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Hematological differences between stingrays at tourist and non-visited sites suggest physiological costs of wildlife tourism

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ABSTRACT

Wildlife tourism alters the environmental conditions in which the focal animal lives, and it is therefore necessary to assess the ability of the animal to adjust to and persist in these novel conditions if the industry is to be sustainable. Here, we report on the physiological responses of southern stingrays (*Dasyatis americana*) which are the focus of intense marine provisioning-tourism in the Cayman Islands. Using stingrays from non-tourist sites about Grand Cayman as a basis for comparison, we show in this natural experiment that tourist-exposed stingrays exhibit hematological changes indicative of physiological costs of wildlife tourism. The novel conditions with which the stingrays must interact include non-natural food, higher injury rates (from boats, conspecifics and predators), and higher parasite loads (from crowding conditions). As a result of this year-round environment, stingrays display sub-optimal health: lower hematocrit, total serum protein concentrations, and oxidative stress (i.e., lower total antioxidant capacity combined with higher total oxidative status). Moreover, they show evidence of attenuation of the defense system: for tourist stingrays only, animals possessing both injuries and high parasite loads also exhibit lowest leukocrit, serum proteins and antioxidant potential, as well as differing proportions of differential leukocytes indicative of suppression (lymphocytes and heterophils) and down-regulation (eosinophils), thus suggesting that the physiological changes of tourist stingrays are in partial response to these stressors. While survival- and reproduction- quantification was not possible in this long-lived marine species, the physiological measures -situated within ecological context, indicate that the long-term health and survival of tourist stingrays have a significant probability of being affected. Consequently, management of the tourism attraction is essential. The indicators chosen in this study reflect general health indices and defense capabilities used across taxa, and represent a tradeoff between ease of collection/analysis and interpretation so that managers can continue the research for monitoring purposes.

1. Introduction

Animals which are the focus of nature-based tourism are exposed to changes in their environment that may influence their survival and reproduction. Their response to these changes depends on whether they perceive humans and their associated activities as a disturbance, predatory threat (Frid and Dill, 2002), refuge, or new food source. Responses within the range of the animal's normal behavioural and physiological repertoire may pose minimal costs (e.g., brown bear, *Ursus arctos*, wildlife viewing; Rode et al., 2006), and in some cases animals can alter their life-history traits to take advantage of the novel conditions created by tourists (Alaskan grizzly bear, *U. arctos*, wildlife viewing; Nevin and Gilbert, 2005). If, however, the new environment causes animals to shift their energetic balance at the cost of maintaining homeostasis, there may be negative impacts on the animal's reproductive effort, survival, and health (e.g., yellow-eyed penguin, *Megadyptes antipodes*, viewing; Ellenberg et al., 2007), particularly for animals exposed to persistent conditions of tourism activities.

Several significant challenges arise when determining the impacts of tourism on marine animals, particularly those that spend their entire life cycle confined to marine waters (unlike seals or penguins). First, marine organisms that do not depend on some above-water-surface resource are often difficult to access and/or observe. The measurement of reproductive success is not always feasible due to the existence of communal nursing grounds or the complete absence of parental care. Similar to terrestrial organisms that are the focus of wildlife tourism, many marine species are long-lived so that tourism effects may be manifested only in

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the long-term, and have large home ranges and migrate over long distances making monitoring and population estimates difficult. Finally, the lack of control populations or baseline estimates for comparison hampers the effectiveness of long-established conservation indicators.

As a result, most studies on the impacts of marine wildlife tourism focus on behavioural changes of the focal species, rather than assessing traditional indicators in conservation biology and wildlife management (animal abundance, food habits, home range size, reproductive success and survival rates; although see Bejder et al., 2006a for an exception). There are difficulties, however, in using deviations in animals' behavioural repertoires to establish cause and effect and/or to demonstrate net cost (Orams, 2004). For instance, many tourism-impact studies rely on wildlife avoidance movements to ascertain energetic costs (Williams et al., 2006), or to establish effective buffer zone distances around viewed animals (Davis et al., 1997). However, sites where avoidance responsiveness is high are not necessarily sensitive areas in need of greater protection; animals in good energetic condition may adopt risk-averse behaviours and initiate avoidance early, whereas animals in poorer condition remain if the cost of escaping is too high (Gill et al., 2001). Alternatively, short-term behavioural responses are insufficient indicators of impacts of anthropogenic disturbance, as moderated responses may not be attributable to habituation but rather due to the absence of sensitive individuals which have already left (Bejder et al., 2006b; Ellenberg et al., 2006).

To fully determine the impacts of tourism, it is imperative to quantify the organism's ability to persist in face of novel selection processes in altered environments (Reznick and Ghalambor, 2001; Stockwell et al., 2003). However, in the absence of the ability to actually determine persistence (i.e., survival and reproduction), a promising alternative or complement to behavioural methods is the use of physiological indicators, the changes in which may be indicative of altered survival and reproductive capabilities. For instance, physiological trade-offs arise when animals have limited resources to allocate between competing life-history traits (Stearns, 1992). Therefore, changes in animals' physiological state may indicate that some important change in their environment has occurred, as well as signify resultant or potential costs. When used in conjunction with other fitness measures, physiological tools can enable the development of effective countermeasures (Hofer and East, 1998; Wikelski and Cooke, 2006) to the effects of wildlife tourism. Indeed, in the absence of population, reproductive and survival estimates, physiological methods are also often the only tools available to assay the perception by an animal of its environment (Wingfield et al., 1997). Moreover, recent advances towards an integrated ecosystem approach to conservation and management have included organismal physiological adaptation as an important link in understanding the relationship between individual- and population-level plasticity (Stevenson et al., 2005); and marine resource management and conservation initiatives are calling upon 'conservation physiology' (Wikelski and Cooke, 2006) to improve fisheries, top pelagic predator conservation (Block, 2005; Young et al., 2006), and in determining the effects of climate-change induced marine acidification (Widdicombe and Spicer, 2008).

Wildlife-tourism impacts on animal physiological defenses have been receiving attention, with recent advances being made. Studies have demonstrated that Galapagos marine iguanas, *Amblyrhynchus cristatus* (Romero and Wikelski, 2002), and adult Magellanic penguins, *Spheniscus magellanicus* (Fowler, 1999), seemed to habituate to tourist disturbances as measured by the stress hormone corticosterone. The chicks of the hoatzin, *Opisthocomus hoatzin*, however, had lower body mass and higher mortality (Müllner et al., 2004), and yellow-eyed penguins (*M. antipodes*; Ellenberg et al., 2007) had higher chick mortality and lower fledgling weight as a result of tourist visitation, using the same hormone as a titer for disturbance. Incubating Royal penguins, *Eudyptes schlegeli*, displayed higher heart rates in the presence of tourists, more so than in the presence of predators (Holmes et al., 2005), and common wall lizards, *Podarcis muralis*, in tourist areas exhibited lower body condition, a higher infection to ticks, lower cell-mediated immune response, and consequently reduced reproductive output (Amo et al., 2006). The ability of physiological measures to reflect health state and predict survival and reproduction of animals exposed to wildlife tourism is therefore immensely effective, and these physiological markers prove reliable tools for evaluating environmental changes including those imposed by tourism. Although conservation physiological approaches have been applied in terrestrial wildlife-tourism settings, we know of no studies to date which have examined animal physiological responses to wildlife tourism confined to the marine environment.

Here, we investigate the physiological responses of the southern stingray (*Dasyatis americana*), the focus of intense tourism activity in Grand Cayman. 'Stingray City Sandbar' (SCS) is an internationally-known tourist attraction approximately 7740 m^2 in area and located in a shallow sound along the island's north coast that began operating in 1984. Year-round, up to 2500 tourists from 40 tour boats can be simultaneously present at any one time at the sandbar feeding, touching, and holding stingrays as part of their marine tourism experience (Shackley, 1998). An estimate of 150 stingrays of both sexes simultaneously aggregate (southern stingrays are normally solitary foragers) at SCS to feed on squid, a non-natural food item, provided by tourists. Corcoran (2006) found that the Grand Cayman tourist stingrays have altered their behaviours in response to the provisioned food including a reduced activity space, strong and persistent site fidelity, and a shift to diurnal behaviors in comparison to stingrays from non-tourist sites. A comparison in serum fatty acid profiles between tourist and non-tourist stingrays suggested that squid is the major food item in the diet of the SCS animals (Semeniuk et al., 2007). Semeniuk and Rothley (2008) have found that as a result of this feeding regime, SCS has now become a permanent habitat for a large population of stingrays which are more likely to have lower body condition (measured as residuals of length–weight relationship), be injured by boats and predators, be susceptible to ecto-dermal parasites, and be engaged in intense interference competition (in the form of conspecific bite marks).

