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RESEARCH ARTICLE

# The membrane pacemaker hypothesis: novel tests during the ontogeny of endothermy

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## ABSTRACT

The 'membrane pacemaker' hypothesis proposes a biochemical explanation for among-species variation in resting metabolism, based on the positive correlation between membrane docosahexaenoic acid (DHA) and metabolic rate. We tested this hypothesis using a novel model, altricial red-winged blackbird nestlings, predicting that the proportion of DHA in muscle and liver membranes should increase with the increasing metabolic rate of the nestling as it develops endothermy. We also used a dietary manipulation, supplementing the natural diet with fish oil (high DHA) or sunflower oil (high linoleic acid) to alter membrane composition and then assessed metabolic rate. In support of the membrane pacemaker hypothesis, DHA proportions increased in membranes from pectoralis muscle, muscle mitochondria and liver during post-hatch development. By contrast, elevated dietary DHA had no effect on resting metabolic rate, despite causing significant changes to membrane lipid composition. During cold challenges, higher metabolic rates were achieved by birds that had lower DHA and higher linoleic acid in membrane phospholipids. Given the mixed support for this hypothesis, we conclude that correlations between membrane DHA and metabolic rate are likely spurious, and should be attributed to a still-unidentified confounding variable.

**KEY WORDS: *Agelaius phoeniceus*, Membrane pacemaker hypothesis, Docosahexaenoic acid, Mitochondria, Ontogeny, Linoleic acid**

## INTRODUCTION

The membrane pacemaker hypothesis proposes that variation in basal (or resting) metabolic rate (BMR) among species is controlled by the physical characteristics of cell membranes (Hulbert and Else, 1999, 2005; Turner et al., 2005). This hypothesis derives, in part, from the observation that many ATP-consuming processes are membrane related (e.g. the $Na^+/K^+$ ATPase pump) and their activities are affected by membrane fatty acyl composition. While multiple aspects of membranes can affect their properties, including head group composition, much of the research focus has been on the composition of the fatty acid moieties of phospholipid membranes (Hulbert and Else, 1999, 2005). There is a correlation between metabolic rate and polyunsaturation of membranes across species, such that high mass-specific metabolic rates are associated with higher proportions of polyunsaturated fatty acids in cell membranes (Hulbert and Else, 1999; Hulbert et al., 2002a). This correlation is particularly strong for docosahexaenoic acid (DHA), which has the greatest number of double bonds of naturally abundant fatty acids (Hulbert and Else, 1999). Moreover, these associations extend to the differences between endotherms and ectotherms: mammals and birds have both higher metabolic rates and higher proportions of DHA in their membranes than reptiles and amphibians (Brand et al., 1991; Else and Wu, 1999; Hulbert and Else, 1999). Membrane fatty acid composition might therefore serve as a 'metabolic pacemaker' (Hulbert and Else, 1999), determining the rates of cellular processes and, ultimately, the overall metabolic rates of individuals.

Support for the membrane pacemaker hypothesis has been mixed (Calhoon et al., 2015). Primary support comes from cross-species correlations of membrane fatty acid composition, particularly in mammals and birds (Hulbert et al., 2002a,b; Turner et al., 2005). However, a phylogenetically controlled cross-species analysis did not support the link between metabolic rate and membrane unsaturation or DHA content in mammals (Valencak and Ruf, 2007). In contrast, a phylogenetically controlled correlation analysis in orchid bees did support the hypothesis, in that hovering metabolic rate was related to the composition of muscle membrane linolenate (18:3ω3), which was the most unsaturated fatty acid present in the bee membranes (Rodríguez et al., 2015). Elegant crossover experiments, in which $Na^+/K^+$ transporters from mammals are placed into membranes of ectotherms (and vice versa), demonstrate that transporter activity is indeed affected by the membrane in which it sits (Else and Wu, 1999; Wu et al., 2004), although it is not necessarily the fatty acid composition that is the critical membrane characteristic that determines activity.

Experiments at the whole-animal level have been few and often fail to support the membrane pacemaker hypothesis. Artificial selection for high BMR did not increase membrane DHA or double bond index in tissues of mice or voles (Brzęk et al., 2007; Stawski et al., 2015). Dietary manipulation, which can be used to alter tissue lipid composition, often does not alter BMR in the way predicted by the membrane pacemaker hypothesis (Pierce et al., 2005; Ben-Hamo et al., 2011; Pannorfi et al., 2012; Dick, 2017).

A natural extension of the membrane pacemaker hypothesis goes beyond BMR, and suggests that maximal metabolic rate and/or exercise performance are affected by membrane fatty acid composition. This has support from observations of high levels of DHA in high-performance muscles, such as rattlesnake shaker muscles and hummingbird pectoralis muscle (Infante et al., 2001). However, experiments in intact animals (via diet manipulation or artificial selection experiments) have had mixed results and mostly fail to support this extended hypothesis (Ayre and Hulbert, 1997; McKenzie et al., 1998; Wagner et al., 2004; Pierce et al., 2005; Price and Guglielmo, 2009; Wone et al., 2013; Dick, 2017).

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**List of symbols and abbreviations**

| | |
|---|---|
| DHA | docosahexaenoic acid |
| dph | days post-hatch |
| $OXPHOS_{CI}$ | oxidative phosphorylation capacity through complex I |
| $OXPHOS_{CII}$ | oxidative phosphorylation capacity through complex II |
| $OXPHOS_{CI+II}$ | oxidative phosphorylation capacity through complexes I and II |
| $\dot{V}_{O_2}$ | oxygen consumption |

To test further the membrane pacemaker theory and its extension, we used a combination of natural and manipulative experiments. We took advantage of a fascinating developmental period in birds – the transition from ectothermy to endothermy – in which basal and maximal metabolic rates change rapidly over a few days. Birds are ectothermic as embryos, and usually develop endothermy (the ability to defend a high body temperature using metabolic means) between pipping and several weeks post-hatching, depending on the species (Price and Dzialowski, 2017). Our model species, the red-winged blackbird [*Agelaius phoeniceus* (Linnaeus 1766)], hatches without endothermic capacity, and develops the higher basal metabolism and aerobic scope required for endothermy around 8 days post-hatching (Sirsat et al., 2016b). This involves an ∼60% increase in BMR (Dzialowski et al., 2016) and the development of aerobic scope over only 1 week.

Based on the membrane pacemaker hypothesis, we predicted that the membranes of red-winged blackbirds would become more enriched in DHA and increase their double bond index during the first week post-hatching, in association with their increasing basal and summit metabolisms. This is thus a 'natural experiment'; that is, it is based on changes that red-winged blackbirds undergo without experimental manipulation. We focused our measurements on pectoralis muscle and liver; both organs are major contributors to BMR, and skeletal muscle is the major contributor to maximal metabolic rate as it is thought to be the primary site of both shivering and non-shivering thermogenesis in birds (Rowland et al., 2015). We examined changes in the total phospholipid fraction and, in muscles, mitochondrial phospholipids, because the membrane pacemaker mechanism could operate by affecting plasma membrane-bound proteins as well as mitochondrial characteristics, such as the activities of oxidative phosphorylation (OXPHOS) complexes and the rate of proton leak (Brookes et al., 1998).

We also tested the membrane pacemaker hypothesis with a manipulative experiment, using diet to alter the membrane fatty acid composition of nestlings. Dietary fatty acids are readily incorporated into most tissue lipids, including the membranes of most organs (Ayre and Hulbert, 1996; Thil et al., 2003; McCue et al., 2009; Price and Guglielmo, 2009). Based on the membrane pacemaker hypothesis, we therefore predicted that supplementation with a DHA-rich diet would increase basal and maximal metabolic rates and advance the development of endothermy, while a DHA-poor diet would decrease metabolism and delay endothermy. Similarly, we predicted that a dietary manipulation would alter mitochondrial membrane composition, and that higher DHA in membranes would increase mitochondrial leak and OXPHOS complex activities.

