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Neonatal nutritional strategy of a viviparous elasmobranch with extremely low reproductive output

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ABSTRACT: Throughout evolutionary history, elasmobranchs have developed diverse reproductive strategies. Little focused work, however, has addressed how neonatal nutritional state is affected by differing degrees of maternal investment associated with these markedly different reproductive strategies. To investigate the effect of maternal investment on the nutritional quality of pups during the early life history of an extremely viviparous elasmobranch, quantitative biomarker analysis including lipids, fatty acids and stable isotopes was conducted. Using the cownose ray *Rhinoptera bonasus* (histotrophic viviparous) as a model, we found that pups were initially born in a positive nutritional state, enriched in physiologically important essential fatty acids and nitrogen and carbon stable isotope values ($\delta^{15}N$ and $\delta^{13}C$), a result of maternal intrauterine transfer. A systematic decrease in some fatty acids and $\delta^{15}N$ values, as well as a decrease in cholesterol with growth, confirmed that these substrates were derived from maternal resources and used in initial metabolic processes following birth. An observed increase in condition factor, plasma essential fatty acids and triglyceride:cholesterol ratio with increasing body size identified a progression towards successful independent foraging with pups not displaying marked nutritional deficiency or fasting phases. Our multi-tracer approach allowed the identification of 2 size classes of young rays (<50 and <70 cm disc width) that displayed distinct physiological states. Since prenatal maternal investment is critical for offspring condition and to promote successful foraging post birth, understanding the trophic ecology and physiological state of pups during their first year is critical to guide management and conservation within nursery grounds.

KEY WORDS: Maternal investment · Nutritional quality · Early life history · Cownose ray · Fatty acid profile · Stable isotope · Energy metabolism · Non-lethal methods

1. INTRODUCTION

The energetic costs of reproduction can be highly variable depending on the reproductive mode an organism adopts (Clutton-Brock 1991). Physiological processes such as egg production and direct provisioning of nutrients to embryos via the placenta typically require high-energy expenditure through dedicated resource allocation (Stearns 1992, Royle et al. 2012). As a consequence, trade-offs between the number and size of offspring and length of gestation are well documented (e.g. Stearns 1992, Zera & Harshman 2001, Roff 2002, Speakman 2008). In terms of allocation of reproductive resources, elasmobranchs (sharks, skates and rays) generally have a long gestation period and small litter size relative to

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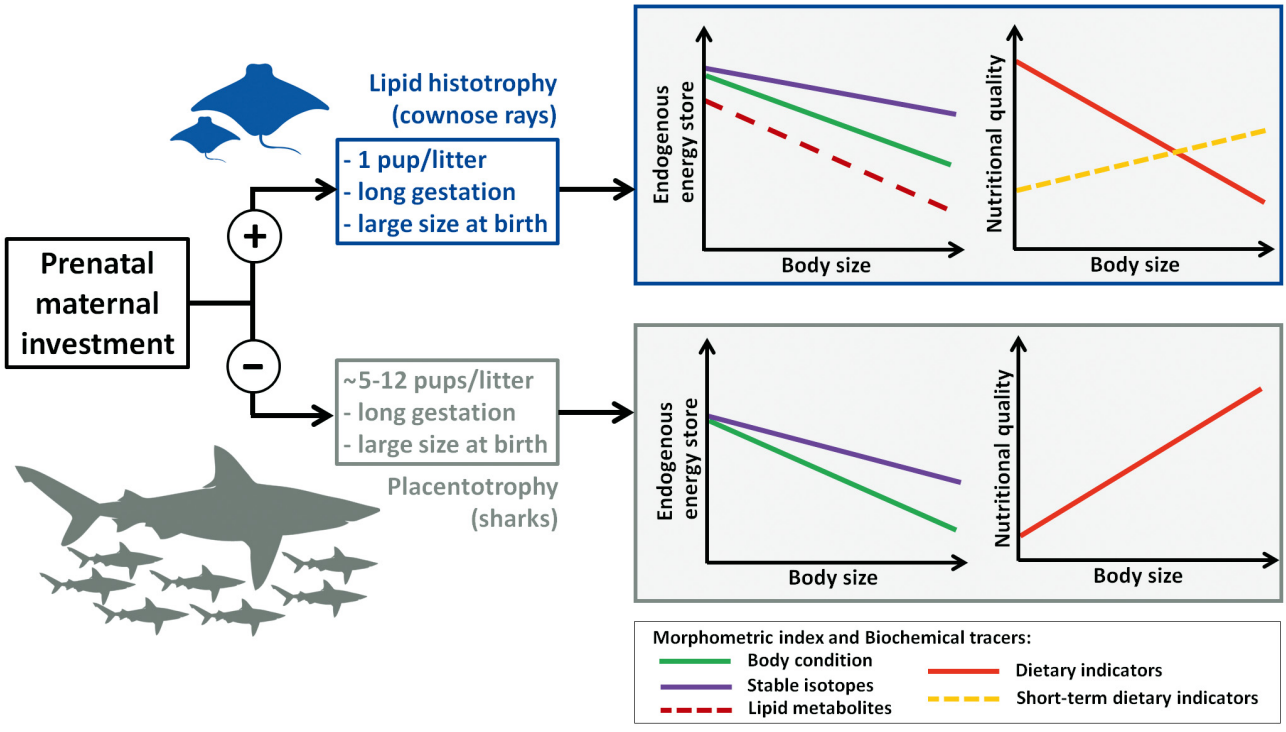

Fig. 1. Conceptual illustration of prenatal maternal investment in terms of endogenous energy stores (body condition, stable isotope values and lipid metabolites) and nutritional quality (fatty acid signatures) for a lipid histotrophic reproductive mode (young-of-the-year cownose rays *Rhinoptera bonasus*) versus placentotrophic sharks (*Carcharhinus obscurus* and *C. leucas*). Information for placentotrophic shark was taken from Hussey et al. (2010) and Belicka et al. (2012)

other aquatic species (e.g. teleosts, Conrath & Musick 2012). Elasmobranchs exhibit diverse reproductive modes, including nutrient provisioning through yolk (lecithotrophy), placenta (placentotrophy), ova (oophagy), embryonic ingestion of siblings (adelphophagy), embryotrophe (embryotrophy) and secretion of lipid-rich uterine fluid (histotrophy), or a combination of the above strategies (Wourms 1981, Hamlett et al. 2005). While numerous studies have anatomically described these reproductive strategies through morphological examination (references in Conrath & Musick 2012, Buddle et al. 2018), several important questions remain unanswered over how these reproductive strategies and the degree of maternal investment associated with them affect offspring fitness.

Unlike some vertebrates, in which food is provided directly to newborns by their parents (e.g. many birds, Royle et al. 2012) or through allocation of postnatal maternal resources (e.g. milk secretion in mammals, Clutton-Brock 1991), elasmobranchs provide no direct postnatal care in the form of exogenous energy sources. Instead, prenatal maternal endogenous sources are provided through yolk, uterine fluid or placental connection (Lechenault et al. 1993, Hussey et al. 2010). In some placental sharks, for example, there is evidence of provisioning neonates with additional maternal resources in the form of an enlarged liver for postnatal use (e.g. Hussey et al. 2010); however, newborns have been shown to be born in a poor nutritional state (Belicka et al. 2012, Wai et al. 2012; our Fig. 1). Although the provisioning of maternal resources is a critical component of life history variation and has direct consequences for both neonatal nutritional condition and consequently offspring survival (i.e. development of foraging skills), neonatal nutritional strategies remain unknown for most elasmobranch species (Conrath & Musick 2012).

