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Parasites and People: Crocodile parasite interactions in human impacted areas of Belize

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Abstract

Despite the recorded cases of crocodilian epidemic mortalities caused by human disturbance of the environment, there still remains a lack of information to understand how anthropogenic disturbances affect crocodilian health.

This study is the first study to investigate the innate immune function, health and parasitism of the American crocodile (*Crocodylus acutus*) and Morelet's crocodile (*Crocodylus moreletii*) in response to human presence and disturbance. More specifically, it focuses on the relationship between the crocodiles and their parasites from various habitats within developed and rural landscapes in Belize. The investigation also examined the host's health response to haemoparasite load, as well as to the presence of the ectoparasite *Paratrichosoma* sp.

Parasite load and presence was used to investigate how parasites affect crocodilian health through hematological analysis. These effects were also investigated in connection to human disturbance. All (100%) blood samples contained haemoparasites. In fact, this is the first study to record such a high haemoparasite infection rate in crocodilians, as well as the first study to report the presence of a haemoparasite in Morelet's crocodile (*Crocodylus moreletii*). However, results indicate that the haemoparasites found in *C. acutus* and *C. moreletii* do not appear to have an adverse effect on the crocodiles, as they did not affect body condition or correlate with a parasite-specific immune response. Across the two species, 34.5% of all sampled individuals showed traces of infection by the ectoparasitic nematode *Paratrichosoma* sp. and the presence of the parasite did not differ between sites, species, salinity or pH. Similarly, the presence of *Paratrichosoma* sp. was not found to correlate with body condition, white blood cell recruitment or eosinophil recruitment, and lends more evidence to the notion that this is a benign parasite in crocodilians.

Haemoparasite load and the presence of haemoparasites or skin parasites did not differ between site classes, sex or species in Belize. There was, however, a significant lower body condition detected among *C. acutus* who inhabited human developed areas compared to *C. moreletii* who were more commonly encountered in rural areas. There was also a correlation between lower body condition and areas of high anthropogenic disturbance and with increased salinity. This suggests that the lowered body condition in American crocodiles may be related to an unidentified anthropogenic stressor.

This investigation uncovered several novel results within the crocodilian research community, nevertheless there is still a need for further studies investigating parasites, immunology, and human disturbance effects in crocodilians.

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Introduction

The natural world is under increasing anthropogenic pressure. Habitat destruction and fragmentation, pollution and over harvesting of natural resources are decreasing global biodiversity at an expedient rate (Aguilar-Stoen and Dhillion 2003, Laurance et al. 2014, Rodriguez-Jorquera et al. 2017).

The anthropogenic threats facing the natural world at large does not omit crocodilians. Of the 27 recognized crocodilian species, 23 species have a conservation action plan created by the International Union for Conservation of Natures (IUCN) Species Survival Commissions Crocodile Specialist Group (CSG). Of these, 11 species are deemed Vulnerable (VU), Endangered (EN), Critically Endangered (CR) or data deficient for classification (DD). The CSG regards habitat destruction, pollution, human driven climate change and over harvesting as some of the threats currently facing crocodilians (CSG 2010). These threats combined with long generation times and low hatchling survival rates add to the growing concern among researchers that some species might not be able to recover a from a serious population decline (Thorbjarnarson and Wang 1999, Xu et al. 2006, Wang et al. 2011). In addition to the direct impacts stated above, the indirect effects of long-term stress caused by human presence may have negative implications on wildlife, such as lowered fecundity, disease and decreased body mass (Milton and Lutz 2003, Murray et al. 2015).

Crocodiles, Order Crocodylia, are aquatic and semi-terrestrial predatorial archosaurs with a long evolutionary history dating back more than 200million years (Seymour et al. 2004, Grigg and Kirshner 2015). The crocodilian family tree includes 27 living species divided by three families; Family Gavialidae (gharial), Family Alligatoridae (alligators and caimans), and Family Crocodylidae (the true crocodiles). All families, sub-families and species have distinct features that separate them, but they all have the same basic shape and lifestyle. Crocodilians are all elongated dorso-ventrally flattened animals, with a strong muscular tail used for propulsion through water, sprawling short legs, scaly skin with bony subcutaneous growths called osteoderms, and strong jaws lined with 40-60 teeth. They all shed and regrow teeth regularly and can have up to fifty sets throughout their life, making the total teeth count up to 3000teeth during a crocodiles lifespan (Seymour et al. 2004, Grigg and Kirshner 2015).

All living crocodilians are aquatic ambush predators utilizing camouflage, stealth and eyes adapted for low light situations to their advantage when they hunt along the shore line at night. Their prey preference is dependent on size, age, habitat choice and species. Prey includes insects, gastropods, shellfish, fish, reptiles, birds, large and small mammals, as well as reports of cannibalism and even predation on humans (Campbell et al. 2013, Grigg and Kirshner 2015, Tellez and Merchant 2015). Grown individuals can also travel considerable distances by land. This combined with their capacity to move very efficiently through water, results in crocodilians potentially inhabiting almost any suitable body of water within their range (Lang 1987, Campos et al. 2003).

Although we have a good general knowledge about crocodilian life history and general ecology, there is still a lack of knowledge regarding the detailed ecology of several species. This is portrayed by the many species listed as DD by the IUCN. It becomes apparent considering the precarious conservation status of many crocodilian species that knowledge gaps need to be addressed in an effort to understand crocodilian ecology and biology.

In Central America the life history and ecology of the American crocodile (*C. acutus*) and the Morelet's crocodile(*C. moreletii*) is largely uncharted, which advocates the gathering of more knowledge about these species for conservation purpose. The lack of ecological and anthropogenic impact data in conjunction with the lack of knowledge of the crocodilian immune system, hampers the ability of conservation managers to properly assess how anthropogenic disturbances affect wildlife.

Parasites and pathogens are experienced by most living organism, and are considered an

important evolutionary driving force as they have the potential of altering fitness, fecundity and behavior of their host (Anderson and May 1978, May and Anderson 1978, Combes 2001, Tellez 2014a). With an evolutionary lineage reaching back more than 200million years, crocodilians are a prime study candidate to investigate the evolutionary relationship between parasites and their hosts (Tellez 2014a). Despite possibly playing a major evolutionary role, crocodilian parasites are still understudied and gaps in our knowledge of their life cycle, evolution and ecological role persist. Contradictory to the definition of a parasite, research conducted on American alligators (*Alligator mississippiensis*) indicate that some host-specific parasites of the digestive system of American alligators may have a beneficial effect on the hosts health (Tellez 2014a). These parasites seem to have a buffering effect on the uptake of heavy metals by the alligators tissues, slowing down the uptake of the heavy metals and reducing toxic impacts on the host organs (Tellez and Merchant 2015). Utilizing this knowledge researchers are looking into using these parasites as early bioindicators for heavy metal toxicity in the environment (Tellez and Merchant 2015).

Another potential bioindicator for ecosystem health is the nematode worm *Paratrichosoma* sp. The presence of the nematode in crocodilians are recognized by a zigzag pattern created by the female in the epidermis of the host. The presence differs between sites and is suspected to be related to some hitherto unknown environmental or behavioral change. Due to the parasite likely having a free-living life stage researchers are investigating the possibility for utilizing the presence of *Paratrichosoma* sp. as biomarkers for changes in habitat quality. Changes in ecosystems would likely affect the nematodes free living stage, which in turn would impact the presence of the parasite in crocodilians. Monitoring these changes could serve as an early indicator of ecosystem disruption (Tellez and Paquet-Durand 2011, Charruau et al. 2017).

Despite the recorded cases of crocodilian epidemic mortalities caused by human environmental disturbance, we still have a poor understanding how anthropogenic disturbances affect crocodilian health. Exactly how human disturbances affect the innate immune system and host-parasite dynamics of crocodiles are one of the areas where knowledge is lacking.

This study examines the innate immune function, health and parasitism of the American crocodile (*Crocodylus acutus*) and Morelet's crocodile (*Crocodylus moreletii*) in response to human presence and disturbance. More specifically, I focus on the relationship between the crocodiles and their parasites from various habitats within developed and rural landscapes in Belize. I also investigate the host's health response to haemoparasite load and the presence of the parasitic nematode *Paratrichosoma* sp. Examining the interaction between the host and its parasite may provide supplemental knowledge on the biodiversity and overall health of an ecosystem (Combes 2001, Tellez 2014a, Tellez and Merchant 2015). Results from this study may in turn be used to detect fluctuations in crocodile health and anthropogenic disturbances, such as pollution and further deterioration of the ecosystem.

Methods

Study site

Belize is a small country (23,000 km²) in the Central American region situated south of Mexico, and bordering Guatemala to the west and south (**Figure 1**).

With forested areas constituting approximately 60% of the total terrestrial area, the second largest barrier reef in the world, and a high degree of biodiversity and endemism, Belize is considered a conservation priority (Aguilar-Stoen and Dhillion 2003, The World Bank 2017, CIA 2018). Currently 20.6% of Belize's total area has been set aside for terrestrial or marine protected areas. The amount of protected lands, combined with the stable warm climate and the high humidity contributes to the high diversity (The World Bank 2017, CIA 2018).

However, the natural resources in Belize are currently facing a rise in anthropogenic pressure and disturbance, especially along the coast (Chomitz and Gray 1999, Duffy 2000, Few 2000, Diedrich 2007, Young 2008, Diedrich 2010, Arkema et al. 2014). Historically, local people have been the primary inhabitants of coastal areas relying on fisheries and waterways for transport. However, the recent increase in the country's tourism industry with holiday homes, hotels and cruise ship traffic has promoted a rapid development of Belize's coastal areas. Today, approximately 40% of Belizeans live and work along the coast, and more than 800,000 tourists visit for recreational purposes every year (Chomitz and Gray 1999, Arkema et al. 2014, Verutes et al. 2017). The main tourist attraction is the tropical climate combined with the sandy beaches, tropical rainforest adventures and diving/snorkeling the coral reefs. All of these activities cause pressure on the waterways, mangroves and coastline, which in turn affect crocodiles as these ecosystems are their preferred habitats.

For this study the sampling locations were divided into two categories: urban/developed/developing areas (*developed* for short), and rural/isolated areas (*rural* for short). *Developed* areas are areas that have been or are being developed for human habitation, industry or other commercial use. This includes cities, towns, villages, industrial areas, waste management plants, transport hubs, and other areas under heavy anthropogenic influence. *Rural* areas are areas with limited or no human impact. These areas include national parks, national reserves, field stations, and inhospitable and remote areas. They may have some infrastructure such as simple boat docks, research stations or simple houses, but use of the area is limited to fishing, hunting and collection of natural resources for sustenance, as well as research or ecosystem management. Yet, illegal hunting and logging may occur within some remote areas in Belize. Areas where industrial scale natural resource extraction is taking place has been included in the *developed* category, regardless of remoteness to permanent settlements.

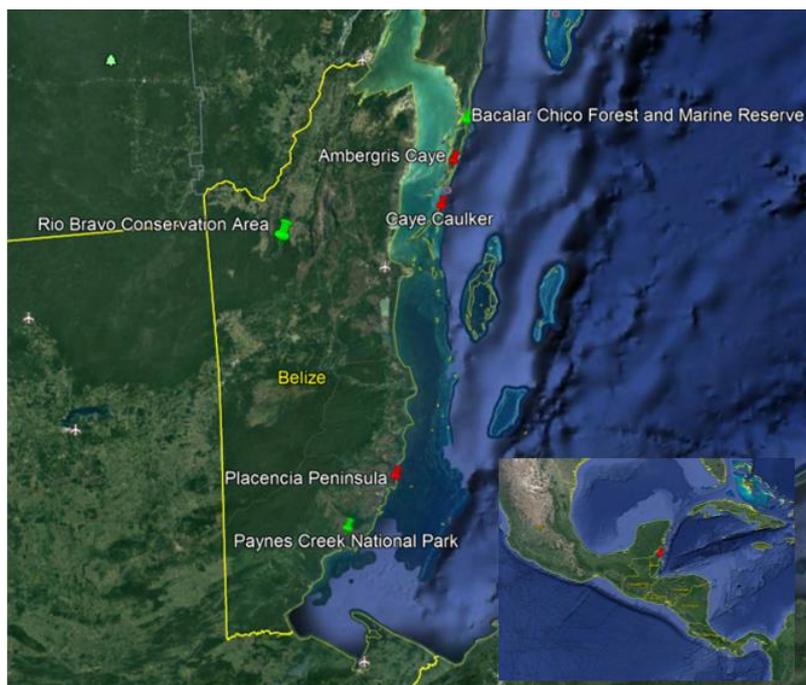


Figure 1: Developed and rural areas sampled in Belize marked with red and green pins, respectively. Inset shows Belize's placement in Central America (red pin). Picture retrieved from Google Earth, Jan 2019.

Developed sites

Placencia Lagoon

Placencia lagoon is situated between the Placencia Peninsula to the east and main land Belize to the west (**Appendix Figure S1**). The Placencia Peninsula is currently under heavy development, primarily for holiday homes and hotels. Some permanent homes are also under development as Placencia has become a popular destination for retirees, especially from the United Kingdom, USA and Canada. The sea south of Placencia Peninsula and the Placencia lagoon has been going through development recently. Norwegian Cruise Lines have spent the past several years planning and developing a lane for their cruise ship anchoring off Harvest Caye to the south east of Placencia Peninsula. This development has included dredging of the sea floor to make space for the ships and is criticized by local residents and conservation groups as it has the potential to disrupt habitats and feeding grounds for crocodiles, manatees and other marine life.

Placencia Lagoon is bordered by mangroves with houses, villages, development sites, canals, and small beaches occurring sporadically. The eastern facing seafront on the other hand, is highly developed and consists mainly of natural and constructed beaches with houses and villages scattered down most of the length of the peninsula.

The Placencia Lagoon with its neighboring tributaries, mangroves, rivers and canals are highly trafficked by smaller boats used for fishing, tourist trips and transport. The smaller rivers connected to the larger lagoon are mainly used for subsistence fishing and transport to hunting-grounds by locals.

Caye Caulker

Caye Caulker (**Appendix Figure S2 and S3**) is a popular tourist destination with several development projects under construction. Among these is the development of a new airstrip for small aircrafts on the south-eastern part of the Cayes planned to be fully operational in early 2019.

Caye Caulker is an island dissected by a narrow human-made canal that separate the northern and southern parts.

The southern part of the Caye is where the ferries and local air traffic from the mainland arrive, where hotels are situated, and where most of the development is taking place. The south-west part of the island is heavily influenced by the tourist sector with large areas of the seafront being modified into white sand beaches, seafront shops and hotels, as well as boat docks.