## MATERIALS AND METHODS

### Animals and feeding

We found red-winged blackbird (*Agelaius phoeniceus*) nests during the nest-building or incubation stages (May–July) in Denton County, TX, USA. Nests were then visited daily to determine exact dates of hatching. Red-winged blackbirds usually lay 3–4 eggs; in nests with 4 eggs, we removed one to standardize the parental feeding effort. Nests were divided into 3 groups: control birds, which were handled and weighed daily but were not dosed, a fish oil group, which received a daily dose of fish oil (enriched in DHA and other ω3 fatty acids; Refined Menhaden Oil, MP Biomedicals, Santa Ana, CA, USA), and a seed oil group, which received a daily dose of sunflower oil (low DHA, high linoleic acid; A&M Gourmet Foods, Toronto, ON, Canada). The fatty acid compositions of the oils are reported in Table 1. We gave single daily doses orally (pipetting into the mouth) beginning 1–2 days post-hatch (dph) at 3% of body mass, but we did not force feed them: we ceased dosing on a given day if and when a hatchling refused to swallow further oil. Dosing continued daily until the day of collection. This daily dosing procedure was employed to provide a semi-natural diet, with most nutrition being provided by the parents. Nests were randomly assigned to treatments in rotating order based on order of nest discovery.

Control birds were collected 1, 3, 5, 7 and 9 dph (we define 0 dph as the hatch day). We also collected some recently fledged birds that were likely between 10 and 17 days old. In addition, we collected 3 adult females by mist-netting at the end of the breeding season. Orally dosed birds were collected at 5, 7 and 9 dph. Upon collection, birds were transported to our laboratory (30 min by vehicle) for measurement of metabolic rate, collection of tissues, etc. All procedures were approved by the University of North Texas Institutional Animal Care and Use Committee, and the birds were collected under Texas Parks and Wildlife Department Permit SPR-0214-034 and US Fish and Wildlife Permit MB02732B-2.

### Metabolic rate and experimental cooling

We measured oxygen consumption using flow-through respirometry as previously described (Sirsat et al., 2016b) for 5, 7 and 9 dph hatchlings from all treatment groups. Metabolic chambers were placed within a custom-built incubation cabinet with automatic

**Table 1. Fatty acid composition of experimental diets**

| Fatty acid name | Fatty acid formula | Seed oil (%) | Fish oil (%) |
|---|---|---|---|
| Myristate | 14:0 | | 9.5 |
| Pentadecanoate | 15:0 | | 0.7 |
| Palmitate | 16:0 | 3.9 | 22.8 |
| Palmitoleate | 16:1ω7 | | 15.6 |
| Heptadecanoate | 17:0 | | 0.5 |
| Heptadecenoate | 17:1 | | 0.1 |
| Stearate | 18:0 | 2.0 | 3.6 |
| Oleate | 18:1ω9 cis | 13.5 | 7.7 |
| Elaidate | 18:1ω9 trans | 0.6 | 3.6 |
| Linoleate | 18:2ω6 | 79.8 | 3.1 |
| γ-Linolenate | 18:3ω6 | | 0.3 |
| Arachidate | 20:0 | | |
| Eicosenoate | 20:1ω9 | | 1.2 |
| Eicosadienoate | 20:2ω6 | | |
| Eicosatrienoate | 20:3ω6 | | 0.2 |
| Arachadonate | 20:4ω6 | | 1.2 |
| Eicosapentaenoate | 20:5ω3 | | 17.7 |
| Docosanoate | 22:0 | 0.2 | |
| Docosahexaenoate (DHA) | 22:6ω3 | | 12.1 |
| Lignocerate | 24:0 | | |
| Nervonate | 24:1ω9 | | 0.2 |

Fatty acid formula presented as (carbon chain length):(number of carbon-carbon double bonds)ω(location of the first double bond relative to the methyl end).

heating and cooling capability. We allowed the birds to acclimate to the initial temperature (35°C) for at least 30 min. This measurement was used to calculate BMR; unless otherwise specified, all metabolic rates presented are body mass-specific. After acclimation, we gradually decreased the cabinet temperature to 15°C at a rate of 10°C per hour while simultaneously measuring oxygen consumption. This coldest temperature of 15°C was likely not cold enough to elicit a true summit or maximal metabolic rate from 7 and 9 dph birds. However, we suggest that the differences among groups at this temperature would correspond to differences in true summit metabolism, and we interpret variation in these cold-induced metabolic rates as representative of variation in maximal metabolic rates.

### Dissection

Immediately following metabolic rate measurements, we measured body mass and then killed the birds by isoflurane anesthesia overdose. We collected blood by cardiac puncture to measure hematocrit. Heart mass (ventricles only) was measured after fat was trimmed and blood clots removed. After weighing, a piece of the liver was stored for later fatty acid analysis. One pectoralis muscle was removed and weighed. A piece of muscle was stored for fatty acid analysis, while other pieces were saved for mitochondrial isolation from fresh tissue [isolation procedures described previously (Price et al., 2017)] and for measuring mitochondrial respiration in permeabilized fibers (see below). We used calipers (±0.01 mm) to measure head length, from the posterior end of the supraoccipital bone to the tip of the beak, with the skin intact. Femur length (from the femoral head to the distal tips of the condyles) was measured after removal of non-bone tissues. Tissues were stored at −80°C until later analysis.

### Mitochondrial respiration

We permeabilized pectoralis muscle fibers from fresh tissue as previously described (Sirsat et al., 2016b). Briefly, we teased apart fibers on ice and permeabilized them with saponin. We blotted and weighed 2–3 mg of fibers and placed them into an oxygen analyzer chamber (Oxygraph-2K, Oroboros Instruments, Innsbruck, Austria). To this we added catalase and $H_2O_2$ to hyperoxygenate the chamber. We then added substrates and inhibitors sequentially for assessment of mitochondrial function. We used glutamate (10 mmol $l^{-1}$) and malate (2 mmol $l^{-1}$) to assess leak respiration in the absence of ADP, representing proton slip and proton leak across the inner mitochondrial membrane. ADP (5 mmol $l^{-1}$) was added to assess the maximal rate of OXPHOS through complex I ($OXPHOS_{CI}$). We tested membrane integrity by adding cytochrome *c* (10 µmol $l^{-1}$); samples were excluded from analysis if this addition increased oxygen flux more than 20% (Sirsat et al., 2016a). We added succinate (20 ml) to assess maximal capacity for OXPHOS through complexes I and II ($OXPHOS_{CI+II}$). Rotenone (0.5 µmol $l^{-1}$) was added to block the transfer of electrons from complex I to ubiquinone, thus representing maximal flux through complex II ($OXPHOS_{CII}$). These were followed by additions of Antimycin A (2.5 µmol $l^{-1}$) to block the transfer of electrons from coenzyme Q to complex III, and *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD; 0.5 mmol $l^{-1}$) and ascorbate (2.5 mmol $l^{-1}$) to assay maximal complex IV activity. Results are reported as oxygen flux (pmol $O_2$ $s^{-1}$) per mg of muscle fiber (wet). We also calculated several ratios from these data. The ratio of leak:OXPHOS represents the portion of oxygen flux that is necessary to overcome proton leak, and is a measure of inefficiency. The ratio of $OXPHOS_{CI}$:$OXPHOS_{CI+II}$ may demonstrate acclimation toward a particular substrate: higher values should be associated with carbohydrate oxidation, while lower values should be associated with lipid oxidation (Sirsat et al., 2016a).

