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SHORT COMMUNICATION

Rapid embryonic accretion of docosahexaenoic acid (DHA) in the brain of an altricial bird with an aquatic-based maternal diet

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ABSTRACT

Docosahexaenoic acid (DHA) is an important and abundant fatty acid moiety in vertebrate brains. We measured brain phospholipid composition during development in red-winged blackbirds (*Agelaius phoeniceus*), an altricial species that breeds in aquatic habitats. We also manipulated diet by feeding nestlings fish oil or sunflower oil. Finally, we assessed selective uptake of yolk by comparing the yolk fatty acid composition of freshly laid eggs and day-old hatchlings. Relative to other altricial species, blackbirds achieved high DHA in brain phospholipids (20% of phospholipid fatty acids in day-old hatchlings). This was not a result of selective uptake from the yolk, but rather a consequence of a high proportion of DHA in the yolk (2.5% of total lipids) at laying. Our dietary study confirmed that nestling brains are sensitive to fatty acid supply. Red-winged blackbirds may be able to advance cognitive development relative to other altricial species owing to their aquatic maternal diet.

INTRODUCTION

The highly unsaturated docosahexaenoic acid (DHA; 22:6ω3) is a member of the ω3 class of fatty acids, which are nutritionally essential for vertebrates. Although DHA is abundant in aquatic and marine ecosystems, terrestrial vertebrates must either obtain DHA preformed from (usually aquatic) dietary sources or must modify – via elongation and desaturation – its precursor α-linolenic acid, an ω3 fatty acid that is much more common in terrestrial ecosystems (Gladyshev et al., 2013; Hixson et al., 2015; Twining et al., 2016). The ability of terrestrial vertebrates to transform α-linolenic acid to DHA varies among species and diets, but recent reviews have suggested that dietary DHA may be a limiting nutrient or 'ecologically essential' for many terrestrial vertebrates (Gladyshev et al., 2013; Twining et al., 2016).

One reason that DHA can be limiting to terrestrial vertebrates is due to the need for large quantities of this fatty acid in membrane phospholipids of the brain, where its unique biophysical properties likely speed synaptic signaling (Jones et al., 1997; Bowen and Clandinin, 2002; Mitchell et al., 2003). This requirement for DHA should be particularly acute during embryonic and post-natal development (Neuringer et al., 1988; Innis, 1991), when the brain is growing rapidly in size and the proportion of DHA in brain phospholipids is simultaneously increasing. In rats, for example, the proportion of DHA in brain phospholipids more than doubles during the last quarter of gestation (Green et al., 1999; Schiefermeier and Yavin, 2002). Similarly, in birds, DHA represents only 8% of brain phospholipids at early embryonic stages but reaches ∼17% – the proportion observed in some adults – prior to hatching in many species (Maldjian et al., 1996; Farkas et al., 2000; Speake and Wood, 2005). These proportional changes, along with rapid brain growth (Graber and Graber, 1965), thus represent a major demand for DHA during development.

Studies in mammals suggest that inadequacy of DHA in the maternal diet can lead to slower cognitive development in the offspring (Mulder et al., 2014; Janssen et al., 2015). Although we know of no such studies in birds, it seems reasonable to hypothesize that it is beneficial for birds to accumulate DHA in the brain early during development, as long as DHA is readily available, so as to advance cognitive development. This, however, is complicated by mode of development and by diet. Under this hypothesis, precocial species – those that hatch at a relatively advanced stage of development and begin walking and feeding independently soon after hatching – are likely to accumulate DHA in the brain prior to hatching. By contrast, altricial species – those that hatch blind, featherless and dependent on parental feeding – may not need early cognitive development, and may be able to wait until the nestling period to accumulate DHA in the brain if DHA is not readily available as an embryo. Regardless of developmental mode, maternal diet must also play a role, because avian embryos are completely dependent on maternally derived yolk as a source for DHA.

