


BRIEF COMMUNICATION

# Differences in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ between embryonic and maternal tissues of the ovoviviparous bluntnose sixgill shark *Hexanchus griseus*

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## Abstract

Stable nitrogen ( $\delta^{15}\text{N}$ ) and carbon ( $\delta^{13}\text{C}$ ) isotope ratios from muscle, liver and yolk were analysed from the mother and embryos of an ovoviviparous shark, *Hexanchus griseus*. Embryonic liver and muscle had similar  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  ratios or were depleted in heavy isotopes, compared to the same maternal somatic and reproductive yolk tissues, but no relationship existed between  $\delta^{15}\text{N}$  or  $\delta^{13}\text{C}$  and embryo length, as expected, because a switch to placental nourishment is lacking in this species. This study expands the understanding of maternal nourishment and embryonic stable isotope differences in ovoviviparous sharks.

## KEYWORDS

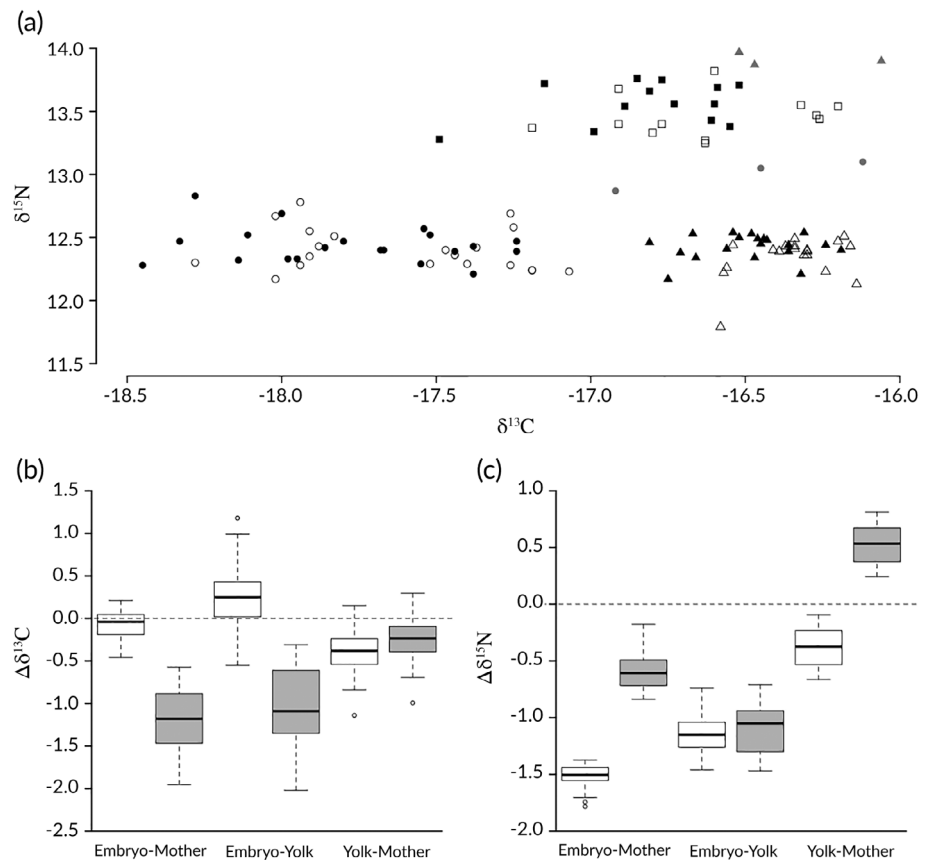
elasmobranch, embryos, liver, maternal provisioning, muscle, stable isotopes, yolk

