

Taking the short- or long-chain route: conversion efficiency of alpha linolenic acid to long-chain omega-3 fatty acids in aerial insectivore chicks

Running head: Tree Swallow ALA to LCPUFA conversion

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1 **Summary Statement**

2 A stable-isotope-labeled tracer reveals the mechanism for omega-3 long-chain polyunsaturated  
3 fatty acid (LCPUFA) limitation in a wild avian insectivore, showing that LCPUFA are an  
4 ecologically essential nutrient.

5  
6 **Abstract**

7 Food availability and quality are both critical for growing young animals. In nature,  
8 swallows (*Tachycineta bicolor*) and other aerial insectivores feed on both aquatic insects, which  
9 are rich in omega-3 long-chain polyunsaturated fatty acid (LCPUFA) and terrestrial insects,  
10 which contain considerably less LCPUFA. Carnivorous mammals and fishes must obtain  
11 LCPUFA from diet, as they have lost the capacity to convert the precursor omega-3 ALA into  
12 LCPUFA. Thus, the relative value of aquatic versus terrestrial insects depends not only on the  
13 fatty acid composition of the prey, but also upon the capacity of consumers to convert ALA into  
14 LCPUFA. We used a combination of stable-isotope-labeled fatty acid tracers to ask if, and how  
15 efficiently, Tree Swallows can deposit newly synthesized LCPUFA into tissue. Our data show  
16 for the first time that Tree Swallows can convert ALA into LCPUFA deposited in liver and  
17 skeletal muscle. However, high Tree Swallow demand for LCPUFA combined with low ALA  
18 availability in natural terrestrial foods may strain their modest conversion ability. This suggests  
19 that while Tree Swallows can synthesize LCPUFA de novo, LCPUFA are ecologically essential  
20 nutrients in natural systems. Our findings thus provide mechanistic support for our previous  
21 findings and the importance of LCPUFA-rich aquatic insects for Tree Swallows and most likely  
22 other aerial insectivores with similar niches.

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## 26 **Introduction**

27           Although dietary resources are crucial for animals throughout their life cycle, energy and  
28 nutrients are particularly critical during early life, especially for animals, like birds, that undergo  
29 rapid determinate growth. For wild birds with young that reach mature size rapidly, timing  
30 breeding phenology with food availability is essential for survival (Lyon et al. 2008). Climate  
31 change is already creating mismatches between the phenology of insect prey and the breeding  
32 timing of insectivores like Pied Flycatchers (*Ficedula hypoleuca*; Both et al. 2006) and Great  
33 Tits (*Parus major*; Nussey et al. 2005). However, even if food resources are available during the  
34 breeding season, food availability in terms of energy alone may not be sufficient for successful  
35 breeding success if available foods lack key nutrients. Recent studies suggest that there is also  
36 the potential for mismatches between the nutritional composition of available food resources and  
37 the complex nutritional needs of growing chicks (Twining et al. 2016a; Twining et al.  
38 submitted).

39           Food quality can be defined in many ways, including caloric density, nutrient  
40 composition, and digestibility. Here, we focus on food quality in terms of the availability of  
41 long-chain polyunsaturated omega-3 fatty acids (LCPUFAs), which we previously found to be  
42 more important for insectivore chick growth performance than was food availability (Twining et  
43 al. 2016a). LCPUFAs, in particular the fatty acids DHA (docosahexaenoic acid, 22:6n-3) and  
44 EPA (eicosapentaenoic acid, 20:5n-3), are important organic compounds for most animals: they  
45 affect a range of important physiological processes from immune function to vision and brain  
46 development (Twining et al. 2016b). However, some species, especially carnivores, are not able  
47 to synthesize LCPUFA. For example, cats, which are strict carnivores, and carnivorous fishes  
48 cannot synthesize any LCPUFA and must obtain them from their diets (Twining et al. 2016b).

49 Omnivorous animals, from humans to many species of birds, can obtain LCPUFA through two  
50 pathways: 1) either directly by consuming food containing EPA and, DHA), or 2) indirectly by  
51 consuming their molecular precursor, the short chain omega-3 PUFA, alpha linolenic acid  
52 (18:3n-3, ALA), and then converting ALA into LCPUFA through the biochemical processes of  
53 elongation and desaturation (Brenna and Carlson 2014).

54 A major dichotomy in LCPUFA availability exists between aquatic and terrestrial  
55 ecosystems: LCPUFA are abundant at the base of aquatic food webs, and aquatic insects  
56 incorporate LCPUFA from aquatic primary producers into tissues (Twining et al. 2016b; Torres-  
57 Ruiz et al. 2007). In contrast, terrestrial plants contain little to no LCPUFA, though they contain  
58 their molecular precursor ALA (Hixson et al. 2015; Twining et al. 2016b), which is either  
59 incorporated into tissue or to a minor degree converted to LCPUFA by terrestrial insects  
60 (Blomquist et al. 1991). As a consequence, aquatic insects are much richer in LCPUFA than are  
61 terrestrial insects. In the wild, avian insectivores in riparian areas, such as Tree Swallows  
62 (*Tachycineta bicolor*), which are considered a model insectivore species, consume a mix of  
63 terrestrial and aquatic insect prey items (McCarty and Winkler 1999a; Winkler et al. 2013). Our  
64 recent studies show that differences in the fatty acid composition between aquatic and terrestrial  
65 insects can have strong consequences on Tree Swallow chick performance (Twining et al. 2016a)  
66 and breeding success (Twining et al. submitted).

