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1 **Measuring cortisol in fish scales to study stress in wild tropical tuna**

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12

13 **Abstract**

14 Cortisol is recognized as a physiological indicator of stress in fish. However, this hormone is typically
15 measured in plasma samples. In this study, cortisol content was evaluated for the first time in the scales
16 of tropical tuna. The sample included 20 skipjack tuna (*Katsuwonus pelamis*) and 25 yellowfin tuna
17 (*Thunnus albacares*) collected in the Atlantic (the Gulf of Guinea off the Ivory Coast) and the Indian
18 Ocean (off Reunion Island), and their scales were analyzed using an ELISA test. The results indicated
19 that on average, cortisol concentration (1) did not show any geographical pattern, (2) was independent
20 of fish size, and (3) was significantly higher in skipjack (mean: $4.75 \pm 6.56 \text{ ng g}^{-1}$) than in yellowfin
21 (mean: $1.65 \pm 1.85 \text{ ng g}^{-1}$), although the difference was mainly due to four skipjack individuals. Larger
22 datasets would be needed to confirm any species difference in cortisol concentration. The particularly
23 high cortisol concentration observed in four individuals may be due to fish from different schools,
24 suggesting the need for future sampling from free-swimming schools to investigate intra- and inter-
25 school variability in cortisol concentration. This study opens the door for future research, including the
26 collection of scales on tagged individuals, to investigate the links between chronic stress and behavior
27 in these species.

28 **Key words** fish scales · cortisol · stress · welfare · skipjack tuna · yellowfin tuna

29 **Introduction**

30 The capacity of fish to cope with environmental challenges is of primary importance in a rapidly
31 changing world. The stress axis plays a central role in the coping capacity of fish, as it diverts energy
32 from growth and reproduction to cope with acute environmental changes (Sadoul and Vijayan 2016).
33 The two stress-related response mechanisms in fish are brain-sympathetic chromaffin cells (BSC) and
34 the hypothalamus-pituitary-interrenal (HPI) axis. The underlying mechanisms of the stress axis in fish
35 have been well described (Barton 2002; Shreck and Tort 2016). At the physiological level, the primary
36 response to a stressor in fish is neuroendocrinal and involves the activation by the central nervous system

37 of the sympathetic nervous system, releasing neurotransmitters and hormones such as catecholamine
38 and corticosteroids (mainly cortisol in fish). Following the stimulus, plasma cortisol increases after a
39 period of time ranging from a few minutes to an hour, depending on the species and the intensity and/or
40 duration of the stimuli (Barton 2002). Cortisol is considered a good quantitative indicator of acute and
41 chronic physiological stress (Ellis et al. 2012). Until recently, this hormone was mainly measured in the
42 plasma of unstressed and stressed fish, providing a basal and high cortisol level. Yet plasma measures
43 of cortisol can only provide insight into the stress level of the fish at a given time. In an ecological
44 context, an integrative value of stress over a long period is much more valuable, allowing a more
45 comprehensive idea of the life history of the animal. Recently, new matrices have emerged to measure
46 cortisol; these are being used as relatively non-invasive markers of stress (Sadoul and Geffroy 2019).
47 Among these markers, fish scales are the most promising matrix as they provide a record of the
48 integrated stress response over time (i.e. from weeks to months, Aerts et al. 2015) and a grid to highlight
49 behavioral ecology regarding exposure to stress factors such as temperature (Goikoetxea et al. 2020),
50 since the metabolic clearance rate in scales is much slower than in plasma (Laberge et al. 2019). Previous
51 studies have shown that intra-specific differences in basal cortisol are genetically driven (Øverli et al.
52 2002a) and are generally aligned with differences in behavior or coping style, in which reactive
53 individuals display higher basal cortisol levels compared to proactive individuals (Koolhaas et al. 1999).
54 In trout, for instance, long-term cortisol administration was shown to decrease both locomotor activity
55 and aggression (Øverli et al. 2002b). This could be transcribed at the scale level with cortisol
56 accumulation.