An interdisciplinary approach combining both physiological measures (i.e. lipids, fatty acids and stable isotopes) and morphometric indices (i.e. size and body condition) can provide important indicators of individual nutritional quality and resource use as well as a non-lethal method to assess the coupling between reproductive mode and maternal provisioning (e.g. Gallagher et al. 2017, Hussey et al. 2017, Meyer et al. 2019). Lipids function as structural components of cell membranes, providing the most concentrated energy resources to organisms; thus, lipid type and quantity directly reflect health-related responses to resource type and availability (Norton et

al. 2001, Tocher 2003). Physiologically important fatty acids (i.e. docosahexaenoic acid [DHA], arachidonic acid [ARA] and eicosapentaenoic acid [EPA]) provide markers to assess nutritional quality, due to their effects on developmental performance and their involvement in key processes from immune function to neural development (Sargent et al. 1995, Arts & Kohler 2009, Tocher 2010). Fatty acids also indicate trophic status, based on the principle that they remain relatively unchanged from prey to predator, and consequently reflect trophic interactions and the base of the food chain where an individual forages (Dalsgaard et al. 2003, Budge et al. 2006). Complementary to fatty acids, the stable isotopes of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) also provide information on resource use and foraging habitat of organisms (e.g. Hobson 1999, Hussey et al. 2012). Because these biomarkers (lipids, fatty acids and stable isotopes) vary in each tissue or body fluid due to tissue-specific metabolic properties and turnover rates (Vander Zanden et al. 2015), the analysis of multiple samples can provide a comprehensive understanding of individual physiological condition as well as temporal reconstructions of resource and habitat use (MacNeil et al. 2005, Matich et al. 2011). Despite the increasing use of these biomarkers, few studies have simultaneously combined multiple physiological measures to examine the nutritional state of elasmobranchs (see McMeans et al. 2012) and specifically for the purpose of examining reproduction and allocation of maternal resources (e.g. Beckmann et al. 2013).

Myliobatoid rays are an interesting model for such studies, given they adopt a highly specialized reproductive strategy characterized by the production and secretion of histotroph (or uterine milk) in the uterine lumen, which corresponds to high nutritional investment (Wourms 1981, Hamlett et al. 1985). Within myliobatoids, the cownose ray *Rhinoptera bonasus* is classified as having one of the lowest lifetime fecundity estimates (<14 throughout life) among all elasmobranchs, as a result of a long gestation period (11–12 mo), large size at birth (approximately half of mother's size) and low reproductive potential (1 embryo female^{-1} yr^{-1}) (Fisher et al. 2013, Grubbs et al. 2016). Such characteristics suggest that maternal investment in cownose ray embryos could be greater than that observed in placental sharks (Wourms 1981; our Fig. 1), through selecting the evolutionary trade-off of having a limited number of pups (Stearns 1992, Roff 2002).

In the current study, we investigated the neonatal nutritional strategy and scope of maternal investment adopted by the cownose ray. Since maternal investment is determined by the energy allocated (i.e. quantity and quality), gestation period and litter size (Stearns 1992, Roff 2002), we hypothesize that the high degree of maternal investment and the transfer of nutrients through histotrophy in cownose rays (Hamlett et al. 1985, Fisher et al. 2013) will confer high-quality nutrition to postnatal pups (Fig. 1). Based on this assumption, we firstly predict that indicators of nutritional condition (i.e. plasma lipids, condition factor and $\delta^{15}N$ values) and nutritional quality (essential fatty acids) will be negatively correlated with body size, reflecting an initial high maternal investment followed by a gradual depletion of reserves (Hussey et al. 2010, Belicka et al. 2012). Secondly, we predict that short- and long-term resource indicators (plasma lipids and fatty acids, $\delta^{15}N$ and $\delta^{13}C$ values) will differ according to body growth, as a result of an ontogenetic shift in diet from dependence on maternal resource allocation to individual foraging. We therefore expect to observe a clear separation among young-of-the-year (YOY, <1 yr old) cownose rays, in relation to the time period newborns are reliant on maternal resources (see Belicka et al. 2012). By adopting an interdisciplinary approach through examining several biochemical tracers in multiple tissues, we provide insight into how maternal investment affects the neonatal nutritional condition and foraging skills of *R. bonasus*, an elasmobranch with extremely low reproductive output.

2. MATERIALS AND METHODS

2.1. Study area and sampling

YOY cownose rays were caught between December 2015 and May 2017 in Bertioga, a protected area on the central coast of São Paulo, southeastern Brazil (Fig. 2) (23° 49′ 35″ S, 46° 5′ 42″ W). Sampling occurred following the incidental capture of individuals by artisanal fishermen using beach seine nets (350 × 11 m, mesh size: 70 mm between opposing nodes) cast 400 to 600 m from the beach and gathered by hand over a period of approximately 40 min (Rangel et al. 2018). Captured specimens were removed from the fishing net, held in 50 l plastic containers filled with seawater (2–3 ind. per box) and identified to species level using dental morphological characteristics, and their corresponding disc width (DW, cm) and body mass (kg) were recorded. Fulton's condition factor was then calculated for all rays sampled using the following equation (Froese 2006):

$$\text{Condition factor} = (\text{body mass} \times \text{DW}^{-3}) \times 100$$

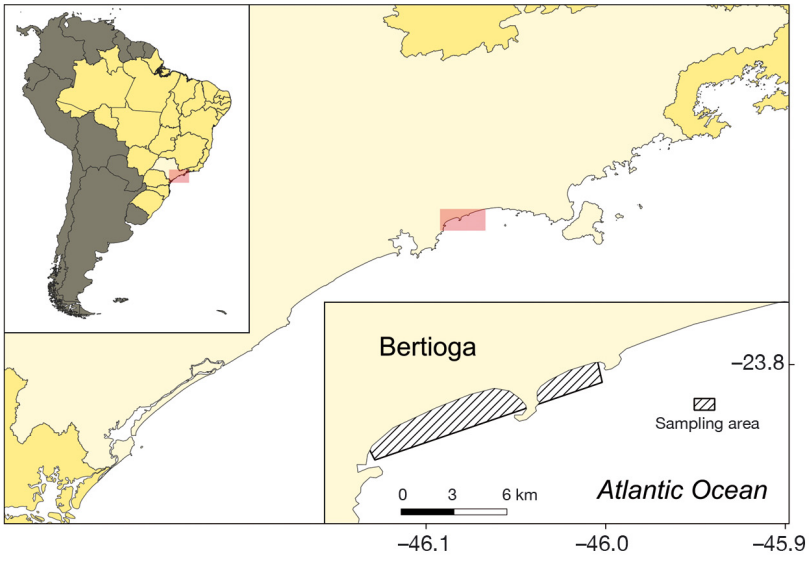

Fig. 2. Location of capture and sampling of cownose rays *Rhinoptera bonasus* in Bertioga, São Paulo, southeastern Brazil

A total of 3 tissues were sampled from each individual where possible for biomarker analyses. A fin sample was excised from the trailing edge of the dorsal fin, ~50 mg muscle plug was taken from the ventral region of the left pectoral fin using a 6 mm biopsy punch following Belicka et al. (2012) and Every et al. (2016), and blood (~1 ml) was collected by caudal venipuncture using pre-heparinized syringes (sodium heparin, 5000 IU, Liquemine). Blood samples were transferred to microtubes placed on ice, which were then centrifuged for 10 min (655.2 × *g*) to isolate red blood cells (RBC) and plasma. Following field sampling, all tissue and plasma samples were placed in cryogenic tubes and stored at −80°C for future analyses.

2.2. Biomarker analysis

Triglyceride (TAG), cholesterol (CHOL) and ketone body β-hydroxybutyrate (β-HB) levels were measured in plasma samples using commercial kits (TAG and CHOL: Labtest®, β-HB: Cayman®) with colorimetric enzymatic reaction using a spectrophotometer ELISA (SpectraMax 250, Molecular Devices), following the manufacturer's guidelines. To examine fatty acid profiles, only individuals caught during the austral summer (December to March) were considered, because of unfavorable environmental conditions in other seasons. Total lipids were extracted from muscle samples (37–74 mg) using a 2:1:0.5 volume of chloroform:methanol:water, following the methods of Folch et al. (1957) and adapted by Parrish (1999). Plasma (100 µl) and lipid extracts from muscle were methylated with acetyl chloride (5% HCl in methanol) and converted into fatty acid methyl esters (FAMEs) (Christie 2003). Fatty acid analysis was then carried out in a Varian gas chromatograph (Model 3900) coupled with a flame ionization detector (FID) and a CP-8400 autosampler. FAMEs were analyzed on a capillary column (Wax 52, 0.25 µm thickness, 0.25 mm inner diameter and 30 m length). Hydrogen was used as the carrier gas at a linear velocity of 22 cm s^{-1}. The column was programmed at 170°C for 1 min, followed by a 2.5°C min^{-1} ramp to 240°C and a final hold time of 5 min. The injector and FID temperatures were 250 and 260°C, respectively. FAMEs were identified by comparing their retention times to those obtained from commercial standards (Supelco 37 Component, Sigma-Aldrich; Mixture ME93, Larodan; and Qualmix PUFA Fish M Natural Menhaden Oil, Larodan). Data are presented as percent of total FAMEs based on peak area analyses.