The northern part of Caye Caulker is less developed than the southern parts. Most of the seafront on the northern part of Caye Caulker is mangrove shallows and narrow sand beaches separating the sea from the forested inland areas. There are, however, some smaller structures found scattered along the coast line such as a simple field station for marine science and some fishing huts and small houses. In the inland of the northern part, there has been some development of raised walkways and other structures, built in an attempt to draw in tourists to the “wilder” side of Caye Caulker.

There was also some dredging going on the eastern side of the northern parts of the Cayes.

Ambergris Caye south

Similar to Caye Caulker, the southern parts of Ambergris Caye is under heavy development for tourism and trade. The northern and the southern parts of the Cayes are separated by a human-made canal and is linked by a tolled bridge (**Appendix Figure S4 and S5**).

Unlike Caye Caulker, however, the top 1/3 of the northern part of Ambergris Caye is a Forest and Marine Reserve and under strict control from rangers and coast guard with no ongoing development.

The southern part of Ambergris Caye is where intense development is taking place, with hotels, holiday homes, as well as industrial sites such as the airport, sewage and waste disposal plants. After advice from local inhabitants and local police, the investigation limited its access to some parts of the southern parts of the island due to high rates of violent crimes, such as robberies and murders. These areas include some areas of the south-western parts of San Pedro town and local residential areas south of San Pedro.

Rural sites

Payne's Creek, Punta Gorda, Toledo District

Payne's Creek National Park in the Toledo district is a 152 km² national park situated in the southern parts of Belize (**Appendix Figure S6**). The national park houses a research station run by the Toledo Institute for Development and Environment (TIDE) and is comprised of a range of habitat types which include inland lakes, river systems, moist grasslands, savannah, tropical rain forest, mangroves and swamps. The large area and the range of habitat types boasts a high degree of biological diversity and is frequented by groups of researchers from many scientific fields. Most of the national park is only accessible by water, by trails or by light four-wheel drive vehicles, which makes most of the park virtually isolated – apart from the rangers and researchers visiting the research station. The area has restricted access through permits and is patrolled by armed rangers from TIDE as well as Belize Forest department and Fisheries department rangers.

There are some fishing and light forestry occurring within the national park. All fishing and forestry require a permit and is exclusively for sustenance use. No industrial scale use is permitted within the

national parks' boundaries.

Within the reserve there is a small body of water, approximately 600m x 50m, known to the rangers as “the crocodile pond”. The pond is bordered by thick forest, steep banks and mangroves. The crocodiles residing here are not at all vary of people or boats and would readily swim up to the canoe. One large crocodile visually estimated to be 10-12ft in length displayed territorial behavior towards the canoe through a head slap next to the vessel. Most of the animals observed were adults, very few subadults and juveniles were registered. This could be due to the small size of the pond and the territorial behavior of crocodiles who readily kill each other in territorial disputes, driving the smaller individuals deeper into the mangroves. The rangers explained that these crocodiles are rarely visited by humans and that the only human activity close to the pond are patrols and some recreational fishing by the rangers themselves. The population in this pond has little to no experience with harassment by humans, which might explain why these isolated animals were seemingly bolder than neighboring populations that encounter humans more frequently.

Rio Bravo Conservation Area, Orange Walk District, Belize

The Rio Bravo Conservation Area in the Orange Walk District is an 800km² conservation and management area situated in the north western parts of Belize, near the Guatemalan border to the west (**Appendix Figure S7**). The conservation area house a research station and eco-lodge managed by the organization Programme for Belize, who also patrol the area. The area stretches across tropical rain forests, wetlands, mangroves, river systems, marshes and steppes, boasting a large variety in both landscape and biodiversity.

To the north, east and south the conservation area borders agricultural areas. Earlier investigations in the wetlands, rivers and lakes of the Rio Bravo Conservation Area found elevated levels of potentially harmful compounds such as organochlorides and heavy metals, and identified pesticide runoff from the neighboring farmlands as the likely culprit (Wu et al. 2000, Rainwater et al. 2002).

Some lakes and wetlands within the conservation area is used for recreational fly fishing and by locals for subsistence hunting and fishing, but commercial fishing is prohibited. There is also some illegal hunting and fishing going on within some areas of the conservation area. The conservation area is otherwise sparsely used by humans, and some areas, such as Habanero pond, are virtually isolated and seldom visited. Interestingly we had a similar experience in Habanero Pond as in the crocodile pond in Payne's Creek, where crocodiles would readily swim up to the canoe to investigate.

Bacalar Chico Forest and Marine Reserve

The far northern 1/3rd of Ambergris Caye is home to Bacalar Chico Forest and Marine Reserve (**Appendix Figure S8 and S9**). This is a remote area which is only reached via a 90minute boat ride along the western coast of Ambergris Caye from San Pedro, or by light 4x4 vehicles along a narrow beach path on the eastern seafront. The area is only accessible through permitted access from the Belize Fisheries Department and The Belize Forest Department.

The eastern seafront is characterized by natural sand beaches, which gradually transforms into grassland, shrubland and forest inland. There are only a few buildings along the beach: The research station Blue Ventures Dive Camp, and a few high-end resorts built before the area was protected.

The western seafront is characterized by mangroves and natural sand beaches, which are interspaced with smaller lagoons, and islets. The Belize Fisheries Department also have their field station on the western coast of Bacalar Chico, and there are a few permitted permanent fish traps along

the coast.

Bacalar Chico's inland is made up by dry palm forests, dense mangrove forests, inland salt water lagoons which are fed by the mangrove rivers, with small sandbanks and sandy islets situated within the internal lagoon.

There are some old docks along the edges of the interior lagoons, as well as some abandoned huts. These were constructed before the protection of the area and some are maintained for use by permitted fishermen.

Bacalar Chico Forest and Marine Reserve is under substantial control and patrolled by both Fisheries and Forest department rangers looking for illegal wildlife and forest product harvest. The area is also patrolled by the coast guard as the natural reserve is bordering Mexico to the north and is occasionally used by drug traffickers as a smuggling route due to the reserves remote location. Despite being regularly patrolled and only legally accessed with a permit, the Forest and Marine Reserve have some limited human activities. These include recreational fly fishing in the shallow lagoons inside the Reserve, as well as some subsistence hunting and fishing by locals. Commercial fishing, however, is strictly prohibited and controls are frequently enforced. There are also some illegal hunting and fishing going on in the area, and crocodiles with old gunshot wounds were recorded.

Study species

American crocodile (Crocodylus acutus)

The American crocodile (*Crocodylus acutus*) is a medium to large sized crocodile species which may exceed lengths of five meters. It ranges from the southern tip of Florida to the north, to the northern parts of Venezuela in its eastern range, and from Mexico to the Northern part of Peru through its western range (Tellez and Paquet-Durand 2011, Grigg and Kirshner 2015, IUCN 2018a). The American crocodile has one of the widest geographical ranges amongst all crocodylians (**Figure 2**).



Figure 2: Range of *C. acutus*. Map retrieved from IUCN's Red List assessment of *C. acutus* (<https://www.iucnredlist.org/species/5659/3043244>)

The American crocodile inhabits coastal marshes, ponds, coastal lagoons, mangroves and estuaries in

salt or brackish water along the coastline. The species has a high tolerance for saltwater due to highly effective salt glands on the tongue. These glands aid the crocodile in dispersing excess salt from the body and prevent desiccation and help sustain proper osmoregulation during extended periods spent in brackish or salt water (Grigg and Kirshner 2015, Balaguera-Reina et al. 2016). The ability to sustain longer periods of activity in salt water is the reason for the species ability to travel long distances at sea and move between habitats.

The American crocodile is regarded as primarily piscivorous, preying on fish and crustaceans (Platt et al. 2013, Sandoval-Hernandez et al. 2017).

The species is regarded as Vulnerable (VU) by the IUCN after historic declines caused by over harvesting for skins and meat. However, the population is increasing throughout most of its range after legal protection (IUCN 2018a). Due to its conservation status trade in live animals or products stemming from the species is highly regulated, and it is classified as an Appendix II-species throughout most of its range by CITES (CITES 2018). Despite the IUCN status the American crocodile still remains one of the most threatened crocodylian species of the Neotropics (Balaguera-Reina et al. 2017). The local population of American crocodiles in Belize are thought to be decreasing and have been upgraded from CITES Appendix II to CITES Appendix I by the Belizean Government. The biggest threats to the American crocodile comes from human development in crocodile habitat along shorelines, accidental and purposeful killings of animals, pollution, and hybridization with the more abundant Morelet's crocodile (*Crocodylus moreletii*) in Belize (CSG 2010, Thorbjarnarson 2010, Tellez et al. 2016).

Morelet's crocodile (Crocodylus moreletii)

The other crocodylian species found in Belize is the Morelet's crocodile, *Crocodylus moreletii*, which is a medium sized crocodylian reaching lengths of three meters. Its range is more limited than that of the American crocodile, and the Morelet's crocodile range from eastern Mexico through Guatemala to southern Belize (**Figure 3**). This species' preferred habitat is freshwater swamps, rivers, ponds and marshes, and may also occasionally be found in brackish water where its range overlap that of the American crocodile (Platt and Thorbjarnarson 2000a, Grigg and Kirshner 2015, Pacheco-Sierra et al. 2016, Tellez et al. 2017, IUCN 2018b).



Figure 3: Range of *C. moreletii*. Map retrieved from IUCN's Red List assessment of *C. moreletii* (<https://www.iucnredlist.org/species/5663/3045579>)

The Morelet's crocodile is an opportunistic feeder that has been found to utilize a variety of food sources. However, its preferred prey seems to be mollusks, gastropods, crustaceans, small mammals and birds rather than the fish rich diet of the American crocodile (Platt *et al.* 2006).

According to the IUCN Red List the Morelet's crocodile is currently listed as Least Concern (LC), but it has, similar to the American crocodile, suffered population decline due to over harvest for skins and meat in the past (Platt and Thorbjarnarson 2000a, Padilla and Weber 2016b, Tellez *et al.* 2017, IUCN 2018b).

The American crocodile and Morelet's crocodile inhabit the Central-American region and experience habitat overlap throughout their range. It is recognized that these two species also hybridize regularly across their range, and that the hybridization may be threatening the individuality of the two species (Tellez and Paquet-Durand 2011, Grigg and Kirshner 2015, Pacheco-Sierra *et al.* 2016). Genetic evidence suggest that these two species are not sister taxa, but that their hybridization is a result of secondary introduction (Pacheco-Sierra *et al.* 2016). Still, due to the close evolutionary relationship among crocodiles, the species can readily interbreed and produce viable offspring. The offspring can possess a myriad of morphological features associated with both crocodile species. The morphological markers, tail scale pattern and nuchal scale arrangement, were used to classify the species in the field as Morelet's, American or as hybrids in this study – following the Crocodile Research Coalitions (CRC) crocodile population survey method manual (Tellez 2011). However, many generations of hybridization and backcrossing may blur the lines between pure species and hybrids, and caution must be taken in classifying the animals (Pacheco-Sierra *et al.* 2016). For this investigation, the morphological markers and habitat choice serve as indicators of species.

Captures, data collection and blood sample analysis

The sampling commenced in the beginning of the wet season in the start of June 2018 and ended in the beginning of August 2018. The CRC assisted in all captures and sample collections. The collection of samples was performed throughout the range of *C. acutus* and *C. moreletii* in Belize, specifically Ambergris Caye (N17°55.18, W87°57.40), Caye Caulker South (N17°44.37, W88°01.26), Caye Caulker North (N17°45.46, W88°01.39) Placencia Lagoon (N16°32.20, W88°22.03), Sittee River (N16°49.18, W88°16.05), Bacalar Chico Marine Reserve (N18°08.45, W87°52.04) and Rio Bravo Conservation Area (N17°40.35, W88°48.45). Additionally, the collection of samples was done in partnership with several national organizations and the Belize Forest Department. Access and assistance to locations and crocodiles for sample collections was provided through the Crocodile Research Coalitions extensive network of wildlife professionals in Belize. All data collection was performed with permits issued by the Belize Forest Department as well as the Belize Fisheries Department.

Before any captures commenced a nocturnal eyeshine survey (NES) was conducted. This was to detect, count and produce estimates of the local and national crocodile populations. Crocodiles have a reflecting structure embedded in their eyes known as the tapetum lucidum. This structure reflects ambient light back to the light sensitive retinal cells in the eyes, increasing the eyes sensitivity to light which allow crocodiles to see in low light situations (Ollivier *et al.* 2004, Grigg and Kirshner 2015). In crocodilians this reflection is seen as glowing red eyes in the beam of a powerful flashlight. By patrolling waterways and shining the water, banks and mangrove edges with a light, the crocodiles in an area can be registered and counted by looking for the reflection from their eyes. This data is used to estimate the population size and density (Thorbjarnarson and Hernandez 1992, Tellez 2011, Fukuda *et al.* 2013, Tellez *et al.* 2016, Tellez *et al.* 2017).

During a NES GPS points of the encountered crocodiles were recorded as close as possible to the

crocodiles' actual position, taking care to limit the disturbance of the animal. If possible, an estimate of the animals' size was also made to gain an indication of the population structure in the surveyed area.

Abiotic factors, such as water temperature, air temperature, date, time and moon waxing, were also registered before departure and after survey conclusion. The abiotic factors were collected and analyzed with NES data in order to investigate the crocodiles habitat choice and environment, and to keep a record of abiotic fluctuations in the environment. This information is also used as a tool to further develop NES and capture survey methods by examining where and when there is highest rate of crocodile encounter, which can streamline the survey methods and increase the success rate of future surveys.

During captures, crocodiles were caught by hand or using a pole noose, and the same method for capture and release of animals were used throughout the sample collection period (Tellez 2011, Mazzotti et al. 2012, Grigg and Kirshner 2015, Brandt et al. 2016). Animals estimated to be shorter than 120cm was caught by hand, whilst animals longer than 120 cm were captured using the pole snare. Animals estimated to be longer than 250cm were restrained by two people operating snares. Lastly the jaws were secured using a zip tie snare closing the mouth. At this point the animal was preferably brought on land, or on the boat if no convenient land was available, for processing. The animals were then blindfolded with a wet rag to calm it down, and the jaws were secured by taping them shut with electrical tape.

At this point snares were removed, and samples collected. All samples were taken as quickly and quietly as possible to minimize stress on the animal before it was released. Sustained stress on a crocodile may result in a buildup of lactic acid which may be harmful and can result in death (Seymour et al. 1987).

