Short communication

Consumption of sea lice by lumpfish (*Cyclopterus lumpus*): qPCR quantification and use of a non-destructive sampling method

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Running title: Evaluation of sea lice gracing by qPCR

Keywords: Lice grazing; lumpfish; growth;

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Abstract

In the present study, it was attempted to quantify sea lice grazing in lumpfish by using a recently published qPCR assay. This method utilizes a small aliquot (10 μL) of the stomach fluid of the lumpfish which can be collected using a sterile, 85 mm long pipette tip. We tested if repetitive sampling of stomach fluid influenced growth and survival in the sampled fish. The test lumpfish were sampled for stomach fluid at 6-16 days intervals for 95 days. No mortalities were recorded during the test period and there were no significant differences in growth compared to the control group. Quantification using the qPCR method was tested by two approaches; one with lumpfish that had grazed on sea lice *ad libitum* and the other with lumpfish that had been intubated with sea lice. The sea lice-specific qPCR assay had an inversely related relationship with the number of eaten lice, especially from stomach samples of lumpfish that naturally fed on sea lice as opposed to intubated fish.

1. Introduction

Global production of Atlantic salmon, *Salmo salar* was 2.07 million tons in 2014 worth over 9 billion Euros (Marine Harvest, 2015), but losses due to salmon louse, *Lepeophtheirus salmonis*, are limiting industry growth and compromising its sustainability (Costello, 2009; Abolofia et al., 2017). Cleaner fish are now commonly used as a biological control for sea lice on farmed salmon in Europe and Canada (Imsland et al. 2014a-c; Powell et al., 2018). In Norway, more than 70% of the fish farms in south and mid Norway used cleaner fish in 2016 and 25% of the farms in the north of Norway (Mortensen et al., 2017). In Northern Norway and the Faroe Islands, lumpfish is particularly of value as a cleaner fish since it is native to Arctic waters and thus continues feeding at low water temperatures (Imsland et al., 2014a, c; Eliasen et al., 2018). It is particularly important to assess the stomach contents and, particularly possible lice grazing, of the lumpfish while they are in the salmon cages, for both research and commercial reasons. It is important to validate its effect in the sea pen and also if there is a need for renewing the lumpfish population in the sea pen as lice graze efficiency of lumpfish is size dependent (Imsland et al., 2016a). In commercial farms, this assessment is predominantly done by sacrificing a number of fish and dissecting their stomach and guts. This is not ideal since the lice-eating lumpfish are identified but at the same time killed and there is a restriction on the sample size that can be collected. In research, gastric lavage has also been used to assess lice-grazing behaviour (Imsland et al., 2014a, c; 2016a; Powell et al., 2018). This is a non-lethal and harmless method where the stomach contents of the lumpfish are flushed out by a stream of water. The limitations of this technique are that people carrying out gastric lavage need to be

trained to ensure that the technique does not lead to excessive stress or death of the fish. In addition, it is time and labour intensive and only about, 100-200 fish can be processed per day. Further, the Norwegian Food Safety Authority recently stated that the flushing technique is in violation of the Norwegian Animal Welfare Act and recommended the use of dissection instead (Mattilsynet, 2016).

A new method of sea lice-grazing assessment has recently been published where a small aliquot (10 µL) of stomach fluid was collected and analysed using a sea lice-specific qPCR (quantitative polymerase chain reaction) assay (Eysturskarð et al., 2016). In the study, the qPCR method was found to have a 95% success rate that is similar to gastric lavage. Unfortunately, while the study claimed that the fish were not harmed during stomach fluid collection, they collected all their material while the fish were euthanized. In addition, only adult male lice were used in the experiment, which is not representative of the lice that are grazed in a salmon cage. Furthermore, all lumpfish were given just one louse each. Thus, they could not test whether one can extract quantitative data from the qPCR results.

In this study, the aim is to fill in these gaps of knowledge and refine this assay as a tool to be used in both research and commercial settings. The study was split into two parts. The first part aimed to study the effect of stomach fluid collection on the growth and survival of the lumpfish, in order to confirm that the technique is not detrimental to the research fish. The second part aimed to quantify the relationship between the number of sea lice ingested to the Ct value (cycle threshold) at different time points so that the qPCR assay will not only be able to confirm the consumption of sea lice but also give an indication on how many sea lice were consumed. .