$\delta^{15}N$ and $\delta^{13}C$ were analyzed in RBC and fin clip samples. These 2 tissues were selected because of their different metabolic turnover rates, with the RBC of young animals having a higher isotopic turnover rate and thus reflecting diet over weeks to months in comparison with a lower turnover rate of fins, which is considered to indicate cumulative diet over months to years (MacNeil et al. 2005, Kim et al. 2012). RBC and fin clip samples were freeze dried and ground to a fine powder, and 400 to 600 µg of material was weighed into tin capsules. Lipid and urea extraction were not undertaken for either tissue based on the recommendation of Kim et al. (2012) for RBC and the unlikely effect of these compounds in fin tissue. The stable isotope values of carbon and nitrogen were then determined by online combustion of samples by continuous flow-isotope ratio mass spectrometry, using an elemental analyzer (Model 1110, Carlo Erba) interfaced to an isotope ratio mass spectrometer (Finnigan, ThermoQuest; Delta Plus, Finnigan MAT). The isotopic composition of carbon and nitrogen was calculated as $\delta X = [(R_{sample}/R_{standard}) - 1] \times 1000$, where R is the molar ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$ in the sample and standard, expressed as delta (δ) per mil (‰). The standards used for nitrogen and carbon

were PDB and atmospheric nitrogen, respectively. Analytical precision was calculated as 0.3 and 0.2‰ for $\delta^{15}N$ and $\delta^{13}C$ values, respectively.

2.3. Nutritional and trophic biomarkers

Condition factor, lipid metabolites (i.e. TAG, CHOL and β-HB), TAG:CHOL ratios and C:N stable isotope ratios were used as indicators to assess the nutritional condition and lipid storage index of YOY *Rhinoptera bonasus* (Weber et al. 2003, Post et al. 2007, Hussey et al. 2009, Sardenne et al. 2016). Essential fatty acids (i.e. DHA, ARA and EPA) were used as descriptive indices of cownose ray nutritional quality and to determine trophic state (Tocher 2003, Arts & Kohler 2009), while the ARA:EPA ratio was used to assess the physiological responses of eicosanoids. The relationships between the muscle DHA:EPA ratio and ARA and $\delta^{15}N$ and $\delta^{13}C$ values were examined. The muscle DHA:EPA ratio is a marker of trophic position and is typically correlated with $\delta^{15}N$ (Dalsgaard et al. 2003, El-Sabaawi et al. 2009, Parrish et al. 2015). Similarly, muscle ARA values and $\delta^{13}C$ have been found to be significantly correlated and indicate if a species inhabits coastal/benthic environments (Sardenne et al. 2017). Odd-chain fatty acids (OFA) and branched-chain fatty acids (BFA) were used as bacterial markers (Dalsgaard et al. 2003).

2.4. Statistical analyses

To analyze metabolite (TAG, CHOL and β-HB), fatty acid and stable isotope values as a function of tissue turnover rate and body growth, generalized linear models (GLMs) were constructed including an interaction between tissue type and DW. Average age-at-length values for *R. bonasus* were obtained from Fisher et al. (2013), in which the mean DW of males and females per age class was calculated and used on a comparative scale in the analysis. Prior to implementing the GLMs, each biomarker category was log transformed and significant model outputs overlapped to identify the corresponding DW thresholds responsible for observed negative and positive effects. The mean of the identified DW threshold values based on significant null effects from the GLM approach were then used to categorize sampled juvenile *R. bonasus* into YOY I (younger) and YOY II (older) size classes. Independent principal component analysis (PCA) simultaneously including tissues with similar turnover rates (i.e. RBC and plasma vs. muscle and fin) was then applied to each size class of *R. bonasus*, using only the significant biomarkers identified by the GLMs. This analysis was conducted as a complementary approach to further investigate differences in the physiological composition of YOY I and YOY II animals, using a subset of animals sampled for which all tissue types were available. Statistical significance was set at $p < 0.05$, and all analyses were conducted in R version 3.5.1 (R Core Team 2018). GLM and PCA analyses were performed using the stats (R Core Team 2018) and vegan (Oksanen et al. 2013) packages, respectively. The correlation between DHA, DHA:EPA ratio and ARA and $\delta^{15}N$ and $\delta^{13}C$ was undertaken using Pearson correlation analysis. Positive and negative Pearson correlation values ranging from 0.1 to 0.29 indicate a weak correlation, from 0.3 to 0.49 a moderate correlation, and from 0.5 to 1.0 a strong correlation (Cohen 1988).

3. RESULTS

A total of 83 YOY cownose rays were analyzed, ranging in body size from 31.5 to 73 cm DW (51.5 ± 8.82 cm DW, mean ± SD) and in body mass from 302 to 4210 g (2094.1 ± 882.98 g). YOY sampled in December had a slightly lower mean DW (46.4 ± 6.97 cm) than those caught in February (52.7 ± 6.86 cm), March (54.5 ± 9.11 cm) and May (55.4 ± 11.41 cm). Condition factor ranged from 0.92 to 1.95 (1.50 ± 0.19, n = 60) and increased significantly with YOY body size (Est. = 0.02, SE = 0.01, $t = 2.39$, $p = 0.019$; Fig. 3a).

YOY rays exhibited substantial variation in raw plasma lipid values, with TAG ranging from 44.7 to 289.4 mg dl^{-1} (144.6 ± 54.63 mg dl^{-1}, n = 31), CHOL ranging from 12.4 to 188.9 mg dl^{-1} (87.6 ± 40.27 mg dl^{-1}, n = 33) and β-HB ranging from 0.14 to 0.78 mg dl^{-1} (0.25 ± 0.14 mg dl^{-1}, n = 20). Ratios of TAG:CHOL were also variable, ranging from 0.41 to 4.13 (2.0 ± 0.99). There was a negative relationship between CHOL and body size (Table 1, Fig. 3b) and a positive relationship between TAG:CHOL ratio and body size (Table 1, Fig. 3c). There was no significant relationship between TAG and body size nor between β-HB and body size (Table 1).

In terms of fatty acids, polyunsaturated fatty acids (PUFA) were dominant in blood plasma (n = 21) and consisted largely of DHA, EPA and ARA, followed by saturated fatty acids (SFA) including C16:0 and C18:0 and monounsaturated fatty acids (MUFA) such as C18:1n9 (Table S1 in the Supplement at www.int-res.com/articles/suppl/m638p107_supp.pdf). Similarly,

