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From egg to hatchling: preferential retention of fatty acid biomarkers in young-of-the-year Port Jackson sharks *Heterodontus portusjacksoni*

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The muscle and liver fatty acid composition of young-of-the-year (YOY) Port Jackson sharks *Heterodontus portusjacksoni* were investigated to determine the effects of a known dietary lipid source *v*. maternal input as demonstrated by egg yolk fatty acid profiles. Ten *Heterodontus portusjacksoni* egg yolks were collected *in situ* and compared with four hatched *H. portusjacksoni* fed a known diet in a controlled feeding experiment of 185 days. This study demonstrated that fatty acids are probably conservatively transferred from egg yolks to YOY *H. portusjacksoni*, while diet did not have a large effect on the fatty acid composition of the liver or muscle.

Key words: chondrichthyan; controlled feeding experiment; diet; lipid; oviparous.

Fatty acid analysis is increasingly being applied to study the feeding ecology of sharks (Schaufler *et al.*, 2005; Pethybridge *et al.*, 2010, 2011*a*; Wai *et al.*, 2011; McMeans *et al.*, 2012, 2013). The fatty acid profile of any organism, including sharks, is dependent on its original fatty acid composition and the cumulative intake of dietary fatty acids, but is also affected by various metabolic processes associated with growth and maturation (Robin *et al.*, 2003). Consequently, the interpretation of fatty acid profiles is hampered by these factors and controlled experiments are required to investigate which fatty acids are preferentially retained through the various metabolic processes. Oviparous chondrichthyans, such as the Port Jackson shark *Heterodontus portusjacksoni* (Meyer 1793), provide an ideal model to compare fatty acid profiles of egg yolks to young-of-the-year (YOY) *H. portusjacksoni* fed a known diet. As *H. portusjacksoni* develops externally without maternal care, all organic requirements are supplied in the

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egg through the yolk (Rodda & Seymour, 2008). This allows a more thorough understanding of which fatty acids are deposited directly or indirectly as a result of diet, and which are deposited as a result of biosynthesis.

Shifts in fatty acid composition occur following hatching as a result of the preferential retention and utilization of specific fatty acids and possible fatty acid synthesis (Heming & Buddington, 1988). In this context, the aims of this study were to demonstrate whether fatty acids were conservatively transferred from egg yolks to YOY *H. portusjacksoni* and also to determine the effect of a known dietary lipid source on the fatty acid profile of YOY *H. portusjacksoni*. Specifically, the aim was to determine whether the fatty acids present in high concentrations in egg yolks, or in the diet, were reflected in the liver and muscle tissues of YOY *H. portusjacksoni* fed a known diet for 185 days, and whether there were patterns reflective of preferential retention of fatty acids in either tissue.

Sixteen *H. portusjacksoni* egg cases were collected at Lassiters Reef (35° 30′ 38″ S; 138° 13′ 01″ E) off Second Valley, South Australia during October 2010. *Heterodontus portusjacksoni* reach sexual maturity at 11–14 years of age for females and 8–10 years of age for males (McLaughlin & O'Gower, 1971), and have an 18 month period between vitellogenesis and ovulation (Tovar-Ávila *et al.*, 2007). Egg cases remain pliable for *c.* 2 weeks after laying before they harden (Rodda & Seymour, 2008). Of the collected egg cases, 11 were identified as freshly laid because of their clean, soft, pliable casings and olive green colour (Rodda & Seymour, 2008). The remaining five egg cases contained full-term embryos. Ten of the fresh eggs and four of the hard eggs were viable and used in the analysis. Additional eggs or full-term embryos were not collected from additional sites or on additional trips to avoid spatiotemporal variations confounding the results. The likelihood of the egg cases originating from the same female was minimal because of the number of eggs found at the location exceeded the annual 10–18 eggs laid per female (McLaughlin & O'Gower, 1971; Gomon *et al.*, 1994; Last & Stevens, 2009) and that egg cases were collected from different areas within the site.

