Journal of FISH BIOLOGY
Published for the Fisheries Society of the British Isles

Journal of Fish Biology (2010) **77**, 769–777
doi:10.1111/j.1095-8649.2010.02693.x, available online at www.interscience.wiley.com

BRIEF COMMUNICATION

Plasma cortisol and 11-ketotestosterone enzyme immunoassay (EIA) kit validation for three fish species: the orange clownfish *Amphiprion percula*, the orangefin anemonefish *Amphiprion chrysopterus* and the blacktip reef shark *Carcharhinus melanopterus*

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(Received 29 July 2009, Accepted 7 April 2010)

Commercially available enzyme immunoassay (EIA) kits were validated for measuring steroid hormone concentrations in blood plasma from three fish species: the orange clownfish *Amphiprion percula*, the orangefin anemonefish *Amphiprion chrysopterus* and the blacktip reef shark *Carcharhinus melanopterus*. A minimum of 5 µl plasma was required to estimate hormone concentrations with both kits. These EIA kits are a simple method requiring minimal equipment, for measuring hormone profiles under field conditions.

Key words: elasmobranch; ELISA; haematocrit; hormone; steroids; teleost.

Steroids are a hormone group produced in the gonads and in the interrenal gland. They are produced in response to a plethora of environmental cues and stimulate changes in cellular metabolism, resulting in a variety of effects on important life-history traits. Steroid hormones, such as testosterone, have important consequences for reproduction, while corticosterone is produced in response to stressful conditions (Sapolsky *et al*., 2000). Due to their trade-offs with immune function, steroid hormones have concomitant effects on other life-history traits such as reproduction and survival, and as such are important physiological variables that can be used to understand an organism's overall fitness (Grossman, 1985; Folstad & Karter, 1992; Mills *et al*., 2010).

Plasma steroid levels can be measured using multiple methods including radioimmunoassay (RIA) (Mills *et al*., 2007, 2008, 2009), high performance liquid chromatography (HPLC) with UV detection, isotope dilution gas chromatography spectrometry (De Brabandere *et al*., 1995) and enzyme-linked immunosorbent assay

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(ELISA) (Barry *et al*., 1993; Ackerman & Iwama, 2001; Sink *et al*., 2008). The choice of technique is often based on expense, equipment and licence limitation, relative speed and ease of use and suitability to the study species. HPLC with UV detection and isotope dilution gas chromatography spectrometry require specialized equipment, while RIA involves radioactive substances (Sink *et al*., 2008). ELISA kits, on the other hand, contain no radioactive agents, a spectrophotometer is the only specialized equipment required and the simple protocol favours their use. Enzyme immunoassay (EIA) kits, however, usually employ human plasma-based standards that are not always suitable for comparison with other non-mammalian species, therefore validation of the kit with each study species is a necessity before use.

Clownfishes and anemonefishes, *Amphiprion* spp., are model species used to answer multiple and disparate questions ranging from connectivity (Jones *et al*., 2005), cooperative breeding (Buston, 2004), sex change (Godwin & Thomas, 1993), size hierarchies (Buston, 2003), mutualism (Holbrook & Schmitt, 2005) and sound production (Parmentier *et al*., 2007). Clownfishes and anemonefishes are also highly valued in the aquarium trade and are used to promote diving tourism. Other key species for diving tourism include sharks, yet their populations are under threat due to fisheries overexploitation (Stevens *et al*., 2000; Baum & Myers, 2004; Campana *et al*., 2006; Robbins *et al*., 2006; Myers *et al*., 2007; Heupel *et al*., 2009). In order to understand the physiological processes associated with anemonefish behaviour, reproduction and survival, and the impacts of overfishing and tourism on shark physiology and life-history traits, it is necessary to measure steroid hormone levels. The aim of the present study was to validate commercially available EIA kits in order to measure plasma 11-ketotestosterone and cortisol in *Amphiprion percula* (Lacepède) and *Amphiprion chrysopterus* Cuvier and 11-ketotestosterone in the blacktip reef shark *Carcharhinus melanopterus* (Quoy & Gaimard).