Stable isotope analysis is an important tool for studying food web interactions and can be especially informative for threatened or poorly studied species (Hussey, MacNeil, *et al.*, 2012). Ratios of nitrogen isotopes ( $\delta^{15}\text{N}$ ) reveal relative trophic position within a food web, whereas ratios of carbon isotopes ( $\delta^{13}\text{C}$ ) provide information about the sources of primary production (*e.g.*, benthic or pelagic) fuelling species in the food web (Hobson, 1999). In elasmobranchs, the slow turnover of tissues impedes the use of these techniques to understand the ecology of young-of-the-year or juvenile sharks (Logan & Lutcavage, 2010; MacNeil *et al.*, 2006), as maternal provisioning can confound stable isotope signatures even after birth (Hussey *et al.*, 2010; McMeans *et al.*, 2009; Olin *et al.*, 2011). Many elasmobranch species exhibit an ontogenetic shift from provisioning to independent feeding as they become more adept at hunting (Belicka *et al.*, 2012; Hussey *et al.*, 2010), but stable isotope analysis can only provide a clear picture of these stages once the effects of maternal provisioning are understood.

Elasmobranch species exhibit a variety of reproductive strategies that could affect stable isotope values in their embryos. Viviparous

species use a pseudo-placenta to nourish developing embryos once yolk reserves are exhausted, whereas oviparous and ovoviviparous elasmobranchs nourish their embryos solely from egg yolk in an external egg or within the uterus, respectively (Conrath & Musick, 2012). In viviparous species, embryos typically have higher  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  compared to maternal somatic and reproductive (yolk, yolk-sac placenta) tissues because of continued maternal provisioning, although variation does exist in this general pattern of enrichment across species, tissues and individuals (McMeans *et al.*, 2009; Olin *et al.*, 2018; Vaudo *et al.*, 2010). Some of this variation is created as embryos switch from yolk to placental nourishment (McMeans *et al.*, 2009; Olin *et al.*, 2018). Isotopic signatures of mothers and embryos have been studied in only two ovoviviparous elasmobranch species to date, the shortnose spurdog *Squalus megalops* Macleay 1881 and the smallfin gulper shark *Centrophorus moluccensis* Bleeker 1860, and no oviparous species. Each ovoviviparous species showed contrasting patterns to viviparous species, as embryos had either similar or lower carbon and nitrogen isotopic ratios compared to mothers (Le Bourg *et al.*, 2014). Stable isotopes in reproductive tissue have been studied in only one species,

**FIGURE 1** Comparisons of stable isotope signatures and fractionation values among embryonic and maternal tissues for *Hexanchus griseus*. (a) The  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values for each tissue from the embryos (black symbols), from the left (open symbols) and right (closed symbols) uteri and from maternal tissue (grey, closed symbols). Boxplot showing the fractionation of (b)  $\Delta\delta^{13}\text{C}$  and (c)  $\Delta\delta^{15}\text{N}$  (embryonic value minus mean maternal somatic value, embryonic value minus paired yolk value or yolk value minus mean maternal value) of each *H. griseus* embryo. (a) (●) Liver, (▲) Muscle, (■) Yolk; (b) (□) Muscle, (▤) Liver; (c) (□) Muscle, (■) Liver



the bonnethead shark *Sphyrna tiburo* L. 1758, which has viviparous reproduction (Olin *et al.*, 2018). More species, especially ovoviviparous ones, need to be studied to understand the variability associated with different reproductive strategies and to support future stable isotope research.

Here, the aim of this study was to expand the understanding of  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  differences between maternal and embryonic somatic tissue (liver and white muscle) in an ovoviviparous elasmobranch species, including a comparison of embryonic and reproductive (yolk) tissues, which has not previously been conducted in sharks with this reproductive strategy. A secondary goal was to compare embryonic  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values based on size, sex and litter in the uterus (right or left) to assess variation in provisioning within the mother as found for other viviparous shark species (McMeans *et al.*, 2009; Olin *et al.*, 2018). It was predicted that without a placental connection, embryos would have contrasting patterns to viviparous species and have similar or lower  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values compared to both maternal somatic and reproductive tissues because, not replaced by consistent maternal nourishment, the heavier isotopes will dilute away from muscles and liver throughout newly developing tissues during embryonic growth.  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  signatures were expected to be similar between the maternal somatic and reproductive tissues. A negative relationship between embryo total length and differences in  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  between embryonic and maternal tissue was also predicted as found for other ovoviviparous species, but no relationship was predicted between length and sex or position (left vs. right) in the uterus.

