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Short communication

Distribution of leukocytes as indicators of stress in the Australian swellshark, *Cephaloscyllium laticeps*

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ABSTRACT

Differential leukocyte counts were taken of blood smears collected from laboratory stressed adult Australian swellsharks *Cephaloscyllium laticeps*. We calculated the granulocyte (combined count of heterophils and neutrophils) to lymphocyte (G/L) ratio to use as a new physiological indicator of stress for sharks. Animals were captured and stressed using commercial fishing gear (monofilament gillnet and mid-water longline) in a laboratory setting, with blood samples collected prior to capture and at predetermined intervals during a subsequent 72 h recovery period. There was a significant increase in the G/L ratio of 291.14 ± 54.13% at 72 h post-capture during recovery from the 6-h gillnet capture plus 15-mintue air exposure. Six hours of longline capture plus 15 min of air exposure also evoked a significant increase in the G/L ratio of 490.32 ± 294.25% (24 h post-capture) and 590.53 ± 277.65% (72 h post-capture). There was no significant change in the G/L ratio for control sharks that did not undergo capture stress but that experienced an identical blood-sampling regime as captured animals. Our study presents findings of stress-induced changes in leukocyte distribution within the peripheral blood of a shark species brought on by lymphopenia (decrease in lymphocytes) and granulocytosis (increase in granulocytes), and confirms this as a useful measure of the relatively rapid onset of stress in these animals.

1. Introduction

The differential leukocyte count has been used as an indicator of physiological stress in a number of vertebrate taxa, including reptiles [1], amphibians [2] and teleosts [3,4]. Most commonly applied to birds, particularly in the poultry industry [5,6] and in ecological field studies [7–9], the leukocyte differential count provides insight into an animal's investment into defence against infection and disease [10–12]. The differential leukocyte count is the classification of the most common circulating white blood cell types in the peripheral blood of an animal, typically granulocytes (neutrophil, heterophil and eosinophil) and lymphocytes. A change in circulating leukocyte proportions defined as lymphopenia (a decrease in lymphocytes) and granulocytosis (an increase in neutrophils and heterophils) can be recorded using the differential leukocyte count, and with a ratio of heterophils/neutrophils to lymphocytes, expressed as the granulocyte to lymphocyte (G/L) ratio. An increase in the G/L ratio is indicative of an individual animal (a shark in this instance) experiencing physiological stress and altering its immune strategy for defence against infection [7,9].

Interpretation of changes in leukocyte proportions must be carefully considered when dealing with relative and absolute ratios. In this study, we only infer an effect of treatment from changes in G/L ratio over time within an individual shark. This change in leukocyte proportions within the peripheral blood of a shark indicates the body is responding to a stressor (i.e., fishing capture and handling) which risks reducing the animal's fitness. By decreasing the numbers of circulating lymphocytes and trafficking them to epithelia of gills, skin and intestine (sites of greatest potential for foreign entry into the body) a shark can mitigate infection from foreign pathogens (e.g. bacteria, fungi, protozoa or viruses) [10–12]. At the same time, the circulating granulocyte populations are increased to attack pathogens that enter the body and are subsequently transported via the circulatory system [10–12]. The combination of these two main macrophage strategies minimises the chance of infection and subsequent immune challenge, while the body attempts to return life-supporting physiological systems to homeostatic equilibrium. However, if an animal's immune strategy is altered for a prolonged period of time, the animal becomes vulnerable to disease and other reproductive, growth and survival consequences brought on by the disintegration of the above life-support systems [12,13].

Little is known about the immunological response of a shark to stress, thus the consequences of fishing capture on its immune

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system strategy are also unknown. Historically, nomenclature of teleost and elasmobranch leukocytes has been inconsistent. However a recent investigation of the hematologic disorders of fish by Clauss et al. [14] and several others preceding it (e.g. [15–18]), have established accurate identification criteria and nomenclature of elasmobranch (sharks and rays) leukocytes. In these cartilaginous fishes, neutrophils and heterophils perform similar functions in the immune system, so when studying their abundance in proportion to lymphocytes, it is usual to combine their numbers and classify them collectively as granulocytes [7,19]. Descriptions of the morphology of immune cells within shark blood [14,15,18], have not recorded changes in the leukocyte distribution as a consequence of stress, but the analysis of leukocyte distribution within the peripheral blood of a shark may provide valuable insight into the function of the immune system in response to stress. There are several advantages to using the leukocyte count in conjunction with or in place of other physiological indicators of stress for sharks. Indicators of stress such as metabolites and electrolytes can show a measurable response within minutes [20], but changes in leukocyte distribution take considerably longer [21]. This is important in allowing the investigator time to handle the animal and obtain a blood sample without confounding results by the act of collecting the sample. Additionally, the method is inexpensive and once blood smears are fixed they can be stored permanently for later or repeated analysis.