Twenty *A. percula* (Lautän Production; www.lautanproduction.com) were kept in aquaria at EPHE, Perpignan, for 10 days at 24° C and on a 12L:12D photoperiod. The mean ± S.E. wet mass of the experimental fish was 1·21 ± 0·03 g (range = 0·67–3·06 g) and the mean ± S.E. standard length (L_S) was 35·05 ± 0·11 mm (range = 26·03–45·07 mm). The fish were placed for 3–4 min in a 2 l seawater anaesthetizing tank containing 1 ml of 10% Eugenol (Merck; http://www.merck.de) diluted in 95% ethanol. Blood samples were taken laterally from the caudal vein using heparinized (Sigma H-0878; www.sigmaaldrich.com) 1 ml plastic syringes fitted with a 30 gauge needle (Godwin & Thomas, 1993).

Thirty *A. chrysopterus* were caught by divers with hand-nets from their host, the magnificent sea anemone *Heteractis magnifica* on the north shore of Moorea (17° 30′ S; 149° 50′ W) in French Polynesia in June 2008. The mean ± S.E. total length (L_T) of the 30 individuals was 93·6 ± 8·9 mm (range = 46·0–154·0 mm). Individuals were tagged (fin-clipped) and released back on their anemones within 5–10 min after capture. Blood (100–250 μl) was drawn from the caudal vein underwater using a heparinized 25 gauge needle and 1 ml plastic disposable syringe. Syringes were kept on ice on the boat.

Twenty-five male *C. melanopterus* were caught from a boat inside and outside the lagoon on the north and west shores of Moorea (17° 30′ S; 149° 50′ W) in French Polynesia in 2008 and 2009. The mean ± L_T was 120·12 ± 9·75 cm (range = 93·0–134·0 cm). Individuals were tagged (fin-clipped) and released back to the sea within 5–10 min after capture. A minimum of 250 μl of blood was drawn

from the caudal vein using a heparinized 15 gauge needle and 10 ml plastic disposable syringe. Syringes were kept on ice on the boat.

Individual blood samples were transferred to 75 μl haematocrit capillaries and centrifuged (Beckman Coulter TJ-25 centrifuge; www.beckmancoulter.com) at 10 000 *g* for 5 min at 4° C and the haematocrit (% of red blood cells in the blood) were measured. The mean ± S.E. haematocrit values were 16·5 ± 0·4, 46·5 ± 4·3 and 29·0 ± 1·8% for *A. percula*, *A. chrysopterus* and *C. melanopterus*, respectively. The supernatant, a yellow plasma layer, was collected without disturbing the white buffy layer or the blood cells. Twenty, 30 and 25 samples were pooled for *A. percula*, *A. chrysopterus* and *C. melanopterus*, respectively and the pools were stored at −80° C.

Plasma 11-KT was measured using an EIA kit (11-keto Testosterone EIA Kit, No. 582751, Cayman Chemicals, SPI BIO; www.spibio.com). Fifty microlitres of the eight standards or 50 μl of the blood plasma samples were added with 50 μl of 11-KT-acetylcholinesterase (AChE) conjugate and 50 μl of 11-KT-specific rabbit antiserum to a 96-well plate. During 18 h at 4° C, 11-KT AChE and sample or standard 11-KT competed for a limited number of 11-KT-specific rabbit antiserum-binding sites, whose complex was attached to the mouse monoclonal anti-rabbit IgG antibody previously attached to the well. The plate was washed five times to remove any unbound reagents and 200 μl of Ellman's reagent, that contains the substrate to AChE, was added to the wells. The plate was placed on an orbital shaker in the dark for 55 min. The intensity of the yellow colour was measured spectrophotometrically (Beckman Coulter AD 340 Spectrophotometer) at 405 nm and was proportional to the amount of 11-KT AChE bound to the well, which was inversely proportional to the amount of free 11-KT present.

Plasma cortisol was measured using an EIA kit (Cortisol EIA Kit, No. 582121, Cayman Chemicals, SPI BIO). The methods were identical to those used for the 11-KT assay except that cortisol-acetylcholinesterase (AChE) conjugate was used and the rabbit antiserum binding sites were cortisol-specific. The plate was placed on an orbital shaker in the dark for 80 min.

Sample 11-KT and cortisol concentrations were determined by interpolation from the standard calibration curve using a common functional model for calibration curves. The data were plotted as % maximum bound (% B Bo^{-1}) *v*. log concentration using a logit-log curve fit as recommended for these kits (www.caymanchem.com/analysis/eia).

