Mother–embryo isotope fractionation in the pygmy devilray *Mobula kuhlii* cf. *eregoodootenkee*

M. K. BROADHURST[1] | C. DOMIT[2] | T. H. TREVIZANI[3] | V. RAOULT[4] | R.B. MILLAR[5]

[1]NSW Department of Primary Industries, Fisheries Conservation Technology Unit, National Marine Science Centre, Coffs Harbour, New South Wales, Australia and Marine and Estuarine Ecology Unit, School of Biological Sciences, University of Queensland, St Lucia, Queensland, Australia

[2]Laboratório de Ecologia e Conservação, Centro de Estudos do Mar, Universidade Federal do Paraná, Paraná, Brazil,

[3]Oceanographic Institute, University of São Paulo, Praça do Oceanográfico 191, Cidade Universitária, São Paulo, Brazil,

[4]School of Environmental and Life Sciences, University of Newcastle, Ourimbah, New South Wales, Australia

[5]Department of Statistics, The University of Auckland, Auckland, New Zealand

Correspondence

M. K. Broadhurst, NSW Department of Primary Industries, Fisheries Conservation Technology Unit, National Marine Science Centre, PO Box 4321, Coffs Harbour, NSW 2450, Australia

Email: matt.broadhurst@dpi.nsw.gov.au

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ABSTRACT

We determined stable-isotope ratios for replicate muscle tissues in 13 gravid *Mobula kuhlii* cf. *eregoodootenkee* (110.4–120.4 cm disc width; W_D), in their embryos (7.0–42.3 cm W_D) and also yolks and histrotroph, to assess the potential implications for juvenile nutrition and habitat use. Irrespective of their development in the uterus, embryos had similar $\delta^{13}C$ values in their muscle tissue as the mothers and both had greater values than in the histotroph. During gestation, $\delta^{13}C$ values increased across all sample types. However, while embryo muscle tissue and the histotroph were associated with increasing ^{15}N levels during embryonic development, this was depleted in the mothers' muscle tissue and yolk. Although speculative, the observed variation in stable-isotope ratios might imply a dietary shift among gravid females during their early gestation. Irrespective of the underlying mechanisms, the results indicate neonates will have relatively greater $\delta^{15}N$ values than post-partum females, which would probably confound juvenile foraging-ecology estimates.

KEYWORDS

batoids, embryos, gestation, histrotroph, *Mobula*, stable isotopes

1 | INTRODUCTION

The family Moblulidae currently comprises eight globally distributed species, most of which are vulnerable to capture in various fishing gears and especially coastal gillnets deployed

throughout the tropic–temperate zone (Couturier *et al.*, 2012; Lawson *et al.*, 2017). Mobulids are assumed to have highly conservative life histories, encompassing low fecundity and long generation times. Seven species have been assessed by the IUCN and all are listed as Threatened with extinction (IUCN, 2018). A necessary prerequisite to conserving mobulid populations is adequate information describing their ecology and biology and especially reproduction, but even basic data are lacking for many species (Couturier *et al.*, 2012; Lawson *et al.*, 2017). Recently, using specimens collected during trials of bather-protection nets off eastern Australia, Broadhurst *et al.* (2018) provided a first assessment of reproduction in the pygmy devilray, *Mobula kuhlii* (Valenciennes 1841) cf. *eregoodootenkee* (Bleeker 1859). The data reiterated the supposition of low reproductive output restricted to a single embryo in the functional left uterus and a non-annual gestation exceeding 10 months (Broadhurst *et al.*, 2018).

Unlike many pelagic elasmobranchs that reproduce by placental viviparity (manifesting as a vascularised placenta-like connection to the uterine wall), mobulids are aplacental viviparous. This reproductive mode involves nutrition delivered to the embryo *via* both a yolk sack (which remains connected throughout the first half of their gestation) and continuous lipid-rich histotroph provided by specialised secretory cells (trophonemata) in the uterine wall (Snelson *et al.*, 2008). The nutritional and therefore trophic relationship between gravid mothers and embryos remains unclear but should be assessed to clarify the requirements for successful embryonic development and the potential for extension into early neonatal stages. The latter is important, because nutritional relationships during gestation might confound subsequent assessments of juvenile feeding strategies or identifying their habitats and movements (McMeans *et al.*, 2009; Olin *et al.*, 2011; 2018). Understanding juvenile life histories is an important component in developing conservation strategies aimed at reducing adverse anthropogenic effects (Couturier et al., 2012).