Egg cases were transported in aerated ambient sea water in an insulated ice box to aquarium facilities at Flinders University, South Australia. The 10 fresh egg cases were sacrificed to analyse yolks for initial fatty acid concentrations. The remaining four egg cases with developed embryos visible inside were maintained in aquaria with ambient recirculating sea water at *c.* 18° C and 38–40 salinity until hatching, which occurred within 7–8 days following collection. The recirculating aquarium systems consisted of four 32 l plastic containers, provided with oxygenated sea water. The tanks were connected to a 32 l sump containing a mechanical filter with standard coarse grade sponge and bio-balls as a biological filtration media. Fifty per cent of the water was changed weekly and *H. portusjacksoni* were fed cockles *Donax deltoides* three times per week to satiation. *Donax deltoides* were all obtained from the Coorong region, South Australia, and maintained at −18° C throughout the study. *Heterodontus portusjacksoni* are known to consume molluscs, including bivalves, which may contribute up to 2·6% by mass to the diet of juveniles (Powter *et al.*, 2010). Bivalves have not been detected in the stomach content of adult and subadult *H. portusjacksoni*, however, high levels of unidentifiable items (*c.* 35%) are common, which could possibly include bivalves (Powter *et al.*, 2010). Water variables including pH 7·9–8·4, ammonia (NH_3:NH_4^+) <0·5 mg l^{-1}, nitrite (NO_2^-) <1·0 mg l^{-1} and nitrate (NO_3^+) < 20 mg l^{-1}, were monitored using an aquarium test kit (API master; www.apifishcare.com) and *H. portusjacksoni* were periodically monitored for total length (L_T) and

mass gain. All research was conducted under Flinders University Animal Welfare permit E301.

Following 185 days in captivity, *H. portusjacksoni* were sacrificed 24 h after feeding by pithing and spinal section. Prior to feeding, the hatched *H. portusjacksoni* measured $195{\cdot}0 \pm 5{\cdot}8$ mm L_T and weighed $44{\cdot}7 \pm 3{\cdot}7$ g (mean ± S.D.). At the conclusion of the experiment, *H. portusjacksoni* measured $241{\cdot}2 \pm 8{\cdot}5$ mm L_T and weighed $96{\cdot}2 \pm 9{\cdot}7$ g. Whole livers and 5–10 g of muscle were collected from the ventral flanks of each individual and frozen at −20° C until analysed. Six whole *D. deltoides*, 10 whole egg yolks, and muscle and liver tissue samples from each individual were homogenized and analysed for fatty acids by the FOODplus Fatty Acid Lab, Urrbrae, South Australia, Australia. Lipids were extracted from diet or tissue samples using a chloroform:methanol (2:1) extraction method as described by Bligh & Dyer (1959). Fatty acids were calculated on a wet mass basis and expressed as $\mu g\, g^{-1}$ and as per cent total fatty acids identified. Fatty acid methyl esters (FAME) were extracted from the tissues and diets, separated and quantified using a gas chromatograph (Hewlett Packard 6890; www.hpl.hp.com) equipped with a flame ionization detection (FID) following the method in Beckmann *et al.* (2013*a*). An external standard (Nu-Chek Prep Inc; www.nu-checkprep.com) was used with 46 different FAME types. Additional fatty acids were identified by the relative locations of other peaks in human blood.

Bray–Curtis similarity matrices were calculated for square-root transformed fatty acid data expressed as $\mu g\, g^{-1}$ to study the differences between dietary items (*D. deltoides*), YOY *H. portusjacksoni* and egg yolks (Primer 6.1.13; www.primer-e.com). The percentage contribution of each fatty acid to the separation between diets and fed *H. portusjacksoni* was assessed using similarity percentage (SIMPER; Clarke, 1993). The similarity of groups was represented by hierarchical cluster analyses based on group-average linking. Differences in fatty acid composition were also analysed using PERMANOVA+ 1.0.3 (Anderson, 2001), with additional pairwise tests conducted using the square root of the PERMANOVA test statistic (a multivariate pseudo *t*-test) under a reduced model using 9999 permutations.

