. However, it is unknown how the infection will affect the wrasse population under farming conditions.

Although *Photobacterium damselae damselae* is considered autochthonous and a normal constituent of aquatic ecosystems, some strains are recognised as opportunistic pathogens in aquaculture, causing haemorrhagic septicaemia and skin ulcers in a wide range of marine fish species at warm water temperatures (Pedersen et al. 2009, Labella et al. 2011, Rivas et al. 2013). Zoonotic potential through wound exposure in both healthy and immunocompromised patients has been occasionally reported for some strains (Rivas et al. 2013).

In the present study, *P. damselae damselae* was isolated from a single fish in one of the most southern waters of the UK during the warmest average water temperatures of the year, approximately 17°C (Cefas 2012). To our knowledge, this is the first report of *P. damselae damselae* isolation in ballan wrasse. The MLST scheme (Fig. 4) placed the ballan wrasse isolate in the subcluster I.a, which includes *P. damselae damselae* isolates from other fish species, including rainbow trout *Oncorhynchus mykiss* and Japanese pilchard *Sardinops melanostictus*. The phylogeny analysis showed that this strain constitutes a novel type of *P. damselae damselae*. Moreover, bioinformatic analysis of this strain revealed that it does not possess the highly virulent pPHDD1 plasmid, or the plasmid-coded phobalysin P (PhylP) and damselysin

(Dly) toxins (Rivas et al. 2011, 2013). The alternative chromosome-encoded toxins, phobalysin C (PhylC) and novel phospholipase of Vibronaceae (PlpV), have been recently described as virulence factors, responsible for pathogenic potential in non-pPHDD1 harbouring strains, causing haemolysis and virulence in sea bass *Dicentrarchus labrax* (Vences et al. 2017). Both of these chromosomally encoded toxins were present in this strain, as shown by the presence of genes $hlyA_{ch}$ and *plpV*. Further work therefore is needed to determine its prevalence within wild ballan wrasse populations, and potential risks to cultured Atlantic salmon at cooler Scottish temperatures.

The mycoplasma *Acholeplasma laidlawii* was identified in cell culture inoculated with wrasse tissue homogenates. Despite being reported as a bacterial mycoplasma contaminant of cell cultures (Low 1974), the irregular nature of cases, spread over 2 separate population groups that were processed weeks apart, suggests that the mycoplasma likely originated from wrasse. *A. laidlawii* has been reported in multiple fish species, but it is thought to be a non-pathogenic microorganism (Francis-Floyd et al. 1998). Filtration removed all mycoplasma infection, and no CPEs were observed. However, some viruses, such as those in the family *Iridoviridae*, can have a diameter greater than the 220 nm filtration size used (Jancovich et al. 2012), and therefore it is possible that an underlying virus causing original CPE was filtered out from the re-inoculation. In future virology studies of ballan wrasse, it should be noted that mycoplasmas could be present and capable of producing misleading results.

No amoebae were isolated from fish gill samples in sterile sea water, but high numbers of fast-moving ciliates were cultured from 42% of sampled ballan wrasse from Cornwall. Molecular identification showed these to be *Pseudocohnilembus persalinus*, a type of free-living scuticociliate that will feed on microorganisms such as bacteria, although under certain conditions, it may initiate feeding on host organism cells and tissues as an opportunistic pathogen (Xiong et al. 2015). One such species has been reported to cause scuticociliatosis in olive flounder *Paralichthys olivaceus* (Kim et al. 2004). However, a challenge trial in olive flounder resulted in low mortality with no pathological changes (Song et al. 2009). The ciliate has also been isolated from the ovarian fluid of mature and healthy freshwater rainbow trout, in which it exhibited an endosymbiont relationship (Jones et al. 2010). As no associated pathology was observed in the sampled ballan wrasse, we predict an endosymbiont relationship is also present.

Neither of the pathogenic agents *Aeromonas salmonicida* or *Neoparamoebae perurans* were isolated from sampled fish; however, due to the severity of each disease and the possible cross-species transmission risk, qPCR assays for *A. salmonicida* and *N. perurans* were used to show that no low-lying infections were present in a subset of the population tested.

