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Application of real-time PCR for specific detection of *Lepeophtheirus salmonis* in fluid samples from lumpfish (*Cyclopterus lumpus*) stomachs

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Abstract The Norwegian Food Safety Authority recently stated that stomach flushing is in violation of the Animal Welfare Act. The objective of our research was to develop a genetic method for the routine identification of *Lepeophtheirus salmonis* in stomach fluid samples taken through the mouth of lumpfish. The established method was based on a previously published real-time PCR assay targeting the mitochondrial cytochrome oxidase I (mtCOI) gene in *L. salmonis*. Lumpfish were fed with *L. salmonis*, then captured upon ingestion and subsequently transferred to an incubation tank. The method was used to test 62 fluid samples collected during the first, second and third days post *L. salmonis* consumption. No DNA extraction or purification was used prior to the real-time PCR. The results were compared with visual examination subsequent to dissection. Visual identification was able to identify 51 *L. salmonis* (82%) whereas real-time PCR was able to detect 59 *L. salmonis* (95%) out of the 62 examined samples. The results indicate that real-time PCR assays can be used for specific detection of *L. salmonis* in fluid samples from the stomach of lumpfish. Furthermore, our study demonstrates that the PCR assay offers a reliable non-lethal alternative to dissection or stomach flushing and the subsequent visual identification of *L. salmonis*.

Keywords Lumpfish (*Cyclopterus lumpus*) · Stomach flushing · *Lepeophtheirus salmonis* · Real-time PCR

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Introduction

The salmon louse (*Lepeophtheirus salmonis*) is an ectoparasitic copepod of Atlantic salmon (*Salmo salar* L.) that causes major economic losses in the Atlantic salmon aquaculture industry (Costello 2009). Although the industry still relies on pharmaceutical delousing methods, various alternatives are being developed to control *L. salmonis* infestations. One alternative method is the use of cleaner fish such as lumpfish (Imsland et al. 2014) or wrasse in co-culture with farmed Atlantic salmon (Deady et al. 1995; Treasurer 2002; Skiftesvik et al. 2014).

Dissection or stomach flushing and subsequent visual identification are currently relied on in the routine identification of *L. salmonis* in the stomach of cleaner fish. Although stomach flushing can be an effective method for investigating the consumption of *L. salmonis*, questions have been raised as to the welfare impact of the procedure. Recently, the Norwegian Food Safety Authority stated that the flushing technique is in violation of the Animal Welfare Act and recommended the use of dissection instead (Mattilsynet 2016).

Dissection allows for complete investigation of the stomach and eliminates the risk that some content is not being flushed during the flushing procedure. Although dissection and the subsequent visual identification are more likely to provide complete information compared to stomach flushing, a non-lethal technique may be a valuable tool for the aquaculture industry for several reasons.

According to Imsland et al. (2014), roughly 28% of lumpfish ingest *L. salmonis*. This clearly indicates that lumpfish are an option for biological delousing, but not all lumpfish appear to consume *L. salmonis*. The number of lumpfish that consume *L. salmonis* varies greatly from one fish farming site to another, and even in the individual site, the number varies (unpublished data). Regular monitoring is necessary in order to improve conditions, and being able to identify the number of lumpfish consuming *L. salmonis* without simultaneously reducing the number of effective cleaners is important.

A non-lethal method can also be used in the selection of livestock, ensuring that only lumpfish that consume *L. salmonis* are used for breeding purposes. It is unclear whether the consuming of *L. salmonis* is a hereditary behaviour. However, it is clear that behavioural traits can be hereditary (Dingemanse et al. 2012), so the notion warrants further investigation. Recently, lumpfish from a specific family were found to be more predisposed to exploiting natural food sources and showed a higher incidence of sea lice grazing (Imsland et al. 2016). As such, a non-lethal technique for identification of *L. salmonis* in stomach samples may be instrumental in identifying potential families of lumpfish consuming *L. salmonis*.

McBeath et al. (2006) developed a genetic method for the routine identification of *L. salmonis* in mixed plankton samples. They used a real-time PCR Taqman®-MGB probe-based assay targeting the mitochondrial cytochrome oxidase I (mtCOI) gene, which allowed specific detection of *L. salmonis* from plankton samples.

This study presents a non-lethal alternative to stomach flushing by using stomach fluid samples taken through the mouth of the lumpfish followed by real-time PCR. The method was used in the testing of fluid samples from the stomach of 62 lumpfish consuming *L. salmonis*.

Materials and methods

Lumpfish (below 37 g) housed in a communal tank (0.25 m^3) were fed with adult male *L. salmonis*, then captured upon ingestion and subsequently transferred to an incubation tank (8–

10 °C). Adult male lice were used in order to obtain a standardised size of *L. salmonis*, minimising the variation of stomach content of each fish. Lumpfish were euthanized in a benzocaine solution (300 mg L^{-1}) (Benzoak® Vet, A.C.D. Pharmaceuticals, Leknes, Norway) before the collection of fluid samples and the subsequent dissection. At dissection, the stomach was opened for visual identification of *L. salmonis*. A total of 62 fluid samples were collected: 48, 8 and 6 fluid samples were collected during the first, second and third day, respectively. As negative controls, six additional fluid samples were collected from lumpfish that had not previously been introduced to *L. salmonis*.