Whilst the animal was under restraint morphometric measurements were registered to the nearest 0.1cm using the guidelines provided by the Crocodile Population Survey Methods Manual (Tellez 2011). The measurements included (**Appendix Figure S10**): Total length (TL), snout-to-vent length (SV), head length (HL), snout length (SL), cranial width (CW), maxillary width (Max W), pre-maxillary width (PMax W), tail girth/width (TW), sex, and weight (Kg). Size category for the animals were registered as: Hatchling, juvenile, sub-adult or adult, after measurement were taken (**Appendix table 1**). All crocodiles were marked with a specific numbered code using scute clippings of the protruding tail scutes after the Crocodile Population Survey Methods Manual guidelines. This way the animal is recognized if it would be recaptured later and information collected on the individual may be revisited for comparison.

Abiotic factors were also sampled for every capture to assess the animal's habitat preference. These included local water pH, water temperature, air temperature and time of capture.

The presence of the parasitic nematode *Paratrichosoma* sp. was also registered. The female parasite creates a zig-zag pattern in the skin of crocodilians. It is believed to be non-harmful, but these parasites may be used as a proxy for ecosystem health, as they likely have a free-living stage that is sensitive to pollutants (Ashford and Muller 1978, Buenviaje et al. 1998, Moravec and Vargas-Vazquez 1998, Moravec 2001, Seijas 2007, Tellez and Paquet-Durand 2011, Padilla and Weber 2016a, Charruau et al. 2017).

Blood samples were collected from 56 individuals, where 49 were registered as *C. acutus*, 6 registered as *C. moreletii*, and 1 registered as a hybrid with traits associated to both Morelets and American crocodiles. Due to conditions in the field, a few of the samples were not usable for further analysis. Blood degenerate easily under sub-optimal conditions, and optimal conditions may be difficult to obtain in field stations without a dedicated lab. Factors such as temperature, time from collection to processing and analysis, as well as humidity, were challenges facing the investigation. In addition, crocodilian blood clots extremely quickly, and in some cases blood even clotted in the needle or the syringe.

Blood sampling of crocodilians is usually done by accessing the post-occipital spinal venous sinus between the cranial plate and first vertebra of the spine. (Huchzermeyer 2003, Myburgh et al.

2014). Due to the vein's proximity to the brain and challenging field conditions (dark, rocking boat or canoe/kayak) in which blood samples were taken, the blood was instead drawn from the caudal vein in the crocodiles' tail to safeguard the animal. The blood was drawn from the lateral side of the tail, puncturing between the lateral tail scutes, approximately 2/3 down the height of the tail in an area where 2/3s of the needle's length would reach center of the tail. This area was found by using the needle as a measuring stick and leading it up the length of the tail from the tip towards the base, locating the spot where 2/3 of the needle would reach the half way point traversing the tail (**Appendix Figure S11 and S12**). Before puncture, the needle entry point was cleaned with water and sprayed with ethanol to avoid any contamination of the animal and of the samples. The bevel was positioned towards the anterior of the animal and inserted in an anterior direction with an approximate 45° angle. By flexing the tail slightly in the opposite direction of the needles entry on larger animals the access to the blood vessels were made easier (**Appendix Figure S12**).

On smaller individuals, where 2/3 of the needle would extend beyond the center of the tail, the blood was collected in an adapted manner. The adaption comprised of inserting the needle as close to the base of the tail as possible, but never closer than four rows of scales down from the end of the cloaca to avoid any direct damage or development of scar tissue around the reproductive organs. If sampling of blood was not successful after three punctures the animal was released to avoid further stress.

During initial trials blood was drawn after morphometric measurements were taken. It was noticed that the rate of sample success and yield was low. After consulting with Dr. Tellez at the CRC and the veterinary specialist group within the CSG, the blood was drawn at the very start of data collection and the success rate went noticeably up with very few attempts resulting in unsuccessful blood sampling. This was likely caused by blood pressure being raised and blood vessels expanding immediately after a capture, whilst after being restrained the blood pressure would lower and the veins would contract (personal communications).

Blood samples were added to 6mL vials containing the anticoagulant ethylenediaminetetraacetic acid (EDTA) for hematological analysis (Stacy and Whitaker 2000, Sykes and Klaphake 2008). The samples were then transported back to the nearest field station or the CRC offices for fixation and staining. Eosinophil analysis, identification of blood cells, parasite identification, and parasite counts were performed at the CRC's headquarters in Placencia, Belize, using a binocular stereoscopic microscope at 100X magnification (see details below).

The release of animals was performed with caution as the animals could be agitated after handling. The animal was either placed on land facing the water and the person or people restraining it would back off swiftly to give the animal an escape route into the water, or it would be gently lowered into the water by the restraining people. On larger animals the tape securing the jaws would be kept on until the animal was completely free of human restraints. A person not involved with the restraining of the animal would hold on tight to a solid rope connected to the tape on the crocodiles' jaw. When the animal would take off, the person holding the rope connected to the jaw would pull with enough force to break the tape on the jaws as soon as the animal was at a safe distance. The loose tape would then be collected for proper disposal.

The animals were never coaxed or chased off and were always allowed a moment to recuperate after being restrained. If the animal did not immediately swim or run off it was kept under observation until it freely moved away.

The nocturnal eyeshine survey and captures were, preferably, performed on two separate nights. This is because crocodiles may react to the disturbance from both NES and captures, resulting in crocodiles retreating into cover or swimming away from the approaching boat. In cases where there was not enough time to return on separate nights, NES was conducted first before looping back to the starting point to start captures (Tellez 2011). This is because NES is less disturbing than captures which often result in splashing from the animal struggling, which might alert other crocodiles in the area and lead to them retreating into hiding.

Body condition calculations

The overall condition of crocodiles can be estimated by using a standard for body condition calculation. This can in turn be used as a proxy for animal health. For crocodilians the Fulton's K is a common calculation used to describe body condition, $K = \left(\frac{W}{L^n}\right) \times 10^b$. Here W is a volumetric measurement and L is a length measurement, whilst n and b are constants. The mass of the animals in Kg was chosen as the volumetric (W) measurement, and Snout-to-Vent length (SV) in cm as the length(L) measurement (Zweig 2003, Mazzotti et al. 2012, Brandt et al. 2016, Shirley et al. 2017). The constant n was set to $n=3$, under the assumption of isometric growth. Body condition was calculated with the assumption of an isometric growth as the investigation aimed at comparing body condition amongst localities. Constant b was set to $b=5$ and was chosen to give a mean $K > 1$, which is the norm in studies utilizing Fulton's K in crocodilian research (Brandt et al. 2016, Shirley et al. 2017).

Blood sample analysis

Before being analyzed the blood was prepared and stained using glass slides and a Sigma-Aldrich modified Giemsa stain for parasite detection.

Blood smears were produced via a common pull-push method. A small drop of blood was placed close to the frosted end of a glass slide using a pipette. Another slide was used to spread the blood smear by holding the "spreader" at approximately a 30-degree angle by the edge of the blood drop on the far end of the blood drop, then pulling back slowly and controlled towards the blood drop. When the blood was spread across approximately 2/3 -3/4 of the main glass slide the spreader was pushed quickly and controlled forward to create a thin and even smear. The goal was to create an even monolayer of blood cells with as little clumping and as little banding as possible (**Figure 4**).

While creating blood smears with reptile blood one must make sure not to put too much pressure on the spreader. Reptile blood cells, like avian blood cells, are more fragile than mammalian blood cells and may break if too much pressure is applied (Sykes and Klaphake 2008, Jenkins-Perez 2012).

To find the best staining method, six blood slides were created for testing. The procedure described below was replicated with three different incubation times for the staining solution. Two slides were incubated with staining solution for five minutes, two slides were incubated for ten minutes and the last two slides were incubated for 45 minutes. The ten minute and 45minute test slides became too darkened with staining solution so that it was difficult and, in some cases, impossible to distinguish and separate blood cells. The slides incubated for five minutes were easier to work with as it was possible to make out different blood cells and parasites in the samples and were used to develop the staining protocol. The blood smear on the glass slide was fixated by bathing them in methanol (99,8%) for ten minutes. After the fixation the blood smears were air dried before the slide was covered in 1,0-1,5mL of Sigma-Aldrich modified Giemsa stain for cellular blood *components and blood parasites* and left to incubate for five minutes. After the five-minute incubation, approximately 1,0-1,5mL of distilled water was added to the layer of staining solution and left to incubate for further 2minutes. The dye was then rinsed off with distilled water and left to dry before analysis.

Both blood smear- and staining preparation methods works better if blood is approximately same temperature as surroundings, and in a dry climate. This was a challenge in a field setting as most field stations in Belize do not have airconditioned rooms for moisture and temperature control. The best blood slide results were obtained in airconditioned room as the air is cooler and drier. The quality of blood slides created outside climate-controlled environments were secured by leaving the slides in an airtight Tupperware-style box with tissue paper to dry out any moisture in the surrounding air. High humidity has shown to create bubbles in blood smear as water condensates on the slide before blood is dry.

After the blood was prepared and stained a Differential White Blood Cell Count (DWBC), parasite counts, and total cell counts was conducted using a modified differentiation count method

(Godfrey Jr et al. 1987).

The prepared and stained sample was scanned at 40X magnification with a stereoscopic microscope to certify the quality of the samples and the staining method. The samples were also controlled to check them for clumping or banding of blood as this would negatively affect the accuracy of the counts (Sykes and Klaphake 2008). A good sample slide for analysis is a glass slide with a smooth monolayer of blood cells spread across the slide in a pattern resembling a thumb print (**Figure 4**).

One field of view was then counted a time at 100x magnification oil immersion (one frame). Care was taken as not to maneuver the slide, other than up and down to gain focus, whilst counting the cells in the frame.

Twenty frames were counted per slide to a minimum 2000 erythrocyte cells. The number of 2000 counted cells were selected to ensure that the samples for analysis were robust enough for further analysis (Godfrey Jr et al. 1987). If twenty counted frames yielded less than 2000 cells, more frames were counted until 2000 cells were reached. Within these additional frames, all the cells were counted even if 2000 cells were surpassed.

Total blood cell counts were registered, as well as erythrocytes, eosinophils, signs of blood parasites and other leukocytes (**Figure 5**). Cells and parasites bordering the field of view were counted as $\frac{1}{2}$. The erythrocytes were recognized as oval shaped cells with a clear border and a deep purple stained nucleus. Different from mammalian erythrocytes, crocodilian erythrocytes are similar to the erythrocytes found in birds which have a nucleus (Claver and Quaglia 2009). The presence of nucleus can make it challenging to separate crocodilian erythrocytes from other blood elements such as thrombocytes (functionally analogous to non-mammalian vertebrates platelets) if one does not have experience or guidance with crocodilian blood (**Figure 5A**) (Jain 1986, Weiss and Wardrop 2011, Shilton 2013).

Leukocytes were counted as a way to measure the crocodiles immune response. Especially eosinophils (**Figure 5D**) were of interest as these are specifically linked to combatting parasite infections in other vertebrates due to their ability to kill metazoan parasites, and the lack of evidence on the same function in crocodilians (Montali 1988, Jain and Jain 1993, Veiga et al. 1998, Sykes and Klaphake 2008, Zimmerman et al. 2010, Weiss and Wardrop 2011). It is worth noting that crocodilians lack the neutrophils found in mammalian blood, and that heterophils is thought to have the analogous task of phagocytosis and killing of bacteria to mediate inflammation (Claver and Quaglia 2009, Brooker et al. 2011, Weiss and Wardrop 2011).

The two leucocytes that are the hardest to differentiate from one another are eosinophils and heterophils. Heterophiles will appear with a more distinct nucleus as their granules are less stained, as well as the granules will be oval to elongated in shape. Unlike heterophils, eosinophils have rounder granules stained a deep pink under the modified Giemsa stain.

Usually there is a 10-20:1 relationship between heterophiles: eosinophils, but most of our knowledge is based on farmed or zoo animals and the information gathered from wild animals may differ from more sanitary environments (Stacy and Whitaker 2000, Lovely et al. 2007, Sykes and Klaphake 2008, Zayas et al. 2011). After counts, ratios were calculated for eosinophil: total blood cell counts, and white blood cells: total blood cell counts.

Haemoparasites were counted and recorded whilst the blood cell count was being performed. Using the modified Giemsa stain, parasites in blood cells appear clearly dark purple inside the cell cytoplasm (**Figure 5B and 5C**). Due to the existence of a nucleus in the erythrocytes of crocodilian, it is possible to distinguish infected blood cells from uninfected blood cells by investigating the nucleus. Crocodilian erythrocyte nuclei are oval in shape with a smooth outside. In cells that are infected by a parasite the nucleus can be displaced to either end of the cell, or the nucleus may be deformed (Lainson et al. 2003, Mihalca et al. 2004). The number of erythrocytes infected with blood parasites and free-floating parasites were recorded. The percentage of infected erythrocytes were calculated from the total count to create a parasite: erythrocyte, haemoparasite: eosinophil ratio and haemoparasite: white blood cell ratio.