1 **2. Materials and methods**

2

3 *2.1. Experimental fish and rearing conditions*

4

5 The lumpfish were produced from fertilised eggs from Senja Akvakultursenter AS,
6 Senja, Troms County, Norway. The eggs were incubated at 9–10°C and the juveniles were
7 initially fed with Gemma Micro (150–500 µm, Skretting, Norway). After 30 days, the
8 juveniles were fed with 500–800 µm dry feed pellets (Gemma Wean Diamond, Skretting,
9 Norway). The fish were vaccinated with ALPHA JECT Marin micro 5 (Pharmaq AS, Oslo,
10 Norway) on 14 September 2017. The health status of the fish was assessed immediately
11 prior to transfer to Gifas, Inndyr, Nordland, Norway in 25 September 2017. Health status
12 was assessed by qPCR screening for known pathogens in lumpfish and salmonids,
13 including *Aeromonas salmonicida*, *Pasteurella spp.*, *Moritella viscosa*, salmnoid
14 alphavirus (SAV), IPN-virus, VHS-virus, Nodavirus and *Paramoeba perurans*. From 25
15 September 25 to 5 October 2017, the juveniles were fed a high protein low fat marine feed
16 (Biomar grower) using Van Gerven 7 L^{-1} feeding automats (The Netherlands). A 50%
17 mixture of 1.5 mm and 2 mm pellets was used during this period. A total of 105 juvenile
18 lumpfish were used in the present trial i.e. N = 60 in Part I (see below) and N = 45 in Part
19 II (see below).

20 The following experiment was approved by the local responsible laboratory animal
21 science specialist under the surveillance of the Norwegian Animal Research Authority
22 (NARA) and registered by the Authority.

23

2.2. Experimental set-up

2.2.1 Part I – Growth, survival and stomach fluid collection

One-week prior to the start of the trial on the 6th of October two duplicate groups of lumpfish with an initial mean (±SD) weight of 35.5 g ± 3.5 (N = 60) were established from the original population and all fish were tagged intraperitoneally with a Trovan® Passive Integrated Transponder (PIT, Melton, United Kingdom) to monitor their growth. After tagging, the weight and length of each lumpfish was recorded along with their individual PIT tag ID and the fish were transferred to four 1.5 m^3 tanks (15 fish in each tank). The fish were allowed to acclimate for a period of one week prior to the start of the trial during which, all tanks were fed Biomar grower 2.0 mm using 12-hour belt feeders (Zeigler Feed, Pennsylvania, USA), at an approximate feeding rate of 2% BW day^{-1}. Lumpfish in two replicate tanks were sampled for stomach fluid during the trial period, with lumpfish in the two other tanks acting as (non-sampled) control group.

All lumpfish from each tank were individually weighed and fork-length recorded along with their individual PIT-tag ID at 6-16 days intervals during the trial period. Specific growth rate (SGR) of individual lumpfish was calculated according to the formula of Houde and Schekter (1981):

$SGR = (e^g - 1) \times 100$

where $g = (\ln(W_2) - \ln(W_1)) / (t_2 - t_1)$ and W_2 and W_1 are weights on days t_2 and t_1, respectively.

At the same dates as fish were weighed, lumpfish in two replicate tanks were collected and 10-40 μL of stomach fluid was sampled using a long 0.2 mL pipette tip (UltraFine™,

FlexTop™, 84 mm long – VWR, Norway). These samples were either stored at -20°C and later used as negative controls in the qPCR assays, or discarded, depending on necessity. The stomach fluid collection procedure was repeated on the same set of fish at 6 to 16 days intervals for 95 days.

All four tanks were supplied with full salinity sea water pumped from 70 m depth at a temperature of between 10.1 and 12.2°C during the trial period and oxygen saturation was maintained above 80% during the whole experimental period. Water temperature and oxygen concentration was recorded daily in each tank using a Handy Polaris 2 probe (OxyGuard International A/S). The study period was from the 13th of October 2017 to the 16th of January 2018 (95 days).

2.2.2 Part II – Optimisation of the PCR method

This part of study contained two approaches; one with lumpfish that had grazed on sea lice *ad libitum* and the other with lumpfish that had been intubated with sea lice.