A 4 m long female bluntnose sixgill shark *Hexanchus griseus* (Bonnaterre 1788) that was found washed ashore dead on 5 February 2019 at Cole's Bay, Vancouver Island, Canada, was sampled. The shark had a total of 72 pups, including one in the cloaca. *H. griseus* is an opportunistic predator that typically occupies deep waters up to 2500 m, although pregnant females migrate to nearshore nursery areas for parturition (Ebert, 1986, 2002, 2003). Currently, its conservation status is uncertain, but the species was protected by Canada's *Species at Risk Act* in 2008 as 'Special Concern' due to incidental capture in fisheries and its late age of maturity (DFO, 2012).

Liver and dorsal white muscle tissue was sampled from the mother and 40 pups, and yolk tissue from 25 pups, subsampled from the 40. All samples were frozen and then dried in a drying oven for a minimum of 48 h before being homogenized into a paste using a mortar and pestle. Because of depletion of  $^{13}\text{C}$  in lipids (Hussey, MacNeil, *et al.*, 2012; Hussey, Olin, *et al.*, 2012), lipid extraction was conducted (modified from Bligh & Dyer, 1959; Olin *et al.*, 2018). Samples were immersed in 2:1 chloroform:methanol, agitated and left 24 h in a 30°C water-bath before being centrifuged and decanted. Each sample was then soaked, agitated, centrifuged and decanted twice more (the liver samples thrice more) before being washed in distilled water and left to dry another 24 h. One pup muscle tissue sample was lost during processing, leaving 39. Samples were then homogenized with a ball-mill grinder (Wig-L-Bug grinder/mixer) or mortar and pestle into a fine powder before 1 mg (mean:  $1.04 \pm 0.03$ ) was weighed and sealed into tin capsules before analysis of  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  using a Thermo Finnigan

**TABLE 1** The coefficients (and *P*-values) from linear regressions of each fractionation value for *Hexanchus griseus* between embryonic liver and muscle tissues, and maternal reproductive yolk tissue and corresponding somatic tissues against total length (in cm), sex (baseline is male) and uterus (baseline is left)

	Total length	Sex (male or female)	Uterus (left or right)
Embryo liver–yolk			
$\Delta\delta^{13}\text{C}$	–0.170 (0.00763)	0.514 (0.00136)	0.507 (0.00695)
	–0.148 (0.114) <sup>a</sup>		
$\Delta\delta^{15}\text{N}$	–0.0762 (0.0343)	0.063 (0.486)	0.112 (0.290)
	–0.101 (0.0507) <sup>a</sup>		
Embryo muscle–yolk			
$\Delta\delta^{13}\text{C}$	–0.0649 (0.328)	0.302 (0.0704)	0.0733 (0.708)
$\Delta\delta^{15}\text{N}$	–0.0140 (0.651)	–0.00207 (0.979)	0.133 (0.148)
Embryo liver–maternal liver			
$\Delta\delta^{13}\text{C}$	–0.0588 (0.215)	0.0831 (0.474)	–0.0960 (0.452)
$\Delta\delta^{15}\text{N}$	–0.0182 (0.386)	0.00336 (0.948)	0.0541 (0.339)
Embryo muscle–maternal muscle			
$\Delta\delta^{13}\text{C}$	0.00513 (0.798)	0.0704 (0.156)	–0.141 (0.00946)
$\Delta\delta^{15}\text{N}$	–0.00509 (0.780)	–0.0295 (0.512)	0.0856 (0.0825)

<sup>a</sup>Without two small (<68 cm) embryos.

Delta Advantage Continuous Flow Isotope Ratio Mass Spectrometer. Standard delta notation ( $\delta$ ) represents the relative abundances of nitrogen ( $^{15}\text{N}/^{14}\text{N}$ ) and carbon ( $^{13}\text{C}/^{12}\text{C}$ ) stable isotopes in parts per thousand:

$$\delta = [(\text{Ratio of sample/ratio of standard}) - 1] \times 10^3$$

Analytical accuracy was within 0.03‰ and 0.13‰, and analytical precision was within 0.17‰ and 0.08‰ for carbon and nitrogen, respectively, based on National Institute of Standards and Technology (NIST; IAEA-N1, IAEA-N2 for nitrogen; IAEA-C-6, NBS-22 OIL for carbon) and DORM-2 (dogfish muscle) standards.

