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Sexing Sirenians: Validation of Visual and Molecular Sex Determination in Both Wild Dugongs (*Dugong dugon*) and Florida Manatees (*Trichechus manatus latirostris*)

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Abstract

Sexing wild marine mammals that show little to no sexual dimorphism is challenging. For sirenians that are difficult to catch or approach closely, molecular sexing from tissue biopsies offers an alternative method to visual discrimination. This paper reports the results of a field study to validate the use of two sexing methods: (1) visual discrimination of sex vs (2) molecular sexing based on a multiplex PCR assay which amplifies the male-specific SRY gene and differentiates ZFX and ZFY gametologues. Skin samples from 628 dugongs (*Dugong dugon*) and 100 Florida manatees (*Trichechus manatus latirostris*) were analysed and assigned as male or female based on molecular sex. These individuals were also assigned a sex based on either direct observation of the genitalia and/or the association of the individual with a calf. Individuals of both species showed 93 to 96% congruence between visual and molecular sexing. For the remaining 4 to 7%, the discrepancies could be explained by human error. To mitigate this error rate, we recommend using both of these robust techniques, with routine inclusion of sex primers into microsatellite panels employed for identity, along with trained field observers and stringent sample handling.

Key Words: dugong, *Dugong dugon*, Florida manatee, *Trichechus manatus latirostris*, visual sex, molecular sex, SRY, ZFX, ZFY

Introduction

Knowing the numbers of male and female free-ranging animals is essential for determining population sex ratios and assessing sex-specific population parameters, including growth, mortality, fecundity, and survivorship. Further, the ability to identify the sex of an animal in studies of social structure and behaviour is important. Fully aquatic marine mammals such as sirenians (dugongs and manatees) present particular challenges because they are not overtly sexually dimorphic in terms of external morphology, size, or colour. Males can be distinguished from females in free-ranging sirenians through visual examination of the genitalia, but this requires that the animal is either closely approached from the ventral (submerged) surface or physically caught and restrained by experienced teams (Bonde et al., 1983; Beck & Reid, 1995; Lanyon et al., 2002, 2006). In the case of dugongs, underwater approach by a human to within visual distance is usually impossible because of the shy nature of the animal and the frequently turbid coastal waters they inhabit. Visual discrimination of males and females in free-ranging Florida manatees is also difficult, except in some situations where their habituation to humans and clear spring-fed waters sometimes makes inspection of the genitalia possible. However, even under ideal conditions, there are few reports on male vs female live sirenians. Further, in cases where capture of sirenians is undesirable or limited through permit regulations (e.g., 1st-y calf-cow pairs, etc.), a molecular genetic method for confirming the animal's sex (e.g., animals assumed to be female because of the presence of a calf) is invaluable in clarifying social relationships.

Molecular techniques offer an advantage over the limitations of determining an animal's sex in the field. Only a small biopsy sample is needed to confirm the sex of a wild animal (e.g., Tringali et al., 2007). Sex determination using molecular methods has been successfully applied to a diversity of marine mammal species that are difficult to approach (e.g., Gowans et al., 2000; Curtis et al.,

2007; Jayasankar et al., 2008). A method of sex assignment using primers to detect the male-specific SRY gene and differentiate the male-specific ZFY gene from its gametologue ZFX is robust to experimental failure and appears to accurately distinguish male and female sirenians (McHale et al., 2008). This study compares the accuracy of conventional field observations to determine the animal's sex (*visual sex*) to the molecular technique developed for distinguishing males and females (*molecular sex*) in both wild dugongs and Florida manatees.