Empirical data generally support this hypothesis. Precocial, semi-precocial and semi-altricial birds hatch with relatively high levels of DHA (∼17%) in their brains (Speake and Wood, 2005). Some of these species (e.g. penguins and coots) can achieve these high levels by the time of hatching because the maternal diet is aquatic or marine based, and thus they produce DHA-rich egg yolks (Speake et al., 2003; Pappas et al., 2007). Other precocial species, such as terrestrial chickens and turkeys, have DHA-poor egg yolks, but instead have the ability as embryos to selectively transfer DHA from the yolk, thus concentrating their small maternal endowment of DHA (Lin et al., 1991; Ding and Lilburn, 1997). Not all precocial birds have this capability, however; ducks, for example, are not able to accumulate high levels of DHA in the brain by the time of hatching if the maternal diet was DHA-poor (Maldjian et al., 1996).

By contrast, altricial birds such as barn swallows and house sparrows hatch with relatively low proportions of DHA in the brain, and brain DHA levels then increase slowly during the nestling period (Speake and Wood, 2005). These birds do not selectively absorb DHA from the yolk as embryos (Speake and Wood, 2005), instead relying on post-natal dietary sources. These data suggest that an altricial developmental pattern is associated with post-natal

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accumulation of brain DHA. However, the altricial species measured to date have been birds that feed in terrestrial ecosystems and therefore have low proportions of yolk DHA. Altricial species with high amounts of DHA available in the maternal diet – such as those that feed in aquatic ecosystems – might instead deposit high amounts of DHA in egg yolks. Embryos, in turn, might accumulate high proportions of DHA in the brain by the time of hatching, despite having an altricial developmental pattern.

Here, we investigated the deposition of DHA in the yolk and brain of an altricial species that feeds in aquatic habitats, the red-winged blackbird (*Agelaius phoeniceus*). Because of its aquatic ecosystem-based diet, we predicted that DHA proportions would be relatively high in its yolk compared to upland altricial birds. In turn, we also predicted that no selective uptake mechanism for DHA would be necessary. Based on our hypothesis that birds should accumulate DHA in the brain early during development if available, we predicted that blackbirds would attain high levels of brain DHA by the time of hatching. Finally, we tested whether nestling brain fatty acid composition is plastic, predicting that brain DHA proportions would be affected by nestling diet.

MATERIALS AND METHODS
Animals, feeding and tissue collection
During the summers of 2015, 2016 and 2017, we conducted regular searches for red-winged blackbird [*Agelaius phoeniceus* (Linnaeus 1766)] nests in marsh habitat at the Lewisville Aquatic Ecosystem Research Facility, Denton County, TX, USA. After locating nests, we visited them daily to monitor progress and determine exact dates of egg laying and hatching. Birds and eggs were collected under Texas Parks and Wildlife Department Permit SPR-0214-034 and US Fish and Wildlife Service Permit MB02732B-2, and all procedures were approved by the University of North Texas Institutional Animal Care and Use Committee.

We collected brains from both embryos and nestlings to determine a timeline of DHA accumulation. Because we required blackbird brains at more time points than contained in the largest clutches (four offspring), and because nest predation was common, we collected birds from many different nests at various ages, and continued collection until we had reached a minimum sample size for each age. We collected brains from five individuals at each embryonic and nestling age, totaling 15 embryos and 60 nestlings, and representing 36 nests. Red-winged blackbirds typically lay clutches of three to four eggs and begin incubating upon laying the penultimate egg, with hatching usually occurring after 11 days of incubation (Yasukawa and Searcy, 1995; E.R.P., unpublished observations). Some eggs were collected after 5 or 8 days of incubation for collection of embryonic brains (denoted e5 and e8). Because blackbirds begin incubation with the penultimate egg, hatching is usually asynchronous, with one egg hatching a day later than the others (Yasukawa and Searcy, 1995). In some nests, we collected this unhatched egg on the day that the rest of the clutch hatched; it was nominally assigned to the 10th day of incubation (e10).