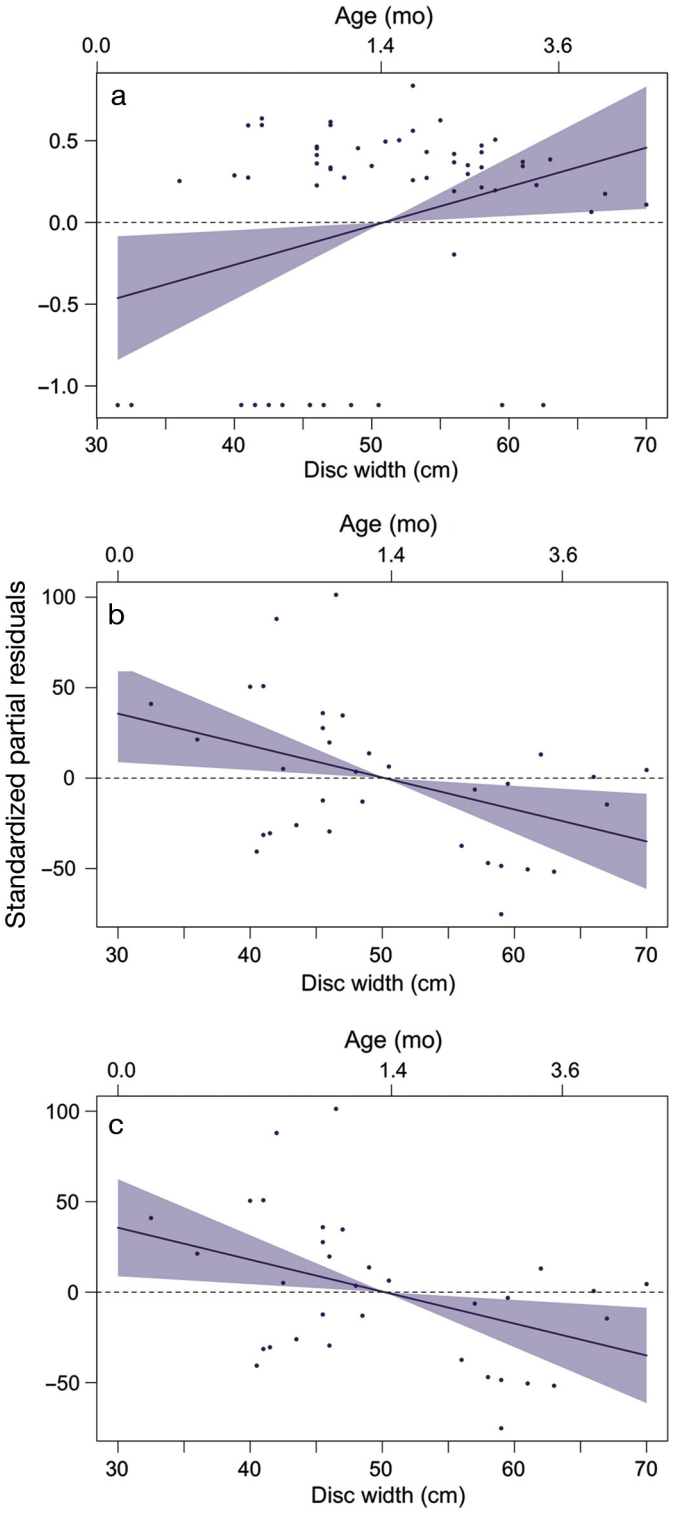

Fig. 3. Regression models of (a) condition factor, (b) cholesterol (CHOL) concentration in plasma and (c) triglyceride: cholesterol (TAG:CHOL) ratio in plasma as a function of disc width. The solid black line represents the regression line, and the horizontal dashed lines and shaded areas represent the null effect and the 95% CIs, respectively

Table 1. Regression models of plasma lipid (triglycerides [TAG], cholesterol [CHOL] and β-hydroxybutyrate [β-HB]) concentrations as a function of disc width (DW). Corresponding coefficient estimates (Est.), SEs, *t*-values and p-values of each model are included. **Bold**: significant values ($p < 0.05$)

Lipid	Variable	Est.	SE	*t*	p
TAG	Intercept	112.89	57.23	1.97	0.058
	DW	0.63	1.12	0.56	0.577
CHOL	Intercept	176.17	34.62	5.09	<0.001
	DW	**−1.76**	**0.68**	**−2.60**	**0.014**
β-HB	Intercept	0.55	0.145	3.75	0.001
	DW	−0.01	0.01	−2.05	0.055
TAG × CHOL	Intercept	−0.48	0.93	−0.52	0.60
	DW	**0.04**	**0.02**	**2.73**	**0.011**

PUFA were dominant in muscle (n = 48), followed by SFA and MUFA. Dominant fatty acids in muscle were C16:0 and DHA, followed by C18:0, C18:1n9 and ARA (Table S2 in the Supplement). YOY size influenced the composition of fatty acids in muscle and plasma (Table 2), with SFA and BFA–OFA decreasing with body size in both muscle and plasma (Fig. 4, see Fig. 6). In contrast, plasma PUFA increased with body size (Fig. 4), including both n3 and n6 PUFA (Table 2, Fig. 5a). For plasma, C16:0, C18:0, C18:1n7 and ARA decreased with body size, while EPA increased with size (Table 2, Fig. 5a,c,d). In muscle, both C18:1n9 and EPA increased with body size, whereas ARA decreased (Table 2, Fig. 5b,d). A negative relationship was found between ARA:EPA ratio and body size in both muscle and plasma (Table 2, Fig. 6a & 6b, respectively). In muscle, the DHA:EPA ratio decreased with body size (Table 2, Fig. 6a).

Stable isotope values (Fig. 7) were significantly correlated with body size in RBC (n = 55) and fin (n = 69; Table 3, Fig. 6). For $\delta^{13}C$ values, there was a negative relationship between RBC values and body size (Fig. 8a); likewise, $\delta^{15}N$ values and C:N ratio of both fin and RBC decreased with increasing body size (Fig. 8). RBC $\delta^{15}N$ values exhibited a strong positive correlation with muscle DHA:EPA ratio (r = 0.60, p = 0.0003) and a moderate positive correlation with muscle DHA values (r = 0.34, p = 0.066). Similarly, fin $\delta^{15}N$ values exhibited a positive correlation with DHA:EPA ratio (r = 0.49, p = 0.0007) and a weak positive correlation with muscle DHA values (r = 0.29, p = 0.062). RBC and fin $\delta^{13}C$ values showed a positive correlation with muscle ARA (r = 0.40, p = 0.031 and r = 0.49, p = 0.001, respectively).

All GLM outputs indicated similar size thresholds (~50 cm DW) to differentiate between the negative

Table 2. Regression models between the families of fatty acids (saturated fatty acids [SFA], monounsaturated fatty acids [MUFA] and polyunsaturated fatty acids [PUFA]) as a function of the interaction between disc width (DW) and tissue type (i.e. muscle and plasma). Corresponding coefficient estimates (Est.), SEs, *t*-values and p-values of each model are included. **Bold**: significant values ($p < 0.05$). EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; ARA: arachidonic acid; BFA: branched-chain fatty acids; OFA: odd-chained fatty acids

Family	Variable	Est.	SE	*t*	p
SFA	Intercept	43.92	3.65	12.01	<0.001
	DW:Muscle	**–0.15**	**0.07**	**–2.08**	**0.041**
	DW:Plasma	**–0.21**	**0.08**	**–3.05**	**0.003**
C16:0	Intercept	23.86	2.13	11.18	<0.001
	DW:Muscle	–0.07	0.04	–1.79	0.077
	DW:Plasma	**–0.13**	**0.04**	**–3.16**	**0.002**
C18:0	Intercept	16.57	1.84	8.99	<0.001
	DW:Muscle	–0.04	0.04	–1.05	0.298
	DW:Plasma	**–0.07**	**0.03**	**–2.02**	**0.047**
MUFA	Intercept	14.15	2.27	6.24	<0.001
	DW:Muscle	0.08	0.04	1.72	0.089
	DW:Plasma	–0.05	0.04	–1.24	0.216
C18:1n7	Intercept	6.08	0.83	7.34	<0.001
	DW:Muscle	–0.01	0.01	–0.22	0.829
	DW:Plasma	**–0.06**	**0.02**	**–3.83**	**<0.001**
C18:1n9	Intercept	4.10	1.42	2.89	0.005
	DW:Muscle	**0.08**	**0.03**	**2.94**	**0.004**
	DW:Plasma	0.01	0.03	0.44	0.662
PUFA	Intercept	32.24	5.72	5.62	<0.001
	DW:Muscle	0.17	0.12	1.47	0.146
	DW:Plasma	**0.32**	**0.11**	**2.89**	**0.005**
PUFA n3	Intercept	17.70	4.52	3.92	<0.001
	DW:Muscle	0.16	0.09	1.83	0.072
	DW:Plasma	**0.24**	**0.08**	**2.78**	**0.007**
PUFA n6	Intercept	13.76	1.97	6.99	<0.001
	DW:Muscle	–0.01	0.04	–0.09	0.927
	DW:Plasma	**0.08**	**0.03**	**2.06**	**0.043**
C20:5n3 (EPA)	Intercept	–0.46	0.95	–0.48	0.627
	DW:Muscle	**0.04**	**0.02**	**2.09**	**0.039**
	DW:Plasma	**0.13**	**0.02**	**7.04**	**<0.001**
C22:6n3 (DHA)	Intercept	16.42	3.77	4.36	<0.001
	DW:Muscle	0.04	0.07	0.56	0.574
	DW:Plasma	0.02	0.07	0.33	0.739
C20:4n6 (ARA)	Intercept	10.59	1.08	9.80	<0.001
	DW:Muscle	**–0.06**	**0.02**	**–3.20**	**0.002**
	DW:Plasma	**–0.07**	**0.02**	**–3.38**	**0.001**
BFA × OFA	Intercept	8.62	1.12	7.65	<0.001
	DW:Muscle	**–0.08**	**0.02**	**–3.96**	**<0.001**
	DW:Plasma	**–0.05**	**0.02**	**–2.65**	**0.010**
DHA × EPA	Intercept	25.44	2.78	9.13	<0.001
	DW:Muscle	**-0.25**	**0.06**	**–4.42**	**<0.001**
	DW:Plasma	0.02	0.05	0.33	0.743
ARA × EPA	Intercept	9.65	0.83	11.60	<0.001
	DW:Muscle	**–0.09**	**0.02**	**–5.61**	**<0.001**
	DW:Plasma	**–0.16**	**0.02**	**–9.79**	**<0.001**

