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Running headline: cortisol manipulation in fishes

ABSTRACT

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

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

INTRODUCTION

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

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

This study is the first comparative study of vegetable shortening and cocoa butter as vehicles for cortisol manipulation in the wild. Both vehicles are particularly suitable for field studies (see Sopinka *et al*., 2015) owing to their low cost and ease of administration. A wild population of juvenile brown trout *Salmo trutta* L. 1758 was used to compare the temporal patterns of circulating cortisol and glucose concentrations resulting from implants of cortisol suspended in vehicles of cocoa butter versus vegetable shortening. Treatment effects were compared to their corresponding sham (vehicle alone) and control (no implant) groups. Additionally, effects on body mass were measured treatment. It was predicted that vegetable shortening implants would result in cortisol being released over a longer period of time and in higher levels, resulting in higher levels of glucose and more mass loss than cocoa butter implants. It was also predicted that sham treatments would not elevate cortisol or glucose concentrations or cause a change in mass compared to control fishes.

MATERIALS AND METHODS

The Villestrup stream is located in north-central Jutland, Denmark. The stream runs for several kilometers across agricultural land, where a number of tributaries join in before reaching the Mariager Fjord. The stream is home to a large population of semi-anadromous *S. trutta* (del Villar-Guerra *et al*., 2014). Three different sites (1 to 2km apart) within the same stream were used. It is unlikely that there are genetic differences among populations so close (Hansen *et al*., 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 3rd (25 fishes per group), 125 fishes at Site 2 on March 4th
96 (25 fishes per group) and 150 fishes at Site 3 on March 5th (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^{-1} ethyl-*p*-aminobenzoate; Sigma,
100 www.sigmaaldrich.com) in stream water, then weighed (±0.1g), measured for total length
101 (±0.1cm), 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^{-1}). Sham fishes were injected with only 0.01 ml g^{-1} 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

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

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

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

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

RESULTS

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

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

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

DISCUSSION

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

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

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

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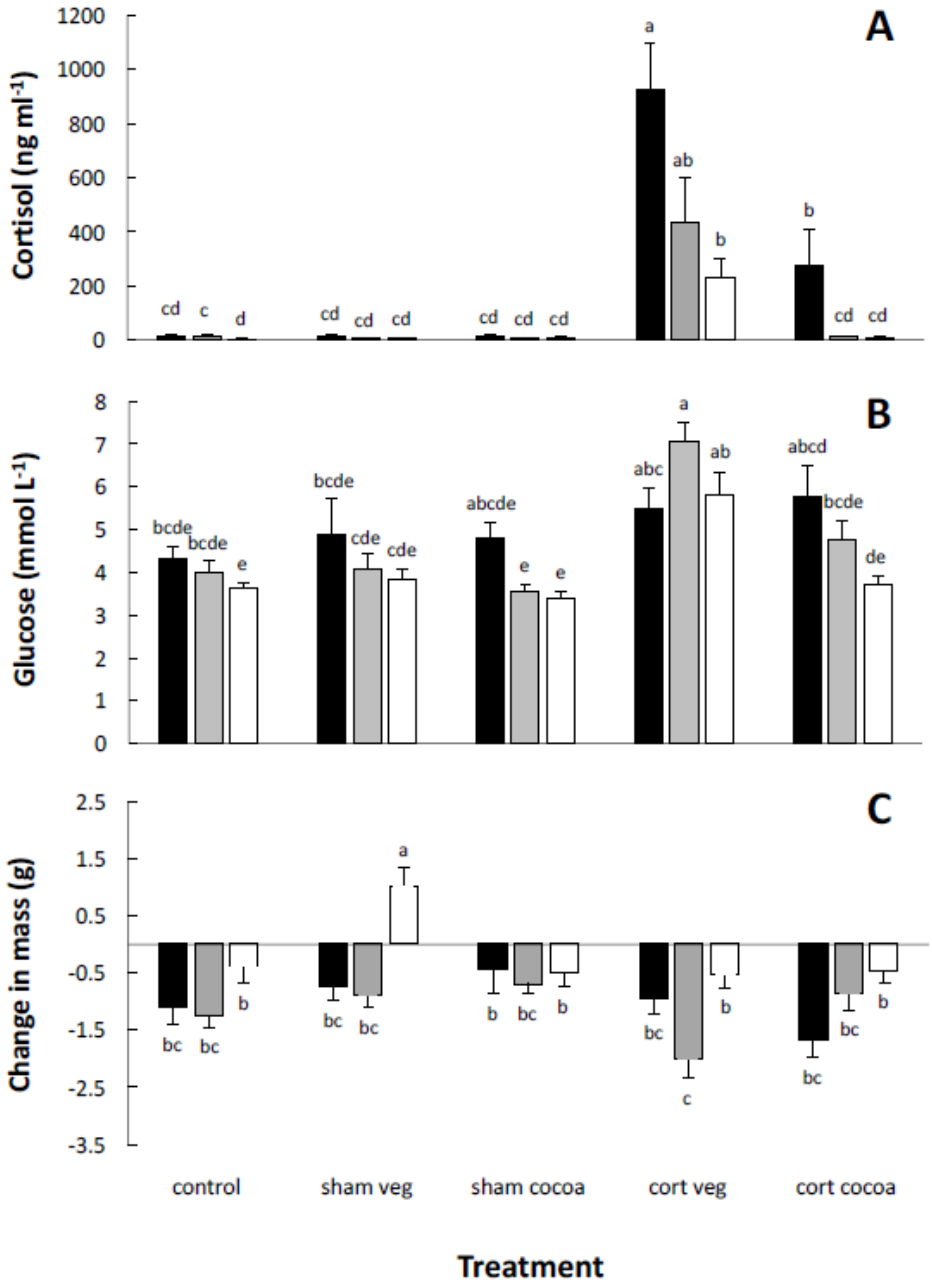
FIGURE CAPTIONS

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

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

367 **Figure 1.**



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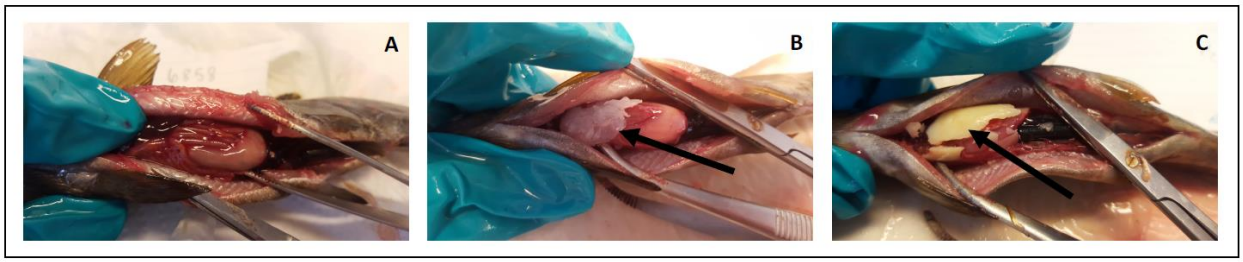
372 **Figure 2.**



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