Materials and Methods

Sample Collection

Molecular assays for documentation of male vs female sirenians were conducted on skin samples from 628 wild dugongs in southeast Queensland, Australia: 524 from Moreton Bay and 104 from Hervey Bay. Dugongs were sampled opportunistically from the shallow eastern seagrass meadows in Moreton Bay between the summers of 2001 to 2007 as part of a mark-recapture population study (Lanyon et al., 2002). Of the 524 dugongs, 460 of all size classes (except 1st-y calves; see below) were captured after a short pursuit (see Lanyon et al., 2006). The captured dugongs were tagged with a titanium numbered turtle tag affixed to the tail fluke, measured for body length (snout to fluke notch in a straight line), and a small skin biopsy was collected from the dorsum using a handheld scraper device. Skin samples were stored in salt-saturated DMSO for individual identification using microsatellite genetic loci (gene-tagging; Broderick et al., 2007) and molecular sex analyses (after McHale et al., 2008). Of the 460 dugongs captured and handled, 454 (98.7%) had sex determined by underwater inspection of the ventral body surface; six were not examined. Dugongs with almost contiguous genital and anal openings were classed as females; whereas those with genital openings located more cranial and closer to the umbilical scar were classed as males (Figure 1a & b). For all dugongs, the unstretched length of the left axillar nipple was measured directly using a small plastic ruler to assess whether nipple size might be an indicator of sex or a corollary of maturity in females. For 86 dugongs, presence or absence of erupted tusks, a possible secondary sex character, was recorded by visual inspection. A further 64 skin biopsies were obtained "remotely" using either a hand-held scraper device or a skin scraper attached to a 2-m pole deployed from a boat positioned adjacent to the dugong (i.e., without capturing the dugong). In these cases, neither identity through physical tag recovery nor sex could be determined at the time of sampling. This sampled group included calves that were neonates or in their first year (distinguished by size and light body colouration) and the adult animals that accompanied them, presumably their mothers. This group also included single animals of all size classes and larger calves and/or juveniles associating closely with adult animals. For these remotely sampled dugongs, visual discrimination of sex was not possible. In Hervey Bay, 104 skin biopsies were assayed for molecular sex. All of these dugongs were sampled from the boat using one of the remote methods, so no other visual data were collected. Identity was determined by gene-tagging, size class through visual estimation, and sex through molecular analysis only. All skin samples were stored in tissue buffer consisting of saturated salt and DMSO, and then frozen.

Skin biopsies were obtained from 100 Florida manatees. Samples were equally divided among the four management units identified for Florida (Langtimm et al., 2004). Sixty-four of these samples were collected from carcasses recovered from Florida between 1998 and 1999. The sex was determined and recorded at necropsy. The other 36 samples were obtained between 2002 and 2005 from live animals while in the water. The researcher swam up to the manatee, visually determined the sex by observing the ventral aspect of the belly, obtained photos (Figure 1c & d), and removed a small piece of skin tissue from the tail margin using a cattle ear notcher tool. In one instance, the sex for the manatee was not recorded. Skin samples were then placed into tissue buffer consisting of saturated NaCl, EDTA, and DMSO. These samples were maintained at room temperature for long-term storage until analysis. Data collected from individuals in Florida are maintained as part of the MIPS (Manatee Individual Photo-identification System). This program contains more than 2,000 individual manatees that have been catalogued based on unique scarring or natural, distinctive features (Beck & Reid, 1995). Subsequent photo-documentation of these animals from year to year is used for mark-recapture techniques to determine population survival estimates (Langtimm et al., 2004).

DNA Extraction and Sex Assignment

A molecular method was used to distinguish males from females using primers to detect the male-specific SRY gene and differentiate the male-specific ZFY gene from its gametologue ZFX (McHale et al., 2008) for each tissue sample. DNA was isolated from ~10 mg of skin by salting out (Miller et al., 1988). This sexing assay targeted SRY, ZFX, and ZFY in 6 μL multiplex PCR amplifications comprising six primers—(1) 0.01μM ZFX-F, (2) 0.05μM ZFY-F, (3) 0.01μM ZFXY, (4) 0.02μM DSRY-F, (5) 0.2μM ESRY-R, and (6) 0.11μM TET-labelled M13—10 ng of genomic DNA, 0.5× Q-solution, and