and positive effects of body length upon metabolite, fatty acid and stable isotope values (Figs. 3–7). A derived mean value of 50.2 cm DW was then used to separate *Rhinoptera bonasus* into YOY I (DW < 50.2 cm, younger than 1.5 mo) and YOY II (DW > 50.2 cm, older than 1.5 mo) size classes for the PCA analysis. PCA results indicated a high degree of separation in terms of physiological status between YOY I and YOY II *R. bonasus* for both blood (RBC and plasma, total explained variance = 80.9%) and muscle (total explained variance = 52.9%) (Fig. 9). The older group (i.e. YOY II) was characterized by higher values of TAG:CHOL ratio and PUFA in blood (Fig. 9a) and more elevated EPA in muscle (Fig. 9b) compared to YOY I.

4. DISCUSSION

Our findings confirm that female *Rhinoptera bonasus*, a model species to understand the scope of maternal investment via lipid histotrophy and its effect on nutritional state of juveniles, contribute a high degree of maternal resources to their young. This maternal head start results in a positive nutritional state of neonate cownose rays at birth, confirmed by plasma lipids, morphometric measures and no marked nutritional deficiency or fasting phase observed in analyzed pups. Equally, fatty acids and stable isotope biomarkers enabled the examination of the degree of maternal investment during the prenatal period through the identification of both the loss of the maternal signature and changes in diet quality due to independent foraging with increasing body size. Additionally, the biomarkers analyzed (plasma lipids, fatty acids and stable isotopes) in our GLMs allowed us to identify 2 YOY size classes in terms of unique size-based tracer profiles. These data provide the first insights into the processes of energy allocation and maternal investment patterns among YOY cownose rays, a species with extremely conservative reproductive life history parameters (low fecundity, long gestation and large size at birth).

Overall, our results indicate that cownose rays possess a different neonatal nutritional strategy from that reported for other elasmobranch species (Duncan & Holland 2006, Hussey et al. 2010, Belicka et al. 2012). Contrary to our prediction, YOY cownose rays exhibited an increase in condition factor and TAG:CHOL ratio (lipid storage

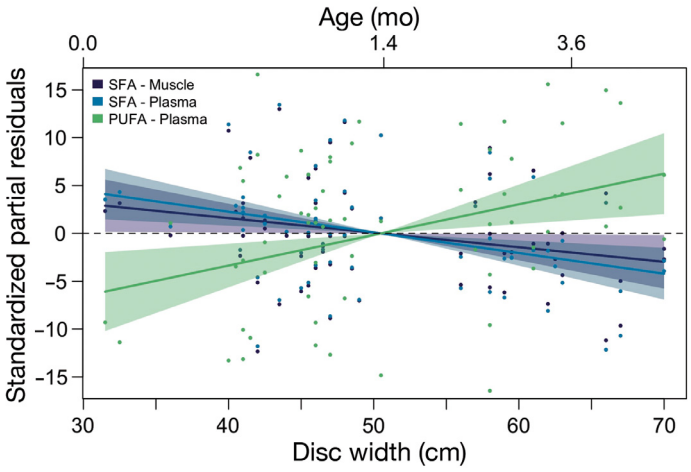

Fig. 4. Regression models of saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA) of species as a function of disc width for muscle and plasma. The horizontal dashed line and shaded areas represent the null effect and 95% CIs, respectively

index) with increasing body size. These results deviate from previous studies, where a negative association between condition factor and body size was found for neonatal scalloped hammerhead *Sphyrna lewini*, dusky shark *Carcharhinus obscurus* and sharpnose shark *Rhizoprionodon lalandii* (Duncan & Holland 2006, Hussey et al. 2010, Corsso et al. 2018). Observed declines in condition factor with increasing body size have been related to the loss or consumption of maternal reserves provisioned by the mothers during gestation, until individuals begin to forage independently (Hussey et al. 2010). The increase in condition factor and TAG:CHOL ratio in *R. bonasus*, together with no changes in TAG concentrations, suggests pups are not born with large amounts of stored resources. In

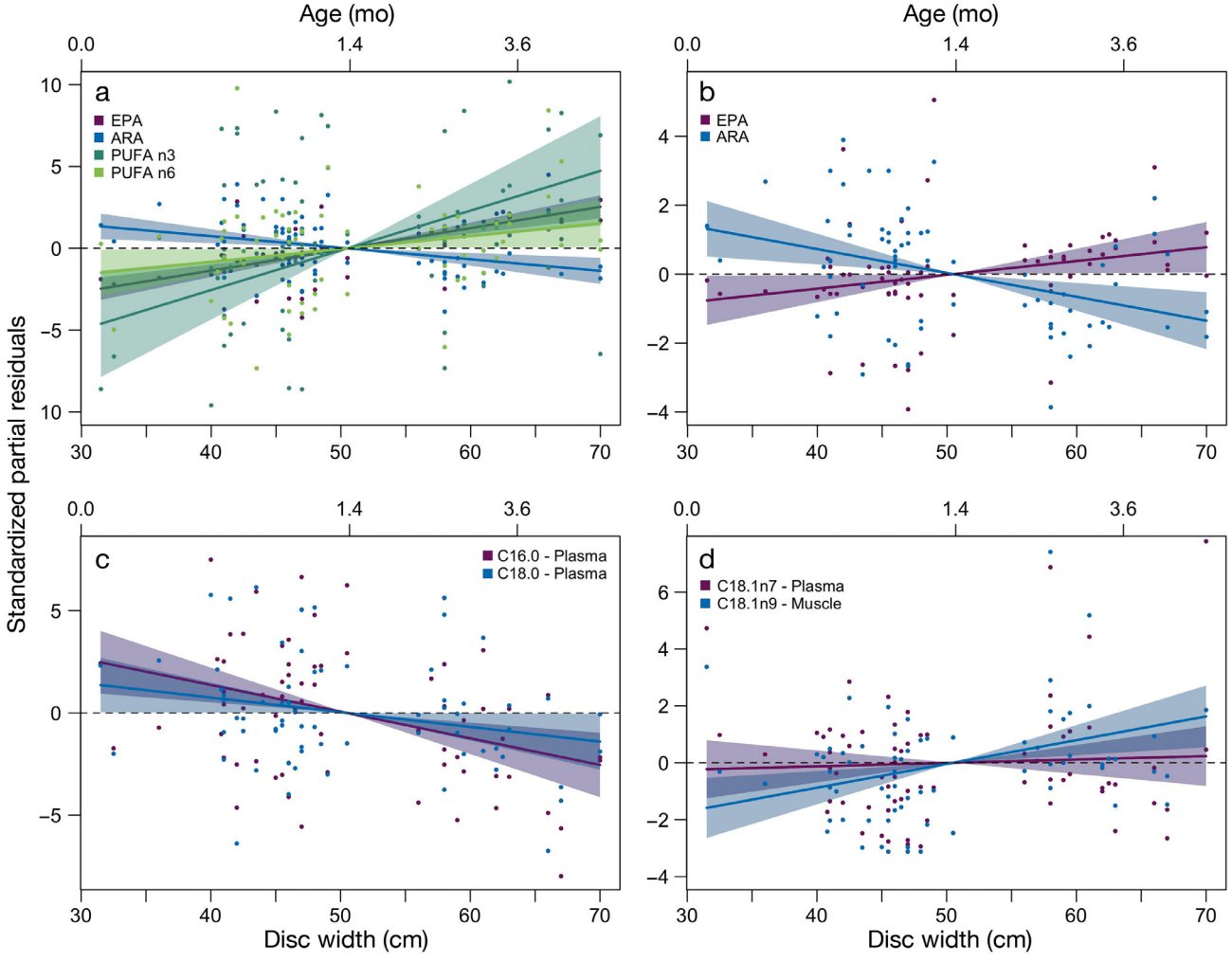

Fig. 5. Regression models of individual (a) polyunsaturated fatty acids (PUFA) in plasma, (b) PUFA in muscle, (c) saturated fatty acids in plasma and (d) monounsaturated fatty acids in plasma and muscle as a function of disc width. The horizontal dashed lines and shaded areas represent the null effects and 95% CIs, respectively. EPA: eicosapentaenoic acid; ARA: arachidonic acid