67 Ultimately, the nutritional value of aquatic versus terrestrial insects depends not only on  
68 the fatty acid composition of these insects, but also upon the capacity of Tree Swallows and  
69 other insectivores to convert ALA into LCPUFA. While strict carnivores, such as cats, have lost  
70 the ability to elongate and desaturate ALA into LCPUFA and must obtain LCPUFA directly  
71 from diet, domestic chickens (*Gallus domesticus*), which are omnivorous, but consume mainly

72 plant-based foods in captivity, appear to be relatively efficient at ALA to LCPUFA conversion  
73 (Twining et al. 2016b), with the capacity to survive and reproduce on LCPUFA-free diets. The  
74 majority of past studies on avian fatty acid requirements have focused on domesticated  
75 omnivorous taxa like chickens (e.g., Cherian and Sim 1991; Lin et al. 1991; Newman et al. 2002;  
76 Cherian et al. 2009). These studies found that domestic adult chickens and their chicks are  
77 capable of converting ALA to LCPUFA and that tissue LCPUFA increase with increased dietary  
78 ALA. Increasing ALA in maternal diet through foods, such as flax seeds, also increases  
79 LCPUFA content in eggs, embryos, and newly hatched chicks (Cherian and Sim 1991).

80         Studies on wild birds have found that both nutritional composition and foraging ecology  
81 affect avian tissue fatty acid composition and avian nutritional needs (McWilliams et al. 2002;  
82 Pierce et al. 2005; Maillet and Weber 2007; McCue et al. 2009). Most studies on wild birds (but  
83 see McWilliams et al. 2002; Pierce et al. 2005) have either looked at seed and fruit-eating  
84 passerines (e.g., McCue et al. 2009), which consume a diet much lower in LCPUFA, or fish- and  
85 shellfish-eating shorebirds, which consume a much higher LCPUFA diet than riparian aerial  
86 insectivores (e.g., McWilliams et al. 2004). Furthermore, past studies on avian fatty acid  
87 requirements have made inferences about conversion efficiency indirectly based on the effects of  
88 dietary fatty acid content on survival, performance, and tissue LCPUFA content, but they have  
89 not established whether ALA to LCPUFA conversion is metabolically possible, as have studies  
90 on humans and other mammals (Brenna et al. 2009).

91         Apart from obligate carnivores, animals generally retain the metabolic capacity to  
92 endogenously biosynthesize LCPUFA from precursors based in part on the LCPUFA content of  
93 their ancestral diets. Thus, terrestrial herbivores must endogenously synthesize effectively all of  
94 their LCPUFA while carnivores must obtain all the LCPUFA in their diets (Castro et al. 2012;

95 Brenna and Carlson 2014; Twining et al. 2016a). On this basis, Tree Swallows and other riparian  
96 aerial insectivores should have limited capacity to convert ALA into the LCPUFA because they  
97 evolved with access to aquatic insects, obviating the need to maintain efficiency in this metabolic  
98 pathway. We previously found that LCPUFA have strong effects on Tree Swallow growth:  
99 chicks on high LCPUFA diets, which approximated diets dominated by aquatic insects, grew  
100 faster, were in better condition, had increased immunocompetence and decreased metabolic rates  
101 compared to chicks on low LCPUFA feeds approximating diets dominated by terrestrial insects  
102 (Twining et al. 2016a). We found similar patterns between dietary LCPUFA and chick  
103 performance in Eastern Phoebes (*Sayornis phoebe*; Twining et al. in prep). In a long-term field  
104 study, we also that found aquatic insects, which, unlike terrestrial insects, are rich in LCPUFA,  
105 are a strong driver of long-term Tree Swallow fledging success in nature (Twining et al. in prep).  
106 Together, these findings suggest that ALA to LCPUFA conversion, if present, is likely  
107 inefficient in Tree Swallows and other riparian aerial insectivore chicks.

108 To understand the physiological importance of LCPUFA for riparian aerial insectivores,  
109 we used rapidly growing Tree Swallow chicks as a model to ask: first, are birds that regularly  
110 consume high LCPUFA dietary resources able to convert ALA into LCPUFA or are dietary  
111 LCPUFA strictly essential nutrients? Second, if birds with access to high LCPUFA resources are  
112 able to convert ALA into LCPUFA, is their conversion and tissue deposition efficiency high  
113 enough to provide them with enough LCPUFA from dietary ALA in natural settings to avoid  
114 performance limitation or are dietary LCPUFA ecologically essential nutrients? We used  
115 enriched <sup>13</sup>C stable isotope fatty acid tracers to trace the metabolic pathway of ALA through  
116 Tree Swallow chicks, adapting this methods from studies on humans and small mammals, to  
117 directly quantify ALA to LCPUFA conversion for the first time in wild birds.

## 118 **Methods**

119 We examined ALA conversion capacity and efficiency in seven wild Tree Swallow  
120 chicks from two sites near Ithaca, New York (Site 1: 42.504434°N, 76.465949°W, Site 2:  
121 42.515459°N, 76.335272°W). At both sites, we briefly removed chicks from the nest and fed  
122 them olive oil with or without dissolved  $\delta^{13}\text{C}$ -enriched ALA (Cambridge Isotope Laboratories,  
123 Cambridge, MA) via syringe. Six chicks were dosed with a  $\delta^{13}\text{C}$ -enriched ALA tracer, serving  
124 as treatment chicks, and one was not dosed with a  $\delta^{13}\text{C}$ -enriched ALA tracer and served as a  
125 natural abundance  $\delta^{13}\text{C}$  control. At the time of dosing, all chicks were approximately 7 days old  
126 and had a mean weight of 10.687 g (standard deviation = 1.204 g). We dissolved 5 mg of  
127  $\delta^{13}\text{C}_{\text{ALA}}$  in 2.5 mL of olive oil creating a 10mg/mL solution of  $\delta^{13}\text{C}_{\text{ALA}}$  in olive oil. Each  
128 treatment chick received 0.25 mL of olive oil with dissolved  $\delta^{13}\text{C}_{\text{ALA}}$  followed by 0.25mL of  
129 olive oil without tracer using same syringe. The control chick received two 0.25 mL syringes of  
130 olive oil that did not come into contact with the tracer.

