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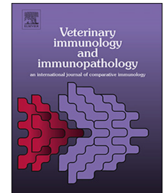
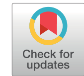


Development of a polyclonal anti-dugong immunoglobulin G (IgG) antibody with evaluation of total plasma IgG in a living dugong (*Dugong dugon*) population



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ABSTRACT

Species-specific antibodies (Ab) for the measurement of immunoglobulins (Ig) are valuable tools for determining the humoral immune status of threatened and endangered wildlife species such as dugongs. However, no studies have reported antibody reagents against dugong immunoglobulin. The object of this study was to develop an Ab with specificity for dugong IgG and apply this tool to survey total IgG levels in plasma samples from a live wild population of dugongs in southern Queensland, Australia. Dugong IgG was isolated from plasma by protein A/G column chromatography and a polyclonal antiserum was successfully raised against the dugong IgG through immunization of mice. The anti-dugong antiserum was reactive with dugong serum but not immunoglobulin from other species such as rats and humans. When tested against a panel of dugong plasma samples, relative IgG levels from dugongs (n = 116) showed biologically relevant relationships with pregnancy status and a principal component of Body Mass Index (BMI)/globulin/fecal glucocorticosteroid (chronic stress) levels combined, which together accounted for 9.2% of the variation in total Ig levels. Together these data suggest that dugongs show variation in total IgG and that this correlates with some physiological parameters of dugong health.

1. Introduction

Immunoglobulins (Ig) are antibodies secreted by B cells as part of the adaptive humoral immune response to invading pathogens (Murphy, 2012). Of the five major classes of immunoglobulins, IgG is the most abundant in the blood of mammals, functioning to neutralize pathogens, activate the complement system and tag microbes for destruction by phagocytic cells (Nimmerjahn and Ravetch, 2008). It is also the earliest form of immune protection provided maternally to the developing fetus (Borghesi et al., 2014). Alterations in total IgG levels in mammals are generally associated with severe immunoglobulinopathies but can also be due to physiologically stressful conditions (Stone and Bovbjerg, 1994; Al-Busaidi et al., 2008; Mishra et al., 2011). Deficiencies in IgG levels are most concerning, as these can result in increased susceptibility to infection (Agarwal and Cunningham-Rundles, 2007).

Environmental factors may also cause declines in IgG levels in mammals and these may include exposure to persistent organic pollutants (Bernhoft et al., 2000; Hultman and Michael Pollard, 2015) or a combination of seasonally related nutritional and environmental stressors (Johnson et al., 1995; Nelson and Drazen, 2000; Al-Busaidi et al., 2008). However, immune effects may differ depending on type and magnitude of environmental stressor, e.g., heavy metal exposure (Rosenberg et al., 1985; Mecdad et al., 2011; Mishra et al., 2011). Whilst IgG levels are not greatly affected by nutritional stressors once adult levels are attained, during the neonatal stages of life, IgG levels are important indicators of malnutrition or nutritional stress that can affect immunity (Sandberg et al., 2000). Adequate nutrients and IgG from maternal colostrum during neonatal stages are essential for protection from disease and for survival, as has been observed in horses (Barton et al., 2006), sheep (Nowak and Poindron, 2006) and cows (Waldner and Rosengren, 2009). However, under nutritional stress, an increase in IgG in neonates is not necessarily a sign of improved overall immunity. In Iberian red deer, nursing calves under nutritional stress had elevated levels of IgG not as a result of poor body condition or high infection, but possibly as a result of compensation for a decline in

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innate immunity and warding off subclinical infection (Landete-Castillejos et al., 2002).

In wild marine mammals, IgG levels have been studied far less than in humans and in other terrestrial mammalian species, and IgG related disorders are not well defined (King et al., 2001; Ross and De Guise, 2007). However, IgG appears to play a particularly important role in neonatal health and survival in several marine mammal species. In wild marine mammal populations, low IgG is generally observed in lactating females and neonates of various seal species (King et al., 1994; Ross et al., 1994), killer whales (*Orcinus orca*) and sea otters (*Enhydra lutris*) (Taylor et al., 2002) and in an orphaned Florida manatee (*Trichechus manatus latirostris*) (McGee, 2012). Thus, during reproductive and certain developmental life stages, marine mammals may be particularly vulnerable to infection.

It is not clear how various environmental stressors including pollutants (Ylitalo et al., 2005; Martineau, 2007), biological pathogens (Jessup et al., 2004; Miller et al., 2008) and loss of food supply (Rosen and Trites, 2000; Atkinson et al., 2008) affect total IgG levels in adult marine mammals (King et al., 2001). However, a negative correlation between IgG and organochlorine levels has been observed in adult polar bears (*Ursus maritimus*) suggesting the possibility of increased susceptibility to infection after contamination (Bernhoft et al., 2000; Lie et al., 2004). Adult Atlantic bottlenose dolphins (*Tursiops truncatus*) and Florida manatees in captive care have shown lower IgG levels compared to wild populations possibly due to relatively lower exposure to pathogens in the controlled environment (Ruiz et al., 2009; McGee, 2012). Further, in wild manatees, IgG levels were relatively higher in groups exposed to coastal flood events and while taking winter refuge amongst the warm water discharges of a power plant, possibly as a result of increased antigenic exposure from coastal pollutants (McGee, 2012). Manatees taking refuge at power plant discharge sites during cold winters may also be exposed to sub-optimal water temperatures (< 20 °C) at times, leading to higher incidences of cold stress (Bossart et al., 2003) and this may also contribute to higher IgG levels (McGee, 2012). Nutritional stress and resultant changes in IgG levels in manatees have not been reported. Further study is needed to understand how total IgG levels may be affected by the various environmental stressors to which marine mammals are exposed (King et al., 2001; Ross and De Guise, 2007).

