Project Update: May 2023

Introduction

Mugger or marsh crocodile (*Crocodylus palustris*) is widely distributed in India and the subcontinent and is mostly found in isolated small pockets. Muggers have a Vulnerable conservation status in IUCN's Red List and are legally protected under Schedule I of the Indian Wildlife (Protection) Act 1972 [1]. Muggers occur in various habitats, including ponds, rivers, canals, and urban drainages. Mugger crocodiles face significant anthropogenic pressure, particularly in the Vadodara district of central Gujarat, India. Records indicate that between 1960 and 2013, a total of 64 attacks by muggers were documented, resulting in 33 fatalities and 31 non-fatal incidents across various regions of Gujarat. More specifically, 52 of these incidents occurred in Vadodara in central Gujarat. In contrast, 50 km away from Vadodara, the Charotar region has low conflict, and humans show high tolerance towards muggers, with a record of only six conflict incidents, so far [2]. The intensity of human-mugger conflict (HMC) becomes acute during breeding seasons, as muggers have onshore denning locations and tend to attack humans to protect their hatchlings [2]. Initially, I had proposed to investigate stress physiology of mugger crocodiles during breeding and non-breeding seasons in the high-conflict region of the Vadodara district. However, following a preliminary survey by our team to assess the potential study sites in central Gujarat, we decided to add a few additional sites in the Charotar region that have a low human population (10,094 as per the 2011 census [3]) in comparison to the Vadodara region (22,813 as per the 2011 census), as well as low HMC. By adding the Charotar region, we will have a more robust understanding of the stress physiology of the muggers within two contrasting regions of central Gujarat.

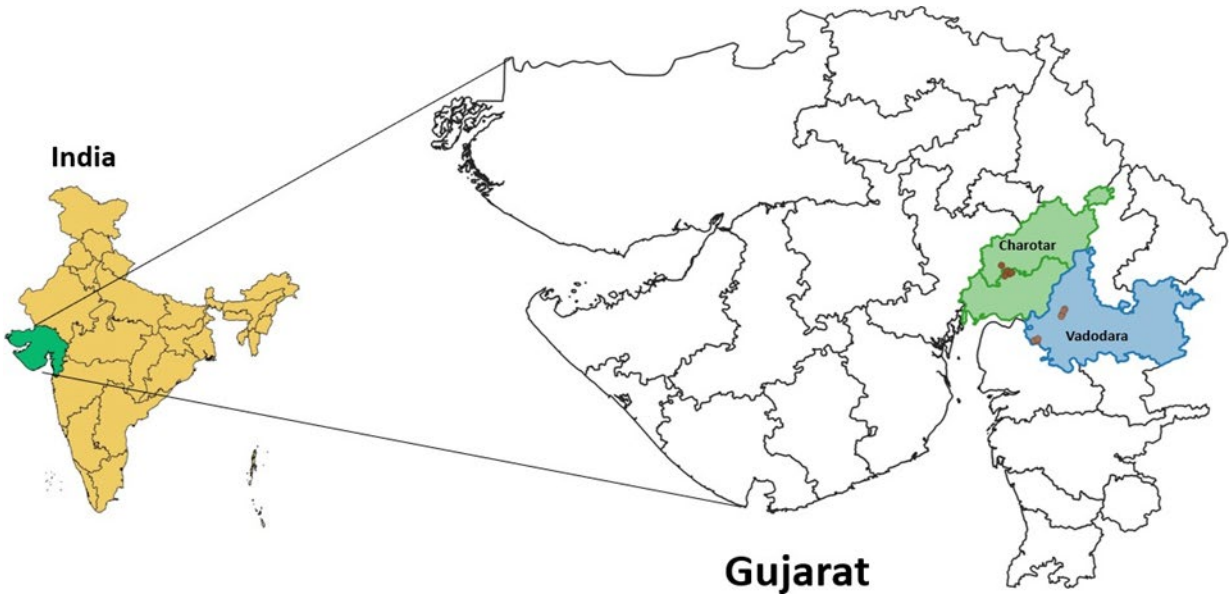

Figure 1[A]: Map representing study sites within Charotar (22.5617° N, 72.9490° E) region, and Vadodara (22.3072° N, 73.1812° E) region, in Central Gujarat, India.

