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

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This study demonstrates that vegetable shortening and cocoa butter are two effective vehicles for intraperitoneal cortisol implants in juvenile teleosts, specifically 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 3, 6 and 9 days post treatment (dpt)], while cocoa butter may be better suited for short-term cortisol elevation (only elevated at 3 dpt). Additionally, plasma cortisol levels were higher with cortisol–vegetable shortening than with cortisol–cocoa butter implants. Plasma glucose levels were elevated 6 and 9 dpt 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.

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Key words: cocoa butter; cortisol implants; *Salmo trutta*; teleost; vegetable shortening.

INTRODUCTION

Cortisol is the primary glucocorticoid stress hormone in fish (Wendelaar Bonga, 1997; Mommsen *et al.*, 1999; Barton, 2002). Not surprisingly, there are hundreds of papers that have measured cortisol in fishes to understand the consequences of different stressors (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 (Slusher, 1966). This allowed researchers to move past simply observing variation in cortisol levels among individuals to performing cause-and-effect studies. Despite its potential ecological relevance (Sopinka

et al., 2015; Crossin *et al*., 2016), however, this technique has been mainly used in the laboratory (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; Doyon *et al*., 2006; Eriksen *et al*., 2006). Studies have also used mini osmotic pumps going back several decades (Theeuwes & Yum, 1976). These, however, are less suitable for field studies owing to expense, as fishes may not be recovered to retrieve the pumps and their higher invasiveness compared with 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. Cocoa butter, however, requires high temperatures to remain in liquid form (*c*. 40° C), potentially resulting in the scalding of organs when injected into a fish and becomes very hard at ambient temperatures in the northern temperate regions, which may lead to damage of the gonads (M. H. Larsen, pers. obs.; McConnachie *et al*., 2012). In contrast, vegetable shortening remains in liquid form at a lower temperature (*c*. 30° C) and remains soft, even in cold water (5° C, K. Birnie-Gauvin and K. S. Peiman, pers. obs.). 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 (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 *v*. vegetable shortening. Treatment effects were compared with their corresponding sham (vehicle alone) and control (no implant) groups. Additionally, treatment effects on body mass were measured. 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 with control fishes.

MATERIALS AND METHODS

The Villestrup Stream is located in north-central Jutland, Denmark. The stream runs for several km across agricultural land, where a number of tributaries join in before reaching the Mariager Fjord (56° 40′ N; 10° 00′ W). The stream is home to a large population of semi-anadromous *S. trutta* (del Villar-Guerra *et al*., 2014). Three different sites (1–2 km 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 comparing responses with treatments within a site. Fishes were captured *via* backpack electrofishing (ELT 60 II GI, 300 v; www.scubla.it) on three separate days in 2016: 125 fishes at site 1 on 3 March (25 fishes per group), 125 fishes at site 2 on 4 March (25 fishes per group) and 150 fishes at Site 3 on 5 March (30 fishes per group). During this period, the temperature of the water in Villestrup was between 6 and 7° C.

Captured fishes were held in a 60 l bin filled with oxygenated fresh stream water. Fishes were anaesthetised in a solution of benzocaine (0·03 g l^{-1} ethyl-p-aminobenzoate; Sigma, www .sigmaaldrich.com) in stream water, then weighed ($\pm$ 0·1 g), measured for total length (L_T, $\pm$ 0·1 cm) and tagged using a 23 mm PIT tag (Texas Instruments RI-TRP-RRHP, 134 Hz, 0·6 g mass in air; www.ti.com). Tags were inserted through a 5 mm incision in the left side of the body, posterior to the pelvic fin. Only $S. trutta$ that were L_T 12−21 cm ($i.e.$ large enough for the PIT tag, but probably still juveniles; Larsen $et al.$, 2013) were used in this study. Fishes were randomly assigned to one of the following five treatment groups: control, sham-vegetable shortening (sham-veg), cortisol−vegetable shortening (cort-veg), sham-cocoa butter (sham-cocoa), cortisol−cocoa butter (cort-cocoa). Cortisol-treated fishes received an intra-coelomic injection (3·8 cm 18-gauge needle) of a suspension of vegetable shortening (100% vegetable shortening; Crisco; www.crisco.com) or cocoa butter (100% pure cocoa butter; NOW Foods; www .nowfoods.com) mixed with hydrocortisone 21-hemisuccinate (Sigma-Aldrich), using a dosage of 0·01 ml vehicle (with a concentration of 0·01 g cortisol ml^{-1}) g^{-1} fish (equivalent to a cortisol dosage of 100 mg kg^{-1}). Sham fishes were injected with only 0·01 ml g^{-1} fish vegetable shortening or cocoa butter. The vegetable shortening and cocoa butter were heated using hot water to a temperature of 37° C 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 1 min fish^{-1}. Overall, fishes were held in tanks for approximately 60 min.

