Aquatic Mammals 2010, *36*(2), 162-171, DOI 10.1578/AM.36.2.2010.162

Use of Alternative Matrices to Monitor Steroid Hormones in Aquatic Mammals: A Review

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Abstract

The measurement of steroid hormones (e.g., androgens, estrogens, progestins, and glucocorticoids) in alternative matrices (e.g., feces, urine, blubber, saliva, blow, milk, and ocular secretion) has been increasingly used in research with aquatic mammals. The aim of this review is to briefly summarize studies using steroid analysis in alternative matrices from captive and free-ranging aquatic mammal species. The analysis of steroid hormones from alternative matrices is a powerful tool to obtain information about reproductive biology and social behavior in free-ranging aquatic mammals, as well as to help in the management of captive animals. However, for a consistent monitoring of steroid hormones in alternative matrices, it is of crucial importance to verify if a chosen matrix and assay reliably reflects physiologic events.

Key Words: cetaceans, sirenians, pinnipeds, mustelids, urine, feces, saliva, immunoassays, blubber

Introduction

The monitoring of steroid hormones has been increasingly used in research with aquatic mammals, for which it is an additional tool used to understand the biology of the studied species.

The reproductive steroids (i.e., androgens, estrogens, and progestins) are currently used in reproductive monitoring (e.g., reproductive status, puberty, and seasonality), whereas glucocorticoids (e.g., cortisol, corticosterone, or their metabolites) are a powerful tool in the monitoring of stressful events.

Hormone concentrations in animals are typically determined from plasma or serum samples. However, for aquatic mammals, blood collection can be a stressful procedure because the animal needs to be captured and/or restrained, which is impracticable for free-ranging animals. Although captive animals can be trained for the procedure, repeated blood collection for longitudinal studies increases the risk of phlebitis (Amaral et al., 2009). Alternative matrices in hormone measurements have been used for several vertebrates (Gross, 1992; Schwarzenberger et al., 1996; Palme, 2005), and it is a promising technique for studies with aquatic mammals. Less invasive alternatives to measure hormone concentrations have been used in several species of aquatic mammals (for references, see Table 1).

The aim of this review is to briefly summarize studies using steroid analysis (e.g., androgens, estrogens, progestins, and glucocorticoids) in alternative matrices from captive and free-ranging aquatic mammal species.

Steroid Metabolism and Excretion

The circulating free steroid hormones in blood can pass through the epithelium of exocrine glands (e.g., salivary, mammary, and lacrimal glands) mostly by passive diffusion (Vining et al., 1983; Nozaki, 2001). Steroid hormones can pass also to the lung mucosa by passive diffusion (Hogg et al., 2009) as well as into a mostly lipid environment, such as adipose tissue (Hoffmann, 1978; Dolezel et al., 1991), due to the lipophilic characteristics of the steroids.

The metabolism of steroids occurs mainly in the liver, but some catabolic activity also occurs in the kidneys. During metabolism, steroids bind with glucuronides or sulfate molecules, becoming water soluble and inactive or less active. The steroid metabolites are excreted in the duodenum by the biliary system and transported with the digesta. During passage through the intestinal system, steroid metabolites can then be further metabolized by intestinal bacteria, deconjugated, and excreted in the feces or re-absorbed into enterohepatic circulation and transported via blood into the kidney for excretion through the urine (Palme et al., 1996; Schwarzenberger et al., 1996; Graham, 2004).

Usually, most metabolites excreted into the urine are conjugated, whereas those excreted into feces are unconjugated. However, the structure of the metabolite (conjugated or unconjugated) and the main route of steroid excretion (feces or urine) can vary considerably among species as well as

Table 1. Studies using steroid analysis in alternative matrices from captive and free-ranging aquatic mammal species; [a] C = captive, W = wild; [b] F = female, M = male; [c] B = blow, Bb = blubber, F = feces, M = milk, OS = ocular secretion, S = saliva, U = urine; [d] A = androgens, E = estrogens, GC = glucocorticoids, P = progestins; [e] ? = data not available; [f] E = enzyme immunoassay, L = liquid chromatography-mass spectrometry, R = radioimmunoassay.