The fluid samples (roughly 10 μL) were collected from the stomach (through the mouth) with a pipette (Thermo Scientific Finnpippette F1 2–20 μL) using disposable tips (35 mm) and stored in 96-well plates at −18 °C at the Aquaculture Research Station of the Faroes (Nesvík, Faroe Islands). The frozen samples were transported to the research park iNOVA (Tórshavn, Faroe Islands) and analysed.

The samples were centrifuged at 3800 rpm (Sigma 4-15; Buch & Holm, Denmark) for 5 min at room temperature. Subsequently, the samples were diluted 10 times in Milli-Q water prior to real-time PCR. The real-time PCR detection of the mtCOI gene specific to *L. salmonis* was carried out according to McBeath et al. (2006). However, no DNA extraction or purification kit was used prior to the real-time PCR.

DNA concentration and 260 nm/280 nm ratio were measured for eight random samples using a BioPhotometer plus (Eppendorf, Hørsholm, Denmark). The fragment was amplified using a QuantiTect PCR kit (Qiagen, Copenhagen, Denmark) and the previously published primers and probe. The primer sequences used were the following: forward: 5′-GACA TAGCTTTCCCCCGCTTA-3′ and reverse: 5′-AGTTCCTGCACCACTTTCTACTAATG-3′. The probe was a TaqMan probe with a 5′ 6FAM dye and a 3′ MGBNFQ quencher (Life Technology, Nærum, Denmark). The 6FAM fluorescent probe sequence that binds to the amplicon was 5′-ACCCTCTTTGAGTTTATTACT-3′. All PCR amplifications were performed in a total volume of 10 μL with 2 μL of the diluted sample and a final concentration of 0.4 μM for the two primers and 0.25 μM for the probe. Only one PCR reaction was performed per individual. The one-step PCR amplifications were conducted using the following conditions: a denaturing step for 15 min at 95 °C, followed by 40 cycles of 94 °C for 15 s and 60 °C for 60 s. Polymerase chain reactions were carried out using a StepOnePlus™ Real-Time PCR System (Life Technology).

Results and discussion

With the exception of the fluid samples collected from six negative control lumpfish and three additional fluid samples collected from the stomach of three lumpfish that had consumed *L. salmonis*, all the fluid samples were positive for the mtCOI gene specific to *L. salmonis*.

By using visual identification, we were able to identify 51 *L. salmonis* (82%), whereas by using real-time PCR, we were able to detect 59 *L. salmonis* (95%) out of 62 examined samples. Overall, the results indicate that the real-time PCR assay can be used for specific detection of *L. salmonis* in fluid samples from the stomach of lumpfish. Furthermore, our study demonstrates that the PCR assay offers a reliable non-lethal alternative to dissection or stomach flushing and the subsequent visual identification of *L. salmonis*.

The average DNA concentration was 30 ± 14 μg/mL (average ± SD; $n = 8$), and the average 260 nm/280 nm ratio was 0.6 ± 0.1 (average ± SD; $n = 8$). The low 260 nm/280 nm

ratio indicates that the samples do not contain pure DNA. This does not really come as a surprise since no DNA purification step was used in the study. However, the quantity and quality of the DNA were sufficient for the real-time PCR analysis used in the study to identify the mtCOI gene specific to *L. salmonis* in the majority of the fluid samples. The Ct values were found to be between 20 and 34 cycles.

L. salmonis were still detectable during the third day of incubation: four *L. salmonis* were visually identifiable whereas the real-time PCR assay detected six out of the six examined. Adult males were used in this study to obtain a consistent size of *L. salmonis*. According to Imsland et al. (2014), adult females are the preferred choice for lumpfish, but they do also consume adult males and pre-adult *L. salmonis*. Further investigations are needed in order to estimate how size, time, temperature as well as sex of *L. salmonis* affect digestion in lumpfish.

In summary, this method was based on a simple pre-treatment without DNA purification followed by real-time PCR and provided a straightforward and reliable *L. salmonis* identification in the majority of the fluid samples. This simple protocol could be useful for selecting lumpfish as cleaner fish for breeding purposes and thereby possibly lowering the number of fish that exhibit no cleaning behaviour within the farmed fish population. It is also a much faster and more efficient sampling method than the frequently time-consuming stomach flushing and dissection for visual inspection. Furthermore, this enables a larger sample size at farming sites and thus improves the knowledge of the conditions. We are of the opinion that sampling of fluid from the stomach and the following real-time PCR may be a suitable non-lethal alternative for detection of *L. salmonis* in the stomach of lumpfish, wrasse as well as in other species of cleaner fish.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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