Although behavioural changes have been noted in the SCS stingrays, it is inconclusive whether they represent long term costs to the animal. Our decision to use physiological indicators was motivated by several factors: comparisons of population size with control populations could not be performed due to the very low recapture probabilities of solitary, control stingrays; reproductive effort (fecundity and pup survival) was not measurable as stingrays give live birth in communal pupping areas around the island; and the southern stingray has an estimated longevity of 26 years (Henningsen, 2002), and therefore mortality was not readily observable. Accordingly, physiological indicators were chosen to reflect the capability of stingrays to persist in response to their altered behaviours, non-natural diet, and grouping costs that result from interactions with tourists. Our hypothesis is that group-living stingrays at the tourist site will exhibit differences in their hematological parameters that are indicative of increased physiological costs, in comparison to solitary stingrays from non-tourist sites. The indicators measured include general-health and defense-system parameters: hematocrit (Hct), leukocrit (Lct), total serum protein concentration (Tsp), differential white blood cell counts, and antioxidant capacity (TAC) and oxidative status (TOS). We therefore predict that tourist-exposed stingrays will show evidence of reduced general health (lowered Hct and Tsp), immunosuppres-

sion (Lct and white blood cell counts) and oxidative stress (low TAC and high TOS) due to the long-term ecological conditions to which they are exposed. We discuss whether the physiological changes represent costs to the stingray, what consequences, if any, they may have on the long-term fitness and survival of the stingray population, and conclude with implications for wildlife management.

2. Materials and methods

2.1. Study species and study site

The southern stingray is a long-lived, common inshore ray frequenting tropical and subtropical shallow bays of the Southern Atlantic Ocean, Caribbean and the Gulf of Mexico. It is an opportunistic forager, feeding on a varied diet of crustaceans and teleosts, and to a lesser extent, on molluscs and annelids (Gilliam and Sullivan, 1993). Although southern stingrays inhabit all shallow bays around the Cayman Islands, it is only in the vicinity of SCS that these stingrays can be found year-round in a dense aggregation of individuals of both sexes. This amassment results from the unregulated quantity of tourist-provisioned squid (*Illex* and *Loligo* spp.), a non-natural diet item shipped in from the North Atlantic and North Pacific (Semeniuk pers. obs., Gina Ebanks-Petrie Director, Cayman Islands Department of Environment pers. comm.). The feeding opportunities (daily, except during the summer months when weekends are excluded) last from early morning until mid afternoon as tour boats continuously deliver tourists (mainly cruise line passengers) for an average 45 min visit to SCS. As a result of this regime, and with no visitor management in place since the site's inception, nearly 170 individuals have been tagged between 2002 and 2005 with a mean yearly recapture rate of 92.5% (0.03 SD; CADS unpublished data, Corcoran, 2006), reflecting their long life span, as well as indicating very strong temporal and spatial fidelity to the feeding site.

We captured immature and adult stingrays at SCS and from three control, non-tourist sites on the southern (opposite) and eastern side of Grand Cayman during May–July 2004 and October–November 2005. Stingrays from the non-tourist sites do not interact with the tourists in SCS (based on acoustic-telemetry tracking data; Corcoran, 2006). Tourist stingrays are accustomed to human presence and were easily captured by hand when they approached for food. Once caught, a stingray was placed in a landing net (1 m diameter) and transferred into a seawater-filled canvas pool (4 m^2) aboard a 24 ft long, 225 hp dusky boat. Control stingrays from non-tourist sites were located visually from atop a 14 ft long 45 hp double hull boat, encircled in a hand-drawn seine net (30 ft long), guided into a landing net, and transferred aboard into the holding pool (average time from first sight to capture: 15 min). Once transferred, binder clips were placed over the barb on the stingray's tail for protection. We then, in an average of 13 min (range: 5–36) from when the stingray was captured, collected blood, and recorded the stingray's identity (stingrays that did not already possess an identification tag were tagged with a passive integrated transponder – PIT), weight, disc width, injuries, dermal parasites count (in the spiracles), and conspecific bite marks (counted in 2004 and noted in 2005). Because this study is part of an overall larger research program investigating the general, physiological and immunological impacts of stingray-provisioning tourism, different indicators were analyzed from different yearly sampling occasions. Due to the stingrays' strong site fidelity and longevity, and the consistent environmental conditions, we did not expect significant yearly differences within tourist and non-tourist groups. For all stingrays (2004 and 2005), blood was drawn from the caudal vein using 21G × 1.5 in. needles into 3 mL serum vacutainers, and samples were kept chilled until their return to the wet lab at Georgetown, C.I. where they were immediately centrifuged. The separated serum was then stored at −70 °C. In 2004, blood samples (ca. 100–150 μl) were collected into two heparinized micro-capillary tubes from the vacutainers upon immediate blood withdrawal and kept cool until centrifugation a few hours later for hematocrit and leukocrit measurement. In 2005, blood smears were made in duplicate on microscope slides from freshly drawn blood, and slides were allowed to air dry. Upon completion of the stingray examination, animals were placed back into the landing net, their tail clip was removed, and they were gently returned to the water. Released, tourist-fed stingrays usually resumed feeding at once, while stingrays from the non-tourist sites swam away from the immediate area. We present data only for the female stingrays, as just 31 (18%) of the 172 tagged stingrays at the tourist site were males. Moreover, as the tourist attraction is currently undergoing ecological (and social) management directives, we focused our research on females as this sex will be the major recipients of any management actions. In addition to their higher relative abundance, females are live-bearers, nourish their embryos via uterine nourishment (i.e., matrotrophy), and have associated low fecundity, thus making females the more instructive target for monitoring purposes as they make superior indicators of change.

2.2. Hematological parameters studied

2.2.1. Hematocrit, leukocrit and total serum protein from 2004 sampling

Hematocrit measures the relative amount of red blood cells in total blood volume, and reflects the intensity of oxygen transport (via hemoglobin in the red blood cells) into tissues (Birchard, 1997). Low values are indicative of bacterial or parasite infections, starvation (Ots et al., 1998), or a scarcity of some micronutrients such as iron, copper, and vitamin B12 (Cho, 1983; Sturkie and Griminger, 1986). Leukocrit, an indicator of the fraction of white-blood cells in total blood volume, can suggest a possible pathogen infection if values are high, or stress-induced immunosuppression if values are low (Barton et al., 2002). Circulating proteins in peripheral blood, measured as total serum proteins, are thought to be an index of total protein reserves in an animal (blood proteins are in a dynamic equilibrium with tissue proteins) and therefore can be used to assess dietary inadequacies. Other vital biological functions of Tsp include: (1) maintenance of osmotic pressure; (2) transport of minerals, hormones, lipids, catabolites and drugs; (3) defense against infection (accumulation of antibodies responding to antigen of bacterial or viral origin); (4) blood clotting and lyses of fibrin; and (5) enzymes and inhibitors of enzymes (Silverman et al., 1986; Řehulka et al., 2005). The time-course response of these parameters in indicating condition/nutritional effects is relatively more rapid (e.g., weeks–months) in comparison to the other parameters measured in this study (Barton et al., 2002).

2.2.2. Leukocytes from 2005 sampling

Differential white blood cell counts determine the percentage of each type of white blood cell in an animal's peripheral blood. The three types of leukocytes (lymphocytes, granulocytes (heterophils and eosinophils), and monocytes) in elasmobranchs (sharks and rays) each have different functions. Lymphocytes (of both the B- and T-types) are found in elasmobranch peripheral blood, and function the same way as in mammalian systems, namely, in being responsible for the production of antibodies (immunoglobulins) and cell-mediated immunity. Heterophils, the most actively phagocytic and pinocytic cells in elasmobranchs, can increase in number resulting from infection, disease, and stressful conditions. Eosinophils, mildly phagocytic, play a role in the control of parasite infection and are involved in immune responses to a variety of

antigens. Monocytes are involved in non-specific immune responses and are highly phagocytic; they also play a role in inflammation and accumulate at the site of injury or infection (Stoskopf, 2000; Luer et al., 2004). Thrombocytes have also been included in our count as 'white-blood cells', as they are speculated to play a role in immune function (phagocytosis), in addition to their blood-clotting function (Walsh and Luer, 2004. The differential cell count reveals if these white-blood cells are present in a normal distribution, or if one cell type is increased or decreased. This information can help identify sources of altered health, as differential cell counts have limited sensitivity and are relatively insensitive to observer-induced biases (Ochs and Dawson, 2008). Substantial alterations in immune status are therefore necessary before significant changes are observed in the relative percentages of white blood cell populations (Gelsleichter et al., 2006).

