

1 **First assessment of the sex ratio for an East Pacific green sea turtle foraging aggregation:**
2 **validation and application of a testosterone ELISA**

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17

18 **Abstract**

19 Determining sex ratios of endangered populations is important for wildlife management,
20 particularly species subject to sex-specific threats or that exhibit temperature-dependent sex
21 determination. Sea turtle sex is determined by incubation temperature and individuals lack
22 external sex-based traits until sexual maturity. Previous research utilized serum/plasma
23 testosterone radioimmunoassays (RIA) to determine sex in immature/juvenile sea turtles.
24 However, there has been a growing application of enzyme-linked immunosorbent assay (ELISA)
25 for wildlife endocrinology studies, but no study on sea turtles has compared the results of ELISA
26 and RIA. This study provides the first sex ratio for a threatened East Pacific green sea turtle
27 (*Chelonia mydas*) foraging aggregation, a critical step for future management of this species.
28 Here, we validate a testosterone ELISA and compare results between RIA and ELISA of
29 duplicate samples. The ELISA demonstrated excellent correspondence with the RIA for
30 providing testosterone concentrations for sex determination. Neither assay proved reliable for
31 predicting the sex of reproductively active females with increased testosterone production. We
32 then applied ELISA to examine the sex ratio of 69 green turtles foraging in San Diego Bay,
33 California. Of 45 immature turtles sampled, sex could not be determined for three turtles because
34 testosterone concentrations fell between the ranges for either sex (females: 4.1 – 113.1 pg/mL.
35 males: 198.4 – 2,613.0 pg/mL) and these turtles were not subsequently recaptured to enable sex
36 determination; using a Bayesian model to predict probabilities of turtle sex we predicted all three
37 ‘unknowns’ were female (> 0.86). Additionally, the model assigned all turtles with their correct
38 sex (if determined at recapture) with 100% accuracy. Results indicated a female bias (2.83F:1M)
39 among all turtles in the aggregation; when focusing only on putative immature turtles the sex

40 ratio was 3.5F:1M. With appropriate validation, ELISA sexing could be applied to other sea
41 turtle species, and serve as a crucial conservation tool.

42

43 **Introduction**

44

45 Understanding the demography of wildlife populations threatened with extinction is
46 essential for developing sound conservation and management plans [1]. Population abundance,
47 survivorship, and sex ratio are among the most fundamental demographic parameters, and these
48 factors provide a context for monitoring population trends and modeling population viability
49 over long time frames [2]. For example, information on the proportion of females in a
50 population can shed light on its reproductive potential. When examined at multiple sites across
51 broad geographic regions and diverse habitats, evaluations of sex ratio can help illustrate sex-
52 related habitat preferences and perhaps identify sex-biased threats that impact a population.
53 Indeed, information on sex ratios is vital for population assessment and management, but can be
54 elusive with species and life stages that do not exhibit external sex-specific characteristics (e.g.
55 seabirds, cetaceans, and sharks; [3–5]).

56 The sex of sea turtles is not distinguishable externally in immature stages. Sex can be
57 distinguished for adult male sea turtles because they have long and muscular tails compared to
58 females [6]. However, using short tail length to determine the sex of adult foraging females has
59 been problematic at times because some turtles of adult size [e.g. as large as 90.5 cm curved
60 carapace length (CCL)] with short tails are actually prepubescent male turtles incorrectly
61 identified as female [7–10]. Therefore, one should be cautious when using external
62 characteristics to determine sex in sea turtles. Furthermore, using molecular tools to determine
63 sea turtle sex is inherently difficult because sex is not genetically determined in sea turtles [9].
64 The sex of a developing sea turtle embryo is influenced by the surrounding environment and is

65 termed temperature-dependent sex determination [11]. Sex is determined during the middle third
66 of incubation (the thermosensitive period) during which cooler temperatures produce males and
67 warmer incubation temperatures produce more females [12–14]. If incubated at a constant
68 temperature, there is a pivotal temperature that will produce a 1:1 sex ratio and this can vary
69 within and between species (27.7 to 31.0 °C, [15]), but variation on either side of that pivotal
70 temperature has the potential to produce embryos of a single sex. Knowledge of sex ratios has
71 practical application considering that some conservation practices (e.g. hatcheries and nest
72 relocation) may alter the thermal environment of sea turtle embryos with unintended
73 consequences (i.e. [16,17]). In addition, information on sex ratios obtained over long time frames
74 can help decipher the impacts of global climate change on populations [18].

75 Efforts to track changes in sea turtle sex ratios often focus on nesting beaches, where the
76 sex of hatchlings (i.e. primary sex ratio) can be inferred from nest sand temperature or direct
77 examination of gonadal histology from expired or sacrificed hatchlings [14,19]. However,
78 because of low survivorship after hatching, the extent to which hatchling sex ratio is conserved
79 in immature and adult life stages is unclear. Thus, characterizing sex ratio at foraging areas,
80 where a larger cross-section of life stages is present, can offer greater insight into the functional
81 sex ratio of a population [20].

82 There are multiple methods to determine the sex of immature turtles [6], including
83 laparoscopy [21], histological examination of gonads from live or dead stranded turtles [22], or
84 hormonal assay for testosterone (T) concentration in blood plasma [23]. However, all of these
85 methods have deleterious impacts to the turtles or are logistically challenging, except blood
86 collection for hormone assay. More specifically, expert training is required to perform the
87 laparoscopy and it is an invasive technique that can be challenging to perform in the field.

88 Histological examination generally requires sacrifice of the animal to obtain the gonadal sample
89 or proficiency in necropsy and gonadal identification. In addition, stranded turtles may not
90 represent an unbiased sample. Therefore, many studies collect blood samples because this
91 technique can be minimally invasive and can be used to determine T concentration, and thus sex.
92 The ease with which blood can be collected allows for adequate and representative sample sizes to
93 make inferences about a population or aggregation.

94 The range of T concentrations for male and female sea turtles can vary between species
95 and even between foraging aggregations within a species [6,24]. It is, therefore, important to
96 determine the T range for individuals from each foraging aggregation and each species.
97 Additionally, some studies using T to determine sex ratio of immature turtles have identified a T
98 concentration range associated with uncertainty— i.e. the sex cannot be determined in these
99 turtles because T concentrations fall between male and female ranges or there is overlap in T
100 concentrations between males and females (e.g. loggerhead and green turtles, [25–27]). Some
101 immature individuals will have T concentrations that overlap, either by chance or possibly due to
102 time of year/water temperature (see [28] for immature loggerheads), while for other
103 populations/species the T concentration range for each sex is more defined (e.g. hawksbills,
104 [29]). Adult-sized turtles can be misidentified as one sex based on T concentrations (i.e.
105 reproductively active female with high T concentrations may be considered an immature or
106 mature male) and hormone assays alone should not be used to determine the sex of turtles near
107 the size threshold for maturity without additional information to corroborate the assay results.
108 Morphological data on carapace size and tail length of sea turtles, as well as information on
109 reproductive status and size at sexual maturity, greatly enhance the interpretation of the results of
110 sex ratio studies. This limitation highlights the need to determine this ‘unknown’ range for each

111 new foraging aggregation and species studied and is integral for studies using T to determine
112 immature sea turtle sex ratios. However, the unknown range can be better defined with greater
113 sample sizes and validation of sex determined by T concentration with known sex via
114 laparoscopy.

115 Circulating T levels have typically been measured by radioimmunoassay (RIA, reviewed
116 by [30]). However, enzyme-linked immunosorbent assay (ELISA), is preferable over RIA
117 because it is faster to complete, easy to use, and does not require radioactive reagents [31].
118 Enzyme-linked immunosorbent assay has been applied in marine wildlife to monitor health,
119 reproduction, and stress response and to determine sex (e.g. fish, marine mammals, and sea
120 turtles, [27,32–35]). However, assay methods must first be validated to confirm reliability with
121 each target species prior to application of a novel hormone assay technology. While this can be
122 accomplished by comparing the results of such tests with internal gonadal examination via
123 laparoscopy [6], a more practical solution is to compare results of new assay systems with results
124 of a validated assay (e.g. RIA, [9,25]) on duplicate samples. To date, however, no sea turtle
125 endocrinology study has yet determined consistencies between ELISA and RIA as has been done
126 for other wildlife species (e.g. big cats, [36]).