Fishes were recaptured via backpack electrofishing after 3, 6 and 9 days post-treatment (dpt), at site 3, site 2 and site 1, respectively. Immediately after shocking, a blood sample (<0·3 ml) was collected from the caudal vasculature using a heparinized 3·8 cm 25-gauge needle and a 1 ml syringe. All samples were collected within 3 min of capture. Fishes were then weighed. Following recovery, fishes were returned to the river and not recaptured. Blood samples were held in water-ice slurry until centrifuged at 2000g for 2 min to separate plasma from red blood cells. Plasma samples were kept at −80° C until analysed. 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 fish potentially experiencing greater variation. This, however, 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 plasma samples (Gamperl $et al.$, 1994). All plasma samples were measured in a single assay. Intra-assay variability (% C.V.) was 7·9%. Plasma glucose levels were determined using an AccuCheck Compact Plus meter system (Roche; www .roche.com), a point-of-care device previously validated for use in teleosts (Stoot $et al.$, 2014).

Statistical analyses were conducted using JMP 12.0.1 (SAS Institute Inc.; www.sas.com). 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 within each category of treatment and day.

RESULTS

Between 9 and 17 fishes were recaptured per treatment group. Fish treated with cortisol suspended in vegetable shortening showed significantly higher plasma cortisol concentrations after 3, 6 and 9 dpt than both sham and the control treatments, with values at day 3 significantly higher than at day 9 [Fig. 1(a); treatment × time, $F_{8,172} = 3·07$, $P < 0·01$]. Cort-cocoa fish at 3 dpt had significantly higher cortisol levels than both
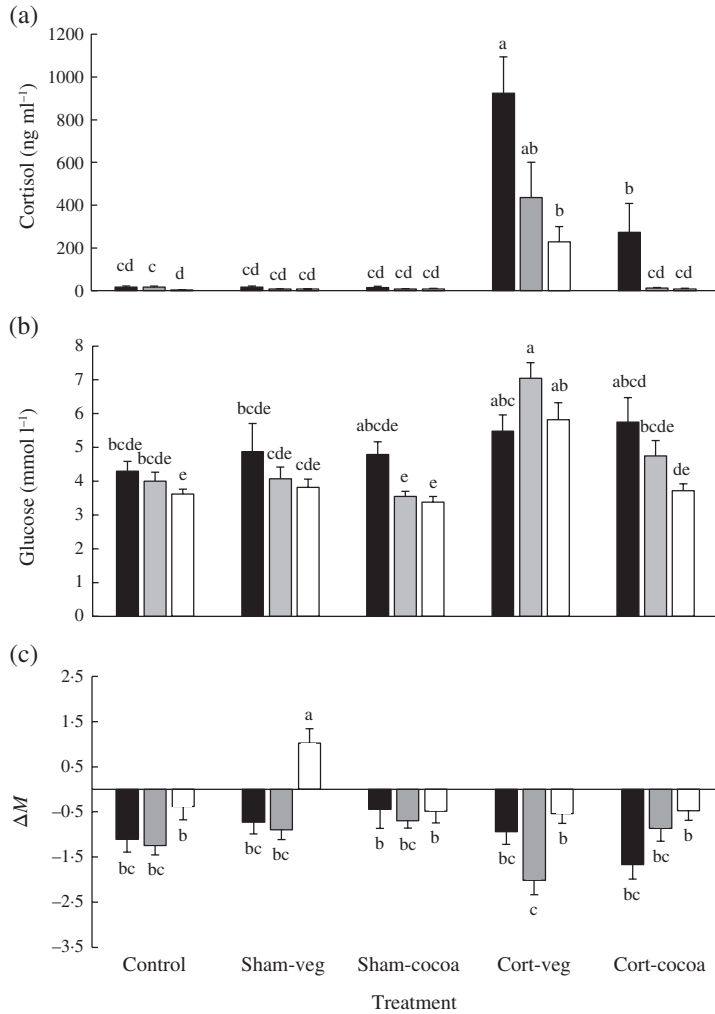
(a)

(b)

(c)

FIG. 1. Mean + S.E. ($n = 9$–17) for (a) plasma-cortisol concentration, (b) plasma-glucose concentration and (c) change in mass (ΔM) 3 days post treatment (dpt; ■), 6 dpt (▨) and 9 dpt (□) of PIT-tagged *Salmo trutta* subjected to one of five treatments: no implant (control); a vegetable shortening implant (sham-veg); a cocoa butter implant (sham-cocoa); $100 \, \text{mg kg}^{-1}$ of cortisol suspended in a vegetable shortening implant (cort-veg); $100 \, \text{mg kg}^{-1}$ of cortisol suspended in a cocoa butter implant (cort-cocoa). Groups that share a letter are not significantly different ($P > 0.05$) from one another.

sham and the control treatments, but values for fish sampled at 6 and 9 dpt did not differ from those for sham or control fishes. At 3 dpt, cort-veg fish exhibited significantly higher plasma cortisol levels than cort-cocoa fish. 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 fish were significantly higher than those for sham and control treatments at 6 and 9 dpt [Fig. 1(b); treatment × time, $F_{8,170} = 2.30$, $P < 0.05$), whereas plasma glucose concentrations in cort-cocoa fishes did not differ

from the sham or control groups on any day. At 6 and 9 dpt, cort-veg fishes had significantly higher glucose concentrations than cort-cocoa fish.

