



## Comparison of vegetable shortening and cocoa butter as vehicles for cortisol manipulation in *Salmo trutta*

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1 **Comparison of vegetable shortening and cocoa butter as vehicles for cortisol manipulation**  
2 **in *Salmo trutta***

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22

23 **Running headline:** cortisol manipulation in fishes

24 **ABSTRACT**

25           Vegetable shortening and cocoa butter have been used as vehicles for cortisol implants in  
26 a wide range of organisms, though no studies have compared the effects of these vehicles on  
27 plasma cortisol and glucose, or change in mass. This study demonstrates that vegetable  
28 shortening and cocoa butter are two effective vehicles for intraperitoneal cortisol implants in  
29 juvenile teleost fish (brown trout, *Salmo trutta*) residing in north temperate freshwater  
30 environments. Each vehicle showed a different pattern of cortisol elevation. Vegetable  
31 shortening was found to be a more suitable vehicle for long-term cortisol elevation (elevated at  
32 days 3, 6 and 9 post-treatment), while cocoa butter may be better suited for short-term cortisol  
33 elevation (only elevated at 3 days post-treatment). Additionally, plasma cortisol levels were  
34 higher with cortisol-vegetable shortening than with cortisol-cocoa butter implants. Plasma  
35 glucose levels were elevated 6 and 9 days post-treatment for fishes injected with cortisol-  
36 vegetable shortening, but did not change relative to controls and shams in cortisol-cocoa butter  
37 fishes. In conclusion, vegetable shortening and cocoa butter are both viable techniques for  
38 cortisol manipulation in fishes in temperate climates, providing researchers with different options  
39 depending on study objectives.

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41 **KEY WORDS:** cocoa butter, cortisol implants, teleost fish, vegetable shortening

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## 47 INTRODUCTION

48 Cortisol is the primary glucocorticoid stress hormone in fish (Mommsen *et al.*, 1989;  
49 Wendelaar Bonga, 1997; Barton, 2002). Not surprisingly, there are hundreds of papers that have  
50 measured cortisol in fishes to understand the consequences of different stressors (reviewed in  
51 Mommsen *et al.*, 1999). Beyond using cortisol as a biomarker of exposure to a stressor,  
52 physiologists started manipulating cortisol in fishes in the 1960s to explore the mechanistic role  
53 of cortisol (e.g., Slusher, 1966). This allowed researchers to move past simply observing  
54 variation in cortisol levels among individuals to performing cause-and-effect studies. However,  
55 despite its potential ecological relevance (Sopinka *et al.*, 2015; Crossin *et al.*, 2016), this  
56 technique has been mainly used in the lab (reviewed in Gamperl *et al.*, 1994). Additionally, the  
57 best vehicle in which to suspend the cortisol for manipulation remains unclear. Past studies have  
58 used saline oil (e.g. coconut oil), cocoa butter, and vegetable shortening to manipulate hormone  
59 levels (Pottinger & Pickering, 1985; Gamperl *et al.*, 1994; Eriksen *et al.*, 2006; Doyon *et al.*,  
60 2006). Studies have also used mini osmotic pumps going back several decades (Theeuwes &  
61 Yum, 1976). However, these are less suitable for field studies owing to expense, as fish may not  
62 be recovered to retrieve the pumps, and their higher invasiveness compared to injections. The  
63 main advantage of cocoa butter and vegetable shortening is that they allow for prolonged,  
64 continuous release of cortisol. They are injected as liquids, and solidify once inside the fish.  
65 However, cocoa butter requires high temperatures to remain in liquid form (approx. 40°C),  
66 potentially resulting in the scalding of organs when injected into a fish, and becomes very hard at  
67 ambient temperatures in the north temperate regions which may lead to damage to the gonads  
68 (personal observation; McConnachie *et al.*, 2012). In contrast, vegetable shortening remains in  
69 liquid form at a lower temperature (approx. 30°C), and remains soft, even in cold water (5°C,

70 personal observation). Gamperl *et al.* (1994) originally suggested that vegetable shortening was  
71 better than cocoa butter at lower temperatures as the hardness of cocoa butter may reduce the  
72 absorption of cortisol.

