

Short communication

## Use of ethanol for preserving steroid and indoleamine hormones in bird plasma

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### Abstract

Endocrinological research on wild animals inhabiting remote areas has been hampered by the need to store plasma samples at subzero temperatures. In an attempt to remedy this logistical issue, we here investigate the use of ethanol as an alternative to freezing for the preservation of steroid and indoleamine hormones in avian plasma. Known quantities of the steroids 5 $\alpha$ -dihydrotestosterone (DHT), testosterone, 17 $\beta$ -estradiol, corticosterone, and the indoleamine melatonin were added to a stripped pool of chicken plasma. Samples were either immediately frozen at  $-40^{\circ}\text{C}$ , or treated with pure ethanol. Ethanol-treated samples were either immediately frozen, or—to simulate storage conditions at various field locations—left sitting at room temperature for one to two months, or incubated at  $36^{\circ}\text{C}$  for one month before all treatment groups were frozen at  $-40^{\circ}\text{C}$ . All samples were then analyzed by radioimmunoassay. For DHT and estradiol there were no differences among treatment groups suggesting that ethanol-treatment is as effective as immediate freezing in preserving plasma steroid concentrations. For testosterone, corticosterone and melatonin ethanol-treated samples differed significantly from immediately frozen samples suggesting that caution is needed when comparing absolute concentrations of hormones between samples preserved in different ways. However, differences among ethanol-treated samples in general were small, demonstrating the feasibility of this preservation method in the field at remote locations.

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### 1. Introduction

Most studies in behavioral and environmental endocrinology are conducted in a laboratory setting, where environmental variables can be controlled for or specifically manipulated. Laboratory studies in behavioral endocrinology are often much more feasible than field studies because the logistical effort to conduct endocrine studies in wild, free-ranging animals can be substantial (e.g. Costa and Sinervo, 2004; Fusani et al., 2005; Goldstein and Pinshow, 2006). However, many questions in behavioral endocrinology cannot be adequately addressed in captive animals. For

example, only a small subset of species can be kept (and bred) in captivity. Thus, studies on captive species represent only a minute fraction of the behavioral, life history and evolutionary diversification present in wild species. Furthermore, because appropriate environmental and social stimuli are often lacking in captivity, behaviors and circulating hormone concentrations in captive species are typically altered compared to their wild counterparts (see examples in Fusani et al., 2005). Thus, many wild species are untapped resources for increasing our understanding of the hormonal mechanisms underlying behavioral, life history and evolutionary diversification (e.g. Wingfield and Farner, 1993; Wingfield and Silverin, 2002; Knapp, 2003; Costa and Sinervo, 2004; Moore and Jessop, 2003; Oliveira et al., 2005; Wilczynski et al., 2005; Wikelski and Cooke, 2006). Endocrinological studies on wild animals clearly are

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important, especially with regard to functional aspects of hormonal mechanisms. However, the need to store and transport plasma samples for hormone analyses at subzero temperatures has hampered many studies on wild animals, especially in remote areas where there is no access to a freezer or dry-ice.

The purpose of this work was to investigate a method for the use of ethanol as an alternative method to freezing for the preservation of the steroid hormones 5 $\alpha$ -dihydrotestosterone (DHT), testosterone, 17 $\beta$ -estradiol and corticosterone, and the indoleamine hormone melatonin in avian plasma. Following some initial validations this method has already been used in some studies on birds (Tarlow et al., 2003; Müllner et al., 2004) and reptiles (Wikelski et al., 2005) in remote (tropical) areas.