127 Despite the acknowledged conservation need for increased information on demography
128 of sea turtles [37,38], sex ratio in foraging habitats has not been examined sufficiently [37]. Here
129 we present findings from three projects: (1) validation of a T ELISA for use with green turtle
130 (*Chelonia mydas*) plasma by comparing results obtained from RIA and ELISA techniques for
131 turtles whose sex was known by laparoscopy, (2) application of the ELISA to a temperate
132 foraging aggregation of green sea turtles in the eastern Pacific Ocean to determine sex ratio, and
133 (3) development of a statistical model to estimate the probabilities of assigned sex for immature

134 turtles which fall within 'unknown' ranges of T assay concentrations. To our knowledge, this is
135 the first study to use hormone assessment techniques to study sex ratio for a sea turtle population
136 in the eastern Pacific Ocean, despite numerous studies on sea turtle biology in the region (e.g.
137 [39–41]).

138 The foraging aggregation of green turtles in San Diego Bay (SDB) has been studied for
139 ~25 years and the congregation of turtles near power plant warm effluent water allowed for easy
140 access to individuals (e.g. [42–47]). It might not be considered to be a large aggregation (mean:
141 37.2 and range: 6-61 individuals, [42]), but it has been continually monitored and extensively
142 sampled and offers a valuable opportunity to test the application of the ELISA on green turtles in
143 the wild. This foraging aggregation of turtles is comprised of ‘black’ green turtles (*Chelonia*
144 *mydas agassizii*) that are part of the threatened East Pacific population [48] and nest primarily in
145 three locations in Mexico (Michoacán, Revillagigedo Archipelago, and Tres Marias Islands,
146 [44]). This study takes advantage of and builds upon a rich set of mark-recapture studies and
147 research projects on a broad range of topics for this aggregation. These data will serve as a
148 baseline for future assessments of sex ratio at this foraging habitat and also provide for
149 comparisons with other studies in this region. Further, we believe validation of the ELISA
150 technique and eventual real-world application to all sea turtle species will increase the speed and
151 ease of hormone analysis for sea turtles. In addition, this study uses physiological tools to answer
152 important ecological questions when traditional ecological metrics cannot be used.

153

154 **Methods**

155 **Ethics Statement**

156 Samples collected from captive sea turtles at SeaWorld were collected as part of the
157 routine veterinary care and are, therefore, exempt from requiring IACUC approval. In addition,
158 the sea turtles housed at SeaWorld were obtained prior to the ESA so these turtles and their
159 offspring do not require a display permit.

160 The blood samples of laparoscoped green turtles used to compare the ELISA and RIA
161 assays were exported to the US under Panama CITES export permits INRENARE 2-94, ANAM
162 SEX/A-077-04, ANAM SEX/A-56-07, ANAM SEX/A-73-08, ANAM SEX7A-056-09 and
163 Bermuda CITES export permit 08BM0016. The US CITES import permit for both Panama and
164 Bermuda is US758093/9. Research in Panama is conducted under Smithsonian Tropical
165 Research Institute IACUC number 2014-0515-2017-2. IACUC is not required for research led by
166 the Bermuda Turtle Project as it is conducted in collaboration with the Bermuda Aquarium,
167 Museum and Zoo (BAMZ). All research on sea turtles in Bermuda water is required to be
168 sanctioned by the Bermuda Government and is permitted at the discretion of the Director of the
169 Department of Conservation Services; the BAMZ falls within the Department of Conservation
170 Services. The BAMZ is accredited by the Association of Zoos and Aquarium and is mandated to
171 have an Animal Welfare and Enrichment Committee as well as a Research and Conservation
172 Committee. Any research on animals involving invasive techniques involving BAMZ staff or
173 resources requires the sanction of both committees.

174 The SDB research was conducted in accordance with the Animal Welfare Act, under
175 Southwest Fisheries Science Center/Pacific Islands Fisheries Science Center IACUC
176 (SWPI2013-04) policy, and permit approvals from the National Marine Fisheries Service (697,
177 988, 1297, 1591, 16803) and State of California Department of Fish and Game Permit (0166).

178

179 **ELISA Validation**

180 **Hormone Extraction**

181 Steroid hormones were extracted from plasma following Wibbels et al. [26]. In brief, 50
182 – 500 μ L of plasma was pipetted into a glass tube (Catalog # 14-961-30, Fisher Scientific, Fair
183 Lawn, NJ) and 4 mL of anhydrous ethyl ether (Fisher Scientific) was added to extract T from the
184 plasma. Then, each glass tube (with plasma and ether) was placed in liquid nitrogen where the
185 plasma layer was frozen and the ether layer (containing the hormones) was decanted into a new
186 glass tube and dried under a steady stream of nitrogen gas. Samples were reconstituted with 1.1
187 mL of acetone (Fisher Scientific) and 1 mL aliquots were air-dried overnight.

188

189 **ELISA Testosterone Assay**

190 Extracted samples were reconstituted in 250 μ L of 0.01 M phosphate buffered saline
191 (PBS, Sigma, St. Louis, MO) with 0.1% bovine serum albumin (BSA, Amresco, Solon, OH). A
192 commercially available T ELISA kit (Catalog # ADI-900-065, ENZO Life Sciences, Plymouth,
193 PA) was used to determine T concentration in each plasma sample and all samples were
194 quantified in duplicate. Data are presented as the mean \pm standard error of mean (SEM) of the
195 duplicate values for each sample. Standards ($n = 5$) of known T concentration (7.81 – 2,000
196 pg/mL) were prepared according to the assay kit protocol and PBS (with BSA) was used as the
197 ‘zero’ standard. The sensitivity of the assay is 5.67 pg/mL and therefore, samples that fell below
198 that concentration could not be detected (ND); however, to allow for detections below this
199 concentration we continued the serial dilution of the standards to create two additional standards
200 (3.905 and 1.9525 pg/mL). Although values within this low T concentration range have high
201 variability, the two additional standards allowed us to obtain the T concentration of a single

202 plasma sample for which the T concentration would be undetectable when simply using the
203 standards suggested in the assay kit protocol. The T ELISA has 100% reactivity with
204 testosterone, 14.64% reactivity with 19-hydroxytestosterone, 7.20% reactivity with
205 androstendione, 0.72% reactivity with dehydroepiandrosterone, and 0.40% reactivity with
206 estradiol (see manual provided in kit).

207 A Tecan spectrophotometer (Model: Sunrise, Phenix Research Products, Candler, NC,
208 USA) was used to read the optical density within each well of the ELISA plate. The resulting T
209 values were computed using a five-parameter logistic curve fitting program (Magellan, Tecan,
210 version 3.11, Phenix Research Products).

211

212 **Assay Precision**

213 Plasma samples from green turtles [7 immature males (determined by T concentration), 1
214 adult male and 5 adult females] housed at SeaWorld San Diego were used for extraction
215 efficiency, quality control and parallelism validations for the assay. All plasma samples were
216 collected at the discretion of the attending veterinarian as part of routine veterinary care. The
217 mean \pm standard deviation (SD) straight carapace length (SCL) was 72.4 ± 14.6 cm (range: 54.6-
218 97.6 cm). Straight carapace length was converted from CCL measurements using an equation
219 [$SCL = (CCL - 0.64) / 1.06$] for the San Diego Bay foraging population of East Pacific green
220 turtles [44].

221 Extraction efficiency was determined by adding (spiking) 10.0 ng of T from the ELISA T
222 assay kit, which was used to make the standards, to a plasma sample of known low T
223 concentration prior to extraction [49–51]. We extracted and quantified the amount of T in this
224 spiked sample as well as in an aliquot of the same sample of known low T concentration without

225 added T (non-spiked). The ratio of the final T measurement minus the amount from the native
226 non-spiked sample over the known quantity of T added was used to estimate extraction
227 efficiency.