Following hatching, neonates were unreceptive to offers of food for ∼2 weeks. Fasting directly after hatching has previously been observed in young dogfish *Scyliorhinus canicula* (L. 1758) (Wrisez *et al.*, 1993), and is probably related to internal yolk stores still present in neonates at hatching (Rodda & Seymour, 2008). *Heterodontus portusjacksoni* egg yolks contained the highest proportions of total lipid, followed by YOY *H. portusjacksoni* liver and muscle, and *D. deltoides* (Table S1, Supporting Information).

The cluster dendrogram showed high variability in the fatty acid profiles of the *H. portusjacksoni* egg yolks with two clusters of eggs (Fig. 1) and SIMPER analysis indicated 16·4% dissimilarity between the clusters. Docosahexanoic acid (22:6n-3, DHA) was the major driver of dissimilarity contributing 14·1% to the average dissimilarity between the two clusters. One cluster (referred to as DHA-deficient eggs) had a mean DHA concentration of $2180{\cdot}9\, \mu g\, g^{-1}$ compared with the other cluster (referred to as viable yolks), which displayed a mean DHA concentration of $11\,663{\cdot}6\, \mu g\, g^{-1}$ corresponding to a 12·8% difference in DHA proportions between the two clusters (Tables S1 and S2, Supporting Information). In addition, DHA-deficient eggs had significantly different fatty acid profiles to viable yolks (PERMANOVA, $t = 3{\cdot}2$, $P < 0{\cdot}01$). Although DHA is an essential dietary fatty acid, it is unclear whether it is used as an energy source or converted to other physiologically important compounds, such as prostaglandins, during early development. Low concentrations of DHA are,

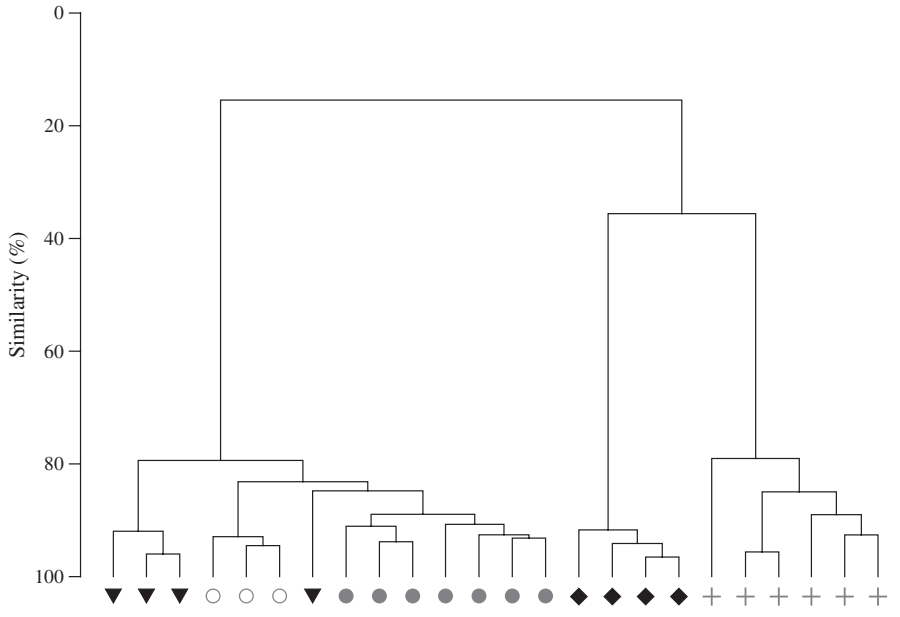

FIG. 1. Cluster dendrogram of fatty acid profiles of *Heterodontus portusjacksoni* egg yolks collected in 2010 at Lassiters Reef, South Australia, and the muscle (♦) and liver (▼) of young-of-the-year fish hatched and fed *Donax deltoides* (+) for 185 days. Samples clustered by group average of ranked Bray–Curtis similarity index based on square-root transformed data expressed as $\mu g\, g^{-1}$ (wet mass). Docosahexanoic acid (DHA)-deficient eggs (○) had significantly different fatty acid profiles to viable eggs (●; PERMANOVA $t = 3{\cdot}2$, $P < 0{\cdot}01$) and displayed a mean DHA composition of 12·8 or 9 482·7 $\mu g\, g^{-1}$ lower than viable eggs.

however, of concern, as this essential fatty acid is required for the growth and functional development of the brain and the nervous system (Bell *et al.*, 1995; Horrocks & Yeo, 1999). The DHA-deficient egg yolks were, therefore, considered to be potentially unviable and removed from further statistical analyses.