To test the study's predictions, a series of paired *t*-tests were first used to compare  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  among yolk, muscle and liver tissues from the same embryo. A series of one-sample *t*-tests were also conducted to compare  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  from embryonic liver, muscle and yolk tissue to mean  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  from appropriate maternal tissue. *P*-values were controlled for multiple comparisons in each series of *t*-tests using a Bonferroni correction. Variability in  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  was compared among tissues using Levene's tests. Finally, likelihood ratio tests on linear regression models were used to determine if size, sex and position in the uterus affected stable isotope differences ( $\Delta\delta^{15}\text{N}$  and  $\Delta\delta^{13}\text{C}$ , i.e., embryonic value minus mean maternal somatic value, embryonic value minus paired yolk value or yolk value minus mean maternal value) between embryo and mother or embryo and yolk. Normality was checked using Shapiro–Wilk tests.

Mean  $\delta^{15}\text{N}$  ( $\pm$  S.D.) of the yolk ( $13.52 \pm 0.17\text{‰}$ ) was significantly greater compared to that of either embryo muscle ( $12.39 \pm 0.14\text{‰}$ ;

$t = -24.44$ ,  $df = 24$ ,  $P < 0.001$ ) or embryo liver ( $12.42 \pm 0.16\text{‰}$ ;  $t = -24.93$ ,  $df = 24$ ,  $P < 0.001$ ) tissue, which did not differ from each other ( $t = 0.90$ ,  $df = 38$ ,  $P > 0.05$ ; Figure 1). Mean  $\delta^{13}\text{C}$  values of both yolk ( $-16.72 \pm 0.30\text{‰}$ ;  $t = -10.84$ ,  $df = 24$ ,  $P < 0.001$ ) and embryo muscle tissue ( $-16.42 \pm 0.17\text{‰}$ ;  $t = -20.72$ ,  $df = 38$ ,  $P < 0.001$ ) were significantly higher (less negative) than those in the corresponding embryo liver tissue ( $-17.69 \pm 0.37\text{‰}$ ). Embryo muscle tissue also had a slightly higher mean  $\delta^{13}\text{C}$  (by 0.30‰) than the corresponding yolk tissue ( $t = 4.70$ ,  $df = 24$ ,  $P < 0.01$ ; Figure 1). The  $\delta^{13}\text{C}$  signature of the liver and yolk tissue of the embryos was approximately twice as variable as that of the muscle tissues (Levene's test,  $F = 14.28$ ,  $df = 2, 101$ ,  $P < 0.001$ ), whereas the variation in  $\delta^{15}\text{N}$  was similar for all these tissues (Levene's test,  $F = 2.33$ ,  $df = 2, 101$ ,  $P > 0.05$ ; Figure 1).