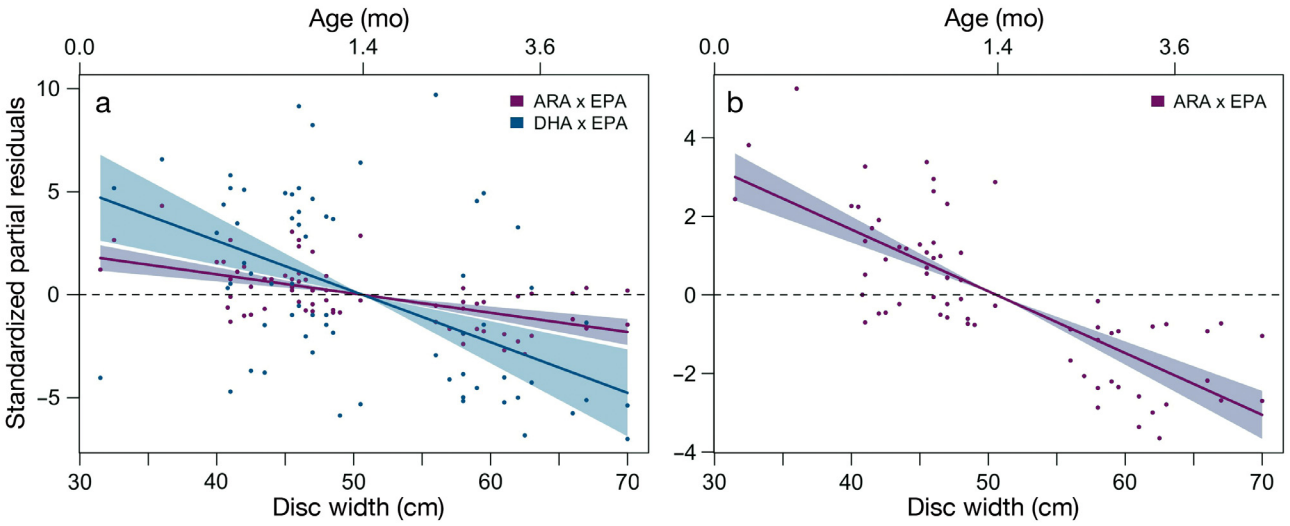

Fig. 6. Regression models of (a) muscle and (b) plasma arachidonic acid (ARA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) percentages as a function of disc width. The horizontal dashed lines and shaded areas represent the null effects and 95% CIs, respectively

Table 3. Regression models of $\delta^{15}N$ and $\delta^{13}C$ values as a function of the interaction between disc width (DW) and tissue type, i.e. fin and red blood cells (RBC). Corresponding coefficient estimates (Est.), SEs, *t*-values and p-values of each model parameters are included. **Bold**: significant values ($p < 0.05$)

Isotope	Variable	Est.	SE	*t*	p
$\delta^{15}N$	Intercept	12.97	0.36	35.41	<0.001
	DW:Fin	**−0.04**	**0.01**	**−5.31**	**<0.001**
	DW:RBC	**−0.05**	**0.01**	**−6.47**	**<0.001**
$\delta^{13}C$	Intercept	−14.19	0.35	−40.85	<0.001
	DW:Fin	−0.01	0.01	−1.92	0.057
	DW:RBC	**-0.04**	**0.01**	**−5.95**	**<0.001**
$\delta^{13}C \times \delta^{15}N$	Intercept	2.73	0.08	33.93	<0.001
	DW:Fin	**−0.03**	**0.01**	**−2.43**	**0.016**
	DW:RBC	**−0.04**	**0.01**	**−2.70**	**0.007**

contrast, the C:N ratio (a proxy for estimating lipid content, Post et al. 2007) declined with increasing body size, suggesting that the samples analyzed (RBC and fin) may not be useful for detecting lipid accumulation in YOY cownose rays due to their low lipid content.

The lower condition factor values found in the early neonatal stage (YOY I) might be a result of rapid growth observed in cownose rays (up to 10 cm DW in the first 4 mo and more than 20 cm yr^{-1}, Fisher et al. 2013), suggesting pronounced catabolism during this initial phase. Corroborating the initial use of body reserves to enable rapid growth, higher concentrations of CHOL and SFA (C16:0 and C18:0) were observed in the plasma of YOY I when compared to YOY II animals. CHOL has been shown to be a good indicator of energy stores via catabolism, because it is involved in cell membrane composition, and is a precursor of steroid hormones, bile acids and vitamin D, which are essential for tissue growth (Nelson & Cox 2014), and SFA are the main fatty acids catabolized for energy (Tocher 2003). Although not significant, possibly due to the number of individuals analyzed (n = 20), a negative trend in plasma β-HB levels (p = 0.055) with body size in cownose rays provides an additional indicator that YOY I were mobilizing lipid reserves and therefore were not feeding on external resources as efficiently as YOY II. In elasmobranchs and other large vertebrates, β-HB is the primary high-energy fuel, with high β-HB plasma concentrations indicating prolonged fasting (Mellish & Iverson 2001, Speers-Roesch & Treberg 2010, Wood et al. 2010).

In terms of nutritional quality, the higher percentages of long-chain PUFA such as DHA and ARA in smaller rays (YOY I) provide a strong indicator of higher nutritional condition at birth, confirming the maternal transfer of these fatty acids during gestation. These long-chain PUFA play important physiological roles, directly influencing growth, reproduction and survival (Tocher 2003, Arts & Kohler 2009, Pethybridge et al. 2011). DHA is the major structural lipid in neurological and visual development and functioning and is found at high percentages in teleost larvae during early life stage development (Izquierdo et al. 2000). ARA is a precursor of eicosanoids by signaling molecules or local hormones and thus has a critical metabolic function in immune and inflamma-

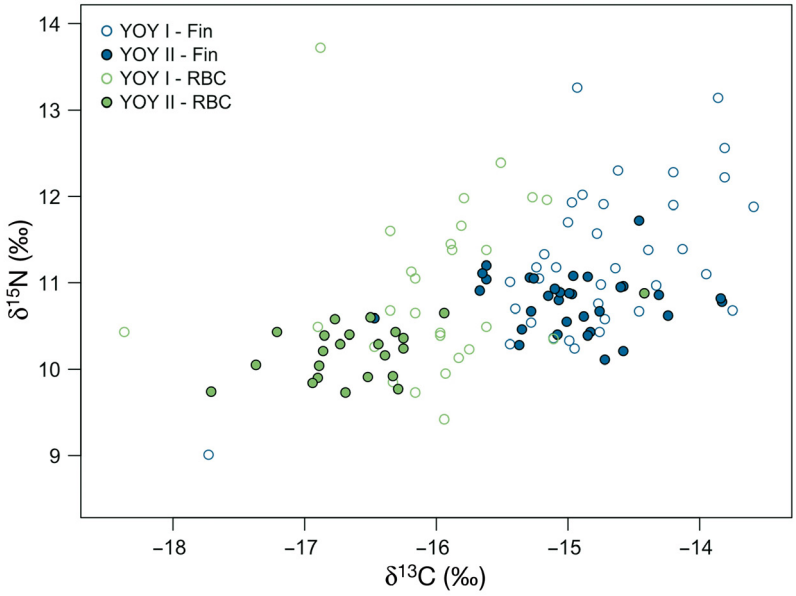

Fig. 7. Stable isotope biplot of $\delta^{13}C$ and $\delta^{15}N$ values for fin and red blood cells (RBC) of young-of-the-year (YOY) I and YOY II cownose rays

