



Physiological markers suggest energetic and nutritional adjustments in male sharks linked to reproduction

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Abstract

Energetic condition is one of the most important factors that influence fitness and reproductive performance in vertebrates. Yet, we lack evidence on how energetic states change in response to reproduction in large marine vertebrates. In the present study, we used a non-lethal approach to assess relationships among reproductive stage, circulating steroid hormones (testosterone and relative corticosteroid levels), plasma fatty acids, and the ketone body β -hydroxybutyrate in male sharks of two species with divergent ecologies, the benthic nurse shark (*Ginglymostoma cirratum*) and the epipelagic blacktip shark (*Carcharhinus limbatus*). We found higher relative corticosteroid levels in adult nurse sharks during the pre-mating period and in blacktip sharks during the mating period. Higher levels of β -hydroxybutyrate were found in adult nurse sharks during the mating period, but concentrations of this ketone body did not significantly vary across reproductive stages in blacktip sharks. We also detected reduced percentages of essential fatty acids during the mating period of both nurse and blacktip sharks. Taken together, our findings suggest that nurse and blacktip sharks differ in their energetic strategy to support reproduction, however, they likely rely on physiologically important fatty acids during mating, to support spermatogenesis.

Keywords Dietary patterns · Reproductive hormones · Trophic markers · Elasmobranchs · Lipid metabolites

Introduction

As long-lived and k-selected species, sharks as a group exhibit relatively slow growth, late sexual maturity, long gestation periods, and reduced fecundity (Cortés 2000;

Dulvy and Forrest 2010). The evolutionary success of sharks, at least in part, is due to their diverse female reproductive modes (e.g. lecithotrophy, placentotrophy, oophagy, and histotrophy; Hamlett et al. 2005) and mating strategies, with polyandry and polygyny, as well as sperm storage and multiple paternity being commonly reported (Pratt and Carrier 2001). Despite their importance for reproductive performance, energetic investments are rarely considered in studies of shark reproduction (e.g. Dudley and Cliff 1993; Hammerschlag et al. 2018; Rangel et al. 2021a). Furthermore, there are no published studies investigating the nutritional quality and dietary patterns of free-living male sharks across life-stages.

In male sharks, energetic investments in reproduction are mainly associated with spermatogenesis, copulation, male-male competition, and where necessary, migration to access mates (e.g. Pratt and Carrier 2001). Like many other vertebrates, seasonal adjustments of sperm production and mating behavior are often driven by changes in hormones that coordinate the timing of breeding and energy allocation (Awruch 2013). Sex hormones, including androgens, progestins, and estrogens, are well-documented players in the regulation of reproductive morphological changes (e.g. testicular

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development and secondary sex characteristics) and associated behaviors (e.g. courtship and territoriality) (Awruch 2013; Becerril-García et al. 2020; Gonzalez et al. 2020). Although studies investigating the role of glucocorticoids in sharks are lacking, these hormones are known to play an important role in regulating the acquisition and mobilization of resources in many vertebrates and are expected to be higher during energetically expensive life-history stages, such as reproduction (e.g. Romero 2002; Crespi et al. 2013; Romero and Wingfield 2015). As such, measuring hormones integrated with nutritional indicators can offer a great opportunity to non-lethally examine patterns of energy regulation across reproductive stages (e.g. Hammerschlag et al. 2018).

Nutritional quality is one of the most important factors that influence reproductive performance in vertebrates, including those related to gamete quality (both eggs and sperm), fecundity, and offspring survival (Izquierdo et al. 2001; Wathes et al. 2007; Bobe and Labbé 2010). In this context, fatty acids have been identified as major essential dietary nutrients that influence reproductive processes through a variety of mechanisms (Izquierdo et al. 2001; Tocher 2010). For example, arachidonic acid (C20:4n6, ARA), an omega-6 polyunsaturated fatty acid (n6 PUFA), is metabolized to form prostaglandins (eicosanoids) that are involved in follicle maturation and steroid production during reproduction in females (e.g. Izquierdo et al. 2001; Lund et al. 2008). Omega-3 polyunsaturated unsaturated fatty acids (n3 PUFAs), e.g. docosahexaenoic acid (C22:6n3, DHA) and eicosapentaenoic acid (C20:5n3, EPA), are well described as important nutrients in successful vertebrate reproduction, as they affect many important physiological processes, such as immune and inflammatory responses, as well as brain and eye development (Tocher 2010). PUFAs also acts as the main component of the cell membranes, including sperm and oocyte, which is important during fertilization (Izquierdo et al. 2001; Bobe and Labbé 2010). Because these important fatty acids cannot be synthesized de novo (Parrish 2009; Colombo et al. 2017), high-quality diets play a key role in vertebrate reproduction.

In the present study, we integrated multiple physiological markers to non-lethally assess if and how energetic state changes in relation to reproductive stage in two species of male shark. For this, we investigated two annual breeders with seasonal sperm production, but with divergent ecologies, the relatively sedentary and benthic nurse shark (*Ginglymostoma cirratum*) and the relatively active and epipelagic blacktip shark (*Carcharhinus limbatus*). Based on previous studies demonstrating that males of both species exhibit temporal decoupling between sperm production and mating behavior (e.g. Dudley and Cliff 1993; Baremore and Passerotti 2013; Rêgo et al. 2015), we expected that energetic state would vary across different reproductive stages. As such, we hypothesized that male sharks of both species

would exhibit higher energetic demand and mobilization during the mating period, given copulatory activities require high energetic investment (e.g. Manire et al. 2007; Valls et al. 2016). We also expected to measure higher nutritional quality during the early stages of the breeding season (i.e. pre-mating period), when male sharks are investing in sperm production.

To test this hypothesis, we used plasma fatty acid profile as short-term dietary biomarkers (McMeans et al. 2012; Beckmann et al. 2014) as a proxy for nutritional quality (Rangel et al. 2020, 2021a, b). Clasper measurements and testosterone concentrations (the main androgen in male sharks) were used to inform sexual maturity and reproductive stage (reviewed by Awruch 2013; Becerril-García et al. 2020). Relative corticosteroid concentrations were used as a proxy for energetic demand, as the role of corticosteroids in regulating the acquisition and mobilization of resources is highly conserved across vertebrates (Romero and Wingfield 2015; Crespi et al. 2013). Finally, we used the ketone body β -hydroxybutyrate as a proxy for mobilization of lipid reserves, as sharks routinely utilize this ketone body as an alternate aerobic fuel source (Speers-Roesch and Treberg 2010). β -hydroxybutyrate typically increases during fasting and starvation in sharks (Speers-Roesch and Treberg 2010; Wood et al. 2010) and has been related to intense physical activity and reproduction (Valls et al. 2016; Moorhead et al. 2020). We, therefore, expected to find increased circulating n3 and n6 PUFAs in adults during the spermatogenesis phase, coincident with increased testosterone levels (i.e. pre-mating) (Fig. 1). We also predicted increased saturated fatty acids (SFA), relative corticosteroid, and β -hydroxybutyrate during the mating period, when sharks are believed to reduce feeding activity (Pratt and Carrier 2001) (Fig. 1).

Materials and methods

Study species

The nurse shark is a large-bodied (maximum 305 cm total length, TL), relatively sedentary shark, exhibiting the lowest metabolic rate measured in any shark species to date (Whitney et al. 2016). Nurse sharks inhabit tropical and subtropical coastal and insular areas within parts of the Atlantic Ocean (Carlson et al. 2021). Female nurse sharks are a yolk-sac viviparous species, exhibiting a biennial cycle but with a shorter gestation period (5–6 months). Males exhibit an annual cycle and mate in June–July (Castro 2000; Pratt et al. 2018). The nurse shark is currently listed as “Vulnerable” by the IUCN (Carlson et al. 2021), mainly due to fishing, where the species is captured by both targeted and as bycatch in commercial, recreational, and artisanal coastal fisheries (Carlson et al. 2021).

