PROJECT REPORT: Validation of a PCR assay as a substitute to gastric lavage and visual inspection of stomach contents when assessing lumpfish delousing behaviour.

This work was undertaken by Enrique Pino Martinez as part of the final dissertation **"Welfare of lumpfish *Cyclopterus lumpus* deployed in salmon farms**", for completion of the MSc in Sustainable Aquaculture, University of Stirling, 2017. It involved staying in The Faroe Islands during one week for lumpfish sampling.

1. Project overview

Lumpfish is a fish species used in the salmon aquaculture industry as cleaner fish against the ectoparasite sea louse (*Lepeiophtherus salmonis*). Ensuring lumpfish health and welfare deployed in salmon pens seems vital to reduce the high mortality rates reported and guarantee the good performance of the species as cleaner fish. During routine lumpfish welfare assessments, stomach contents are commonly studied in order to characterize diet preferences and delousing efficiency. This requires either the use of gastric lavage, a technique recently banned by the Norwegian Food Safety Authority as in violation of the Animal Welfare Act (Eysturskarð *et al*. 2016), or a visual inspection of stomach contents after sacrifice. However, researchers and industry members acknowledge the benefits of a method that allowed the assessment of delousing behaviour without euthanizing the lumpfish or compromising the animal welfare. In this regard, a non-lethal methodology has been recently published by scientists in Faroe Islands to allow the identification of sea lice in stomach fluid samples taken through the mouth of anaesthetized lumpfish (Eysturskarð *et al*. 2016). These samples are analyzed using a real-time PCR assay that targets the mitochondrial cytochrome oxidase I (mtCOI) gene in *L. salmonis*. According to the authors, this PCR assay offers a reliable non-lethal alternative to dissection or gastric lavage and can be used to study sea lice ingestion. **Validation of this technique and assessment of the primers specificity for *L. salmonis* against other sea lice species (*Caligus elongatus*) were thus included as a main aim of the Masters project dissertation**.

2. Aims of this study

- Investigate whether the PCR assay described by Eysturskarð *et al*. (2016) could replace the visual assessment of stomach contents, as a non-lethal alternative for the routine study of sea lice *L. salmonis* ingestion by lumpfish.

- Assess the specificity of different sets of primers for *L. salmonis* against other species of sea louse (*C. elongatus*).

3. Materials and methods

Lumpfish sampling took place in the Faroe Islands between 22nd and 29th of June 2017. Stomach fluid samples from 36 individuals were collected with Vygon® PVC paediatric graduated feeding tubes, 1.2 mm internal diameter and 40 cm length, connected to 1ml syringes. The tubes were carefully introduced by hand in the lumpfish stomach through the mouth, until the end of the stomach was reached. The depth up to which tubes were introduced varied with the size of the lumpfish. Volume samples of stomach fluid ranged between 50 and 500 µL, depending on the amount of fluids contained in the fish guts, and were transferred into 1.8 ml Eppendorf® polypropylene tubes. These were labelled and placed in dry ice, prior to their storage in a -80ºC freezer for posterior analysis.

The **primers** used included those utilized by Eysturskarð *et al*. (2016) and designed by McBeath *et al*. (2006), plus three more primers designed specifically for this study. Primers were designed carrying out an alignment between the mtCOI gene FASTA sequences of *L. salmonis*, *C. elongatus*, and *Tigriopus japonicus* (a copepod closely related to *L. salmonis* as shown in Tjensvoll *et al*. 2005). One forward and two reverse primers were then selected based upon the greatest differences observed in the 3' ends among the three mtCOI sequences. Additionally, primers were selected in order to obtain short amplicons so that quantification with qPCR could be carried out if required. Primers used are shown in table 1.

Table 1. Primer combinations used, base sequences, size of the amplicon (bp) and optimal annealing temperature estimated (ºC).

Primer set	Forward 5´ - 3´	Reverse 5´ - 3´	Amplicon size (bp)	Ann. T(ºC)
1	AGCTGTCTCAGCCGGGAGCA	TAATGCCCTTATAAGCAATAAACTC	233	60
2	AGCTGTCTCAGCCGGGAGCA	ATGAAGGCATGAGCAGTTACAATA	73	67
3	GACATAGCTTTCCCCCGCTTA	AGTTCCTGCACCACTTTCTACTAATG	102	60

Eight **PCR trials** in total were performed using the previous sets of primers, two with primer set 1, three with primer set 2, three with primer set 3. 42 PCR cycles were run, after which the amplicon was separated by gel electrophoresis at 2.5% agarose and observed under the UV light. 10 stomach samples were analyzed, of which only one had been confirmed to contain *L. salmonis* DNA after a visual inspection of stomach contents. Four controls were included in

every trial: + *L. Salmonis* DNA, + *C. elongatus* DNA, - *Cyclopterus lumpus* DNA, - No DNA**.** All the DNA was extracted with DNeasy® DNA Tissue kit (Qiagen).