228 For quality control, an adult male green turtle plasma sample with known (determined by
229 initial ELISA measurement) high T concentration (90,800 pg/mL, diluted 1:10) was extracted
230 and T concentration was determined in each assay for this same sample to confirm both T
231 within-assay (intra-assay) and between-assay (inter-assay) variation.

232 The assay was validated for use with green turtle plasma by demonstrating parallelism,
233 where the slopes of plotted curves from serial dilutions of the hormone standard provided in the
234 ELISA kit were compared to serial dilutions of pooled plasma extracts (n = 4) of unknown T
235 concentration. Serial dilutions of the standards and pooled plasma extracts were assayed in
236 duplicate and triplicate, respectively.

237

238 **ELISA and RIA Comparison**

239 To validate the ELISA T assay against an already-validated RIA we determined the T
240 concentrations of 30 duplicate plasma samples previously analyzed using RIA (Table in S1
241 Table) and compared the results of the two assays using a correlation analysis. Plasma samples
242 were obtained from known sex (via laparoscopy) wild-caught immature and adult green turtles [n
243 = 30; mean \pm SD (range), 58.2 \pm 20.7 cm (28.5-104.6) cm SCL] captured in nets in
244 developmental habitats (Bermuda and Panama) or along a migratory corridor (Caribbean
245 Panama) as part of ongoing research programs. Blood samples were collected and the animals
246 were laparoscoped to determine sex and maturity status, as well as to calibrate the RIA (methods

247 described in [10]). Information about the sex (via laparoscopy) of these turtles was unknown to
248 the researchers performing either the RIA or ELISA assays.

249 **RIA Testosterone Assay**

250 RIA samples were extracted using previously published methodologies described above
251 [26] and then T concentration was determined using a previously published and validated in-
252 house RIA protocol ([23,26]; but see [28,52,53]). All samples were quantified in duplicate.
253 Seven standards of known radioinert T concentration (19.5 – 1,250 pg/100 μ L) were used to
254 generate a standard curve included with each assay and 0.05 M TRIS buffer (Sigma-Aldrich, St.
255 Louis, MO) was used as a ‘zero’ standard. The sensitivity of the assay was approximately 10
256 pg/mL. The antiserum used in the T RIA (T3-125, Esoterix, Calabasas, CA) has 100% reactivity
257 with testosterone, moderate cross-reactivity with dihydrotestosterone and delta-1-testosterone,
258 and low cross-reactivity with several additional androgens. The resultant hormone concentration
259 therefore denoted total T.

260 **Data Analysis**

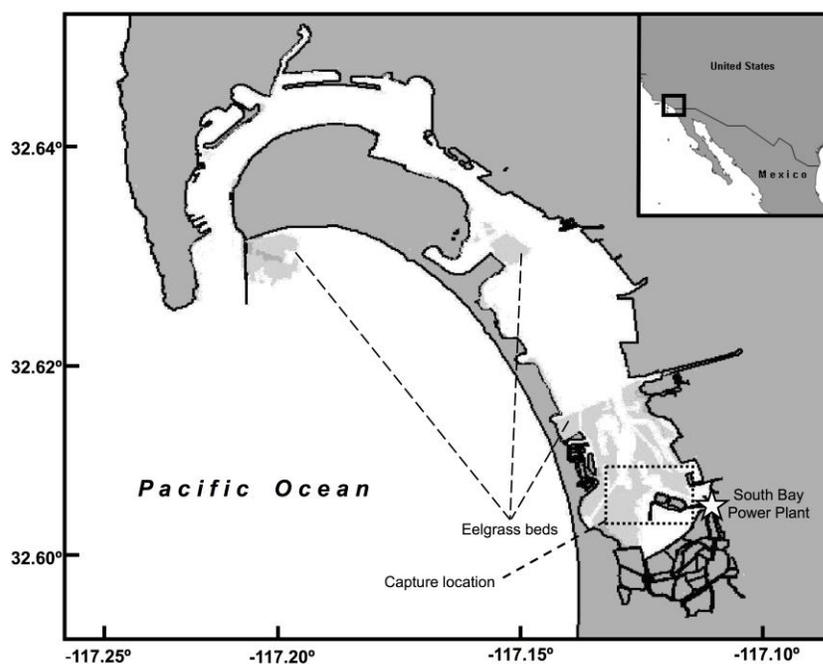
261 We compared T concentrations from the ELISA against the previous results from RIA
262 and to reduce heteroscedasticity (i.e., increasing T concentration produced an increase in
263 measurement variance) all T concentrations were log-transformed prior to statistical analysis. To
264 identify parallelism, we performed a correlation analysis to compare slopes of the log-
265 transformed binding curve, specifically the central linear portion (~ 0.5 B/B₀).

266

267 **ELISA Measurements of San Diego Bay Turtles**

268 **Study Site**

269 San Diego Bay (SDB; Fig. 1), a temperate foraging area for green turtles, is characterized
270 by eelgrass (*Zostera marina*) beds, benthic marine algae, salt marshes, and invertebrate
271 communities [54]. The northern part of this 25-km long bay is urbanized with heavy boat and
272 shipping traffic, whereas in the south there is an ecological reserve [55] where the turtles spend
273 the majority of their time [56]. Monthly average sea surface temperatures (SST) within SDB
274 range from 12.8 – 18.3°C in the winter months and maximum monthly average SST in the
275 summer months was from 22.2 – 26.4°C [57]. However, turtles were mostly captured in the
276 discharge channel of the south bay power plant (Fig. 1) where the monthly average SST was
277 much warmer in winter (20.0 – 23.9°C) and the maximum monthly average SST was extremely
278 warm during the summer (31.7°C) compared to the rest of the bay [57].



279
280 **Fig. 1. San Diego Bay foraging area of East Pacific green sea turtles.**

281

282 **Turtle capture and measurement**

283 Between 1990 and 2014, green turtles were captured with entanglement nets (100 m x 8
284 m, mesh size = 40 – 60 cm knot-to-knot) in the southern portion of San Diego Bay. Straight
285 carapace length was measured (± 0.1 cm) from the nuchal notch to the posterior-most portion of
286 the rear marginal scutes using a forester’s caliper. Turtles were weighed to the nearest kg using a
287 500-kg electronic balance and tagged with Inconel tags (Style 681, National Band and Tag
288 Company, Newport, Kentucky) in the first large proximal scale of one of the front flippers. We
289 measured tail length (TL; ± 1.0 cm) from the tip of the tail to the trailing edge of the plastron
290 using a flexible measuring tape. Turtles smaller than 90.0 cm SCL were considered immature
291 (growth rates were zero or negative for turtles greater than this size, indicating adulthood, [44]).
292 Individuals with tail length > 30 cm and SCL > 90.0 cm were considered putative adult males,
293 and those with short tails and SCL > 90.0 cm were considered putative adult females. The mean
294 (range) female size at nesting is 76.8 (56.0 –95.6) cm SCL at Michoacán [58] and 88.5 (75.8 –
295 102.0) cm SCL at Revillagigedo Archipelago [59]. Straight carapace length was converted from
296 CCL measurements using the previously described equation [44]. Genetic studies indicate that
297 the SDB aggregation includes individuals from several Mexican breeding sites with more turtles
298 likely originating from the Revillagigedo Archipelago than Michoacán ([60], Dutton et al. in
299 prep). However, because male and female green sea turtles may mature at different sizes and
300 over a range of sizes [10], we acknowledge that mature female turtles < 90 cm SCL would be
301 incorrectly classified as immature. Nevertheless, because the primary goal of this study is to
302 determine sex of immature turtles, using the relatively large 90 cm SCL delineation should
303 prevent misidentification of large immature males as adult females. We collected blood samples
304 from 69 individual green turtles in SDB, with multiple blood samples collected from 19 turtles

305 that were captured on several occasions ($n = 96$ total samples). Sex was determined for 39 turtles
306 upon recapture based on SCL and tail length. The mean \pm SD (range) SCL of the turtles used in
307 this study was 82.6 ± 17.1 cm (45.4 – 109.3 cm).