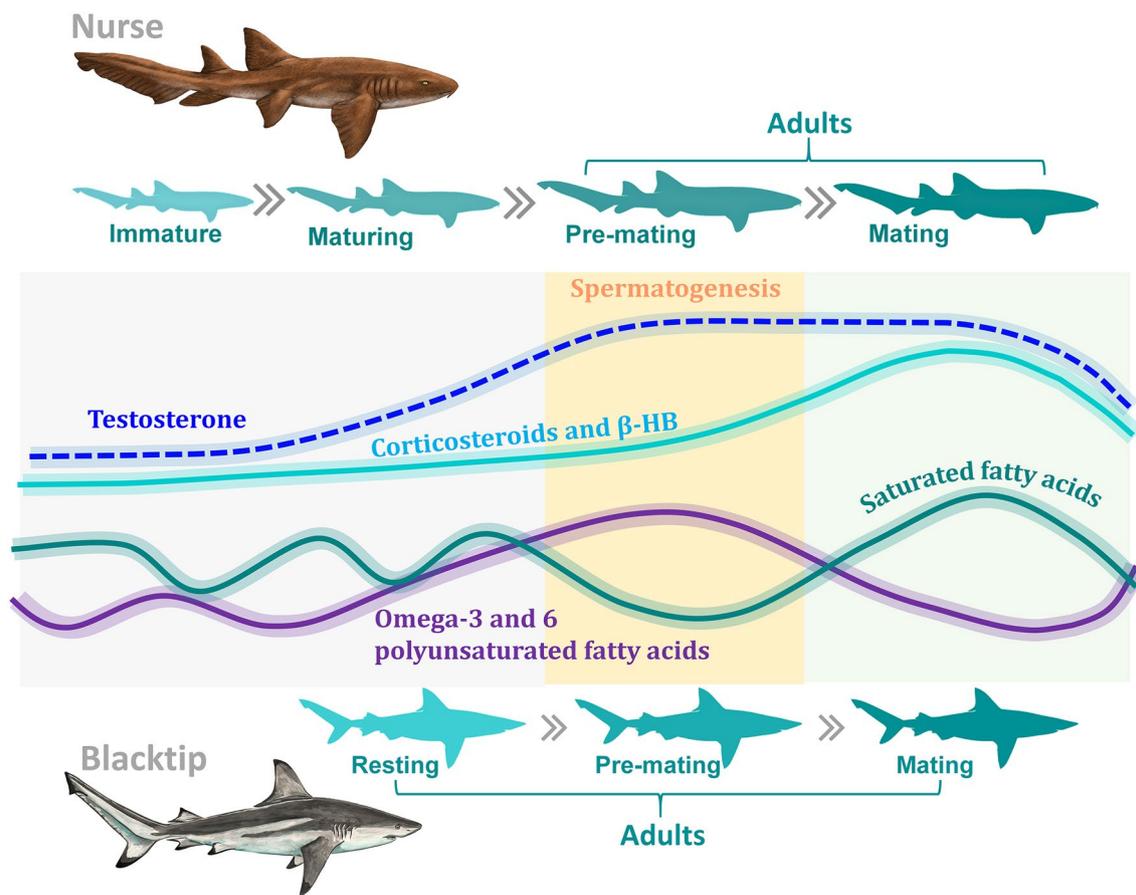


Fig. 1 Conceptual figure of predicted changes on profiles of plasma fatty acids, steroid hormones (testosterone and relative corticosteroid levels) and ketone body β-hydroxybutyrate (β-HB) across reproductive stages of male nurse sharks *Ginglymostoma cirratum* and blacktip sharks *Carcharhinus limbatus*. We predicted these results based on our hypothesis that male sharks of both species would have higher

nutritional quality during the early stages of the breeding season (i.e. pre-mating period) when they are investing in sperm production, and higher and energetic demand and energy mobilization during the mating period, since copulatory activities required high energetic investment. Illustration of nurse shark courtesy Kelly Quinn, and blacktip shark courtesy Alexandre Huber

The blacktip shark is a medium-sized species, with a maximum length of approximately 200 cm TL (Ebert et al. 2021). The blacktip shark is cosmopolitan in tropical and subtropical waters, found mainly on continental and insular shelves (Ebert et al. 2021). Female blacktips are placental viviparous, with a reported annual reproductive cycle in populations inhabiting southern Florida (Verkamp 2019). In males, the breeding season extends from February to June and mating period from April to August (Castro 1996; Baremore and Passerotti 2013). While blacktips tagged off northeast Florida display seasonal migrations (Kajiura and Tellman 2016), the population off southeast Florida may be year-round residents (Tinari and Hammerschlag 2021). The blacktip shark is harvested in both commercial and artisanal fisheries worldwide, and is currently listed as Near Threatened by the International Union for the Conservation of Nature Red List (IUCN, Burgess and Branstetter 2009).

Sampling sites and capture

Sampling occurred opportunistically between 2015 and 2019 within Biscayne Bay, Florida, USA (25.61°N, 80.17°W) as part of an ongoing standardized shark survey (Tinari and Hammerschlag 2021). Sharks were captured using circle-hook drumlines, a passive fishing technique that allows the captured sharks to swim (as described by Gallagher et al. 2014). Briefly, gear consisted of a submerged weight with two attachment points: (1) a line running to the surface with buoy floats and (2) a swivel connecting a 23-m monofilament ganglion line that terminated with a baited 16/05°-offset circle hook. To access the time each shark has been on the line, a hook timer (Lindgren Pitman HT600) was connected between the proximal end of the monofilament line and the weight. Hooking duration was not guaranteed for all sharks captured due to gear malfunction and damage of hook times. Drumlines were deployed (10–40 m deep, between

10:00 and 16:00 h) to soak for 1 h before being checked for shark presence. Following capture, sharks were secured by hand to a platform. Once landed, a water pump moving fresh seawater was inserted into the mouth of blacktip sharks to actively pump water over the shark's gills while temporarily immobilized. In the case of nurse sharks, this was not done given their low metabolic rates and propensity to clamp down on objects in their mouth. While sharks were secured, blood samples were obtained, sex was recorded and morphological measurements were taken (cm), including shark total length (TL) as well as inner clasper length, outer clasper length, and clasper width for a subset of males. Sharks were then tagged for identification (in the sulcus between the dorsal fin and the body) and released. The entire procedure from the moment of capture (removal of the drumline from the water) to release, varied from 5 to 10 min. No mortality was reported during the sampling period of the present study. Procedures and animal husbandry were approved by the University of Miami Institutional Animal Care and Use Committee (Protocol 15-238) and research permits from Florida Fish and Wildlife Conservation Commission, Biscayne National Park and National Marine Fisheries Service.

Blood collection and physiological analysis

Approximately 10 mL of blood was collected from the caudal vein of each shark and immediately centrifuged (3500 rpm, $410 \times g$) for 2 min. Plasma was then removed and stored frozen at -80°C for future hormonal analyses. Plasma levels of testosterone, corticosteroids and ketone body β -hydroxybutyrate were quantified in duplicate by hormone enzyme-linked immunoabsorbent assays (ELISA) using commercial kits (Cayman Chemical Company, MI, USA), with colorimetric enzymatic reaction using a spectrophotometer ELISA (SpectraMax 250, Molecular Devices). For corticosterone assay the plasma dilution selected was 1:5 for both species, and for testosterone was 1:100 for nurse sharks and 1:1000 for blacktip sharks (diluted with Cayman Assays buffer). All hormone assays were validated for nurse and blacktip sharks by conducting tests of parallelism. The mean intra-assay coefficients of variation were 8% (nurse) and 15.1% (blacktip) for testosterone, and 12% (nurse) and 13.1% (blacktip) for corticosterone.

An assay for 1α -hydroxycorticosterone (1α -OH-B, the primary corticosteroid in elasmobranchs) is not commercially available, thus we measuring relative corticosteroid concentrations using a corticosterone ELISA assay. The corticosterone kit assay has been previously validated to quantify relative 1α -OH-B, by exploiting the cross-reactivity of the corticosterone antibody with 1α -OH-B concentrations (Evans et al. 2010) and by excluding other corticosteroids by mass spectrometry (Lyons et al. 2019). However, as this approach will not deliver precision for concentrations

of 1α -OH-B, and we did not identify other corticosteroids (e.g. cortisol, cortisone, corticosterone, 11-deoxycortisol, 11-dehydrocorticosterone) as did by (Lyons et al. 2019), we assumed that the corticosterone ELISA would reflect corticosteroid concentrations. Therefore, the results are referred to as relative corticosteroid concentrations. Since corticosteroids are related to stress responses in sharks (e.g. Ruiz-Jarabo et al. 2019; Iki et al. 2020), a regression analysis was employed to the first test for possible effects of hooking duration (i.e. hook time) on relative corticosteroid concentrations. However, no significant correlation was found between relative corticosteroid concentrations and hook time in both nurse ($n=30$, $R=0.23$, $p=0.216$) and blacktip sharks ($n=20$, $R=0.13$, $p=0.587$) (Fig. S1). Therefore, we were able to confirm that relatively corticosteroid concentrations measured were not reflective of hooking duration stress.