57 Tuna are present in all oceans, and as top predators they play a major ecological role in offshore pelagic
58 ecosystems. Worldwide, tuna species are exploited by both artisanal and industrial fisheries, using
59 different types of fishing gear. Two tuna species, skipjack (SKJ, *Katsuwonus pelamis*) and yellowfin
60 (YFT, *Thunnus albacares*), are among the most landed species in the world (FAO 2018). Although the
61 biology and ecology of tuna have been extensively investigated, particularly to provide scientific advice
62 for fishery management and the conservation of stocks, studies measuring cortisol in tuna are scarce and
63 limited to only one species (Atlantic bluefin tuna, *Thunnus thynnus*) in one environment (Kirchhoff et
64 al. 2011; Addis et al. 2013; Tracey et al. 2016). By measuring the basal levels of plasma cortisol, these
65 studies showed a rapid accumulation of cortisol in response to an intense one-off event (capture) and
66 focused on the short-term stress reaction to capture. In general, captured fish are exposed to stressful
67 stimuli, e.g. physical trauma and physiological stress (Skomal 2007), which is worth investigating
68 mainly in an ethical context. Our study chose another line of approach, using, for the first time, fish
69 scales as a matrix to measure cortisol in two wild marine fish, skipjack and yellowfin tuna (male and
70 female), in order to describe the causal effects of size and location on this indicator.

71 **Materials and methods**

72 The sample for the study was based on individuals from two tuna species, YFT and SKJ, captured by
73 handline around anchored fish-aggregating devices (FADs) in the Atlantic (Gulf of Guinea off the Ivory
74 Coast) and the Indian Ocean (off Reunion Island) (Fig. 1A and B) on 10–11 July and 26–29 September
75 2018, respectively. The total time between the bite on the hook and the killing of a tuna was between 2
76 and 10 minutes. To minimize the pain, we euthanized the animals as soon as they were boarded, with a
77 blow to the head that immediately killed the tuna (following European directive 2010/63/UE). The
78 number of fish sampled was $n=22$ in the Gulf of Guinea (16 YFT, 6 SKJ) and $n=23$ off Reunion Island
79 (9 YFT, 14 SKJ).

80 Onboard, the fork length (FL in cm, from the tip of the upper jaw to the caudal fin fork) and body mass
81 (W in kg) were measured by scientists. Scale samples (under the pectoral fin) were taken by scraping
82 the flank with a blunt scalpel and these were collected in plastic Eppendorf tubes, which were
83 immediately immersed in liquid nitrogen and stored at -80°C until analysis. The handlers wore gloves
84 during the entire process to avoid external contamination.

85 The cortisol extraction from the scale samples ($n=45$) was adapted from two different protocols (Aerts
86 and De Saeger 2017; Carbajal et al. 2018). Scales were washed by adding 1.5 mL of isopropanol in each
87 tube, agitated with a ceramic ball mill at 10 Hz for 3 min in a Retsch MM 400 tissue lyzer, and
88 centrifuged at 14,000 g and 4°C for 1 min. The supernatant was then discarded. This washing process
89 was repeated three times to fully remove the covering mucus. The samples were then stored at -20°C
90 for 1 h and lyophilized (2 h freezing phase down to -40°C , followed by a rapid increase up to 40°C
91 lyophilization phase in a 1000 mbar vacuum overnight). Once dried, subsamples were aliquoted in a
92 new 2 mL Eppendorf tube and precisely weighed (mean weight and SE: 95.5 ± 9 mg). One ceramic mill
93 ball was then added to each tube and samples were minced to powder in the tissue lyzer for 1 min at 30
94 Hz. Cortisol extraction was performed by adding 1 mL of methanol to each tube, vortexed for 10 sec
95 and shaken overnight (18 h) at room temperature. Then the samples were centrifuged at 12,000 g for 3
96 min, and the supernatant transferred to a borosilicate glass evaporation tube. This last step was repeated
97 after adding 1 mL of methanol to ensure maximum cortisol extraction. The contents of the glass tube
98 were then evaporated to dryness by nitrogen flow at 40°C using a MULTIVAP® Nitrogen evaporator
99 (Organomation®). All samples were then reconstituted in 150 μL of 0.01% Bovine Serum Albumine
100 (BSA) in Phosphate Buffered Saline solution (PBS – 2.7 M KCl; 0.137 M NaCl; 1.8 mM KH_2PO_4 ;
101 Na_2HPO_4), vortexed 3 times for 15 seconds and stored at 4°C until the ELISA assay. The cortisol was
102 quantified using a competitive ELISA measurement described in Faught et al. (2016) and adapted from
103 a protocol validated for zebrafish (*Danio rerio*) (Yeh et al. 2013). This involved coating 96-well plates
104 with a cortisol monoclonal antibody ($1.6 \mu\text{g}\cdot\text{mL}^{-1}$; East Coast Bio, ME, USA) in PBS and storing them
105 at 4°C for 16 hours. The cross-reactivity of the antibody is 0.4% for cortisone, 7.7% for 11-deoxycortisol
106 and 21.9% for corticosterone (Sadoul and Geffroy 2019). While this is a high level of cross-reactivity
107 with corticosterone, it should be noted that levels of this hormone have been reported to be relatively