### Fatty acid composition

We measured the fatty acid compositions of phospholipids from pectoralis muscle and from isolated muscle mitochondria, and of both phospholipids and neutral lipids from the whole liver. We homogenized 20–50 mg of tissues with 1 ml 70°C isopropanol. Another 1 ml of isopropanol was added and the homogenates were incubated at 70°C for 30 min. We then added 1 ml chloroform and allowed the homogenates to extract overnight at 4°C. We separated the lipid and aqueous phases by adding 0.5 ml chloroform and 1 ml 1 mol $l^{-1}$ KCl. The aqueous phase was aspirated and discarded, and the KCl wash was repeated. After again aspirating the aqueous phase, the lipid phase was evaporated under nitrogen gas ($N_2$). To these extracted lipids we added 0.5 ml chloroform for passage through a filter (Millex SLGVX13NL, EMD Millipore, Billerica, MA, USA). We separated this filtrate into a neutral lipid fraction (mostly triglycerides) and a polar lipid fraction (mostly phospholipids) with solid phase extraction tubes as previously described (Price et al., 2017). After drying the lipid fractions under $N_2$, we derivatized them with 2 ml 1 mol $l^{-1}$ methanolic HCl at 85°C for 1.5 h to create fatty acid methyl esters. To this we added 1 ml hexane and 1 ml KCl, and transferred the resulting lipid phase to an autosampler vial for evaporation of the hexane solvent. We added dichloromethane as a running solvent for the gas chromatography–mass spectrometry (GC–MS).

We used gas chromatography (model 6890) for fatty acid methyl ester separation in conjunction with mass spectrometry (model 5973) for quantification using selected ion monitoring (Agilent Technologies, Santa Clara, CA, USA). The gas chromatograph was equipped with an Agilent DB-5 ms column (30 m, 0.25 mm diameter, 0.25 µm film thickness). The injection volume was 2 µl in an inlet at 260°C. The chromatograph was run in pulsed splitless mode with helium as the carrier gas and constant pressure of 8 psi. Inlet pulse pressure was 25 psi until 0.5 min, and purge flow was 15 ml $min^{-1}$ at 0.5 min. The ramping method was as follows: hold 3 min at 40°C, ramp to 175°C at 9°C $min^{-1}$, hold 2 min, ramp to 265°C at 2.5°C $min^{-1}$, hold 1 min, ramp to 300°C at 10°C $min^{-1}$, then hold 5 min. The GC–MS transfer line temperature was 280°C. Peaks were quantified based on external standard curves created from a serial dilution of a mixed standard (Supelco 37 Component FAME Mix, Millipore-Sigma, St Louis, MO, USA). We periodically ran sample blanks, and these did not show any peaks that interfered with the peaks we report.

Fatty acids and most indexes thereof are presented as mass percent; however, the double bond index and peroxidation index are based on molar percent. The 'PUFA balance' index shows the percentage of polyunsaturated fatty acids that are ω3 fatty acids. The 'double bond index' shows the number of double bonds per 100 fatty acyl chains and is calculated as: [(1×%monoenoic)+(2×%dienoic)+(3×%trienoic)+…+(6×%hexaenoic)]. The peroxidizability index was calculated as: [(%monoenioc×0.025)+(%dienoic×1)+(%trienoic×2)+(%tetraenoic×4)+(%pentaenoic×6)+(%hexaenoic×8)] (Pamplona et al., 1998).

### Statistics

For body size measurements and organ masses, we used ANOVA with Tukey's *post hoc* test to test for differences among diet groups. To analyze metabolic rate data across a range of temperatures, we performed a linear mixed effects analysis using the 'lmer' function

in the 'lme4' package in R (Bates et al., 2015). Dietary treatment and ambient temperature were fixed effects in the model, while individual was a random effect. Visual inspection did not reveal obvious deviations from homoscedasticity or normality in residual plots. Significance of fixed effects and interactions were determined using a likelihood ratio test comparing full models against a model without the effect in question. *Post hoc* analysis was performed using the glht function from the multcomp package (Hothorn et al., 2008). Body temperature was analyzed in a similar manner to metabolic rate. Differences in fatty acid percentages among age groups were assessed for the control birds using ANOVA and Tukey's *post hoc* tests after arcsine square root transformation. At a given age, differences among dietary treatments (in fatty acid proportions or mitochondrial oxidation measurements) were assessed using ANOVA with Tukey's *post hoc* tests. We aimed for a minimal sample size of 6 for all groups; actual sample sizes are reported in each table and figure. Fatty acid composition of adult tissues ($N$=3) are not included in statistical analyses, but are presented for reference.

## RESULTS

### Body size and organ mass

Most body and organ measurements were similar between the oil dietary groups, but both of these groups tended to be smaller than controls (Table 2). Body mass was smaller for the diet-manipulated birds, significantly so at 7 dph ($F_{2,31}$=5.46, $P$=0.009). Pectoralis mass also tended to be smaller for the diet-manipulated birds than the controls, and this was significant at 7 dph ($F_{2,20}$=6.0, $P$=0.009). These patterns were not evident in heart mass nor liver mass (Table 2). However, skeletal measurements showed that the diet-manipulated birds tended to be structurally smaller than controls, with head length significantly smaller at all measured ages ($P$<0.0037 for all ages) and femur length smaller at 9 dph ($F_{2,22}$=3.9, $P$=0.035) (Table 2).

### Metabolic rate

The blackbirds mostly maintained metabolic rate in the face of decreasing ambient temperature at 5 dph, and achieved an endothermic response (raising metabolic rate with declining ambient temperature) by 7 dph, regardless of diet treatment (Fig. 1, left panels).

At 5 dph, there was no main effect of dietary treatment on mass-specific $\dot{V}_{O_2}$ ($\chi^2_2$=0.957, $P$=0.6); however, there was a significant interaction between treatment and temperature ($\chi^2_2$=10.2, $P$=0.006), such that the fish oil group was significantly lower than controls at low temperatures ($P$=0.041) (Fig. 1).

At 7 dph, there was no main effect of diet on mass-specific $\dot{V}_{O_2}$ ($\chi^2_2$=2.3, $P$=0.325), but there was a significant interaction with ambient temperature ($\chi^2_2$=35, $P$<0.0001), with both oil treatments having lower mass-specific $\dot{V}_{O_2}$ than controls at low temperatures ($P$<0.001) (Fig. 1).

At 9 dph, there was no main effect of treatment on mass-specific $\dot{V}_{O_2}$ ($\chi^2_2$=1.8, $P$=0.4), but there was a significant interaction with ambient temperature ($\chi^2_2$=13.6, $P$=0.0011) such that the fish oil group was lower than the seed oil group at low temperatures ($P$=0.002) (Fig. 1).