Figure 4: Prepared and stained test blood slides. Only the slide on the far left is decently prepared. Number two (from left) has a layer too thin, three is banded, four is blotched, five and six have moisture damage, and seven has been stained too long (45minutes)

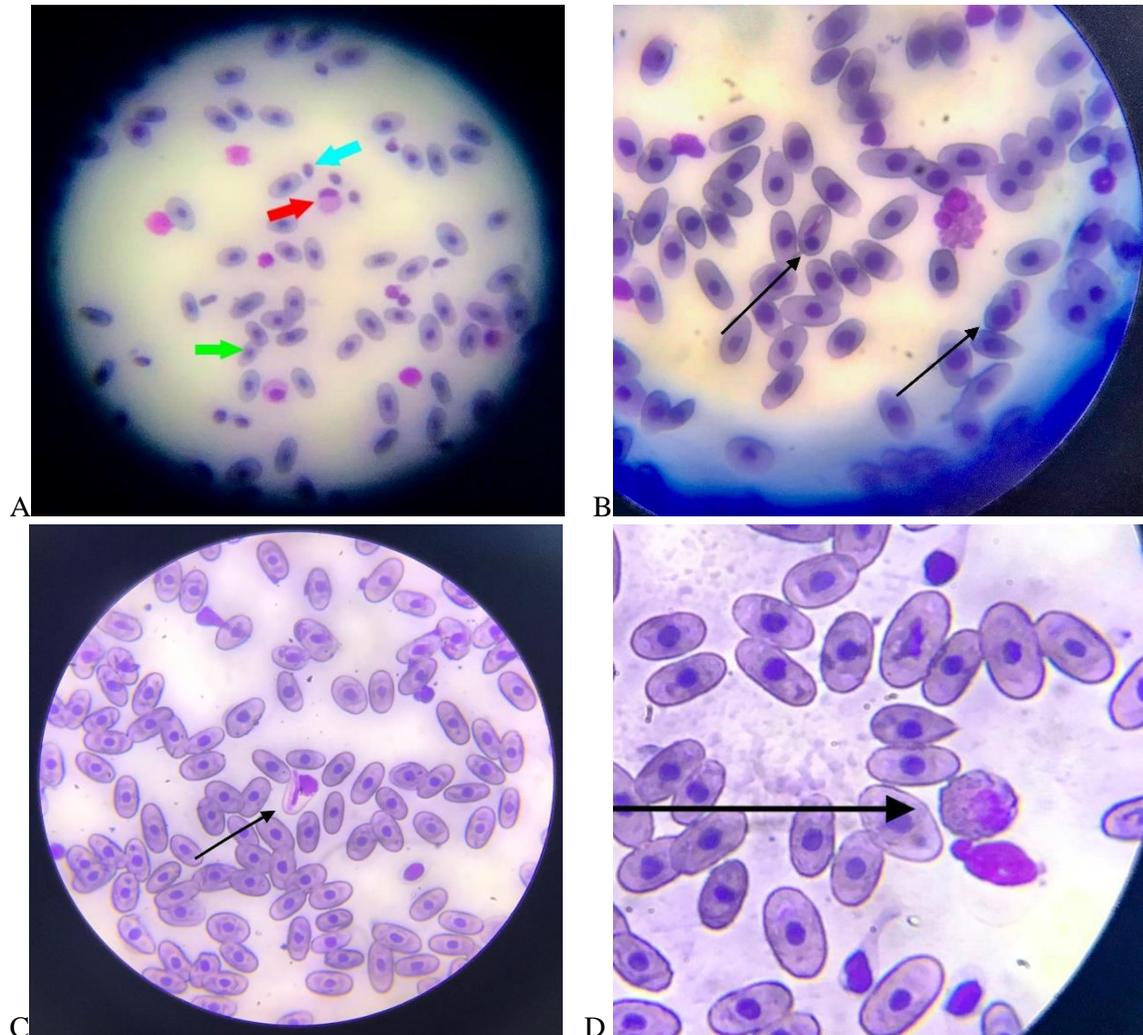


Figure 5: A: Blue arrow pointing towards thrombocyte, red arrow pointing towards a heterophil, green arrow pointing towards erythrocyte. B: Arrows pointing towards developed blood parasite inside erythrocyte. C: Arrow pointing towards blood parasite inside granulocyte. D: Arrow pointing to eosinophil with round granules

Statistical analysis

A total of 84 crocodiles were captured across Belize during this investigation. Among the samples collected, 33 went through hematological analysis and parasite investigation. Of the analyzed samples, 19 were used to differentiate between eosinophils and heterophils, whereas 14 samples were used for non-differentiating white blood cell counts due to difficulties differentiating the granulocytes, heterophiles and eosinophils, in the sample.

Whilst controlling the dataset, one sample had several blood: parasite ratios between two and three times higher than any other sample in the collection. After performing a Grubb's test, it was confirmed that this sample was an outlier on all hematological parameters chosen for this investigation. This sample was therefore removed from the analysis.

The numeric variables being used in the statistical data analysis were all continuous and was controlled for normal distribution using a Ryan- Joiner test, similar to Shapiro-Wilks, for normality. The results showed that the data was not normally distributed and suitable non-parametric tests were applied. All the statistical analysis was run using MiniTab v.18 and the $\alpha =$ was set to 0.05.

All analyzed blood samples showed signs of being infected with haemoparasites, displaying patterns similar to other *Hepatozoon* found in other crocodylians (Lainson et al. 2003).

To investigate if the ratio of infected red blood cells was influenced by site class, species or sex, a Kruskal-Wallis test was applied with parasite: red blood cell ratio as the response variable, and the categorical variables site class, species, and sex as explanatory variables. Box plots were constructed for all three analyses.

Habitat and proximity to humans and their effect on crocodile body condition was analyzed using a Kruskal-Wallis test. Next, a Kruskal-Wallis test was used to test if there was any difference in body condition between the species *C. acutus*, *C. moreletii*, and their hybrids. In addition, a Chi-square test was applied to examine whether there was any difference between the species in which site classes they were encountered.

Since there is a suspected higher density of humans close to coastal areas, and *C. acutus* is expected to frequent salt and brackish water more often than *C. moreletii*, a scatterplot was created for data on salinity (ppt) and body condition and a Pearson's correlation test was applied on the variables.

Building on the salinity data, a Kruskal Wallis test was applied to salinity (ppt) as a response variable against site class as well as against species as the explanatory variables.

The haemoparasite ratio influence on crocodile health was examined using a Pearson's correlation test to test for a correlation between parasite: red blood cell ratio and the explanatory variables body condition, white blood cell: total blood cell ratio, and eosinophil: total blood cell ratio. These were chosen as measures for overall health, activation of a broad innate immune response and activation of a parasite-specific innate immune response to parasites respectively.

To study the effect of the immune response in crocodylians on the health of the animals, scatterplots were created and Pearson's correlation test was applied. Using the body condition as the response variable, as a proxy for overall health, the analysis was performed with the two explanatory variables eosinophil: total blood ratio and white blood cell: total blood cell ratio. These were chosen as measures for as a measure for parasite-specific innate immune response and as a measure for a broad scale innate immune response respectively. To investigate if the site class had any effect on the immune response Kruskal-Wallis tests were applied with site class as the response variable, using white blood cell: total blood cell ratio and eosinophil: total blood cell ratio as explanatory variables.

Finally, the presence of *Paratrichosoma* sp. was analyzed. The data on occurrence was tested against site class and species using a Chi-square test. A binary logistic regression was applied to investigate how the variables pH, salinity (ppt) and body condition affected the presence of *Paratrichosoma* sp.

Lastly a Kruskal-Wallis test was applied to investigate the effect of *Paratrichosoma* sp. on the health and general immune response in the crocodiles using white blood cell: total blood cell ratio and eosinophil: total blood ratio as explanatory variables.

Results

Haemoparasite ratio: differences between site classes, species and sex

Thirty-three samples (100%) showed traces of being infected by haemoparasites. These consisted of fully developed worms and developing worms within the erythrocyte cell (**Figure 4B**)

There was no statistically significant difference in the ratio of infected erythrocytes between the sites ($p = 0.062$), although rural areas did show a somewhat higher ratio of infected erythrocytes (**Figure 6**).

Similarly, there was no significant difference in the ratio of infected erythrocytes between the species and the hybrids ($p = 0.554$), but the hybrids appeared to have a slightly higher prevalence than the two true species (**Figure 7**). Analyzing the ratio of infected erythrocytes between sexes also produced a non-significant result ($p = 0.361$). However, females seemed to have a higher haemoparasite: red blood cell than males (**Figure 8**).

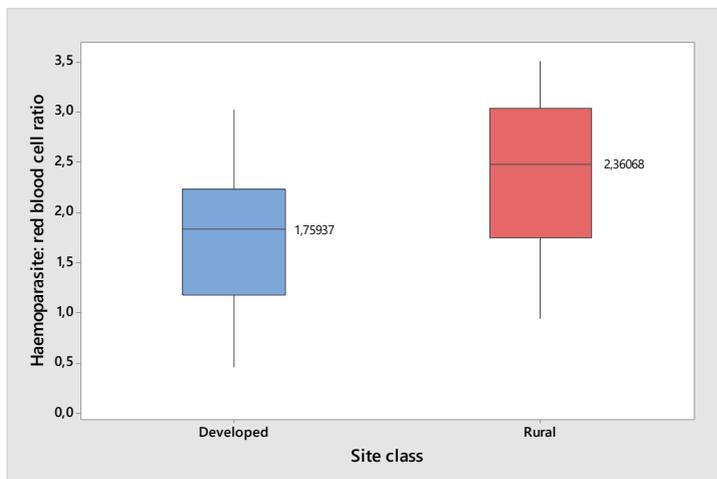


Figure 6: Boxplot depicting the haemoparasite: red blood cell ratio for site class. Means of haemoparasite: red blood cell ratio to the right of boxes in the graph. Developed $n=23$, Rural $n=9$

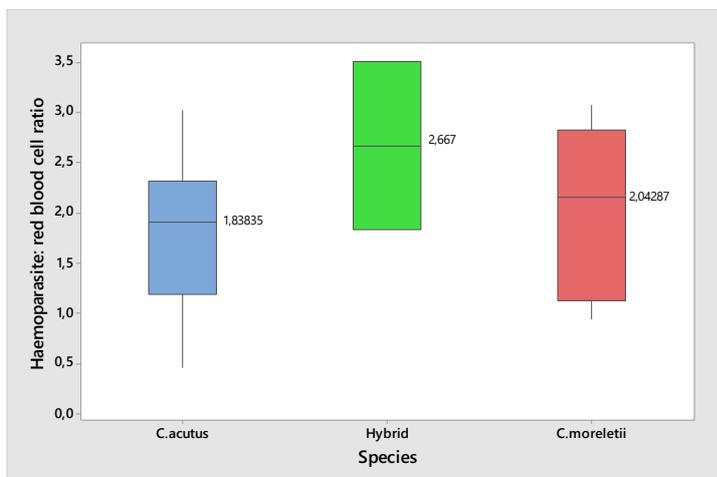


Figure 7: Boxplot depicting the haemoparasite: red blood cell ratio for the species. Mean haemoparasite: red blood cell ratio to the right of each databox. C. acutus ($n=24$), Hybrid ($n=2$), C. moreletii ($n=6$)

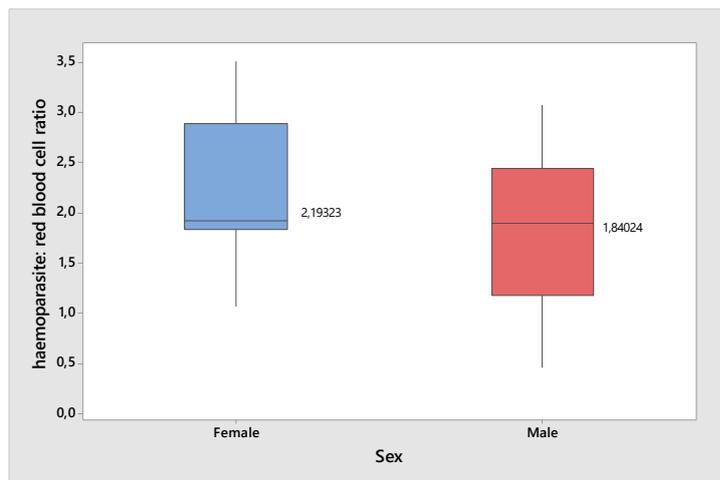


Figure 8: Boxplot depicting the difference in haemoparasite: red blood cell ratio between sexes. Mean parasite: red blood cell ratio to the right of each databox. Female (n=8), Male (n= 24).

Biotic and abiotic factors effect on body condition

There is a statistical difference in body condition between the site classes sampled ($p=0.025$), with individuals sampled in rural areas displaying noticeably higher mean body condition numbers ($rural = 3.358$, $developed=2.465$) (Figure 9).

Body condition also differed significantly between species ($p=0.005$), with Morelet's crocodiles and hybrids showing significantly higher mean body conditions, 3.789 and 3.769 respectively, compared to American crocodiles body condition of 2.376 (Figure 10).

When species and site class are analyzed the results shows that there is a significant difference in the encounter of the species among the site classes ($p= 0.001$, $Pearson's \chi^2=16.857$). The American crocodile was encountered more often in developed areas compared to rural. For *C. moreletii* the trend is opposite and the majority of Morelet's crocodile were encountered in rural areas. The encounter rate of hybrids is seemingly equal between rural and developed areas, but with a $n=5$ the sampling pool is too small to state anything definitive.

The scatterplot for body condition and salinity showed a downwards sloping trend. The Pearson's correlation test revealed a significant weak downward correlation between the two variables ($p= 0.006$, $r= -0.369$) (Figure 11).

A Kruskal-Wallis analysis also revealed significant differences in mean salinity between developed ($mean\ salinity_{Developed}=29.703\ ppt$) and rural sites ($mean\ salinity_{Rural}= 14.270\ ppt$) ($p= 0.001$) (Figure 12).

Looking at the salinity in the areas the different species were caught, the data reveals that *C. acutus* is encountered more often in saline waters ($mean\ salinity_{C.acutus}=31.5$), whilst *C. moreletii* is encountered most often in fresh water ($mean\ salinity_{C.moreletii}=2.381$) ($p=0.001$). Investigating the plot reveals that although the different crocodiles have a strong affinity for high or low saline waters, both species do occur in both fresh and salt water (Figure 13). The boxplot suggest that hybrids are found across a wider salinity gradient ($mean\ salinity_{Hybrid} =13$).

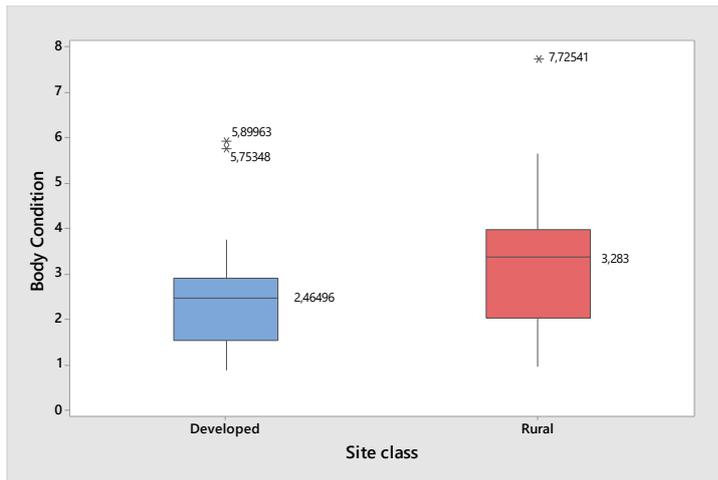


Figure 9: Boxplot depicting the difference in body condition between site classes. Mean body condition to the right of each databox. Developed n=30, Rural n=25.