308

309 **Blood collection and handling**

310 We collected blood samples (maximum volume < 5 mL/kg) from the dorsal cervical
311 sinus [61] using a 1.5 inch, 21 gauge vacutainer needle (Becton, Dickinson and Company,
312 Franklin Lakes, NJ) and a 10 mL sodium heparin vacutainer blood collection tube (Becton,
313 Dickinson and Company). Although blood collection immediately after capture is ideal to reduce
314 influence of capture stress on circulating hormone concentration, it was not always possible due
315 to logistical constraints (large majority collected within one hour). Samples were kept chilled on
316 ice packs until centrifugation ($3000 \times g$ for 10 min) after field work completion that day.
317 Following centrifugation, plasma was aliquoted into 2 mL cryovials (Corning Inc., Corning, NY)
318 and stored at -80°C until the assays were conducted.

319

320 **Estimating Probability of Sex Assignment**

321 We developed a statistical model for the relationship between T and various covariates,
322 including sex. The log-transformed and standardized observed T quantities in subsamples from
323 the c^{th} capture of the i^{th} individual were treated as a random sample ($n = 2$) from a normal
324 distribution with a mean ($\mu_{i,c,j}$) and variance (σ^2), where the variance was treated as equal among
325 all individuals. The mean ($\mu_{i,c,j}$) was assumed to be a linear function of covariates, including sex,
326 size (SCL), day of capture (DOY), and tail length (TL). The full model included all covariates:

327 $\mu_{i,c,j} = \beta_0 + \beta_{\text{sex}} \times (\text{sex}_i) + \beta_{\text{SCL}} \times (\text{SCL}_{i,c}) + \beta_{\text{DOY}} \times (\text{DOY}_{i,c}) + \beta_{\text{TL}} \times (\text{TL}_{i,c})$. We standardized

328 (mean = 0 and SD =1) all covariates, except sex. For combining the data from SDB (n = 69) and
329 Bermuda/Panama (n = 30), we used common parameters for all but DOY, considering a
330 possibility that seasons may affect the T concentrations differently between temperate and tropic
331 foraging areas. In addition, it cannot be assumed that East Pacific and Atlantic green turtles have
332 the same seasonal pattern of reproduction or age/size at maturity; samples from these two groups
333 were used to demonstrate correlation between the results of the assays, as well as to ground-truth
334 the sex determinations. Turtles with unknown sex were given missing values for the sex
335 covariate and were predicted in the analysis. Specifically, the sex of each turtle [known sex via
336 laparoscopy or tail length/T concentration at additional captures (Bermuda/Panama n = 30 and
337 SDB n = 47) or unknown sex] was modeled as a Bernoulli trial with unknown probability
338 [62,63]. Because of the lack of other variables that can inform the probability of sex but which
339 were not already in the main model (e.g., tail lengths and T concentration), the probability of sex
340 (expressed as the probability of being male) was assumed to be uniformly distributed between 0
341 and 1. We also considered the following models with fewer covariates: (1) Sex+DOY+SCL, (2)
342 Sex+DOY, (3) Sex+SCL, (4) Sex+SCL+TL, (5) Sex+DOY+TL, and (6) Sex only. Performance
343 of the models was compared using Deviance Information Criteria (DIC), a metric for model
344 comparison in Bayesian statistics where smaller values indicate a better fit [64].

345 Posterior distributions of the parameters were obtained through Markov chain Monte
346 Carlo (MCMC) sampling using JAGS language (v. 3.4.0, [65]) and executed in the R statistical
347 environment (v.3.1.2, [66]) via the RJAGS package [67]. We ran five independent chains of
348 10,000 burn-in steps, followed by 20,000 steps to sample from the joint posterior distribution.
349 The Gelman-Rubin convergence diagnostics statistic was used to determine the convergence of
350 the chains. During the MCMC sampling, the number of times the samples were drawn from each

351 sex was counted. The proportions then were treated as the probability of each sex. The
352 appropriateness of the model to the observed data was determined via posterior predictive
353 simulations, in which possible data were simulated using the random samples from the joint
354 posterior distribution of the parameters. If the majority of observed data points were within the
355 ranges of simulated data, the model was assumed to be appropriate [64]. JAGS and R code used
356 in this analysis are available upon request.

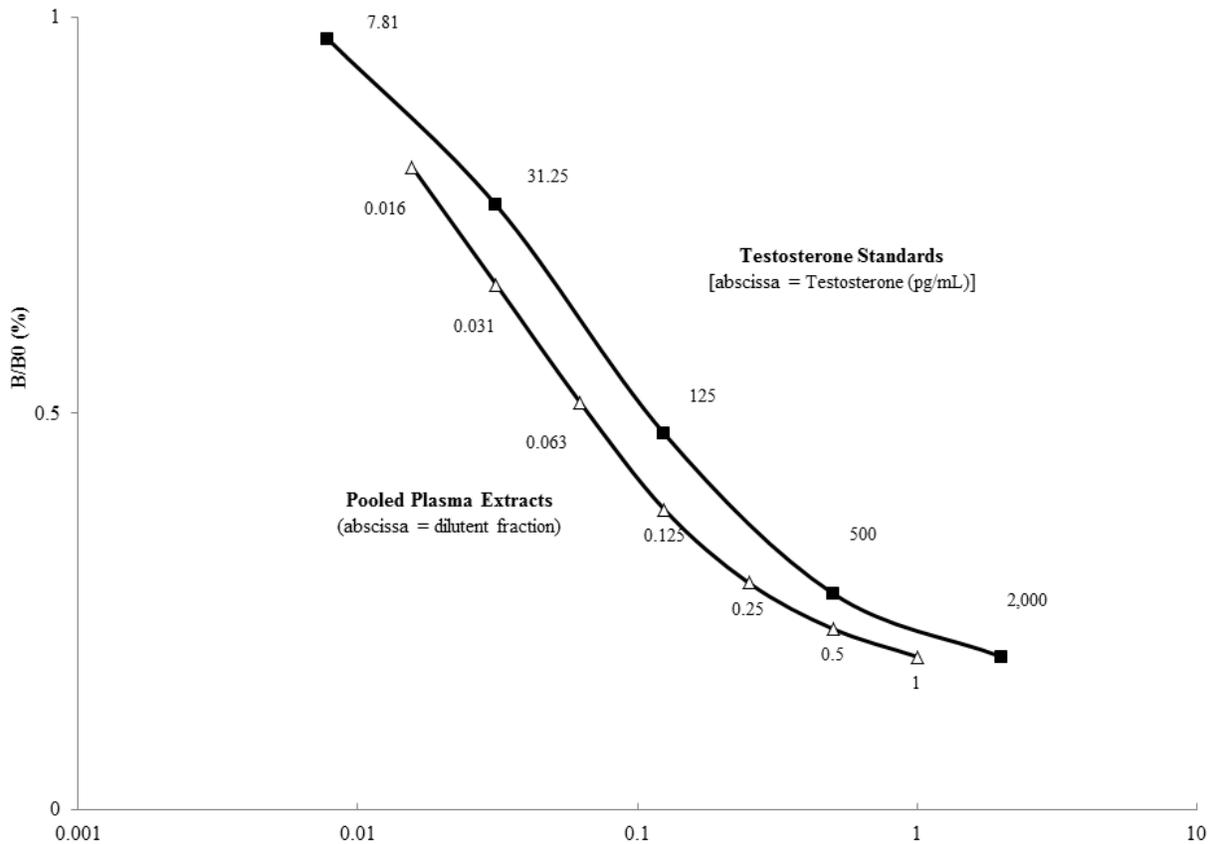
357

358 **Results**

359 **ELISA validation**

360 **Assay Precision**

361 Mean extraction efficiency was 90.7% and the mean intra- and inter-assay coefficients of
362 variation were 6.7% and 17.4%, respectively (n = 11 assays). We confirmed that the T ELISA
363 measured the same antigen in the standard controls and plasma extracts because the slopes of
364 curves from the known standard controls and serial dilutions of pooled plasma extracts
365 demonstrated parallelism (Fig. 2).