Species	Habitat[a]	Sex[b]	Matrix[c]	Hormone[d]	Extraction method[e]	Assay[f]	Reference
Mustelidae							
River otter	C	M, F	F	GC	95% ethanol	E	Rothschild et al., 2008
Lontra canadensis	C	M, F	F	P, E, A	40% methanol	E	Bateman et al., 2009
	C	M, F	F	P, E, A	Anydrous ether/Benzene: hexane	R	Gross, 1992
Asian small-clawed otter	C	M	F	P, E, A	40% methanol	E	Bateman et al., 2009
Aonyx cinereus	C	F	F	P, E	?	R	Sobel, 1996
	C	F	F	P, E	?	R	Binczik, 1993
Sea otter	C	F	F	P, E	90% ethanol	R	Da Silva & Larson, 2005
Enhydra lutris	C	F	F	P, E	90% ethanol	R	Larson et al., 2003
Alaskan sea otter	C	M	F	GC	90% ethanol	R	Wasser et al., 2000
Enhydra lutris kenyoni							
Neotropical otter	C, W	M	F	A	90% ethanol	R	Guilherme et al., 2001
Lontra longicaudis							
Eurasian otter	W	M, F	F	P, A	90% methanol	E	Kalz et al., 2006
Lutra lutra							
Sirenia							
Amazonian manatee	C	M	F, U, S, OS	A	90% methanol	R	Amaral et al., 2009
Trichechus inunguis	C	M	F	A	100% ethanol	R	Pimentel, 1998
	C	F	F	P, E	100% methanol/Petroleum ether	R	Nascimento, 2004
Florida manatee	C, W	M	F	A	100% ethanol/ethyl ether	R	Larkin et al., 2005
Trichechus manatus latirostris	C, W	M, F	F	P, E, A	100% ethanol/ethyl ether	R	Larkin, 2000
	C, W	M, F	F	GC	100% ethanol/ethyl ether	R	Donnelly & Larkin, 2008
Dugong	W	M, F	F	E, A	90% ethanol	R	Lanyon et al., 2005
Dugong dugon	C	F	U	P, E	--	R	Wakai et al., 2002
Cetacea							
False killer whale	C	F	S, OS	P	Petroleum ether	R	Atkinson et al., 1999
Pseudorca crassidens							
Killer whale	C	F	U	P, E	--	R, E	Robeck et al., 2004
Orcinus orca	C	F	U	P, E	--	R	Walker et al., 1988
	C	F	U	P, E	--	R	Robeck et al., 1993

Species	Habitat[a]	Sex[b]	Matrix[c]	Hormone[d]	Extraction method[e]	Assay[f]	Reference
Beluga whale	W	F	Bb	P	?	R	Dupré et al., 2003
Delphinapterus leucas	C	F	U	P, E	--	?	Steinman et al., 2007
	C, W	?	F, S	GC	Diethyl ether	E	Biancani et al., 2009b
Bottlenose dolphin	C	M	S, B	A	SPE cartridge	L	Hogg et al., 2005
Tursiops truncatus	C	M, F	S	GC	--	R	Pedernera-Romano et al., 2006
	C	F	U	P, E	--	E	Robeck et al., 2005
	C	F	M	P	--	R	West et al., 2000
	C	F	F	P, E	Petroleum ether/diethyl ether	R	Biancani et al., 2009a
Short-beaked common dolphin	W	F	Bb	P	100% ethanol/hexane	E	Kellar et al., 2006
Delphinus delphis	W	M	Bb	A	100% ethanol/hexane	E	Kellar et al., 2009
Long-beaked common dolphin	W	F	Bb	P	?	?	Trego & Kellar, 2007
Delphinus capensis							
Northern right-whale dolphin	W	F	Bb	P	100% ethanol/ hexane	E	Kellar et al., 2006
Lissodelphis borealis							
Pacific white-sided dolphin	W	F	Bb	P	100% ethanol/ hexane	E	Kellar et al., 2006
Lagenorhynchus obliquidens	C	F	U	P, E	--	E	Robeck et al., 2009
Spotted dolphin	W	F	Bb	P	?	?	Kellar & Trego, 2007
Stenella attenuata	W	F	Bb	P	?	?	Trego & Kellar, 2007
Spinner dolphin	W	F	Bb	P	?	?	Trego & Kellar, 2007
Stenella longirostris							
Estuarine dolphin	W	F, M	Bb	P, E, A	Ethanol: acetone/diethyl ether	R	Rocha, 2001
Sotalia guianensis							
Franciscana dolphin	W	F, M	Bb	P, E, A	Ethanol: acetone/diethyl ether	R	Rocha, 2001
Pontoporia blainvillei							
Dall's porpoise	W	F	Bb	P	?	?	Trego & Kellar, 2007
Phocoenoides dalli							
Humpback whale	W	M, F	B	P, A	SPE cartridge	L	Hogg et al., 2009
Megaptera novaeangliae	W	M, F	Bb	P	?	R	Sheridan et al., 2003
Northern right whale	W	M, F	B	P, A	SPE cartridge	L	Hogg et al., 2009
Eubalaena glacialis	W	M, F	F	GC	90% methanol	R	Hunt et al., 2006
	W	M, F	F	P, E, A	90% methanol	R	Rolland et al., 2005
	W	M, F	Bb	P	?	R	Sheridan et al., 2003
Minke whale	W	F	Bb	P	Ethanol: acetone/diethyl ether	R	Mansour et al., 2002
Balaenoptera acutorostrata							