Validation of both kits comprised 1) parallel displacement of serially diluted plasma to the standard curve, 2) accuracy from spike recovery and 3) precision from intra- and inter-assay variabilities.

Parallelism was evaluated by measuring 11-KT and cortisol concentrations in pooled plasma samples, serially diluted in EIA buffer provided with the kits. For *A. percula*, two sets of dilution ratios were prepared, both were used for validation of the 11-KT assay, whereas only the first set was required for validation of the cortisol assay. The first dilution set ratios were 1:10, 1:32, 1:100, 1:302, 1:585, 1:1210, 1:3732 and 1:12 109 and the second dilution set ratios were 1:3, 1:6, 1:10, 1:18·5, 1:32, 1:59, 1:100, 1:185, 1:302, 1:585 and 1:1216. For *A. chrysopterus* and *C. melanopterus*, one set of dilution ratios was prepared for both species: 1:1·8, 1:3·18; 1:6, 1:10, 1:31·875, 1:100, 1:318·75 and 1:1000. The % BBo^{-1} for each sample dilution and for the standards was plotted against their relative log dilution

and the shapes of the resulting curves were compared. These curves must be parallel to support the assumption that the antibody-binding characteristics of standard and sample are similar enough to allow the determination of antibody levels in the diluted plasma sample. An ANCOVA was carried out to determine the homogeneity of slopes between the sample dilutions and those of the kit's standards. In addition, regression analysis of the diluted sample was used to determine the dilution factor that corresponds to 50% of antibody bound.

Accuracy was assessed by the determination of spike recovery performed by adding a pre-determined hormone concentration (see Table I for each concentration per species and per kit) to each of four plasma sample dilutions, in duplicate. The per cent recovery is the concentration of the spiked sample minus the spike concentration, as a proportion of the unspiked sample. Spike recovery detects errors due to either sample manipulation, which may sometimes result in the loss of an analyte leading to erroneously low measured values, or the introduction of compounds by the sample or sample matrix that interfere with accurate measurement. Therefore, spike recovery assists in accounting for the per cent loss of an analyte and may detect interfering substances.

Precision was assessed by examining intra- and inter-assay variability of samples with different hormones levels. Intra-assay variability was determined by evaluating between four and 23 plasma samples (according to species and assay; see Table II) in duplicate within the same run of the assay. Inter-assay variability was determined by evaluating between three and eight samples (according to species and assay; see Table II) in two or three runs of the assay. Variabilities or coefficients of variation (C.V.) of repeated measures of samples were assessed. C.V. (y) was calculated according to the formula: $y = 100z\ \overline{x}^{-1}$, where $z =$ S.D. A kit was considered to have good precision if the C.V. was $<20\%$ as per the guidelines, for example Plikaytis *et al.* (1994) and Sukovaty *et al.* (2006).

Pooled plasma from the three species was screened with eight dilutions of the 11-KT kit's standards. A characteristic S-shaped curve was observed for 12 dilutions of *A. percula's* pooled plasma [Fig. 1(a)]. As only the linear part is of interest, however, the curve using seven dilutions of pooled plasma was used and was found to run parallel to that obtained using standards provided with the 11-KT kit [Table I and Fig. 1(b)]. Five dilutions of pooled plasma from *A. chrysopterus* and five dilutions of pooled plasma from *C. melanopterus* were screened with the 11-KT kit's standard curve. The curves obtained were parallel to those using 11-KT standards [Table I and

TABLE I. ANCOVA on homogeneity of slopes for sample dilution *v.*, standard dilution curves for 11-ketotestosterone (11-KT) and cortisol kits in, *Amphiprion percula, Amphiprion chrysopterus* and *Carcharhinus melanopterus*. The dilution factor (dilution) for 50% of antibody bound determined from regression analyses (Figs 1 and 2) is also given

Assay	Species	d.f.	Mean square	*F*	*P*	Dilution factor
11-KT	*A. percula*	1,47	0·002	0·180	>0·05	1:18·7 (0·054)
	A. chrysopterus	1,35	0·015	1·991	>0·05	1:8·7 (0·115)
	C. melanopterus	1,33	0·011	1·264	>0·05	1:187·5 (0·005)
Cortisol	*A. percula*	1,55	0·018	0·676	>0·05	1:425·9 (0·002)
	A. chrysopterus	1,36	0·004	0·379	>0·05	1:137·4 (0·007)