131 After dosing, we labeled chicks with non-toxic children's nail varnish before returning  
132 them to their nests for parental care and feeding. All chicks were sacrificed at approximately 48  
133 hrs post-dose per United States Fish and Wildlife Service migratory bird scientific collection  
134 permit #MB757670 and New York State Department of Environmental Conservation scientific  
135 collection permit #1477. All animal work was approved under Cornell Institutional Animal Care  
136 and Use Committee #2001-0051. After sacrificing chicks, we removed their livers and pectoral  
137 muscles for analyses.

138 Next, we performed compound-specific  $\delta^{13}\text{C}$  analysis and fatty acid composition  
139 analysis. Briefly, we extracted liver and pectoral muscle fatty acid methyl esters (FAMES) using  
140 a modified one-step method (Garces and Mancha 1993). We quantified fatty acid composition

141 using a BPX-70 (SGE Inc.) column and a HP5890 series II gas chromatograph-flame ionization  
142 detector (GC-FID). Chromatogram data were processed using PeakSimple. Response factors  
143 were calculated using the reference standard 462a (Nucheck prep). FAMEs were identified using  
144 a Varian Saturn 2000 ion trap with a Varian Star 3400 gas chromatography mass spectrometer  
145 run in chemical ionization mass spectrometry mode using Acteonitrile as reagent gas. We used  
146 gas chromatography combustion isotope ratio mass spectrometry (GCC-IRMS) to measure the  
147  $\delta^{13}\text{C}$  signatures of ALA, EPA, and DHA (Goodman and Brenna 1992; Plourde et al 2014).  
148 Briefly, an Agilent 6890 GC was interfaced to a Thermo Scientific 253 isotope-ratio mass  
149 spectrometer via a custom-built combustion interface. Peaks were confirmed to be baseline  
150 separated, and calibrated against working standards with isotope ratios traceable to international  
151 standards calibrated to VPDB (Caimi et al. 1994; Zhang et al. 2009).

152 Conversion from ALA to LCPUFA requires a complex series of biochemical reactions of  
153 varying efficiency in each organ, including transport across and deposition into membranes.  
154 From measurement of the amount of labeled LCPUFA in tissue, we derive an apparent  
155 conversion efficiency (CE) reflecting all processes leading to the deposition of newly  
156 synthesized LCPUFA. This parameter, derived from experimental measurements, can also be  
157 understood as net deposition of labeled LCPUFA in tissue, rather than a mechanistic conversion  
158 rate that would be measured for a single enzymatic reaction. In experimental animals, this  
159 process is sensitive in the short-term to diet, with lower expression of genes involved in fatty  
160 acid conversion with LCPUFA feeding, as well as subject to competition due to the various fatty  
161 acids in the diet. We assessed ALA to EPA and DHA conversion efficiency by calculating  
162 conversion levels according to Sheaff et al. (1995). Atom Percent Excess (APE) as:

163

164  $APE_X = AP_{X-C(tr)} - AP_{X-C(ctl)}$  (1)

165 where AP are calculated directly from the measured  $\delta^{13}C_X$ , X is a fatty acid (ALA, EPA, DPA,  
166 DHA), and tr and ctrl refer to chicked dosed with tracer (tr) or not dosed (Ctrl).

167 Total Label, or the concentration of tracer per unit weight of tissue, was calculated as:

168  $Total\ X\ Label/unit\ of\ tissue = APE_{ALA} \cdot (concentration\ of\ ALA / unit\ of\ tissue)$  (2)

169 Finally, we calculated a tissue-specific apparent conversion efficiency (aCE) for fatty  
170 acids as:

171  $aCE_{LCPUFA} = (Total\ LCPUFA\ Label / Total\ ALA\ Label) \cdot 100\%$  (3)

172 where LCPUFA refers to either DHA or EPA.

173 To understand how ALA-derived EPA and ALA-derived DHA accumulation varies  
174 between tissues where conversion activity occurs (i.e., liver) and those where it is expected to be  
175 lower (i.e., pectoral muscle), we divided liver CE by muscle CE for both EPA and DHA.

176 To understand the significance of our measured ALA conversion efficiency on  
177 performance, we applied our calculations of apparent CE to the mean ALA content of nestling  
178 bird feeds and wild aquatic and terrestrial insects to estimate potential EPA and DHA synthesis.

179 We calculated potential EPA and DHA (LCPUFA) synthesis from ALA as:

180  $LCPUFA_{ALA} = CE_{LCPUFA} \cdot ALA\ content\ of\ food\ item$  (4)

181 And potential total EPA and DHA (LCPUFA) as:

182  $LCPUFA_{Total} = LCPUFA_{ALA} + LCPUFA\ content\ of\ food\ item$  (5)

183 We then calculated the fraction of EPA and DHA (LCPUFA) from diet versus ALA  
184 conversion for each prey item as:

185  $LCPUFA_{conversion} = LCPUFA_{ALA} / LCPUFA_{Total}$  (6)

186  $LCPUFA_{diet} = LCPUFA\ content\ of\ food\ item / LCPUFA_{Total}$  (7)

187 To understand how ALA-derived EPA and ALA-derived DHA accumulation varies  
188 between tissues where conversion activity occurs (i.e., liver) and those where it is minimal (i.e.,  
189 pectoral muscle), we divided liver CE by muscle CE for both EPA and DHA.