108 low in fish (at least 10 times lower than cortisol, as measured in plasma) (Kiilerich and Prunet 2011), so
109 the specificity of the monoclonal antibody used here and in other fish studies (Yeh et al. 2013; Faught
110 et al. 2016; Best et al. 2017) should only marginally change results. Standards were obtained by serially
111 diluting hydrocortisone (Sigma) in PBS (0–25 ng.mL⁻¹), and 50µL of either standards or samples were
112 added to the wells in duplicate. Each well then received cortisol conjugated with horseradish peroxidase
113 (HRP) and filled with a detection solution that turns blue when in contact with HRP. Sulfuric acid (1N)
114 was used to stop the reaction. Absorption was read at 450 nm (EL800 Universal Microplate Reader,
115 BIO-TEK INSTRUMENTS, INC.). Cortisol ELISA concentrations (in ng.mL⁻¹) were normalized by the
116 sample weight of each sample (expressed in ng of cortisol per g of scales). The intra-assay CV for the
117 two plates was below 10%. The standard curve was performed with 0.5, 1, 2, 5, and 10 ng.mL⁻¹, and the
118 cortisol concentration measured in the scales was correlated with expected cortisol values for plate 1
119 ($r^2=0.95$) and plate 2 ($r^2=0.98$).

120 We first compared cortisol concentration in scales between species caught at the same site and tested
121 for a potential fish-size effect. Then we pooled all the data together to test whether the cortisol
122 concentration in scales varied as a function of size, location or species in the same linear mixed model.
123 We added the ELISA plate as a random factor, as samples were run on separate ELISA plates. The
124 interaction was removed from the model when not significant. To ensure that all fixed effects were taken
125 into account similarly, we used a type II Analysis of Variance with Satterthwaite’s method. All statistical
126 analyses were performed using R software 3.2.2 (R Development Core Team 2013) and package “lme4”
127 for linear mixed models (Bates et al. 2015). Maps were designed using the “ggmap” package (Kahle
128 and Wickham 2013).

129 **Results and discussion**

130 We did not detect significant differences in cortisol concentration in scales as a function of fish length
131 for either species (YFT: $t=-0.24$; $p=0.8$; SKJ: $t=-0.14$; $p=0.9$) (Table 1). Within each site, the cortisol
132 concentration in SKJ scales was higher (although not significantly) than that in YFT scales ($p=0.17$ in
133 the Gulf of Guinea and $p=0.09$ off Reunion Island), so that when pooling all individuals together in the
134 model accounting for all fixed effects (Cortisol ~ Location + Length + Species + (1|PlateScales)),
135 cortisol concentration was significantly higher ($t=1.97$; $p=0.0558$) in SKJ scales compared to YFT
136 scales, independently of their size and location (Fig. 2). However, it is worth noting that this pattern was
137 driven by 4 SKJ individuals between 54 and 59 cm in FL (3 individuals from Reunion Island and 1
138 individual from the Gulf of Guinea). According to the sampling size of this study, neither the
139 size:species interactions nor the size:location interactions were found to be significant ($p=0.5$ & 0.6
140 respectively).