Fatty acid profiles were analyzed in plasma (100 μL) by direct transmethylation, without lipid extraction, as described by Parrish et al. (2015). Briefly, the samples were homogenized and directly transmethylated in 3 mL of methanol: dichloromethane: concentrated hydrochloric acid (10:1:1 v/v) solution for 2 h at 80 – 85°C . After this process, was added 1.5 ml of Milli-Q® water and 1.8 ml of hexane and dichloromethane (4:1 v:v), and then the tubes were mixed and centrifuged at 2,000 rpm for 5 min. The upper layer was removed, transferred to 2 ml injection vials and the volume reduced under a nitrogen stream. Fatty acid analysis was carried out in a Varian gas chromatograph (Scienc 436) coupled with a flame ionisation detector and a CP 8410 auto-sampler. Hydrogen was used as a carrier gas at a linear velocity of 1.4 mL/min cm/s, and the capillary column used was CP Wax, 0.25 μm thickness, 0.25 mm inner diameter, and 30 m length. The column was programmed at 170°C for 1 min, followed by a $2.5^\circ\text{C}/\text{min}$ ramp to 240°C and a final hold time of 5 min. The injector and flame ionisation detector temperatures were 250 and 260°C , respectively. Fatty acid methyl esters were identified by comparing their retention times to those obtained from commercial standards (Supelco, 37 components; Sigma-Aldrich; Mixture, Me93, Larodan and Qualmix, PUFA fish M, Menhaden Oil, Larodan). The data are presented as % of total fatty acid methyl-esters based on peak area analyses.

Reproductive status

We used clasper measurements, testosterone plasma levels, and published length at maturity data to classify the reproductive stage of both nurse and blacktip sharks.

Based on published data for nurse sharks, 50% of males at 214 cm TL were sexually mature (Castro 2000), with the mating period occurring from June to July (Castro 2000; Pratt and Carrier 2001; Pratt et al. 2018). Given that the

lowest testosterone plasma concentration found in mature nurse sharks during the mating period was 2.5 ng mL^{-1} , individuals with $< 214 \text{ cm TL}$ with testosterone level higher than 1.0 ng mL^{-1} were considered as maturing males. Mature male nurse sharks were obtained from March to July, corresponding to the breeding season, i.e. pre-mating period (March—May) and mating period (June and July; Castro 2000; Pratt et al. 2018). Therefore, nurse sharks were classified as: (1) immature ($< 214 \text{ cm TL}$ with testosterone $< 1.0 \text{ ng mL}^{-1}$), (2) maturing ($< 214 \text{ cm TL}$ with testosterone $> 1.0 \text{ ng mL}^{-1}$), (3) mature ($> 214 \text{ cm TL}$) during the pre-mating period (March—May) and (4) during the mating period ($> 214 \text{ cm TL}$, between June—July).

For blacktip sharks, only mature males were analyzed. Based on published data, the breeding season extends from January to August, with the mating period occurring from May to August (Castro 1996; Baremore and Passerotti 2013). We classified mature male blacktip sharks as (1) non-breeders (i.e. resting period) those sampled from October to December, (2) breeders during the pre-mating period, sampled from January to April and (3) breeders during the mating period, sampled from May to August.

Fatty acid markers

The essential fatty acids, i.e. DHA, ARA and EPA, as well as the sum (PUFA, n3 and n6 PUFA), ARA/EPA, and n3/n6 ratios were used to compare the indices of shark nutritional quality (Tocher 2003; Arts & Kohler 2009) and to infer physiological responses of eicosanoids (Tocher 2003). Despite being subject to biosynthesis when transferred from prey to predator, fatty acids mostly remain relatively unchanged, permitting use as nutritional biomarkers (Dalsgaard et al. 2003; Iverson 2009; Budge et al. 2006). In terms of trophic markers, DHA was used as an indicator of dinoflagellates, while C16:1n7/C16:0 as an indicator of diatoms (Parrish et al. 2000, Budge et al. 2006). Additionally, ARA and C18:2n6 values have been found to be a useful indicator of whether a species inhabits coastal/benthic environments (Sardenne et al. 2017), and the odd chain fatty acids (OFA) and branched-chain fatty acids (BFA) as biomarkers of heterotrophic bacteria (Dalsgaard et al. 2003). Fatty acids that accounted for less than 0.5% were excluded from statistical analyses.

Data analysis

Linear regression was used to assess for relationships between testosterone plasma levels and clasper measurements across reproductive stages. Testosterone values were log-transformed before analyses to meet assumptions of normality. Potential differences among clasper measurements, hormones, fatty acid profiles, and the ketone body

β -hydroxybutyrate were statistically compared across reproductive stages of the nurse (i.e. immature, maturing, and mature during the pre-mating period and mating period) and blacktip sharks (i.e. resting, pre-mating, and mating periods) using one-way Analysis of Variance (ANOVA) with Tukey post-hoc test for parametric data or Kruskal–Wallis H tests followed by Dunn post-hoc tests for non-parametric data. As nurse sharks have a well-defined mating period (June and July; Castro 2000), all physiological variables were compared among breeding months for adults (i.e. May, June, and July).

Discriminant analyses were performed to identify which combination of fatty acids better discriminated between male reproductive stages in both species. Fatty acids that accounted for less than 0.5% of total fatty acids were excluded from statistical analyses. Statistical significance was declared at $p < 0.05$, and all analyses were conducted in SigmaStat 3.10 (SystatSoftware, Inc.; www.systat.com), PAST 3.12 (EFB; www.essential-freebies.de; Hammer et al. 2001) and R software (version 4.0.2).

Results

Nurse shark

A total of 75 male nurse sharks were analyzed, comprising 21 immature (mean \pm S.D., $122.8 \pm 38.4 \text{ cm TL}$), 28 maturing ($175.7 \pm 30.7 \text{ cm TL}$), and 26 mature during the breeding season, including 14 during the pre-mating period ($237.9 \pm 9.6 \text{ cm TL}$) and 12 during the mating period ($242.8 \pm 8.8 \text{ cm TL}$). All clasper measurements were positively related to testosterone concentrations (Figs. 2a–c) and differed among reproductive stages, with higher values found in adult males during both the pre-mating and mating periods (Figs. 2d–f) (Supplemental Table S1). Testosterone concentrations also differed among all reproductive stages (Fig. 2g). Immature animals had the lowest testosterone concentrations followed by maturing males, then mature males during the mating season, followed by males during the pre-mating season (Fig. 2g). Testosterone concentrations also exhibited temporal shifts in adult males, decreasing from May to June to July (Figs. 3, 4a).

Relative corticosteroid levels were higher in mature males during the pre-mating period compared to immature and maturing males. However, relative corticosteroid levels did not differ between the pre-mating and mating period (Fig. 2h), nor among months (Fig. 4b; Supplemental Table S1). Concentrations of the Ketone body β -hydroxybutyrate were higher in males during the mating period compared to all other reproductive stages (Fig. 2i), and gradually increased from May to July in adult males (Fig. 4c; Supplemental Table S1).