190 To understand the potential impact of our measured ALA conversion efficiency on  
191 growth performance, we applied our calculations of conversion efficiency to the mean ALA  
192 content of nestling bird feeds and wild aquatic and terrestrial insects to estimate potential EPA  
193 and DHA synthesis. Insects were collected from eight sites around Ithaca, NY, using a  
194 combination of emergence traps, pan traps, and targeted hand-netting (Twining, unpublished).  
195 We determined insect fatty acid composition following the same methods described above for  
196 Tree Swallow tissue. We then calculated potential EPA and DHA (LCPUFA) synthesis from  
197 ALA as:

$$198 \text{LCPUFA}_{\text{ALA}} = \text{CE}_{\text{LCPUFA}} \cdot \text{ALA content of food item} \quad (8)$$

199 And potential total EPA and DHA (LCPUFA) as:

$$200 \text{LCPUFA}_{\text{Total}} = \text{LCPUFA}_{\text{ALA}} + \text{LCPUFA content of food item} \quad (9)$$

201 We then calculated the fraction of EPA and DHA (LCPUFA) from diet versus ALA  
202 conversion for each prey item as:

$$203 \text{LCPUFA}_{\text{conversion}} = \text{LCPUFA}_{\text{ALA}} / \text{LCPUFA}_{\text{Total}} \quad (10)$$

$$204 \text{LCPUFA}_{\text{diet}} = \text{LCPUFA content of food item} / \text{LCPUFA}_{\text{Total}} \quad (11)$$

205 We calculated each of these measures for the following food items: 1) aquatic mayflies  
206 (Ephemeroptera Heptageniidae), 2) aquatic stoneflies (Plecoptera Perlidae), 3) aquatic  
207 dragonflies (Odonata Anisoptera), 4) terrestrial beetles (Coleoptera), 5) terrestrial flies (Diptera  
208 Brachycera), 6) terrestrial moths and butterflies (Lepidoptera), and 7) terrestrial bees  
209 (Hymenoptera Apidae).

210 We compared stable isotope values between our treatment chicks (n=6) and control chick  
211 (n=1) using one sample t-tests for both muscle and liver using control chick values as  $\mu_0$ . We  
212 used paired two-sample t-tests to compare conversion efficiency and tissue deposition between  
213 liver (n=7) and muscle (n=7) for treatment chicks. To compare raw ALA and EPA, potential  
214 ALA-derived EPA and DHA, and total potential EPA and DHA between aquatic and terrestrial  
215 insects, we also used two sample t-tests. We also used General Linear Models (GLM) to  
216 compare raw ALA and EPA, potential ALA-derived EPA and DHA, and total potential EPA and  
217 DHA between all insect groups using insect group as a factor. We performed post-hoc Tukey  
218 contrasts on all GLMs to determine which insect groups were significantly different from each  
219 other. All statistical analyses were performed in R version 3.3.3 (R core team).

## 220 **Results**

221 We first asked if LCPUFA synthesis supports our previous results showing that LCPUFA  
222 are strictly essential nutrients for Tree Swallows. We found evidence that Tree Swallow chicks  
223 can derive LCPUFA from ALA:  $\delta^{13}\text{C}_{\text{EPA}}$  and  $\delta^{13}\text{C}_{\text{DHA}}$  values of liver and muscle from chicks fed  
224  $\delta^{13}\text{C}$ -enriched ALA were significantly higher than controls (one sample t-tests: t-value = 4.62, df  
225 = 5, p-value < 0.01 for liver; t-value = 5.75, df = 5, p-value < 0.01; Table 1). Although all chicks  
226 fed  $\delta^{13}\text{C}$ -enriched ALA showed evidence of ALA to LCPUFA conversion and deposition of  
227 ALA-derived LCPUFA in tissues, we found substantial individual variation in conversion  
228 efficiency between individuals across both field sites, especially for DHA (Table 1). We found  
229 that ALA-derived EPA and ALA-derived DHA were significantly higher in liver, where most  
230 conversion activity is likely to occur, than in pectoral muscle, where LCPUFA are deposited  
231 (two sample t-test: t-value = 4.99, df = 5.42, p-value < 0.01 for EPA; t-value = 2.56, df = 5.23, p-  
232 value < 0.05 for DHA; Table 1).

233 We next asked if LCPUFA are ecologically essential nutrients for Tree Swallows based  
234 on measured conversion efficiencies and tissue deposition levels combined with the fatty acid  
235 composition of potential insect prey (Table 2). Aquatic insects, especially mayflies and  
236 stoneflies, had much higher percentages of raw EPA than did terrestrial insects (two sample t-test  
237 for aquatic versus terrestrial EPA:  $t$ -value = 8.05,  $df$  = 13.96,  $p$ -value < 0.01; Table 3) while  
238 terrestrial insects, especially terrestrial moths, butterflies, and bees, had much higher percentages  
239 of ALA than did aquatic insects or terrestrial beetles and terrestrial flies (Figure 1a; two sample  
240 t-test for aquatic versus terrestrial ALA:  $t$ -value = -2.08,  $df$  = 42.81,  $p$ -value < 0.05; Table 3).

241 We estimated that Tree Swallows could derive significantly more potential EPA and  
242 DHA from ALA in terrestrial moths, butterflies, and bees than from other terrestrial or aquatic  
243 insects (Figure 1b; Table 4). However, total EPA (raw EPA plus potential EPA from ALA) from  
244 aquatic insects was still significantly higher than the largely ALA-derived total EPA from  
245 terrestrial moths, butterflies, and bees (two sample t-test for aquatic versus terrestrial total EPA:  
246  $t$ -value = 8.05,  $df$  = 14.07,  $p$ -value < 0.01; Figure 1c; Table 5). Aquatic insect EPA derived  
247 primarily from diet (Figure 2) because aquatic insects had the significantly higher raw EPA  
248 values than did any terrestrial insects (Figures 1). Unlike aquatic insects, terrestrial insects,  
249 especially terrestrial moths, butterflies, and bees, had the potential to provide Tree Swallows  
250 with EPA primarily from ALA conversion (Figure 2).