TABLE II. Percentage spike and recovery of 11-ketotestosterone (11-KT) and cortisol in *Amphiprion percula, Amphiprion chrysopterus* and *Carcharhinus melanopterus*

Assay	Species	*n*	Spike (pg ml^{-1})	Mean ± S.E. spike recovery
11-KT	*A. percula*	4	50	116·1 ± 22·1
	A. chrysopterus	4	25	89·8 ± 16·0
	C. melanopterus	4	100	123·6 ± 16·2
Cortisol	*A. percula*	4	5000	111·5 ± 14·0
	A. chrysopterus	4	1000	107·9 ± 20·5

Fig. 1(c), (d)]. Regression analyses enabled the appropriate dilution factors for 50% of antibody bound for all three species to be determined for the 11-KT kit (Table I). All three species tested with the 11-KT kit showed high accuracy determined from spike and recovery (89·8–123·6%; Table II) and high precision determined from intra- and inter-assay variabilities (8·7–14·8%; Table III).

11-KT concentrations of aquarium and field, *A. percula* and *A. chrysopterus*, based on the mean ± S.E. of the pooled plasma from 20 and 30 individuals were 0·35 ± 0·02 and 0·11 ± 0·00 ng ml^{-1}, respectively. These means are comparable to 11-KT levels previously recorded using radioimmunoassay for *Amphiprion melanopus* Bleeker (mean ± S.E. plasma 11-KT value estimated from figures: males = 0·63 ± 0·06 ng ml^{-1} and females = 0·23 ± 0·02 ng ml^{-1}; Godwin & Thomas, 1993). 11-KT concentration of field *C. melanopterus*, based on the mean ± S.E. of the pooled plasma from 25 individuals was 1·82 ± 0·10 ng ml^{-1}. This mean is comparable to values previously recorded using radioimmunoassay for adult male bonnetheads, *Sphyrna tiburo* (L.) (mean ± S.E. range of plasma 11-KT values = 0·07 ± 0·91 to 2·21 ± 0·09 ng ml^{-1}; Manire *et al*., 1999).

Pooled plasma from the two *Amphiprion* species were also screened for parallelism with eight dilutions of the cortisol kit's standards. For *A. percula* and *A. chrysopterus*, seven and four dilutions of pooled plasma respectively, were found to run parallel to the cortisol standard curve (Table I and Fig. 2). Regression analyses enabled the appropriate dilution factors for 50% of antibody bound for both clown anemonefish species to be determined with the cortisol kit (Table I). *Amphiprion percula* and *A. chrysopterus* also showed high accuracy and precision with the cortisol kit determined from spike and recovery (107·9–111·5%; Table II) and intra- and inter-assay variabilities, respectively (9·9–19·4%; Table III).

Cortisol concentrations of aquarium and field *A. percula* and *A. chrysopterus* based on the mean ± S.E. of the pooled plasma were 18·98 ± 1·59 and 21·53 ± 2·46 ng ml^{-1}, respectively. These means are comparable with cortisol levels recorded using radioimmunoassay for *A. melanopus* (mean plasma cortisol values: males = 17 ± 4 ng ml^{-1} and females = 16 ± 3 ng ml^{-1}; Godwin & Thomas, 1993).

In conclusion, 11-KT EIA assay kits were tested on two *Amphiprion* species, *A. percula* and *A. chrysopterus* and *C. melanopterus*, and cortisol EIA assay kits were tested for use on *A. percula* and *A. chrysopterus*. The dose–response curves were parallel to the kits standards [Figs1(b)–(d) and 2(a), (b) and Table I], high accuracy was obtained from spike recovery determination (Table II) and high precision was obtained from intra- and inter-assay variabilities (<20%; Table III) for both kits with