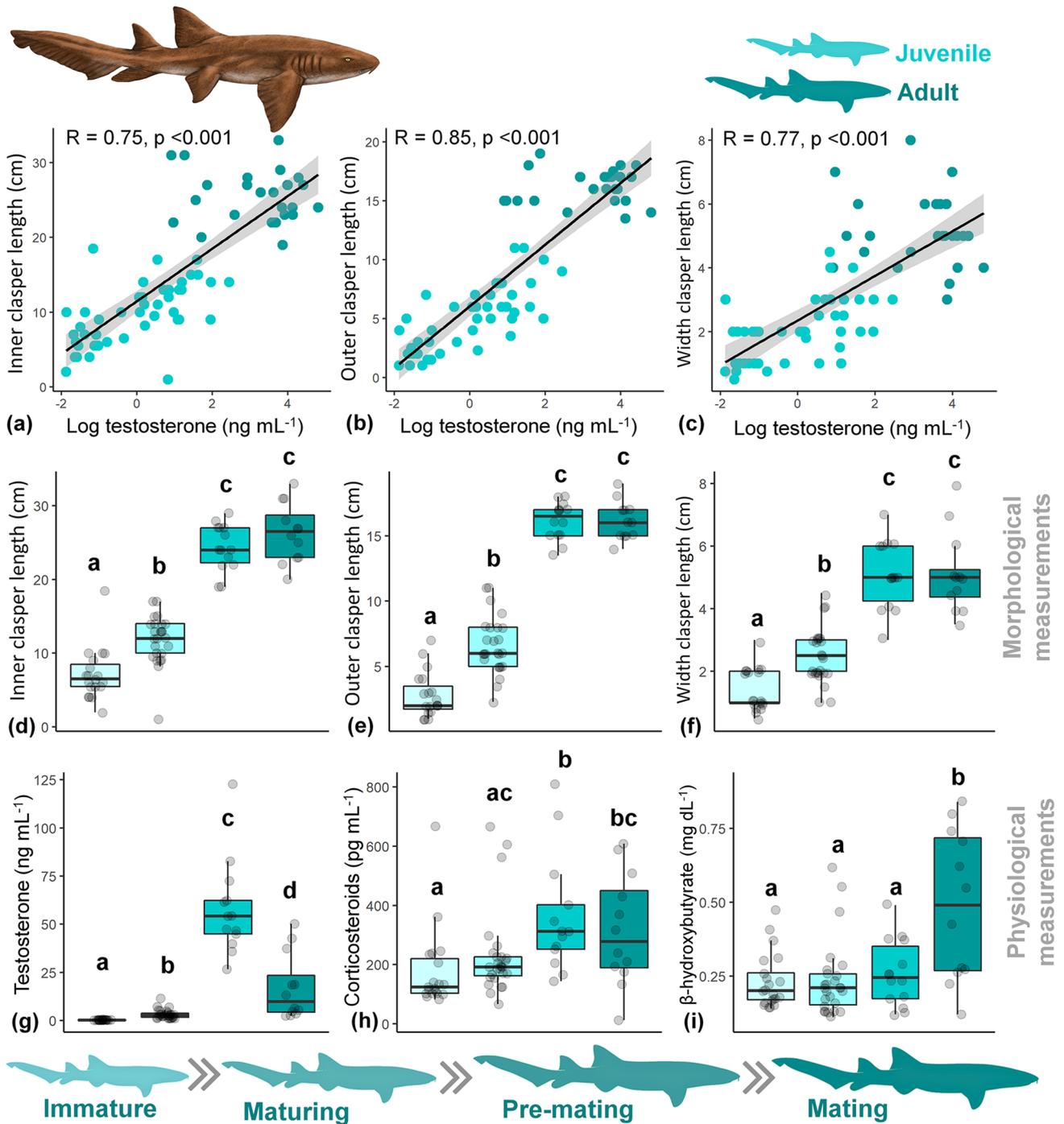


Fig. 2 Linear regression models and comparison across reproductive stages of nurse sharks (*Ginglymostoma cirratum*), including immature males ($N=21$), maturing males ($N=28$), mature males during the pre-mating period ($N=14$) and mating period ($N=12$). **(a-c)** relationship between inner, outer and width clasper length (cm) and testosterone (ng mL^{-1}). Comparison across reproductive stages of **(d)** inner

clasper length (cm), **(e)** outer clasper length (cm), **(f)** width clasper length (cm), **(g)** testosterone levels, **(h)** relative corticosteroid levels and **(i)** ketone body β -hydroxybutyrate levels. Significant difference among reproductive stages is denoted with different superscripts above bars (ANOVA followed by Tukey's post hoc or Kruskal-Wallis followed by Dunn post hoc)

Blood plasma comprised mainly SFAs (C16:0 and C18:0) for all life-stages, followed by PUFAs, mainly DHA and ARA, and monounsaturated fatty acids (MUFAs, mainly

C18:1n9) (Supplemental Table S2). Adults had higher proportions of C18:1n7 than immature males (Fig. 3b), while males during the pre-mating period had higher proportions

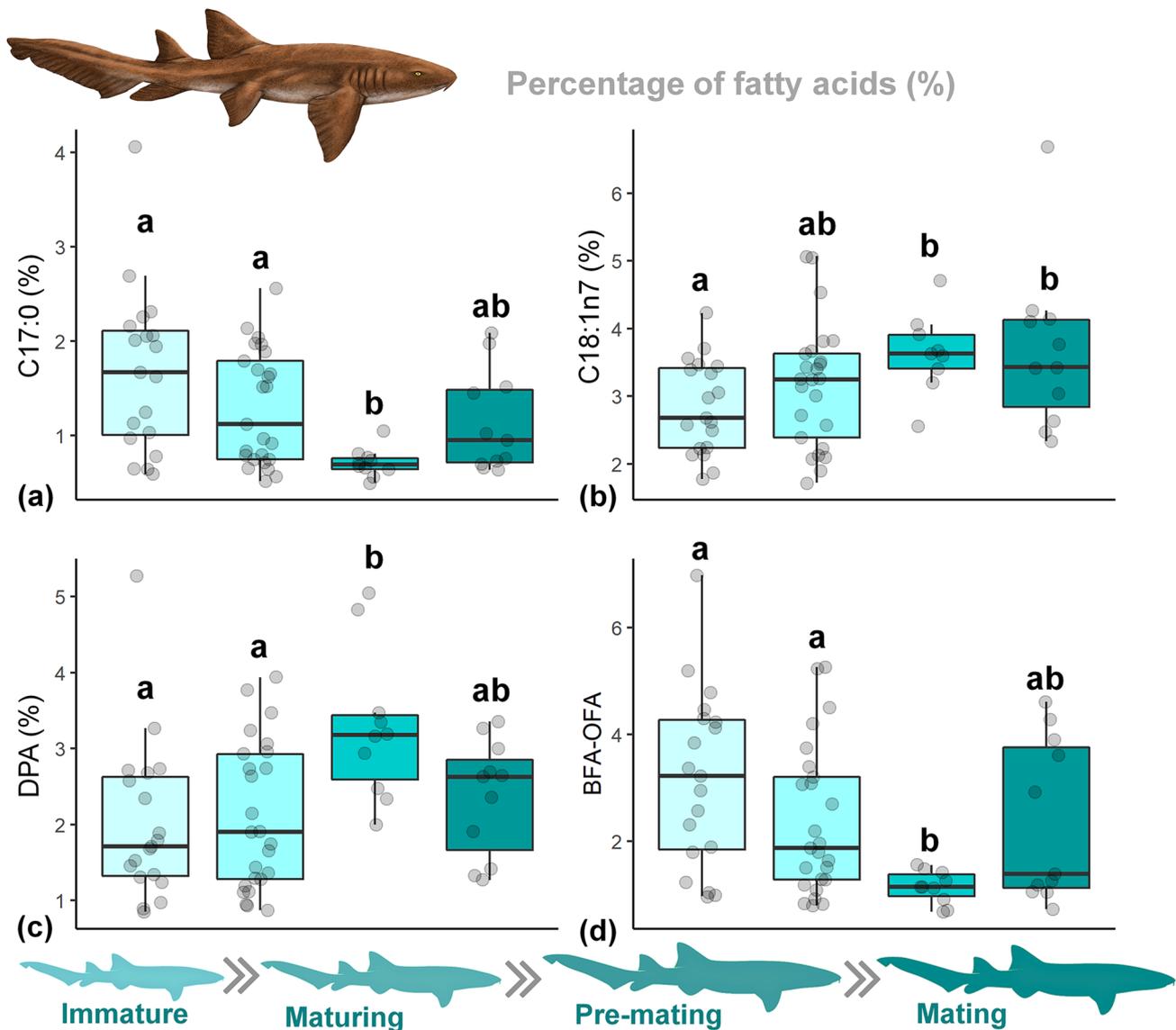


Fig. 3 Comparison of fatty acids throughout reproductive stages of nurse sharks (*Ginglymostoma cirratum*), including immature males ($N=21$), maturing males ($N=28$), mature males during the pre-mating period ($N=14$) and mating period ($N=12$). **(a)** C17:0, **(b)** C18:1n7, **(c)** DPA (C22:5n3, docosapentaenoic acid), **(d)** BFA:

branched-chain fatty acids; OFA: odd fatty acids. Significant difference among reproductive stages is denoted with different superscripts above bars (ANOVA followed by Tukey's post hoc or Kruskal-Wallis followed by Dunn post hoc)

of DPA compared to immature and maturing sharks (Fig. 3c). For fatty acids biomarkers, BFA-OFA, including C17:0, were lower in males during the pre-mating period compared to immature and maturing sharks (Figs. 3a and d) (Supplemental Table S2). A temporal shift in fatty acid profiles was detected in adult males (Fig. 5). Total PUFAs, total n3 PUFAs, EPA, DPA, DHA, and the n6 PUFA ARA decreased from May to July (Fig. 4) (Supplemental Table S2).