One method for assessing trophic relationships between mothers and embryos (and identifying nursery habitats) is *via* ecological tracers, including isotope ratios of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$), collectively known as stable-isotope (SI) analyses (De Niro & Epstein, 1978; Jenkins *et al.*, 2001; McMeans *et al.*, 2009). Much of the previous relevant isotopic-fractionation literature (*i.e.*, describing the allocation of maternal resources to embryos), concerns mammals with clear relationships between isotopic signatures of mothers and their offspring during breastfeeding for various terrestrial and marine species (Jenkins *et al*., 2001; Miller *et al*., 2011).

Fewer studies have assessed elasmobranch mother–embryo fractionation, but these imply considerable variability. For example, among placentatrophic species, greater $\delta^{13}C$ and $\delta^{15}N$ values were noted in the embryos of the Atlantic sharpnose shark *Rhizoprionodon terraenovae* (Richardson 1837) and scalloped hammerhead *Sphyrna lewini* (Griffith & Smith 1834) than their mothers, but $\delta^{13}C$ and $\delta^{15}N$ values were depleted in the embryos of blacktip *Carcharhinus limbatus* (Valenciennes 1839) and bonnethead sharks *Sphyrna tiburo* (L. 1758), respectively (McMeans *et al.*, 2009; Vaudo *et al.*, 2010; Olin et al., 2018). By comparison, Bourg *et al.* (2014) recently recorded similar or lower $\delta^{13}C$ and $\delta^{15}N$ values in muscle tissue between embryos and mothers for the aplacental shortnose spurdog *Squalus megalops* (Macleay 1881) and smallfin gulper shark *Centrophorus moluccensis* Bleeker 1860.

The underlying physiological causes of such species-specific variability in ^{13}C and ^{15}N enrichment/depletion among embryos within and between reproductive modes is poorly understood and clearly precludes predicting relationships among non-assessed elasmobranchs such as *M. kuhlii* cf. *eregoodootenkee*. Considering the above, the objectives here were to use opportunistically sourced, gravid *M. kuhlii* cf. *eregoodootenkee* to quantify SI signatures among mother and embryo muscle tissues and yolk, and histrotroph in the

uterus during embryonic development and to test the hypothesis of no confounding effects of any mother-embryo fractionation on subsequent juvenile foraging-ecology estimates.

2 | MATERIALS AND METHODS

2.1 | Fish samples

The study was completed using 13 gravid *M. kuhlii* cf. *eregoodootenkee* specimens caught in five bather-protection gillnets (described by Broadhurst *et al.*, 2018) deployed during 2017 off northern New South Wales in 5–13 m of water (28.77^{o} S, 153.60^{o} E to 29.10^{o} S; 153.44^{o} E). Dead specimens were removed from the gillnets and stored at –20^{o}C before being sexed, weighed (all to the nearest 10 g) and measured for disc width (W_D; nearest 1 mm). A sample of muscle (*c.* 5 g; *i.e.*, tissue with a yearly-turnover rate and therefore a good indicator of long-term dietary patterns; Kim *et al.* 2012), was removed from the dorsal musculature on the right pectoral fin before the abdomen wall was incised and all organs were removed.

Any yolks and, in some cases, ancillary follicles (all considered as yolk), along with replicate samples of histotroph were collected from gravid females, before the single embryo was removed from the functional left uterus. Although there are no data considering the reproductive mode, yolk presumably has a *c.* 6 month turnover rate and because histotroph is continuously supplied by the mother it should have a much shorter (*e.g.*, < 1 month) turnover. The embryo was measured for W_D and weighed and a muscle sample collected (as above for the mother). In total, 44 tissue samples were taken.