Results showed significant differences in the fatty acid profiles (PERMANOVA, d.f. = 3, pseudo-$F = 183{\cdot}4$, $P < 0{\cdot}001$) and pairwise tests disclosed significant differences in the fatty acid profiles of the *H. portusjacksoni* liver, muscle, egg yolks and *D. deltoides* (Table I). The 92·5% average dissimilarity between the composition of fatty acids in viable *H. portusjacksoni* egg yolks and *D. deltoides* was driven by 16:0, DHA, arachidonic acid (ARA, 20:4n-6), 18:1n-9, docosapentaenoic acid (DPA, 22:5n-3), 18:0 and 18:1n-7, which contributed a combined 28·3% to the average dissimilarity. The concentration of all these fatty acids was higher in viable *H. portusjacksoni* egg yolks than in *D. deltoides* (Table S2, Supporting Information). The proportions of 18:0 were higher in *H. portusjacksoni* liver, while 22:6n-3 was higher in *D. deltoides* compared with egg yolks (Table S1, Supporting Information). The fatty acid profiles of *H. portusjacksoni* liver were more similar to viable egg yolks than to their *D. deltoides* diet. This was demonstrated by the low levels of dissimilarity between the fatty acid profiles of *H. portusjacksoni* egg yolks and liver with 17·6% compared with egg yolks and *D. deltoides* with 92·5% dissimilarity (Table I).The composition of *H. portusjacksoni* muscle fatty acids and viable egg yolks showed a

TABLE I. PERMANOVA comparing fatty acid composition ($\mu g\, g^{-1}$) in *Heterodontus portusjacksoni* egg yolks collected in 2010 at Lassiters Reef, South Australia, and the muscle and liver of young-of-the-year fish hatched and fed *Donax deltoides* for 185 days. The average dissimilarity of groups was measured using SIMPER

	PERMANOVA		SIMPER
Groups compared	*t*	*P*	Average dissimilarity (%)
Viable egg yolks, *D. deltoides*	10·1	<0·01	92·5
Liver, *D. deltoides*	13·1	<0·01	91·8
Viable egg yolks, muscle	18·1	<0·01	73·5
Liver, muscle	13·8	<0·05	70·0
D. deltoides, muscle	17·4	<0·001	64·4
Viable egg yolks, liver	3·0	<0·01	17·6

higher level of dissimilarity at 73·5% compared with muscle and *D. deltoides* with 64·4% (Table I). This was probably a result of the high levels of lipid observed in *H. portusjacksoni* egg yolks and liver tissue compared with muscle and *D. deltoides*. High lipid levels in *H. portusjacksoni* egg yolks (16·09%) and liver (11·89%) compared with muscle (0·75%) and *D. deltoides* (0·76%) corresponded to higher overall levels of fatty acids (Table S1, Supporting Information). While proportions of fatty acids may be similar, the absolute amount of fatty acids varied considerably.

While *H. portusjacksoni* egg yolks contained the highest concentration of ARA, the muscle contained the highest proportion of ARA (Tables S1 and S2, Supporting Information). High levels of eicosapentaenoic acid (20:5n-3, EPA) relative to ARA are normally retained in the membrane phosphoglycerides of fish and EPA competitively inhibits the production and efficacy of eicosanoids derived from ARA (Lands, 1989). In this study, higher concentrations and proportions of ARA compared with EPA were detected in *H. portusjacksoni* liver and muscle (Tables S1 and S2, Supporting Information). The YOY *H. portusjacksoni* examined in this study grew rapidly, suggesting high levels of eicosanoid production are normal for growth and development. Hence, studies of fishes sustaining the eggs and newly hatched larvae indicate that ARA concentrations are up to seven times higher than in the normal body lipids of metamorphosed fishes, indicating the high biological importance of ARA (Falk-Petersen *et al.*, 1989; Tocher, 2003). In the muscle and plasma of Greenland sharks *Somniosus microcephalus* (Bloch & Schneider 1801) preferential retention of ARA has previously been reported (McMeans *et al.*, 2012). Physiological compounds such as eicosanoids, which are associated with the release of ARA from membrane phospholipids, are important during larval development even at low physiological concentrations (McPhail *et al.*, 1984; Smith, 1989; Bell *et al.* 1994; Sargent *et al.* 1999; Koven *et al.*, 2001). While dietary concentrations of ARA may influence fatty acid composition of tissues, particularly in the muscle, it appears that the physiological role of ARA may make it unsuitable as an indicator of diet.