2.2.3. Oxidative stress from 2005 sampling

The last physiological response investigated was oxidative stress. Cellular metabolism generates reactive oxygen (and nitrogen) species (ROS) that can damage cell structures, deplete energy, and cause early apoptosis (programed cell death). To counteract the harmful effect of ROS, organisms rely on antioxidants in the form of endogenously produced enzymes and low-molecular weight molecules, and exogenous, food derived antioxidants (Hõrak et al., 2007). Oxidative stress results when there is an imbalance between the production of ROS and the biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage. Oxidative stress can occur during times of environmental stress and/or high energy demand, and these processes are associated with the appearance of and increase in the severity of many diseases (Martínez-Álvarez et al., 2005). The processes that lead to the occurrence of oxidative stress vary significantly over large gradients and at different temporal scales in many environmental factors (Lesser, 2006); however, a build-up of oxidative stress in excess over the organism's lifespan is hypothesized to contribute to early ageing and shortened life span (Finkel and Holbrrok, 2000). Thus, to maintain proper cellular homeostasis, a balance must be struck between reactive oxygen production and consumption by antioxidants. Determination of a system's capability to prevent oxidative stress is accomplished by measuring total antioxidant capacity (TAC) as well as total oxidative status (TOS), and contrasting the magnitude of the ratio under differing environmental challenges.

2.3. Laboratory analyses

2.3.1. Hematological preparation

After coagulation on ice for 4–6 h, blood samples in vacutainers were centrifuged at 5500 rpm for 10 min. Serum was separated from sedimented cells, aliquoted into Eppendorf tubes, and frozen at −70 °C. Serum samples were then transported on dry ice to Simon Fraser University for subsequent analysis of total serum protein (Tsp) and TAC/TOS. Microcapillary tubes (two per individual) were centrifuged for 5 min at 11,500 rpm, and hematocrit (Hct) and leukocrit (Lct) were twice measured for each tube with a sliding caliper to the nearest 0.1 mm (coefficient of variation in duplicated measurements: 2.4% and 7.1%, respectively). Averages are reported. Blood smears prepared for determining the contribution of different leukocyte cell populations were stained with Wright's–Giemsa stain (Sigma Chemical Co. St. Louis, MO) and shipped to Florida International University for quantification.

2.3.2. Serum protein quantification

The total protein concentration in the sera was determined by the Bradford protein (BioRad, Hercules CA). Briefly, 20 μl of the diluted sera were placed in the flat bottomed 96-well plate in triplicate and the protein concentration determined following the manufacturer's instructions. The optical density was read at 595 nm in a plate reader. Protein concentrations (mg/mL) were obtained from a standard curve made with gamma-globulin, as elasmobranchs are not thought to possess albumin (the typical standard; Metcalf and Gemmell, 2005), and we wished to use a purified preparation of the protein being assayed for comparative purposes.

2.3.3. Differential white blood cell count

Differential immune cell counts were performed using a compound microscope via oil immersion (1000×). Circulating concentrations of total white-blood cells (WBCs) were performed by a single observer and estimated by enumerating the number of leukocytes (and thrombocytes) per 100 cells (red plus white) in duplicate and subsequently averaged. In a separate count (again, performed in duplicate on different sections of the microscope slide and then averaged), the contribution of each leukocyte population (lymphocytes, heterophils, monocytes, eosinophils and thrombocytes) was determined as a percentage per 100 white-blood cells counted (r^2 between mean duplicate counts = 0.98).

2.3.4. Total antioxidant capacity/total oxidative status

Total antioxidant capacity (TAC) was measured according to a modification of the commercially available Randox TEAC (Trolox equivalent antioxidant capacity) assay (Erel, 2004). The reduced ABTS molecule (a free radical standard) is oxidized to ABTSS+ using hydrogen peroxide in acidic medium (the acetate buffer, 30 mmol L^{-1}, pH 3.6), where the colour is spontaneously and slowly bleached. Antioxidants present in the sample accelerate the bleaching rate to a degree proportional to their concentrations. This reaction can be monitored spectrophotometrically and the bleaching rate is inversely related with the TAC of the sample. The reaction rate is calibrated with Trolox, a water-soluble vitamin E analogue widely used as a traditional standard for TAC measurement assays. Samples were tested in triplicate and assay results are expressed in mmol Trolox equivalent/L in reference to a standard curve.

The total oxidative status (TOS; Erel, 2005) assay uses two reagents: ferrous ion-o-dianisidin complex and xylenol orange. Oxidants present in the sample oxidize the ferrous ion-o-dianisidin complex of the reagent to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion then makes a coloured complex with xylenol orange in the acidic medium. The colour intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample, which was tested in triplicate. The assay is calibrated with hydrogen peroxide and the results are expressed in terms of micromolar hydrogen peroxide equivalent per liter (μmol H_2O_2 equivalent/L^{-1}).

2.4. Statistical analyses

The interpretations of the hematological parameters requires discretion, because a particular pattern can arise for a variety of reasons (Adamo, 2004; Matson et al., 2006). Additionally, the bi-directionality of change in certain parameters necessitates the incorporation of ecological context in the form of intrinsic (i.e., 'condition') and extrinsic (i.e., tourist 'treatment') variables (Beldomenico et al., 2008). Therefore, for the aggregate health indicators (Hct, Lct, Tsp and oxidative stress) we created a 'fitness' variable to assign a general health score to individual stingrays. This was done by applying a principle components analysis to stingray parasite load, number of injuries (fresh wounds and other injuries, 2005) and number of conspecific bite marks (2004) to generate a single principle component (PC) of stingray fitness for each

year. This action allowed us to then use the PC as a covariate in our models to ascertain whether an animal simultaneously exhibiting high amounts of injuries *and* parasites (i.e., poorer condition) determined to a certain extent the pattern of stingray general health. To investigate the relationship between the PC and its original variables to define the directionality of the PC scores, we used linear and quadratic curve estimation regressions.

Hematocrit, Lct, Tsp and TAC/TOS responses were each analyzed in a least-squares, multiple regression model using treatment (non-tourism vs. tourism) as a factor variable, and fitness PC, disc width (cm) and body size metric (residuals of log-transformed disc-width and weight variables) as continuous, independent variables. Starting with all of the independent variables, we used backward deletion of least significant terms until only significant terms remained. A Student's *t*-test was used to determine if total WBC counts differed among sites: (1) when all five cell types are grouped and (2) when thrombocytes are not included in the cell counts.

Because different leukocytes have cell-specific responses to differing stressors – for example, dermal wounding promotes lymphophilia (Boyce et al., 2000) whereas parasite infection decreases lymphocyte circulation (Feldman et al., 2000), we explored how number of injuries and parasite loads as individual covariates influenced the proportion of individual cell types between treatments, using least-squares, multiple regression. Lastly, we used linear and quadratic curve estimation regressions within treatments to investigate any (non)linear relationships between stingray physiological responses and body condition and stingray fitness PC, as we wanted to more fully explore the effects of the treatment-specific differences in condition, parasite loads, and injuries. We performed all statistical analyses using JMP IN 6.0 (SAS Institute Inc., 2005) employing two-tailed tests of probability. We report the significance at both the 5% and 10% levels following the recommendations of Field et al. (2004) and Fidler et al. (2006), who caution against interpreting non-statistical results (at the 5% level) in null-hypothesis significance testing as 'no effect' in conservation science. As per their recommendations, we also report Hedge's effect size and power for the results significant at the 10% level. As appropriate, original variables (both dependent and independent) were transformed to meet the assumptions of normality for parametric tests, and then back-transformed (dependent variables) to obtain the mean (±SE).

Lastly, the effect of capture time was investigated for the 2005 physiological parameters as accurate times were recorded in this sampling event. We found no significant effect of capture stress on the differential leukocytes nor total antioxidant capacity, with the exception of monocytes and total oxidative species (TOC), in which the trend found was opposite to expected; i.e., control animals with longer capture times had fewer monocytes and TOC, suggesting these animals were fit specimens, capable of evading capture more easily. We therefore did not correct for capture time, as we felt it had no direct effect on the stingrays' physiology, nor did it greatly contribute to improving the overall *r*-squared values. We were also unconcerned about capture stress on the Hct, Lct, and Tsp parameters from 2004, as previous studies demonstrated no capture or restraint stress effects with regards to hemodilution or hemoconcentration in elasmobranchs (Hoffmayer and Parsons, 2001; Manire et al., 2001).