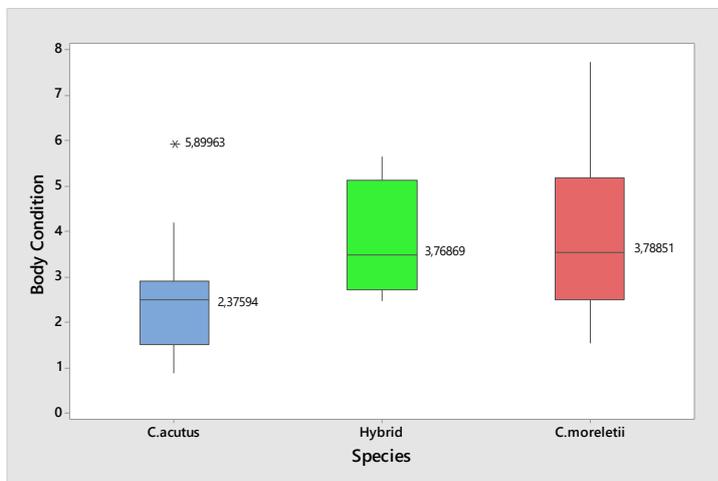


Figure 10: Boxplot depicting the difference in body condition between site classes. Mean body condition to the right of each databox. C. acutus n=37, Hybrid n=4, C.moreletii n=14.

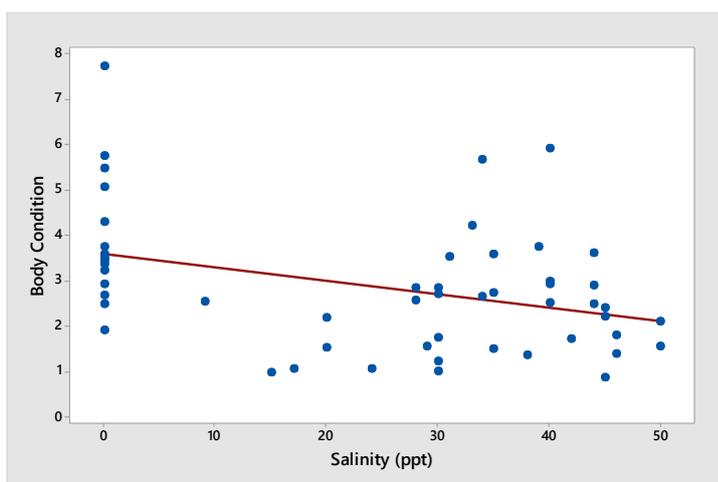


Figure 11: Scatterplot depicting the relationship between body condition and Salinity (ppt). n=53. Freshwater sites with 0ppt salinity lie to the left in the plot

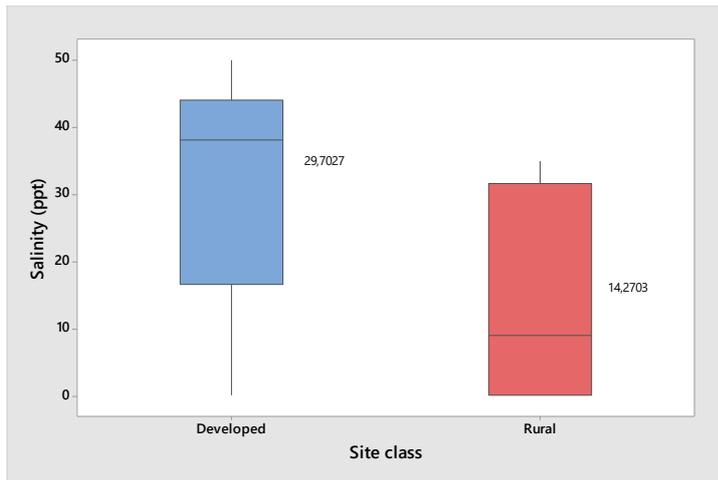


Figure 12: Boxplot depicting the difference in salinity (ppt) between site classes, developed and rural. Mean salinity for each site class is noted to the right of each box. Developed n=37, rural n=37

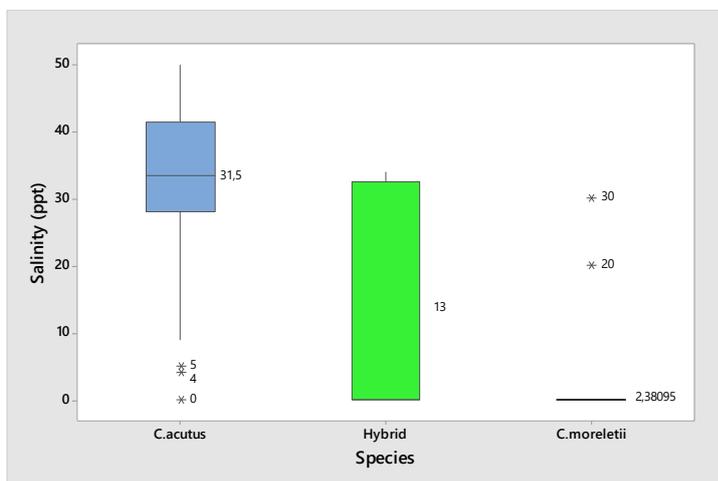


Figure 13: Boxplot depicting the difference in salinity (ppt) between species encounters. Mean salinity for each species is noted to the right of each box. C.acutus n= 48, Hybrid n= 5, C.moreletii n=21

Haemoparasites and their effect on crocodilian health

The ratio of infected erythrocytes does not seem to be correlated with body condition ($p=0.986$, $r=0.004$), and there is no clear pattern to be observed (**Figure 14**). No significance was found in the correlation analysis between haemoparasite: red blood cell ratio and white blood cell: total blood cell ratio ($p=0.193$, $r= -0.236$), although the scatterplot depicts a slight negative relationship (**Figure 15**). Analysis of the relationship between haemoparasite: red blood cell ratio and eosinophil: total blood cell ratio was also not statistically significant ($p=0.651$, $r=0.114$) (**Figure 16**).

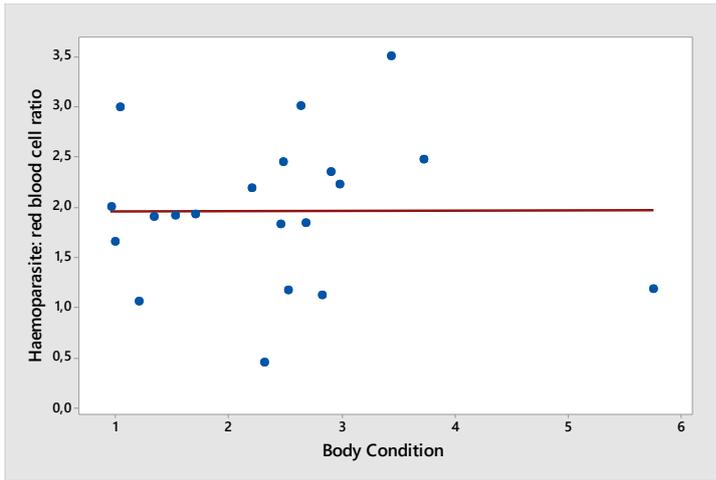


Figure 14: Scatterplot depicting the relationship between haemoparasite: red blood cell ratio versus body condition. $n=20$

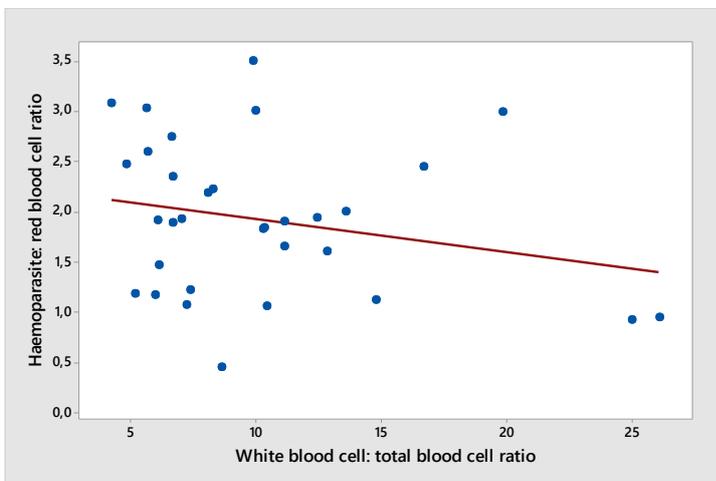


Figure 15: Scatterplot depicting the relationship between haemoparasite: red blood cell ratio versus white blood cell: total blood cell ratio. $n=32$

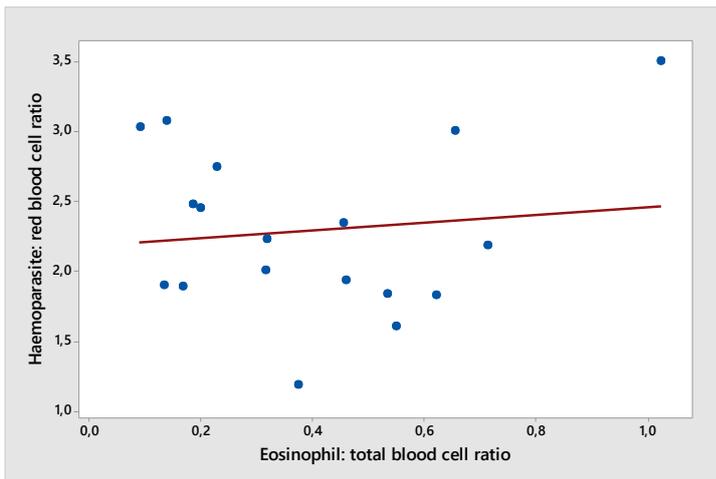


Figure 16: Scatterplot depicting the relationship between haemoparasite: red blood cell (RBC) ratio versus eosinophil: total blood cell ratio. $n=18$

Innate immune response and its relation to body condition

There was no significant correlation between body condition and eosinophil ratio in the total blood count among the crocodiles ($p=0.115$, $r=0.364$) (Figure 17).

However, there was a significant moderate negative correlation between body condition and the white blood cell ratio in the total blood count ($p=0.038$, $r= -0.467$) (Figure 18).

The control for difference in the immune response to the site classes returned with non-significant differences in the eosinophil: total blood cell ratio ($p=0.929$) and the white blood cell: total blood cell ratio ($p=0.414$), suggesting that the immune response did not differ between the site classes.

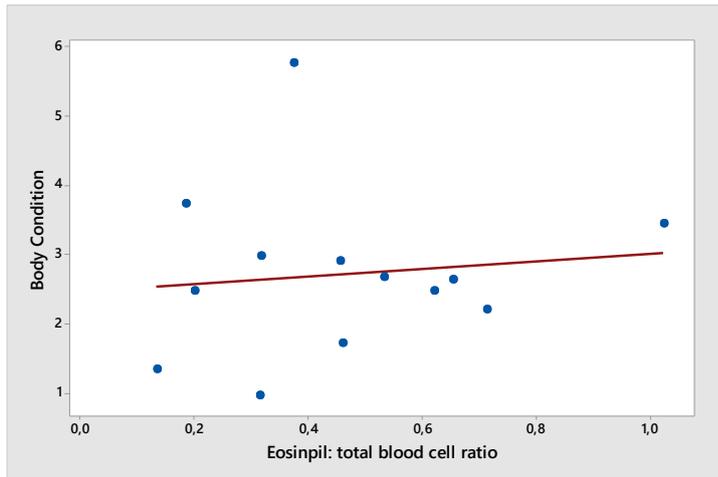


Figure 17: Scatterplot of the relationship between body condition and eosinophil: total blood cell ratio showing a positive relationship, $n=13$.

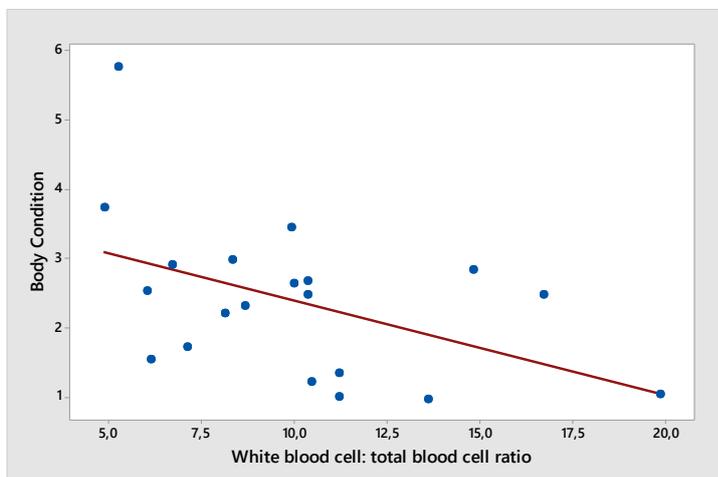


Figure 18: Scatterplot of the relationship between body condition and white blood cell: total blood cell ratio showing a negative relationship, $n=20$

Presence of *Paratrichosoma* sp. and its effect on crocodilian health

Among *C. acutus*, the proportion of infected individuals with *Paratrichosoma* sp. in this investigation came out to 33.3% of captured animals. The *C. moreletii* had a slightly higher proportion of 37.5% of animals being infected, but this was shown to not be a significant difference between the species ($p=0.492$, Pearson's $\chi^2=1.419$).

Paratrichosoma sp. presence was not determined by any of the tested parameters. The results from the

analysis of *Paratrichosoma* sp. presence between site class returned with a non-significant result ($p=0.542$, $\chi^2=0.372$). Similarly, the test for association between the presence of *Paratrichosoma* sp. and species was also deemed non-significant ($p=0.492$, $\chi^2=1.419$).

Results from the binary logistic regression could not explain any relationship between the presence of *Paratrichosoma* sp. or any of the explanatory variables, salinity ($p=0.829$), pH ($p=0.133$) and body condition ($p=0.122$).

There was no significant difference in the white blood cell count: total blood cell ratio between infected and non-infected crocodiles ($p=0.414$). Yet, there is a slightly higher average white blood cell count: total blood cell ratio among subjects not infected with the parasites compared to the infected crocodiles (**Figure 19**).