After hatching, we left nests *in situ*, and nestlings were identified via non-toxic marker and later by metal leg bands. In clutches of four eggs, we removed one egg or neonate hatchling to standardize parental feeding effort to three offspring. To test whether diet affects brain fatty acid composition, we randomly divided hatchlings into three groups: control birds were weighed daily but were not dosed, the fish oil group received a single daily dose of fish oil (high DHA and other ω3 fatty acids; Refined Menhaden Oil, MP Biomedicals, Santa Ana, CA, USA), and the seed oil group received a single daily

dose of sunflower oil (low DHA, high 18:2ω6; A&M Gourmet Foods, Toronto, ON, Canada) (oil fatty acid compositions reported in Price et al., 2018). All nestmates received the same treatment but were collected at different ages. After weighing, oil doses were given orally (pipetting slowly into the mouth) beginning 1–2 days post-hatch (dph) at a dose of 30 µl per gram body mass, but limited to the quantity the birds would swallow before rejecting further oil in a given dosing (i.e. we did not force feed them). The single daily dose protocol was designed to provide a semi-natural diet, with most of the nutrition provided by the parents.

Control nestlings were collected at 0, 1, 3, 5, 7 and 9 dph (we define 0 dph to be the day of hatching). Four recently fledged birds were also caught by hand. These did not come from known nests, and we estimated them to be 10–17 dph. We were also able to catch three adult (after second-year) females at the end of the season by mist-net; we present brain lipid composition of these adults for reference. Orally dosed birds were collected at 5, 7 and 9 dph. We transported all birds and eggs from the field to our laboratory at the University of North Texas for dissection. Embryos and nestlings were euthanized by isoflurane overdose followed by decapitation. The birds were then immediately dissected and their brains (cortex) were collected and flash-frozen in liquid nitrogen and then stored at −80°C for later analysis.

To determine whether blackbirds exhibit selective uptake of yolk fatty acids, we compared the composition of yolk from freshly laid eggs with the residual yolk remaining in the internalized yolk sacs of 1 dph hatchlings. Six eggs from unique nests were collected on the day they were laid for determination of initial yolk lipids. Yolks from fresh eggs were separated from albumen and then transferred to a cryo-vial and flash frozen. The residual yolk sacs from 1 dph hatchlings (*n*=7 from unique nests) were carefully removed and then pierced and blotted inside a cryo-vial to collect the residual yolk before flash freezing and storage at −80°C.

Lipid analysis
Lipids were extracted and separated into total lipids, a neutral lipid fraction (mostly triglycerides) and a polar lipid fraction (mostly phospholipids), as previously described (Price et al., 2018). For yolk samples, we measured the fatty acid composition of total lipids, neutral lipids and polar lipids; for brain samples, we discarded the total and neutral lipid fractions so as to focus on membrane lipids, where DHA is proposed to be functionally important. After drying the lipids under N_2, we derivatized lipids with 2 ml 1 mol l^{-1} methanolic HCl at 85°C for 1.5 h to create fatty acid methyl esters. After cooling, we added 1 ml hexane and 1 ml KCl, and then transferred the lipid phase to an autosampler vial for drying. We added dichloromethane as a running solvent. Fatty acid separation, identification and quantification were performed using gas chromatography and mass spectrometry using calibration curves derived from fatty acid methyl ester standards as previously described (Price et al., 2018).

Data presentation and statistics
We present fatty acids as mass percents of the total fatty acids in a given fraction (total lipids, triglycerides or phospholipids). Fatty acid proportions were arcsine square root transformed prior to conducting statistical tests (Zar, 1999). Differences in fatty acid composition between freshly laid eggs and residual yolk of 1 dph hatchlings were detected using Student's *t*-tests assuming unequal variance. For presentation in figures, relative uptake was calculated as the average proportion of a fatty acid in the fresh yolk divided by the average proportion of that fatty acid in residual yolk. Differences

among dietary treatments were determined using ANOVA and Tukey's HSD *post hoc* tests. Because some embryos and nestlings were nestmates when comparing across ages, we investigated the effect of age on brain fatty acid composition using a mixed effects analysis ('lmer' function of the package 'lme4' in R; Bates et al., 2015) with age as a fixed factor and nest as a random factor. Significance of the age effect was tested using a likelihood ratio test comparing the full model with a null model without age as a factor. We performed a *post hoc* analysis comparing ages using the glht function in the 'multcomp' package (Hothorn et al., 2008). Statistical tests were performed using R version 3.2.2 (https://www.r-project.org/). Significance was accepted at $P<0.05$.