3. Results

3.1. Stingray condition and fitness metrics

Although size ranges overlapped ($range_{tourist}$ = 37–130 cm; $range_{non\text{-}tourist}$ = 40–104 cm), female stingrays were significantly larger, both in disc width and weight, at the tourist site than females sampled from the non-tourist sites for both the 2004 and 2005 years (25–75% median $quartiles_{tourist}$ = 99–100 cm, 32–33 kg; $quartiles_{non\text{-}tourist}$ = 76.5–78 cm, 14.5–15 kg (Semeniuk and Rothley, 2008). However, despite the larger size, growth trajectories (i.e., log-transformed disc width and weight relationship) were not significantly different between the two locations, indicating that tourist-fed stingrays are not significantly heavier for a given size. Body condition, however, measured as residuals of the logarithmic relationship between disc width and weight for the tourist and non-tourist sites combined, was lower for stingrays at the tourist site (Semeniuk and Rothley, 2008).

For the 2004 data year, a principle component analysis on the correlation between number of parasites, injuries (e.g., predator-detection/defense, susceptible-to-infection, and motility-impairment injury types), and conspecific bite marks (all corrected for stingray disc width) returned a significant factor with an eigenvalue >1 that explained 44.6% of the original variation. This stingray fitness metric loaded positively for parasite load and injuries, but negatively for conspecific bite marks, so an intermediate score corresponds to a stingray simultaneously exhibiting intermediate amounts of parasites, injuries, and bite marks; a low score denotes a stingray in good condition, and a high score represents poor condition with respect to a stingray possessing high injuries and parasites. The 2005 PC analysis on the correlation between number of parasites, 'fresh injuries' (including open wounds from conspecific bite marks) and other, non-fresh injuries (e.g., predator-detection/defense and motility-impairment injury types; again, all corrected for disc width) returned a significant factor with an eigenvalue of 1.28 that explained 42.7% of the original variation. The 2005 stingray fitness metric loaded positively for both injury variables, and had a positive quadratic relationship with the parasite-load variable; therefore, an intermediate score denotes low parasites and an intermediate number of injuries, and a high score signifies a poor-condition stingray with a high number of parasites and injuries (open wounds and other).

3.2. Relationships between physiological indicators and tourism treatment, stingray fitness

3.2.1. Hematocrit

Tourist stingrays had significantly lower hematocrit than non-tourist stingrays ($F_{1,102}$ = 9.13, P < 0.005; $n_{tourist\ \ stingrays}$ = 67, $\bar{x} = 0.294 \pm 0.004$ SE; $n_{non\text{-}tourist\ \ stingrays}$ = 37, $\bar{x} = 0.312 \pm 0.005$ SE; Fig. 1). There were no linear or quadratic effects nor second-order interactions of stingray size, body condition, or fitness metric 2004 on the proportion of packed red cell volume overall or within each treatment (all P's > 0.143), and they were subsequently removed from the model.

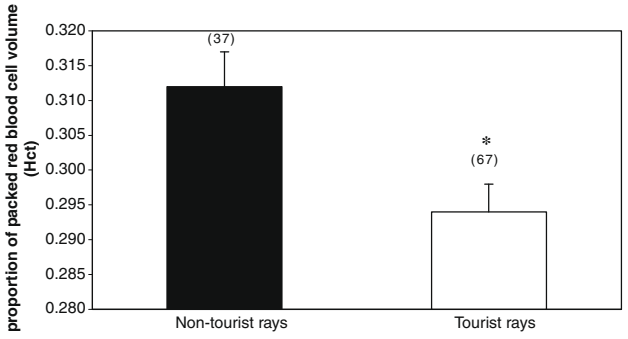

Fig. 1. Bar graph showing significant difference (*) in mean (+SE) hematocrit between tourist and non-tourist stingrays.

3.2.2. Leukocrit

There was a highly significant, negative relationship between leukocrit and disc width, even with other variables and their interactions included in the model which were not significant (i.e., treatment, body condition, fitness PC 2004 and their second-order interactions; overall model: $F_{8,69} = 4.67$, $P < 0.0001$; $\beta_{\text{disc width}} = -0.167$, $t = -4.66$, $P < 0.0001$; all other variables $P > 0.18$). After taking the residuals of Lct standardized for stingray disc width, we found no effect of treatment, but a significant, negative linear effect of the fitness metric on residual Lct ($t = -2.03$, $P = 0.046$). Investigating this further, we found the relationship between Lct and fitness was driven by tourist stingrays solely, and displayed a significant, negative linear trend ($F_{1,53} = 5.21$, $P = 0.027$, $r^2 = 0.09$; residual Lct = −0.003–0.002 × fitness PC 2004; non-tourist stingrays: $P = 0.73$; Fig. 2), denoting lowest Lct was associated with highest number of parasites and injuries.

3.2.3. Total serum protein

Both treatment and stingray disc size had a significant effect on total serum protein (Tsp; overall model: $F_{2,108} = 6.57$, $P = 0.002$), with larger stingrays having significantly higher concentrations of serum protein ($\beta_{\text{disc width}} = 13.25$, $t = 2.45$, $P = 0.016$), and tourist stingrays having significantly lower Tsp than non-tourist stingrays ($\beta_{\text{treatment(non-tourist stingrays)}} = 2.05$, $t = 3.49$, $P < 0.001$; $n_{\text{tourist stingrays}} = 70$, least-squared $\bar{x} = 41.2$ mg/mL ± 0.67; $n_{\text{non-tourist stingrays}} = 41$, least-squared $\bar{x} = 45.3$mg/mL ± 0.89; Fig. 3a). No other variables in the model were significant. Taking the residuals of Tsp standardized for disc width, we found a significant, non-linear effect of stingray fitness metric 2004 on residual Tsp for tourist stingrays only (($F_{2,53} = 3.77$, $P = 0.029$, $r^2 = 0.125$; residual Tsp = $-2.38 - 0.23 \times$ fitness PC 2004 + 1.33(fitness PC 2004 − 0.015)^2), with animals which simultaneously exhibited parasites, bite marks and injuries having had the lowest total serum protein concentration (Fig. 3b).

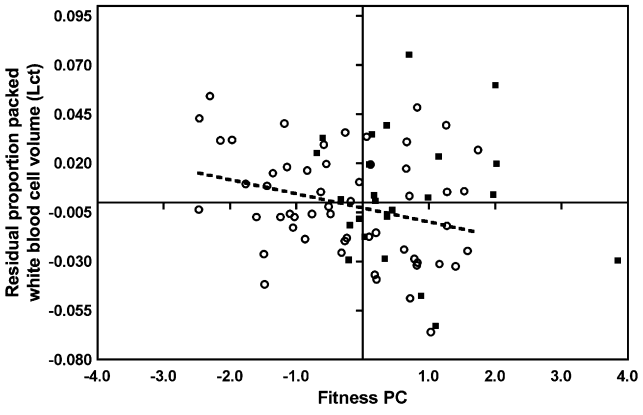

Fig. 2. Negative linear relationship between fitness PC 2004 (a high score denotes high injury and parasite load and low conspecific bite marks) and leukocrit (controlled for stingray size) for tourist stingrays only. No relationship found for non-tourist stingrays. ■ = Non-tourist stingrays; ○ = tourist stingrays; and (- -) = tourist stingray trendline.

3.2.4. Differential white-blood cells

Overall, the proportion of summed white-blood cells (lymphocytes, heterophils, eosinophils, monocytes and thrombocytes) out of the total peripheral blood cell count did not differ between treatments ($t = 0.12$, $P = 0.72$; $n_{\text{tourist stingrays}} = 46$, $\bar{x} = 0.183 \pm 0.008$; $n_{\text{non-tourist stingrays}} = 49$, $\bar{x} = 0.188 \pm 0.007$); however, this non-difference may be attributed to a higher proportion of thrombocytes in tourist stingray peripheral blood, since when thrombocytes were excluded, the proportion of remaining leukocytes in the total white blood cell count was significantly lower at the 10% significance-level in tourist stingrays than in non-tourist stingrays ($t = 1.68$, $P = 0.09$, power = 0.39, effect size = 0.35; $\bar{x}_{\text{tourist stingrays}} = 0.741 \pm 0.01$; $\bar{x}_{\text{non-tourist stingrays}} = 0.776 \pm 0.01$).