Muscle samples were rinsed in deionised water for *c.* 5 min to remove urea following Burgess and Bennett (2017) and along with histotroph and yolk were freeze-dried and

homogenised. A *c.* 1.5 mg mass of each dried tissue sample was weighed, placed in a tin capsule and pelletised. Sub-samples of tissue were then analysed following international standards by the Stable Isotopes Analysis Lab, Australian Rivers Institute at Griffith University, Australia (www.griffith.edu.au/__data/assets/pdf_file/0024/516840/ARI-SIL-Information-June-2018.pdf) for $\delta^{13}C$ and $\delta^{15}N$ using an isotope-ratio mass spectrometer (Hydra 20-22; Sercon Ltd.; www.serconlimited.com) and elemental analyser (Europa EA-GSL; Sercon). Stable-isotope ratios were reported in delta (δ) notation as parts per thousand (‰) based on the equation: $\delta x‰ = 103(R_{sample}R_{standard}^{-1}) - 1$, where $x = {}^{13}C$ or ${}^{15}N$ and R is the isotope ratio of ${}^{13}C:{}^{12}C$ or ${}^{15}N:{}^{14}N$, respectively. Supplementary standards of ammonium sulphate (IAEA-N1 and IAEA-N2) and sucrose (IAEA-CH-6) were also used in each process at the end of each lane. The standard deviation across all the standards measured during stable-isotope analysis ($n = 33$) was 0.1‰.

It is well established that lipids are depleted in ^{13}C relative to carbohydrates and proteins and if they are not removed can cause $\delta^{13}C$ to vary among tissues and individuals (Post *et al.*, 2007). However, the consequences of chemical lipid extraction in elasmobranch tissues are not well understood and can also affect $\delta^{15}N$ (Burgess & Bennett, 2017). Post *et al.* (2017) implied that lipid extraction is unnecessary if the C:N ratio is < 3.5. Therefore, in the present study, $\delta^{13}C$ values were not corrected during assay. Rather, C:N ratios were assessed prior to statistical analyses. Most C:N ratios were < 3.5 in the mother and embryo muscle tissues and histotroph, but variably elevated in yolk (mean ± SD = 7.4 ± 4.3). The latter were excluded from the analyses of $\delta^{13}C$ because the high concentrations of lipids could have produced measurements significantly depleted relative to true values (Hussey *et al.*, 2012).

2.2 | Statistical analyses

The $\delta^{13}C$ and $\delta^{15}N$ data were separately investigated using linear mixed models (LMM), implemented using the lmer package in R (Kuznetsova *et al.*, 2019; www.r-project.org). Initial models included the fixed effect of sample type (mother and embryo muscle, histotroph and for $\delta^{15}N$, the yolk), embryo W_D (*i.e.*, reflecting the embryonic developmental stage) and the interaction of these two effects. The interaction term was eliminated if not statistically significant at the 5% level and similarly for the main effects. The mother was included as a random effect.

To ensure robustness of the type 1 error rate to the modest sample size (n = 44), the LMMs were fitted using restricted maximum likelihood (Millar, 2011) and the Wald F-test used to test significance of terms (Luke, 2017). When the F-test found a significant effect of sample type, significant pairwise differences among the individual sample types were identified using the Benjamini–Hochberg–Yekutieli procedure to control the false discovery rate (FDR) of multiple comparisons (Benjamini & Hochberg, 1995).

3 | RESULTS

The gravid females were mostly caught in April (n = 8), followed by December (n = 2) and one each in March, May and November. The W_D and masses ranged between 110.4 and 120.4 cm (mean ± SD = 114.3 ± 3.0 cm W_D) and 14.8–21.5 kg (18.5 ± 2.3 kg), respectively. Yolk or follicles were collected from all but two gravid females, while histotroph was restricted to five specimens. The embryos were 7.0–42.3 cm W_D (20.3 ± 11.0 cm W_D) and 0.01–1.1 kg (0.2 ± 0.3 kg) and comprised six of each sex (and one that was not sexed). The smallest embryos were sampled in March 2017 and the largest 9 months later in December.