Species	Habitat[a]	Sex[b]	Matrix[c]	Hormone[d]	Extraction method[e]	Assay[f]	Reference
Pinnipedia							
Weddell Seal	W	M, F	U	GC	--	R	Constable et al., 2006
Leptonychotes weddellii							
Pacific Harbor seal	C	M, F	F	GC	50% ethanol	R	Gulland et al., 1999
Phoca vitulina richardii							
Hawaiian monk seal	C	F	S	P, E	--	R	Pietraszek & Atkinson, 1994
Monachus schauinslandi	C	M	S	A	--	R	Theodorou & Atkinson, 1998
California sea lion	C	M, F	F	GC	100% methanol	R	Petrauskas et al., 2008
Zalophus californianus	W	M, F	S	GC	?	R	Labrada-Martagón et al., 2007
Steller sea lion	C	M, F	S	P, A	--	R	Harmon, 2001
Eumetopias jubatus	C		F	GC	90% methanol	R	Hunt et al., 2004
	C, W	M, F	F	GC	100% methanol	R	Mashburn & Atkinson, 2004
	C, W	M, F	F	GC	100% methanol	R	Mashburn & Atkinson, 2007
	C	M, F	F	GC	100% methanol	R	Mashburn & Atkinson, 2008
	C	F	F	GC	100% methanol	R	Petrauskas et al., 2006
	C	M, F	F	GC	100% methanol	R	Petrauskas et al., 2008
	C	M	F	A	?	R	Litz et al., 2005

between steroids within the same species (Palme et al., 1996; Schwarzenberger et al., 1996).

The salivary steroid concentrations are well correlated with the level of free steroids in the serum (Vining et al., 1983; Pietraszek & Atkinson, 1994; Theodorou & Atkinson, 1998; Harmon, 2001; Nozaki, 2001; Pedernera-Romano et al., 2006). However, the time delay from steroid synthesis to metabolite excretion in feces or urine varies considerably among species (Palme et al., 1996; Schwarzenberger et al., 1996; Graham, 2004), and this information is vital when correlating hormones with a physiologic and/or behavioral event. The time delay in urine samples is usually only a few hours, but in feces, it is closely related to the passage rate of food through the gastrointestinal tract, resulting in transit times varying considerably between species (Palme et al., 1996; Schwarzenberger et al., 1996; Graham, 2004). There are no studies of gut transit times for cetaceans, but for other aquatic mammals, this passage rate is between 1 h to 9 d, where mustelids show the shortest times and sirenians the longest times (Lomolino & Ewel, 1984; Markussen, 1993; Krockenberger & Bryden, 1994; Lanyon & Marsh, 1995; Itavo et al., 1996; Martensson et al., 1998; Grellier & Hammond, 2006; Larkin et al., 2007; White et al., 2007).

Sample Collection

Before choosing which biologic sample should be used for endocrine monitoring, some points need to be considered: the means of collection, the habitat of the animal (i.e., captive or free-ranging), whether or not a given matrix will answer the research questions, and the number of steps necessary before the hormonal assay occurs.

In captive aquatic mammals, sample collections may be facilitated with animal training, minimizing animal distress and manipulation. The animals can be trained to position the head out of the water and/or open the mouth for saliva collection (Pietraszek & Atkinson, 1994; Theodorou & Atkinson, 1998; Atkinson et al., 1999; Harmon, 2001; Hogg et al., 2005; Pedernera-Romano et al., 2006), to blow in a cup on cue (Hogg et al., 2005), and to float on the back for urine collection after abdominal compression (Walker et al., 1988; Robeck et al., 1993, 2004, 2005, 2009; Colbert et al., 2001; Wakai et al., 2002; Lima et al., 2005; Steinman et al., 2007). For sirenians, if the animals are not trained, urine samples can be collected from males with the animal positioned laterally and applying pressure with the fingertips near the urogenital area, or from females, by placing a metal dish under the genital aperture and waiting a couple of minutes (Amaral et al., 2009). Fecal material can be collected from the floor immediately after defecation (for pinnipeds and mustelids) (Gulland et al., 1999; Da Silva & Larson, 2005; Mashburn & Atkinson, 2008; Petrauskas et al., 2008; Bateman et al., 2009) or floating on the water (for sirenians) (Pimentel, 1998; Larkin et al., 2005; Donnelly & Larkin, 2008).