Any difference in the eosinophil: total blood cell ratio between crocodiles infected and not infected with the parasite was also non-significant ($p=0.657$), although the boxplot shows slightly higher eosinophil: total blood cell ratio in infected animals (**Figure 20**)

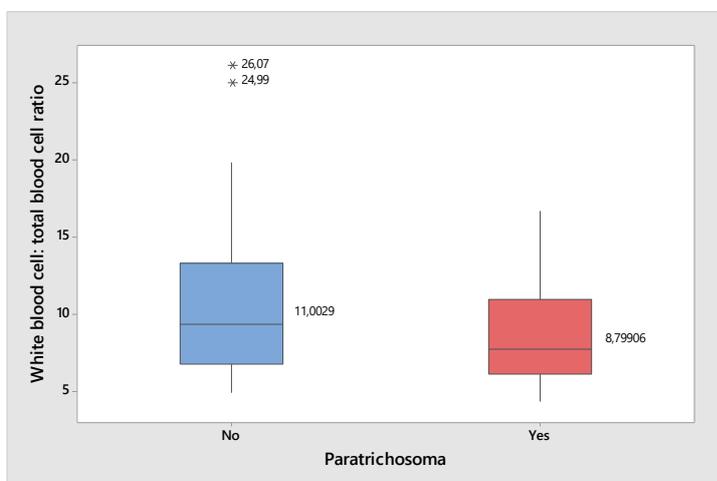


Figure 19: Boxplot depicting the eosinophil: total blood cell ratio between crocodiles with and without traces of *Paratrichosoma* sp. Mean eosinophil: total blood cell ratio noted on the right of each box. No $n=20$, Yes $n=12$

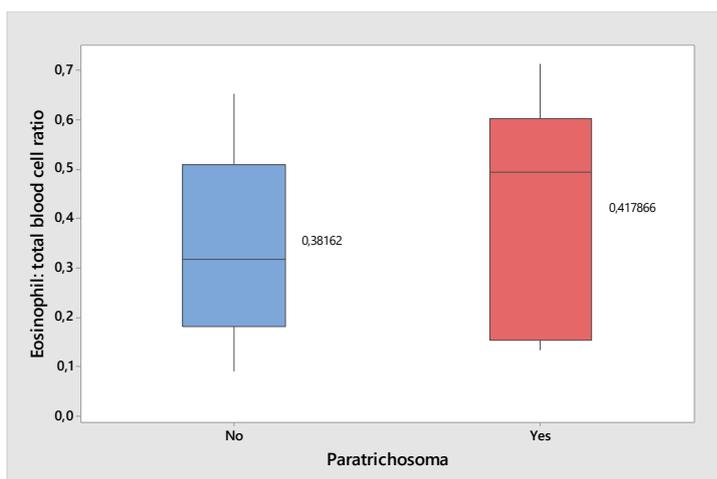


Figure 20: Boxplot depicting the eosinophil: total blood cell ratio between crocodiles with and without traces of *Paratrichosoma* sp. Mean eosinophil: total blood cell ratio noted on the right of each box. No $n=10$, Yes $n=8$

Discussion

Haemoparasite ratio; Differences between site classes, species and sex

All 33 analyzed samples showed signs of being infected with *Hepatozoon* spp., which yields a 100% presence of blood parasites in this study. This is also the first study to report the presence of haemoparasites in *C. moreletii* across its range.

Of six species of *Hepatozoon* recognized to infect crocodylians, only one species of the blood born parasite has been found in the American crocodile (*C. acutus*), the *Hepatozoon crocodilorum*. Until now, none had been detected in the Morelet's crocodile (*C. moreletii*) (Smith 1996, Lainson et al. 2003, Tellez 2014b). The parasites discovered in the blood slides from *C. acutus* and *C. moreletii* bore similar patterns to those produced by *Hepatozoon* spp. recorded in other crocodylian species, but could not be determined to species level due to the scope of this study (Lainson et al. 2003). Although infection by *Hepatozoon* spp. is common among wildlife, and reptiles in particular, it has not been registered a prevalence of 100% among wild crocodylians before this study (Davis et al. 2011). The reason for the high prevalence is unclear but could be caused by exposure to the parasites vector in both habitats and/or the overlapping habitats of the crocodiles facilitating the spread. High prevalence is connected to high encounter rate of the parasites vector. This suggests that there is a shared behavior of the two crocodylian species which facilitate equal exposure to the vector (Combes 2001). Further investigations into the cause of the high prevalence is warranted.

There was no significant difference between haemoparasite: red blood cell ratio between site classes ($p = 0.062$). This suggests that the haemoparasite load was not higher in rural areas than in developed, and that the vector likely exist in both site classes. Despite the infection pathway still being uncertain, the overlapping range and habitat of *C. acutus* and *C. moreletii*, as well as their capacity for long range travel increase the chances of crocodiles encountering the same parasites vector, thus increasing the chance for infection (Smith 1996, Platt and Thorbjarnarson 2000a, b, Thorbjarnarson 2010, Viana et al. 2010, Platt et al. 2013, Tellez 2014b, Grigg and Kirshner 2015, Pacheco-Sierra et al. 2016, Tellez et al. 2016, Tellez et al. 2017, IUCN 2018a, b).

There was also no statistical difference in the parasite: red blood cell ratio between species ($p=0.554$), suggesting that the haemoparasite load did not differ between American crocodiles and Morelet's crocodiles. This suggests that the vector does not discriminate between the two crocodylian species. This could also be caused by the parasites conceivable adaption to withstand the immune system response from both species, or that the immune system response to this parasite is similar across these species. A few studies investigating the co-evolution between crocodylians and their parasites, suggest that the parasites of crocodylians have a long and specified relationship with their host species (Brooks 1979, Tellez 2014a). With the close evolutionary relationship and shared habitat between *C. moreletii* and *C. acutus* there is also a possibility of a shared relationship between the blood parasite and both crocodile species. This can also be observed in **Figure 13** as both species are found in freshwater and salt water habitats, producing hybrid offspring found in habitats of high and low salinity.

The haemoparasite load between sexes follows the same trend indicating that there was no significant difference in the ratio of parasite infected red blood cells between the sexes ($p=0.361$). Interestingly, previous work found a higher ratio of *Hepatozoon* sp. in female American alligators than in males. (Davis et al. 2011). The bias towards heavier parasite load in females may be due to behavioral differences between the sexes, which could cause females to encounter the parasite vector more frequently. Females are more sedentary than males, especially during the nesting period. This may potentially increase exposure to the parasites vector, and therefore the prevalence of parasites. This, however, is yet to be examined.

Biotic and abiotic factors effect on body condition

Body condition was noticeably better in rural compared to developed areas ($P=0.025$). Additionally, there was a significant negative correlation between body condition and salinity ($p=0.010$, $r=-0.344$). An increase in salinity and anthropogenic disturbance was therefore correlated with a decrease in body condition. These trends are likely due to behavior and habitat choice, rather than the salinity's effect on crocodile physiology and were explored further.

The Morelet's crocodile and hybrids had higher mean body condition scores ($m=3.789$, $h=3.769$) compared to the American crocodiles (2.376). American crocodiles were also encountered more often in developed areas compared to rural. For the Morelet's crocodile the trend was opposite and the majority of Morelet's crocodile were encountered in rural areas ($p=0.001$). The higher occurrence of American crocodiles in developed areas is likely linked to their preferred habitat in saline environments, as most developed areas are found along the coast. It was therefore not surprising to find the American crocodile was encountered more often in saline waters ($mean\ salinity\ C.acutus=31.5$, $mean\ salinity\ C.moreletii=2.381$), whilst *C. moreletii* was encountered most often in fresh water ($p=0.001$). The encounter rate of hybrids is seemingly equal between rural and developed areas but the sample size too small ($n=5$) to say anything definitive. That hybrids are found in both rural and developed areas is not surprising as the two interbreeding species have overlapping habitats.

American crocodiles inhabiting more disturbed and saline environments along the coast, experience more anthropogenic stress as coastal areas experience higher degree of development compared to rural areas in Belize (Arkema et al. 2014). A 2014 analysis showed that 40% of Belizeans live and work along the coast, and more than 800,000 tourists visit the country every year, many of which visit and use the coast and ocean for recreational purposes (Chomitz and Gray 1999, Arkema et al. 2014, Verutes et al. 2017). Investigations into the spatial ecology of American crocodiles suggest that the species prefer to spend their time away from human settlements. However, direct or indirect anthropogenic impact on the American crocodile in Belize is impossible to avoid due to their preferred habitat overlapping zones with high human activity (Arkema et al. 2014, Balaguera-Reina et al. 2016, Tellez et al. 2016, Tellez et al. 2017). Future projections show that, with the current rate of coastal development, the coastal systems of Belize will be under moderate to severe stress in the near future (Arkema et al. 2014, Verutes et al. 2017). The higher anthropogenic impact on these coastal areas stem from an increase in industry, transport and recreational use by locals and tourists. This may, in turn, have a negative effect on the body condition of animals living in the coastal areas. Crocodiles may allocate energy towards combatting stressors, such as increased mobility, longer foraging times due to destruction of prey habitat, or immunological responses due to pathogens and environmental toxins such as pollutants (Veiga et al. 1998, Oliveira et al. 2017). The exposure to higher levels of anthropogenic stress may be what is contributing to a lower body condition in the American crocodile and should be followed up by further studies.

Haemoparasites and their effect on crocodilian health

The effects of haemoparasites on their crocodilian hosts are poorly understood (Veiga et al. 1998). Findings from this study suggests that haemoparasites have a limited effect on crocodilians, since the ratio of parasite effected erythrocytes did not appear to affect body condition ($p=0.986$), broad innate immune response ($p_{white\ blood\ cell\ ratio}=0.193$), or the parasite-specific immune response ($p_{eosinophil\ ratio}=0.651$).

Presence of haemoparasites has been associated with decreased hematocrit numbers in several species as erythrocytes are destroyed by the parasite. This in turn leads to reduced oxygen transport resulting in a depressed cellular function, organ damage and reduced body condition (Habib et al. 2003, Amo et al. 2005). However, it is still uncertain how big an effect this has on reptiles in general and crocodilians in particular as no apparent effect has been observed in this examination (Amo et al. 2005, Knotkova et al. 2005).

In the mammalian innate immune system, the eosinophils are tasked with combatting parasitic infections, and eosinophil counts are therefore a good estimator for parasite infection. In crocodilians on the other hand, the function of the eosinophils is uncertain, as there is a lack of knowledge on their role in the innate immune response to parasites (Montali 1988, Veiga et al. 1998, Zimmerman et al. 2010, Brooker et al. 2011). Experimental work has shown that American alligators (*Alligator mississippiensis*) can react to simulated parasite infections by recruiting eosinophils, but the same response has not yet been recorded in wild individuals with naturally occurring parasite infections (Montali 1988, Zimmerman et al. 2010).

Another leukocyte found in crocodiles is the heterophil. These are thought to be analogous to the neutrophils in mammals, being recruited to suppress other microbial invasions and mediate inflammation (Jain and Jain 1993, Sykes and Klaphake 2008, Claver and Quaglia 2009, Brooker et al. 2011, Weiss and Wardrop 2011). Crocodiles, turtles, birds, and some species in Order Squamata possess both eosinophils and heterophils, but their exact function in the immune response is still uncertain (Jain 1986, Montali 1988, Jain and Jain 1993, Sykes and Klaphake 2008, Weiss and Wardrop 2011, Charles-Smith et al. 2014). Laboratory experiments using chickens, show that the bird's response to a foreign pathogen differ from the mammalian response. When exposed to a simulated parasite infection, the bird's recruitment was that of either both eosinophils and heterophils, or of one or the other. This suggest that these granulocytes may have a different function in the immune system in birds than eosinophils and neutrophils in mammals (Huynh and Chubb 1987, Abdul-Careem et al. 2008, Zimmerman et al. 2010). The crocodilian response to parasites seems to differ from mammals and other vertebrates and may not be measurable by the recruitment of eosinophils alone.

The close evolutionary relationship between birds and crocodiles, the lack of evidence of eosinophil response to parasite interaction, and the similarity in granulocyte composition may suggest that future research should seek inspiration in the immunology and hematology of birds and reptiles for future studies.

Innate immune response and its relation to body condition

There was no significant correlation between body condition and eosinophil ratio in the total blood count amongst the sampled crocodiles ($p=0.115$). There was, however, a significant moderate negative correlation between body condition and the white blood cell ratio in the total blood count among the crocodiles ($p=0.038$, $r=-0.467$). Referring to the differences in body condition found between site classes, controlling for immune response between site classes was performed yielding a non-significant result ($p_{\text{eosinophil ratio}}=0.929$, $p_{\text{white blood cell ratio}}=0.414$), suggesting that immune response did not differ between site classes.

The cause of a decrease in body condition in relation to an increase in the white blood cell: total blood cell ratio is uncertain. It is however possible that the broader immune response is a response to an unknown stressor that is causing the decrease in body condition. This could result in a trade-off in the form of decreased activity levels to allocate resources to combatting an infection resulting in less time spent foraging for prey. Yet, it could also be a trade-off in less resources being allocated to body growth and maintenance (Veiga et al. 1998, Combes 2001, Bonneaud et al. 2003, Navarro et al. 2003, Oliveira et al. 2017). A heterophile: lymphocyte ratio or corticosteroid analysis would be able to measure the chronic stress response, but data for this type of analysis is lacking in this study and needs to be addressed in further studies (Cotter 2015, Davis and Maney 2018). The recruitment of a broader innate immune response could have several explanations, such as bacterial infections, other pathogens, environmental or anthropogenic stress, and/or environmental toxins, as well as a response to parasites (Montali 1988, Ewenson et al. 2001, Murray et al. 2015). The findings presented here show that there is a correlation between crocodile body condition and the recruitment of white blood cells in an immune response, as well as a decrease in body condition in

habitats with higher anthropogenic disturbance. This may suggest that the higher level of disturbance could negatively affect crocodiles as they experience higher level of stress over longer periods of time. However, due to the uncertainties in the function of granulocytes in crocodilian immune response and the lack of lymphocyte and corticosteroid data, the cause of the correlation between decreasing body condition with increasing white blood cell: total blood cell ratio remains unclear.

Presence of Paratrichosoma sp. and its effect on crocodilian health

Of all crocodiles captured during this investigation, 34,5% had traces of being or having been infected with the nematode worm *Paratrichosoma* sp. with no significant difference between the crocodile species ($p=0.492$).

This is very similar to a previous study on the Mexican Caribbean islands north of Belize where the proportion of infected animals was 30.3% (Charruau et al. 2017). This is not surprising since Belize share their population of both Morelet's and American crocodiles with southern Mexico. However, previous studies into the presence of *Paratrichosoma* sp. in *C. acutus* in Caye Caulker and *C. moreletii* in Chiquibul Forest in Belize showed much higher infection rates (92% and 79%, respectively) (Tellez et al. 2016, Tellez et al. 2017). The reasons for this contrasting result are unclear but could be due to a seasonal effect of prevalence since the studies took place in different seasons. Other biotic and/or abiotic variations that produce parasite fluctuations may also play a role. Another hypothesis is that these parasites have a free-living life stage that may be sensitive to pollution, and that fluctuations in the presence and load of *Paratrichosoma* sp. differ according to pollution in the environment (Tellez and Paquet-Durand 2011). However, none of the tested abiotic variables could explain the prevalence of *Paratrichosoma* sp. among the crocodiles sampled ($site\ p=0.542$; $salinity\ p=0.829$; $pH\ p=0.133$).