In summary, the wrasse sampled in the present study, despite harbouring a number of parasites, were in a healthy condition, and no listed disease agents were detected. Consequently, there are no immediate concerns for the continued mass translocation of wild-caught ballan wrasse from the southwest of England to Scottish Atlantic salmon farms. However, the dynamic nature of disease occurrence in fish populations and the continued trade in wrasse species require careful monitoring of these species (Peeler & Feist 2011). We did discover a potential threat in the isolation of *P. damselae damselae* as an observed cryptic infection. Further studies are planned to investigate the virulence of this isolated strain to both ballan wrasse and Atlantic salmon. The parasites identified in this population were not associated with any serious pathological changes, but they may pose a threat to juvenile wrasse, in particular *C. centrodonti*, *Cryptocaryon*-like and *Eimeria* spp. It is very difficult to predict how transfer of parasitic fauna will affect community structure, both directly and indirectly (Prenter et al. 2004), so careful monitoring will be needed during the continued mass translocation of wrasse.

Acknowledgements. This work was funded by the Department for Environment, Food and Rural Affairs FB002A and F1172A contracts. We thank the Fish Health Inspectorate for their support and Loch Duart for funding the supply of wild ballan wrasse.

LITERATURE CITED

Alba P, Caprioli A, Cocumelli C, Ianzano A and others (2016) A new multilocus sequence typing scheme and its application for the characterization of *Photobacterium damselae* subsp. *damselae* associated with mortality in cetaceans. Front Microbiol 7:1656

Altschul SF, Gish W, Miller W, Myers EW & Lipman DJ (1990) Basic Local Alignment Search Tool. J Mol Biol 215:403–410

Alvarez-Pellitero P (2008) Fish immunity and parasite infections: from innate immunity to immunoprophylactic prospects. Vet Immunol Immunopathol 126:171–198

Andrews S (2010) FastQC: a quality control tool for high throughput sequence data. www.bioinformatics.babraham.ac.uk/projects/fastqc/

Bankevich A, Nurk S, Antipov D, Gurevich AA and others (2012) SPAdes: a new genome assembly algorithm and