Because the dietary oil groups had smaller pectoralis muscles than the control birds, and due to the importance of pectoralis muscle in affecting summit metabolic rate (Rowland et al., 2015; Price and Dzialowski, 2017), we also analyzed the effects of diet on metabolic rate normalized to pectoralis mass (Fig. 1, right panels). There was no main effect of diet treatment on pectoralis mass-

**Table 2. Body measurements for control and dietary treatment groups during development**

| | 1 dph | 3 dph | 5 dph | 7 dph | 9 dph |
|---|---|---|---|---|---|
| Body mass (g) | | | | | |
| Control | 5.75±0.49 (8) | 12.44±0.77 (8) | 20.36±0.74 (16) | **25.89±0.99 (16)$^a$** | 28.68±1.53 (14) |
| Seed oil | n.m. | n.m. | 18.88±0.78 (9) | **22.14±0.66 (10)$^b$** | 26.27±2.24 (6) |
| Fish oil | n.m. | n.m. | 18.98±1.14 (6) | **22.03±1.14 (8)$^b$** | 22.25±2.01 (6) |
| Pectoralis mass (mg) | | | | | |
| Control | 66±7.0 (7) | 213±1.7 (2) | 372±25 (5) | **671±97 (5)$^a$** | 1018±243 (3) |
| Seed oil | n.m. | n.m. | 297±24 (9) | **481±33 (10)$^b$** | 857±78 (6) |
| Fish oil | n.m. | n.m. | 314±35 (6) | **454±34 (8)$^b$** | 684±82 (6) |
| Heart mass (mg) | | | | | |
| Control | 45±2.5 (7) | 102±5.4 (7) | 146±5.3 (15) | 203±10 (15) | 218±11 (13) |
| Seed oil | n.m. | n.m. | 152±11 (9) | 178±9.1 (10) | 225±24 (6) |
| Fish oil | n.m. | n.m. | 151±13 (6) | 173±12 (8) | 204±20 (6) |
| Liver mass (mg) | | | | | |
| Control | 250±23 (7) | 573±47 (7) | 827±34 (16) | 1063±38 (16) | **965±41 (13)$^a$** |
| Seed oil | n.m. | n.m. | 865±36 (9) | 995±45 (10) | **1178±51 (6)$^b$** |
| Fish oil | n.m. | n.m. | 790±45 (6) | 1014±56 (8) | **1051±81 (6)$^{a,b}$** |
| Femur length (mm) | | | | | |
| Control | 12.7±1.12 (6) | 17.3±0.43 (7) | 21.8±0.33 (15) | 24.1±0.37 (16) | **24.6±0.51 (13)$^a$** |
| Seed oil | n.m. | n.m. | 21.0±0.30 (9) | 22.8±0.37 (10) | **23.7±0.91 (6)$^{a,b}$** |
| Fish oil | n.m. | n.m. | 21.7±0.60 (6) | 22.9±0.47 (8) | **22.0±0.67 (6)$^b$** |
| Head length (mm) | | | | | |
| Control | 19.1±0.45 (7) | 23.5±0.29 (7) | **27.3±0.27 (16)$^a$** | **29.9±0.35 (16)$^a$** | **32.1±0.46 (13)$^a$** |
| Seed oil | n.m. | n.m. | **26.2±0.32 (9)$^b$** | **28.7±0.22 (9)$^b$** | **30.5±0.57 (6)$^b$** |
| Fish oil | n.m. | n.m. | **26.7±0.42 (6)$^{a,b}$** | **28.3±0.33 (8)$^b$** | **29.2±0.50 (6)$^b$** |
| Hematocrit | | | | | |
| Control | n.m. | 0.37±0.03 (4) | **0.40±0.01 (14)$^a$** | 0.44±0.02 (16) | 0.51±0.03 (11) |
| Seed oil | n.m. | n.m. | **0.46±0.03 (6)$^b$** | 0.47±0.03 (7) | 0.46±0.03 (5) |
| Fish oil | n.m. | n.m. | **0.49±0.02 (4)$^b$** | 0.50±0.02 (6) | 0.40±0.03 (5) |

Data are means±s.e.m. ($n$). n.m., 'not measured'. For a given variable, different letters indicate significant difference ($P$<0.05; all are shown in bold) among treatments at that age.

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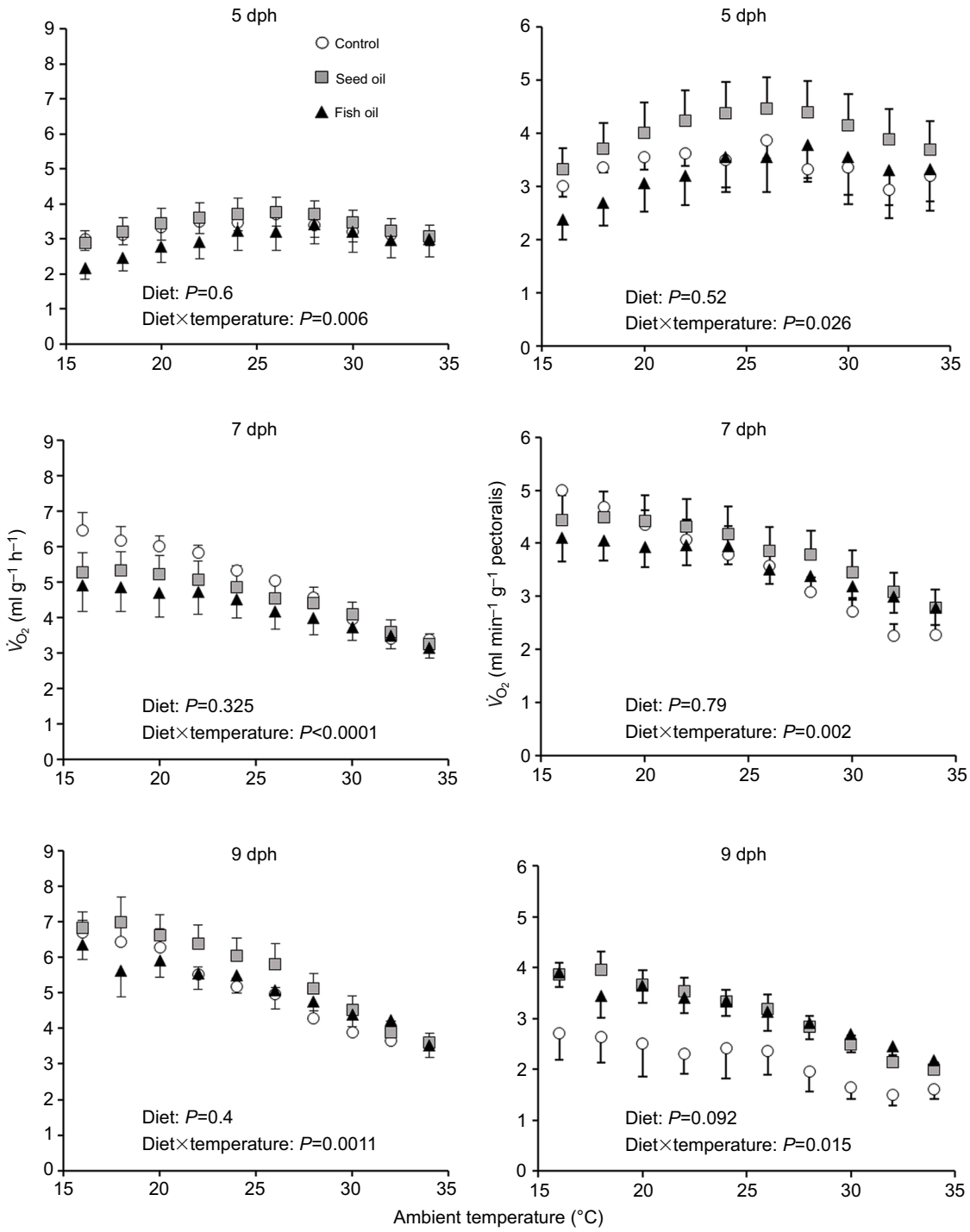


**Fig. 1. Body mass-specific metabolic rate and pectoralis mass-corrected metabolic rate of red-winged blackbird nestlings exposed to experimental cooling.** Nestlings were tested at 5, 7 or 9 days post-hatch (dph) after treatment with dietary seed oil, fish oil or controls. Data are means; whiskers show 1 s.e.m. Sample sizes were as follows: $N_{5dph\ Control}$=9, $N_{7dph\ Control}$=9, $N_{9dph\ Control}$=6, $N_{5dph\ Seed}$=9, $N_{7dph\ Seed}$=10, $N_{9dph\ Seed}$=6, $N_{5dph\ Fish}$=6, $N_{7dph\ Fish}$=8, $N_{9dph\ Fish}$=6. *P*-values are for the main effect of the dietary treatment, or the interaction of diet with ambient temperature.

specific $\dot{V}_{O_2}$ ($P$>0.092 for all ages), but there were significant interactions with ambient temperature at all ages ($P$<0.026 for all), with the seed oil group tending to have the highest pectoralis mass-specific $\dot{V}_{O_2}$, especially at low temperatures (Fig. 1, right panels).