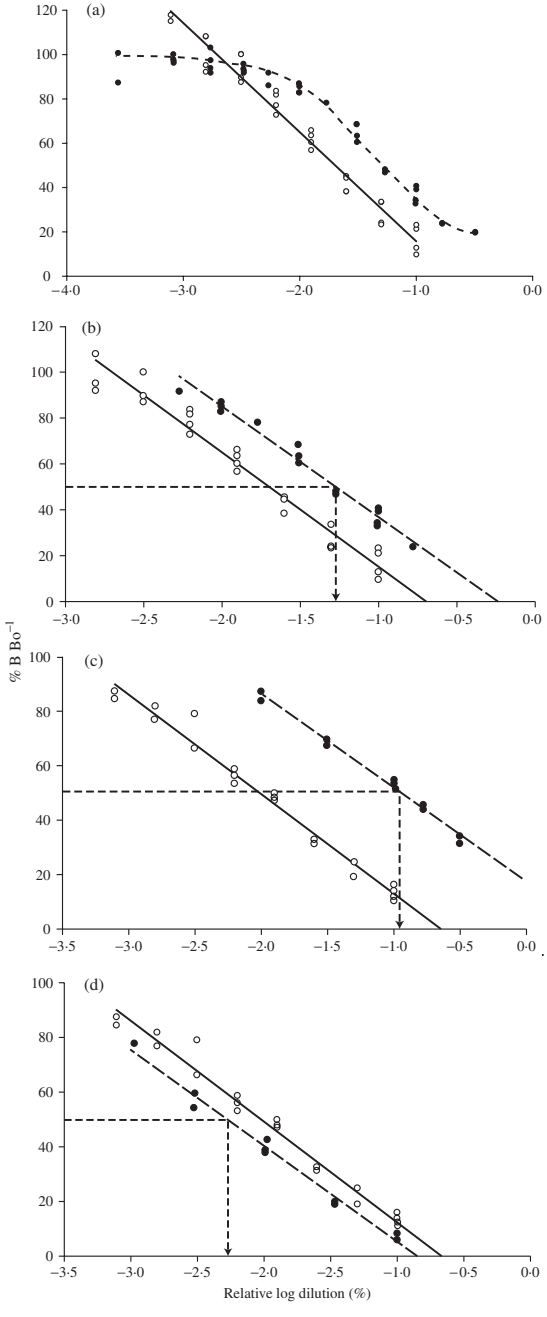

FIG. 1. Dose–response curves for 11-ketotestosterone (11-KT) obtained using eight kit standards and pooled plasma from (a) *Amphiprion percula*, (b) *Amphiprion percula* [simple linear regression: kit standards $y = -49{\cdot}26x - 33{\cdot}52$ ($r^2 = 0{\cdot}97$, $n = 29$, $P < 0{\cdot}001$) and samples $y = -48{\cdot}19x - 11{\cdot}27$ ($r^2 = 0{\cdot}97$, $n = 17$, $P < 0{\cdot}001$)], (c) *Amphiprion chrysopterus* [kit standards $y = -36{\cdot}71x - 24{\cdot}08$, ($r^2 = 0{\cdot}98$, $n = 21$, $P < 0{\cdot}001$) and samples $y = -34{\cdot}32x + 17{\cdot}73$ ($r^2 = 0{\cdot}99$, $n = 13$, $P < 0{\cdot}001$)] and (d) *Carcharhinus melanopterus* [kit standards $y = -36{\cdot}71x - 24{\cdot}08$ ($r^2 = 0{\cdot}98$, $n = 21$, $P < 0{\cdot}001$) and samples $y = -35{\cdot}02x + 19{\cdot}59$ ($r^2 = 0{\cdot}99$, $n = 11$, $P < 0{\cdot}001$)]. Dashed line and arrow represents 50% bound (see Table I for corresponding dilution factors). ●, pooled species plasma; ○, 11-KT kit standards.