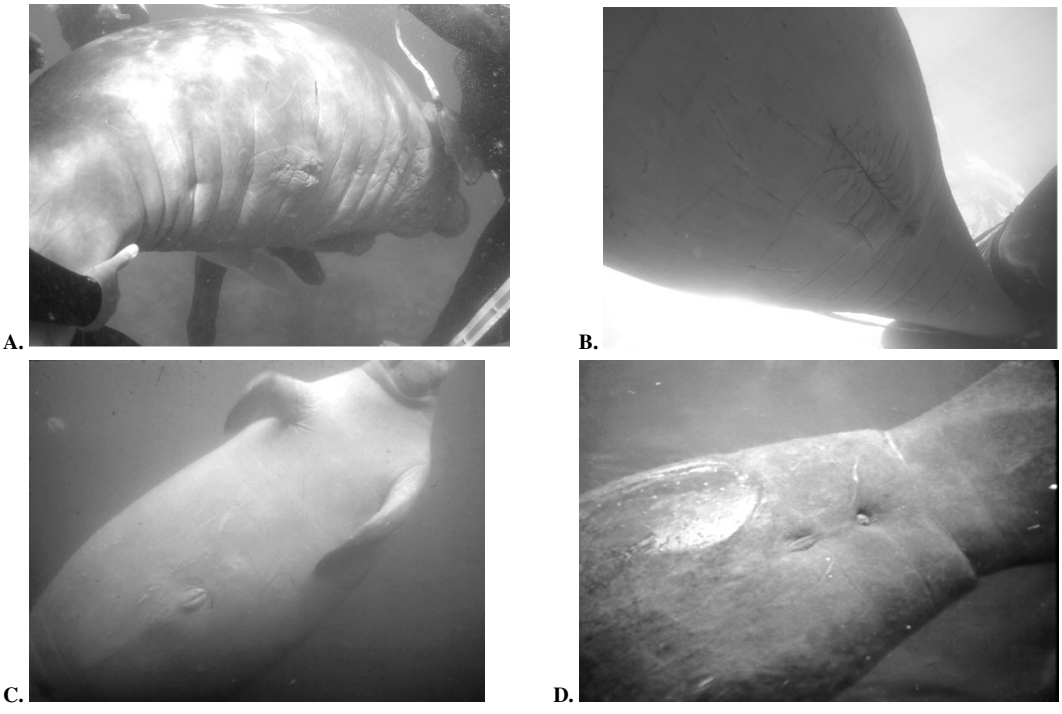

Figure 1. Photographs of the ventrum of (a) a male dugong, (b) a female dugong, (c) a male manatee, and (d) a female manatee, showing relative distance between more caudal anus and cranial genital opening

3 µl of QIAGEN's multiplex master mix containing 3 mM $MgCl_2$ (McHale et al., 2008). Cycling conditions were 94° C for 15 min and 35 cycles of 94° C for 30 s, 58° C for 30 s, and 72° C for 30 s, with a final extension at 72° C for 10 min. Amplicons were diluted 25-fold with ultrapure Milli-Q water and 6-fold with ABI Hi-Di Formamide before capillary electrophoresis. Alleles were sized against an internal size standard (GeneScan – 500 LIZ) and scored using *GeneMapper*® software (Version 3.7, ABI). Male sirenians were readily distinguishable from females by presence of three amplicon in males (155 bp from SRY, 230 bp from ZFX, and 242 bp from ZFY) compared to a single product in females (230bp from ZFX). Molecular sex of each sample was determined without knowledge of the sex determined by visual examination in both dugong and manatee tissue sets, including some replicate tissue samples whose identity was not disclosed during molecular sexing.

Results

Dugongs

Of the 454 dugongs captured and assigned a sex by observations in the field, 434 (96%) showed complete congruence between visual and molecular sexing methods. Secondary sex characters were only used as an accessory confirmation since they were not consistently present within size classes. In male dugongs, erupted tusks were recorded in 100% of animals > 260-cm body length, but also in 54% of apparent subadults (241 to 260 cm) and 10% of juveniles (two individuals of 229 and 239 cm, respectively). The only female dugongs to have erupted tusks were adults (> 260 cm), which is consistent with Marsh et al. (1984), and the 22% of adult females with these were all ≥ 267 cm long. This confirmed that erupted tusks are not necessarily indicative of sex in dugongs. Further, other studies (Marsh et al., 1984; Kwan 2002) have suggested that tusk eruption may succeed testicular competence. If this is the case that erupted tusks indicate maturity in dugongs, attainment of maturity in some males may occur at smaller than expected body sizes. All adult females with tusks had nipples ≥ 5 cm long. Nipples > 3 cm long were only recorded in females > 240-cm body length (i.e., subadults). All juvenile females had nipple buds 1 to 2 cm long. This suggests that nipple length is likely to be greater in more mature females. All male dugongs had nipple buds ≤ 2 cm long, except for one adult (277-cm body length) with 3-cm nipples and well-erupted tusks.

Eighteen dugongs (4%) had discrepancies between visual sex and molecular sex assigned in

the field. In eight cases, molecular sex was confirmed by visual sex during subsequent recapture of the same individuals. Each of these also had a photographic record of the genital region that corroborated molecular sex. In ten cases, the individual was captured only on a single occasion, and since there was no evidence to corroborate or refute either the molecular or visual sex, it was assumed that molecular sex was probably correct. In a further three cases, the initial assessment of molecular sex appeared to be in error. However, samples from multiple recaptures and a review of the co-amplified DNA genotypes indicated that the wrong individual had inadvertently been tested. The inclusion of the sexing primers into the genotyping panel is a powerful way to detect human handling errors.