Despite achieving an endothermic response by 7 dph, the blackbirds did not achieve homeothermy at low ambient temperatures until 9 dph (Fig. S1). At 5 dph, there was no main effect of diet treatment on body temperature ($\chi^2_2$=1.2, $P$=0.55), but there was an interaction with ambient temperature ($\chi^2_2$=14.7, $P$=0.04). At days 7 and 9, there were no main effects of diet nor interaction with ambient temperature ($P$>0.1 for all tests) (Fig. S1).

### Fatty acid composition

In pectoralis muscle phospholipids, the polyunsaturated fatty acids increased with age, as a proportion, particularly during the first few days after hatching (Table S1). This was accompanied by declines in

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both the saturated and monounsaturated proportions. The increase in polyunsaturated fatty acids was driven by linoleic acid (18:2ω6), which more than doubled over the first few days, prior to the onset of endothermy (Fig. 2). Between 5 and 9 dph, the period during which endothermy was attained, the DHA proportion nearly doubled (Fig. 2). This caused a slight but non-significant increase in polyunsaturated fatty acids during this later period. Other indices of fatty acid composition, such as double bond index and peroxidizability index, increased over the nestling period, with significant differences between 1 dph and some older ages (Table S1).

Diet had a large effect on individual fatty acids of muscles without having major effects on the proportion of polyunsaturated fatty acids as a group (Table S1). The proportion of DHA was approximately 4-fold higher than controls in the fish oil-treated birds (Fig. 2), whereas the proportion of linoleic acid was about 50% lower in the fish oil group compared with controls or the seed oil group (Fig. 2). Similarly, the PUFA balance, double bond index and peroxidizability index were significantly increased in the fish oil treatment group (Table S1).

The fatty acid composition of mitochondria mirrored that of the skeletal muscle from which they were isolated (Table S2, Fig. 3). The proportion of polyunsaturated fatty acids increased, primarily between 3 and 5 dph, which occurred at the expense of the saturated and monounsaturated proportions (Table S2). This was driven by an increase in linoleic acid between 3 and 5 dph (Fig. 3). The DHA proportion rose steadily, more than doubling between 3 and 9 dph (Fig. 3). The proportion of total polyunsaturated fatty acids did not vary significantly after 5 dph. As in whole muscle, the double bond index and peroxidation index increased steadily over the nestling period, and there were significant differences between 3 dph and later ages (Table S2). Diet again had a large effect, with dietary fish oil causing significant changes in the DHA and linoleate proportions (Table S2, Fig. 3). Although diet did not have significant effects on the total polyunsaturated fatty acids, other measures of membrane composition, including PUFA balance, double bond index and peroxidation index, were highly elevated in the fish oil group (Table S2).

In the liver phospholipids, the DHA proportion increased distinctly between 1 and 3 dph, and then stayed relatively constant at about 18% of the phospholipids (Table S3, Fig. 4). Total polyunsaturated fatty acids, PUFA balance, double bond index and peroxidation index followed a similar pattern with age (Table S3). By contrast, the linoleate proportion stayed relatively constant and high (around 20%) throughout development (Fig. 4). Both dietary oils had major effects on DHA, linoleate and arachidonate (20:4ω6).

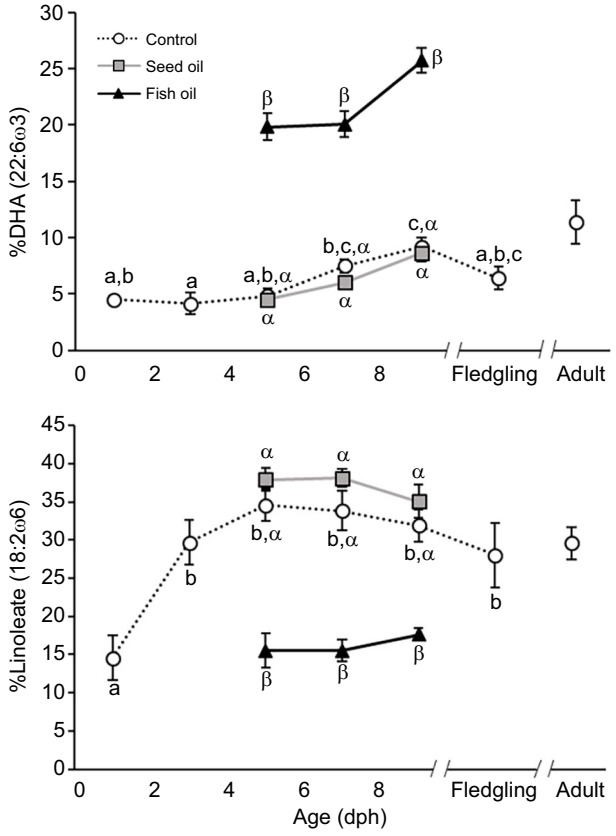


**Fig. 2. Proportion of docosahexaenoic acid (DHA) and linoleate in pectoralis muscle phospholipids during development in red-winged blackbirds.** Data are means±s.e.m. and are mass percents of total phospholipid fatty acids. Latin letters indicate significant differences (ANOVA) among ages within the control group ($P$<0.05). Greek letters indicate significant differences (ANOVA) among diet treatments for a given age ($P$<0.05). Sample sizes are 6 for all juvenile groups, except for the control group at 7 and 9 dph, for which $N$=7. Adults ($N$=3) were not included in statistical analyses.

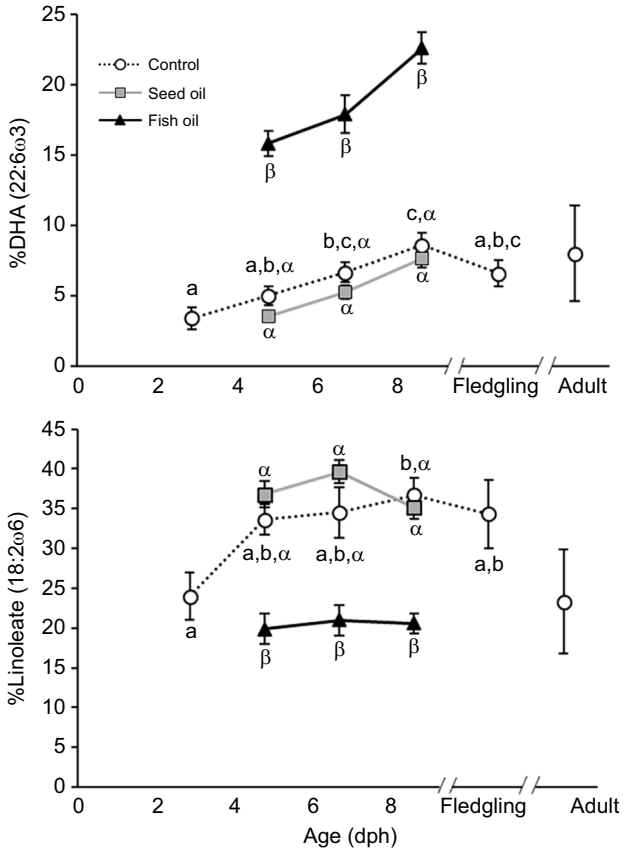


**Fig. 3. Proportion of DHA and linoleate in mitochondrial phospholipids isolated from pectoralis muscle.** Data are means±s.e.m. and are mass percents of total phospholipid fatty acids. Latin letters indicate significant differences (ANOVA) among ages within the control group ($P$<0.05). Greek letters indicate significant differences (ANOVA) among diet treatments for a given age ($P$<0.05). Sample sizes are 6 for all juvenile groups, except for the 3 dph birds and fledglings, for which $N$=4 and 3, respectively. Adults ($N$=3) were not included in statistical analyses.