3.2.4.1. Lymphocytes. There was a significant interaction effect of treatment and parasite load (overall model: $F_{3,91} = 5.78$, $P = 0.001$; $\beta_{\text{treatment} \times \text{parasite load}} = -0.045$, $t = -3.71$, $P < 0.001$), and a parasite load effect on the proportion of lymphocytes ($\beta_{\text{parasite load}} = -0.027$, $t = -2.25$, $P = 0.027$). Further within-treatment analysis revealed that while lymphocytes decreased with increasing parasites in non-tourist stingrays (linear regression: $F_{1,47} = 17.5$, $P < 0.001$, $r^2 = 0.27$), there was no relationship among tourist stingrays (Fig. 4a), as they maintained a constant and lower ($\text{median}_{\text{tourist stingrays}} = 0.475$ vs. $\text{median}_{\text{non-tourist stingrays}} = 0.52$) proportion of these white-blood cells ($F_{1,44} = 1.27$, $P = 0.27$, $r^2 = 0.03$).

3.2.4.2. Heterophils. Heterophils were significantly and positively affected by stingray size and number of fresh injuries (overall model: $F_{3,91} = 5.72$, $P = 0.001$; $\beta_{\text{disc width}} = 0.34$, $t = 3.25$, $P < 0.001$; $\beta_{\text{fresh injury number}} = 0.11$, $t = 5.08$, $P < 0.001$), and tourist stingrays had a significantly lower proportion of these cell types ($\beta_{\text{treatment}} = 0.06$, $t = 3.81$, $P < 0.001$; back-transformed, least-squared $\bar{x}_{\text{tourist stingrays}} = 0.126 \pm 0.016$ SE; back-transformed, least-squared $\bar{x}_{\text{non-tourist stingrays}} = 0.219 \pm 0.025$ SE). In addition, there were significant interaction effects between treatment and disc width and

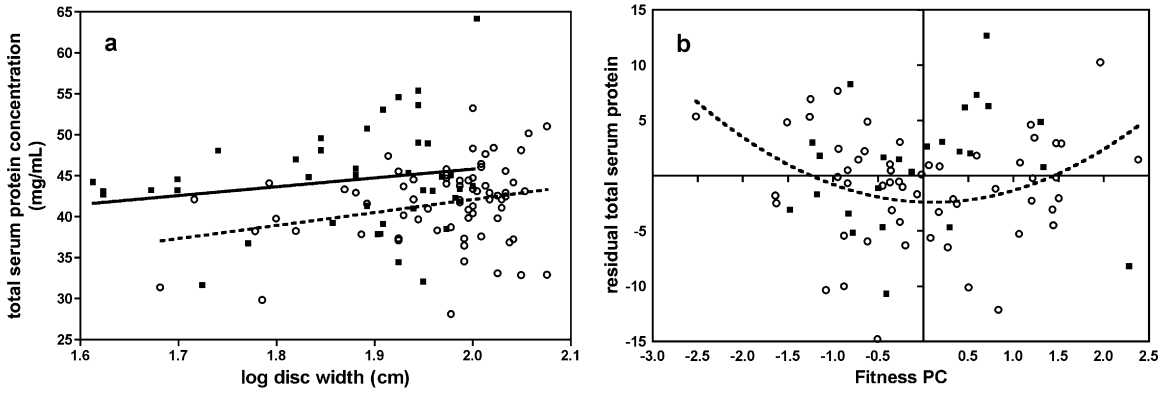

Fig. 3. (a) Negative linear relationship between ($\log_{10}$) disc width (cm) and total serum protein concentration (mg/mL) for both tourist and non-tourist stingrays. (b) Non-linear relationship between fitness PC 2004 (an intermediate score denotes intermediate parasite load and injuries and high conspecific bite marks) and total serum protein concentration (corrected for stingray size) for tourist stingrays only. No relationship found for non-tourist stingrays. ■ = Non-tourist stingrays; ○ = tourist stingrays; (–) = non-tourist stingray trendline; and (- -) = tourist stingray trendline.

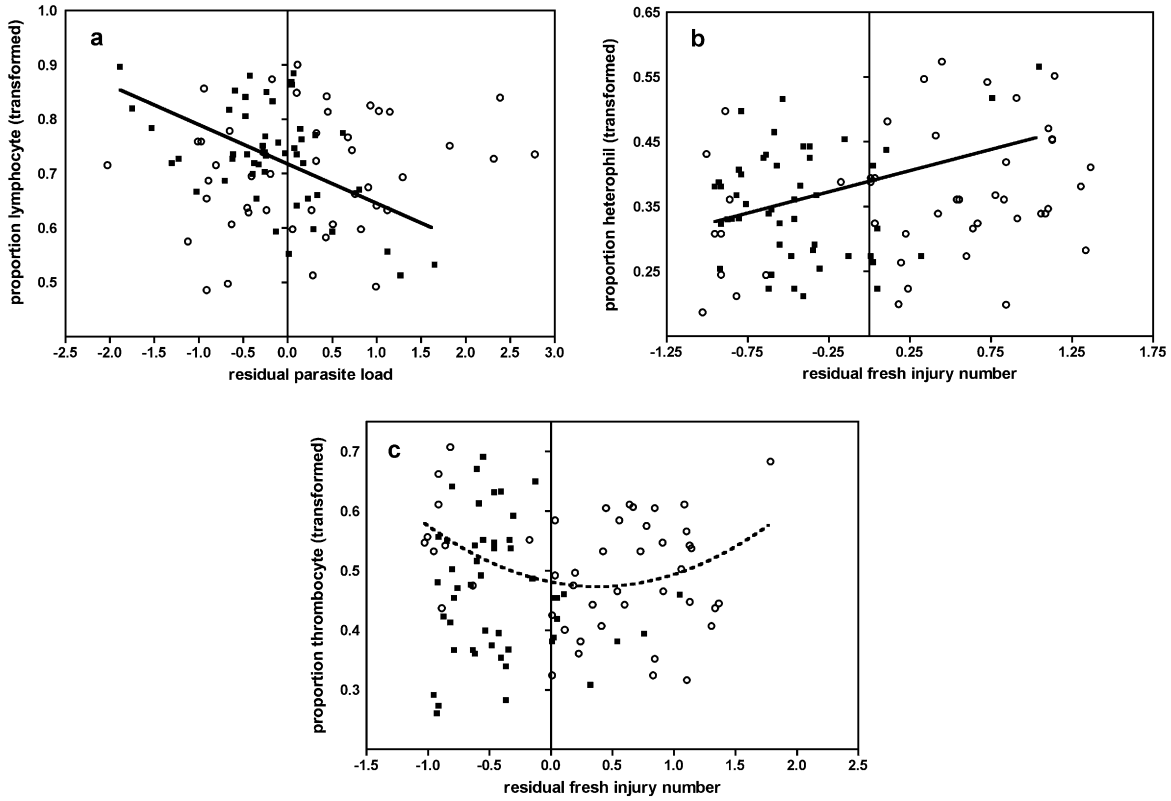

Fig. 4. (a) Negative linear relationship between parasite load (corrected for stingray size, 2005) and proportion of lymphocytes for non-tourist stingrays only. No relationship found for tourist stingrays. (b) Positive linear relationship between injury number (corrected for stingray size) and proportion of heterophils for non-tourist stingrays only. No relationship found for tourist stingrays. (c) Non-linear relationship between fresh injury number (corrected for stingray size, 2005) and proportion of thrombocytes for tourist stingrays only. No relationship found for non-tourist stingrays. ■ = Non-tourist stingrays; ○ = tourist stingrays; (–) = non-tourist stingray trendline; and (- -) = tourist stingray trendline.

fresh-injury numbers ($\beta_{\text{treatment} \times \text{disc width}} = 0.403$, $t = 3.83$, $P < 0.001$; $\beta_{\text{treatment} \times \text{fresh injury number}} = 0.077$, $t = 3.51$, $P < 0.001$). A within-treatment analysis revealed that non-tourist stingrays were more responsive to an increase in the number of fresh injuries than tourist stingrays (non-tourist stingrays: $F_{1,47} = 4.53$, $P = 0.038$, $r^2 = 0.09$, proportion heterophils = 0.389 + 0.065 × fresh injury number; tourist stingrays: $F_{1,44} = 3.48$, $P = 0.069$, $r^2 = 0.07$; proportion heterophils = 0.35 + 0.036 × fresh injury number; Fig. 4b).