Thus, we have selected two study sites (Fig 1) in central Gujarat, India, for the ongoing project. One region is Charotar, a rural area with a low human population, low HMC, lentic ecosystem, and non-industrial area. The other region is Vadodara, an urban area with a high human population, high HMC, lotic ecosystem, and an industrial area. Both regions are 40-50 km apart from each other. In the mid-review report, we are presenting preliminary data on stress physiology for both the regions during the breeding seasons of the muggers.

Figure 1[B]: Image displaying representative habitats of Vadodara and Charotar region.

Objectives completed so far:

1. The ongoing project has now developed, validated, and standardised a non-invasive method to measure fecal glucocorticoid metabolite (fGCM) concentrations in mugger crocodiles.
2. The developed tool has been applied to monitor the well-being of muggers, living under free-ranging conditions across diverse habitats within central Gujarat, India.

Methods

Study design for objective 1

We decided to use the assay that targets the 5b-3a-ol-11-one structure (11-oxoaetiocholanolone Enzyme ImmunoAssay (EIA)) to measure fGCM levels in mugger crocodiles. The selected assay has been previously validated for use in Nile crocodiles (*Crocodylus niloticus*) [4], that belong to the same genus as the muggers. For biological validation of the selected hormone assay for our target species, mugger crocodiles, we conducted a study on captive mugger crocodiles housed at the Madras Crocodile Bank Trust in Chennai, India. We collected scat samples (n=11) opportunistically during the annual medical checkups of captive muggers that were restrained during the process. Restrain and capture has been shown to be a stressor for most large vertebrates, triggering the hypothalamo-pituitary-adrenal (HPA) axis to secrete glucocorticoids, a stress-indicating hormone [4,6]. For comparison, we also collected scats (n=11) pre-capture from the same focal group of individuals. We collected scats for 15 days pre-capture and 15 days post-capture [4]. All the scat samples were stored at -20°C until analysis and were brought in ice packs to the lab at Ahmedabad University for further analysis.

Study design for objective 2

We collected scats from free-ranging mugger crocodiles within each of the two regions, Vadodara and Charotar (Fig 1) in central Gujarat, India. Scat sampling sites in each of the two regions were selected based on the feasibility of scat collection and after conducting a preliminary observation (N= 7 days, from 6 am to 6 pm) on assessing the duration of basking behaviour (Fig 2) for both Charotar and Vadodara sites. Anecdotes from our captive work done in 2021-2022 demonstrated that muggers mostly defecate during basking, thus, scat collection timing should be limited before or after the basking durations to avoid disturbing the basking mugger. Observation for the basking survey started when the first animal came out to bask and ended at sunset.

Figure 2: The image taken during the basking study shows the basking of muggers, a typical behavior exhibited by cold-blooded animals, in one of the sites of the Vadodara region.

We did a random and cross-sectional sampling for scats [5]. Based on our captive data, we observed that muggers eat once in 2 weeks and defecate up to 7 days after feeding. We visited each site consecutively for 6 days for scat collection, and then we did not revisit the site in order to avoid pseudo-replication. Our observations indicated that muggers start basking at 10 am and continue till 4 pm. Thus, we decided to collect scats early in the morning, between 6-10 am, so that no animal is disturbed during the

basking. The evening slot from 4-7 pm was not chosen to avoid risk due to low ambient light.

Each scat sample was labeled with detailed information about the time of collection, location, and any other pertinent remarks (Fig. 3). Scats were stored in an ice box at the field site, and within 5 hours, they were transferred to -20°C. Only fresh samples were collected, and this was ensured by performing a 'wet test'. We broke a scat from the middle and used a thin paper strip to check the freshness of the sample. If the paper strip did not become wet due to moisture, we did not collect the sample. Post-scat collection, we covered the sample with sand to avoid confusion during the next round of sampling at the same site. We collected 107 fresh scat samples from 1st-31st December 2022 from both the regions and stored them at -20°C for further analysis. The samples were brought to the lab at Ahmedabad University in ice packs.

Figure 3: Scat collection and labeling during fieldwork.