RESULTS AND DISCUSSION
Yolk fatty acid composition and selective uptake during embryonic development

In support of our prediction, red-winged blackbird eggs were endowed with substantial proportions of DHA ($2.5\pm0.4\%$ of total lipids), which was primarily due to the high concentration of DHA in yolk phospholipids (Table 1). The fresh yolk fatty acid profile of these blackbirds differs somewhat from that previously reported for red-winged blackbirds collected from Saskatchewan (Surai and Speake, 2008), including the proportion of DHA, which was higher in our birds (2.5% versus 1.6%). This difference in yolk composition presumably represents differences in natural maternal diet between the two studied populations. However, in both studies, DHA represented a relatively high proportion of yolk lipids in comparison with other birds such as flickers, bluebirds, tits and swallows, which eat primarily terrestrial insects and in which DHA represents less than 1% – and often less than 0.5% – of yolk lipids (Surai and Speake, 2008; Toledo et al., 2016). As a marshland species, red-winged blackbirds eat primarily aquatic insects during the breeding season (Yasukawa and Searcy, 1995), which, unlike terrestrial insects, tend to be enriched with DHA (Gladyshev et al., 2013; Hixson et al., 2015). It is therefore likely that red-winged blackbird mothers had relatively abundant quantities of DHA to deposit in yolks owing to this aquatic ecosystem-based diet.

The proportions of several fatty acids were significantly different when comparing fresh yolk with residual yolk (Table 1). This implies that during embryonic development, yolk fatty acids were utilized at different rates. Some fatty acids were more prevalent in the residual yolk compared with their proportions in yolk from fresh eggs, and thus were selectively retained. These include the saturated fatty acid stearate (18:0) (8.0 ± 0.65 versus $16.2\pm3.1\%$; $t_{7.1}=2.9$, $P=0.022$). Others had lower proportions in residual yolk than in yolk from freshly laid eggs, thus exhibiting selective uptake (Table 1), such as the short-chain monounsaturated palmitoleate (16:1ω7) (2.7 ± 0.69 versus $0.34\pm0.18\%$; $t_{10.7}=4.5$, $P<0.001$). In the total lipid fraction, DHA (22:6ω3) was neither highly taken up nor highly retained in the yolk relative to other fatty acids ($t_8=0.06$, $P=0.95$; Fig. S1). This resulted from a slight (and non-significant) selective uptake of DHA in yolk phospholipids ($t_{8.4}=1.5$, $P=0.17$) combined with selective retention of DHA in yolk neutral lipids ($t_{8.6}=6.2$, $P<0.001$; Table 1).

Interestingly, the pattern of relative uptake from yolk was similar to that which occurs during mobilization of fatty acids from adipocytes. As in adipocytes, the relative uptake pattern was related to properties of the fatty acids: all else constant, shorter fatty acids are usually more readily mobilized than longer ones, and fatty acids with more double bonds tend to be more readily mobilized than those with fewer (Raclot, 2003; Price et al., 2008) (Fig. S1). In adipocytes, this has been attributed to enzymatic preferences of lipases as well as easier access of lipases to less hydrophobic fatty acids in the lipid droplet (Raclot, 2003). It is not known what mechanism is responsible for selective fatty acid removal from yolk; in chickens, yolk is predominantly taken up by the yolk sac membrane through a bulk yolk engulfment process (Speake et al., 1998), which does not lend itself to an obvious selective mechanism.