## 2. Methods

### 2.1. Sample preparation

Pilot experiments comparing freezing, ethanol, sodium azide, methanol, and formaldehyde treatment had suggested that ethanol is an easily available substance with very little toxicity that is well-suited for the preservation of testosterone in avian plasma (M. Hau and J.C. Wingfield, unpubl. data). For this evaluation we used freeze-dried chicken plasma because this allowed us to reconstitute the plasma with a smaller amount of water to then add standard hormones and end up with the original and desired volume of plasma. With an ordinary plasma pool this would not have been possible. Freeze-dried chicken plasma (Sigma P-3266; reconstitution volume 5 ml) was reconstituted with 2.5 ml ddH<sub>2</sub>O and stripped with 1.5 ml activated charcoal (Sigma C-5510; 12.5 g charcoal and 10 ml dextran (Sigma D-4751: 0.2 g in 10 ml ddH<sub>2</sub>O) in 1 l of phosphate buffered saline). Then, for each of the five hormones investigated (testosterone, 5 $\alpha$ -DHT (DHT), 17 $\beta$ -estradiol, corticosterone, and melatonin) five groups of six replicates each were aliquotted from the stripped plasma pool (for melatonin only five replicates were used). Each replicate consisted of 60  $\mu$ l stripped chicken plasma plus 20  $\mu$ l of the respective hormone solution in assay buffer (hormone concentrations: 2 ng/ml for DHT, estradiol and melatonin, 4 ng/ml for testosterone and 40 ng/ml for corticosterone), resulting in a total amount of either 40 pg DHT, estradiol, and melatonin, 80 pg testosterone, or 800 pg corticosterone per replicate. These amounts equal plasma concentrations of 0.5 ng/ml for DHT, estradiol, and melatonin, 1 ng/ml for testosterone and 10 ng/ml for corticosterone; levels that are commonly found in samples from free-ranging passerine birds.

The five treatment groups for each hormone (25 groups in total) received the following treatments: samples of group 1 were capped and frozen immediately at  $-40^{\circ}\text{C}$  (Plasma group). All replicates in groups two to five received 750  $\mu$ l of pure ethanol (resulting in a plasma-ethanol ratio of 1:9.4), were capped and vortexed. Group 2 (Ethanol group) was then frozen at  $-40^{\circ}\text{C}$ . Group 3 was kept at room temperature (at 20–25  $^{\circ}\text{C}$ ) for 1 month and then frozen (Ethanol+RT-1mo group), group 4 was kept at 36  $^{\circ}\text{C}$  for one month and then frozen (Ethanol+36  $^{\circ}\text{C}$ -1mo group), and group 5 was kept at room temperature for 2 months (Ethanol+RT-2mo group) and then frozen.

We also report results of a previous experiment that we conducted with two doses for testosterone (a low dose of 0.1 ng/ml and a high dose of 1.0 ng/ml). The six replicate samples for this experiment received the same treatment as above, but we used 100  $\mu$ l of plasma and a smaller amount of ethanol for storage (200  $\mu$ l instead of 750  $\mu$ l) and different storage vials (screwcap cryovials with a silicon seal). Using this smaller amount of ethanol and these particular vials we found that most of the ethanol had evaporated during the treatment period. For the other hormones (DHT, estradiol, corticosterone and melatonin) this led to a degradation of hormones in ethanol treated samples stored at RT or 36  $^{\circ}\text{C}$ . Surprisingly however, testosterone did not degrade and hence we decided to present the

results to demonstrate that the ethanol treatment gives similar results for samples of different hormone concentration.

### 2.2. Radioimmunoassay

Concentrations of the steroids DHT, testosterone, estradiol, corticosterone, and the indoleamine melatonin were each determined by direct radioimmunoassay (RIA; Goymann et al., 2001, 2006; Fusani and Gwinner, 2004). All samples for each hormone were analyzed in a single assay. For the steroids, all replicate samples were extracted twice with freshly distilled dichloromethane after overnight equilibration at 4  $^{\circ}\text{C}$  of the replicate with 1500 dpm of the respective tritiated steroid (Perkin Elmer, Wellesley, MA, USA) to estimate the extraction recoveries. The organic phase was then separated from the aqueous phase by plunging the extraction tubes into a methanol-dry-ice bath (holding the tubes at an angle of approximately 60  $^{\circ}$  and rotating them so that the water phase freezes to the side wall of the tube) the dichloromethane phase decanted and dried under a stream of nitrogen in a water bath at 40  $^{\circ}\text{C}$ . The extracts were resuspended in 300  $\mu$ l phosphate buffered saline containing 1% gelatine (PBSG) and left overnight at 4  $^{\circ}\text{C}$  to equilibrate. Melatonin was extracted from the plasma with chloroform after overnight equilibration (4  $^{\circ}\text{C}$ ) with 1500 dpm of tritiated melatonin (Amersham, Buckinghamshire, UK) to estimate the recoveries. The extracted samples were dried with nitrogen at 40  $^{\circ}\text{C}$  and redissolved in 200  $\mu$ l of tricine buffer and left overnight at 4  $^{\circ}\text{C}$  to equilibrate. Samples were then washed with petroleum benzine (Merck no. 1.1775; boiling point 40–60  $^{\circ}\text{C}$ ) to remove residual fats. An aliquot (80  $\mu$ l) of the redissolved samples was transferred to scintillation vials, mixed with 4 ml scintillation fluid (Packard Ultima Gold) and counted to an accuracy of 2–3% in a Beckman LS 6000  $\beta$ -counter to estimate individual extraction recoveries. The remainder was stored at  $-40^{\circ}\text{C}$  until RIA was conducted.