The $\delta^{13}C$ and $\delta^{15}N$ values from the various samples were –19.6 to –15.5‰ and 10.4 to 13.7‰, respectively (Figure 1). Significant main effects in the selected LMM (r^2 = 0.53) for

$\delta^{13}C$ values included both sample type and embryo W_D (but with no interaction; $P < 0.01$; Figure 1a,b). The FDRs separating sample type showed a significantly lower mean value of $\delta^{13}C$ in histotroph than in muscle tissue for either the mothers or embryos ($P < 0.05$), which had the same $\delta^{13}C$ values ($P > 0.05$; Figure 1a). The main effect of embryo W_D had a positive coefficient, manifesting as slightly greater $\delta^{13}C$ values in all three sample types throughout gestation (Figure 1b).

The LMM for $\delta^{15}N$ values ($r^2 = 0.78$) returned a significant interaction between sample type and embryo W_D that presented as negative associations between embryo W_D and the muscle tissue of the mother and her yolk, but positive associations for embryo muscle tissue and histotroph ($P < 0.001$; Table 1 and Figure 1c). The FDR comparisons revealed significant differences in the slopes (*i.e.*, the effect of embryo W_D) between the mother and embryo muscle-tissue samples and between embryo muscle tissue and yolk ($P < 0.05$; Figure 1c). Only the muscle samples from the mothers and embryos had significantly different intercepts (FDR, $P < 0.05$; Figure 1c).

4 | DISCUSSION

This study represents the first assessment of mother–embryo isotope fractionation in a mobulid and one of the few for viviparous elasmobranchs in general (McMeans *et al.*, 2009; Vaudo *et al.*, 2010; Le Bourg *et al.*, 2014; Olin *et al.*, 2018). Collectively, these studies reiterate considerable species-specific variability in fractionation, which now encompasses not only the potential for isotope enrichment–depletion in embryos, but concurrent ^{15}N depletion in the mother as the embryo develops and grows in the uterus. Although the data are few and therefore should be treated with some caution, the observed variation among $\delta^{13}C$ and especially $\delta^{15}N$ signatures in the assessed gravid *M. kuhlii* cf. *eregoodootenkee* can

be discussed according to: (1) the likely requirements during embryonic development; (2) a possible diet shift by gravid females; and (3) the implications for future studies on juvenile foraging ecology.

As might be expected, among the four sample types the yolk had lowest mean $\delta^{13}C$ values (and therefore the greatest C:N ratio), which should at least partially reflect the preferential selection of lighter isotopes during lipid synthesis (McMeans *et al.*, 2009). Of note is that, while required to support embryonic development throughout at least 50% of gestation (Hamlet, 2005), histotroph had much lower C:N ratios (< 3.5) and $\delta^{13}C$ values, which were also significantly lower than in the muscle tissues of the mothers and embryos.

While individual values of $\delta^{13}C$ varied (by –0.7 to 2‰) in both mother and embryo-muscle tissues, mean levels were very similar and certainly followed the same trend of enrichment during gestation (shown by the significant main effect of embryo W_D). There are very few comparative studies, but it is noteworthy that Bourg *et al.* (2014) recorded similar $\delta^{13}C$ signatures between embryos and mothers for *S. megalops* and *C. moluccenis*, which are also aplacental. By comparison, relatively greater muscle $\delta^{13}C$ values were observed in the embryos of the placentatrophic *R. terranovae*, *S. lewini* and *S. tiburo* but depleted in *C. limbatus* (McMeans *et al*., 2009; Vaudo et al., 2010; Olin et al., 2018). Such variable relationships warrant further assessment to more clearly ascertain any reproduction-mode trends.

Unlike $\delta^{13}C$ values, there was a significant interaction between sample type and embryo development for $\delta^{15}N$ values. Previous studies have similarly observed different $\delta^{15}N$ values between mothers and embryos, which have been attributed to increases among the latter (McMeans *et al.*, 2009; Vaudo *et al.*, 2010). The differences here manifested as concurrent increases in $\delta^{15}N$ values in the histotroph and embryonic muscle tissues, but corresponding reductions in the yolk and maternal muscle tissues. While enrichment among near-term

embryos might not be sufficient to reach the expected mean difference of 3.2‰ proposed by McMeans *et al.* (2009) to suggest successive trophic steps, the data nevertheless support such a trend. Specifically, the progressively greater $\delta^{15}N$ values in histotroph (constantly maintained by the maternal muscle tissue), concurrent with the depleting yolk (possibly driven by isotopic routing for the heavier isotopes; Pecquerie *et al.*, 2010) probably reflect the change in nutrient source for the embryo as their yolk is completely consumed by mid-term and the histotroph supports their second half of development.