Regarding the discriminant analyses, the first three discriminant functions distinguished the life and reproductive

stages (Figs. 6, 7a and b, Supplemental Table S4), with Axis 1 accounting for 59.2%, Axis 2 for 24.6% and Axis 3 for 16.2% of the variation (eigenvalues: Axis 1 = 0.97; Axis 2 = 0.41; and Axis 3 = 0.27). The first function separated immature and maturing males from adults (pre-mating and mating periods), mainly due to differences in the proportions of DHA, ARA, DPA and C18:0. The second function, however, separated adults during the pre-mating period from those during the mating period, mainly due to differences in C18:1n9, C18:0, and DHA.

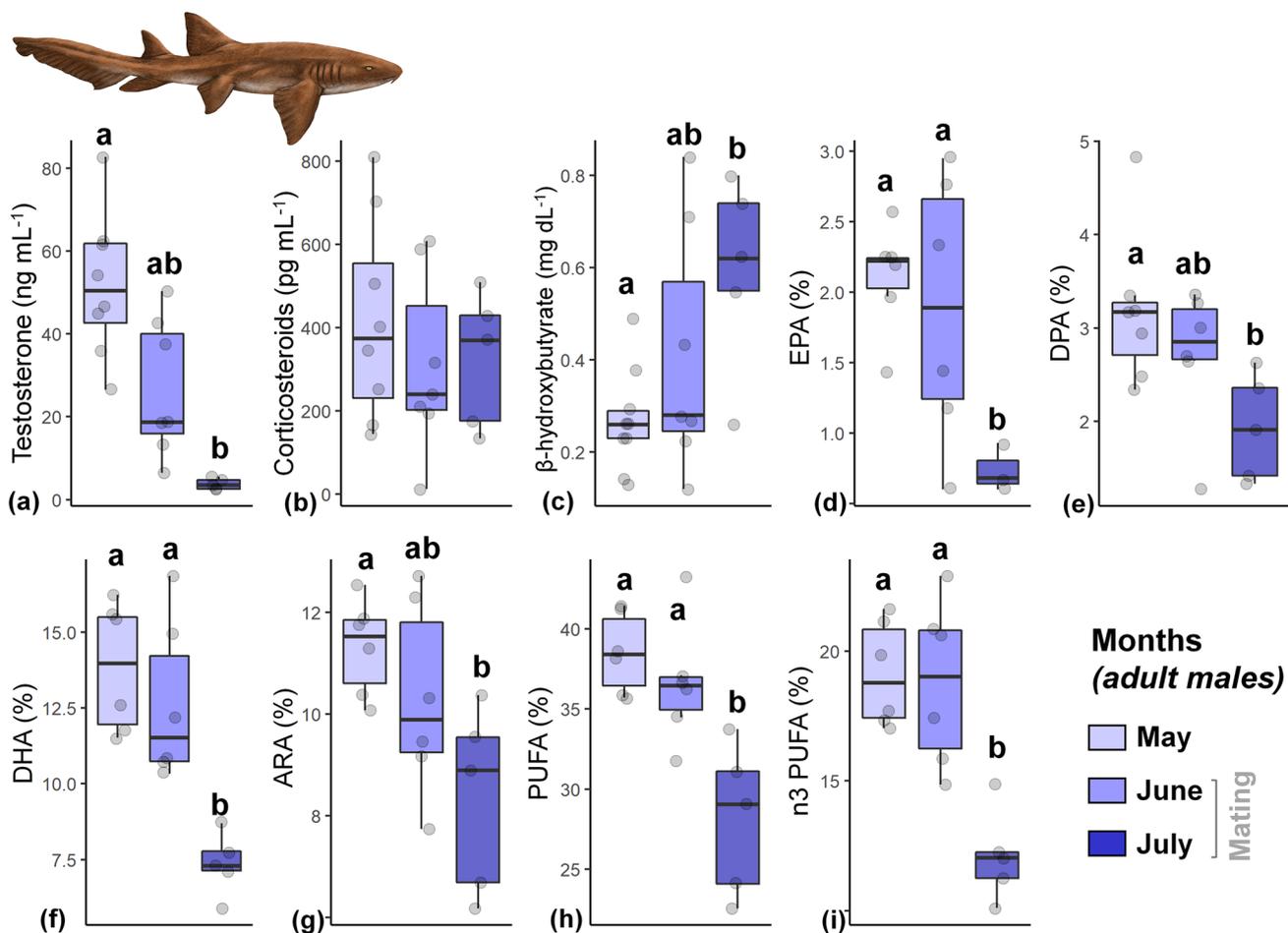


Fig. 4 Comparison of physiological variables throughout breeding months (May ($N=9$), June ($N=7$), and July ($N=5$)) of nurse sharks (*Ginglymostoma cirratum*), (a) testosterone, (b) relative corticosteroid levels, (c) ketone body β -hydroxybutyrate levels, fatty acids: (d) EPA (C20:5n3, eicosapentaenoic acid), (e) DPA (C22:5n3, docosapentaenoic acid), (f) DHA (C22:6n3, docosahexaenoic acid), (g)

ARA (C20:4n6, arachidonic acid), (h) Σ PUFA (polyunsaturated fatty acids), and (i) Σ n3 PUFA (omega-3 polyunsaturated fatty acids). Significant difference among reproductive stages is denoted with different superscripts above bars (ANOVA followed by Tukey's post hoc or Kruskal-Wallis followed by Dunn post hoc)

Blacktip shark

A total of 41 adult male blacktip sharks were analyzed, comprising 16 during the resting period, and 25 during the breeding season, including 11 during the pre-mating period and 14 during the mating period (Supplemental Table S1). While inner and outer clasper lengths did not differ among reproductive stages (Figs. 5a and b), clasper width values were higher in males during the pre-mating and mating period, compared with males during the resting period (Fig. 5c). None of the clasper measurements were significantly related to testosterone concentrations (Supplemental Table S1). Adult males had higher concentrations of testosterone during the breeding season (pre-mating and mating periods) compared to males during the resting period (Fig. 5d; Supplemental Table S1). Relative

corticosteroid levels were higher in males during the mating period compared to sharks during the resting and pre-mating periods (Fig. 5d; Supplemental Table S1), while concentrations of the Ketone body β -hydroxybutyrate did not differ among reproductive stages (Fig. 5e; Supplemental Table S1).

Plasma fatty acid profiles of blacktip sharks were comprised mainly of PUFAs during the resting period, while SFAs were the most abundant fatty acids measured during the mating period (Supplemental Table S3). Males during the mating period exhibited higher proportions of total SFA (including C16:0 and C18:0) and total MUFA (including C18:1n9) compared to males during the resting period (Fig. 6). In contrast, males during the resting and pre-mating periods exhibited higher proportions of n3 PUFA (including EPA, DHA, and the n3/n6 ratio) compared to those