Uncertainties around the infection pathway of *Paratrichosoma* sp. still linger as the knowledge of the effect of biological and abiotic factors on the parasite and their ecology remains unclear (Tellez and Paquet-Durand 2011). Information from this investigation is supported by previous data from the region that pH, salinity, body condition, anthropomorphic disturbance or species does not seem to have much effect on the presence of the parasite (Charruau et al. 2017). Still, one investigation from the Yucatan, Mexico, found a slight increase in the presence of the parasite with increasing salinity (Charruau et al. 2017). This, however, can also be explained by evaporation of water during periods of dry weather. This will decrease water levels resulting in higher concentration of salt as well as constraining the habitat of crocodiles and parasites, increasing the density and encounter rate of the two.

Information gathered from this investigation also support previous studies in their claims that *Paratrichosoma* sp. appears to be a benign parasite, as no effect on body condition ($p=0.122$) or immune response in crocodiles infected by *Paratrichosoma* sp. was detected here ($p_{white\ blood\ cell\ ratio}=0.414$, $p_{eosinophil\ ratio}=0.657$) (Tellez and Paquet-Durand 2011, Tellez et al. 2016, Tellez et al. 2017). Clearly, continuous monitoring and long-term studies are needed to uncover the causes for presence and load of *Paratrichosoma* sp. in crocodiles in the region. Further research into the ecology and life cycle of *Paratrichosoma* sp. is also considered important as the scarring the females create on the epidermis of the crocodile decrease the sales value of the skin in the crocodile farming industry (Moravec and Vargas-Vazquez 1998).

Conclusions

Studies into hematology, immunology and parasitology of wild crocodilians is a scientific frontier with limited information available. However, with increasing pressure on the crocodilians around the world, it is important to investigate these avenues. This study is one of few investigating the relationship between haemoparasites and their crocodilian hosts, as well as the haemoparasite effects on the crocodile innate immune response and body condition. During this study 100% of the analyzed crocodile blood samples showed signs of being infected. This is not only a haemoparasite prevalence that has not yet been recorded in crocodiles anywhere else, but it is also the first record of haemoparasites in Morelet's crocodile (*Crocodylus moreletii*). Although all analyzed crocodiles were infected with the blood borne parasite the results also uncovered that the crocodiles of Belize do not seem to be adversely affected by the blood parasites.

Crocodilians classical assignment to the reptiles, Class Reptilia, is currently under review. This is due to crocodiles physiology and genetics being more closely comparable to that of the other living group of archosaurs, birds (Class Aves) (Seymour et al. 2004, Ezcurra et al. 2014, Farmer 2015, Grigg and Kirshner 2015, Mannion et al. 2015). Results from this study lends more support to this hypothesis, as it appears that eosinophils may not have as prominent a role in combatting parasite infections as it has in mammalian vertebrates. In addition, the crocodiles and birds have similar immune cell assemblage with heterophils and eosinophils, whereas only some squamates possess eosinophils.

American crocodiles living in habitats under high anthropogenic stress showed signs of lower body condition than the Morelet's crocodiles residing in rural areas. Combined with the findings that there was no significant difference in haemoparasite prevalence between site classes, and that neither haemoparasites nor *Paratrichosoma* sp. seem to have an adverse effect on the crocodiles health, this suggest that some unknown stressor in the environment of the American crocodile is the cause of lowered body condition. The correlation of lower body condition and proximity to human developed habitats suggest that this may have an anthropogenic source.

Being able to detect changes in the crocodilian health at an early stage before it affects their body condition is crucial, as crocodilians are apex predators in their ecosystem and important markers for a functioning ecosystem. Using their parasites as biomarkers is a promising less-invasive technique for detecting habitat degradation and other stressors in their environment. For example, parasites of the digestive system have already shown promising signs of being biomarkers for heavy metals in the environment. And *Paratrichosoma* sp. seems to be related to environmental or behavioral changes and is another pathway researchers are examining as a useful biomarker for habitat quality and alteration.

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Appendix

Tables

Table 1: Size classes for crocodiles in Belize

	<i>Crocodylus acutus</i>	<i>Crocodylus moreletii</i>
Hatchling	<50cm	<50cm
Juvenile	51-120cm	51-100cm
Subadult	121-180cm	101-150cm
Adult	>181cm	>150cm

Table 2: List of equipment used during blood draws, blood smear preparation and staining

• 300x Vacutainer Plus 6ml EDTA purple k2, blood collection tube
• 200x Vacuette, no additive 6 ml Premium, blood collection tube
• 200X Needle 0,70x40mm 22Gx1½” regular bevel
• 100X Needle dental 0,50x40mm 25Gx1½” regular bevel
• 200X Needle 1,20x40mm 18Gx1 ½” regular bevel
• 500x Object glass 2406 76x26mm with matted tip
• 500x Pipette 3,5ml Pasteur graded til 3 ml
• 300x syringe luer lock 10ml
• 200x Syringe luer lock 3ml
• CAPP Bravo 0,1-2µL Micropipette
• 1000x 10µL Pipettepoints
• Dye rack for 24 slides
• 300x 1,5mL Eppendorff tubes with lid
• 1L modified Giemsa dye (https://www.sigmaaldrich.com/catalog/product/sial/48900?lang=en&region=NO)
• Methyl rinsing alcohol
• FreeStyle Freedom Lite human glucose meter, and code 16 measuring strips.

Supplementary figures

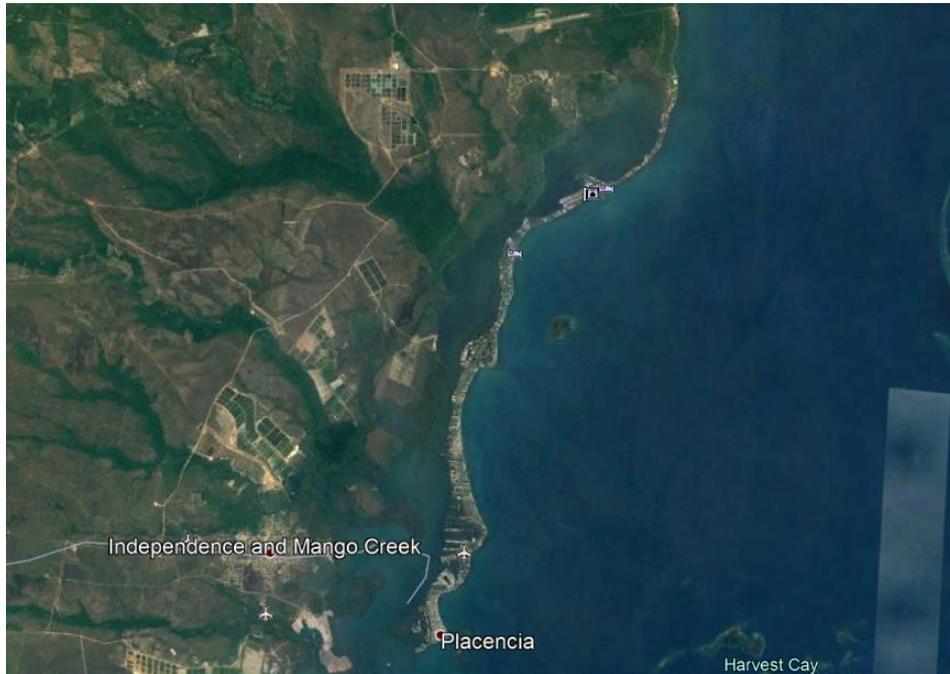


Figure S1: Placencia peninsula. Placencia lagoon is the body of water separating the peninsula from main land. Picture taken from Google Earth, Jan 2019

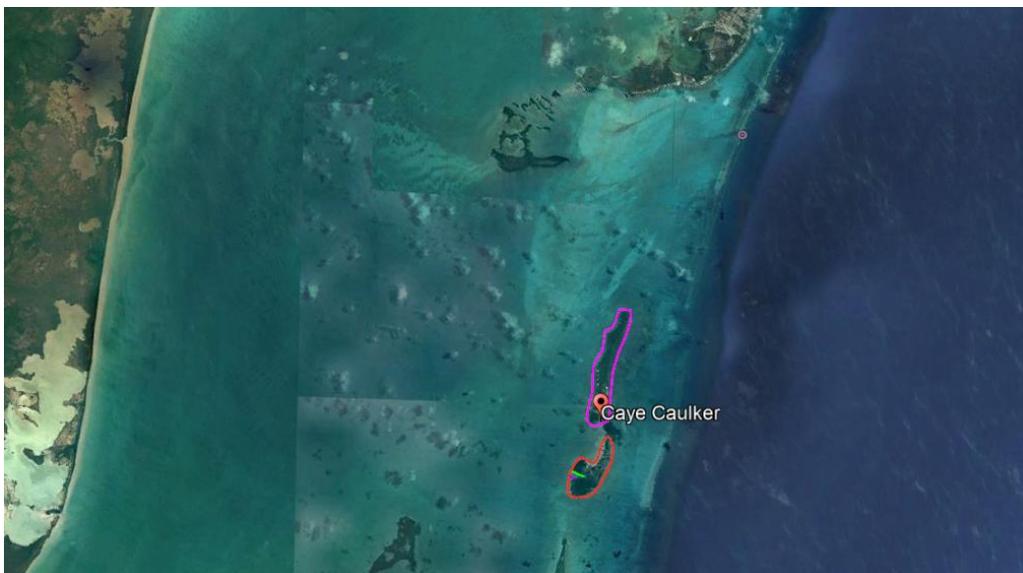


Figure S2: Caye Caulker. North part of island shown in pink, Southern part shown in Red. Picture retrieved from Google Earth, Jan 2019



Figure S3: Caye Caulker. North and south separated by a channel (represented by blue line). Picture retrieved from Google Earth, Jan 2019

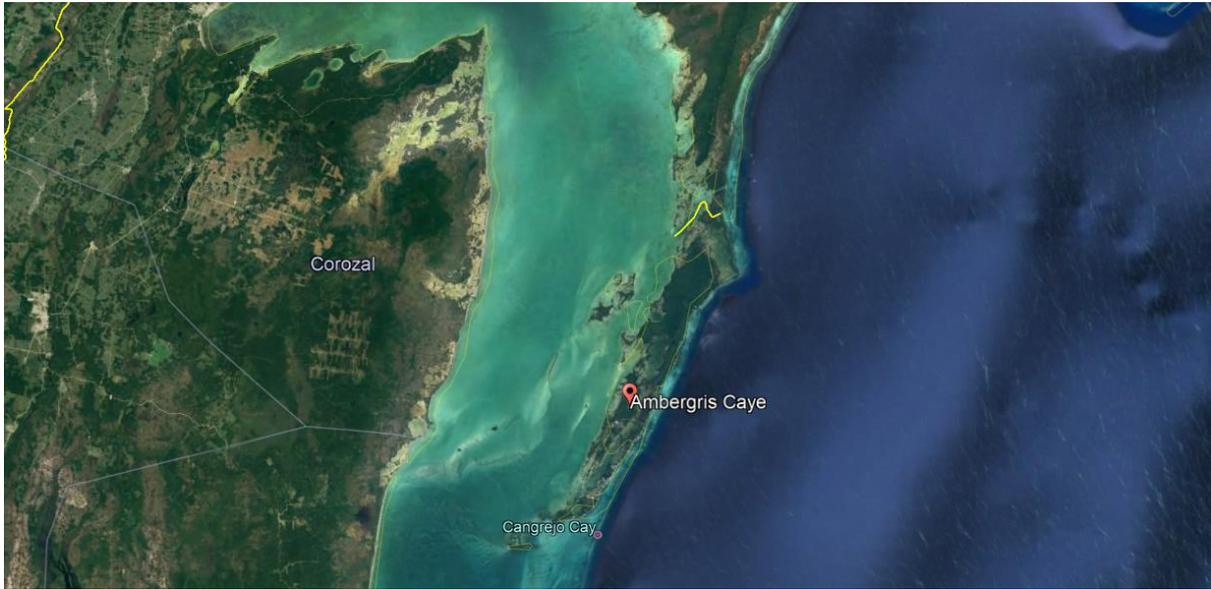


Figure S 4: Ambergris Caye. Picture retrieved from Google Earth, Jan 2019

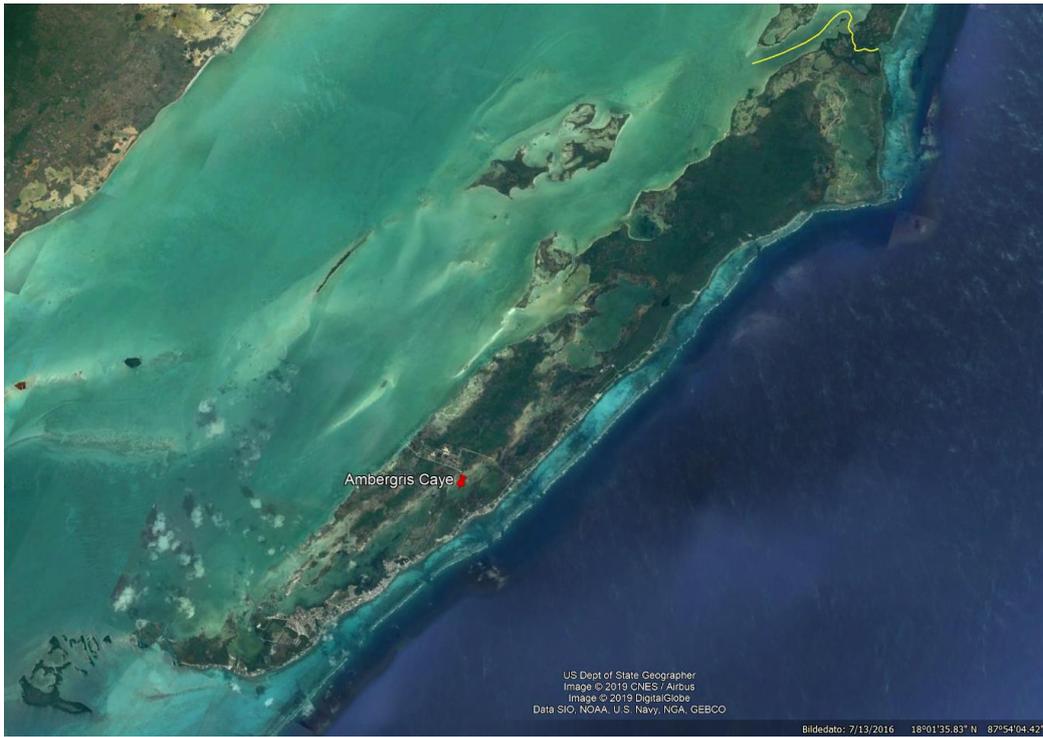


Figure S5: Ambergris Caye. South part of the Caye is heavily developed, whilst northern part is a forest and marine reserve. Picture retrieved from Google Earth, Jan 2019