73 This study is the first comparative study of vegetable shortening and cocoa butter as  
74 vehicles for cortisol manipulation in the wild. Both vehicles are particularly suitable for field  
75 studies (see Sopinka *et al.*, 2015) owing to their low cost and ease of administration. A wild  
76 population of juvenile brown trout *Salmo trutta* L. 1758 was used to compare the temporal  
77 patterns of circulating cortisol and glucose concentrations resulting from implants of cortisol  
78 suspended in vehicles of cocoa butter versus vegetable shortening. Treatment effects were  
79 compared to their corresponding sham (vehicle alone) and control (no implant) groups.

80 Additionally, effects on body mass were measured treatment. It was predicted that vegetable  
81 shortening implants would result in cortisol being released over a longer period of time and in  
82 higher levels, resulting in higher levels of glucose and more mass loss than cocoa butter  
83 implants. It was also predicted that sham treatments would not elevate cortisol or glucose  
84 concentrations or cause a change in mass compared to control fishes.

85

## 86 **MATERIALS AND METHODS**

87 The Villestrup stream is located in north-central Jutland, Denmark. The stream runs for several  
88 kilometers across agricultural land, where a number of tributaries join in before reaching the  
89 Mariager Fjord. The stream is home to a large population of semi-anadromous *S. trutta* (del  
90 Villar-Guerra *et al.*, 2014). Three different sites (1 to 2km apart) within the same stream were  
91 used. It is unlikely that there are genetic differences among populations so close (Hansen *et al.*,  
92 2002), but even if there are, they are unlikely to have any biological significance especially when

93 comparing responses to treatments within a site. Fishes were captured via backpack  
94 electrofishing (ELT 60 II GI; 300 volts; Scubla, Remanzacco, Italy) on three separate days in  
95 2016: 125 fishes at Site 1 on March 3<sup>rd</sup> (25 fishes per group), 125 fishes at Site 2 on March 4<sup>th</sup>  
96 (25 fishes per group) and 150 fishes at Site 3 on March 5<sup>th</sup> (30 fishes per group). During this  
97 period, the temperature of the water in Villestrup was between 6 and 7°C.

98 Captured fishes were held in a 60 l bin filled with oxygenated fresh stream water. Fishes  
99 were anesthetized in a solution of benzocaine (0.03 g l<sup>-1</sup> ethyl-*p*-aminobenzoate; Sigma,  
100 www.sigmaaldrich.com) in stream water, then weighed ( $\pm 0.1$ g), measured for total length  
101 ( $\pm 0.1$ cm), and tagged using a 23mm PIT tag (Passive Integrated Transponder tag, Texas  
102 Instruments, RI-TRP-RRHP, 134Hz, 0.6g mass in air, Plano, Texas, USA). Tags were inserted  
103 through a 5mm incision in the left side of the body, posterior to the pelvic fin. Only trout that  
104 were 12-21cm in length (large enough for the PIT tag, but likely still juveniles; Larsen et al.  
105 2013) were used in this study. Fishes were randomly assigned to one of the following treatment  
106 groups: (1) control, (2) sham-vegetable shortening (sham-veg), (3) cortisol-vegetable shortening  
107 (cort-veg), (4) sham-cocoa butter (sham-cocoa), (5) cortisol-cocoa butter (cort-cocoa). Cortisol-  
108 treated fishes received an intracoelomic injection (1.5inch 18-gauge needle) of a suspension of  
109 vegetable shortening (100% vegetable shortening, Crisco, OH, USA) or cocoa butter (100% pure  
110 cocoa butter, NOW Foods, IL, USA) mixed with hydrocortisone 21-hemisuccinate (Sigma-  
111 Aldrich, St. Louis, MO, USA, Product #H2882-1G), using a dosage of 0.01 ml vehicle (with a  
112 concentration of 0.01g cortisol per ml) per 1 g of fish (equivalent to a cortisol dosage of 100mg  
113 kg<sup>-1</sup>). Sham fishes were injected with only 0.01 ml g<sup>-1</sup> fish vegetable shortening or cocoa butter.  
114 The vegetable shortening and cocoa butter were heated using hot water to a temperature of 37°C  
115 and 40 °C, respectively. All fishes were recovered (i.e., until full equilibrium was reached) in a

116 60 l tank of benzocaine-free fresh stream water following tagging. Cortisol-treated fishes were  
117 recovered separately from sham and control fishes to prevent any cross-treatment contamination  
118 of cortisol, and all fishes were then released at the site of capture. The tagging, weighing,  
119 measuring and injecting process took less than one minute per fish. Overall, fishes were held in  
120 tanks for approximately 60 minutes.