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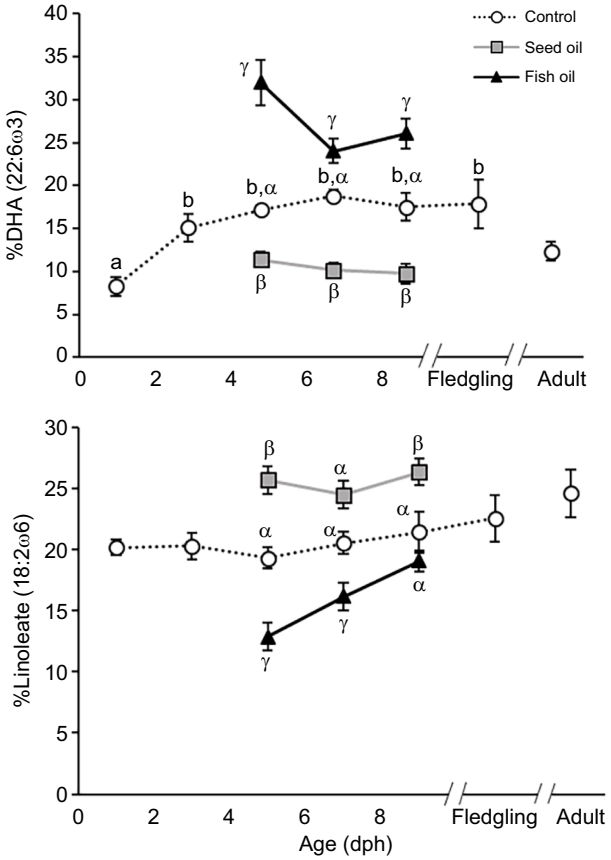


**Fig. 4. Proportion of DHA and linoleate in liver phospholipids during development in red-winged blackbirds.** Data are means±s.e.m. and are mass percents of total phospholipid fatty acids. Latin letters indicate significant differences (ANOVA) among ages within the control group ($P$<0.05). Greek letters indicate significant differences (ANOVA) among diet treatments for a given age ($P$<0.05). Sample sizes are 6 for all groups except for adults ($N$=3), which were not included in statistical analyses.

The seed oil-fed birds had lower proportions of DHA and greater proportions of linoleate at most ages, whereas the fish oil-fed birds had higher DHA and lower linoleate and arachidonate at most ages (Table S3, Fig. 4). Overall polyunsaturation was affected to only a small degree by the diets (Table S3). The experimental diets also had significant effects on various indexes; the seed oil decreased the PUFA balance index, double bond index and the peroxidation index, whereas fish oil increased these indexes (Table S3).

Interestingly, the fatty acid composition of the neutral lipids of the liver did not undergo any significant changes through development (Table S4). However, dietary treatment had substantial and significant effects on the proportions of several fatty acids, with seed oil increasing the proportion of linoleate and decreasing DHA, and fish oil having opposite effects (Table S4). The double bond index was not affected by diet, but the PUFA balance index was raised by fish oil and diminished by the seed oil diet, and the peroxidation index was increased by the fish oil diet (Table S4).

### Mitochondrial respiration in permeabilized fibers

In general, dietary treatment had no effect on mitochondrial function (Fig. 5). At 5 dph, there were no significant differences among treatments for any state ($P$>0.099 for all, Fig. 5). There were similarly no differences in our calculated ratios ($P$>0.6 for all, Fig. 5). At 7 dph, there were no differences among treatments ($P$>0.15 for all states), nor for the ratios ($P$>0.74 for all ratios). At 9 dph, only the $OXPHOS_{CII}$ measurement differed among treatments ($F_{2,13}$=4.5; $P$=0.034) ($P$>0.077 for all others) (Fig. 5). The ratios at 9 dph did not differ by treatment ($P$>0.35 for all).

## DISCUSSION

The development of endothermy and several of its mechanistic correlates have previously been described for red-winged blackbirds (Olson, 1992; Sirsat et al., 2016b). Therefore, we will not discuss this ontogeny other than to note that an endothermic response was reached at a slightly younger age in our study compared with a previous study from our laboratory (Sirsat et al., 2016b). This may have been due to differences in the populations sampled (Texas, USA in the current study versus Michigan, USA in the previous study), and may be associated with the somewhat larger body mass and organ sizes of the birds in our study.

### The ontogeny of membrane lipid composition supports the membrane pacemaker hypothesis

In agreement with predictions of the membrane pacemaker hypothesis, DHA proportions increased in the membranes of pectoralis muscle, muscle mitochondria and liver in association with the development of endothermic capacity. Other measures of fatty acid composition such as the total polyunsaturates and the double bond index also increased with age, in agreement with predictions from the membrane pacemaker hypothesis. For muscle and muscle mitochondria, the increase in membrane DHA was most prominent during the period in which endothermic capacity is reached and aerobic scope develops (5–9 dph). In the liver, the increase in DHA occurred earlier (1–5 dph), during a period in which blackbirds undergo a large increase in mass-specific BMR (Dzialowski et al., 2016) but have little aerobic scope.

An interesting feature of our data is the high proportion of DHA in the liver phospholipid fraction (Fig. 4). The value for 1 dph hatchlings is not unusual compared with several poultry species (Surai et al., 1999). However, unlike chickens, in which the DHA proportion had not changed much by 5 dph (Noble and Cocchi, 1990), the proportion of DHA in liver phospholipids increased to near 18% in our control blackbirds. This high concentration rivals that of the brain of adult birds, which is notoriously DHA rich (Farkas et al., 2000). Nonetheless, this appears to be temporary, because the DHA proportion was much lower in adult livers.

Although these results are consistent with the membrane pacemaker hypothesis and its extension to maximal metabolic rate, there are several alternative interpretations. For example, Speake and Wood (2005) also observed a developmental increase in muscle membrane DHA in altricial swallow hatchlings, but attributed this to a transient need for this fatty acid during critical developmental periods of myoblast fusion and myogenesis. This explanation is not easily applied to our data, however, because adults appear to have higher levels of DHA than hatchlings (Fig. 2).