366

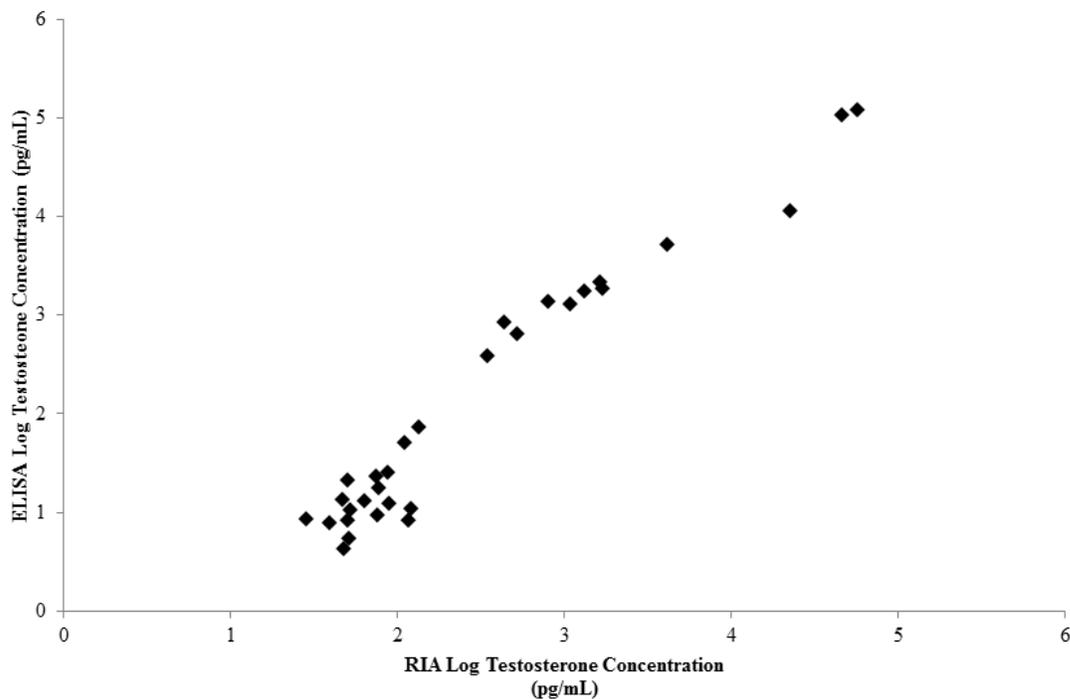
367 **Fig. 2. Results from a linearity assessment of a testosterone enzyme-linked immunosorbent**
 368 **assay (ELISA) with green sea turtle plasma extracts (six immature males).** Serial dilutions
 369 of a pool of extracted green turtle plasma samples (hallow triangles) show parallelism with serial
 370 dilutions of testosterone standards (filled squares). Linearity suggests that the assay is measuring
 371 the same antigens in the plasma extracts as in the testosterone standards, and therefore, that the
 372 ELISA is valid for use with green sea turtle plasma extracts. % B/B0 = % bound/maximum
 373 bound

374

375 ELISA and RIA Comparison

376 There was excellent correspondence ($R = 0.97$, Fig. 3) between RIA- and ELISA-derived
 377 T concentrations for the samples that were analyzed by both methods. Additionally, sex
 378 determination based on ELISA-derived T concentrations agreed with the sex determined via
 379 laparoscopy for all immature turtles. However, occurrence of high T values in some

380 reproductively active females may lead to incorrect predictions of sex in both the RIA and
381 ELISA assays. For example, one adult female captured along a migratory corridor in Panama had
382 high T values (RIA: 1,333.0 pg/mL and ELISA: 1760.2 pg/mL) and was observed via
383 laparoscopy to have shelled eggs. This turtle was incorrectly predicted it to be a male in both
384 assay techniques (RIA and ELISA). It is known that high T values in reproductively active
385 female greens are typical and, therefore, can make it impossible to distinguish between
386 reproductively active females and immature/mature males. However, this poses a problem for
387 foraging aggregations only if reproductively active females are present.



388
389 **Fig. 3. A correlation analysis between radioimmunoassay (RIA) and enzyme-linked**
390 **immunosorbent assay (ELISA) testosterone concentrations of duplicate green sea turtle**
391 **plasma samples.** The high correlation coefficient ($R=0.97$) indicates that the ELISA method is
392 as a comparable method to RIA for determining immature green sea turtle sex.
393

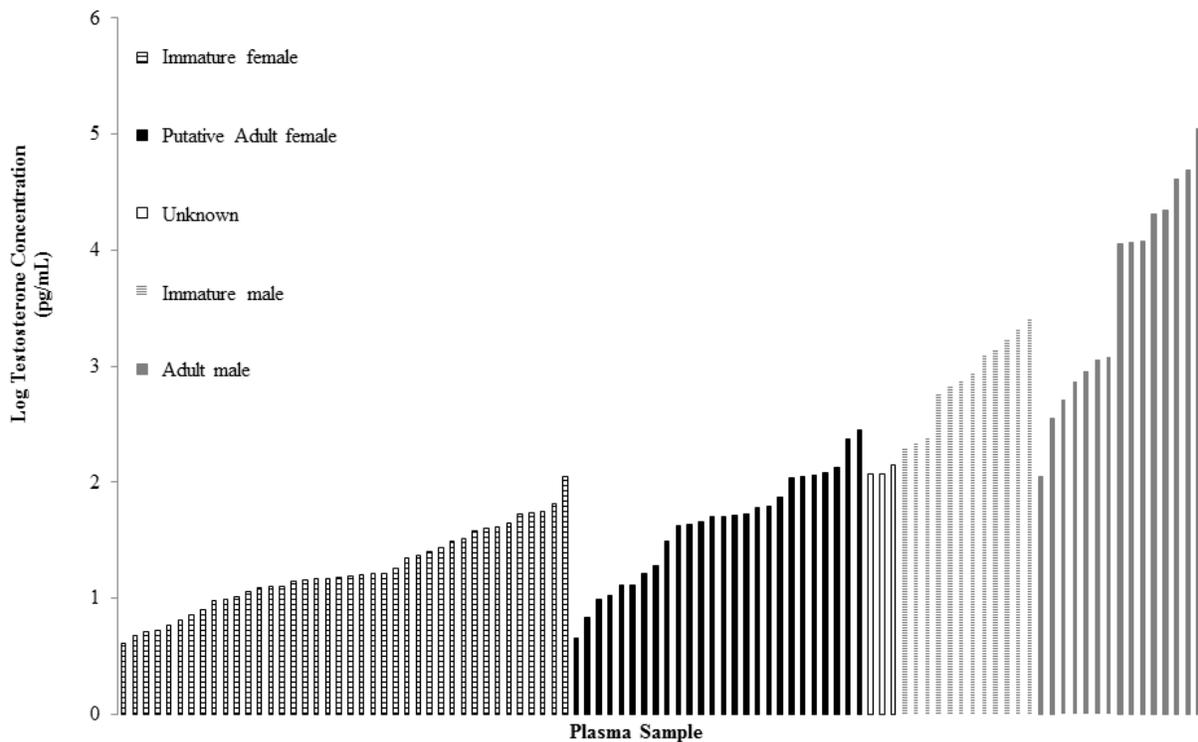
394 **ELISA Measurements of San Diego Bay Turtles**

395 The range of T concentrations for all turtles in SDB was 4.15 – 112,094.2 pg/mL (Fig. 4,
396 Table in S2 Table). The plasma samples of adult and immature male green turtles, in general,
397 had higher T concentrations (112.4 – 112,094.2 pg/mL) than immature females and putative
398 adult females (4.1 – 281.2 pg/mL, Table 1, Fig. 4). However, they had overlapping values in the
399 range of 112.4 - 281.2 pg/ml. Similar to Bolten et al. [49] we identified a T concentration range
400 ('unknown' range) where the sex could not be determined for immature turtles because T
401 concentrations did not fall within the T concentration ranges for either sex, but rather fell in a
402 range in between immature male and female ranges. Of the 45 immature turtles (55 total plasma
403 samples) examined in this study the female range was 4.1 – 113.1 pg/mL and the male range was
404 198.4 – 2,613.0 pg/mL. Three immature turtles had T concentrations (116.4, 119.0, 139.1
405 pg/mL) that fell within the unknown range of 113.1 – 198.4 pg/mL (Table 1, Fig. 4).