The dissimilarity between *H. portusjacksoni* egg yolks and muscle, and egg yolks and *D. deltoides* was also driven by DPA. Higher proportions and concentrations of DPA were measured in *H. portusjacksoni* egg yolks than in muscle tissue, *D. deltoides* and liver tissue (Table S1). Lower DPA levels in *H. portusjacksoni* tissues compared

with egg yolks suggest that this fatty acid is catabolized for energy and not retained in tissues. DPA is predominantly supplied in the diet to marine fishes, however, many species have a limited ability to synthesize DPA from other fatty acid precursors *via* desaturation and elongation (Tocher, 2003). The liver of the individual *H. portusjacksoni* that grew the most during the experiment had high concentrations of DPA, 16:0, 16:1n-7, 18:0, 18:1n-9, 18:1n-7, 20:2n-9 and DHA. This resulted in the liver of this individual being more similar to viable egg yolks than to the liver of the remaining three *H. portusjacksoni* (Fig. 1).

The dissimilarity in fatty acid profiles between *H. portusjacksoni* egg yolks and muscle tissues and egg yolks and liver was driven by the concentrations of 16:0, ARA, 18:1n-9 and 18:1n-7. In addition, differences in the *H. portusjacksoni* egg yolk and muscle tissue fatty acid profiles were driven by DHA, 22:5n-3 and 18:0. Except for 18:0 and DHA, all of the aforementioned fatty acids had higher concentrations and proportions in egg yolks than in liver or muscle. The proportions of DHA observed in *H. portusjacksoni* liver were higher than in the egg yolks and in *D. deltoies*, suggesting some dietary input. Previous controlled experiments have indicated that high proportions of DHA are reflected in *H. portusjacksoni* liver and muscle fatty acid profiles (Beckmann *et al.*, 2013*a*, *b*). The level of DHA within *in utero* shark embryos has also been shown to increase throughout embryonic development (Pethybridge *et al.*, 2011*b*). This indicates that developing embryos require high concentrations of DHA, which are being used for anabolic processes as fast as it is being taken up. This is probably due to the role of DHA in developing visual and neural tissues, which account for a relatively great proportion of total body mass in larval stages (Sargent *et al.*, 2002). The reliance of young sharks on maternal resources has previously been demonstrated in dusky sharks *Carcharhinus obscurus* (LeSueuer 1818), where tissue concentrations of essential fatty acids increased once the initial period of rapid growth had passed (Hussey *et al.*, 2010). Higher proportions of 18:0 were also observed in *H. portusjacksoni* muscle than in egg yolks and higher concentrations in the liver than in egg yolks. As 18:0 can be synthesized *de novo* (Tocher, 2003) it is difficult to determine whether high proportions of 18:0 observed in *D. deltoides* are reflected in tissues.