Another possibility is that the changes in polyunsaturated fatty acids (including both DHA and linoleate) represent a limitation of the yolk fatty acid composition, a limitation that is released once the hatchlings begin to eat and thereby obtain these essential nutrients. However, red-winged blackbird yolk contains about 23% linoleate and 2.5% DHA as a proportion of total fatty acids ($N$=6; E.R.P., B.J.V. and E.M.D., unpublished data). The level of linoleate is therefore higher in the yolk than it is in 1 dph hatchling muscle or liver membranes, suggesting that there was no limitation from the yolk composition. The DHA proportion was higher in both the muscle and the liver of 1 dph hatchlings compared with yolk. Birds

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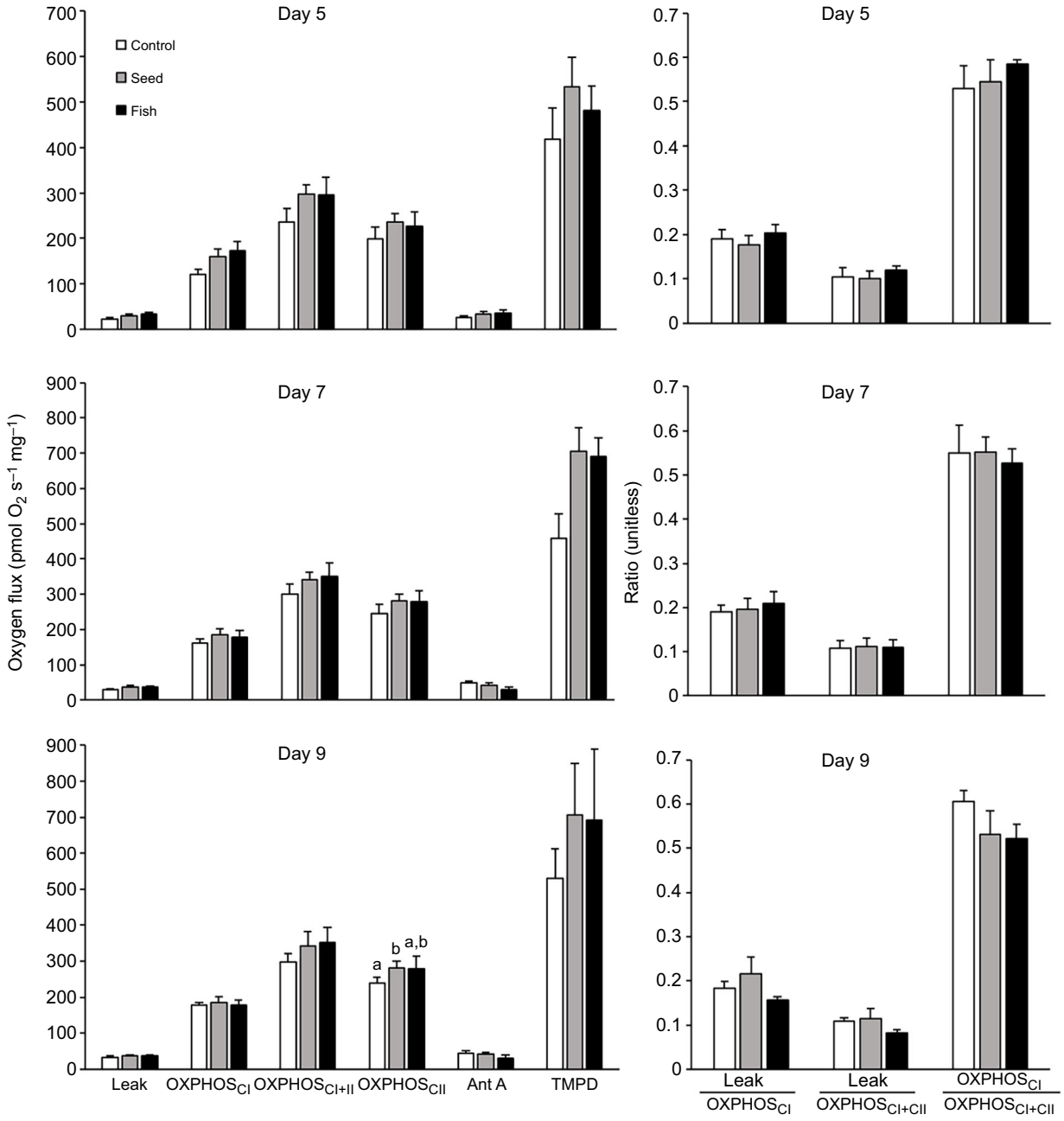


**Fig. 5. Mitochondrial function at several ages in nestling red-winged blackbirds using permeabilized pectoralis muscle fibers.** Left panels show oxygen flux when various electron transport chain complexes are isolated (see Materials and methods for description). Right panels show ratios of several states. Letters indicate significant differences (ANOVA) among treatment groups ($P$<0.05). Data are means+s.e.m. Sample sizes were as follows: $N_{5dph\ Control}$=6, $N_{7dph\ Control}$=8, $N_{9dph\ Control}$=5, $N_{5dph\ Seed}$=7, $N_{7dph\ Seed}$=9, $N_{9dph\ Seed}$=6, $N_{5dph\ Fish}$=6, $N_{7dph\ Fish}$=8, $N_{9dph\ Fish}$=5.

and reptiles have the ability to sequester and biomagnify DHA during embryonic development (Speake et al., 2003; Speake and Wood, 2005; Pappas et al., 2007), but this still leaves open the possibility that yolk DHA content represented a limitation to tissue concentrations.

An obvious candidate for explaining changes in membrane composition is homeoviscous adaptation (Hazel and Williams, 1990). Under this hypothesis, as the birds develop toward higher and consistently high body temperatures, membrane saturation should increase to maintain fluidity characteristics. However, our data are not consistent with this alternative hypothesis, as the proportion of saturated fatty acids generally decreased during development in all tissues, offset by an increase in polyunsaturated fatty acids, such that the double bond index increased during nestling development.

### Our dietary manipulation experiment contradicts the membrane pacemaker hypothesis

The dietary manipulations had the desired effect of altering the DHA content and double bond index of muscle and liver membranes. Both oil treatment groups tended to have smaller body masses and pectoralis masses than controls. We speculate that the smaller pectoralis mass in the dietary treatments was due to lower dietary protein; our addition of oil to their daily diets may

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have led to satiation and lower consumption of their naturally proteinaceous food compared with controls. This may also explain their smaller skeletal measurements, although the skeleton tends to be resistant to dietary alteration (Killpack and Karasov, 2012). Nonetheless, body and organ masses were similar between the 2 oil treatments, so they should serve as good comparisons for each other.

Neither dietary oil treatment advanced the onset of endothermy versus controls, although our sampling time scale may have been too coarse to detect a change. More critically, and contrary to our prediction, the fish oil diet did not increase metabolic rate compared with controls or with the birds that received seed oil. In fact, the fish oil group tended to have the lowest metabolic rate of all three groups, particularly during cold challenges. This was true at all ages, and for both whole-animal metabolic rate (not shown) and mass-specific metabolic rate (Fig. 1). Even when controlling for the smaller pectoralis mass of the dietary groups, the fish oil group had similar or lower metabolic rates than the seed oil group at all ages (Fig. 1). The results of our dietary manipulation experiment therefore contradict the membrane pacemaker hypothesis. These findings are similar to those of Pierce et al. (2005) and Dick (2017), who manipulated tissue phospholipid fatty acid composition via diet but did not observe an effect on BMR in birds, and with Price and Guglielmo (2009), who found a negative effect of muscle DHA on maximal metabolic rate. In contrast, Wagner et al. (2004) saw a positive effect of muscle DHA on critical swimming speed in fish.

Although dietary fish oil increased the DHA proportion and double bond index in all tissues, it had little effect on the total polyunsaturated fatty acids, indicating that there may be some homeostatic response to dietary fatty acid composition. If the total polyunsaturated proportion is the main determinant in controlling membrane processes, this might indicate that our dietary manipulation was not a valid test of the membrane pacemaker hypothesis. Although we do not know exactly which membrane characteristics may affect membrane-bound protein activity, the double bond index and DHA in particular have been proposed to be more important than total polyunsaturated fatty acids in controlling metabolism (Hulbert et al., 2002a,b); thus, we suggest that this was a valid test of the hypothesis. It is also possible that there were other homeostatic mechanisms, such as changes in phospholipid head-group composition or molecular species composition, which could have offset any changes to membrane properties caused by diet-induced alterations of membrane fatty acid composition. Such homeostatic mechanisms were beyond the scope of this study.