To understand HMC and dependency of humans on adjoining freshwater habitats that support the mugger crocodile populations, we did an interview survey with the local community to understand their dependency on natural water bodies for both Charotar and Vadodara regions. A total of 120 interviews were taken for both the regions (Fig. 4) (Data sheet attached with the report).

Figure 4: Interview survey with the local community

Scat sample analysis to measure fGCM

All the collected scat samples were subjected to drying at 80°C in a hot air oven. Post-drying, the samples were sieved and pulverised for the extraction process [5]. The extraction was done by weighing 0.1 g of the dry powder and mixing it with 3 ml of 80% methanol, vortexed for 3 min, and then centrifuged at 1500 rpm for 10 min. After centrifugation, 2-2.5 ml of supernatant was collected in centrifuge tubes and stored at -20°C for further analysis.

EIA analysis for fGCM measurements

The EIA kits targeting the 5b-3a-ol-11-one structure (11-oxo etiocholanolone EIA) for measuring fGCM were purchased from Dr Rupert Palme's lab (Department of Biomedical Sciences, University of Veterinary Medicine, Vienna) [6]. Cross-reactivity details are described by Palme and Möstl [8]. The microtiter plates were coated using anti-rabbit-IgG (R2004, Sigma-Aldrich, USA) and stored at -20°C until assay. On Day 1 of the assay, the IgG- coated plates were incubated overnight at 4°C with either standard or sample, along with antibody and tracer (labeled 11-oxo etiocholanolone). On day 2, after discarding the contents of the well, plates were washed with Tween20 (Merck 822184, USA) wash buffer solution, and the assay was terminated by adding streptavidin peroxidase conjugate (Roche 11089153001, USA) solution, followed by the addition of tetramethylbenzidine peroxide (Fluka 87748, USA) substrate solution. To stop

the enzyme activity, sulphuric acid (Merck 100731, USA) solution was added, and the reading was taken at 450 nm. The detailed procedure of the assay is described in Palme and Möstl [8].

Statistical analysis

Data used for analysis was checked for normality, and appropriate tests were conducted for a post-normality check. The data that was found to be non-normal were log-transformed to meet the normality. We used Welch's two-sample t-test for data with unequal variance and the student two-sample t-test for data with equal variance. The level of significance was kept at ≤0.05. All the statistical analyses were performed using the R programming software (version 4.2.3).

Results

Objective 1

The assay sensitivity was 2.04 pg per/well. The standard range of the assay was 2.04pg to 500 pg per/well. The EC50 (half maximal effective concentration) value (Mean±SEM) for the assays performed was 32.6±3.9. The inter-assay and intra-assay coefficients of variation calculated were 20.23% and 14.99%. The data were tested for normality using the Shapiro- Wilk test. The data was found to be normally distributed. Hence parametric tests were performed.