In the first approach 30 juvenile lumpfish from the original population (see 2.1 above) were placed in a large tank with flow-through water. These fish were starved for 1-2 days in order to prime their appetite. Ice-cubes prepared by freezing adult sea lice (*Lepeophtheirus salmonis* only) in freshwater, containing about 100 lice in each, were then presented to the lumpfish. The fish were observed until the ice-cube melted and the uneaten lice sank to the bottom of the tank. Lumpfish that were observed eating sea lice were caught with a net and transferred to a smaller tank (1 m^3). Six hours after the fish were collected the lice-eating fish were taken out of the tank and killed with an overdose of Benzoak

1 (Benzocain 200 mg ml^{-1}) and stomach fluid was collected with a pipette as described above.
2 The fluid was stored in a sterile 1.5 mL Eppendorf tube and immediately frozen at -20°C.
3 Samples were sent for PCR analysis at BioVivo Technologies AS, Bodø, Norway.
4 In the second approach, the assay was tested with 15 juvenile lumpfish that were
5 intubated with (force-fed) lice. First, 5 lumpfish were collected and lightly sedated
6 (Benzocain 50 mg ml^{-1}). Each of them was intubated with 3 adult sea lice (*Lepeophtheirus*
7 *salmoni*s only). This was done by picking up one sea louse at a time with blunt forceps and
8 transferring into the innermost part of the lumpfish's mouth. The louse was then ingested
9 by a gag reflex which happens spontaneously when a foreign object is placed so deep into
10 the mouth. This process was repeated until the fish ingested 3 lice, each time making sure
11 that the lice were properly ingested by visually checking the buccal cavity. This process
12 was repeated with two more sets of 5 fish each and one set was fed 6 lice per fish and the
13 other 9 lice per fish. Six hours after intubation, the fish were sampled and killed with an
14 overdose of Benzoak (Benzocain 200 mg ml^{-1}) and stomach fluid was collected using a
15 long pipette (as described above) and stored at -20°C. Afterwards, the stomach and
16 intestines of each fish were dissected. The number of lice found in the stomach/intestines
17 was recorded.
18
19 *2.2.3 Part III – Stomach fluid analysis by PCR*
20
21 Real-time PCR assays were carried out as described in McBeath et al. (2006) and
22 Eysturskarð et al. (2016). DNA fragment amplification was achieved using qScript XLT
23 cDNA SuperMix (Quantabio, Massachusetts, USA) and the published primers and probes.

1 All qPCR reactions were accomplished in a total volume of 12 µL, including 2 µL diluted
2 sample and a final concentration of 900 nM for the primers and 200 nM for the probe. All
3 reactions were run in duplicate or triplicate wells. Negative (no template) controls were
4 included in each plate. Samples from Part I were used as biological negative controls, i.e.
5 stomach fluid from fish that were never exposed to lice. The temperature program was set
6 up as follows: a denaturing step for 15 min at 95°C, followed by 40 cycles of 94°C for 15
7 s and 60°C for 60 s. The LightCycler® 96 Instrument (Roche Diagnostics Norway AS,
8 Oslo, Norway) was used to run the quantitative polymerase chain reactions.
9
10 *2.3. Statistics*
11 All statistical analyses were conducted using Statistica™ 13.3 software. Kolmogorov-
12 Smirnov test (Zar, 1984) was used to assess for normality of distributions. The
13 homogeneity of variances was tested using the Levene's F test (Zar, 1984). Possible
14 differences in growth performance were tested with two-way analysis of variance
15 (ANOVA) where replicates are nested within the experimental groups (i.e. sampled vs.
16 non-sampled fish). Significant differences revealed in ANOVA were followed by Student-
17 Newman-Keuls (SNK) post hoc test to determine differences among experimental groups.
18 Possible relationship between number of lice eaten and Ct values was tested with a linear
19 regression. A significance level (α) of 0.05 was used if not stated otherwise.
20

3. Results

3.1. Growth and survival

No fish died during the trial period. No differences in mean weight between the control group and stomach fluid sampled group were seen at any time point during the trial period (SNK post hoc test, $P > 0.25$, Fig. 1). Initial mean growth rate was 3.8 % day^{-1} in both groups and declined with increasing size in both groups and final SGR was 1.9 % day^{-1} in both groups. SGR did not differ between the two groups during the trial period (SNK post hoc test, $P > 0.45$).

3.2 qPCR – ad libitum feeding

Thirteen fish (size range 42-107 g) were observed eating sea lice and successfully caught and transferred to a smaller tank. The lice eating fish were sampled 6 hours post-consumption of sea lice. On dissecting the fish, no sea lice were found in 25% (4 lumpfish) of the sampled population. This could be due to either a mistake while catching the fish in the feeding tank, regurgitation of the lice after catching the fish or digestion of the lice beyond the stomach.