Standard curves were set up in duplicates by serial dilution of stock standard solutions in a range of concentration between 0.39–200 pg standard hormone for DHT, testosterone and estradiol, 0.19–100 pg for melatonin and 1.95–1000 pg for corticosterone. The respective antiserum was added to the standard curve, the controls and to duplicates of the respective sample fractions (100  $\mu$ l). After 30 min the respective tritiated hormone labels were added and samples incubated for 20 h at 4  $^{\circ}\text{C}$  (or at 25  $^{\circ}\text{C}$  for DHT). Bound and free fractions were separated at 4  $^{\circ}\text{C}$  by adding 0.5 ml dextran-coated charcoal (4 g charcoal (Sigma C-5510) in 1 l PBSG assay buffer with 2 ml dextran solution, as described above). After 14 min incubation with charcoal samples were spun (3600g, 10 min, 4  $^{\circ}\text{C}$ ) and supernatants decanted into scintillation vials at 4  $^{\circ}\text{C}$ . After adding 4 ml scintillation liquid (Packard Ultima Gold) vials were counted.

Standard curves and sample concentrations were calculated with Immunofit 3.0 (Beckman Inc., Fullerton, CA), using a four parameter logistic curve fit. The lower detection limit of the standard curves was determined as the first value outside the 95% confidence intervals for the zero standard ( $B_{\text{max}}$ ) and was 10 pg/ml for DHT, 7 pg/ml for testosterone, 5 pg/ml for estradiol, 59 pg/ml for corticosterone and 18 pg/ml for melatonin. The intra-assay coefficient of variation was 0.6% for DHT, 1.3% for testosterone, 15.9% for estradiol, 6.6% for corticosterone, and 5.8% for melatonin. Hormone concentrations were adjusted for individual recoveries for each hormone after extraction with dichloromethane. Mean recoveries were 76% for DHT, 91% for testosterone, 86% for estradiol, 94% for corticosterone, and 80% for melatonin. For the second testosterone assay (two doses) the intraassay coefficient of variation was 5.7% and mean recoveries were 79%.

### 2.3. Statistics

Because we had added a certain amount of each respective hormone to the replicates of each treatment group we determined and statistically compared the amounts of hormones instead of their concentrations. All data are presented as mean amounts of the respective hormone  $\pm 95\%$  CI. The accuracy of the measurement was calculated as percent (%) of the hormone amount recovered compared to the amount that was initially added. The different treatment groups were compared with Systat 11 (Systat Software, Erkrath, Germany) using one-way ANOVAs followed by Bonferroni corrected post-hoc tests.

### 3. Results

The amount of plasma DHT did not vary among treatment groups ( $F_{4,25} = 0.628$ ,  $p = 0.647$ ,  $r^2 = 0.09$ ; Fig. 1a). The assay overestimated the amount of DHT by 13–20% compared to the amount that was actually added to the stripped chicken plasma (Table 1).

For testosterone, there was a significant difference between treatment groups ( $F_{4,25} = 6.287$ ,  $p = 0.001$ ,  $r^2 = 0.50$ ; Fig. 1b). The Ethanol+RT-1mo, the Ethanol+36°C-1mo and the Ethanol+RT-2mo group had significantly larger

amounts of testosterone than the Plasma and the Ethanol groups (post-hoc tests, all  $p$ 's < 0.02). Interestingly, the accuracy for testosterone in these latter groups was higher than those of the Plasma and Ethanol groups (Table 1).

For estradiol there was a strong trend for differences between treatment groups ( $F_{4,25} = 2.540$ ,  $p = 0.065$ ,  $r^2 = 0.29$ ; Fig. 1c). Responsible for this trend was the Ethanol+36°C-1mo group which tended to have larger amounts of estradiol than all other groups. The accuracy for estradiol was lowest for the Plasma group (81% of added estradiol) and highest for the Ethanol+36°C-1mo group (92%; Table 1).