251 In contrast to our findings for EPA, aquatic insects contained only trace amounts of DHA  
252 and no terrestrial insects contained any detectable DHA (Figure 1a). Therefore, regardless of  
253 conversion efficiency, over 95% of total DHA was derived from conversion, rather than diet for  
254 all insect prey (Tables 1-2). Due to their high ALA content, terrestrial moths, butterflies, and  
255 bees had the potential to supply significantly more DHA than aquatic insects or other terrestrial

256 insects (Figure 1; Table 5). Our estimates for DHA from EPA-rich aquatic insects are likely  
257 conservative because our  $^{13}\text{C}$ -ALA tracer allowed us to measure ALA to DHA conversion as  
258 well as ALA-derived EPA conversion to DHA, but not direct EPA to DHA conversion (i.e., Tree  
259 Swallows could have converted additional unlabeled EPA to DHA).

## 260 **Discussion**

261 Food quantity in terms of energy and food quality in terms of limiting nutrients are the  
262 major drivers of survival and performance in all living things everywhere, and especially in  
263 developing animals in the wild. We previously found that food containing LCPUFA reflective of  
264 aquatic insects improves multiple metrics of performance in Tree Swallow chicks in the  
265 laboratory (Twining et al. 2016a) and that the biomass of LCPUFA-rich aquatic insects is a  
266 strong predictor of Tree Swallow chick fledgling production in nature (Twining et al. submitted).  
267 In the present study, we sought to understand these costs to chick performance further by  
268 determining if and how Tree Swallow chicks can efficiently convert ALA into LCPUFA and  
269 deposit it into tissues. Our ALA tracer-based results reveal that Tree Swallow chicks are able to  
270 derive LCPUFA from ALA within liver and deposit ALA-derived LCPUFA into both liver and  
271 pectoral muscle. This evidence for ALA to LCPUFA conversion and deposition of ALA-derived  
272 LCPUFA suggests that performance costs in chicks on low LCPUFA diets (Twining et al. 2016a;  
273 Twining et al. in prep) likely arise from a combination of the energetic cost of converting ALA  
274 to LCPUFA as well as indirect LCPUFA limitation when ALA itself is in limited supply.

275 Our evidence that ALA to LCPUFA conversion is possible in Tree Swallows (Table 1)  
276 reveals that increased chick performance on diets with higher percentages of LCPUFA is likely  
277 due to a combination of direct LCPUFA limitation as well as the energetic savings accrued by  
278 receiving LCPUFA directly from of EPA-rich aquatic insects in diet. In our previous study, we

279 found that Tree Swallow chicks suffered performance declines when feed on diets with 6.25%  
280 ALA, 1.47% EPA, and 1.42% DHA compared to diets with 1.82% ALA, 3.74% EPA, and 3.44%  
281 DHA (Twining et al. 2016a). While all insect taxa that we examined contain more than 1.82%  
282 ALA (the level of ALA in our higher performance dietary treatment), terrestrial Diptera provide  
283 less than the 3.74% EPA and 3.44% DHA levels in our high performance dietary treatment  
284 (Figure 1; Table 2), even at maximum measured deposition levels of 22.1% for EPA and 34.1%  
285 DHA (Table 2). Terrestrial insects that did contribute more total EPA than that of our high  
286 performing lab treatments provided substantially less EPA than aquatic insects (Figure 1). Only  
287 terrestrial butterflies and moths had the potential to provide more than sufficient total (raw plus  
288 potential from ALA) EPA and DHA (Figure 1). However, terrestrial butterflies and moths,  
289 beetles, and bees are all rare in Tree Swallow insect boluses fed to chicks, which are dominated  
290 by true flies (Diptera; McCarty and Winkler 1999; Winkler and others unpublished). Aquatic and  
291 terrestrial flies are likely the dominant prey in Tree Swallow boluses they are highly abundant  
292 and relatively easy to catch compared to less abundant and faster flying prey like butterflies  
293 (McCarty and Winkler 1999). Thus, it is highly unlikely that terrestrial insects alone can supply  
294 chicks with sufficient ALA-derive LCPUFA, making LCPUFA-rich aquatic insects ecologically  
295 essential for Tree Swallow chicks.

296 Strictly essential nutrients are nutrients that animals are unable to synthesize from their  
297 molecular precursors and must derive directly from diet. LCPUFA are thus not strictly essential  
298 for Tree Swallows because Tree Swallows can synthesize limited amounts of LCPUFA from  
299 precursors. However, LCPUFA-rich prey like aquatic insects appear to be crucially important,  
300 ecologically essential nutrients for Tree Swallows in natural systems because the ALA content of  
301 terrestrial insects and ALA to LCPUFA conversion efficiency are insufficient to supply chicks

302 with the LCPUFA that they demand. In addition, ALA-derived LCPUFA levels were much  
303 higher in liver where much ALA to LCPUFA conversion occurs than in pectoral muscle (Table  
304 1), where LCPUFA are deposited and used, but not synthesized. Our findings in Tree Swallows  
305 contrast with observations in chickens: chickens increase tissue LCPUFA with increased dietary  
306 LCPUFA, but are able to thrive even without dietary LCPUFA (Cherian and Sim 1991).