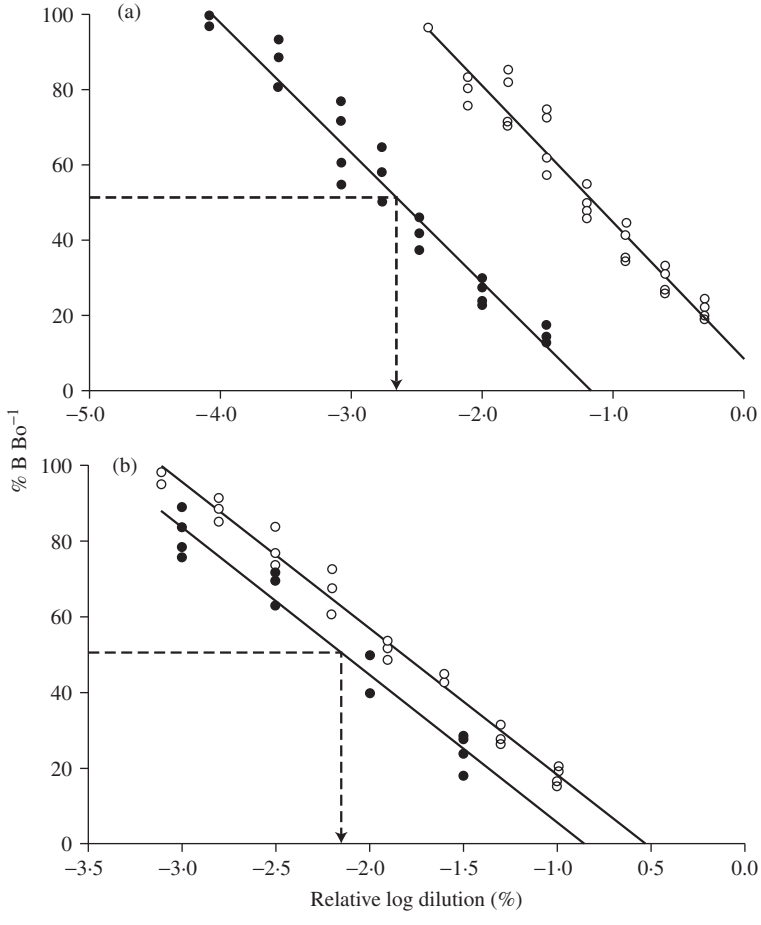

FIG. 2. Dose–response curves for cortisol obtained using eight kit standards and pooled plasma from (a) *Amphiprion percula* [kit standards $y = -36{\cdot}39x + 8{\cdot}27$ ($r^2 = 0{\cdot}95$, $n = 29$, $P < 0{\cdot}001$) and samples $y = -34{\cdot}52x - 40{\cdot}76$ ($r^2 = 0{\cdot}95$, $n = 25$, $P < 0{\cdot}001$)] and (b) *Amphiprion chrysopterus* [kit standards $y = -38{\cdot}66x - 20{\cdot}56$ ($r^2 = 0{\cdot}99$, $n = 23$, $P < 0{\cdot}001$) and samples $y = -38{\cdot}92x - 33{\cdot}22$ ($r^2 = 0{\cdot}95$, $n = 12$, $P < 0{\cdot}001$)]. Dashed line and arrow represents 50% bound (see Table I for corresponding dilution factors). ●, pooled sample plasma; ○, cortisol kit standards.

the two *Amphiprion* species, and for the 11-KT kit with *C. melanopterus*. Consequently, these kits can be confidently used for measuring 11-KT and cortisol in *A. percula* and 11-KT in *C. melanopterus*. In addition, only small quantities of blood plasma were required, the minimum quantity of blood plasma required being 5 or 7 µl if the samples are to be tested in duplicate. This is of particular interest for the small-bodied, *A. percula* (mean $L_S = 35{\cdot}05$ mm). *Amphiprion percula* has an average haematocrit of 16·5%, therefore the total blood volume required is 6 and 8 µl, for measuring samples singly or in duplicate, respectively. The small volume of blood required for hormone determination is an additional advantage for using this EIA kit and furthermore, multiple sampling on the same individual may also be performed.

TABLE III. Intra- and inter-assay variabilities (coefficient of variation C.V.) for 11-ketotestosterone (11-KT) and cortisol in *Amphiprion percula, Amphiprion chrysopterus* and *Carcharhinus melanopterus*

Hormone	Species	Intraplate C.V. (*n*)	Interplate C.V. (*n*)
11-KT	*A. percula*	14·8 (22)	12·6 (7)
	A. chrysopterus	8·7 (6)	9·6 (3)
	C. melanopterus	14·2 (7)	14·6 (3)
Cortisol	*A. percula*	9·9 (23)	13·1 (8)
	A. chrysopterus	16·6 (4)	19·4 (4)

We are grateful to G. Simon, R. Beldade, D. Miles and the Ocean Bridges programme funded by the Partnership University Fund of the French American Cultural Exchange for funding this project.

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