its applications to single-cell sequencing. J Comput Biol 19:455–477
Barta JR, Martin DS, Liberator PA, Dashkevicz M and others (1997) Phylogenetic relationships among eight *Eimeria* species infecting domestic fowl inferred using complete small subunit ribosomal DNA sequences. J Parasitol 83: 262–271
Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30:2114–2120
Bron JE, Treasurer JW (1992) Sea lice (Caligidae) on wrasse (Labridae) from selected British wild and salmon-farm sources. J Mar Biol Assoc UK 72:645–650
Brooker AJ, Papadopoulou A, Gutierrez C, Rey S, Davie A, Migaud H (2018a) Sustainable production and use of cleaner fish for the biological control of sea lice: recent advances and current challenges. Vet Rec 183:383
Brooker AJ, Skern-Mauritzen R, Bron JE (2018b) Production, mortality, and infectivity of planktonic larval sea lice, *Lepeophtheirus salmonis* (Krøyer, 1837): current knowledge and implications for epidemiological modelling. ICES J Mar Sci 75:1214–1234
Cano I, Ferro P, Alonso MC, Sarasquete C, Garcia-Rosado E, Borrego JJ, Castro D (2009) Application of in situ detection techniques to determine the systemic condition of lymphocystis disease virus infection in cultured gilt-head seabream, *Sparus aurata* L. J Fish Dis 32:143–150
Cefas (The Centre for Environmental Fisheries & Aquaculture) (2012) Cefas Station 24: Weymouth. www.cefas.co.uk/cefas-data-hub/sea-temperature-and-salinity-trends/presentation-of-results/station-24-weymouth/
Chen W, Sun HY, Xie MQ, Bai JS, Zhu XQ, Li AX (2008) Development of specific PCR assays for the detection of *Cryptocaryon irritans*. Parasitol Res 103:423–427
Colorni A, Burgess P (1997) *Cryptocaryon irritans* Brown 1951, the cause of 'white spot disease' in marine fish: an update. Aquar Sci Conserv 1:217–238
Connor TR, Loman NJ, Thompson S, Smith A and others (2016) CLIMB (the Cloud Infrastructure for Microbial Bioinformatics): an online resource for the medical microbiology community. Microb Genom 2:e000086
Daoudi F (1987) Coccidies et coccidioses de poissons méditerranéens: systématique, ultrastructure et biologique. PhD thesis, Académie de Montpellier, Université Montpellier II des Sciences et Techniques du Languedoc, Montpellier
Dixon PF, Ngoh GH, Stone DM, Chang SF, Way K, Kueh SLF (2008) Proposal for a fourth aquabirnavirus serogroup. Arch Virol 153:1937–1941
Dopheide A, Lear G, Stott R, Lewis G (2008) Molecular characterization of ciliate diversity in stream biofilms. Appl Environ Microbiol 74:1740–1747
Essam HM, Abdellrazeq GS, Tayel SI, Torky HA, Fadel AH (2016) Pathogenesis of *Photobacterium damselae* subspecies infections in sea bass and sea bream. Microb Pathog 99:41–50
FAO (2018) The state of world fisheries and aquaculture 2018 — meeting the sustainable development goals. FAO, Rome
Faust E, Halvorsen KT, Andersen P, Knutsen H, André C (2018) Cleaner fish escape salmon farms and hybridize with local wrasse populations. R Soc Open Sci 5:171752
Feist SW, Longshaw M (2008) Histopathology of fish parasite infections — importance for populations. J Fish Biol 73:2143–2160
Francis-Floyd R, Reed P, Gibbs P, Shotts E, Bolon B, Coleman W, Klinger R (1998) Isolation of *Acholeplasma laidlawii* from centrarchids in a central Florida lake. J Aquat Anim Health 10:252–258
Fringuelli E, Gordon A, Rodger H, Welsh M, Graham D (2012) Detection of *Neoparamoeba perurans* by duplex quantitative Taqman real-time PCR in formalin-fixed, paraffin-embedded Atlantic salmonid gill tissues. J Fish Dis 35:711–724
Gibson DR (1995) Potential pathogens of wrasse (family: Labridae) from Scottish coastal waters. PhD dissertation, University of Stirling
Gulla S, Duodu S, Nilsen A, Fossen I, Colquhoun DJ (2016a) *Aeromonas salmonicida* infection levels in pre- and post-stocked cleaner fish assessed by culture and an amended qPCR assay. J Fish Dis 39:867–877
Gulla S, Lund V, Kristoffersen AB, Sørum H, Colquhoun DJ (2016b) VapA (A-layer) typing differentiates *Aeromonas salmonicida* subspecies and identifies a number of previously undescribed subtypes. J Fish Dis 39:329–342
Haugland GT, Olsen AB, Rønneseth A, Andersen L (2017) Lumpfish (*Cyclopterus lumpus* L.) develop amoebic gill disease (AGD) after experimental challenge with *Paramoeba perurans* and can transfer amoebae to Atlantic salmon (*Salmo salar* L.). Aquaculture 478:48–55
Helgesen KO, Romstad H, Aaen SM, Horsberg TE (2015) First report of reduced sensitivity towards hydrogen peroxide found in the salmon louse *Lepeophtheirus salmonis* in Norway. Aquacult Rep 1:37–42
Hempleman C (2017) Cleaner fish: Scotland overview. In: International Cleaner Fish Summit 2017. http://scottishaquaculture.com/wp-content/uploads/2017/05/ICFS17_Session1.pdf
Jancovich JK, Chinchar VG, Hyatt A, Miyazaki T, Williams T, Zhang QY (2012) Family Iridoviridae. In: King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ (eds) Virus taxonomy. Classification and nomenclature of viruses. Ninth Report of the International Committee on Taxonomy of Viruses, Elsevier, Amsterdam, p 193–210
Jolley KA, Maiden MCJ (2010) BIGSdb: Scalable analysis of bacterial genome variation at the population level. BMC Bioinformatics 11:595
Jones SRM, Prosperi-Porta G, LaPatra SE (2010) First isolation of *Pseudocohnilembus persalinus* (Ciliophora: Scuticociliatida) from freshwater-reared rainbow trout, *Oncorhynchus mykiss*. J Parasitol 96:1014–1016
Jones PG, Hammell KL, Gettinby G, Revie CW (2013) Detection of emamectin benzoate tolerance emergence in different life stages of sea lice, *Lepeophtheirus salmonis*, on farmed Atlantic salmon, *Salmo salar* L. J Fish Dis 36: 209–220
Karlsbakk E, Olsen AB, Einen ACB, Mo TA and others (2013) Amoebic gill disease due to *Paramoeba perurans* in ballan wrasse (*Labrus bergylta*). Aquaculture 412–413:41–44
Keeling SE, Brosnahan CL, Johnston C, Wallis R, Gudkovs N, McDonald WL (2013) Development and validation of a real-time PCR assay for the detection of *Aeromonas salmonicida*. J Fish Dis 36:495–503
Kim SM, Cho JB, Lee EH, Kwon SR, Kim SK, Nam YK, Kim KH (2004) *Pseudocohnilembus persalinus* (Ciliophora: Scuticociitida) is an additional species causing scuticociliatosis in olive flounder *Paralichthys olivaceus*. Dis Aquat Org 62:239–244
Labella A, Berbel C, Manchado M, Castro D, Borrego JJ