3.2.4.3. Eosinophils. The proportion of eosinophils significantly increased with both stingray size and parasite load (overall model: $F_{3,91} = 6.54$, $P = 0.0005$; $\beta_{\text{disc width}} = 0.204$, $t = 3.37$, $P = 0.001$; $\beta_{\text{parasite load}} = 0.021$, $t = 2.77$, $P = 0.007$); also, tourist stingrays had significantly lower proportion of eosinophils than did non-tourist stingrays ($\beta_{\text{disc width}} = 0.024$, $t = 3.27$, $P = 0.002$; back-transformed, least-squared $\bar{x}_{\text{tourist stingrays}} = 0.076 \pm 0.010$ SE; back-transformed, least-squared $\bar{x}_{\text{non-tourist stingrays}} = 0.105 \pm 0.009$ SE).

3.2.4.4. Monocytes. There was a significant effect at the 10% level of treatment only on the proportion of monocytes, with tourist stingrays having a higher proportion of these cell types ($F_{1,93} = 3.56$, $P = 0.059$, effect size = 0.39, power = 0.46; back-transformed, least-squared $\bar{x}_{\text{tourist stingrays}} = 0.027 \pm 0.010$ SE; back-transformed, least-squared $\bar{x}_{\text{non-tourist stingrays}} = 0.020 \pm 0.009$ SE).

3.2.4.5. Thrombocytes. Thrombocyte proportion also had a significant treatment effect, with tourist stingrays having a higher proportion of cells significant at the 10% level than non-tourist stingrays ($F_{1,93} = 3.33$, $P = 0.067$, effect size = 0.38, power = 0.44; back-transformed, least-squared $\bar{x}_{\text{tourist stingrays}} = 0.251 \pm 0.015$ SE; back-transformed, least-squared $\bar{x}_{\text{non-tourist stingrays}} = 0.214 \pm 0.015$ SE). Within-treatment analyses showed that for tourist stingrays only, there was a (non-linear) relationship between the proportion of thrombocytes and the number of fresh injuries, corrected for stingray size, with lowest thrombocyte number corresponding to lowest injuries ($F_{2,43} = 3.17$, $P = 0.05$, $r^2 = 0.129$; transformed proportion thrombocytes = $0.474 - 0.0016 \times$ residual fresh injury number + $0.054(\text{residual fresh injury number} - 0.366)^2$; Fig. 4c).

3.2.5. Oxidative stress

3.2.5.1. Total antioxidant capacity. There was a significant effect of treatment and body condition on the concentration of serum TAC (overall model: $F_{2,91} = 8.48$, $P < 0.001$; $\beta_{\text{treatment}} = 0.056$, $t = 3.69$, $P < 0.001$; $\beta_{\text{body condition}} = -0.631$, $t = -2.33$, $P = 0.022$), with animals from the tourist site having significantly lower concentrations of antioxidants ($n_{\text{tourist stingrays}} = 49$, least-squared $\bar{x} = 0.455 \pm 0.020$ SE; $n_{\text{non-tourist stingrays}} = 45$, least-squared $\bar{x} = 0.565 \pm 0.021$ SE). The negative relationship between TAC and body condition, although significant in the overall model, was driven entirely by stingrays from the non-tourist site ($F_{1,43} = 6.48$, $P = 0.015$, $r^2 = 0.131$; [TAC] = $0.566 - 0.820 \times$ body condition; Fig. 5a), since within-treatment analyses showed no relationship for tourist stingrays ($P = 0.96$). Instead, the fitness PC 2005 variable significantly explained a portion of the TAC concentration in a non-linear way for tourist stingrays only, with highest TAC corresponding to low parasite and intermediate injury numbers ($F_{2,44} = 3.82$, $P = 0.03$, $r^2 = 0.148$; [TAC] = $0.471 - 0.043 \times$ fitness PC 2005 $- 0.028(\text{residual fresh injury number} - 0.052)^2$; Fig. 5b). There was no effect of disc width or higher order interactions.

3.2.5.2. Total oxidative status. TOS concentrations were significantly higher among tourist stingrays (overall model: $F_{2,86} = 4.51$,

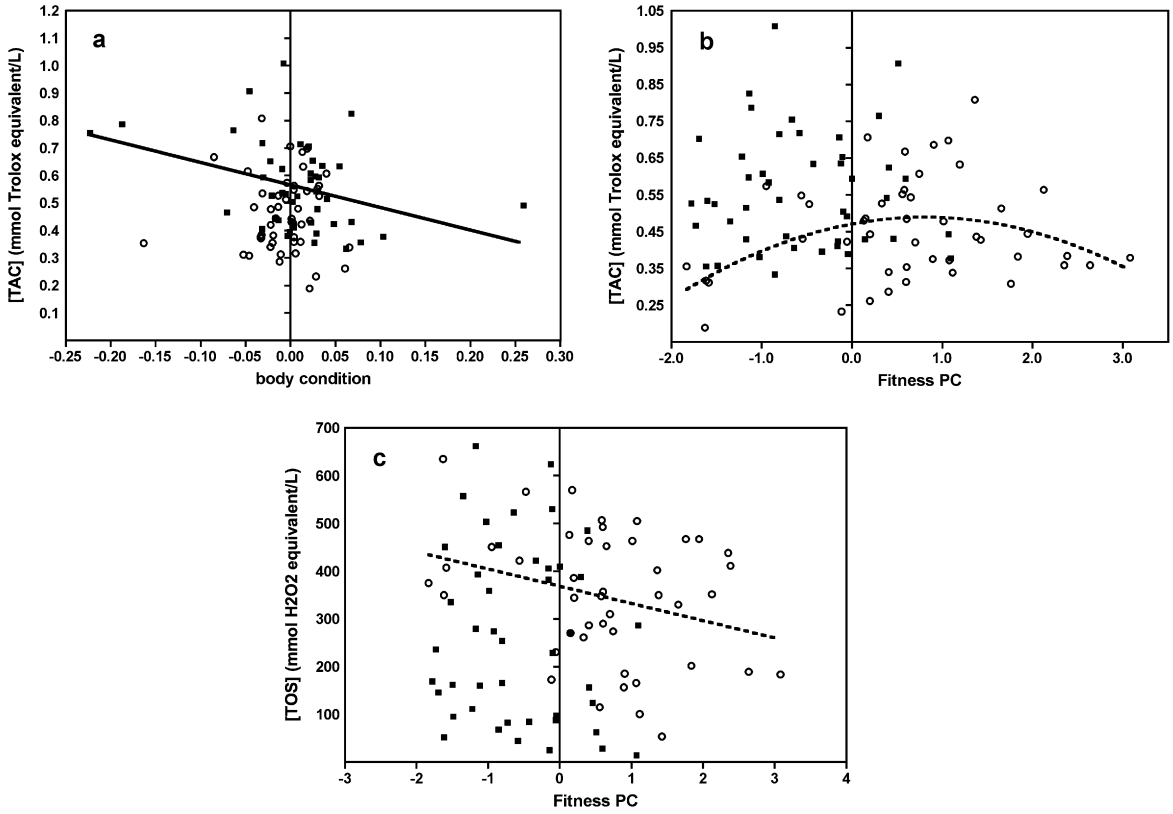

Fig. 5. (a) Negative linear relationship between body condition (residuals of length–weight relationship) and total antioxidant capacity (TAC). No relationship found for tourist stingrays. (b) Non-linear relationship between fitness PC 2005 (intermediate score denotes low parasites and intermediate number of injuries, fresh and other) and TAC for tourist stingrays only. (c) Negative linear relationship between fitness PC 2005 (high score denotes high injuries (fresh and other) and high parasite load) and total oxidant status for both tourist and non-tourist stingrays. ■ = Non-tourist stingrays; ○ = tourist stingrays; (–) = non-tourist stingray trendline; and (- -) = tourist stingray trendline.

$P = 0.014$; $\beta_{\text{treatment}} = 59.65$, $t = 2.99$, $P = 0.0036$; $n_{\text{tourist stingrays}} = 47$, least-squared $\bar{x} = 364.6 \pm 25.88$ SE; $n_{\text{non-tourist stingrays}} = 44$, least-squared $\bar{x} = 245.3 \pm 26.23$ SE), and also decreased with increasing fitness PC 2005 (i.e., highest score representing high parasites and injuries, both fresh and other; $\beta_{\text{fitness PC}} = -32.03$, $t = -1.82$, $P = 0.072$; Fig. 5c). However, this relationship with stingray fitness metrics was driven solely by tourist stingrays ($F_{1,53} = 4.14$, $P = 0.048$, $r^2 = 0.09$; [TOS] = 368.8 − 36.1 × fitness PC 2005; non-tourist stingrays: $P = 0.51$). There was no effect of body condition, disc width, or higher order interactions.