141 Cortisol in scales has been proven to be an effective marker of chronic stress in various fish species
142 (Aerts et al. 2015; Carbajal et al. 2019, Laberge et al. 2019; Hanke et al. 2020). Yet the mechanisms of
143 cortisol clearance in scales are not well described, and to our knowledge only one recent study on

144 goldfish (*Carassius auratus*) has provided insights regarding its rate (Laberge et al. 2019). That study
145 demonstrated that following a cortisol treatment, plasma levels went back to initial values after only 3
146 days, while it took 27 days to return to the basal level in scales. This implies that cortisol is somehow
147 catabolized at a much slower rate in scales than in plasma, likely because this steroid is embedded in
148 calcified structures that protect it. The cortisol values found for both species in our study were well
149 within the range of those reported for other fish species, e.g. European seabass (*Dicentrarchus labrax*)
150 (from 1.7 to 3.4 ng g⁻¹, Aerts and De Saeger 2017) and goldfish (*Carassius auratus*) (from 0.25 to 2.10
151 ng.g⁻¹, Carbajal et al. 2018). Although our study relied on a small dataset, the cortisol concentrations in
152 scales were significantly higher in skipjack (mean: 4.75 ± 6.56 ng.g⁻¹) than in yellowfin (mean: 1.65 ±
153 1.85 ng.g⁻¹) for individuals of the same size. Interestingly, the cortisol concentration did not increase
154 with fish size (i.e. age), which argues for a slow – but active – renewal of cortisol in this matrix, as
155 demonstrated by Laberge et al. (2019), rather than an accumulation over time.

156 Adult skipjack and juvenile yellowfin tuna (the size classes sampled in our study) are known to share
157 many anatomical, physiological and behavioral similarities. They have similar swimming modes,
158 thermal biology, increased rate functions (e.g. standard metabolic rate, aerobic capacity, heart rate and
159 gut clearance) and cardiac physiology (Graham and Dickson 2004), which distinguish them from many
160 other teleost species. They have the same vertical habitat utilization (Schaefer et al. 2009) and usually
161 feed on the same prey species (Jaquemet et al. 2011), although some diet differences have been observed
162 in specific oceanic regions (Olson et al. 2016). They both form large schools and associate with floating
163 objects, but differences in their fine-scale FAD-associative behavior have been observed (Rodriguez-
164 Tress et al. 2017). Considering the strong similarities between these two species, one might expect
165 similar cortisol metabolism, resulting in similar concentrations of cortisol in scales. Larger datasets are
166 needed to confirm the differences between these two species. In parallel, experiments on captive tuna
167 should be conducted in order to investigate the time evolution of cortisol concentration over several
168 days and weeks. If a species difference in cortisol concentration is confirmed by larger datasets, this
169 may be explained by species-specific cortisol metabolism. This would imply intrinsic differences in the
170 production and/or clearance of cortisol (dependent upon cortisol-binding proteins, target tissue
171 receptors, tissue uptake and catabolism of cortisol) (Mommensen et al. 1999). The production/clearance
172 of cortisol and the rate of scale formation (and therefore the associated integration of cortisol) in these
173 two species have not yet been investigated and represent a perspective for future studies.