406

407 **Table 1: Mean \pm standard error of mean (SEM) and (range) testosterone concentrations (pg/mL) of plasma samples (n = 96)**
 408 **obtained from different maturity states of female and male green turtles (n = 69) that forage in San Diego Bay, CA.** Note:
 409 multiple blood samples were collected from 19 turtles captured on several occasions and, therefore, the results of those additional
 410 samplings are integrated within the maturity state at the time of capture based on size [straight carapace length (SCL)].
 411
 412

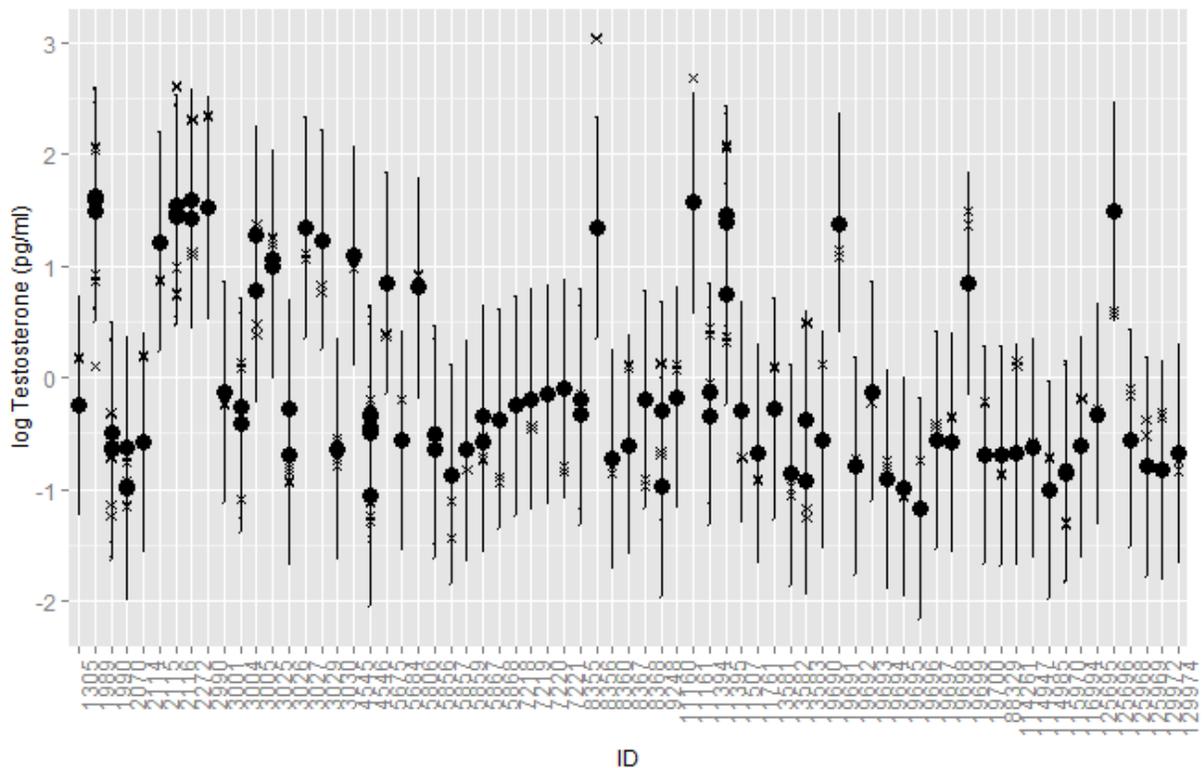
| | Adult (> 90 cm SCL) | | Immature (< 90 cm SCL) | | |
|-------------------------------|---|---|--|--|------------------------------------|
| | Putative Females (n = 21) | Males (n = 9) | Females (n = 32) | Males (n = 10) | Unknown (n = 3) |
| Testosterone Concentration | 67.8 \pm 13.4 (4.6 – 281.2) | 18,939.0 \pm 7,737.3 (112.4 – 112,094.2) | 23.9 \pm 3.4 (4.1 – 113.1) | 1,046.7 \pm 223.1 (198.4 – 2,613.0) | 124.9 \pm 7.2 (116.4 – 139.1) |
| Plasma Samples | n = 26 | n = 15 | n = 40 | n = 12 | n = 3 |



413
 414
 415 **Fig. 4. Mean testosterone concentration (pg/mL) of plasma samples (n = 96) collected from**
 416 **immature and putative adult green sea turtles (n = 69) captured in San Diego Bay, CA.**
 417 Maturity was based on straight carapace length (> 90 cm) and sex was determined by
 418 testosterone levels and validated where possible by tail length, turtle size, and testosterone
 419 concentration upon future captures of the same individuals. Note: some turtles were recaptured
 420 and are represented by multiple data points
 421

422 **Estimating Probability of Sex Assignment**

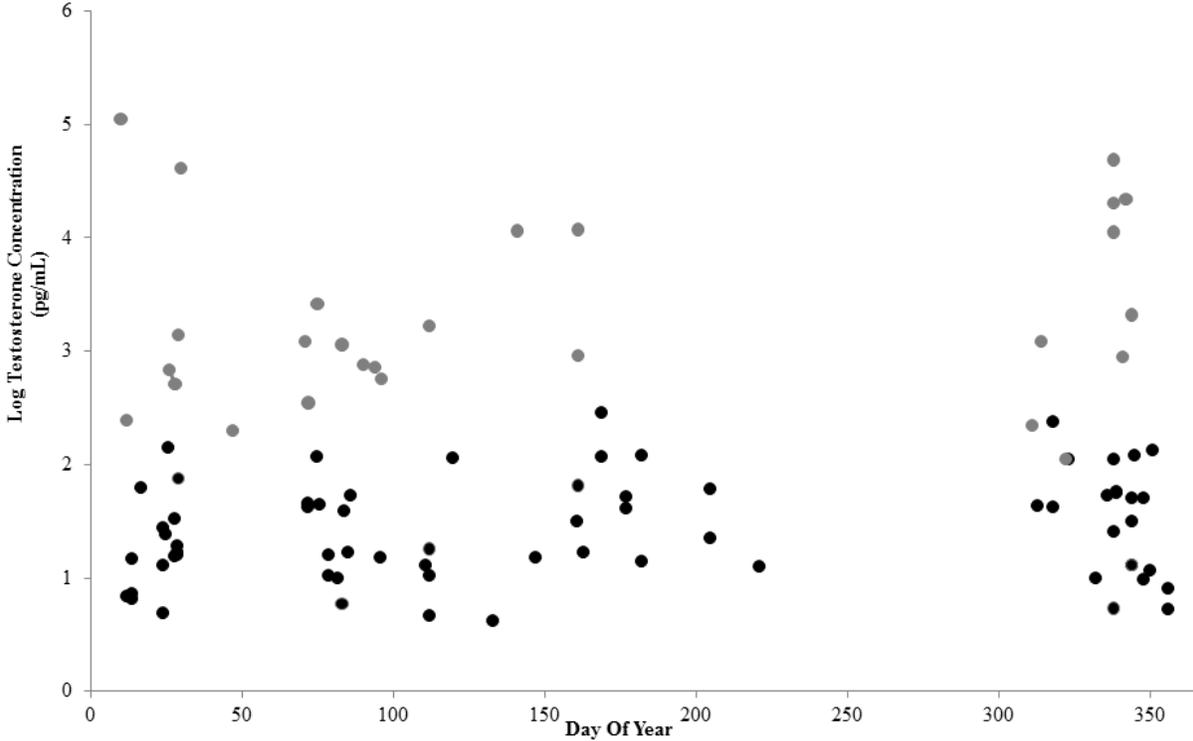
423 Convergence was reached in the MCMC sampling for all models (Gelman-Rubin Rhat
 424 statistic = 1.0). DIC indicated that Sex+SCL+DOY and Sex+SCL were the best two models for
 425 predicting sex probability; the difference in DIC between these models was 1.32. We used the
 426 best model (Sex+SCL+DOY) for parameter inference and this model was determined to be
 427 appropriate based on the results of posterior predictive simulations. The majority of observed
 428 concentrations (except 3 plasma samples collected from adult male turtles) were within 95%
 429 confidence intervals of simulated data (Fig. 5) for each individual.