Although several fatty acids showed significant selectivity of uptake from the yolk, this was not the case for DHA. Like other birds that have marine or aquatic diets and lay DHA-rich eggs, blackbirds exhibited little propensity to selectively remove DHA from the yolk during embryonic development (Fig. 1). In this, they were also similar to the altricial house sparrow and barn swallow. In contrast to blackbirds, however, house sparrows and barn swallows lay DHA-poor eggs and exhibit accretion of DHA in the brain relatively late during the nestling period (Speake and Wood, 2005). In terrestrial species that exhibit high selectivity for DHA uptake

Table 1. Fatty acid composition of yolks from freshly laid eggs and residual yolk from red-winged blackbird hatchlings

Fatty acid*	Total lipids		Phospholipids		Neutral lipids	
	Fresh	1 dph	Fresh	1 dph	Fresh	1 dph
14:0	**0.33±0.05**	**0.12±0.10**	n.d.	Trace	0.52±0.13	0.62±0.15
16:0	**30.4±0.97**	**20.79±1.21**	**28.9±0.76**	**21.6±2.3**	**33.4±1.9**	**18.3±1.3**
16:1	**2.7±0.69**	**0.34±0.18**	0.43±0.26	0.24±0.08	**3.2±0.71**	**0.50±0.16**
17:0	**0.32±0.05**	**0.12±0.07**	0.25±0.04	0.33±0.08	0.40±0.04	0.28±0.05
18:0	**8.0±0.65**	**16.2±3.1**	**12.0±0.76**	**21.1±1.0**	6.9±0.71	8.3±0.37
18:1ω9 cis	**27.7±1.2**	**33.8±2.0**	23.1±0.84	20.7±1.2	**30.3±1.2**	**44.0±1.5**
18:1ω9 trans	1.2±0.15	1.6±0.24	1.1±0.23	1.4±0.32	**2.0±0.34**	**3.7±0.54**
18:2ω6	23.7±0.90	20.1±2.1	**22.8±1.5**	**16.7±1.8**	21.9±2.1	19.5±1.1
20:0	n.d.	n.d.	**0.15±0.15**	**1.1±0.26**	n.d.	Trace
20:4ω6	2.5±0.37	3.5±0.49	**4.5±0.22**	**8.1±1.3**	**0.72±0.09**	**1.4±0.23**
20:5ω3	0.59±0.08	0.78±0.23	0.92±0.14	1.4±0.25	**0.16±0.04**	**0.62±0.1**
22:0	n.d.	trace	**0.34±0.17**	**1.7±0.31**	n.d.	Trace
22:6ω3 (DHA)	2.5±0.42	2.5±0.23	5.3±0.34	4.2±0.66	**0.40±0.08**	**1.99±0.35**
ΣSaturated	39.0±1.5	37.4±3.8	41.7±1.4	46.7±3.0	**41.2±2.3**	**27.8±1.2**
ΣMonounsaturated	31.7±1.6	35.7±2.0	24.7±1.1	22.3±1.0	**35.6±1.3**	**48.2±1.6**
ΣPolyunsaturated	29.3±1.1	26.8±2.4	33.6±1.7	30.6±3.4	23.2±2.2	23.9±0.8