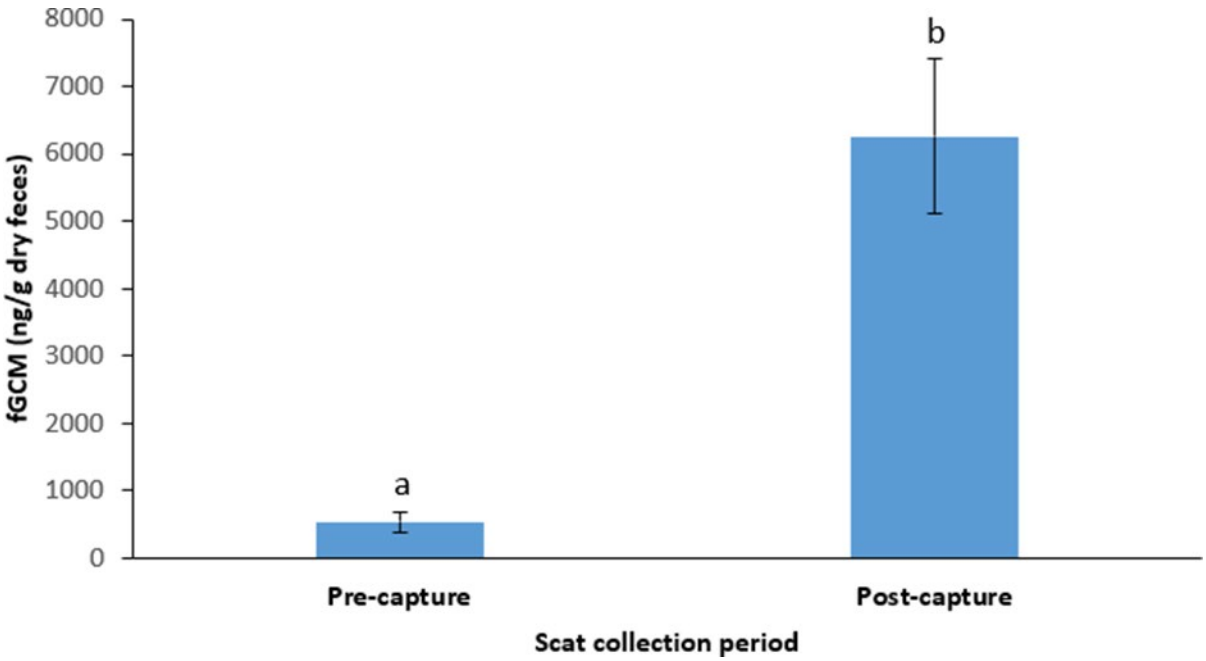

Figure 5: Levels of fGCM (Mean±SEM, ng/g dry feces) in captive muggers during the biological validation study. Different alphabets represent significant differences (P≤0.05).

The results of the biological validation for the selected assay demonstrated a

statistically significant difference (Welch's two sample t-test) with an 11-fold increase ($P<0.05$) in fGCM levels (Mean±SEM) between pre-capture (540.9±149.2 ng/g dry faeces, n=11) and capture (6259.7±1150.5 ng/g dry faeces, n=11) samples (Figure 5).

Objective 2:
HMC and basking duration (Mean±SEM) showed a significant difference between the two regions ($P\leq0.05$, Welch's two sample t-test) (Fig. 6). The Charotar region exhibited higher basking duration (498.6±18.44 min), four times greater than that observed in the Vadodara region (146.85±15.74 min).