430

431 **Fig. 5. Posterior simulations of mean testosterone concentrations (in the natural logarithm**
 432 **space) for the 96 green sea turtle plasma samples used in this study.** Filled circles indicate
 433 medians, vertical lines indicate 95% confidence intervals, and X symbols indicate observed
 434 values. Note: some turtles were recaptured and are represented by multiple data points. Three
 435 adult male turtles (IDs 2116, 8356, 11161) have values outside of the 95% confidence intervals
 436

437 All turtles were given probabilities of being male at < 0.14 or > 0.98 (Fig. 6). One turtle
 438 (ID 1989) was a known adult male (SCL = 99.6 cm) yet indicated a low average T concentration
 439 during winter (112.4 pg/ml, November 1998). Subsequent captures of this turtle over 11 years,
 440 however, resulted in high average T concentrations (11,184.2 and 728.5 pg/ml). These results
 441 indicated that T concentrations can vary within individuals or perhaps when water temperatures
 442 are cooler during winter. Additionally, sex may be difficult to assign, based solely on observed
 443 T, for green turtles with concentrations between 100 and 300 pg/mL. With sufficient samples,



453

454 **Fig. 7. Bivariate analysis to examine if the date (day of year) of plasma sample collection**
 455 **influences log transformed mean testosterone concentrations (pg/mL) obtained from**
 456 **putative immature and adult green sea turtles which forage in San Diego Bay, CA. Grey**
 457 **circles represent plasma samples collected from male turtles and black circles represent female**
 458 **turtles. No strong trend was found.**

459

460 Treating all turtles with probability of male > 0.5 to be males, the sex ratio for the SDB
 461 foraging ground was estimated to be 2.83F:1M (51 females and 18 males), whereas samples
 462 obtained from putative immature turtles (< 90 cm SCL) had a 3.5F:1M (35 females and 10
 463 males) sex ratio.

464

465 **Discussion**

466

467 We used a commercially available ELISA T assay in a novel application to a wild
468 aggregation of green sea turtles and provide the first estimate of sex ratio at a foraging ground in
469 the eastern Pacific Ocean. In addition to this field application, we developed a Bayesian
470 approach to determine probability of sex from T measurements of individuals of known and
471 unknown sex. Taken together, these study components will advance the practicability of sea
472 turtle hormonal studies and allow for a broader understanding of sea turtle sex ratio in the eastern
473 Pacific Ocean and globally.

474

475 **ELISA validation**

476 Similar to Cocci et al. [27], we have demonstrated that enzyme immunoassay is a reliable
477 and convenient tool for the rapid assessment of sea turtle sex, and therefore, sex ratio. The only
478 limitation we encountered was that the assay was not reliable for predicting the sex of
479 reproductively active females. This has also proven to be the case for RIA assays (Owens pers.
480 comm.), and in both cases it is important to be able to identify and exclude these animals from
481 the analysis. Our assay precision was similar to other laboratories that follow the same extraction
482 procedure, but use RIA. For example, extraction efficiency ranges of 73.0-97.1%, intra-assay
483 variation ranges of 4.9-13.3%, and inter-assay variation ranges of 13.8-23.9% [29,52,68–70]
484 have been observed in previous studies employing RIA. Validation of the commercially
485 available ELISA kits for use in sea turtle endocrinology studies is advantageous for researchers
486 worldwide that do not have laboratory access to perform RIA assays, which require radioactive
487 material licenses. The validation of the T ELISA for sea turtles, and comparison to results
488 obtained using RIA, will substantially broaden the application of sex ratio analyses for multiple
489 species in many foraging areas worldwide that consist of cohorts of different age and maturity

490 states and likely represent multiple genetic sources. Similar to an endocrinology study of East
491 Pacific green turtles on the relationship of hormones and physiologic/environmental factors [35],
492 the T ELISA (in addition to other biochemical and phenotypic markers) could also inform
493 maturity or senescence and provide an indication of reproductive status of individuals within a
494 population [71], one of several key demographic missing links for the SDB and other foraging
495 populations [37]. In the future, the ELISA could also be applied to other relevant hormones
496 which have thus far been quantified in sea turtles using RIA (e.g. corticosterone to examine
497 stress response).

498

499 **ELISA Measurements of San Diego Bay Turtles**

500 Assuming that the sex ratio was constant over time, the SDB foraging population on the
501 whole is female biased. Available data from past and current studies of all life stages indicate sea
502 turtle sex ratios are female-biased at most sites (Table 2) However, compared to other foraging
503 locations globally, SDB has a more highly female-skewed total population and immature sex
504 ratio (3:5F:1M; Table 2). Comparison of the present study to previous studies of sex ratios of
505 immature green turtles at foraging grounds worldwide (Table 2) found nearly no bias (Hawaii
506 and Bahamas) or moderately to heavily female-biased populations (Australia and Malaysia).

507

508 **Table 2: Sex ratios of different life stages (hatchling, immature, and adult) of green turtles**
509 **at various locations worldwide.** Hatchling sex ratios are from nesting beaches while immature
510 and adult sex ratios are from foraging grounds. NA: not applicable. CCL: curved carapace
511 length. *: 1,199 turtles sexed, but number of turtles for each life stage was not provided

| Life Stage | Sexing Method | Location | Proportion of Females (%) | Sex Ratio (F:M) | Number of Turtles | Source |
|-------------------|--------------------------------------|--------------------------------|----------------------------------|------------------------|--------------------------|---------------|
| Hatchling | Incubation duration | Sri Lanka | 57.2% | | NA | [72] |
| | Nest temperature & gonad histology | Taiwan | 84% | | 215 | [73] |
| | Nest temperature | Heron Island, Australia | 94% | | NA | [74] |
| | Incubation duration | Alagadi Beach, Northern Cyprus | 86–96% | | NA | [75] |
| | Nest temperature & metabolic heating | Ascension Island | 52.5–99.8% | | NA | [76] |
| Immature | Blood hormones | Hawaii | | 1.0: 0.96 | 66 | [70] |
| | Laparoscopy | Shoalwater Bay, Australia | | 1.74:1.0 | 738 (<65.1 cm CCL) | [77] |
| | Blood hormones | Inagua, Bahamas | | 1.4:1.0 | 111 | [49] |
| | Laparoscopy and blood hormones | Heron Island, Australia | | 2.0:1.0 | 200 | [20] |
| | Laparoscopy | Clack Reef, Australia | | 2.2:1.0 | * (>65.1 cm CCL) | [78] |
| | Laparoscopy | Shoalwater Bay, Australia | | 3.26:1.0 | 637 (>65.1 cm CCL) | [77] |
| | Blood hormones & mark-recapture | San Diego, California | | 3.5:1.0 | 45 | Present Study |
| | Laparoscopy | Sabah, Malaysia | | 4.0:1.0 | 75 | [79] |
| | Laparoscopy | Clack Reef, Australia | | 4.2:1.0 | * (<65.1 cm CCL) | [78] |
| Adult | Tail Length | Gulf of Carpentaria, Australia | 2.0% | | 42 | [80] |

| | | | | | | |
|------------------------------------|---|-----|----------|-----|---------------|-----|
| Tail Length | Masirah Island, Sultanate of Oman | 47% | | 242 | [81] | 512 |
| Laparoscopy | Shoalwater Bay, Australia | | 1.78:1.0 | 620 | [77] | |
| Laparoscopy | Clack Reef, Australia | | 2.1:1.0 | * | [78] | |
| Blood hormones & mark-recapture | San Diego, California | | 2.83:1.0 | 30 | Present study | |