Initially, mass for cortisol-treated fish did not differ from their sham or the control group (all $P > 0.05$). Sham-veg fish sampled 9 dpt gained mass while all other groups lost mass [Fig. 1(c); treatment $\times$ time, $F_{8,170} = 2.94$, $P < 0.01$].

Plasma cortisol and glucose concentrations were positively related in day 9 cort-veg treatment ($R^2 = 0.60$, $n = 15$, $P < 0.05$) No other correlation was significant (all $P > 0.05$).

DISCUSSION

Cortisol implants ($100\,\mathrm{mg\,kg^{-1}}$) generated a significant elevation in plasma cortisol concentration using either vegetable shortening or cocoa butter as a vehicle. The use of vegetable shortening as a vehicle, however, caused a greater elevation of cortisol concentration than cocoa butter after 3 days and this elevation lasted longer. Moreover, plasma cortisol concentration probably remained high for more than 9 days in fish 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) probably 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 northern temperate fish species, as indicated by the peak cortisol levels 3 dpt. The outer cortisol probably 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 fish peaking earlier than the first sampling time (3 dpt). Unfortunately, there is no way to distinguish between the two possibilities with the data here. The conclusion however, remains the same: vegetable shortening appears to be a more appropriate vehicle for studies seeking long-term cortisol elevation, while cocoa butter may be better suited for short-term cortisol elevation, at least in northern temperate regions.

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

Increased conversion of stored energy reserves to glucose during gluconeogenesis may also lead to a loss in mass. Additionally, cortisol tends to suppress appetite leading to a reduction in food intake and this would also be expected to result in mass loss
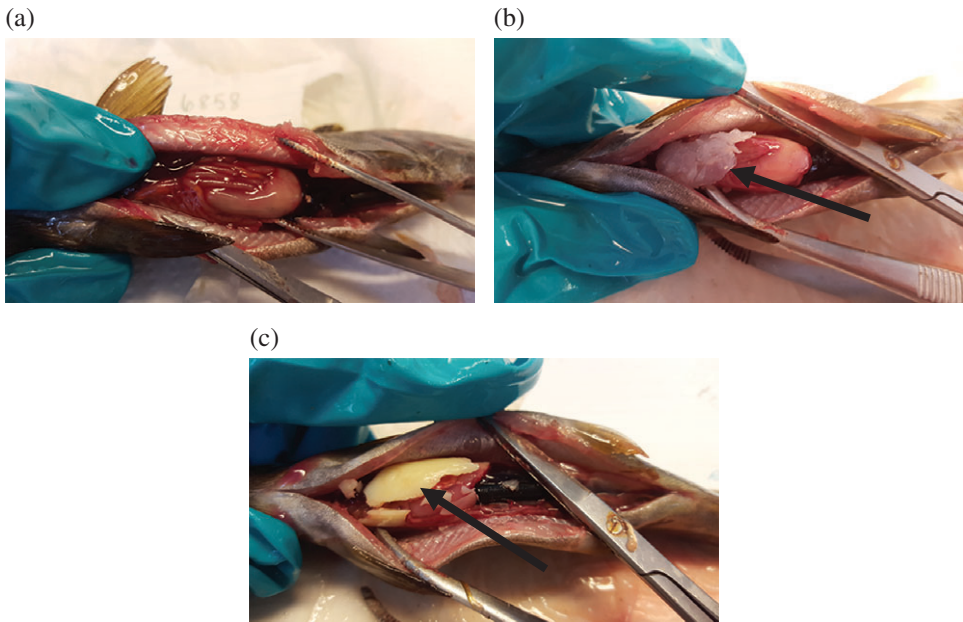
(a)

(b)

(c)

FIG. 2. Representative images of the dissection of *Salmo trutta* post-treatment to illustrate the different implant vehicles (⬆): (a) control, (b) vegetable shortening implant and (c) cocoa-butter implant.

(Madison *et al.*, 2015). The 9 days of the cortisol treatment examined in the present study did not have a significant effect on change in mass relative to that observed in control or sham-treated fish, suggesting that the physiological effects of elevated cortisol take more time to manifest as changes in mass. Previous studies in similar systems have reported decreased growth rates of cortisol-treated fish over 2 weeks and longer (Madison *et al.*, 2015; Midwood *et al.*, 2015, 2016; Birnie-Gauvin *et al.*, 2017; Peiman *et al.*, 2017). Sham-veg fish at 9 dpt 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 fish 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 northen temperate regions and that sham treatments with the vehicle alone do not result in growth impairment compared with controls over the short-term, as previously observed in reproductive female *S. trutta* following cocoa-butter sham implants (Hoogenboom *et al.*, 2011). It was noticed, however, that cocoa butter implants had sharp edges, which could result in internal organ damage, a potentially deleterious effect that has not previously been noted. Cortisol levels peaked 3 dpt 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 fish that received vegetable shortening but not cocoa butter implants. Thus, in northern 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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