*Fatty acid formulae represent (carbon chain length):(number of C=C double bonds)ω(location of the first double bond relative to the methyl end).
Data are means±s.e.m. Values in bold were significantly different among ages, within a fraction ($P<0.05$). $N_{fresh}=6$ for all fractions. $N_{1dph}=7$ for total lipids and 6 for the other fractions.
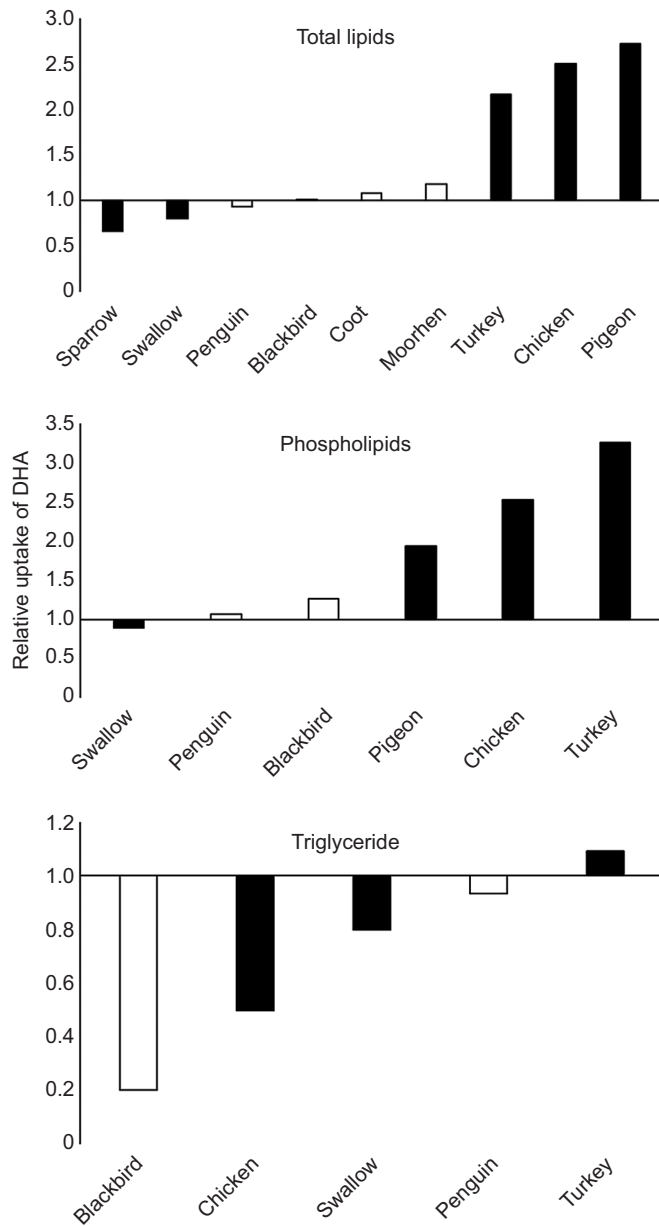
Fig. 1. Relative uptake of docosahexaenoic acid (DHA) from the total lipids, the phospholipid fraction and the triglyceride fraction of yolk from several bird species. Dark bars represent birds with terrestrial diets that lay DHA-poor eggs, whereas light bars denote birds with marine or aquatic diets and DHA-rich eggs. Values greater than unity indicate selective uptake of DHA from the yolk, whereas values less than unity indicate selective retention of DHA. Not shown here is the process of selective uptake of the phospholipid class relative to triglycerides, which can also contribute to the high selective uptake of DHA in turkeys, chickens and pigeons (Vansheel et al., 1981; Noble and Cocchi, 1990; Ding et al., 1995) but has not been measured here. Data are calculated from: Noble and Moore (1964, 1965); Vansheel et al. (1981); Lin et al. (1991); Ding et al. (1995); Ding and Lilburn (1997); Groscolas et al. (2003); Speake et al. (2003); Speake and Wood (2005); Pappas et al. (2007); and the present study. For the chickens of Lin et al. (1991), we used data from animals fed the control diet, and for the turkeys of Ding and colleagues (1995) and Ding and Lilburn (1997), we averaged selective uptake calculated for the two breeding lines.

from the yolk, selective DHA uptake is driven partly by selective uptake of DHA from the phospholipids but not from the triglycerides (Fig. 1). Although we saw the same trend in our
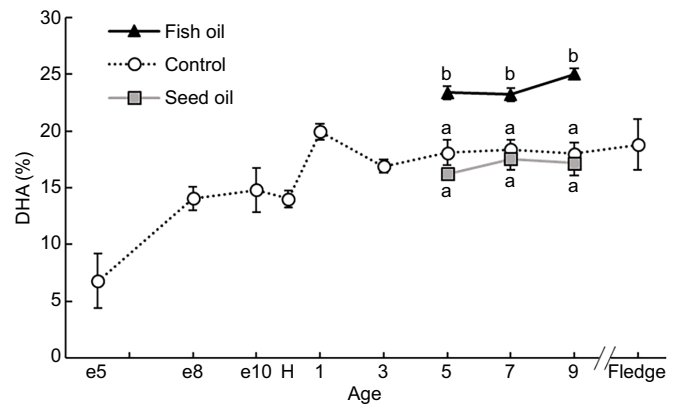