307 Past studies directly measuring ALA to LCPUFA conversion efficiency have focused on  
308 humans and other laboratory mammals (Brenna et al. 2009). Human studies uniformly show that  
309 ALA conversion to EPA is significant and in the few percent range, while conversion to DHA is  
310 barely above trace levels (Brenna et al. 2009). Our measured conversion efficiency in Tree  
311 Swallow liver (Table 1), where presumably most LCPUFA synthesis occurs, is broadly similar to  
312 that found in human blood (Burdge et al. 2004). However, ALA-derived LCPUFA levels in Tree  
313 Swallow pectoral muscle (Table 1), which is a more comparable tissue to blood in that LCPUFA  
314 synthesis is low or zero in both, were lower than levels in human blood (Burdge et al. 2004).  
315 Studies in rat pups suggest that ALA to LCPUFA conversion occurs at much higher efficiency  
316 than those we found in Tree Swallows even when LCPUFA are present in diet (Sheaff et al.  
317 1995). However, although these studies suggest that ALA conversion is possible in humans and  
318 other mammals throughout their lifetime, most LCPUFA still come directly from dietary  
319 LCPUFA unless dietary sources are kept artificially low (Brenna et al. 2009).

320 Within the existing literature on ALA to LCPUFA conversion on humans, it is well  
321 known that ontogeny, sex, and reproductive status have important effects on conversion  
322 efficiency. For example, human females of reproductive age are more efficient at ALA to  
323 LCPUFA conversion than are human males of the same age (Burdge et al. 2002), which  
324 researchers have interpreted as an adaptation for provisioning human infants with LCPUFA

325 (Brenna et al. 2009). Studies in humans also suggest that infants themselves are more efficient at  
326 conversion than are adults or older children, although infant conversion efficiency may be  
327 insufficient for optimal development in the absence of preformed dietary LCPUFA (Brenna et al.  
328 2009). Even among human infants, those at earlier gestational ages appear to have higher  
329 LCPUFA conversion efficiency than do infants at later gestational ages (Carnielli et al. 2007).

330 Like human infants, the Tree Swallow nestlings that we studied are entirely reliant upon  
331 parental feeding. Nutritional demands are greatest for altricial temperate passerines like Tree  
332 Swallows during the nestling period when they undergo rapid growth, often doubling in mass  
333 every few days (Zach and Mayoh 1982). Thus, the conversion efficiency that we report for Tree  
334 Swallow nestlings is likely near maximum for the species under the diet conditions provided  
335 them. Unlike human mothers, who provision their fetuses and nursing infants with a constant  
336 supply of their own digested nutrients including LCPUFA (Brenna et al. 2009), or even precocial  
337 species of birds like chickens who provision embryos with high levels of ALA and LCPUFA in  
338 eggs (Lin et al. 1991; Speake and Wood 2005), wild altricial birds like Tree Swallows invest  
339 little LCPUFA into eggs (Speake and Wood 2005; Twining et al. in prep). Tree Swallows also  
340 complete nearly all somatic growth, including growing the brain, eyes, and other nervous tissues,  
341 within approximately three weeks. This means that nestling Tree Swallow chicks must acquire  
342 all of their LCPUFA within a very short time window, creating high selective pressure for high  
343 ALA to LCPUFA conversion efficiency and tissue deposition during the nestling period. As in  
344 humans, further studies on Tree Swallow adults are necessary to confirm how ALA to LCPUFA  
345 conversion efficiency varies throughout the life cycle. Tree Swallow chicks must be sexed with  
346 karyotypes and therefore we did not sex the chicks in this study. Studies comparing adult Tree

347 Swallow males and females, which can be readily sexed in the field, will lend additional insights  
348 into the complete LCPUFA needs of these model avian insectivores throughout their life cycles.

349 Previous studies on birds and other animals in natural systems have not investigated ALA  
350 to LCPUFA conversion mechanistically using stable isotope tracers. Thus, unfortunately, it  
351 remains unclear if LCPUFA are either strictly or ecologically essential for many wild animals,  
352 including other birds. Most researchers have made inferences about conversion efficiency based  
353 on data on the effects of dietary LCPUFA content on performance or survival (e.g., Sargent et al.  
354 1999) or tissue fatty acid composition (e.g., Cherian and Sim 1991). However, if conversion  
355 efficiency is low, costs to performance and survival may either be due to dietary LCPUFA  
356 limitation or the costs associated with converting ALA to LCPUFA. In contrast, if conversion  
357 efficiency is zero, then all costs to performance and survival must be due to direct limitation,  
358 making LCPUFA strictly essential. Direct measurements of conversion efficiency are clearly  
359 necessary to distinguish these possibilities.

360 Understanding whether LCPUFA are strictly essential, ecologically essential, or non-  
361 essential nutrients for birds and other wild animals is crucial for informing conservation efforts.  
362 In order to develop successful species management plans, environmental managers must  
363 understand the full suite of food and habitat resources that wild animals require throughout their  
364 life cycle. For example, our findings in Tree Swallow chicks suggest LCPUFA-rich aquatic  
365 insects and habitats are ecologically essential resources during a critical ontogenetic period. As a  
366 consequence, human activities that alter the composition and resulting nutritional quality of  
367 insect prey, such as land use change and pesticide use, as well as phenological shifts in insect  
368 emergence due to climate change have the potential to create nutritional mismatches for Tree  
369 Swallows (Twining et al. submitted). We hope that our field-based adaptation of an enriched

370 stable isotope tracer method from human clinical studies is a starting point towards ultimately  
371 developing a general understanding of fatty acid nutritional ecology in wild animals.

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### **Author Contributions**

CWT and JTB conceived the ideas and designed methodology; CWT and PL collected the data; CWT, PL, and JTB analyzed data; CWT led the writing of the manuscript and JTB, ASF, and DWW contributed critically to the drafts and all authors gave final approval for publications.

### **Competing Interests**

No competing interests declared.