At the 6 h sampling point, the cycle numbers (Ct values) were positively correlated with the number of ingested sea lice (linear regression, $\beta = -0.81$, $R^2 = 0.65$, $P < 0.01$, Fig. 2A, N=9), where the higher the number of ingested lice was, the lower was the Ct value.

3.3 qPCR – intubation

The fish used in this part of the study ranged between 67 and 175.5 g in weight. Out of the 15 fish intubated with lice, 8 fish gave positive Ct values. A few lice were observed in the tanks, indicating that the lumpfish might have regurgitated the intubated sea lice. The results showed that the number of sea lice intubated was negatively related to the Ct value (linear regression, $\beta = -0.39$, $R^2 = 0.39$, $P < 0.05$, Fig. 2B, N=8).

The samples taken from lumpfish that were not exposed to sea lice were all negative on the PCR assay – providing a good control that the assay is specific to sea lice DNA.

1 **4. Discussion**

2

3 The present study shows that regular stomach fluid collection using a long pipette tip is
4 safe for the lumpfish and has no negative effect on growth and survival. The first approach
5 used in the present study to assess the PCR assay was to feed lumpfish sea lice by placing
6 an ice cube containing sea lice in the tank and catching lumpfish that are observed
7 consuming lice. The results obtained were promising since the cycle number had an inverse
8 proportional relationship to the number of lice consumed. However, this trend would
9 perhaps have been clearer with higher replication. This was not possible to achieve due to
10 the low proportion of lice grazers amongst the lumpfish. Thus, in the second approach,
11 lumpfish was force-feed (intubated) sea lice. The results showed an inverse relationship
12 between the Ct value and the lice number. However, the intubation data was weakened as
13 it was not possible to measure consistent Ct values in some of the fish due to high variation
14 within the triplicate well values. Variation between triplicate PCR reactions may be due to
15 pipetting error. Additionally, if the concentration of the target DNA is very low, it may also
16 lead to errors in the well replicates since it becomes more likely that the reagents are
17 amplifying the wrong DNA fragment (McInerney et al. 2014).

18 Assessing stomach contents of lumpfish has normally been undertaken by gastric
19 lavage (GL). This non-destructive method has been used in previous sea-cage based studies
20 (e.g. Imsland et al., 2014a, c, 2016a-b) and is an important analytical method to determine
21 feeding behaviour in this species. These studies have shown that there is variation in sea
22 lice grazing among individuals and populations and that this variation may be genetically
23 influenced (Imsland et al., 2016b). There has been concern that using GL may have a

negative effect on welfare of lumpfish. Previous studies undertaken by our research group have shown no negative effects on growth or incidences of mortality or reduced health status for fish that have routinely been assessed with GL (P. Reynolds, GIFAS, unpublished data). This method has been used on other fish species (Stehlik et al., 2015; Braga et al., 2017) with no detrimental effects. However, given that the Norwegian Food Safety Authority recently stated that GL is in violation of the Norwegian Animal Welfare Act and recommended the use of dissection instead (Mattilsynet, 2016), we have been assessing other suitable methods to determine the lice grazing potential for this species as gastric lavage is time consuming and due to concerns to welfare. The qPCR method to detect sea lice DNA in the stomach fluids of lumpfish would provide a quicker and less intrusive method compared to gastric lavage. However, the method is not fully established yet, but based on present data, and further refinement, it could be fully validated and used in the future as a suitable method to assess grazing efficacy for lumpfish and perhaps other cleaner fish species.

It is early days for the complete optimization of this assay but present results have aided in getting closer to this goal. Regarding optimizing an assay which can yield quantitative results regarding lice grazing, present findings indicate that it is best to use fish that have ingested sea lice in a "natural" way as opposed to intubation.

In the present trial starvation was used to prime feeding in lumpfish. Alternatively, sea lice ice-cubes could be placed daily in the tanks up to one week before the actual collection of sea lice-eating lumpfish as recent research has indicated that sea lice eating can be enhanced by feeding juvenile lumpfish live Artemia nauplii together with frozen sea lice (Frogg, 2018). Smaller-sized lumpfish (<50 g) should also be used for the

experiment since they are known to have a stronger preference towards lice grazing than larger individuals (Imsland et al., 2016).