121 Fishes were recaptured via backpack electrofishing after 3, 6 and 9 days post-treatment,  
122 at Site 3, Site 2 and Site 1, respectively. Immediately after shocking, we collected a blood sample  
123 (<0.3 ml) from the caudal vasculature using a heparinized 1.5-inch 25-gauge needle and a 1 ml  
124 syringe. All samples were collected within 3 minutes of capture. Fishes were then weighed.  
125 Following recovery, fishes were returned to the river, and not recaptured. Blood samples were  
126 held in a water-ice slurry until centrifuged at 2000 g for 2 minutes to separate plasma from red  
127 blood cells. Plasma samples were kept at -80°C until analyzed. Environmental conditions should  
128 not be a confounding factor here, as the 3 day sampling was within the 6 day sampling, and both  
129 were within the 9 day sampling period. Hence, all fishes were exposed to the same conditions,  
130 with day 9 fishes potentially experiencing a greater variation. However, this does not affect  
131 treatment effects within a single time point, which is the focus of this study.

132 Plasma cortisol concentration was determined using a commercial radioimmunoassay kit  
133 (ImmunoChem Cortisol <sup>125</sup>I RIA kit; MP Biomedicals, www.mpbio.com). This assay was  
134 previously validated for use with teleost fish plasma samples (Gamperl *et al.*, 1994). All plasma  
135 samples were measured in a single assay. Intra-assay variability (%CV) was 7.9%. Plasma  
136 glucose levels were determined using an AccuCheck Compact Plus meter system (Roche, Basel,  
137 Switzerland), a point-of-care device previously validated for use in teleost fishes (Stoot *et al.*,  
138 2014).

139 Statistical analyses were conducted using JMP v12.0.1 (SAS Institute Inc.,  
140 Buckinghamshire, UK). Cortisol and glucose values were log-transformed to achieve normality  
141 of residuals. Two-way ANOVAs were used to evaluate differences in cortisol, glucose and  
142 change in mass among treatment groups over the three sampling times. A Tukey-Kramer *post-*  
143 *hoc* test was used to determine which groups differed, which is conservative with unequal  
144 sample sizes as is the case here. Spearman correlations (to reduce the effect of outliers) were  
145 used to determine whether cortisol levels were related to glucose levels among individuals using  
146 within each category of treatment and day.

147

## 148 **RESULTS**

149 Between 9 and 17 fishes were recaptured per treatment group. Fishes treated with cortisol  
150 suspended in vegetable shortening showed significantly higher plasma cortisol concentrations  
151 after 3, 6 and 9 days post-treatment than both sham and the control treatments, with values at day  
152 3 significantly higher than at day 9 (Fig. 1A; treatment  $\times$  time,  $F_{8,172} = 3.07$ ,  $P = 0.0029$ ). Cort-  
153 cocoa fishes at day 3 had significantly higher cortisol levels than both sham and the control  
154 treatments, but values for fishes sampled at days 6 and 9 did not differ from those for sham or  
155 control fishes. At day 3, cort-veg fishes exhibited significantly higher plasma cortisol levels than  
156 cort-cocoa fishes. Cortisol concentrations for fishes in the sham treatment were similar to fishes  
157 in the control group across all time points. Glucose concentrations in cort-veg fishes were  
158 significantly higher than those for sham and control treatments at days 6 and 9 (Fig. 1B;  
159 treatment  $\times$  time,  $F_{8,170} = 2.30$ ,  $P = 0.023$ ), whereas plasma glucose concentrations in cort-cocoa  
160 fishes did not differ from the sham or control groups on any day. On days 6 and 9, cort-veg  
161 fishes had significantly higher glucose concentrations than cort-cocoa fishes.