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## Tables and Figures

Table 1 – ALA to EPA and DHA conversion efficiency in Tree Swallow chick liver and pectoral muscle and ratio of liver to muscle conversion efficiency

Tissue Type	Mean	Standard Deviation	Maximum	Minimum
Liver EPA	16.47%	5.38%	22.15%	9.93%
Liver DHA	18.06%	13.76%	34.05%	0.23%
Muscle EPA	5.32 %	1.11%	6.68%	3.45%
Muscle DHA	3.5%	2.09%	6.17%	0.80%
Liver/Muscle EPA	3.17	0.99	4.46	1.49
Liver/Muscle DHA	4.61	2.78	6.61	0.29

Table 2 – Potential EPA and DHA from ALA and potential total EPA at average, maximum, and minimum conversion efficiency. All data are expressed as percent of total fatty acids.

<b>Food Item</b>	<b>EPA<sub>ALA</sub> Avg.</b>	<b>EPA<sub>ALA</sub> Max.</b>	<b>EPA<sub>ALA</sub> Min.</b>	<b>EPA<sub>Total</sub> Avg.</b>	<b>EPA<sub>Total</sub> Max.</b>	<b>EPA<sub>Total</sub> Min.</b>
Aquatic Mayflies	1.14	1.53	0.69	16.13	16.53	15.86
Aquatic Dragonflies	0.48	0.64	0.29	6.47	6.63	6.28
Aquatic Stoneflies	0.87	1.18	0.53	21.40	21.71	21.06
Terrestrial Beetles	0.41	0.55	0.25	5.18	5.32	5.02
Terrestrial Flies	0.39	0.53	0.24	2.83	2.97	2.68
Terrestrial Butterflies and Moths	4.47	6.01	2.69	6.65	8.19	4.87
Terrestrial Bees	3.00	4.03	1.81	3.00	4.03	1.81
<b>Food Item</b>	<b>DHA<sub>ALA</sub> Avg.</b>		<b>DHA<sub>ALA</sub> Max.</b>		<b>DHA<sub>ALA</sub> Min.</b>	
Aquatic Mayflies	1.25		2.36		0.02	

Aquatic Dragonflies	0.52	0.99	0.01
Aquatic Stoneflies	0.96	1.81	0.01
Terrestrial Beetles	0.45	0.84	0.01
Terrestrial Flies	0.43	0.81	0.01
Terrestrial Butterflies and Moths	4.90	9.23	0.06
Terrestrial Bees	3.29	6.20	0.04

Table 3: GLM results for raw ALA and EPA in insects

<b>Model: Raw ALA</b>			
	t-value	p-value	Contrasts
Intercept	11.49	< 0.01	Terrestrial Beetles, T. Flies, Aquatic Mayflies, A. Stoneflies, and A. Dragonflies < T. Bees < T. Moths and Butterflies
Aquatic Mayflies	-4.99	< 0.01	
Aquatic Dragonflies	-3.22	< 0.01	
Aquatic Stoneflies	-4.68	< 0.01	
Terrestrial Beetles	-6.89	< 0.01	
Terrestrial Flies	-7.06	< 0.01	
Terrestrial Butterflies and Moths	4.00	< 0.01	
Null deviance: 4707.33 on 44 degrees of freedom			
Residual deviance: 760.28 on 38 degrees of freedom			
<b>Model: Raw EPA</b>			
Intercept	0	Not significant	Terrestrial Bees < T. Beetles
Aquatic Mayflies	18.16	< 0.01	Aquatic Dragonflies, T. Bees, T. Flies, T.

Aquatic Dragonflies	2.28	< 0.05	Moths and Butterflies, and T. Beetles < A. Mayflies A. Dragonflies, T. Beetles, T. Flies, T. Moths and Butterflies, and T. Bees < A. Stoneflies
Aquatic Stoneflies	13.39	< 0.01	
Terrestrial Flies	1.97	Not significant	
Terrestrial Beetles	3.57	< 0.01	
Terrestrial Moths and Butterflies	1.67	Not significant	
Null deviance: 3636.90 on 44 degrees of freedom			
Residual deviance: 232.52 on 38 degrees of freedom			

Table 4: GLM results for potential ALA-derived EPA and DHA in insects

<b>Model: Potential ALA-derived EPA</b>			
	t-value	p-value	Contrasts
Intercept	11.49	< 0.01	Aquatic Dragonflies, A. Stoneflies, A. Mayflies, T. Beetles, and T. Flies < T. Bees < T. Butterflies
Aquatic Mayflies	-4.99	< 0.01	
Aquatic Dragonflies	-3.22	< 0.01	
Aquatic Stoneflies	-4.68	< 0.01	
Terrestrial Beetles	-6.89	< 0.01	
Terrestrial Flies	-7.06	< 0.01	
Terrestrial Butterflies and Moths	4.00	< 0.01	
Null deviance: 127.69 on 44 degrees of freedom			
Residual deviance: 20.62 on 38 degrees of freedom			
<b>Model: Potential ALA-Derived DHA</b>			

Intercept	11.49	< 0.01	Aquatic Dragonflies, A. Stoneflies, A. Mayflies, T. Beetles, and T. Flies < T. Bees < T. Butterflies
Aquatic Mayflies	-4.99	< 0.01	
Aquatic Dragonflies	-3.22	< 0.01	
Aquatic Stoneflies	-4.68	< 0.01	
Terrestrial Beetles	-6.89	< 0.01	
Terrestrial Flies	-7.06	< 0.01	
Terrestrial Butterflies and Moths	4.00	< 0.01	
Null deviance: 153.54 on 44 degrees of freedom			
Residual deviance: 24.80 on 38 degrees of freedom			