174 In our dataset, however, the difference in cortisol concentration observed between the two species was
175 mainly due to four skipjack individuals. Interestingly, comparable high values were also detected in
176 some extremely stressed European seabass (Aerts and de Saeger 2017) and goldfish (Laberge et al.
177 2019). These high concentrations of cortisol in scales may be the consequence of specific stressful
178 events experienced by these individuals, such as attacks by predators or fasting periods that might have
179 occurred over the preceding days or weeks. Understanding why some individuals of the same species

180 and the same size, in the same fish aggregation around a FAD, have significantly higher concentrations
181 of cortisol (about five times higher) would be valuable to study on a physiological and behavioral level.
182 Considering that the clearance rate of cortisol in scales is very slow, it may be that individuals with high
183 concentrations of cortisol belong to different schools than the fish with lower cortisol concentrations.
184 Tuna aggregations at FADs usually consist of several schools (Josse et al. 2000; Dagorn et al. 2007;
185 Moreno et al. 2007). As fish from the same school are likely to have a relatively long common history
186 (although the stability of a tuna school appears to vary quite a lot, see Bayliff 1988 and Hilborn 1991),
187 it can be assumed that fish from the same school have comparable concentrations of cortisol as they
188 have usually experienced the same events. This hypothesis could be investigated by comparing intra-
189 and inter-school variability of cortisol concentrations in individuals of the same species sampled from
190 different free-swimming schools caught by purse seiners (as sampling tuna associated with FADs does
191 not ensure that they are from the same school).

192 **Conclusion**

193 This preliminary study provides the first measurements of cortisol in the scales of wild tuna. While
194 further studies are needed to better understand the mechanisms of scale cortisol clearance and
195 metabolism in the selected species (e.g. a comparison of the cortisol level in scales and in other
196 biological matrices: mucus, plasma, muscle, etc.), this data offers a good start for designing new
197 experiments. In the same way Willis and Hobday (2008) suggested taking bioelectrical impedance
198 analysis (BIA) measurements to measure the condition of tuna during tagging operations, we suggest
199 sampling a few scales from individuals during conventional or electronic tagging. This would be a quick
200 and harmless operation that should not disrupt tagging operations and would allow measuring the
201 markers of chronic stress (as opposed to the stress due to capture, measured by cortisol in plasma) of
202 tagged individuals. Comparing the large-scale movements (from conventional or archival tags) or the
203 fine-scale movements within arrays of FADs (from acoustic tags) for fish with different concentrations
204 of cortisol would provide key insights into the ecology of these species, particularly the possible links
205 between stress response and behavior.

206

207 ***Supplementary Information.*** *Not applicable.*

208 ***Acknowledgments***

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210 funding from Montpellier University of Excellence (MUSE) and the Marine Biodiversity and
211 Conservation (MARBEC) research group.

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214 Ecosystems Observatory, which is ISO 9001:2015 certified.

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222 ***Authors' contributions***

223 E.R.O. and B.G. designed the study, E.R.O., Y.B. and T.B. collected the samples, and M.D., B.S, B.G.
224 and E.R.O. performed the analyses. All authors prepared the manuscript and approved its submission.

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227 Excellence (MUSE) and the Marine Biodiversity and Conservation (MARBEC) research group.

228 ***Code availability.*** *Not applicable.*

229 ***Data availability.*** *Not applicable.*

230 ***Declarations***

231 ***Ethics approval***

232 All sampling and animal handling respected good scientific practice and animal welfare rules. To
233 minimize suffering, we euthanized the animals as soon as they were boarded. The number of fish
234 sampled was limited to the strict number necessary.

235 ***Consent to participate.*** *Not applicable.*

236 ***Consent for publication.*** *Not applicable.*

237 ***Conflict of Interest/ competing interests.*** The authors declare that they have no conflict of interest
238 concerning this manuscript and no competing financial interests that could influence the work reported
239 in this paper.