162 Initially, mass for cortisol-treated fishes did not differ from their sham or the control  
163 group (all  $P > 0.50$ ). Sham-veg fishes sampled on day 9 gained mass while all other groups lost  
164 mass (Fig 1C, treatment  $\times$  time,  $F_{8,170} = 2.94$ ,  $P = 0.0042$ ).

165 Plasma cortisol and glucose concentrations were positively related in day 9 cort-veg  
166 treatment ( $R^2 = 0.60$ ,  $n=15$ ,  $P = 0.037$ ) No other correlation was significant (all  $P > 0.093$ ).

167

## 168 **DISCUSSION**

169 Cortisol implants ( $100\text{mg kg}^{-1}$ ) generated a significant elevation in plasma cortisol  
170 concentration using either vegetable shortening or cocoa butter as a vehicle. However, the use of  
171 vegetable shortening as a vehicle caused a greater elevation of cortisol concentration than cocoa  
172 butter after 3 days, and this elevation lasted longer. Moreover, plasma cortisol concentration  
173 likely remained high for more than 9 days in fishes that received cortisol-vegetable shortening  
174 implants, as found by Pickering & Duston (1983). In contrast, cocoa butter implants had short-  
175 lasting effects on plasma cortisol levels, with circulating concentrations returning to control  
176 levels by 6 days post-treatment. The soft texture of vegetable shortening (Fig. 2), even at low  
177 temperatures (solidifies at  $20^\circ\text{C}$ , but remains soft at lower temperatures – e.g., it was  $6-7^\circ\text{C}$   
178 during this study) likely allows for more effective (i.e., faster) release of the cortisol. Cocoa  
179 butter, however, becomes very hard even at fairly high temperatures (solidifies at  $20^\circ\text{C}$ ), which  
180 may prevent long-lasting release of cortisol in north temperate fish species, as indicated by the  
181 peak cortisol levels 3 days post-treatment. The outer cortisol likely gets released quickly, but the  
182 hardness of the cocoa butter prevents the release of the inner cortisol. Alternatively, it is possible  
183 that cocoa butter releases cortisol more readily than vegetable shortening, leading to the implant  
184 being depleted of cortisol more rapidly and the cortisol values in cocoa butter-treated fishes

185 peaking earlier than the first sampling time (3 days). Unfortunately, there is no way to  
186 distinguish between the two possibilities with our data. The conclusion however, remains the  
187 same: vegetable shortening appears to be a more appropriate vehicle for studies seeking long-  
188 term cortisol elevation, while cocoa butter may be better suited for short-term cortisol elevation,  
189 at least in north temperate regions.

190 Cortisol increases the rate of gluconeogenesis (reviewed by Mommsen *et al.*, 1999). An  
191 increase in plasma glucose following treatment with cortisol implants therefore would be  
192 consistent with the known physiological effects of cortisol. Plasma glucose concentrations were  
193 found to be higher than those of sham and control treatments at both day 6 and 9 in cort-veg  
194 fishes. In contrast, plasma glucose was never elevated above sham or control treatment fishes in  
195 cort-cocoa fishes, in agreement with the shorter-lasting physiological effect of cocoa butter than  
196 vegetable shortening on cortisol levels. Additionally, cortisol caused an increase in glucose  
197 levels earlier in the cort-cocoa treatment (day 3) than in the cort-veg treatment (day 9), further  
198 supporting the hypothesis that the cocoa butter vehicle generates a shorter and faster response  
199 than vegetable shortening.

200 Increased conversion of stored energy reserves to glucose during gluconeogenesis may  
201 also lead to a loss in mass. Additionally, cortisol tends to suppress appetite leading to a reduction  
202 in food intake, and this would also be expected to result in mass loss (Madison *et al.*, 2015). The  
203 9 days of the cortisol treatment examined in the present study did not have a significant effect on  
204 change in mass relative to that observed in control or sham-treated fishes, suggesting that the  
205 physiological effects of elevated cortisol take more time to manifest as changes in mass.  
206 Previous studies in similar systems have reported decreased growth rates of cortisol-treated  
207 fishes over two weeks and longer (Madison *et al.*, 2015; Midwood *et al.*, 2015; Midwood *et al.*,

208 2016; Birnie-Gauvin *et al.*, 2017; Peiman *et al.*, 2017). Sham-veg fishes at day 9 showed a  
209 significant increase in mass, which may have resulted from the vegetable shortening itself  
210 starting to be absorbed internally, while in the cort-veg fishes this effect may have been offset by  
211 glucose metabolized by cortisol. Indeed, it was only in this latter group that cortisol and glucose  
212 were positively related. The mechanism by which this occurred is unknown and its biological  
213 significance remains evasive.