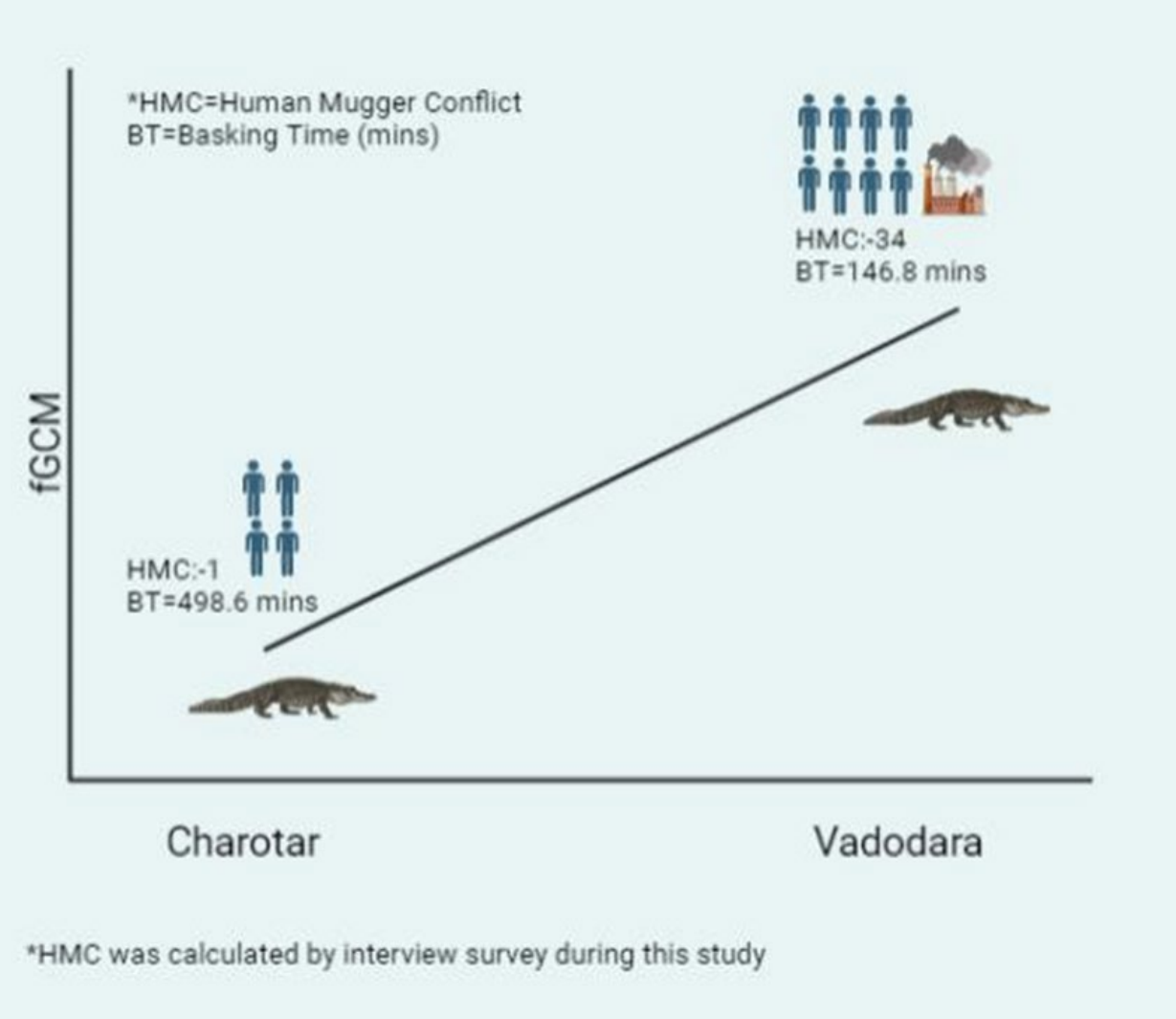

Figure 6: Schematic representation of the overall findings of the study in terms of HMC and basking duration of free-ranging muggers in between the two-study region in central Gujarat, India.

So far, out of 107 scat samples collected from free-ranging muggers, we have analysed 50 samples. Our preliminary analysis showed that the mean concentration of fGCM was 915.25±162.99 (Mean±SEM, n=50) ng/g dry faeces for free-ranging muggers, a slightly higher fGCM concentration when compared to the captive muggers during the pre-

capture stage. Additionally, we found significant differences (P≤0.05, Student two sample t-test) in fGCM levels (Mean±SEM) between Charotar (412±76.75) and Vadodara (1174.17±232.29) regions (Figure 7).