Table 5: GLM results for potential total ALA and EPA in insects

<b>Model: Total Potential EPA</b>			
	t-value	p-value	Contrasts
Intercept	3.56	< 0.01	Terrestrial Flies and T. Bees < T. Moths and Butterflies
Aquatic Mayflies	17.36	< 0.01	T. Beetles, T. Flies, T. Bees, T. Moths and Butterflies, and Aquatic Dragonflies < A. Stoneflies < A. Mayflies
Aquatic Dragonflies	1.38	Not significant	
Aquatic Stoneflies	12.50	< 0.01	
Terrestrial Beetles	1.57	Not significant	
Terrestrial Flies	-0.13	Not significant	
Terrestrial Butterflies and	3.08	< 0.01	

Moths			
Null deviance: 3292.18 on 44 degrees of freedom			
Residual deviance: 214.44 on 38 degrees of freedom			
<b>Model: Total Potential DHA</b>			
Intercept	11.49	< 0.01	Terrestrial Beetles, T. Flies, Aquatic Dragonflies, A. Stoneflies, A. Mayflies < T. Bees < T. Moths and Butterflies
Aquatic Mayflies	-4.99	< 0.01	
Aquatic Dragonflies	-3.22	< 0.01	
Aquatic Stoneflies	-4.67	< 0.01	
Terrestrial Beetles	-6.89	< 0.01	
Terrestrial Flies	-7.06	< 0.01	
Terrestrial Butterflies and Moths	4.00	< 0.01	
Null deviance: 153.50 on 44 degrees of freedom			
Residual deviance: 24.80 on 38 degrees of freedom			

Figure 1: ALA, EPA, and DHA content of (a) food sources, (b) potential EPA and DHA from ALA to LCPUFA conversion, and (c) potential total ALA, EPA, and DHA content from food sources and ALC to LCPUFA conversion

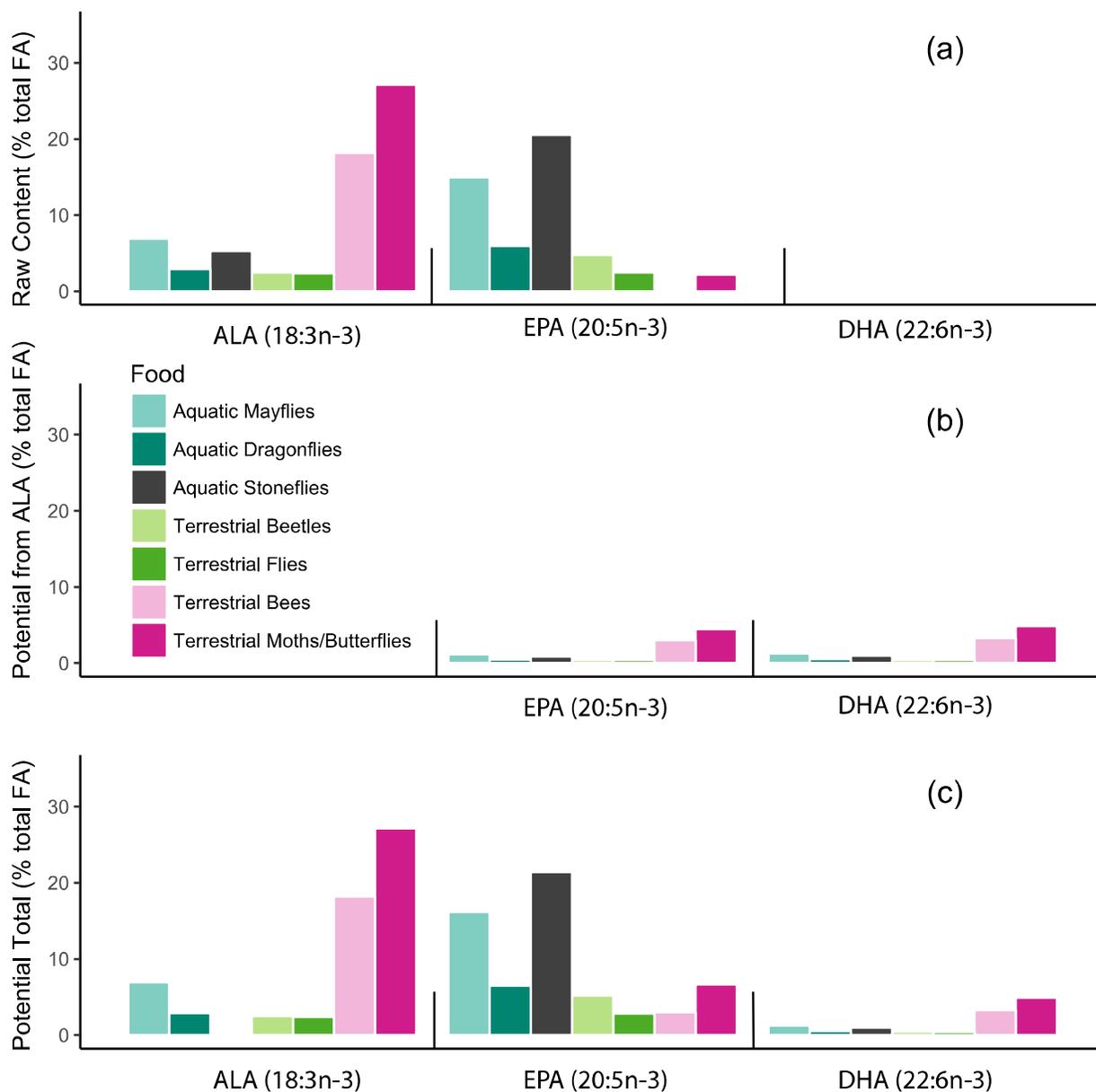


Figure 2: Fraction of EPA directly from diet or from ALA to LCPUFA conversion. LCPUFA derived from diet is gray and that from conversion is black.