4. Discussion

4.1. Interpretation of results

We explored whether the cumulative effects of the tourist stingrays' altered behaviours, non-natural diet, and associated grouping costs had any hematological modifications indicating that there may be some significant physiological costs being incurred by stingrays in tourist-visited areas. Comparing the condition of stingrays from tourist and non-tourist sites, we found marked physiological differences. Tourist stingrays had lowered hematocrit, lowered total serum proteins, differential leukocrit and leukocyte reactions (adjusted for body size), and exhibited oxidative stress, all of which likely indicate that tourist stingrays are subjected to negative physiological consequences of visitation, and suggest that their ability to persist has been affected. Because the general variation in physiological responses of anthropogenic impacts is attributed to: differences in the predictability of the duration of the stressors, the number and temporal pattern of stressors, the damaged-induced mortality rate from inadequate homeostatic maintenance, the mortality rate from the stressor if no resources are allocated to combat it, and the ability of the organism to recover (Schreck, 2000; McNamara and Buchanan, 2005), it is important to take into account the ecological context of the study system. Accordingly, we also found that parasite loads and injuries (bite marks, fresh wounds and other types) explained a proportion of the variation in our hematological variables, suggesting that the physiological changes of tourist stingrays were in partial response to these stressors.

Stingrays were, on average, larger at the tourist site (although the minimum range overlapped); however, we do not believe size – as a proxy for age – to be the principle driving factor explaining the physiological differences. For hematological variables for which stingray disc width was a significant factor, controlling for size still resulted in significant effects of fitness PC's; moreover, if there was a significant linear relationship between the dependent variable and stingray size in non-tourist stingrays, the same relationship did not hold for tourist stingrays, and was in the opposite direction (for example, as Tsp increased with size in control stingrays, tourist stingrays had lower Tsp). Lastly, previous research by Semeniuk and Rothley (2008) demonstrated that SCS stingrays were equally injured across size categories, and that the largest females did not have the highest number of conspecific bite marks. Therefore, we believe other factors, such as injuries and parasite loads, independent of stingray size, have more of an effect on our measured physiological variables. We acknowledge these variables could not completely explain the observed patterns, and other tourism-related causative factors, while not explored in this paper (e.g., internal parasites and bacterial pathogens, water pollution

(fouled by boat fuel or sunscreens), and increased predation pressure), could also be responsible for the variability in stingray physiology.

Next, the range of times needed to capture and sample the stingrays could have added to the observed data variability. We do not believe this to be of relevant concern, since while we had only rough estimates of capture times from the 2004 sample year, our more precise estimates in 2005 revealed no significant effects on the hematological variables, and in fact, in some instances, were in the direction opposite to what would be predicted if the animals were exercise-stressed (i.e., an increase in monocytes and oxidative stress rather than a decrease; Vider et al., 2001). While we are confident that tourism activity has a significant effect on the physiological state of the focal animals at the tourist site, we also acknowledge that having only one site allocated as a 'treatment' variable may unintentionally overlook other explanatory variables that are not tourism related. For instance, tourist stingrays may be subjected to more polluted conditions (Ebanks-Petrie, 1993) than the control populations, and continual exposure to such conditions may explain at least some of the variation displayed between tourist and non-tourist individuals. Although recent work by Cayman Island research officers monitoring North Sound have measured bacteriological and nutrient levels to be on par with ocean water (John Bothwell, CI Department of Environment, unpublished data), there may still be unknown environmental factors affecting the North Sound stingrays that may be partially contributing to the variation seen, although we still maintain tourism to be the largest contributor. Lastly, the overall low r^2-values for both the control and tourist sites suggest that variation in general stingray physiology is influenced by multiple mediators and systems in a complex and non-linear fashion; and denotes there are enormous individual differences in the response to stressors, based upon the experience of the individual in early and in adult life (McEwen, 2008).

While hematocrit values of elasmobranchs are generally quite low (<1/3 cell volume, Stoskopf, 2000), the lowered hematocrit of tourist stingrays can be indicative of parasites and infection (e.g., Jones and Grutter, 2005). In our model, however, there was no effect of body condition, ecto-dermal parasite load, or injuries on packed red blood cell volume (although this does not negate the possibility of internal parasites). Low Hct values can also be due to reproductive anemia (Williams et al., 2004), caused by reduced physical exercise (Gallaugher et al., 1995); or conversely, increased through capture and handling (Wells et al., 1986). We do not believe these alternative explanations can explain our results. Firstly, the female stingrays we examined were a mixture of sexually mature (>75 cm disc width) and immature in both treatments (Henningsen, 2000), and there was no effect of body size on Hct; therefore reproductive status had no bearing on the results. An acoustic tracking study of stingrays at the tourist site furthermore revealed that tourist stingrays have similar rates of movement ($km\ h^{-1}$) when compared to stingrays from other areas around Grand Cayman (Corcoran, 2006), and consequently, the lowered Hct in tourist stingrays cannot be due to reduced physical activity. Lastly, although non-tourist stingrays required tracking before capture, as mentioned above, studies of the capture and handling of sharks have found no evidence of hemodilution or hemoconcentration in response to capture and restraint (Hoffmayer and Parsons, 2001; Manire et al., 2001). Finally, lowered hematocrit can be caused by a scarcity of micronutrients such as iron, copper, and vitamin B12 (Cho, 1983; Sturkie and Griminger, 1986). Squid, the non-natural diet, is lower in iron and B12 and higher in copper than in shellfish (King et al., 1990; Kongkachuichai et al., 2002), the natural diet on which southern stingrays feed (Gilliam and Sullivan, 1993). Although we have no direct evidence, there is a significant possibility of diet-induced anemia in our system. Regardless of the cause, because lowered hematocrit is an aggregate, general indicator of poor health state and nutritional condition of animals in the wild (e.g., Verhulst et al., 2004; Huitu et al., 2007), we believe it also reflects the general, poorer state of tourist stingrays, as well.

Leukocrit is used as a general indicator to assess health and immunocompetence of a wide variety of animals, and low values can indicate stress-induced immunosuppression (e.g., McLeay and Gordon, 1977). Given the significantly higher numbers of ecto-dermal parasites and injuries of tourist stingrays compared to control, non-tourist animals (Semeniuk and Rothley, 2008), the negative relationship between Lct (corrected for the stingray size) and increasing injuries and parasite load (fitness PC 2004) for tourist stingrays only (Fig. 2) is not surprising. Similarly, the lower total serum protein concentration of tourist stingrays was also partially explained by stingray fitness (2004), with individuals simultaneously possessing parasites, injuries and bite marks demonstrating lowest Tsp (Fig. 3b). Total serum protein is also a general indicator, with low values indicative of a range of health issues such as dietary inadequacies, immune deficiency and disease (e.g., Adams et al., 2003). The low values of Lct and Tsp associated with 'poor' fitness scores, coupled with the incidence of hypoproteinaemia (e.g., Ots et al., 1998), suggest sub-optimal health and a downregulation in the defense mechanism of tourist stingrays.

Further substantiation of altered physiological defenses was found in the white blood cell differentials. The differences in the proportion of the various leukocytes (including thrombocytes) was influenced by stingray size, parasite load, and fresh-injury numbers. Interestingly, the direction and magnitude of these covariates differed between treatments. For instance, the proportion of lymphocytes, which play a role in cell-mediated immunity and antibody production, decreased with increasing parasite loads in non-tourist stingrays, perhaps in favour of the corresponding measured rise in heterophils – phagocytic and pinocytic cells – which increased with fresh injuries (Fig. 4a and b, respectively). In tourist stingrays, however, this same relationship did not hold: the percentage of lymphocytes, while marginally smaller than in non-tourist stingrays, was relatively unresponsive to parasites, and heterophils, which were significantly lower in proportion than in non-tourist stingrays, were not as responsive to the number of injuries. Differences in immune response continued with the other cell types: eosinophils which play a role in parasite and antigen control, expectedly increased with parasite load regardless of treatment, but were still significantly lower in tourist stingrays; and monocytes and thrombocytes (both involved in non-specific immune responses, although the latter has more of a role in blood clotting) were proportionally higher (at the 10% significance level) in tourist stingrays. Within this latter group, thrombocytes were lowest when individuals displayed the lowest number of parasites (Fig. 4c). To sum, it appears that with regards to cell-mediated immunity, the responses of tourist stingrays do not match the suite of responses of control stingrays when exposed to similar, albeit fewer, stressors. There is evidence that some of the physiological responses are indicative of suppression (i.e., low and unresponsive – lymphocytes and heterophils), up-regulation (monocytes and thrombocytes), and down-regulation (eosinophils). Variations in differential cell counts suggestive of immunosuppression have been shown in other studies (see Barker et al., 1994 and Lepak and Kraft, 2008 for examples in teleosts); and in addition to the differential reaction between treatments, the lower ratio of leukocytes to thrombocytes in tourist stingrays (at the 10% level) also suggest that cell-mediated immunity has been attenuated in tourist stingrays.