513 It is unlikely that reproductively active females with high T concentrations confounded
514 our results significantly as all turtles considered immature or adult males based on high T
515 concentration were re-captured with distinguishable male-sized tails. However, two immature
516 turtles (considered to be male based on T) were caught a single time and measured 85.9 cm SCL
517 and 59.3 cm SCL with T concentrations of 688.7 pg/mL and 2613.0, respectively. It is unlikely
518 that either of these were mature female turtles becoming reproductively active prior to the onset
519 of the peak breeding season (Michoacán: September to December, [82]; Revillagigedo
520 Archipelago: October to November [59]) and migration to Mexican nesting grounds because
521 they were sampled in November and March, respectively. Nonetheless, if these two immature
522 male turtles were indeed reproductively active female turtles it would increase the female-biased
523 sex ratio of immature turtles in the SDB aggregation to 3.7F:1.0M.

524 Female biases in sea turtle foraging populations may be driven by a number of factors.
525 The most likely explanation for a female-biased foraging ground is that this bias results from
526 female-biased hatchling sex ratios because foraging grounds represent a concentration over many
527 years of the sex ratio of the rookeries from which the turtles were hatched [15]. However,
528 estimating sex ratios in hatchlings remains difficult (or not possible) without sacrificing the
529 animal, so using the T ELISA to estimate sex ratios in immature turtles at foraging grounds is an
530 effective method to detect changes or signs of skewed ratios. Also, coupled with genetic mixed-
531 stock analyses, the sex ratio on foraging grounds can be used to estimate the sex ratio at source
532 rookeries (Jensen et al. in review). Although only small volumes of plasma can be collected from
533 hatchlings due to their size, ELISA sensitivity should be sufficient to determine hatchling sex
534 and may provide a less-invasive approach for determining hatchling sex ratios. A potentially

535 critical result is the facilitation of studies to identify the degree to which climate change may
536 impact sex ratios of annual hatchling cohorts (if applied over multiple nesting seasons).

537 Another possible explanation for a female-bias at foraging grounds is differences in
538 habitat preference between males and females. Previous research in other reptiles (hatchling
539 crocodiles and juvenile snapping turtles) has found that thermal behavior was influenced by
540 incubation temperature [83,84]. For example, hatchling crocodiles incubated at higher
541 temperatures were introduced into thermal gradients and they selected a gradient which
542 maintained a higher body temperature compared to their counterparts incubated at a lower
543 temperature. Both studies suggested that temperature selection may influence thermal habitat
544 choice and it was possibly due directly or indirectly to incubation temperature, and thus, the
545 animals' sex. Because the water temperature in the SDB was artificially increased due to the
546 power plant effluent near our capture location, it is possible more females were captured due to
547 their selection of warmer water temperatures (or other covariates). Therefore, male turtles may
548 forage at different locations within the SDB (or at other foraging sites in the East Pacific not yet
549 identified) associated with lower water temperatures. Nonetheless, we believe the plasma
550 samples used for this study provided a representative sample for the SDB population, and further
551 investigation is required to support sex differences in habitat preference for sea turtles.

552 An alternative explanation for the female-biased SDB population is that female turtles
553 exhibit different migratory periodicity than males. In Australia, the remigration interval to
554 breeding grounds for adult female green turtles (5.8 years, [85]) is much longer than for males
555 (2.08 years, [86]); therefore, there may be proportionately fewer males available for sampling in
556 the foraging aggregation because they are instead at the breeding grounds. While the remigration
557 interval for adult male green turtles in the East Pacific is unknown, females migrate to Mexican

558 nesting grounds (where SDB turtles have been tracked by satellite telemetry, [60]) every 1.8 to
559 3.0 years [87,88]. Perhaps females in SDB forego migration in some years while males may
560 migrate to nesting beaches more frequently, thereby leaving a greater number of females in the
561 bay on average. However, this rationalization is unlikely because of the high female bias in the
562 SDB immature turtles that do not migrate for mating.

563

564 **Estimating Probability of Sex**

565 An additional novel outcome of this study was a statistical model that provides
566 probability of sex for turtles that exhibit intermediate T concentration. The model requires data
567 on T concentrations from individuals of known sex. Other covariates, such as size, tail lengths,
568 and a seasonal index (e.g. day of year), may be useful in providing precise estimates. In our
569 analyses, however, tail lengths were not useful—perhaps because of missing data in some
570 individuals or because an alternative tail measurement parameter might be more suitable for
571 determining sex in the SDB population, similar to findings for loggerhead sea turtles [89]. The
572 probabilistic outcome is helpful in estimating a sex ratio because there is no need to establish
573 arbitrary thresholds for T concentrations. Previous studies that determined sex ratio using RIA
574 only (without validation by laparoscopy) still reported immature animals of unknown sex due to
575 plasma T concentrations falling within the unknown range (e.g., [49,70]). Conversely, the
576 statistical model can provide the probability of sex for immature turtles. Moreover, the model
577 can be applied to other populations and species, thereby enhancing baseline information on sex
578 ratios to inform management decisions for conserving endangered sea turtles.

579 We assumed normal distributions for the observed standardized and log-transformed
580 ELISA T concentrations and believe this was a reasonable approximation of the data. The mean

581 of the normal distribution was treated as a linear function of covariates, which may be improved
582 by including products and other functions of covariates if sufficient samples are collected from
583 each individual. In our data, repeated samples from the same individuals were not collected
584 within a year to justify inclusion of more complex functions in the model. Investigation into
585 possible effects of collection date upon T concentration did not show a strong pattern (Fig. 7),
586 suggesting that there was no seasonal effect, however, to determine possible seasonal
587 fluctuations in T titers, it is best to repeatedly sample the same individuals within a year [28].

588

589 **Conservation implications**

590 Female-biases in breeding populations may be beneficial for species recovery due to an
591 increase in the number of breeding females, and therefore, population growth potential [90]. For
592 example, a female bias may explain the population increase of the threatened Pacific Mexico
593 green turtle population [48] since it was protected in 1978 [91].

594 Climate change scenarios indicate that the problem of near complete feminization for
595 certain rookeries of different sea turtle species could occur within the next ten to fifteen years
596 [92,93] or longer (by 2070, [19]; 159 years, [90]) without phenomenological shifts or other
597 behavioral adaptations. Current sex ratio baseline information will be informative for predicting
598 climate warming conservation concerns for sea turtles, and sex ratio information for each sea
599 turtle species is vital for inferring population status and the survivorship of each sex. Indeed,
600 Labrada-Martgón et al. [71] emphasized how physiological approaches (hormone
601 determinations) can provide valuable demographic information for marine vertebrate
602 conservation. Knowledge of population sex ratios and their inclusion in management plans will
603 create a more comprehensive approach to the conservation of sea turtles globally.

604

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619

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903 **Supporting Information Captions**

904 **S1 Table. Morphometrics of and testosterone concentration of plasma samples collected**
905 **from 30 green sea turtles captured in Panama and Bermuda.** F: female and M: male.

906

907 **S2 Table. Morphometrics of and testosterone concentration of plasma samples collected**
908 **from 69 green sea turtles captured in San Diego Bay, California.** Note: some turtles were
909 recaptured multiple times. F: female, M: male, and U: unknown sex.