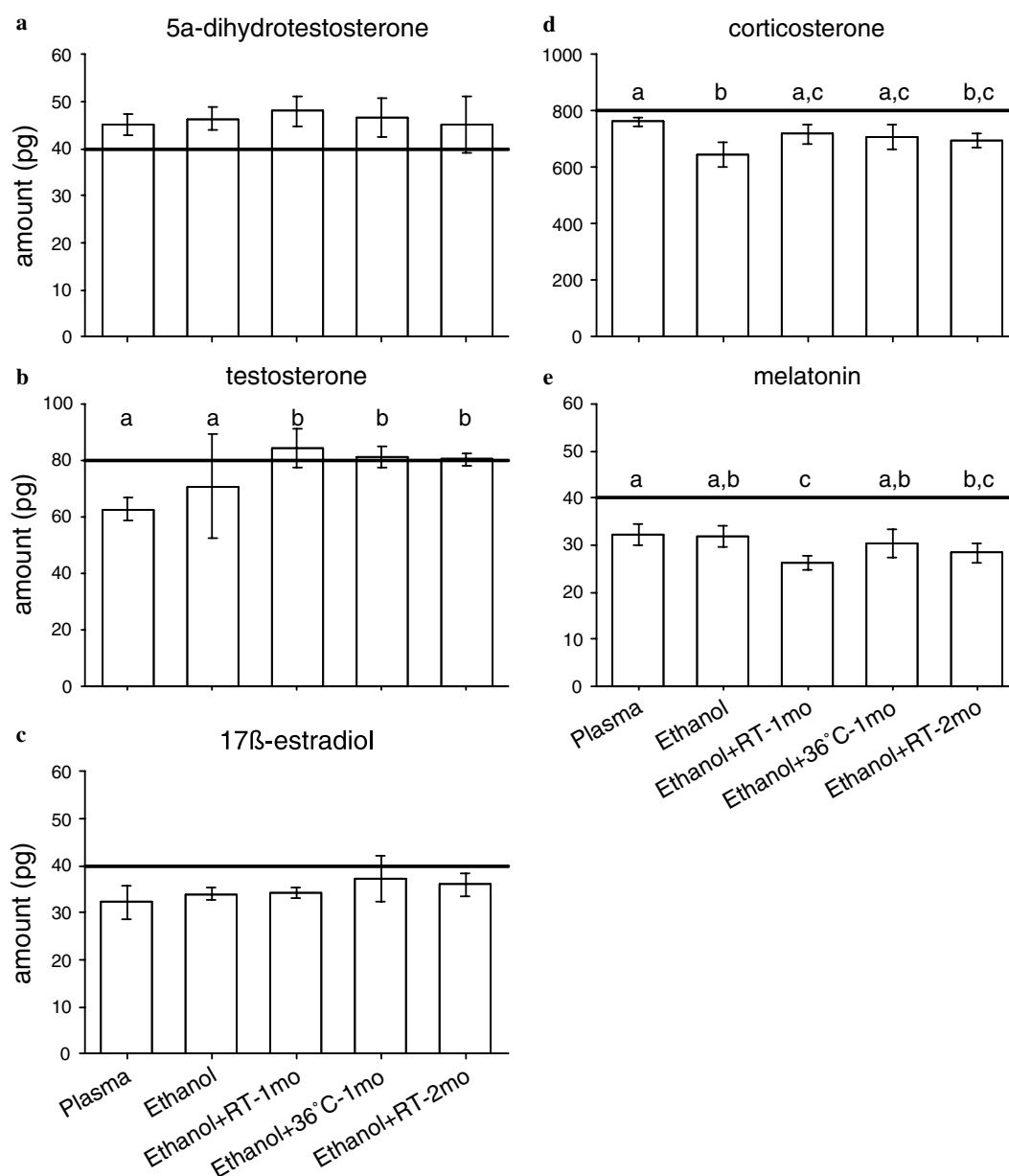


Fig. 1. Mean ( $\pm 95\%$  CI) plasma amounts of 5 $\alpha$ -DHT (a), testosterone (b), 17 $\beta$ -estradiol (c), corticosterone (d), and melatonin (e) in stripped chicken plasma preserved with or without Ethanol (EtOH) at different ambient temperature and for different periods of time ( $N = 6$  per treatment group, except for melatonin where  $N = 5$  per treatment group). Plasma: immediately frozen. Ethanol: ethanol added and immediately frozen. Ethanol+RT-1mo: ethanol added and stored at room temperature for one month before freezing. Ethanol+36°C-1mo: ethanol added and stored at 36°C for one month before freezing. Ethanol+RT-2mo: ethanol added and stored at room temperature for 2 months. Solid line indicates amount of hormone initially added to each replicate. Different letters indicate significant differences between groups as determined by post-hoc Bonferroni tests.