4. Results and conclusions

A summary of results is displayed in table 2

Table 2. Summary of positive results for *L. salmonis* in the eight PCR assays run with 3 different sets of primers. Percentage of detection of sea lice for every sample and expected detection is shown at the end.

	PCR ASSAYS									
SAMPLE	**1 (a)**	**1(b)**	**2(a)**	**2(b)**	**2(c)**	**3(a)**	**3(b)**	**3(c)**	**% detection**	**% expected**
01	-	-	-	-	-	-	-	-	0.00%	0.00%
03	-	-	-	-	-	-	-	-	0.00%	0.00%
06	-	-	-	Y	Y	-	-	Y	37.50%	0.00%
41	-	-	Y	Y	Y	-	-	Y	50.00%	0.00%
44	-	-	-	Y	Y	Y	Y	Y	62.50%	0.00%
49	-	-	Y	Y	Y	-	-	Y	50.00%	0.00%
93	-	-	-	Y	Y	-	-	Y	37.50%	0.00%
96	-	-	Y	-	Y	-	Y	-	37.50%	0.00%
101	Y	Y	Y	Y	Y	Y	Y	Y	100.00%	100.00%
105	-	-	Y	-	Y	-	Y	-	37.50%	0.00%
+ (DNA L.s.)	Y	Y	Y	Y	Y	Y	Y	Y	100.00%	100.00%
(DNA C.e.)	Y	Y	Y	Y	Y	Y	Y	Y	100.00%	0.00%
- (DNA C.l.)	-	-	-	-	Y	Y	Y	Y	50.00%	0.00%
- (No DNA)	-	-	-	Y	-	Y	Y	Y	50.00%	0.00%

PCR trials demonstrated that the **methodology described by Eysturskarð *et al*. (2016) can be used to determine sea lice in stomach fluid samples**, with 100% detection of samples positive for *L. salmonis* (+ control and stomach sample 101). However, several unexpected bands were obtained for some stomach samples collected from lumpfish that did not contain *L. salmonis* in the stomach after visual inspection. Various reasons might explain this: the ingestion of *L. salmonis* or *C. elongatus* several days before but the DNA remained (Eysturskarð *et al*. 2016), ingestion of the parasite´s larvae (not detected in the visual inspection), amplification of DNA from other copepods abundant in the zooplankton (Debes *et al*. 2008), amplification of random sequences after the high number of cycles performed, or contamination of the samples. This recommends the refinement of the technique before its routine use.

The *C. elongatus* control was also detected 100% of times, demonstrating that **none of the primer sets are species-specific for *L. salmonis***. This lack of primer specificity also requires further research before the method can be routinely implemented in the industry.

This technique could already be useful when avoiding sacrificing the fish is strictly necessary, i.e. in case of implementation of a selective breeding program targeting the best sea lice grazers (Eysturskarð *et al*. 2016). However, overall this method is not recommended yet for routine determination of sea lice ingestion by lumpfish until the primers are properly validated and the lack of specificity solved.

5. Costs

A summary of the expenses for the whole project is detailed in table 3.

Table 3. Summary of the costs incurred during the project carried out in the Faroe Islands.

CONCEPT	COST (£)
Accommodation	704
Travel expenses	575.9
Subsistence allowance	104.97
Dry ice	58.3
Transaction fees	27.43
Bench fees	580
TOTAL	**2050.6**

6. References

Debes, H., Eliasen, K., & Gaard, E. (2008). Seasonal variability in copepod ingestion and egg production on the Faroe shelf. *Hydrobiologia*, 600(1), 247-265.

Eysturskarð, J., Johannesen, Á., & Eliasen, K. (2016). Application of real-time PCR for specific detection of *Lepeophtheirus salmonis* in fluid samples from lumpfish (*Cyclopterus lumpus*) stomachs. *Aquaculture International*, 1-5.

McBeath, A. J., Penston, M. J., Snow, M., Cook, P. F., Bricknell, I. R., & Cunningham, C. O. (2006). Development and application of real-time PCR for specific detection of *Lepeophtheirus salmonis* and *Caligus elongatus* larvae in Scottish plankton samples. *Diseases of Aquatic Organisms*, *73*(2), 141-150.

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