Embryo muscle ( $t = -67.28$ ,  $df = 38$ ,  $P < 0.001$ ) and liver tissue ( $t = -23.47$ ,  $df = 39$ ,  $P < 0.001$ ) were both lower in  $\delta^{15}\text{N}$  compared to the corresponding maternal tissues, by 1.53‰ and 0.59‰, respectively (Figure 1). Although the  $\delta^{13}\text{C}$  of the embryo muscle tissue was not significantly lower in  $\delta^{13}\text{C}$  compared to maternal muscle tissue ( $t = -2.52$ ,  $df = 38$ ,  $P > 0.05$ ), the embryo liver tissue was significantly lower in  $\delta^{13}\text{C}$  compared to maternal liver tissue, with an average difference of 1.20‰. ( $t = -20.41$ ,  $df = 39$ ,  $P < 0.001$ ) (Figure 1). The difference between maternal somatic tissue and yolk was generally smaller and less negative than that of embryos to yolk or embryos to maternal tissues, especially for  $\delta^{15}\text{N}$  (Figure 1). Compared to maternal muscle tissue, the  $\delta^{15}\text{N}$  of the yolk tissue was significantly lower in  $\delta^{15}\text{N}$ , but only by 0.40‰ on average ( $t = -11.59$ ,  $df = 24$ ,  $P < 0.001$ ), whereas compared to maternal liver tissue, yolk had significantly higher  $\delta^{15}\text{N}$ , by 0.51‰ on average ( $t = 14.86$ ,  $df = 24$ ,  $P < 0.001$ ; Figure 1). For  $\delta^{13}\text{C}$ , yolk had significantly lower  $\delta^{13}\text{C}$  compared to both maternal muscle tissue ( $t = -6.14$ ,  $df = 24$ ,  $P < 0.01$ ) and maternal liver tissue ( $t = -3.72$ ,  $df = 24$ ,  $P < 0.001$ ), but only by 0.37‰ and 0.22‰ on average, respectively (Figure 1).

The only relationship between  $\Delta\delta^{15}\text{N}$  or  $\Delta\delta^{13}\text{C}$  and total length were declines in both for the difference between embryonic liver and yolk, reflecting greater depletion of the heavier isotopes in larger embryos; however, after removing the two smallest (<68 cm total length (TL)) embryos the trend was no longer significant (Table 1). The negative relationships between  $\Delta\delta^{15}\text{N}$  or  $\Delta\delta^{13}\text{C}$  of embryonic muscle and total length found by Le Bourg *et al.* (2014), who sampled a larger size range, in the ovoviviparous *S. megalops* were likely caused by the dilution of a finite supply of heavier isotopes from the yolk into the growing embryos and other developing tissues. In contrast, the small to absent relationships observed likely reflect that these embryos were already near size-at-birth and so, without a shift to placental nourishment (McMeans *et al.*, 2009; Olin *et al.*, 2018), the heavy isotopes available in their yolk had reached their final concentrations throughout the embryos. The size range was small (67.7–73.0 cm TL), limiting the trends that could be observed, and given the effect of the smallest embryos on this trend, a larger range of sampled sizes would likely find a significant relationship between embryo size and  $\Delta\delta^{15}\text{N}$  and  $\Delta\delta^{13}\text{C}$ . Male embryos had a significantly greater  $\Delta\delta^{13}\text{C}$  for the difference from embryonic liver to yolk, but no other relationship existed with sex (Table 1). Embryos also showed greater  $\Delta\delta^{13}\text{C}$  in the right

uterus comparing embryonic liver to yolk and embryonic to maternal muscle, possibly related to differences in the mother's diet between the fertilization and yolk formation for each litter or to inherent variability in isotopic values between individuals (Table 1) (Barnes *et al.*, 2008).

This study's prediction that embryonic  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  of *H. griseus* would be similar or lower in  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  than in its maternal tissues was confirmed. Indeed, it was found that  $\delta^{15}\text{N}$  was even lower in embryonic muscle and liver tissues of *H. griseus*, and  $\delta^{13}\text{C}$  was lower in embryonic liver tissues, compared to the corresponding maternal tissues, than observed in the two previously studied ovoviparous sharks, *S. megalops* and *C. moluccensis* (Le Bourg *et al.*, 2014). The only major difference observed for those ovoviparous sharks was enrichment in  $^{13}\text{C}$  in the embryonic livers of *C. moluccensis* compared to maternal livers (Le Bourg *et al.*, 2014), opposite to the higher  $\delta^{13}\text{C}$  that was observed in the liver tissue of the *H. griseus* mother, likely reflecting varying changes to the mother's diet since yolk production (Bosley *et al.*, 2002; Hesslein *et al.*, 1993; Le Bourg *et al.*, 2014). The lower  $\delta^{15}\text{N}$  in *H. griseus* embryonic muscle tissue could have arisen from a long-term decrease in the trophic level of the mother after provisioning the yolk, which was also reflected in the lower  $\delta^{15}\text{N}$  of the maternal liver relative to the maternal muscle tissue. The small increase in  $\delta^{15}\text{N}$  in the yolk compared to the maternal liver could also indicate recent dietary changes in the mother. Nonetheless, physiological changes caused by pregnancy could also be responsible (Clark *et al.*, 2016; Hussey *et al.*, 2011). The same embryonic *H. griseus* tissues ( $\delta^{15}\text{N}$  muscle and liver;  $\delta^{13}\text{C}$  liver) were also depleted in heavier isotopes relative to the yolk tissue, which contrasts with the increase of  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  in yolk and yolk-sac placenta in the viviparous *S. tiburo* (Olin *et al.*, 2018). The small difference in  $\delta^{13}\text{C}$  of *H. griseus* embryonic muscles compared to yolk was less than the 1‰ increase expected from an individual's diet (DeNiro & Epstein, 1978). Overall, the difference in stable isotope values exhibited in *H. griseus*, even if in different directions than those observed in viviparous species, did still have similar magnitudes to those species (~1.0‰–1.5‰), suggesting common physiological limits to this difference across shark species (McMeans *et al.*, 2009; Olin *et al.*, 2018; Vaudo *et al.*, 2010).