tory responses (Tocher 2003, Arts & Kohler 2009, Gladyshev et al. 2018). In contrast, previously studied placental sharks are generally born with low percentages of ARA and DHA, indicating that lipids with low levels of PUFA are provisioned during pregnancy or that a larger amount of these fatty acids are used by pups during intrauterine development (Belicka et al. 2012, Wai et al. 2012). Unlike placental sharks, which show an increase in muscle long-chain PUFA with growth (Belicka et al. 2012, Wai et al. 2012), cownose rays maintained an elevated percentage of DHA, while the percentages of ARA declined with body size. For cownose rays, where maternal resources are invested in a single pup, the lipid-rich histotroph could allow selective mobilization of fatty acids from maternal reserves, as seen in marine mammals through transfer by milk (e.g. Iverson et al. 1995). Consequently, it would appear there is a strong relationship between elasmobranch reproductive mode and gestation period, litter size and the quality and quantity of nutrients invested by the mother (Clutton-Brock 1991, Stearns 1992).

The high maternal provisioning of long-chain PUFA found in cownose rays may be due to the importance of these fatty acids for metabolism in young individuals. Long-chain PUFA, in particular DHA, are correlated with metabolic rate and are proposed as pacemakers for animal metabolism (Hulbert 2007, Hulbert & Abbott 2012). They are also attributed to adaptations for migration and fast continuous swimming (Weber 2009, Gladyshev et al. 2018), which would be consistent with the observed lifestyle of this species. Cownose rays are highly mobile, with an annual migratory cycle between summer pupping/mating and winter habitats (Omori & Fisher 2017, Ogburn et al. 2018). Additionally, since cownose rays are benthopelagic animals, they exhibit continuous movement shifts between foraging on benthic communities and movements in the pelagic zone (Smith & Merriner 1985, Ajemian & Powers 2012). Previous work comparing 2 cownose ray species within a single nursery habitat showed that *Rhinoptera brasiliensis* was less enriched in physiologically important essential fatty acids in comparison with *R. bonasus*. This suggests that congeneric cownose ray species differ in their maternal investment strategy, maybe as a result of their different lifestyles and/or trophic relationships (Rangel et al. 2019). Future comparative studies exploring the metabolic rate and movement patterns of congeneric cownose ray species could help elucidate such trends.

The higher values of the ARA:EPA ratio found in muscle and plasma of smaller cownose rays (YOY I), and the subsequent decrease with growth, suggest high eicosanoid production and/or a possible change in the physiological responses of eicosanoids. Both ARA and EPA act as precursors in eicosanoid synthesis and compete for the same enzymes; however, ARA-derived eicosanoids are more physiologically active compared to the EPA-derived form and have been described as essential for early life stages (Tocher 2003). For example, in teleost larvae, ARA is responsible for improving survival and stress resistance (Sargent et al. 1995, Atalah et al. 2011) and has also been found at high levels in YOY Port Jackson sharks *Heterodontus portusjacksoni* (Beckmann et al. 2014).

Understanding the maternal–fetal transfer of nutrients is fundamental for our interpretation of biomarkers in young free-swimming individuals, especially considering their use in trophic ecology studies (Vaudo et al. 2010, Olin et al. 2011, 2018). Here, we found that YOY cownose rays are born enriched in certain trophic biomarkers, supporting an initial dependency on maternal resources. The isotopic values of RBC (faster turnover rate) and fin (slower turnover rate) revealed that cownose rays were born enriched in

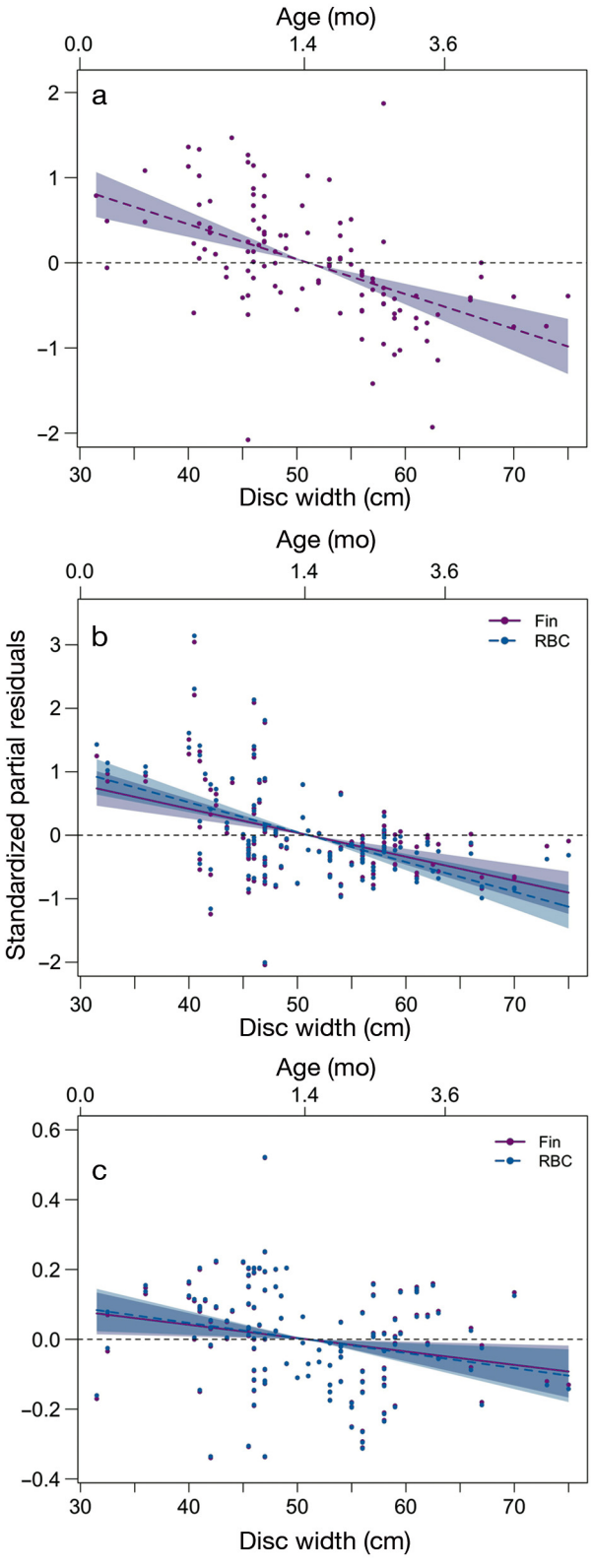

Fig. 8. Regression models of (a) $\delta^{13}C$, (b) $\delta^{15}N$ and (c) $\delta^{13}C \times \delta^{15}N$ as a function of disc width for the 2 analyzed tissues (fin and red blood cells [RBC]). The horizontal dashed lines and shaded areas represent the null effects and 95% CIs, respectively

^{15}N and ^{13}C (i.e. a maternal signature) and exhibited a gradual decline in isotope values with growth. This declining trend in $\delta^{15}N$ with size was previously reported for other shark and ray species, e.g. bull sharks *Carcharhinus leucas* (Olin et al. 2011, Matich et al. 2015) and Brazilian cownose rays *R. brasiliensis* (Rangel et al. 2019). Based on previous studies, we propose 2 possible explanations for the observed $\delta^{15}N$ trend: (1) neonates could show an enrichment in ^{15}N compared to their mothers (e.g. Olin et al. 2011, Borrell et al. 2016) if gestating females use endogenous substrates during reproduction and there is isotopic enrichment of young through the allocation of protein resources from maternal tissues (i.e. capital breeders, e.g. Borrell et al. 2016) or (2) $\delta^{15}N$ discrimination between maternal and fetal tissues is nonexistent, as observed for species where females use concurrent energy intake during reproduction (i.e. income breeders, Jenkins et al. 2001). Enriched isotope values in neonate cownose rays in comparison with mothers were not observed in an estuarine system located at low latitudes (Poulakis et al. 2017), suggesting that cownose rays might adopt an income breeder strategy, i.e. nutrient allocation recently acquired by the mother at least during the uterolactation stage. Future controlled experimental studies comparing stable isotope fractionation between maternal and embryonic tissues could reveal the mechanisms of resource use for reproduction (Jenkins et al. 2001, Borrell et al. 2016).