The final evidence of compromised defenses in tourist stingrays come from the oxidative stress findings. A rise in reactive oxygen species is not necessarily problematic if cells are able to

defend themselves against ROS damage through a compensatory increase in antioxidant potential. In particular, ROS can play a positive role in the activation of protective signaling pathways provided they are not produced in excess, i.e., beyond the capacity of antioxidants to counteract their production (Finkel and Holbrrok, 2000). However, tourist stingrays not only exhibited a significantly higher concentration of total oxidative species, but significantly lower total antioxidant capacity as well (Fig. 5c and b, respectively). The assay used in this study measures small molecule antioxidants (AO) such as ascorbic acid, uric acid, glutathione, and polyphenol AO. Nonetheless, cells in many vertebrates also defend themselves through the use of enzymes such as superoxide dismutases and catalases. Elasmobranchs, however, have a limited enzymatic antioxidant system in their sera, and compensate for this deficiency by relying on small molecular AO instead, such as vitamin K, urea, and glutathione (Rudneva, 1997). Therefore, our results should reflect an accurate assessment of the degree of oxidative stress experienced. Oxidative stress in fish can be caused by nutritional deficiencies, environmental factors, xenobiotics, immune responses to injury, parasite infestations, and increased energy demand and workload (see Martínez-Álvarez et al., 2005 for a review). At the non-tourist sites, animals in the best body condition had lowest TAC; this relationship did not hold for stingrays from the tourist site. Instead, animals that simultaneously possessed the lowest number of parasites (and intermediate number of injuries – fitness PC 2005) had the highest TAC. Additionally, the TOS decrease with a rise in 'poor' fitness PC (i.e., higher parasites, and fresh and other injuries) for tourist stingrays may be speculatively explained by the significant reduction in circulating heterophils (unlike for the non-tourist stingrays) that usually remain in chronic wounds for longer than they do in acute wounds and which produce reactive oxygen species and enzymes (Schönfelder et al., 2005). Regardless, TOS concentrations were still higher overall, suggesting additional sources of oxidative damage – such as the possibility of ischemia, a lack of oxygen from being removed from the water, which can also be a contributing factor to the higher oxidative status of the rays (e.g., Hermes-Lima and Zenteno-Savin, 2002). In combination with lowered TAC, the TOS findings demonstrate a cumulative effect of oxidative stress which can presumably lead to premature cellular ageing and shortened stingray lifespan.

4.2. Sources of physiological costs at 'Stingray City Sandbar'

McNamara and Buchanan (2005) modeled the optimal tradeoff of resource allocation between competing demands of combating a stressor and bodily maintenance, and predicted that the longer the stress period is expected to last, damage to self maintenance (e.g., reduced physiological reserves of essential nutrients, minerals or energy; increased levels of oxidative stress; or reduced condition of protective body covering) will build up to high and unacceptable levels unless resources are put into maintenance and thus fewer into combating the stressor. Consequently, as the duration of the stressor increases, the probability of death from both poor condition and the stressor increase at an accelerating rate, with the stressor becoming proportionately more important as a threat of mortality. This is because the longer the stress period lasts, the more resources are allocated toward maintaining condition.

Our findings provide evidence that in tourist stingrays, which are continually exposed to the impacts of tourism, both self-maintenance and protection from the stressor may be compromised due to their novel environmental conditions: unnatural food, high injury rates and increased parasite loads. Energy and nutrient pools are used by the organism for maintenance, repair, and growth and reproduction. If a stressor degrades the quality or quantity of available food, it may compromise maintenance and repair processes as well as limit the energy available for growth or reproduction (Adams, 1990). Squid, the predominant diet item of fed stingrays, is a non-natural food with a different composition of minerals and vitamins than the natural shellfish diet; and the tourist stingrays have a drastically different ratio of dietary omega-3:omega-6 fatty acids when compared to the ratio found in non-tourist stingray serum (Semeniuk et al., 2007). Essential fatty acid requirements for different fish species reflect dietary and metabolic adaptations to distinctive habitats and ecosystems (Sargent et al., 1999; Bell and Sargent, 2003). The imbalance of essential nutrients and fatty acid ratios from the tourist stingray's diet – important for disease resistance, stress-management and gamete quality – may be hindering the capability of stingrays to allocate their resources into proper maintenance. The low hematocrit, serum protein, and total antioxidant capacity, all influenced by diet, also support this hypothesis.

Likewise, chronically high injury rates and increased parasite loads have influenced, to a certain extent, the majority of the physiological traits measured. The incidence of oxidative stress coupled with dampened physiological responses may resultantly increase the stingrays' vulnerability to additional or future stressors such as modified physicochemical regimes, changes in food and habitat availability, increased predation risk, and increases in infectious pathogens (Schreck, 2000; Barton et al., 2002). The tourist stingrays' altered defense system may also enhance their susceptibility to impacts arising from changing environmental conditions such as oil spills, increased hurricane intensity, and climate change, which may ultimately prove lethal.

4.3. Physiological change and fitness costs

The question remains as to whether the physiological differences detected in this system will translate into negative consequences for reproduction and/or survival. There are few studies that have evaluated the correlations between physiological parameters and fitness components, but the available research supports this likelihood (e.g., Romero and Wikelski, 2001; Verhulst et al., 2004; Cabezas et al., 2007). In our system, while direct evidence is still unknown, the probability of reduced survival seems likely to be quite high. The tourist system may therefore act as an "ecological trap" by enticing the stingrays to exploit an attractant with an immediate payoff (i.e., an easily exploitable food source) that may generate relatively greater fitness costs longer term (Schlaepfer et al., 2002). Alternatively, stingrays can be allocating the surplus resource to faster growth rates and/or reproduction, in which case, the fitness benefits would outweigh the costs. This may certainly explain why stingrays are larger at the tourist site, but would not explain why for a given size, tourist stingrays had smaller mass. In addition, since larger females have larger litter sizes (Henningsen, 2000), females could also be trading off the physiological costs incurred with higher fecundity, although we would have again expected residuals of the length–weight relationship for tourist stingrays to be positive, not negative (Semeniuk and Rothley, 2008). The potential positive impacts of tourism consequently cannot be supported at this time. Accordingly, we purport that based on previous research and current physiological evidence, wildlife tourism for the current Cayman Island stingrays frequenting SCS acts as an ecological trap – i.e., maladaptive decisions resulting in lowered fitness. Furthermore, should the following generations of stingrays born from SCS stingrays seek out the tourist site themselves, the site may then not be sustainable. From a management perspective, long-term monitoring and management of the Stingray City Sandbar is essential.

5. Conclusion

Education and awareness of the risks posed to stingrays are a key tactic in mitigating the negative impacts of tourism (Semeniuk et al., 2009). Furthermore, measures should be taken to alleviate crowding conditions (leading to injuries and parasite transmission) at SCS by limiting the number of people and boats, or by expanding the site into nearby areas to accommodate the current level (although this decision would have to be adaptively monitored). Less food provisioned to the stingrays would also alleviate the stingray aggregation, and ensure that the stingrays resume foraging naturally and solitarily, further away from the tourist site. Additionally, safety devices on boat propellers, such as cages and guards, can also aid in reducing injuries. We explore how these management plans can affect stingray population size and life expectancy in a forthcoming simulation study on the system dynamics of the tourist–stingray relationship (unpublished data).

The discrepancies detected among different physiological indicators when assessing the physiological and condition-related indicators of environmental impacts emphasize the importance of using multiple single indicators (Adamo, 2004; Matson et al., 2006) and of an appropriate control (Barton et al., 2002) when defining best measures for fitness. This undertaken in our study, we also employed indicators that varied in their ease of collection and interpretation, and those that are fairly robust to capture and handling, to allow for the reproduction of our methods by managers for monitoring purposes. Future assessment should consider baseline (control) as well as tourism-induced parameters for key monitoring purposes, integrating both physiological and general fitness (injury rates, open wounds, parasite loads) indicators as a basis for limits of acceptable change. We duly acknowledge that while we tout the advantages of investigating stress-physiology in marine wildlife over behavioural studies, we understand that there exist some complications involved in procuring the data. Whenever possible, however, finer scale and multi-level analyses of disturbance effects will provide a more complete understanding of the actual costs to the animal, especially in the absence of long-term population data.

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