The fatty acid profiles of the liver of YOY *H. portusjacksoni* were most dissimilar to the fatty acid profile of the *D. deltoides* diet. In comparison, the *D. deltoides* and *H. portusjacksoni* muscle fatty acid profiles grouped more closely. The 91·78% average dissimilarity between the concentrations of fatty acids observed in *D. deltoides* and *H. portusjacksoni* liver and the 64·4% dissimilarity between *D. deltoides* and *H. portusjacksoni* muscle was driven by DHA, 16:0, 18:0 and 18:1n-9. In addition, the dissimilarity between *D. deltoides* and *H. portusjacksoni* muscle was driven by 18:1n-7. Higher concentrations of the aforementioned fatty acids occurred in the liver than in the *H. portusjacksoni* muscle or *D. deltoides*. The *H. portusjacksoni* muscle, however, contained higher proportions of 16:0 than the liver and *D. deltoides*. Overall, higher proportions of SFA were observed in *H. portusjacksoni* muscle compared with the liver, *D. deltoides* and egg yolk. This is consistent with previous studies where deep-water chondrichthyan muscle was typified by high proportions of saturated fatty acids, as well as high relative levels of PUFA (Pethybridge *et al.*, 2010). *Donax deltoides* contained the highest proportions of DHA followed by *H. portusjacksoni* liver, while viable egg yolks contained the highest proportions of 18:1n-9 followed by the muscle. *Heterodontus portusjacksoni* muscle contained the highest proportions of 18:1n-7 followed by viable egg yolks, liver and *D. deltoides*.

This study indicated that fatty acids are conservatively transferred from egg yolks to YOY *H. portusjacksoni*. The fatty acid profiles of *H. portusjacksoni* liver were more similar to viable egg yolks than to their *D. deltoides* diet, indicating that diet was not having a large effect on the fatty acid composition of *H. portusjacksoni* liver. Muscle and viable egg yolk fatty acids from *H. portusjacksoni* showed a higher level of dissimilarity than muscle and *D. deltoides*, however, this was correlated with lipid levels rather than direct dietary effects. High concentrations of ARA in egg yolks were reflected in the liver and proportionally in the muscle of *H. portusjacksoni* and preferential retention of ARA may be indicative of eicosanoid production which is normal for growth and development in young fish. High proportions and concentrations of DPA were measured in egg yolks but were not, however, reflected in *H. portusjacksoni* tissues, suggesting that DPA is catabolized for energy. Higher proportions of DHA were observed in *H. portusjacksoni* liver than in egg yolks, suggesting some dietary input. High proportions of SFA were detected in *H. portusjacksoni* muscle and this may be indicative of the domination of cell-membrane phospholipids resulting in this tissue being less responsive to dietary change than would be expected in the storage fats (Jobling, 2003). Combined with rapid growth and development, the fatty acid profiles of YOY *H. portusjacksoni* are very difficult to relate to diet and are more probably reflective of maternal inputs and indicative of limited foraging ability during this time. This was a short-term study and although sample size was relatively small, the variability within each group was also small and this suggests that the low sample size is unlikely to have affected reliability of the results. Further research assessing the changes in the fatty acid profiles through different life history stages is needed to investigate the effects of metabolic rate related to reproduction. Furthermore, investigations and hatching of DHA-deficient eggs could reveal whether eggs are in fact viable and what effect low levels of DHA have on *H. portusjacksoni* development.

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Supporting Information

Supporting Information may be found in the online version of this paper:

Table S1. Selected fatty acid percentages of total fatty acids identified in *Heterodontus portusjacksoni* egg yolks collected in 2010 at Lassiters Reef, South Australia, and young-of-the-year fish hatched and fed *Donax deltoides* for 185 days. Means ± S.D. are expressed as per cent total fatty acids detected. Docosahexanoic acid-deficient (DHA def.) eggs had significantly different fatty acid profiles to viable eggs (PERMANOVA, $t = 3.2$, $P < 0.01$) and displayed a mean DHA proportion of 12.8% lower than viable eggs.

Table S2. Selected fatty acid concentrations expressed as $\mu g\, g^{-1}$ (wet mass basis) in *Heterodontus portusjacksoni* egg yolks collected in 2010 at Lassiters Reef, South

Australia, and young-of-the-year fish hatched and fed *Donax deltoides* for 185 days. Means ± S.D. are expressed in $\mu g\ g^{-1}$ (wet mass) of total fatty acid detected. Docosahexanoic acid-deficient eggs (DHA def.) had significantly different fatty acid profiles to viable eggs (PERMANOVA, $t = 3.2$, $P < 0.01$) and displayed a mean DHA concentration of 9482.7 $\mu g\ g^{-1}$ lower than viable eggs.

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