IgG levels have not been measured previously in dugongs and how their levels may be affected by life history variables and environmental stressors is not known. In southern Queensland Australia, dugongs in Moreton Bay are located in close proximity to a capital city (Brisbane) and major shipping port. There is a threat of terrigenous runoff including industrial and agricultural pollutants, which may or may not affect the dugongs' seagrass habitats located several kilometres offshore (Lanyon et al., 2002; Chilvers et al., 2005). These dugongs are also located at the southern limit of their range and may be exposed to seasonal declines in water temperature and reduced forage (Lanyon, 2003; Lanyon et al., 2005), which can affect chronic stress levels (Burgess et al., 2013). Further, extreme weather events may also affect dugongs indirectly through degradation of their seagrass habitat; for example, the catastrophic flood event in Queensland in summer 2010–2011 resulted in the confirmed dead stranding of 240 dugongs although the number indirectly attributed to the actual flood event through malnutrition is uncertain (Meager and Limpus, 2012). Evaluation of total IgG levels in relation to these types of events and other factors is warranted, and will help improve our understanding of total IgG as a health biomarker.

Prior to this study no species-specific antibodies existed for dugongs. To assess the relative IgG levels in an Australian population of wild dugongs and interrogate possible associations with other health parameters, we have generated a polyclonal antibody against dugong IgG. Our study shows variability within total dugong IgG levels and a relationship between IgG level and life history/physiological parameters.

2. Methods

2.1. Sample collection

Serum and plasma samples were collected during annual out-of-water health assessments of dugongs in Moreton Bay, Queensland Australia (latitude 27° 20.09′ to 27° 24.87′ S; longitude 153° 21.26′ to 153° 23.84′ E) from 2008 to 2014 (Lanyon et al., 2010, 2012, 2015). Health assessments were also conducted in Hervey Bay, 300 km to the north (latitude 25° 12.05′ to 25° 13.62′ S; longitude 152° 38.60′ to 152° 39.89′ E) in 2011. Health assessments occurred in late autumn to early winter in all years. In 2012, samples were also collected in spring. Blood samples were collected from dugongs of both sexes and all size classes except dependent calf and attendant cow (Burgess et al., 2012a). Blood was collected from the brachial arteriovenous plexus of the pectoral flipper via a 21 gauge needle connected to a 20 cm extension set and luer-fitted Vacutainer® collar (BD, Becton Dickinson, Franklin Lakes, New Jersey 07417, USA) into 10 mL red top clot activator (for serum) and 10 mL green top lithium heparin (for plasma) BD Vacutainer® tubes. Serum and plasma were stored at −80 °C. Blood was also collected into 4 mL lavender top EDTA tubes and sent to IDEXX Laboratories Brisbane for same day hematological analysis. Apparent health of dugongs was classified on a five point scale (poor, fair, good, very good, excellent) based on general body appearance, body girths (fatness or emaciation as evidenced by bony protuberances such as spinal processes), skin condition (including structural integrity of epidermis, scarring and barnacle load) and overall demeanor (after Lanyon et al., 2010, 2015). Whilst on deck, dugongs were sexed and females examined by ultrasound for possible pregnancy (after Lanyon et al., 2010, 2015). Twelve pregnant dugongs were identified within the cohort. Fecal samples were collected for endocrinology to determine reproductive maturity (sub-adult or adult), and to confirm pregnancy status of females (Burgess et al., 2012a, 2012b), and for chronic stress levels via fecal glucocorticoid metabolite (fGC) measurement (after Burgess et al., 2013). Straight body length (snout to fluke notch, cm) was taken with the dugong lying in a prone position on deck, and girths were measured at each of axilla, maximum, anal and peduncle positions. Prior to release, each dugong was weighed to the nearest 0.5 kg in a suspended stretcher (Lanyon et al., 2010). Health assessments were conducted under The University of Queensland Animal Ethics Permit no. ZOO/ENT/344/04/NSF/CRL, Moreton Bay Marine Parks Permit no. QS2004/CVL228 and QS2008/CVL228, and Scientific Purposes Permit no. WISP01660304 and WISP14654414.

2.2. Isolation of dugong IgG

Serum (500 μL) from a field-captured, apparently healthy adult male dugong was applied to protein A/G binding columns (Thermo Scientific™ Nab Protein A/G Spin Kit # 89950, Pierce Biotechnology, Rockford, IL, USA) typically used for the isolation of mammalian IgG (Kania et al., 1997; Nollens et al., 2007). Dugong serum was passed through the protein A/G column, and IgG-containing fractions were eluted as per the manufacturer's instructions. The flow-through of unbound dugong serum and three consecutive IgG elution fractions were collected and stored at −20 °C.

2.3. Production of mouse anti-dugong IgG polyclonal Ab (dugong IgG Ab)

The concentration of dugong IgG from each of three consecutive IgG elution fractions was estimated with a NanoDrop Lite spectrophotometer at 280 nm (Thermo Scientific™, Pierce Biotechnology, Rockford, IL, USA) using the mass extinction coefficient for IgG. Purified dugong IgG (100 μg) diluted in phosphate buffered saline (PBS) was mixed with 20 μg of Quil-A® adjuvant (Brenntag Nordic A/S, Frederikssund, DK) for immunization. Three mice were each immunized on Day 0 and again on Day 24 and then two weeks later were

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