(2011) *Photobacterium damselae* subsp. *damselae*, an emerging pathogen affecting new cultured marine fish species in southern Spain. In: Aral F, Do Z (eds) Recent advances in fish farms. IntechOpen, London, p 136–152

Lovy J, Piesik P, Hershberger PK, Garver KA (2013) Experimental infection studies demonstrating Atlantic salmon as a host and reservoir of viral hemorrhagic septicemia virus type IVa with insights into pathology and host immunity. Vet Microbiol 166:91–101

Low IE (1974) Isolation of *Acholeplasma laidlawii* from commercial, serum-free tissue culture medium and studies on its survival and detection. Appl Microbiol 27: 1046–1052

Marine Harvest (2017) Salmon farming industry handbook. Marine Harvest, Bergen

Molnár K, Ostoros G, Dunams-Morel D, Rosenthal BM (2012) *Eimeria* that infect fish are diverse and are related to, but distinct from, those that infect terrestrial vertebrates. Infect Genet Evol 12:1810–1815

Munro LA, Wallace IS (2017) Scottish Fish Farm Production Survey 2016. Marine Scotland Science, Edinburgh

Munro ES, McIntosh RE, Weir SJ, Noguera PA and others (2015) A mortality event in wrasse species (Labridae) associated with the presence of viral haemorrhagic septicaemia virus. J Fish Dis 38:335–341

OIE (2018) Viral haemorrhagic septicaemia. In: Manual of diagnostic tests for aquatic animals

Pedersen K, Skall HF, Lassen-Nielsen AM, Bjerrum L, Olesen NJ (2009) *Photobacterium damselae* subsp. *damselae*, an emerging pathogen in Danish rainbow trout, *Oncorhynchus mykiss* (Walbaum), mariculture. J Fish Dis 32:465–472

Peeler EJ, Feist SW (2011) Human intervention in freshwater ecosystems drives disease emergence. Freshw Biol 56:705–716

Powell A, Treasurer JW, Pooley CL, Keay AJ, Lloyd R, Imsland AK, Garcia de Leaniz C (2018) Use of lumpfish for sea-lice control in salmon farming: challenges and opportunities. Rev Aquacult 10:683–702

Prenter J, MacNeil C, Dick JTA, Dunn AM (2004) Roles of parasites in animal invasions. Trends Ecol Evol 19:385–390

Price MN, Dehal PS, Arkin AP (2010) FastTree 2 — Approximately maximum-likelihood trees for large alignments. PLOS ONE 5:e9490

Riley A, Jeffery K, Cochrane-Dyet T, White P, Ellis J (2017) Northern European wrasse — summary of commercial use, fisheries and implications for management. https://www.gov.scot/binaries/content/documents/govscot/publications/foi-eir-release/2018/03/foi-18-00461/documents/foi-18-00461-documents-release-2-pdf/foi-18-00461-documents-release-2-pdf/govscot:document/FoI-18-00461%20%20Documents%20for%20release%202.pdf (accessed 1 July 2018)