Another aspect of present study that should be improved is the quality of the stomach fluid sample. Variations in volume, consistency and colour of this sample was experienced which could affect the outcome of the qPCR assay. In upcoming trials, an attempt to collect the stomach fluid using flocked swabs (FLOQSwabs, Copan Flock Technologies, Italy) will be done. These swabs are made of a highly absorptive polymer that gets readily saturated when exposed to a liquid. The sample can also be easily eluted out of the swab by a gentle twirl in a buffer solution. Since the swab has a soft tip/applicator, it is unlikely that the stomach fluid will be contaminated with blood. In addition, it should be easier to collect a consistent volume of stomach fluid using a swab.

5. Conclusions

Stomach fluid sampling is safe for lumpfish, even when carried out regularly. The sea lice-specific qPCR assay has an inversely related relationship with the number of eaten lice, especially from stomach samples of lumpfish that "naturally" fed on sea lice as opposed to intubated fish.

Acknowledgements

The authors would like to thank the technical staff at GIFAS aquaculture research station at Inndyr for valuable assistance prior to and during the experimental period. Financial support was given by The Research Council of Norway (LUSINFER 256199).

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19

1 **Figure legends**

2

3 Fig.1**.** Mean weight (g) of lumpfish sampled for stomach fluid at regular intervals for 95

4 days and in unsampled (control) fish. Values represent means ± SD. No statistical

5 differences were found.

6

7 Fig. 2. Detection of sea lice DNA using qPCR in lumpfish stomach fluid fed sea lice *ad*

8 *libitum* (A, N=9) or by intubation (B, N=8). Significant regression lines are shown (linear

9 regression, $P < 0.05$).

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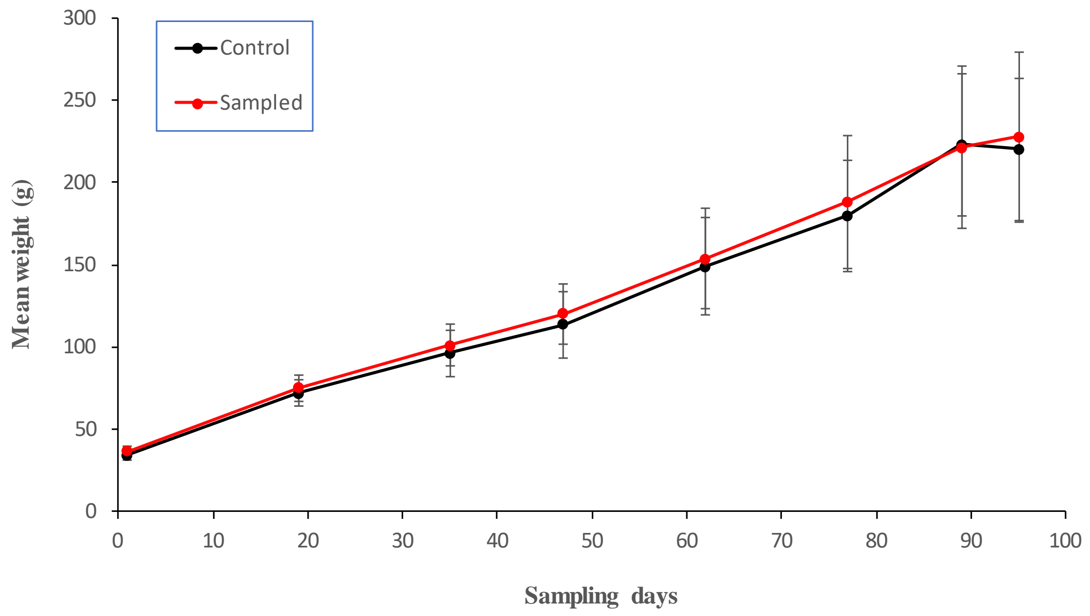

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3 Fig. 1. Imsland, Micallef et al