Table 1  
Average measurements as percent (%; = accuracy) of hormone added to chicken plasma depending on the treatment

Treatment group	DHT	T	E2	Cort	Mel
Plasma	113	78	81	96	80
Ethanol	116	88	85	81	80
Ethanol+RT-1mo	120	105	86	90	66
Ethanol+36 °C-1mo	117	101	93	89	76
Ethanol+RT-2mo	113	100	90	87	71

Also for corticosterone, there were significant differences between treatment groups ( $F_{4,25} = 9.929$ ,  $p < 0.001$ ,  $r^2 = 0.61$ ; Fig. 1d), with the Ethanol group and the Ethanol+RT-2mo group having significantly lower amounts than the Plasma group. For corticosterone, the accuracy was highest for the Plasma group (Table 1).

There were differences among treatment groups in the amounts of plasma melatonin ( $F_{4,20} = 8.861$ ,  $p < 0.0003$ ,  $r^2 = 0.64$ ; Fig. 1e), with the Ethanol+RT-1mo, the Ethanol+RT-2mo treatment groups measuring significantly less than the Plasma, the Ethanol, and the Ethanol+36 °C-1mo groups (post-hoc tests: all  $p$ 's  $< 0.04$ ). The accuracy for melatonin was highest for the Plasma and Ethanol groups (80%) and lowest for the Ethanol+RT-1mo group (66%; Table 1).

For testosterone we additionally investigated whether different concentrations of this hormone were affected differently by ethanol storage. There was no significant difference between the five treatment groups neither for the high dose group (1 ng/ml testosterone:  $F_{4,24} = 0.710$ ,  $p = 0.59$ ,  $r^2 = 0.11$ ; Fig. 2), nor for the low dose group (0.1 ng/ml testosterone:  $F_{4,24} = 1.957$ ,  $p = 0.13$ ,  $r^2 = 0.24$ ; Fig. 2), suggesting that different concentrations of testosterone were not affected differently by the different treatments.

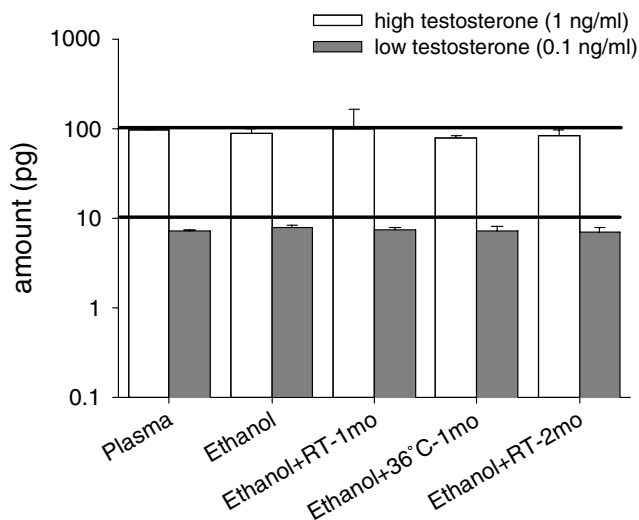


Fig. 2. Mean ( $\pm 95\%$  CI) plasma amounts of a high (1 ng/ml) and a low concentration (0.1 ng/ml) pool of testosterone ( $N = 6$  per treatment and concentration group). The solid lines indicate the amount of hormone initially added to each replicate. Please note logarithmic scale of y-axis.

#### 4. Discussion

In this study we investigated the use of ethanol as a method for preserving four steroids and one indoleamine hormone in avian plasma. For two steroids, 5 $\alpha$ -DHT and 17 $\beta$ -estradiol, the ethanol-treatment groups showed no significant differences to the traditional freezing of avian plasma. For testosterone, corticosterone and melatonin, ethanol-treated samples differed from immediately frozen samples.