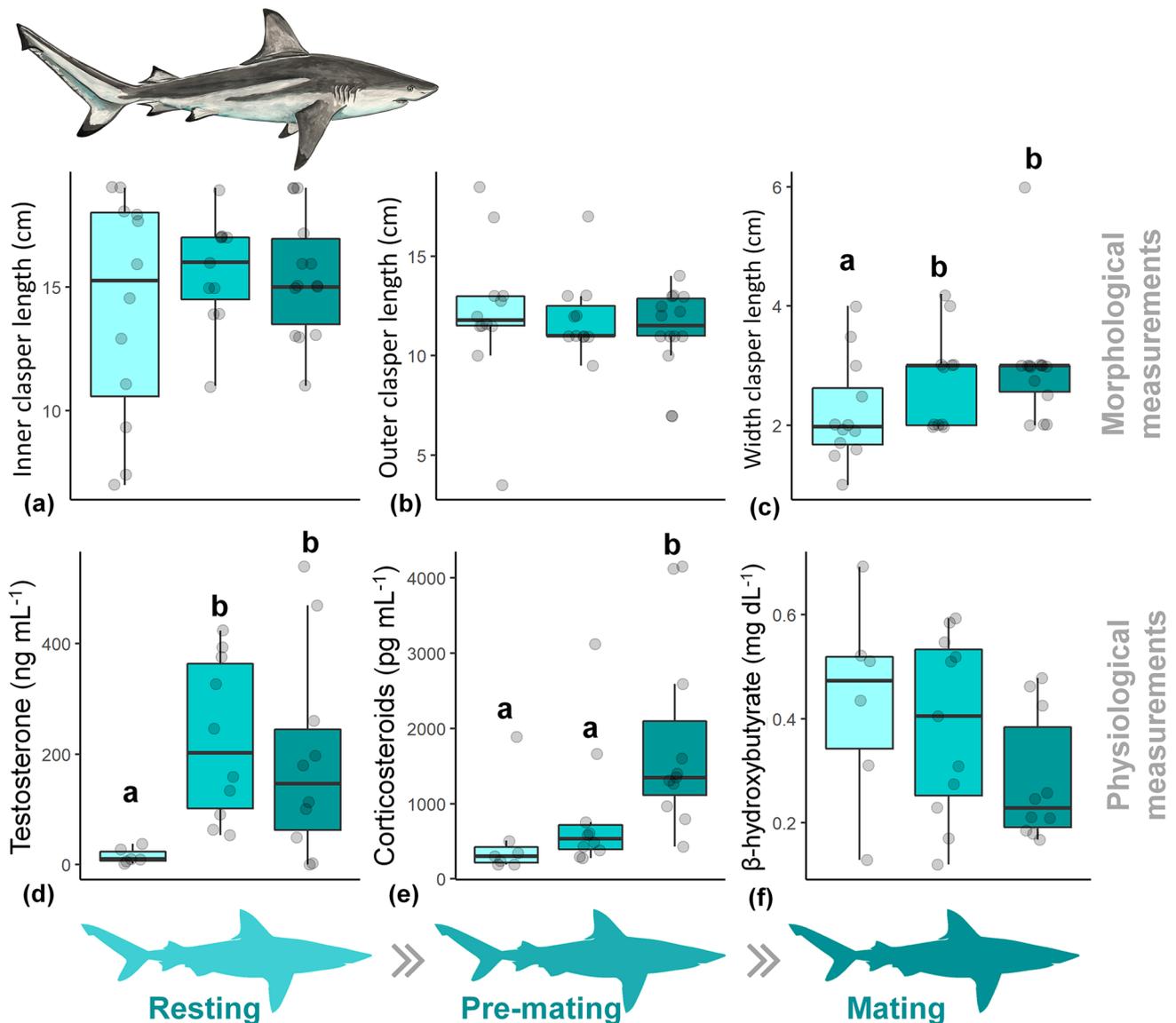


Fig. 5 Comparison throughout the reproductive stages of blacktip shark (*Carcharhinus limbatus*), including males during the resting period ($N=16$), pre-mating period ($N=11$), and mating period ($N=14$). **(a)** inner clasper length (cm), **(b)** outer clasper length (cm), **(c)** width clasper length (cm), **(d)** testosterone levels, **(e)** relative cor-

ticosteroid levels and **(f)** ketone body β -hydroxybutyrate levels. Significant difference among reproductive stages is denoted with different superscripts above bars (ANOVA followed by Tukey's post hoc or Kruskal–Wallis followed by Dunn post hoc)

during the mating period (Fig. 6). In terms of biomarkers, BFA-OFA (including C17:0) were higher in resting males, whereas breeders during the mating period had the highest ARA/EPA ratio (Fig. 6).

Discriminant analyses revealed that the first two discriminant functions distinguished the reproductive stages (Figs. 7c and d; Supplemental Table S4), with Axis 1 accounting for 67.9% and Axis 2 for 32.0% of the variation (eigenvalues: Axis 1 = 1.5 and Axis 2 = 0.7). The first function separated males during the resting and pre-mating periods from those during the mating period, mainly due to differences in the

proportions of C16:0, DHA, and C18:1n9. The second function separated males during the resting period from breeders during the pre-mating period, mainly due to C17:0, C16:0, DHA, and C18:1n9 (Figs. 7c and d, Supplemental Table S4).

Discussion

The integration of multiple physiological markers in this study shed new insights into how the energetic state varies across reproductive stages in male sharks. Consistent with

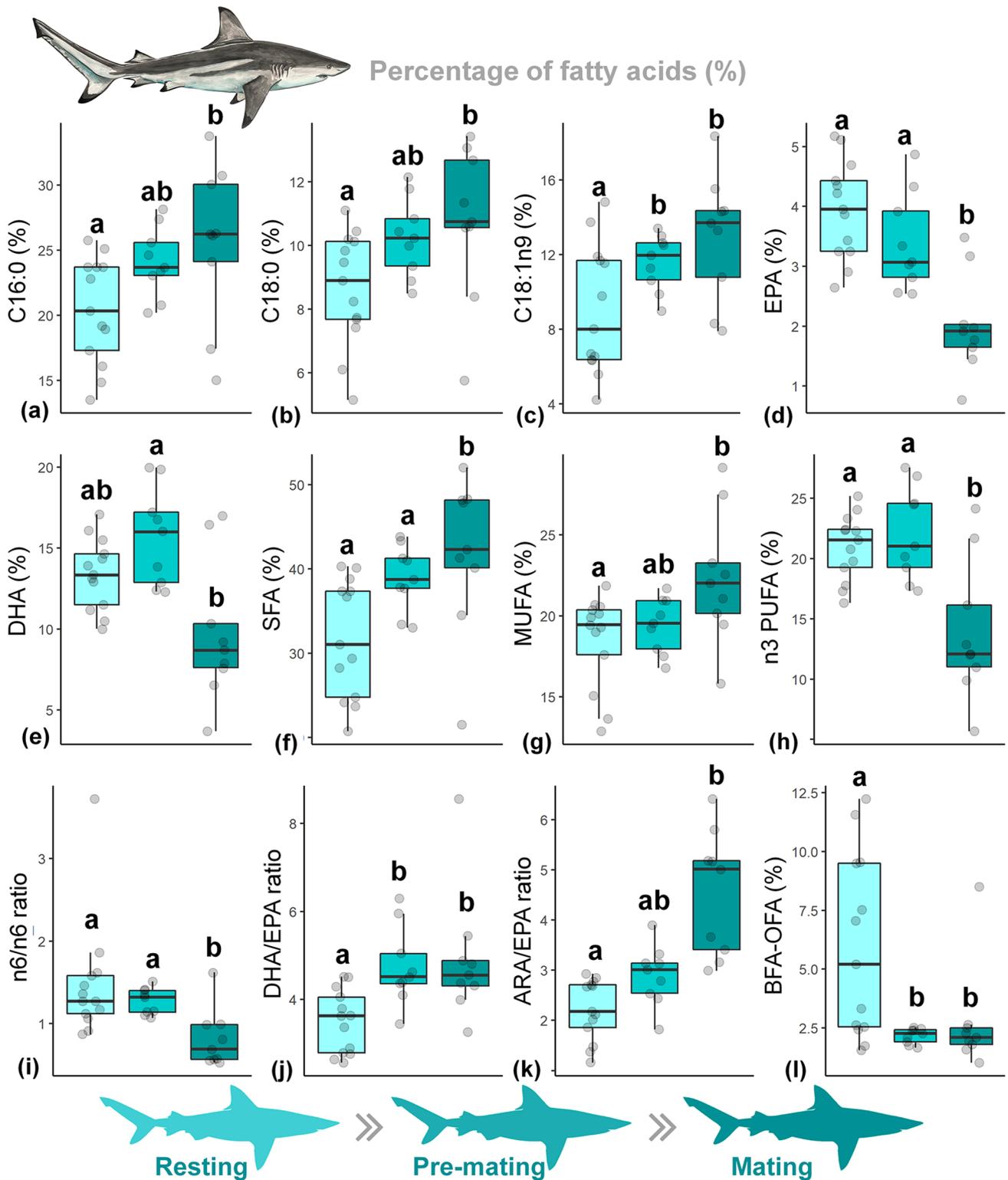


Fig. 6 Comparison of fatty acids throughout reproductive stages of blacktip shark (*Carcharhinus limbatus*), including males during the resting period ($N=16$), pre-mating period ($N=11$), and mating period ($N=14$). **(a)** C16:0, **(b)** C17:0, **(c)** C18:0, **(d)** C18:1n9, **(e)** EPA, **(f)** DHA (C22:6n3, docosahexaenoic acid), **(g)** Σ SFA (saturated fatty acids), **(h)** Σ MUFA (monounsaturated fatty acids), **(i)** Σ n3