To date there has been no investigation into how the shark immune system responds to stress, acute or otherwise. Sememiuk et al. [22] used the differential leukocyte count method to investigate the degree of stress experienced by southern stingrays *Dasyatis americana* (Order: Rajiformes) in response to intense marine tourism. Larger and freshly wounded Southern stingrays were found to have significantly more heterophils than smaller and uninjured individuals, while stingrays interacting with tourists had a lower proportion of lymphocytes compared to the undisturbed individuals [22]. There have also been few investigations into how leukocytes respond to stress in bony fish species. Immunomodulation has been demonstrated following feeding of cortisol to coho salmon (*Oncorhynchus kisutch*) in a study by Maule and Schreck [23]. Lymphopenia and neutrophilia are have also been reported in several stressed freshwater species [3,4,24], and reviewed in Barton and Iwama [25]. Similarly, the lack of knowledge surrounding the immune response to stressors of cartilaginous fishes, particularly sharks, calls for greater investigation.

We measured changes in the distribution of leukocytes in the peripheral blood of adult Australian swellsharks, *Cephaloscyllium laticeps* in response to stress of fishing capture, with the following aims: (1) to validate the method of differential leukocyte analysis for a shark species, (2) quantify physiological stress by changes in leukocyte distribution, and (3) identify and compare the magnitude of lymphopenia and granulocytosis with other taxa.

2. Methods

2.1. Animals and husbandry

Australian swellsharks (*C. laticeps*) are a common by-catch species in southeastern Australian fisheries [26,27], and were collected by a commercial fisherman using Danish seine gear in the coastal waters near Queenscliff, Victoria, Australia (Latitude: 38°16′S; Longitude: 144°39′E). Animals were transported to research facilities in Queenscliff and placed in circular 19,000 l holding tanks connected to an open seawater system at ambient temperature. After introduction to the captivity tanks, all sharks were treated identically. The sharks were fed within 48 h of collection and were left undisturbed to acclimate for at least five days prior to experimentation. Experiments commenced within seven days of initial capture. They were fed on a diet of chopped pilchards *Sardinops neopilchardus* twice per week (3% of body mass).

2.2. Experimental design

Using an experimental protocol adapted from Frick et al. [28] we replicated capture by commercial gear in a controlled laboratory setting and collected blood samples before capture and during a recovery period. We obtained reference blood samples from animals that did not experience the capture technique and compared leukocyte counts over time with those from the experimentally captured animals. We used commercial gillnet, and longline gear with capture durations of 6-h for each type followed by removal from the gear and exposure to air in a fish-tub for 15-min. In summary, a section of gillnet (monofilament, stretched mesh diameter 100–127 mm [4–5 inch size] depending on shark size) was suspended mid water in a 5, 000 l circular aquaculture tank. To ensure immediate entanglement, the shark was manually 'gilled' ($n = 9$ animals) by bringing the gillnet to the surface and placing the shark's head into the mesh, ensuring the net slipped over its head and was secured around the gills. Longline capture was conducted using the same technique as per the gillnet group, where instead of gillnet mesh, a length of fishing line (breaking strength 55.4 kg [120 lbs]) with a hook (Mustad 5/0 suicide) was positioned so that the hook was hanging mid-water in the centre of the tank. To ensure immediate hooking, the shark was manually 'hooked' ($n = 8$ animals) in the mouth where the jaw cartilage is thinnest. These capture durations and air exposure are representative of standard fishing practice in the fisheries where this species is usually encountered [26]. An 'unstressed' control group ($n = 7$ animals) did not undergo any laboratory capture, but did experience the same blood sampling and experimental handling procedures as the sharks in the capture groups.

Stress resulting from capture was assessed through a series of blood samples taken over time. Blood was collected immediately before experimental capture (pre-treatment sample), and a second sample was taken 72 h post-capture from the gillnet gear, while samples were collected prior to capture, then at 24 h and 72 h for the longline capture group. During the post-capture sampling period, the shark was kept in a circular 5000 l recovery tank. Each shark was used in one experiment only and was released back into the ocean several days post experimentation, after veterinary inspection.

2.3. Blood sampling

All blood samples (1.0 ml) were collected from the caudal vein using a heparinised needle (1.2 × 38 mm) with a 3 ml syringe. A dip net was used to bring the shark up to surface, where it was removed from the net while holding the mouth closed to prevent reflexive ingestion of air or water. It was positioned ventral side up (with gill region submerged) so the blood could be easily drawn from the caudal vein, with the whole sampling event (including dip netting) usually taking less than 70 s.

2.4. Differential leukocyte count and preparation

For each blood sample, single blood smears were prepared on microscope slides. The smear was left to air-dry in a plastic container with a lid for 24 h before fixing in 100% methanol for 10 min and being stained. The staining procedure adapted from Bain and Lewis [29] consisted of 15 min in freshly diluted May-Grunwald (standard solution diluted 1:1 with water, Australian