Fig. 2. Proportion of docosahexaenoic acid (DHA) in brain phospholipids over embryonic and post-hatch development in red-winged blackbirds. Eggs hatch on the 11th day of incubation (age 0 on the x-axis, indicated by 'H'). DHA levels were higher in nestlings that had been fed fish oil since 1 dph, but were unchanged in nestlings fed sunflower seed oil. Fledglings were estimated to be 10–17 days old. Data are means±s.e.m. Different letters indicate significant differences among dietary treatments at each age. N=5 for all groups except fledglings, for which N=4. See also Table S1.

blackbird data, it may be that higher selectivity for DHA within the DHA-rich phospholipid fraction or the high selectivity for phospholipids relative to triglycerides (Vanheel et al., 1981; Noble and Cocchi, 1990; Ding et al., 1995) (not measured in the present study) that imparts the overall selectivity for DHA (in total lipids) in those species. Overall, our data support our prediction that owing to their aquatic diet, blackbirds would not need a selective uptake mechanism for DHA.

The timing of brain DHA accretion

DHA increased as a proportion of brain phospholipids during embryonic development (χ^2_9=48, P<0.0001), reaching 20±0.69% by 1 dph (Table S1; Fig. 2). The DHA proportion then stayed relatively constant throughout the nestling period at around 18% of brain phospholipids (Fig. 2). The developmental increase in DHA during embryonic ages was largely offset by decreases in palmitic acid (16:0) (χ^2_9=20.7, P=0.014), stearic acid (18:0) (χ^2_9=36, P<0.0001) and linoleic acid (18:2ω6) (χ^2_9=23, P=0.0053; Table S1). Adults had high levels of brain DHA (28.3±0.71%; Table S1).

Dietary supplementation with fish oil caused a marked increase in brain DHA at all measured ages (ANOVA: $F_{2,12}$>13, P<0.001 for all ages; Tukey: P<0.002 for all ages; Fig. 2). Supplementation with sunflower seed oil caused only a minor and non-significant decrease in brain DHA (P>0.254 for all ages; Fig. 2), but increased the proportion of linoleic acid in brain membranes (ANOVA: $F_{2,12}$>6.3, P<0.0122 for all ages; Tukey: P<0.027 for all ages; Table S1).

In contrast to terrestrial-feeding altricial birds (Speake and Wood, 2005), red-winged blackbirds reached high levels of brain DHA by the first post-natal day (and in fact by the time of hatching) and maintained this through the fledgling stage. This level of DHA (~18%) is similar to that found in precocial, semi-precocial and semi-altricial neonates (Speake and Wood, 2005). This result supports our prediction that blackbirds would achieve high proportions of DHA in the brain early in their development. Given blackbird yolk composition and lack of selective DHA uptake, we submit that this advanced timeline for brain DHA accretion is a product of the high amount of DHA deposited in blackbird eggs, itself a product of the aquatic ecosystem-based, DHA-rich diet of blackbird mothers. Maternal diet is known to have

effects on both yolk DHA composition and the resulting levels of neonatal brain DHA in birds (Anderson et al., 1989; Cherian and Sim, 1992; Maldjian et al., 1996; Speake and Wood, 2005). Furthermore, our dietary study demonstrates that the proportion of DHA in the neonatal blackbird brain is plastic and affected by dietary DHA supply. Given the similarity in lipid transport from the gut and from the yolk sac membrane in birds (Speake et al., 1998), it is likely that the DHA proportion of the blackbird embryonic brain is affected by the composition of the fatty acid supply from the yolk as well.

Currently, there is little information on the importance of early accretion of DHA into the avian brain. Other altricial species measured to date hatch with low levels of brain DHA and even fledge with low levels compared with precocial birds (Speake and Wood, 2005), suggesting that early brain DHA accretion is not critical. However, low brain DHA levels have the potential to delay cognitive development, as indicated by studies in mammals (Mulder et al., 2014; Janssen et al., 2015). Our data suggest the intriguing possibility that aquatic-ecosystem-based species such as the red-winged blackbird can advance cognitive development relative to other altricial species owing to their aquatic maternal diets. Future studies should focus on the cognitive effects of DHA levels in the avian brain.

Supplementary information
Supplementary information available online at
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