PUFA (omega-3 polyunsaturated fatty acids), **(j)** n3/n6 PUFA ratio, **(k)** ARA/EPA ratio, ARA (C20:4n6, arachidonic acid) and **(l)** BFA: branched-chain fatty acids; OFA: odd fatty acids. Significant difference among reproductive stages is denoted with different superscripts above bars (ANOVA followed by Tukey's post hoc or Kruskal–Wallis followed by Dunn post hoc)

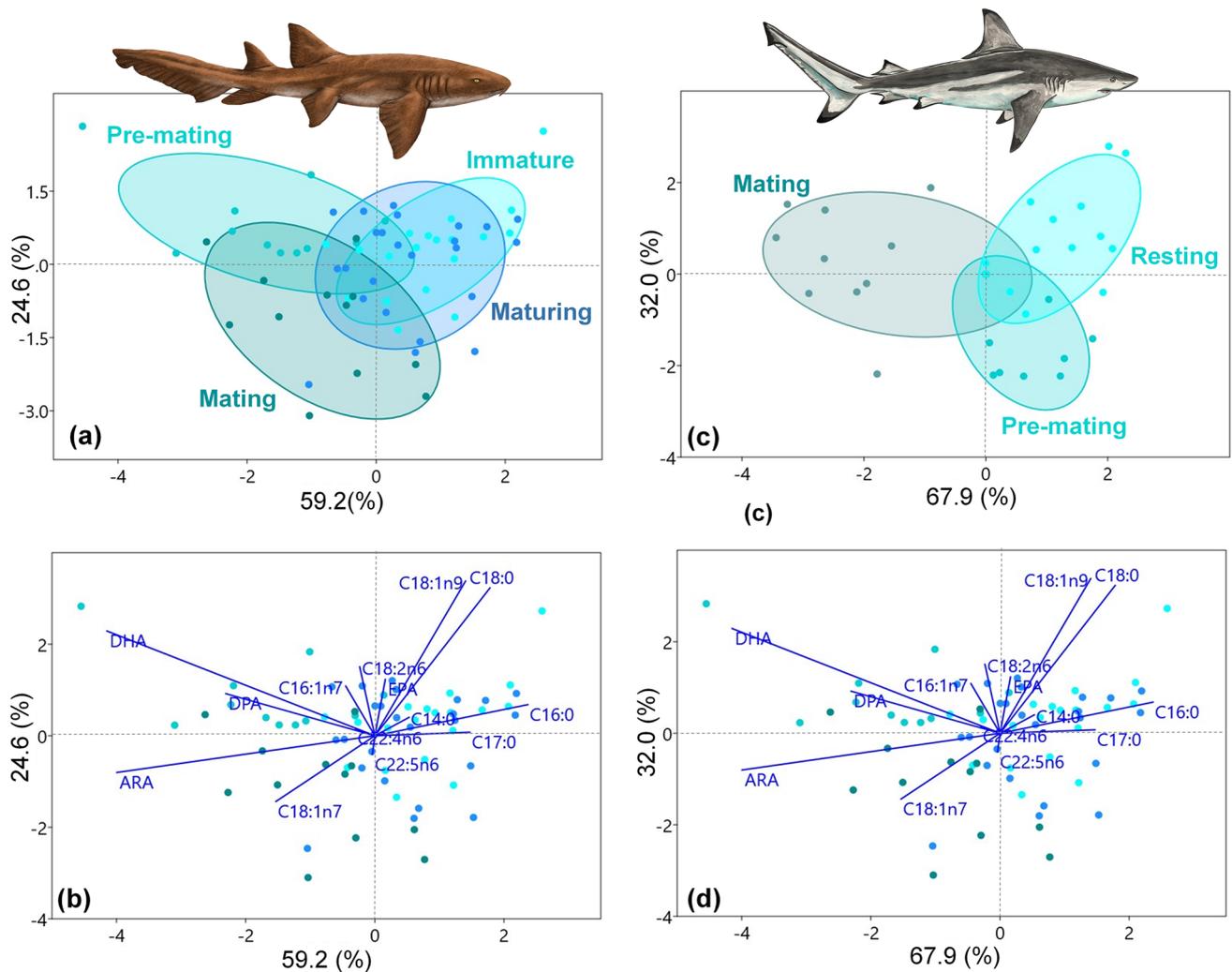


Fig. 7 Linear discriminant function analyses of selected fatty acids (based on their abundance in all samples) of **(a–b)** nurse sharks (*Ginglymostoma cirratum*), including immature males ($N=21$), maturing males ($N=28$), mature males during the pre-mating period ($N=14$) and mating period ($N=12$); and **(c–d)** blacktip shark (*Car-*

charhinus limbatus), including males during the resting period ($N=16$), pre-mating period ($N=11$), and mating period ($N=14$). EPA: eicosapentaenoic acid, DHA docosahexaenoic acid, ARA arachidonic acid. The 70% ellipses similarly of seasons in each site is provided

our predictions, nutritional state (in terms of fatty acids) decreased in both nurse and blacktip sharks during the mating period. Increased relative corticosteroid levels were found during the mating period in male blacktip sharks and during the pre-mating and mating periods in nurse sharks. While β -hydroxybutyrate levels did not differ across reproductive stages in blacktip sharks, higher levels of this energetic substrate were found in adult nurse sharks during the mating period as predicted. Understanding how energetic condition changes across reproductive stages in wild animals may provide an improved capacity to detect breeding strategies (Soulsbury 2019) and monitor impacts from environmental disturbance (e.g. trophic mismatch vulnerability; Williams et al. 2017).

Patterns of testosterone concentration measured in both nurse and blacktip sharks were consistent with previous studies describing a pronounced seasonal change in their testicular development and sperm production (Castro 1996; 2000; Baremore and Passerotti 2013; Verkamp 2019). While the highest values of testosterone were found in mature nurse sharks (> 214 cm TL), intermediate values in maturing males (and associated smaller claspers) may correspond with puberty, when testosterone acts promoting testicular development and secondary sex characteristics (Gelsleichter et al. 2002). Mature individuals during the pre-mating period exhibited higher testosterone levels that declined during the mating period. Similar patterns have been observed in other shark species with temporal decoupling between sperm production and mating behavior, such

as bonnethead shark *Sphyrna tiburo* (Manire and Rasmussen 1997) and Atlantic sharpnose shark *Rhizoprionodon terraenovae* (Hoffmayer et al. 2010). In blacktip sharks, clasper width and testosterone levels increased during the breeding season (i.e. pre-mating and mating periods) and decreased during the resting period, a time of testicular regression, also consistent with previous studies (Castro 1996; Baremore and Passerotti 2013; Verkamp 2019). However, despite morphological evidences for temporal decoupling in sperm production and mating for this species (Dudley and Cliff 1993; Castro 1996; Baremore and Passerotti 2013), we did not find differences between pre-mating and mating periods. This suggests the possible role of testosterone in stimulating copulatory behaviors in blacktips as found in other shark species (e.g. Awruch 2013).

A temporal decoupling in sperm production and mating may allow males to direct energy investment to one process at a time, i.e. spermatogenesis and mating activity (male–male competition and courtship) (Hoffmayer et al. 2010; Awruch 2016). Although both nurse and blacktip sharks seemed to exhibit temporal decoupling in sperm production between the pre-mating and mating periods, the relative corticosteroid and β -hydroxybutyrate data suggest that they may have differed in their energetic strategy. Specifically, while nurse sharks exhibited elevated β -hydroxybutyrate levels during the pre-mating period, blacktip sharks exhibited elevated relative corticosteroid levels during the mating period. One possible explanation is that nurse sharks may utilize β -hydroxybutyrate as an energetic substrate to fuel copulatory activities, though mobilization of lipid storage is recently suggested (Moorhead et al. 2020). This corroborates previous observations in the field, where no evidence of feeding was observed during nurse shark mating (Pratt and Carrier 2001), suggesting males may rely on stored endogenous resources to fuel mating behaviors. In contrast, blacktip sharks may utilize more carbohydrates during mating, as corticosteroids are associated with enhanced glycolysis and gluconeogenesis (e.g. Ruiz-Jarabo et al. 2019). Although not significant, possibly due to relatively low sample size, a decreasing trend in plasma β -hydroxybutyrate levels during the mating period of blacktips provides preliminary support for this hypothesis.