Similarly, the muscle DHA:EPA ratio was enriched in smaller cownose rays (YOY I) and showed a similar pattern to that found for $\delta^{15}N$ values. In terms of trophic transfer, DHA is mainly synthesized by dinoflagellates and minimally modified during ingestion and assimilation processes; consequently, it is transferred and selectively retained in consumers (Dalsgaard et al. 2003). As a result, the DHA:EPA ratio has a positive correlation with $\delta^{15}N$ and trophic level (El-Sabaawi et al. 2009, Parrish et al. 2015, Sardenne et al. 2017). The $\delta^{13}C$ values and ARA levels of young rays were positively correlated, indicating foraging in benthic coastal systems and, together with DHA and the DHA:EPA ratio, showed a similar pattern to that observed for $\delta^{15}N$ and $\delta^{13}C$ (Sardenne et al. 2017). Similar to trends previously described using stable isotopes (e.g. Olin et al. 2011, 2018), the DHA:EPA ratio and ARA appear to be good indicators to assess the loss of the maternal signal in elasmobranch species. In addition, changes in fatty acid and stable isotope values could be attributed to the YOY II beginning to forage on different prey items in different habitats when compared to their mothers.

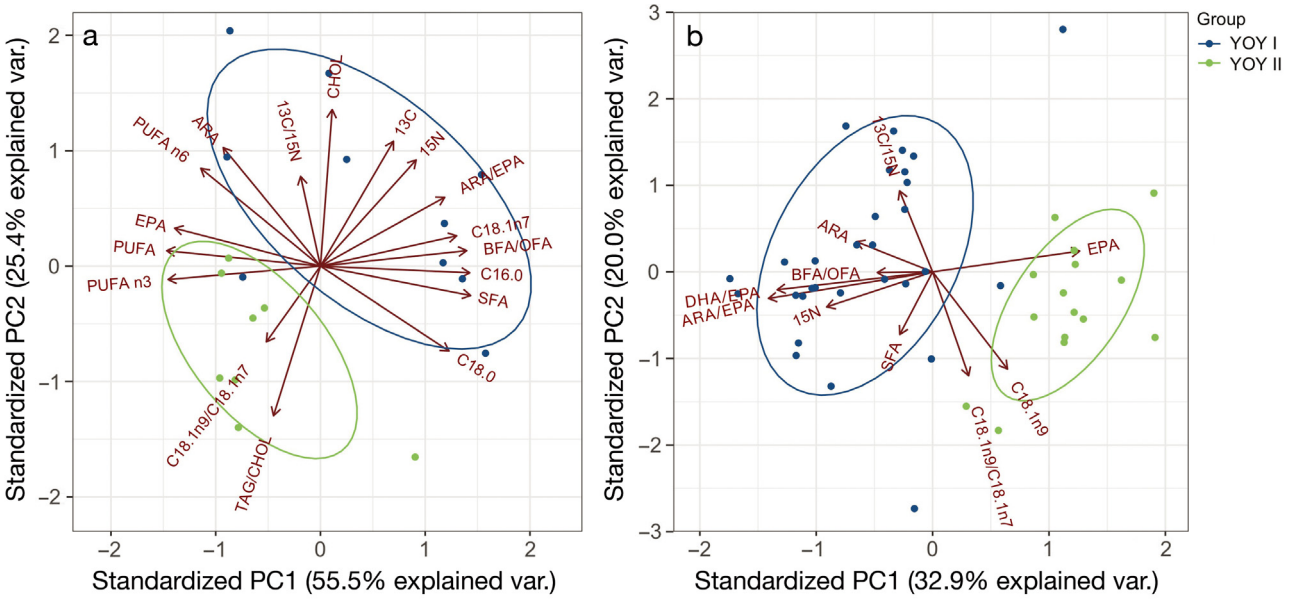

Fig. 9. Principal component (PC) analysis of biochemical composition, using only the significant biomarkers identified by the generalized linear models of (a) short turnover (plasma and red blood cells) and (b) long turnover (muscle and fin) tissues for young-of-the-year (YOY) I and YOY II cownose rays, including the interacting effects of metabolites, fatty acids and stable isotopes. ARA: arachidonic acid; CHOL: cholesterol; EPA: eicosapentaenoic acid; BFA: branched-chain fatty acids; OFA: odd-chain fatty acids; SFA: saturated fatty acids; TAG: triglycerides; PUFA: polyunsaturated fatty acids; DHA: docosahexaenoic acid

Future telemetry studies would reveal such behavioral patterns.

The plasma fatty acid profiles confirm that the foraging skills of *R. bonasus* improve at larger sizes and support our initial hypothesis that YOY rays differ in their nutritional status according to growth. Since plasma acts as a carrier of dietary fatty acids to other tissues and has a faster transient composition, it is often used as a short-term indicator of diet (Käkelä et al. 2009, McMeans et al. 2012). In addition, given elasmobranchs use ketone bodies as oxidative fuels, it is expected that the plasma fatty acid profile will reflect what was acquired directly from diet (Speers-Roesch & Treberg 2010, McMeans et al. 2012). In this study, increases in n6 and n3 PUFA, together with a reduction of SFA and BFA–OFA (bacterial markers), indicate that larger pups (YOY II) feed more independently, accessing prey with a greater nutritional quality. Unlike placental sharks, which exhibit poor foraging skills at this early life stage and are almost exclusively dependent on maternal reserves allocated in the liver (Hussey et al. 2010, Belicka et al. 2012), juvenile cownose rays have been found to ingest large amounts of food relative to body weight (Ajemian & Powers 2012). These findings corroborate our data identifying that high-quality maternal investment in cownose rays allows newborn pups to quickly become independent and successful foragers. A similar hypothesis has been proposed for the postweaning food provisioning in mammals. The nutritional hypothesis assumes that maternal resource provisioning during critical phases provides nutritional benefits for pups, i.e. a more rapid transition to independent and successful foragers (Brown et al. 2004, Geipel et al. 2013). Nevertheless, further studies comparing species that utilize different reproductive strategies are required to better evaluate this hypothesis.

Finally, our integrated biomarker approach allowed the identification of 2 YOY cownose ray size classes with distinct physiological states, with implications for management and conservation initiatives. Similarly, Belicka et al. (2012) identified size groups of bull sharks using some of the biomarkers used in the present study (fatty acids and $\delta^{13}C$); however, the small sharks (<100 cm total length) were in a poor nutritional state, while older sharks (immature but >100 cm total length) were found to be maternally independent through developed foraging skills. Characterizing the transition phases between maternal dependence to independent foraging is critical for identifying the functional ecological role of YOY animals and to develop effective monitoring tools for nursery habitats. Moreover, our results provide a physiological basis that could be used in mechanistic models to predict the responsiveness and potential impacts associated with human activities and environmental change on the fitness of young elasmobranchs.

5. CONCLUSIONS

Our results support the hypothesis that the lipid histotrophic strategy of cownose rays, together with a long gestation period and production of a single large pup, confers a high level of maternal investment, hence producing young that are in a positive nutritional condition at birth. In response to high maternal investment, cownose ray pups are born enriched in biochemical tracers such as DHA, ARA and $\delta^{15}N$, as a result of maternal intrauterine transfer. The gradual loss of these biomarkers, as well as a decrease in CHOL and SFA with increasing body size, indicates a high-energy demand during early life (neonatal phase). Importantly, cownose ray pups did not display a deficiency in essential fatty acids, which suggests adequate foraging skills or rapid development of foraging skills after birth. Through the use of a combination of biomarkers, it was possible to assess the neonatal nutritional strategy of cownose rays via a non-lethal approach to understand how maternal resource allocation affects physiological traits during the early life history of a viviparous elasmobranch. This biomarker framework has the potential to assess maternal investment strategies across a range of elasmobranchs with distinct reproductive strategies.

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