In free-ranging animals, the most commonly used matrix in pinnipeds and mustelids are the feces, which can be collected from the ground (Guilherme et al., 2001; Kalz et al., 2006; Mashburn & Atkinson, 2007). For cetaceans, the main matrix is the blubber collected from projectile biopsies (Sheridan et al., 2003; Kellar & Trego, 2007; Kellar et al., 2009). There are reports of urine being collected from ice, as well as of saliva collection in wild pinnipeds (Constable et al., 2006), feces collection in sirenians (Donnelly & Larkin, 2008), and blow and feces collection in large cetaceans (Rolland et al., 2005; Hunt et al., 2006; Hogg et al., 2009).

In order to identify each sample, it is important to observe the animal during defecation; however, genetic analysis has been applied in combination with fecal steroids analysis for identifying individual animals (Hunt et al., 2006; Kalz et al., 2006). For saliva samples, close attention must be paid to water contamination and the collection method. Since cotton materials can interfere with the concentration of some steroids (Shirtcliff et al., 2001; Gröschl & Rauh, 2006), an alternative is the use of a metal spoon or synthetic materials during saliva collection (Theodorou & Atkinson, 1998; Amaral et al., 2009).

All samples need to be frozen (-20° C or less) until assay to avoid steroid degradation. However, if the researcher does not have access to a freezer, the samples can be maintained on ice or stored in alcohol (for fecal samples). It is of critical importance that steroid degradation studies are conducted for each matrix and for each species prior to any long-term biological study being conducted (Hogg et al., 2005; Touma & Palme, 2005).

Materials and Methods

Lyophilization of fecal samples is strongly recommended in order to avoid contamination from water. A vacuum lyophilization machine is the best option to dry the feces, but an oven can be used if strenuous validation of the technique is made due to fecal fauna potentially affecting hormone metabolism in the fecal sample. Urine, saliva, ocular secretion, and milk require centrifugation to remove particulates and/or proteinaceous components that may interfere with measurement.

Before analysis, steroid metabolites need to be extracted from fecal and blubber samples. The extraction process should be kept as simple

as possible because additional steps increase the variation of determined concentrations (Palme, 2005). For fecal samples, a relatively simple extraction procedure using a high concentration of alcohol (methanol or ethanol) proved best suited for most animals (Table 1). However, for blubber samples, the extraction process has been reported as more laborious (Mansour et al., 2002; Kellar et al., 2006, 2009).

There is an abundance of commercially available antibodies for urinary conjugates; however, depending on the chosen hormonal assay, the urine samples need to undergo hydrolysis to break the conjugations. The hydrolysis can be acid (using sulfuric or chloridric acids) or enzymatic (using glucuronidase and/or sulfatase enzymes), the latter being used specifically to break the conjugations. In addition, urinary steroids need to be indexed by urinary creatinine to compensate for variations in water intake and clearance rates.

Usually, milk, saliva, and ocular secretion samples do not need hormonal extraction before hormonal assay; however, in some instances, it is better to concentrate the sample before analysis.

Hormonal Assays

There are various ways to quantify steroid hormones in alternative matrices. Typically, immunoassays (e.g., radioimmunoassays and enzyme immunoassays) are most commonly used (Table 1).

Commercially available radioimmunoassay kits developed for serum or plasma are specific to steroids. Some brands have cross-reaction with a number of steroid metabolites and have been successfully used. However, hydrolysis of urine samples, or a modification in the assay protocol to increase the sensitivity for salivary samples, is often required (Amaral et al., 2009). Urinary steroids can also be measured with steroid-conjugated antibody in an in-house radioimmunoassay, making the hydrolysis step unnecessary. Radioimmunoassays are expensive (i.e., commercial assays) and produce radioactive residues. Therefore, the use of enzyme immunoassays with group-specific antibodies is gaining popularity.