In the absence of additional data, the mechanisms contributing towards the suggested depletion in ^{15}N among the gravid females during gestation remain speculative. One possible explanation was a dietary shift and if so, this might have occurred in the early stages of pregnancy, considering muscle tissue provides a representative signal of diets over long periods (Kim *et al.* 2012; Vander Zanden *et al.*, 2015). Specifically, given the large proportion of gravid females caught in bather-protection nets observed by Broadhurst *et al*. (2018), these animals may relocate inshore for either mating or to maximise the success of their single embryo in areas with more abundant prey resources than deeper pelagic or epipelagic areas where many mobulids are hypothesised to range (Couturier *et al.*, 2012). Food availability probably contributes towards aggregative behaviour among mobuilids (Dewar *et al.*, 2008) and any variation in diet might register as depleted ^{15}N. Although speculative, the observed consistent increase in $\delta^{13}C$ values (*i.e.*, as a main effect, irrespective of sample type) may have also manifested from a dietary shift requiring a need for lipid reserves, which would affect the ratio of isotopes. Notwithstanding the above, if gravid females had transitioned to a diet depleted in ^{15}N during their early gestation, there might be similar consequences for their histotroph and therefore embryos—unless there was isotopic routing or fractionation occurring during histotroph production. Tagging and

tracking studies would help in deciphering regional migrations and improve clarity on the possible causes for the isotopic variation observed here.

While the mechanisms contributing towards the observed isotopic differences remain speculative, as in previous studies, there was strong evidence of greater $\delta^{15}N$ values in the muscle tissues of embryos than in their mothers, which would confound subsequent assessments of neonate diets and therefore ultimately their habitat use (McMeans *et al.*, 2009; Vaudo *et al.*, 2010; Le Bourge *et al*., 2014). Such dietary confounding might also extend to post-partum females but further data are required to determine the mechanisms affecting ^{15}N depletion in gravid *M. kuhlii* cf. *eregoodootenkee* and whether this is a consequence of nutritional exchange during their gestation, or simply a dietary shift, which would then be implicit among the broader aggregated populations occupying the same area.

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Caption to fig

FIGURE 1 (a) Mean (±SE) $\delta^{13}C$ values for three sample types: embryonic and maternal muscle tissues and histotroph (=, > and < indicate differences detected in false-discovery-rate pairwise comparisons tests; $P < 0.05$). (b) Regressions of embryo disc width (W_D, cm) against $\delta^{13}C$ (combined across the three sample types). (c) $\delta^{15}N$ for four sample types (embryonic and maternal muscle tissues, histotroph and yolk) taken from 13 gravid *Mobula kuhlii* cf. *eregoodootenkee*: ‑‑▽‑‑, embryonic muscle; - -O- -, histotroph; —▼—, maternal muscle; ···●···, yolk.

TABLE 1 Summaries of Wald *F*-values for linear mixed models explaining variability in $\delta^{13}C$ and $\delta^{15}N$ values in 13 gravid *Mobula kuhlii* cf. *eregoodootenkee* due to sample type (from each gravid female's muscle tissue, histotroph and their embryo muscle tissue for $\delta^{13}C$ and also yolk for $\delta^{15}N$) and embryo disc width (W_D, cm). The r^2 values for each main-effects model were 0.53 and 0.78, respectively,

Variable	**$\delta^{13}C$**		**$\delta^{15}N$**	
	F	**df**	***F***	**df**
Sample type (S)	13.51***	2	14.86***	3
Embryo disc width (EW_D)	10.96**	1	0.15	1
Sample × Embryo W_D	3.11	2	5.85***	3

**$P < 0.01$; ***$P < 0.001$.

Figure