The use of diet to manipulate membrane composition can have non-target effects that could influence metabolic rate via other mechanisms (Price, 2010; Pierce and McWilliams, 2014). For example, diet can affect the composition of stored lipids, an effect observed in our liver neutral lipid data. The composition of stored triglycerides then has the potential to affect maximal metabolic rate due to differences in mobilization and catabolic rates for various fatty acids (Price et al., 2008, 2011; Price and Guglielmo, 2009). Thus, it is conceivable that elevated DHA in the membranes of the fish oil group had the effect of increasing metabolic rate, but that this was washed out by a greater effect decreasing metabolism via other aspects of physiology that were affected by diet. It is also notable that the DHA-poor seed oil did not significantly diminish the proportion of DHA in muscle membranes in comparison to controls (Fig. 2, top), suggesting that there may be an important developmental trajectory for muscle membrane DHA that is relatively resistant to dietary scarcity. Nonetheless, the simplest explanation is that membrane lipid composition did not affect metabolic rate in line with the membrane pacemaker hypothesis.

### The high maximal metabolic rate of the seed oil group may have been mediated via the effects of linoleate on SERCA activity

Although BMR was generally similar among dietary treatments, an exciting finding was that the seed oil group often reached higher cold-induced metabolic rates than the other groups, particularly compared with the fish oil group, with which it was well size matched. This result joins several other studies in vertebrates that have found higher maximal metabolic rates or exercise performance when fed linoleate-rich diets (Ayre and Hulbert, 1997; McKenzie et al., 1998; Pierce et al., 2005; Price and Guglielmo, 2009). Artificial selection for high maximal metabolic rate can result in higher linoleate proportions in muscle membranes (Wone et al., 2013; Stawski et al., 2015), which suggests that this effect may be mediated by diet-induced alterations of membrane composition. Relatedly, a positive correlation was found between muscle membrane linoleate and the maximum running speed of mammals (Ruf et al., 2006).

Linoleate has been proposed as an important mediator of sarcoplasmic reticulum $Ca^{2+}$ ATPase (SERCA), the pump that clears $Ca^{2+}$ from the muscle cytosol after each contraction (Ruf and Arnold, 2008). The hypothesis is based on correlations between cardiac sarcoplasmic reticulum linoleate content and SERCA activity (Swanson et al., 1989; Giroud et al., 2013), and seasonal changes in cardiac muscle phospholipids in hibernators (Ruf and Arnold, 2008; Giroud et al., 2013), which require higher SERCA activity in the face of low body temperatures during torpor.

While this hypothesis has focused on cardiac SERCA, skeletal muscle SERCA (a different isoform) may interact similarly with membrane linoleate. With respect to the ontogeny of endothermy, the activity of muscle SERCA can be important for two thermogenic pathways. As a key enzyme for $Ca^{2+}$ handling, SERCA activity is important for muscle contraction associated with shivering. Additionally, the uncoupling of SERCA from $Ca^{2+}$ cycling via sarcolipin has been proposed as an important mechanism for non-shivering thermogenesis, particularly in birds (Rowland et al., 2015). Thus, we propose that the effect of seed oil on improving cold-induced metabolic rate occurred due to the effects of membrane linoleate on SERCA activity. This could also explain the steep developmental rise of linoleate in the muscle membranes of control birds [a similar rise in muscle linoleate also occurs in swallow hatchlings (Speake and Wood, 2005)]. Further studies should investigate this putative mechanism during the ontogeny of endothermy.

### Dietary manipulation had no effect on mitochondrial respiration

Because our mitochondrial data are normalized per milligram of muscle fiber, we prefer to assess proton leak relative to OXPHOS respiration to help account for differences in mitochondrial density. Across vertebrates, proton leak in liver mitochondria differs between endotherms and ectotherms, and in endotherms is correlated with mitochondrial membrane DHA or fatty acyl unsaturation (Brand et al., 1994, 2003; Brookes et al., 1998). However, despite a difference in muscle mitochondrial proton leak: OXPHOS between endotherms and ectotherms, there is not a

developmental increase in this ratio during the avian ontogeny of endothermy (see discussion in Sirsat et al., 2016a; Price and Dzialowski, 2017). This is true despite the changes in muscle mitochondrial fatty acid composition, documented here, that birds undergo during this period.

Similarly, we found no major differences in muscle mitochondrial OXPHOS capacity or leak:OXPHOS among our dietary groups. Previous dietary manipulations have had varied and mostly minor results on mitochondrial function (Martin et al., 2015), including higher proton leak in association with linoleate-rich diets and membranes (Gerson et al., 2008; Crescenzo et al., 2012), no effect of diet (Dudognon et al., 2014) or complex effects that are related to minor membrane components (Guderley et al., 2008; Martin et al., 2013). Overall, the differences in cold-induced metabolic rate we observed in different treatment groups do not seem to have been mediated by mitochondrial function nor fatty acid composition. However, because we do not have an independent measurement of mitochondrial density or the electron transport system, we cannot exclude the possibility that the mitochondria have compensated (e.g. via proliferation or head-group adjustments) for diet-induced changes in fatty acid composition (Martin et al., 2015). The muscle mitochondria also did not become 'geared' toward the lipid substrate, because the ratio of $OXPHOS_{CI}$:$OXPHOS_{CI+II}$ did not decrease significantly in the oil treatment groups relative to controls. This may indicate that the mitochondria are relatively invariant with respect to substrate, or that the mitochondria were already acclimatized to oxidizing primarily lipid substrates in the control condition.

## Perspectives on the membrane pacemaker hypothesis

As if a microcosm for membrane pacemaker research, we found mixed support for the hypothesis. Our natural ontogeny experiment joins cross-species correlation analyses in supporting the membrane pacemaker hypothesis (Hulbert et al., 2002a,b; Turner et al., 2005). But our diet experiment joins a host of manipulative experiments that fail to support it (Pierce et al., 2005; Brzęk et al., 2007; Ben-Hamo et al., 2011; Pannorfi et al., 2012; Stawski et al., 2015; Dick, 2017). How can we rectify these mixed results? To us, the simplest solution is to note that correlation and association do not imply causation. Correlations of membrane DHA with metabolic rate might arise for various reasons, and these variables may not be functionally connected. From our viewpoint, alternative explanations for cross-species correlations and ontogenetic associations are multiple and easier to accept than alternative explanations for manipulative experiments, which have increased BMR without increasing membrane DHA (Brzęk et al., 2007; Stawski et al., 2015), and have increased membrane DHA without altering BMR (Pierce et al., 2005; Ben-Hamo et al., 2011; Pannorfi et al., 2012; Dick, 2017; present study).

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

Conceptualization: E.R.P., T.S.S., E.M.D.; Methodology: E.R.P., T.S.S., S.K.G.S., T.C., B.J.V., E.M.D.; Validation: E.R.P.; Formal analysis: E.R.P.; Investigation: E.R.P., T.S.S., S.K.G.S., T.C., B.J.V., E.M.D.; Resources: B.J.V., E.M.D.; Data curation: E.R.P.; Writing - original draft: E.R.P.; Writing - review & editing: E.R.P., T.S.S., B.J.V., E.M.D.; Visualization: E.R.P.; Project administration: E.M.D.; Funding acquisition: E.M.D.

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### Supplementary information

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