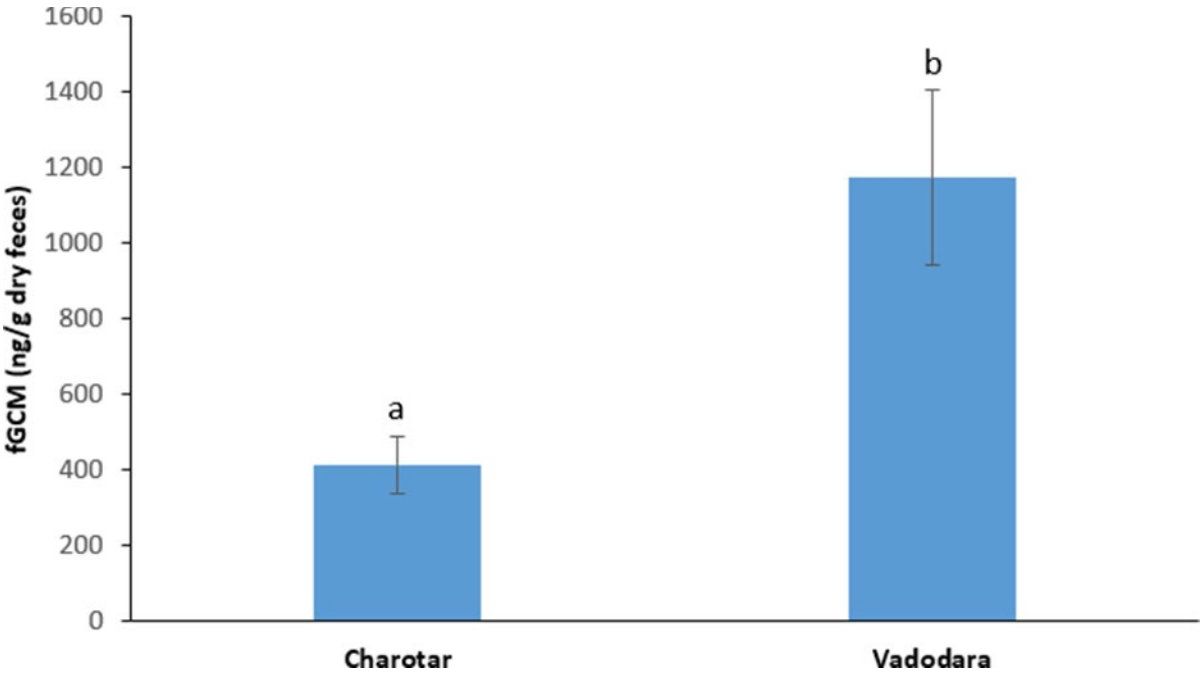

Figure 7. Levels of fGCM (Mean±SEM, ng/g dry feces) for free-ranging muggers for Charotar and Vadodara regions within central Gujarat. Different alphabets represent significant differences (P≤0.05).

Main findings based on the preliminary analyses

- The biological validation showed 11-fold increase in fGCM levels during the restraining procedure (pre-capture<post-capture), confirming the high specificity of the selected assay towards measuring the targeted cortisol metabolite (11-oxo etiocholanolone) released in muggers as a consequence of a stressful event.

- The results of the interview survey showed that the Vadodara region had a higher incidence of HMC when compared to Charotar (Vadodara>Charotar), and similarly, we found, the basking duration of mugger crocodiles was lower in the Vadodara region than Charotar sites (Charotar>Vadodara)

- The scat samples analysed so far to measure the fGCM levels showed high concentrations of fGCM in the Vadodara region in comparison to other Charotar (Vadodara>Charotar).

The next phase of our project

- Currently, we are analysing the remaining scat samples. Further analysis of the scat samples will help in making robust conclusions on understanding the stress

physiology of mugger crocodiles across diverse habitats within central Gujarat, India.

- We also plan to collect scats during the non-breeding season (May-June 2023) from the same locations and to compare if the fGCM values are different in muggers during breeding versus non-breeding seasons, and across different regions during the non- breeding time.

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Dr. Ratna Ghosal	Ahmedabad University	Supervisor (Professor)
Tathagata Bhowmik	Ahmedabad University	Intern (Ph.D. student)
Rohith Srinivasan	Ahmedabad University	Intern (Master's student)

Ronak Trivedi	Institute of Science and Technology for Advance Studies and Research, Charotar.	Intern (Master's student)

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Interview based questionnaire

- Pointers: One person throughout will conduct all the interviews.
- Mostly in Gujarati or rarely in Hindi
- 5 person/ site (Approx. 20 for Kheda and 40 for Vadodara)

General information

1. Basic information
    a. Name, age and gender
    b. Occupation
    c. Education
    d. Residential occupancy in years
    e. Native or migrant

Human-crocodile conflict-specific information

2. Has a mugger attacked you? (yes/no)
    i. 1If yes:
        1. When
        2. Where
        3. Why (qualitative)
3. Has a mugger attacked anyone else (acquaintance or relative)? (yes/no)
    i. If yes:
        1. When
        2. Where
        3. Why (qualitative)
        4. Is this direct observation or indirect information?
4. Any incidence of a crocodile attack on livestock or pets? (yes/no)
    i. If yes:
        1. When
        2. Where
        3. Why(qualitative)?
5. Do you guard your livestock near water bodies? yes/no
6. How do people deal with nuisance muggers? (qualitative)
7. Do you know of any incidents wherein the nuisance mugger has been removed?
    a. Where?
    b. When (date or year)?
    c. By whom (NGO or Forest department)?
8. Do people kill or poison nuisance muggers? (If yes, how)