Liquid chromatography-mass spectrometry, which is able to detect low concentrations of hormones, has been used in the monitoring of steroid hormones from blow samples, (Hogg et al., 2005, 2009). However, it requires expensive equipment and extensive equipment calibration.

Validation

For consistent monitoring of the steroid hormones in alternative matrices, it is of crucial importance to carefully validate the techniques used.

It is necessary to carry out an analytical validation to verify the sensitivity, precision, parallelism, and cross-reaction of the assay. The sensitivity shows the minimum concentration that the assay could detect. The use of high-level and low-level controls before and after the samples into the assays is the primary method of monitoring the precision of the laboratory and method used. For parallelism, serial dilutions of pool samples need to be measured and compared with the standard curve. The cross-reaction verifies that the assay reacts only with the substances of interest. This information is typically supplied by an antibody manufacturer (Palme, 2005).

After assay validation, it is necessary to verify if a chosen matrix can reflect reliable physiologic events. There are four ways to verify this: (1) radioactively labeled steroids, (2) biological validation, (3) physiological validation, and (4) correlation with anatomical findings.

The infusion of radioactively labeled steroids is useful in determining the route of excretion, the time course of excretion, and the type of metabolic steroids excreted. However, this method is not currently being used because of the dangers inherent in using radioactive substances (Palme et al., 1996; Graham, 2004). For aquatic mammals, there is a report of a radioactively labeled steroid infusion in a river otter (*Lontra canadensis*) (Gross, 1992).

Biological validation means to pharmacologically induce physiological changes in circulating steroid levels and to evaluate if these changes are reflected in measured concentrations of steroids in the chosen matrix. Usually it is carried out by a hormonal challenge with ACTH (adrenocorticotropic hormone) and dexamethasone for glucocorticoids, and GnRH (gonadotropin-release hormone) for reproductive steroids (mostly androgens). There are reports of hormonal challenges being used in some species of pinnipeds (Gulland et al., 1999; Hunt et al., 2004; Mashburn & Atkinson, 2004, 2007, 2008), sirenians (Amaral et al., 2009), and mustelids (Wasser et al., 2000).

Physiological validation means to collect samples before and after a known physiological event (e.g., capture or immobilization as a stressful event; mating behavior or pregnancy as a reproductive event) and to determine if these observations are reflected in measured concentrations of steroids in the chosen matrix or to correlate with blood samples (Binczik, 1993; Pietraszek & Atkinson, 1994; Sobel, 1996; Theodorou & Atkinson, 1998; Atkinson et al., 1999; West et al., 2000; Wakai et al., 2002; Larson et al., 2003; Sheridan et al., 2003; Mashburn & Atkinson, 2004; Da Silva & Larson, 2005; Litz et al., 2005; Labrada-Martagón

et al., 2007; Petrauskas et al., 2008; Rothschild et al., 2008; Bateman et al., 2009).

Correlating with anatomical findings means to measure the steroids in the chosen matrix and to correlate with anatomical findings from necropsy (e.g., presence of corpus luteum, fetus, and testis diameter) (Rocha, 2001; Mansour et al., 2002; Dupré et al., 2003; Kellar et al., 2006, 2009; Trego & Kellar, 2007) or clinical exam (Walker et al., 1988; Robeck et al., 1993, 2004, 2005, 2009; Pietraszek & Atkinson, 1994; Harmon, 2001; Steinman et al., 2007). Correlation with anatomical findings and physiological validation are the most widely used validation techniques, having been applied in both captive and free-ranging animals. Consequently, captive animals, stranded carcasses, and fishery by-caught animals are very useful for the validation of hormone assay techniques.

Conclusion

In summary, valuable information can be collected about the reproductive biology and social behavior, as well as captive management, of aquatic mammals through the analysis of steroid hormones from alternative matrices. However, the choice of biological matrix depends on many factors such as the means of sample collection, the habitat of the animal, if the given matrix will answer the specific research questions, and the laboratory procedures. Previous information from steroid monitoring in closely related species may serve as a helpful reference. However, since the route of excretion of steroids can vary considerably among species, as well as between steroids within the same species, it is strongly recommended that a careful validation of assay methods be undertaken in order to generate accurate results. Moreover, different matrices as well as different laboratory techniques (e.g., collection, extraction, and assay) could produce different numeric results and must be considered when comparing studies. Therefore, research involving captive animals as well as carcasses is vital to developing more precise and accurate techniques.

Acknowledgments

I would like to thank Licia Lavor, Justin D. Gregg, and the three anonymous reviewers for the revisions and suggestions made to the manuscript.

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