*H. griseus* embryonic tissues also had greater variability in  $\delta^{13}\text{C}$  than  $\delta^{15}\text{N}$ , opposite to the variation within litters of *S. tiburo* (Olin *et al.*, 2018), and it is unclear if this variation relates to the opportunistic diet of *H. griseus* or other mechanisms; for instance, the inherent physiological variability of stable isotopes could simply be greater in this species than others (Barnes *et al.*, 2008). Incomplete lipid extraction could cause underestimation of  $\delta^{13}\text{C}$  and add variability in at least some samples, and this likely occurred for some embryonic liver samples, as their C:N ratio was high (mean: 5.10, range: 4.35–5.74) and had a negative relationship with  $\delta^{13}\text{C}$  (slope =  $-0.54$ , Figure S1, Table S1, Supporting Information). Despite a lower C:N (mean: 3.13, range: 3.06–3.27), a small negative relationship between yolk C:N and  $\delta^{13}\text{C}$  (slope =  $-0.13$ , Figure S1, Table S1, Supporting Information) suggests that incomplete lipid extraction may have occurred in the yolk samples as well. Nonetheless, the incomplete extraction did not lead to great variation in  $\delta^{13}\text{C}$  in other ovoviparous shark species (Le Bourg *et al.*, 2014). Variation in  $\delta^{15}\text{N}$  because of urea retention

(Kim & Koch, 2012) was not a major concern because a significant positive relationship between C:N and  $\delta^{15}\text{N}$  existed only in the muscle (slope = 0.23, Figure S1, Table S1, Supporting Information). Even this disappeared with the removal of two outliers with low C:N and overall had minimal impact on overall conclusions (slope = 0.035, Table S1, Supporting Information). Samples from more mothers of this species would help elucidate the causes and scale of the variation in  $\delta^{13}\text{C}$ , and the lack of  $\delta^{15}\text{N}$  variability, and understand the extent of inherent, within-species isotopic variability in this shark (Barnes *et al.*, 2008).

In conclusion, the predictions of this study that without continuous maternal input like a pseudo-placenta, growing embryos of ovoviparous sharks show a reduction in heavier stable isotopes relative to both their maternal somatic and reproductive tissues, particularly when fully developed and close to parturition were confirmed (Le Bourg *et al.*, 2014). This relative reduction in heavy isotopes during development also occurred in the yolk, as it had similar or smaller  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values compared to maternal liver and muscle, and also between embryos and yolk, extending what is known for ovoviparous sharks. More tissues also need to be examined to assess whether this depletion applies to the whole embryo, or if heavier isotopes are directed to particular tissues during development. The variability in  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values that was found in the same litter will help inform expected variation among individuals of *H. griseus* in food web studies, and results of this study establish a baseline of maternal effects on stable isotope ratios for comparison when studying neonates and juveniles just beginning to feed independently.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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