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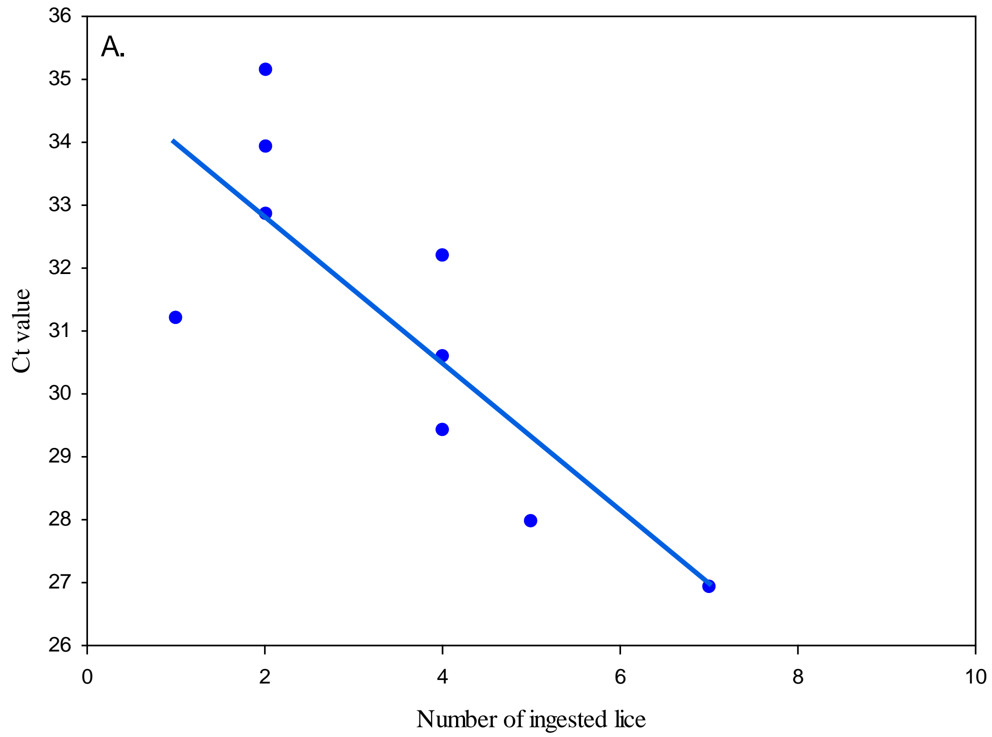

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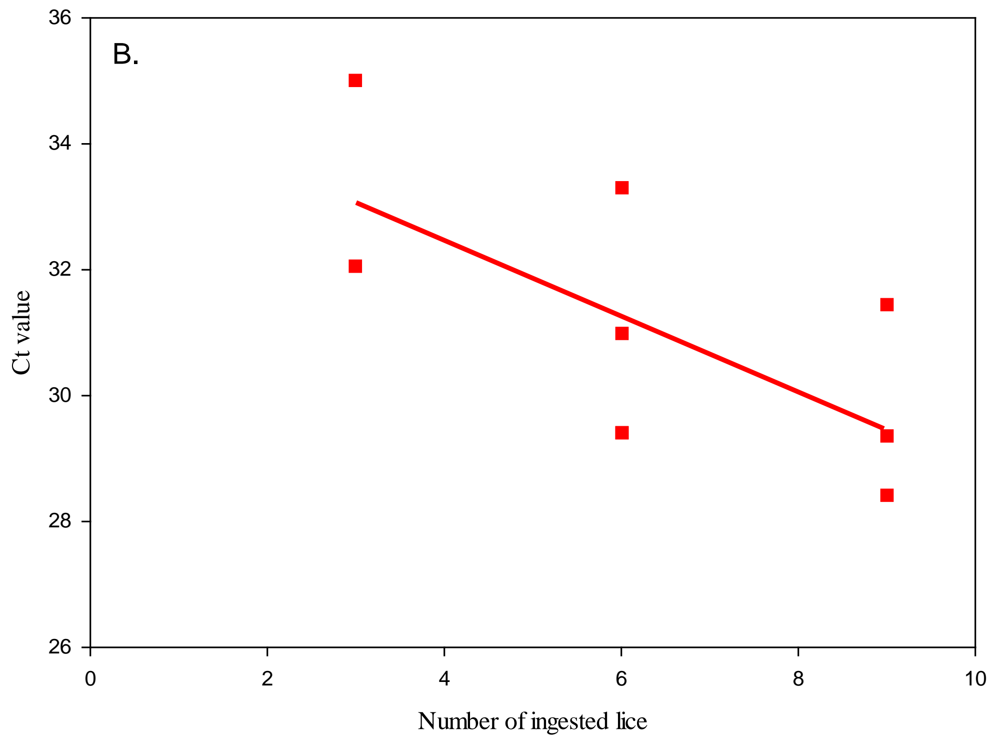

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3 Fig. 2. Imsland, Micallef et al.

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