214         The present study showed that vegetable shortening and cocoa butter are two effective  
215 vehicles for cortisol implants in north temperate regions, and that sham treatments with the  
216 vehicle alone do not result in growth impairments compared to controls over the short-term, as  
217 previously observed in reproductive female *S. trutta* following cocoa butter sham implants  
218 (Hoogenboom *et al.*, 2011). However, it was noticed that cocoa butter implants had sharp edges,  
219 which could result in internal organ damage, a potentially deleterious effect which has not  
220 previously been noted. Cortisol levels peaked 3 days post-treatment for both vegetable  
221 shortening and cocoa butter implants, and cortisol levels remained elevated for 9 days with the  
222 vegetable shortening implant. Maximum cortisol levels achieved in this experiment are beyond  
223 the physiological range for salmonids (Donaldson, 1981; Gamperl *et al.*, 1994). If the goal of the  
224 study requires cortisol levels within the normal physiological range, a lower dosage of cortisol  
225 may be appropriate. Glucose levels were affected by cortisol in fishes that received vegetable  
226 shortening but not cocoa butter implants. Thus, in north temperate regions, vegetable shortening  
227 is a more appropriate vehicle for studies seeking longer-term cortisol elevation, while cocoa  
228 butter may be better suited for studies looking for short-term cortisol elevation, providing  
229 researchers with different options depending on study objectives.

230

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237

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343



344 **FIGURE CAPTIONS**

345

346 **Fig 1.** PIT-tagged brown trout (*Salmo trutta*) were subjected to one of 5 treatments; control (no  
347 implant), sham-veg (given a vegetable shortening implant), sham-cocoa (given a cocoa butter  
348 implant), cort-veg (given 100mg kg<sup>-1</sup> of cortisol suspended in a vegetable shortening implant)  
349 and cort-cocoa (given 100mg kg<sup>-1</sup> of cortisol suspended in a cocoa butter implant), and were re-  
350 captured at 3 (black bars), 6 (grey bars) or 9 (white bars) days post-treatment. (A) Plasma  
351 cortisol concentration, (B) plasma glucose concentration, and (C) change in mass are presented  
352 as a function of treatment group and sampling day. Values are means + SEM, *N* = 9 to 17.  
353 Groups that share a letter are not significantly different from one another (see text for details).

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355 **Fig 2.** Representative images of the dissection of brown trout (*Salmo trutta*) post-treatment to  
356 illustrate the different implant vehicles; (A) control, (B) vegetable shortening implant, and (C)  
357 cocoa butter implant. Arrows point to the implants. Vegetable shortening remained soft at 3, 6  
358 and 9 days post-treatment. Cocoa butter implants were hard to the touch at 3, 6 and 9 days post-  
359 treatment, with some implants showing sharp edges.