Testosterone concentrations were significantly higher in samples that were stored in ethanol for one or two months at room temperature or at 36 °C (Ethanol+RT-1, Ethanol+RT-2, and Ethanol+36 °C-1mo groups) than in immediately frozen plasma samples with or without ethanol (Plasma and Ethanol groups). Interestingly, the assay accuracy, i.e. the degree to which the measured amounts approximated the amount of hormone initially added, deviated positively (more hormone was measured than initially added) for the Ethanol+RT-1, Ethanol+RT-2, and Ethanol+36 °C-1mo groups compared to the immediately frozen Plasma and Ethanol groups. Possibly, prolonged storage of plasma in ethanol increased the extractability of testosterone or ethanol prevented the degradation of testosterone when samples are thawed before the analysis. The opposite effect seemed to be the case for corticosterone, for which accuracy was highest when plasma samples were frozen immediately. However, neither testosterone nor corticosterone seemed to degrade when stored in ethanol for prolonged periods of time. Melatonin concentrations were significantly lower in ethanol-treated samples stored for one or 2 months at room temperature (Ethanol+RT-1mo and Ethanol+RT-2mo groups) compared to immediately frozen plasma samples. Melatonin is sensitive to photodegradation (Andrisano et al., 2000; Tursilli et al., 2006) and the Ethanol+RT-1mo and Ethanol+RT-2mo samples were not entirely protected from light, although they were kept in a darker part of a room. In contrast, the immediately frozen Plasma and Ethanol samples were kept in a dark freezer from the beginning, the Ethanol+36 °C-1mo samples were kept in a drying oven which was not entirely dark, but darker than the treatment groups that were kept at room temperature. The melatonin levels of the Plasma, Ethanol, and Ethanol+36 °C-1mo samples were significantly higher than those of the former Ethanol+RT-1mo and Ethanol+RT-2mo samples, suggesting that the impact of small amount of light may have been sufficient to degrade some of the melatonin in the samples kept at RT with some light.

For testosterone we also reported data investigating the use of ethanol storage for two different hormone concentrations (high and low dose, i.e. 1 ng/ml and 0.1 ng/ml). For both concentrations of testosterone there was no difference between treatment groups. The results of this experiment are not directly comparable to the one reported above, since we used a smaller amount of ethanol and since the silicon-sealed cryovials used in this experiment resulted in evaporation of the ethanol. Nevertheless, we reported these

results to show that ethanol storage works similarly for low and high concentrations of testosterone. Interestingly, in contrast to DHT, estradiol, corticosterone and melatonin (data not shown), testosterone did not seem to degrade when most of the ethanol had evaporated from the samples.

When storing samples in ethanol three technical issues need to be considered. First, for an accurate determination of the final hormone concentration (in ng/ml) it is necessary to exactly measure the volume of the plasma aliquot before the ethanol is added. Hence, a high-precision syringe (e.g. Hamilton syringe) to measure the volume of plasma is required in the field. Second, the evaporation of ethanol is a serious issue. As mentioned above, silicon-sealed cryovials failed to prevent ethanol from evaporating. Loss of sample volume due to evaporation was especially prevalent in the Ethanol+36°C-1mo and the Ethanol+RT-2mo groups. In the validation presented here, we used polypropylene screwtop vials with a rubber-seal (No. 294980702, Zefa, Harthausen, Germany) which more effectively prevented evaporation of ethanol. Third, samples should be kept in a dark place, especially when analyzing melatonin.

We conclude that ethanol can be used to preserve the four steroids investigated here and melatonin in avian plasma. Ethanol-treated samples are not different from frozen plasma samples for 5 $\alpha$ -DHT and 17 $\beta$ -estradiol. For testosterone, corticosterone and melatonin, differences exist between plasma and ethanol-treated samples. However, differences among ethanol-treated groups are minor and even extended periods of warm ambient temperatures had little effect on hormone concentrations, suggesting that ethanol can be used to store avian plasma. Nevertheless, direct comparison between samples stored as frozen plasma and in ethanol should be avoided, especially for testosterone and corticosterone. Since steroids and indoleamines are identical across vertebrate taxa it is likely that ethanol-treatment can also be used for preserving steroids and melatonin in other vertebrates (see Wikelski et al., 2005 for an example in reptiles).

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