The sources of error in assigning visual sex (above) are unclear. However, nine of these 18 individuals (including two dugongs on each of four days) had been sexed by the same person and evidence was later obtained to suggest that this person was not adept at distinguishing sexes. The remaining nine errors all occurred in the first 5 y of the program, with no errors recorded in the most recent 3 y. This might suggest that inexperience or unfamiliarity with the animals played a role. Further, of the animals incorrectly assigned visual sex, more molecular females were assigned a visual sex of male (11) compared to molecular males being labeled female (7). It is possible that in the case of true females, the umbilicus was mistaken for a male genital opening with a resultant assignment as male.

Only two of the first ten dugongs captured in the program had a definite visual sex recorded. A further two were of uncertain sex because the animals were not sufficiently restrained to allow observers to get a good look at the ventral surface—one visual sex was confirmed by molecular sex and the other refuted. For the remaining six dugongs with no visual sex recorded at capture, three were assigned a molecular sex of female and three of male; however, there was no other visual information available to verify sex because tusk eruption and nipple length were unrecorded.

Twenty-five of the 64 dugong tissue samples collected remotely (i.e., without capture) were assigned a sex using the molecular assay. A further 39 were originally assumed to be adult females because of their association with a calf or juvenile. Of these, 38 (99%) were confirmed as female through molecular sex: 17 adults were associated with calves in their first season; 15 adults with assumed 2nd-y calves, and 6 with larger calves/juveniles. Only one dugong assumed to be a female because of its association with a smaller dugong was determined by molecular methods to be male. However, this smaller dugong was recorded in field notes as "large 2+ y," suggesting that it was not a dependent calf.

Skin biopsies from 104 dugongs remotely sampled in Hervey Bay in 2006 to 2007 were analysed. These included 28 adults (body length estimated as > 250 cm) that were assigned the visual sex of female because of their close association with one smaller animal (calf or juvenile) in 27 cases, or with two small calves (possibly twins) in one case. All but one (96%) of these visually assigned females (with a juvenile) was confirmed as female through molecular sex. The other 76 dugongs of unknown visual sex were assigned molecular sex. These results indicate that females can be discriminated reliably through association with very young calves, while caution should be exercised in assigning sex of animals accompanying larger calves or juveniles.

Florida Manatees

Of the 100 tissue samples collected from Florida manatees throughout the state, only 81 screened successfully for molecular sex. The other 19 samples, from retrieved carcasses, were of too poor a quality (degraded) to get a good molecular result. In 75 of the 81 samples that screened (93%), molecular sex and visual sex were congruent. During this study, discrepancies in visually determined sex and molecular sex were apparent in six cases (7%). Of these, three manatees were reassigned a different visual sex after reexamination of the original photographic records; two manatees had had visual sex recorded incorrectly when transcribed from the original data sheets to the database; and the other was a badly decomposed carcass whose visual sex could have been misidentified in the field. In the first five cases, mismatch between visual and molecular sex could be accounted for by human error. In the last case, however, there was not enough field evidence to override the molecular sex assignment. A continuation of field effort may allow for better field determination methods for these individuals.

Discussion

The high level of agreement between visual and molecular sex (93 to 96%) indicated that field techniques for discriminating sex of Florida manatees and dugongs were generally good. Of all the biopsy samples analysed in this project, only three calls for molecular sex were suspect, and a review of data from multiple recaptures and their co-amplified DNA profiles indicated that the wrong individual had inadvertently been tested. Error rates may be minimised or eliminated by more stringent sample handling procedures in the

laboratory and in the field and the incorporation of the sexing primers into the genotyping panel.

The 4 to 7% error rate in assigning visual sex to each species is of sufficient concern to warrant a review of the way we sex sirenians in the field, especially when the option of molecular sex is unavailable. To counteract the human error involved, we suggest the use of the following approaches: field personnel experienced in discrimination of sexes, multiple personnel to sex each animal, multiple observations for the target individual over time, routine photography of the genital region, and a record of secondary sexual characteristics regardless of size class and perceived sex (e.g., emergent tusks in adult male dugongs, calf association, nipple length). These approaches are already standard practice within the existing MIPS and the University of Queensland dugong programs. It should be cautioned that this sample size is too small to estimate error rates for the entire MIPS (over 2,000 known individuals in Florida). A proper analysis would need to be conducted using data collected from the four management units, and there is a great deal of variation between them in the way data are collected under various field conditions. Importantly, skin biopsy collection with routine inclusion of sex primers into the microsatellite primer panel employed for identity is also recommended as standard procedure. Subsequently, the quality of tissue sample needs to be high to ensure a good molecular result. It should be noted, however, that these additional techniques of integrating genetic sexing are expensive, and sample acquisition is often logistically difficult from some areas. Strengthening the ability to accurately determine sex of sirenians in the field will improve estimates of sex-specific parameters for gauging population structure, population status, mortality risks, reproduction, and other life history information.

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