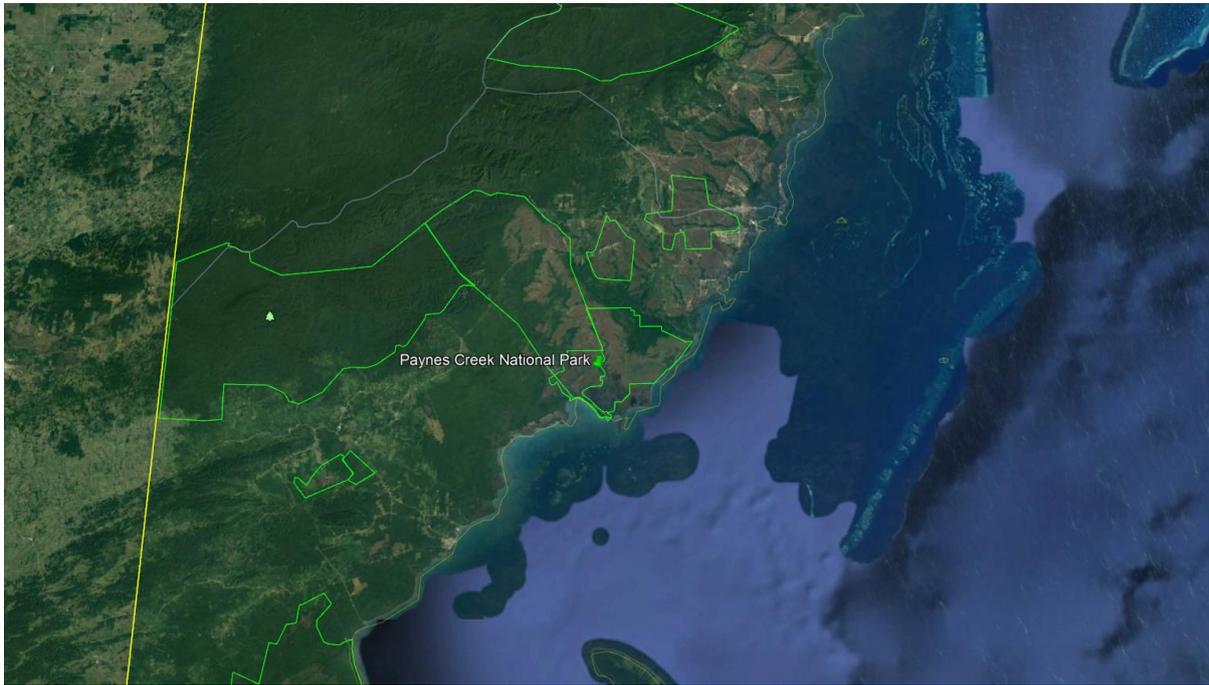


Figure S6: Payne's Creek National Park, Toledo District. Picture retrieved from Google Earth, Jan 2019

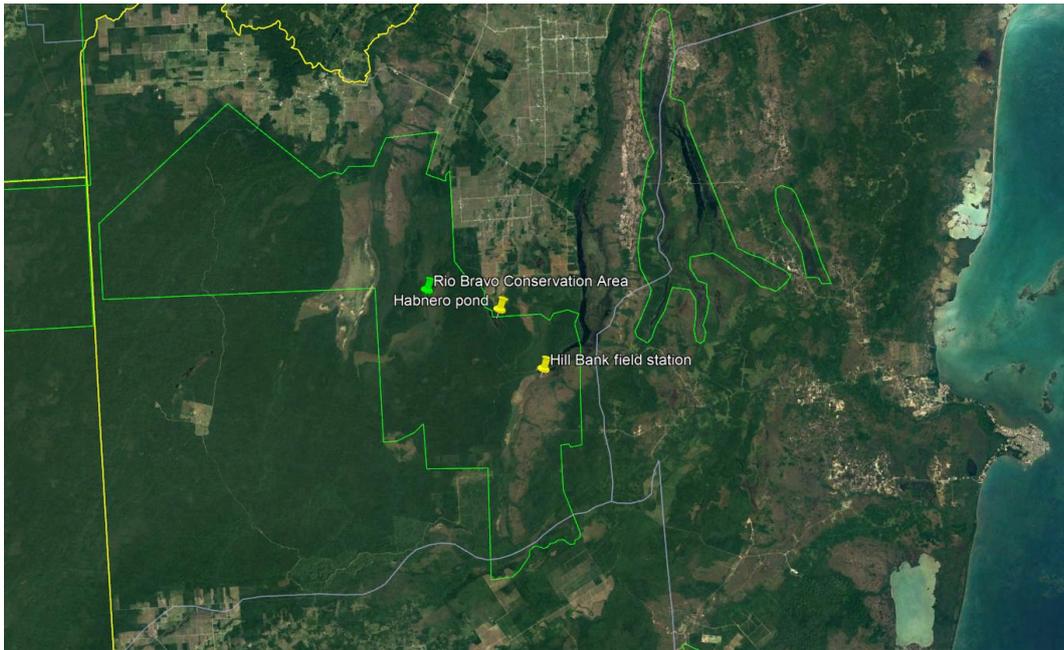


Figure S7: Rio Bravo Conservation Area outlined in green. Yellow pins mark the two main sampling area starting points. Picture retrieved from Google Earth, Jan 2019

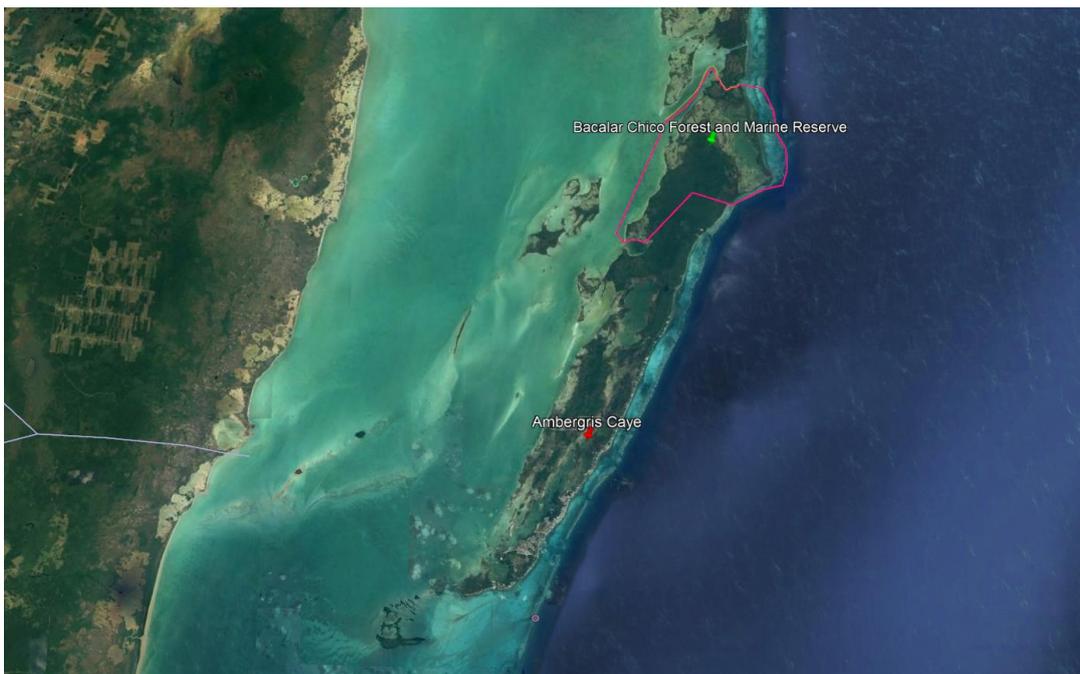


Figure S8: Bacalar Chico Forest and Marine Reserve. Northern part of Ambergris Caye, bordering Mexico to the North. Pink lines outline the reserve. Picture retrieved from Google Earth, Jan 2019

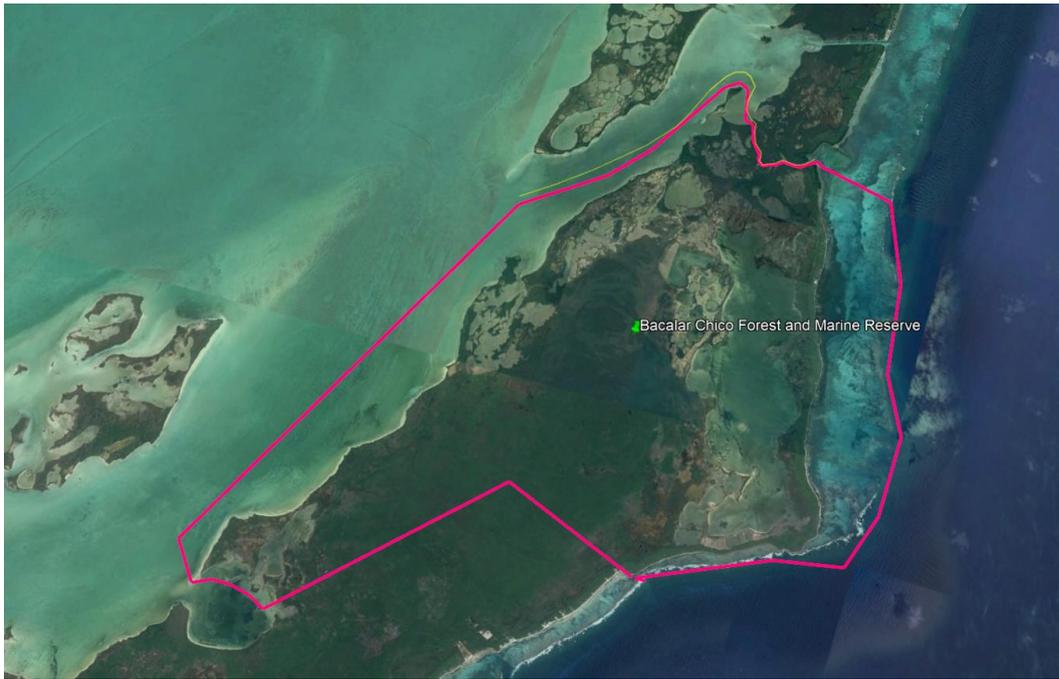


Figure S9: Bacalar Chico Forest and Marine Reserve. Northern part of Ambergris Caye, bordering Mexico to the North. Pink lines outline the reserve. Picture retrieved from Google Earth, Jan 2019

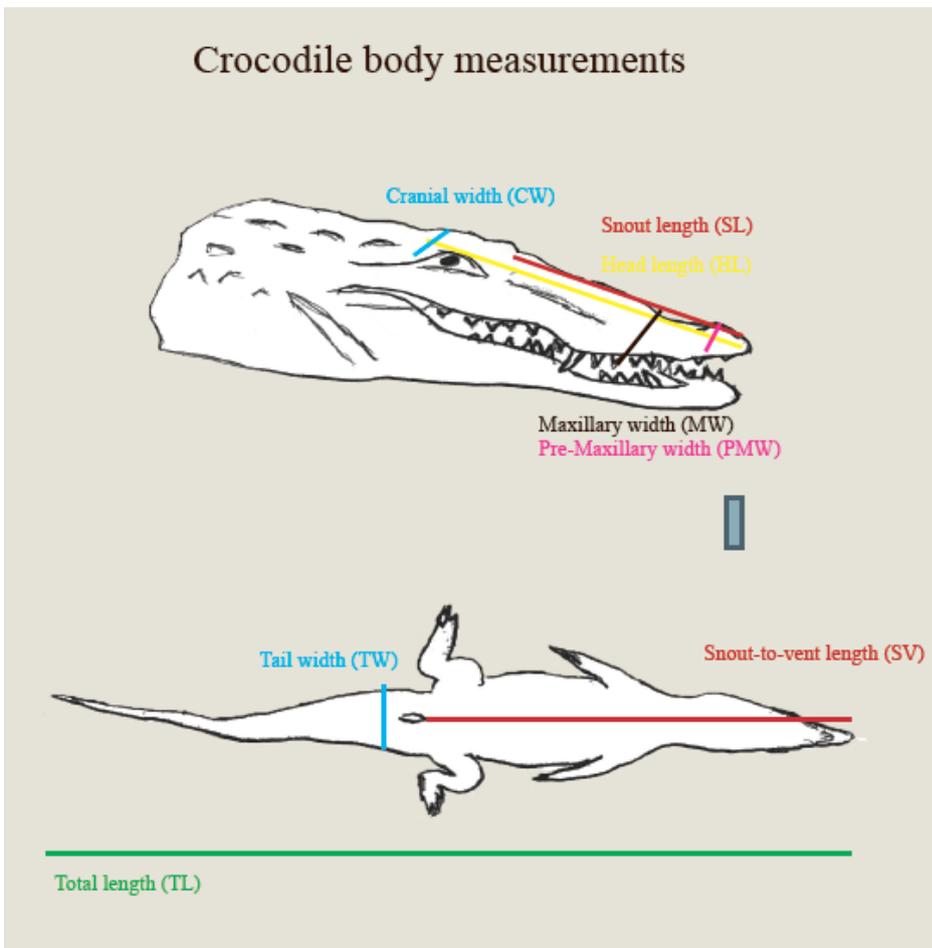


Figure S10: Morphometric measurement sites

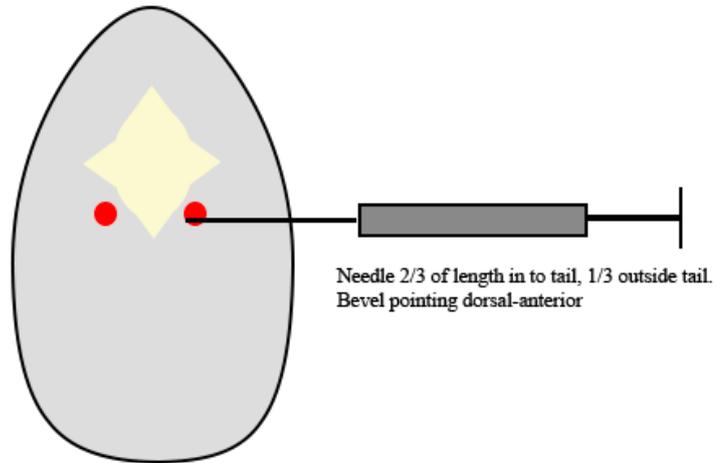


Figure S11: Caudal bleeding of tail. Large grey area depicts cross section of tail, off-white section in middle depict spine, red dots are blood vessels from which blood was drawn. Flattened side of drawing represent ventral side of tail.



Figure S 12: Illustrative photo of a blood draw on an adult *Crocodylus acutus*. The slight flexion of tail would help gain access to the blood vessel by creating space between the vertebrae of the tail.



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