Based on our results, it is plausible that nurse sharks are capital breeders, i.e. using stored endogenous resources (typically lipids) to finance reproduction, whereas blacktip sharks are income breeders, utilizing exogenous resources (typically carbohydrates) to finance reproduction (Soulsbury 2019). Corroborating this hypothesis for blacktip sharks, increased plasma SFAs (including C16:0 and C18:0) and MUFAs (including C18:1n7 and C18:1n9) were found during the mating period, suggesting an increase of de novo biosynthesis of fatty acids in the liver (i.e. from carbohydrate or protein precursors; Budge et al. 2006). This is because de

novo biosynthesis is inhibited during fasting (Budge et al. 2006). Also, increased biosynthesis of fatty acids may fuel increased activity levels associated with courtship and mating behaviors, since SFAs are the main fatty acids catabolized for energy (Tocher 2003).

Another possible explanation for the observed pattern of β -hydroxybutyrate and corticosteroid levels may be related to the intensity and overall duration of physical activity during the mating behavior (Clark 2012; Soulsbury 2019). For example, in other vertebrates, males performing sustained mating behavior typically use lipids, whereas those performing short bouts of intense activity more often use carbohydrates (Soulsbury 2019). Although mating behavior in blacktips has not been observed, it is well described for the nurse shark (Castro 2000; Pratt and Carrier 2001; Colbachini et al. 2020). During the mating period, male nurse sharks actively “patrol” for potential mates, frequently mating multiple times daily, while competing with other males for access to females (Pratt and Carrier 2001). Further research involving additional physiological markers coupled with behavior observations would be valuable for testing these hypotheses.

Male nurse sharks had relatively low testosterone and relative corticosteroid concentrations, at levels comparable to those found in benthic species, such as the demersal stingray *Hypanus sabinus*, in both androgens (0.0–36.0 pg ml⁻¹; Snelson et al. 1997) and corticosteroids (median maximum of 550.0 pg ml⁻¹; Manire et al. 2007). On the other hand, the concentrations of both testosterone and relative corticosteroids in male blacktip sharks were relatively high, comparable to levels found in other active and epipelagic shark species. For instance, a mean of 182.0 ng ml⁻¹ of circulating testosterone (Manire and Rasmussen 1997) and a median of 3000.0 pg ml⁻¹ of circulating corticosteroid has been previously reported in the bonnethead shark (Manire et al. 2007). Accordingly, a relationship may exist between levels of these two hormones and a species ecology, however, comparative studies are needed to test this phenomenon.

Consistent with findings in other vertebrates (e.g. teleosts, Izquierdo et al. 2001; mammals, Wathes et al. 2007), males of both nurse and blacktip sharks exhibited a decrease of physiologically important omega-3 fatty acids during the mating period, suggesting the use of these physiologically important fatty acids during mating, such to support spermatogenesis. Moreover, DHA was the main fatty acid responsible for separating breeders during the pre-mating period from other stages, when presumably males are investing in sperm production. This n3 PUFA has an important structural function, as it is found in high proportions in sperm of several teleost species (e.g. Pérez et al. 2000; Baeza et al. 2014, 2015).

Other important n3 PUFA, EPA, decreased during the mating period in both shark species, which resulted in an increased ARA/EPA ratio in blacktip sharks. EPA has a

physiological role in modulating the synthesis of androgens and is related to the volume of sperm produced in teleosts (Baeza et al. 2015). Additionally, in some species, e.g. in European eels (*Anguilla anguilla*), the proportions of n3 and n6 PUFAs remain constant in the testes during spermiation, suggesting some mobilization of these fatty acids from the liver for testicular maintenance (Baeza et al. 2015). If a similar phenomenon occurs in male sharks, it would indicate that species may allocate physiologically important fatty acids to support reproduction, whether through dietary and/or non-dietary origin (e.g. mobilized from storage tissues). Moreover, as both nurse and blacktip sharks store sperm prior to copulation (e.g. Dudley and Cliff 1993; Baremore and Passerotti 2013; Rêgo et al. 2015), these fatty acids may have some role in semen production or sperm maintenance and activation (e.g. Baeza et al. 2015).

Shifts found in the proportions of ARA in nurse sharks imply that this fatty acid is also critical to male reproduction. ARA was an important fatty acid in discriminating maturity in nurse sharks, exhibiting a gradual increase from immature to mature sharks during the pre-mating period. ARA has been demonstrated to stimulate testicular steroidogenesis in teleost fishes by regulating the cholesterol transfer within mitochondrial membrane (e.g. Hu et al. 2010). ARA is also directly involved in testosterone production through elevating cyclic adenosine monophosphate (cAMP) levels (Mercuré and Van der Kraak 1995). Experimentally, increased dietary ARA has also been found to significantly increase androgen production in male teleosts (e.g. *Solea senegalensis*, Norambuena et al. 2013), and therefore, it has several important implications for reproduction. One plausible reason for the lack of significance of ARA among reproductive stages in blacktip sharks might be related to high levels of testosterone maintained during the mating period. Consistent with these results, decreases in n3/n6 ratios were found in blacktip sharks during the mating period, while no differences were observed in n3/n6 ratio in nurse sharks across reproductive stages and breeding months.

Regarding trophic markers, the bacterial markers, including C17:0 and C18:1n7, were found in higher proportions in immature nurse sharks and resting blacktips. Increased bacterial marker contribution found during these stages is likely to be a result of greater intake of benthic/demersal prey since these fatty acids are markers for heterotrophic bacteria associated with sediments and suspended organic material (Kelly and Scheibling 2012). Moreover, given that some of the immature nurse sharks were captured close to urbanized areas, higher proportions of bacterial markers maybe related to anthropogenic influence, such as the eutrophication process and increased production of organic materials (e.g. Le Moal et al. 2019; Rangel et al., 2021b). Other biomarkers, such as the DHA, were the most abundant PUFA in both nurse and blacktip sharks across all reproductive stages,

suggesting a greater dependency on marine food webs based on dinoflagellates (Dalsgaard et al. 2003). Despite this, high proportions of ARA and relatively low ratio of n3/n6 found in both species imply they are utilizing food webs influenced by freshwater, commonly found in coastal shark species (e.g. Every et al. 2016). Future studies comparing these two sympatric shark species in relation to their diet and habitat use patterns are needed to characterize these possible trophic dynamics.

Our multiple physiological markers approach allowed us to better understand the relationship between nutritional and reproductive stages in male sharks. However, due to opportunistic sampling and because we used a non-lethal approach and correlative analyses, our study has several limitations. This includes the inability to identify dietary and/or non-dietary origin of fatty acids, seasonal patterns, and specific mechanisms through the breeding period. Since we used the corticosterone ELISA assay as a proxy for the potential effects of corticosteroids, further comparative studies are needed to identify which specific corticosteroids are present in nurse and blacktip sharks at physiologically relevant concentrations, including the adrenocorticotrophic hormone levels. Another study limitation is that we did not consider other factors that may be influencing the physiological variables measured here, such as location, season, temperature, time of day, capture stress, and urbanization. More extensive sampling integrated with other nutritional markers will help to clarify the changes observed in nutritional status across male reproduction.

Conclusion

Our study presents evidence of energetic and nutritional adjustments in male sharks linked to reproduction. Though more research is needed to identify specific mechanisms, decreased nutritional quality (in terms of essential fatty acids) found during the mating period of both blacktip and nurse sharks, indicating the possible use of physiologically important fatty acids to support reproduction, such as spermatogenesis and/or higher activity behaviors related to mating. In general, while both omega-3 and -6 seemed to be important for nurse shark reproduction, only omega-3 differed across reproductive stages of blacktip sharks. Despite similar trends and shifts in fatty acid profile for both blacktip and nurse sharks, these species appeared to differ in their energetic strategy to finance reproduction, maybe a result of their divergent ecologies (sedentary and benthic = nurse shark versus active and epipelagic = blacktip) and energy resource-use strategies for reproduction. This knowledge is particularly important for predicting how these on other species may respond physiologically to environmental disturbance, such as trophic mismatch vulnerability.

Author contribution statement

BSR envisioned the study. NH and JAS conducted fieldwork and collected blood samples. RGM acquired resources for laboratory analyses. BSR performed laboratory and statistical analyses and wrote the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable institutional and/or national guidelines for the care and use of animals were followed.

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