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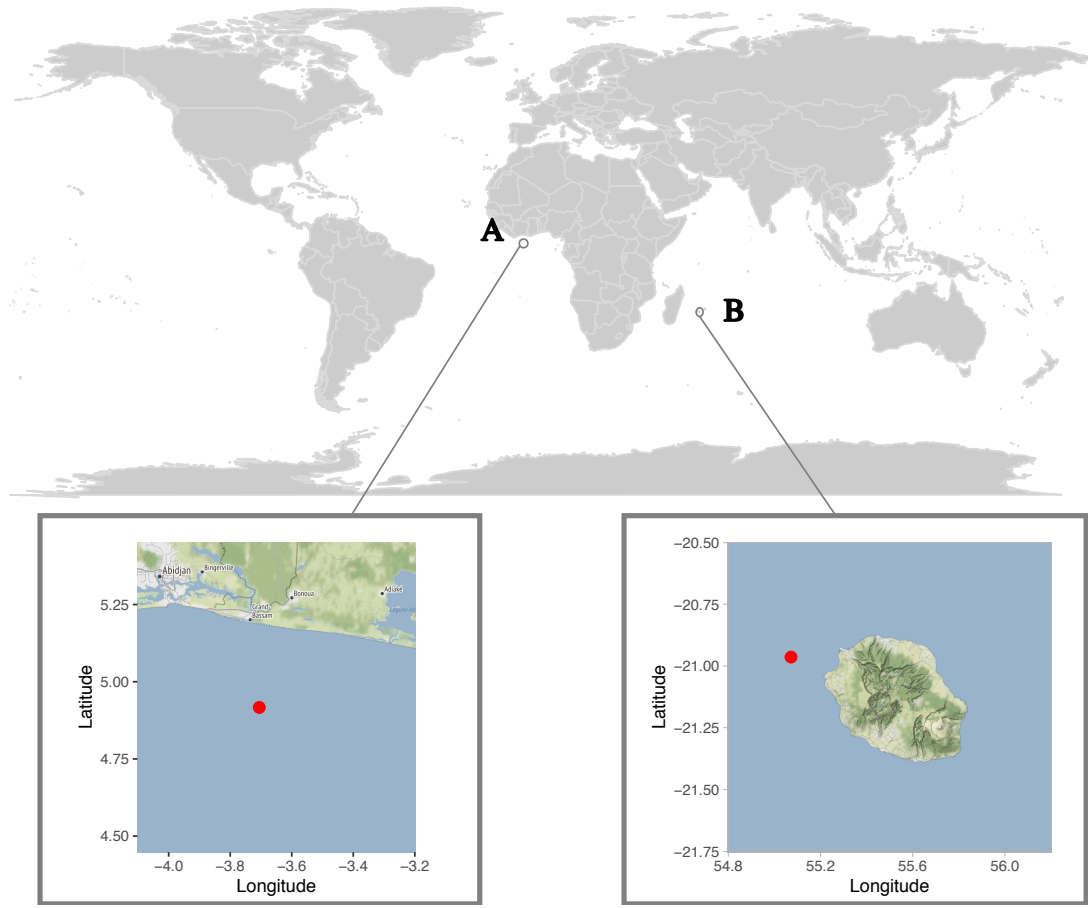
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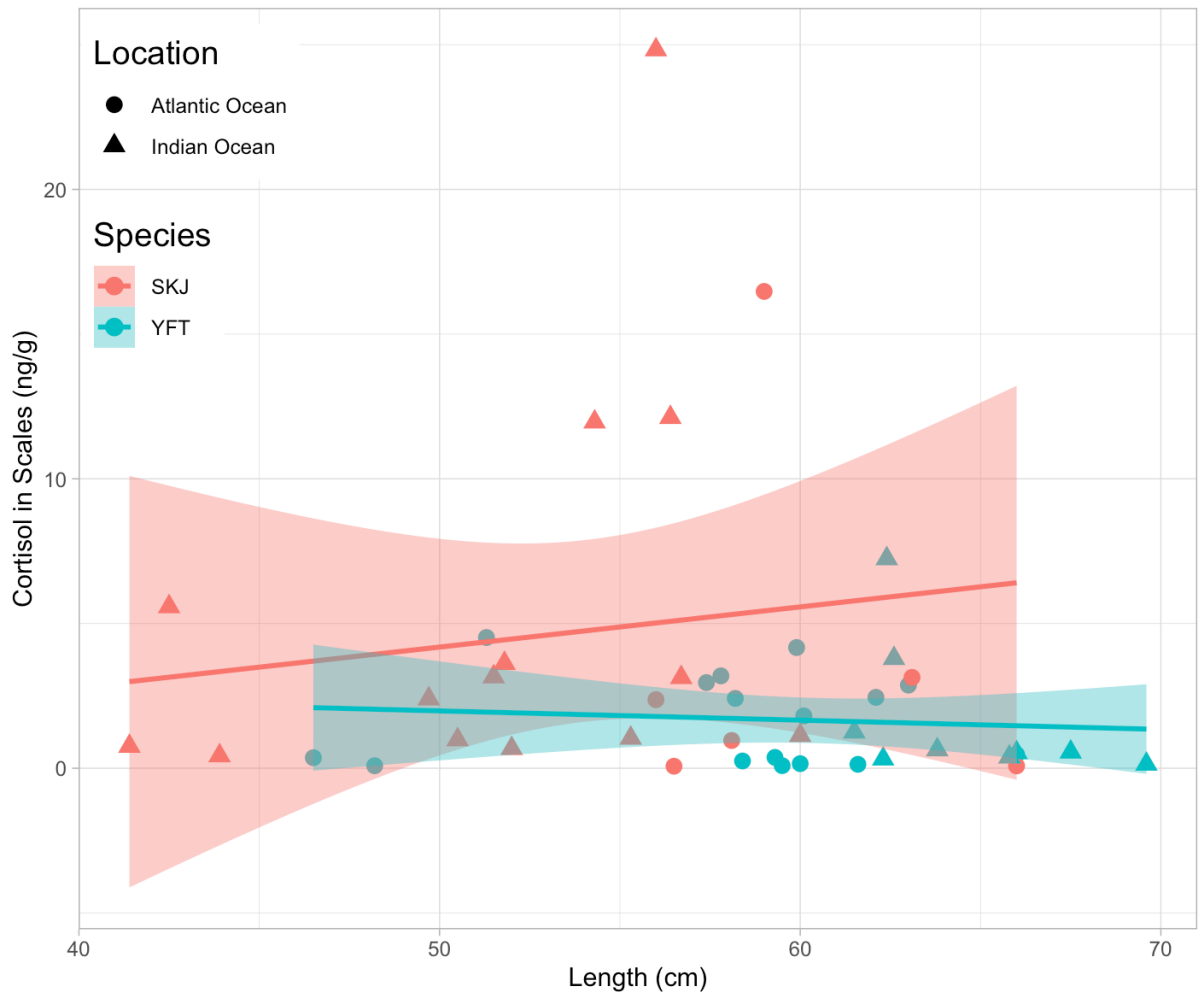
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Fig. 1. Tuna species, skipjack (*Katsuwonus pelamis*) and yellowfin (*Thunnus albacares*) were sampled in A) the Gulf of Guinea (Atlantic Ocean), 27 nautical miles off Abidjan (Ivory Coast) on July 2018 and B) La Réunion Island (Indian Ocean), 10 nautical miles from shore, on September 2018. Red dots indicate the sampling location of the Fish Aggregating Devices (FAD) where tunas have been captured.



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Fig. 2. Cortisol concentrations ($\text{ng}\cdot\text{g}^{-1}$) measured in the scales of the two tuna species *K. pelamis* (SKJ) and *T. albacares* (YFT), in the Atlantic and Indian oceans, correlated to the fish length (in cm).

261 **Table 1.** Biometrics data (number, length, weight) of the two tuna species (sp), skipjack (SKJ,
 262 *Katsuwonus pelamis*) and yellowfin (YFT, *Thunnus albacares*)

	sp	nb	♂	♀	ND	length (cm) min-max	length (cm) average ± SD	weight (kg) min-max	weight (kg) average ± SD
Gulf of Guinea	YFT	16	7	6	3	46.5-66	58.08±5.22	1.6-4.6	3.13±0.87
Gulf of Guinea	SKJ	6	3	3	0	56-66	59.78±3.95	2.9-4.4	3.52±0.52
La Réunion Island	YFT	9	3	6	0	61.5-69.6	64.61±2.77	4.1-5.5	4.51±0.45
La Réunion Island	SKJ	14	4	10	0	41.4-60	51.57±5.62	1.2-4.0	2.45±0.79

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