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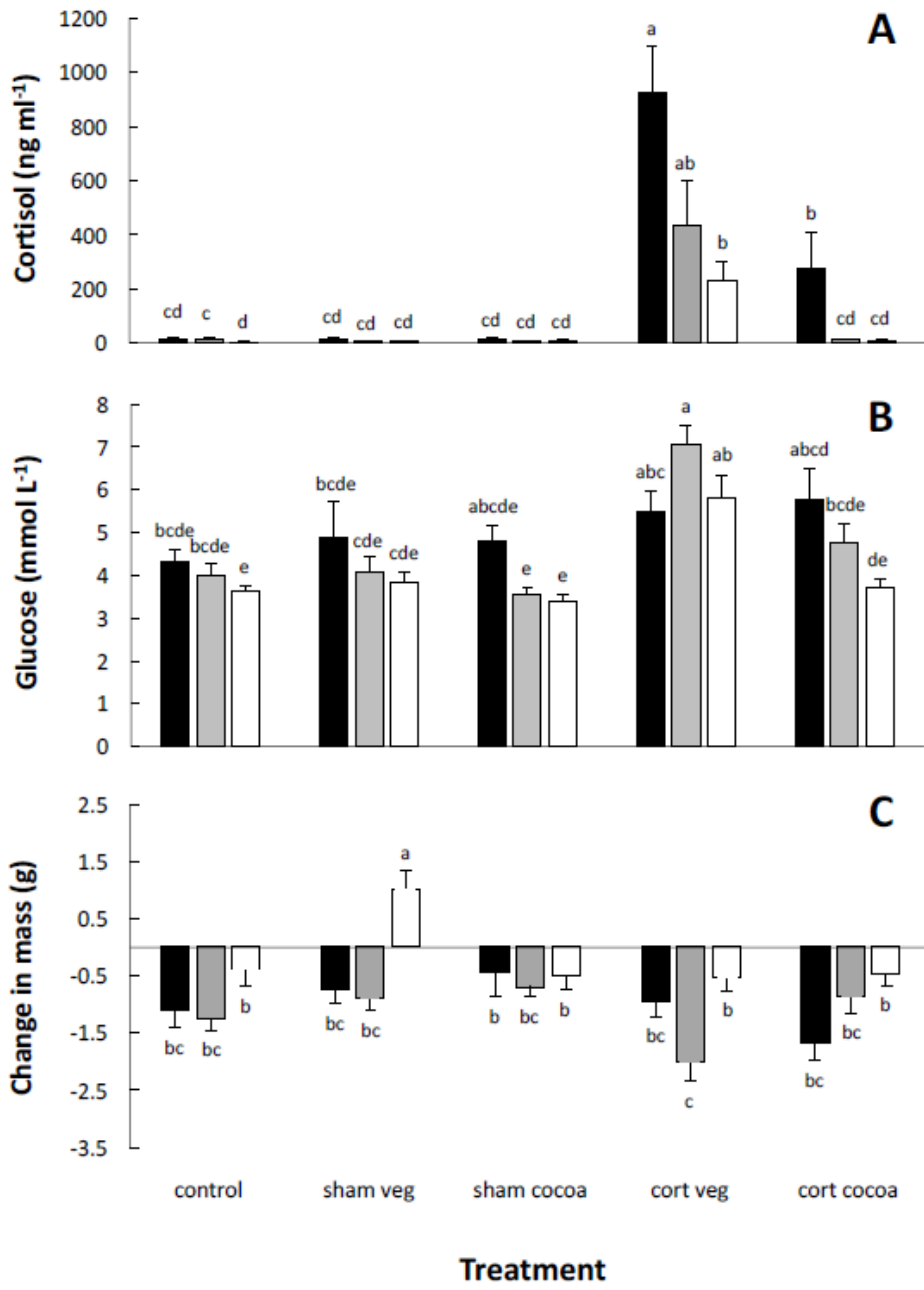
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367 **Figure 1.**



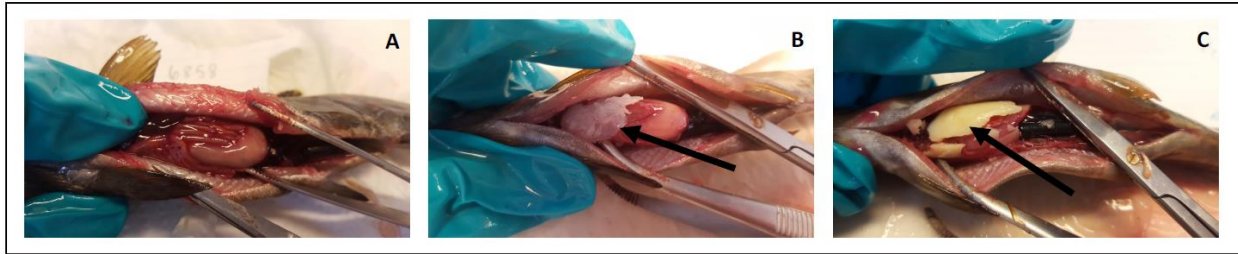
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372 **Figure 2.**



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