Rimstad E, Basic D, Gulla S, Hjeltnes B, Mortensen S (2017) Risk assessment of fish health associated with the use of cleaner fish in aquaculture. Opinion of the Panel on Animal Health and Welfare of the Norwegian Scientific Committee for Food and Environment. VKM report 2017: 32. Norwegian Scientific Committee for Food and Environment (VKM), Oslo

Rivas AJ, Balado M, Lemos ML, Osorio CR (2011) The *Photobacterium damselae* subsp. *damselae* hemolysins damselysin and HlyA are encoded within a new virulence plasmid. Infect Immun 79:4617–4627

Rivas AJ, Lemos ML, Osorio CR (2013) *Photobacterium damselae* subsp. *damselae*, a bacterium pathogenic for marine animals and humans. Front Microbiol 4:283

Ruane NM, Rodger HD, McCarthy LJ, Swords D and others (2014) Genetic diversity and associated pathology of rhabdovirus infections in farmed and wild perch *Perca fluviatilis* in Ireland. Dis Aquat Org 112:121–130

Seemann T (2014) Prokka: Rapid prokaryotic genome annotation. Bioinformatics 30:2068–2069

Song JY, Kitamura SI, Oh MJ, Kang HS, Lee JH, Tanaka SJ, Jung SJ (2009) Pathogenicity of *Miamiensis avidus* (syn. *Philasterides dicentrarchi*), *Pseudocohnilembus persalinus*, *Pseudocohnilembus hargisi* and *Uronema marinum* (Ciliophora, Scuticociliatida). Dis Aquat Org 83: 133–143

Suau A, Bonnet R, Sutren M, Godon JJ, Gibson GR, Collins MD, Dore J (1999) Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Appl Environ Microbiol 65:4799–4807

Sun HY, Zhu XQ, Xie MQ, Wu XY, Li AX, Lin RQ, Song HQ (2006) Characterization of *Cryptocaryon irritans* isolates from marine fishes in mainland China by ITS ribosomal DNA sequences. Parasitol Res 99:160–166

Treasurer JW (1997) Parasites of wrasse (Labridae) in inshore waters of west Scotland stocked as cleaner fish of sea lice (Caligidae) on farmed Atlantic salmon. J Fish Biol 50:895–899

Vences A, Rivas AJ, Lemos ML, Husmann M (2017) Chromosome-encoded hemolysin, phospholipase, and collagenase in plasmidless isolates of *Photobacterium damselae* subsp. *damselae* contribute to virulence for fish. Appl Environ Microbiol 83:1–16

Waterhouse RM, Tegenfeldt F, Li J, Zdobnov EM, Kriventseva EV (2013) OrthoDB: a hierarchical catalog of animal, fungal and bacterial orthologs. Nucleic Acids Res 41: D358–D365

Waterhouse RM, Seppey M, Simão FA, Manni M and others (2018) BUSCO applications from quality assessments to gene prediction and phylogenomics. Mol Biol Evol 35: 543–548

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S Ribosomal DNA amplification for phylogenetic study. J Bacteriol 173:697–703

Wick RR, Judd LM, Gorrie CL, Holt KE (2017) Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. PLOS Comput Biol 13:e1005595

Wiedmer S, Erdbeer A, Volke B, Randel S, Kapplusch F, Hanig S, Kurth M (2017) Identification and analysis of *Eimeria nieschulzi* gametocyte genes reveal splicing events of gam genes and conserved motifs in the wall-forming proteins within the genus *Eimeria* (Coccidia, Apicomplexa). Parasite 24:50

Wood DE, Salzberg SL (2014) Kraken: ultrafast metagenomic sequence classification using exact alignments. Genome Biol 15:R46

Xiong J, Wang G, Cheng J, Tian M and others (2015) Genome of the facultative scuticociliatosis pathogen *Pseudocohnilembus persalinus* provides insight into its virulence through horizontal gene transfer. Sci Rep 5: 15470

Yoshinaga T, Dickerson HW (1994) Laboratory propagation of *Cryptocaryon irritans* on a saltwater-adapted *Poecilia* hybrid, the black molly. J Aquat Anim Health 6:197–201

Editorial responsibility: Lori Gustafson, Fort Collins, Colorado, USA

Submitted: April 15, 2019; Accepted: July 1, 2019
Proofs received from author(s): September 16, 2019