

1 **Concentration and ratio of essential fatty acids influences the inflammatory**
2 **response in lipopolysaccharide challenged mice**

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25 **Summary**

26
27 The goal of this study was to evaluate the role of both the % of dietary, 18-carbon PUFA
28 (2.5%, 5% and 10%) and the n-6:n-3 ratio (1:1, 10:1 and 20:1) on the acute inflammatory
29 response. Mice were fed diets for 8 weeks and injected intraperitoneally with LPS to induce
30 acute inflammation. After 24 hours mice were sacrificed and plasma cytokines measured.
31 Diets significantly affected the erythrocyte PUFA composition and the effect of PUFA ratio
32 was more prominent than of PUFA concentration. The % dietary PUFA affected feed
33 efficiency ($p<0.05$) and there was a PUFA \times ratio interaction with body fat ($p<0.01$). In mice
34 fed high %kcal from PUFA, those given a low n-6:n-3 ratio had more body fat than those fed
35 a high ratio. Of the twelve cytokines measured, eleven were significantly affected by the %
36 PUFA ($p<0.05$), whereas five were affected by the ratio ($p<0.05$). For seven cytokines, there
37 was a significant PUFA \times ratio interaction according to a two way ANOVA ($p<0.05$). These
38 data indicate that dietary polyunsaturated fatty acids can affect LPS induced-inflammation.

39

40 **Keywords**

41 Omega-3
42 Omega-6
43 Polyunsaturated fatty acids (PUFA)
44 Inflammation
45 Lipopolysaccharide (LPS)

46

47 1. Introduction

48

49 Essential fatty acids are required nutrients in animal diets because they cannot be

50 synthesized *de novo*. The two 18 carbon essential fatty acids (EFA), linoleic acid (18:2n-6;

51 LA) and linolenic acid (18:3n-3; ALA) can be further elongated and desaturated to create

52 long chain unsaturated fatty acids such as arachidonate (20:4n-6; AA), eicosapentaenoate

53 (20:4n-3; EPA) and docosahexenoate (22:6n-3; DHA) [1]. As the typical American diet

54 contains a substantial excess of LA (n-6) over ALA (n-3), there is a greater proportion of AA

55 (n-6) compared to EPA (n-3) in tissue long chain PUFA, which may influence inflammatory

56 processes [2]. The 20 carbon PUFA species AA, EPA and dihomo-gamma-linoleate (20:3 n-6;

57 DGLA) serve as signaling molecules (eicosanoids) when they are enzymatically released

58 from membranes [3]. In response to stimuli, the fatty acid is cleaved from the cell membrane

59 via a phospholipase (such as phospholipase A2)[4]. The process by which these 20 carbon

60 fatty acids are recognized and cleaved from the membrane seems to be nonspecific for the

61 particular class (n-6 vs. n-3) and thus the fatty acids released reflect their relative membrane

62 proportions. The 20 carbon PUFA can then be acted upon by cyclooxygenases (COX) or

63 lipoxygenases (LOX) which result in prostaglandins, prostacyclins and thromboxanes (COX

64 metabolites) or leukotrienes (LOX metabolites). The net effect of eicosanoid release is

65 complex, as it depends on the relative proportions of the different eicosanoids present, as

66 well as the sensitivity of the tissues that sense them. In general, the eicosanoids generated

67 from arachidonic acid (AA) are considered to be more potent mediators of inflammation

68 than those generated from EPAs [5]. However, even COX metabolites of AA, such as PGE₂ can

69 have both pro-inflammatory and anti-inflammatory activity [5]. Thus, dietary ratios of the

70 EFAs directly impact tissue structural lipid composition and consequently systemic
71 inflammatory and immune processes [2].

72 One reason intakes of EFA are so high in the US that replacing saturated fatty acids with
73 EFA in the diet leads to lower total and LDL cholesterol levels, with LA being the most
74 effective fatty acid in achieving this effect [6]. Thus, dietary advice given to Americans has
75 been to replace saturated fats with foods containing more EFA. Over the last 100 years, an
76 increase of vegetable oils in Western diets has affected both total PUFA intake and the n-6:n-
77 3 ratio. Blasbalg and colleagues [7] estimated that LA consumption increased from 2.23 to
78 7.21% of daily calories from 1909 to 1999 and in this same time frame, the ratio of dietary
79 n-6 to n-3 fatty acids changed from 5.4 to 9.6. It has been suggested that the increased LA
80 intake and high n-6:n-3 ratio may negatively affect health through excessive eicosanoid
81 signaling [8].

82 Adding n-3 PUFA to the diet is generally considered as an effective strategy for reducing
83 inflammation, and these molecules may affect inflammation in several ways (reviewed by
84 Calder [4]). For example, high dietary intake of EPA results in the displacement of AA in
85 membrane phospholipids which reduces the pool of available AA for eicosanoid synthesis.
86 In addition, EPA inhibits the hydrolysis of AA from immune cell membranes. Furthermore,
87 when fatty acids are cleaved from membranes enriched in EPA by PLA₂, the freed EPA
88 competes with free AA for conversion by COX and LOX enzymes. Lastly, EPA-derived
89 eicosanoids compete with those derived from AA at target cell binding sites, and the
90 individual eicosanoids have different effects. While the rationale behind replacing AA with
91 EPA is based on the weaker activity of the eicosanoids derived from EPA, there are also
92 metabolites from EPA as well as DHA that actively promote inflammation resolution.

93 Specialized pro-resolving mediators (SPM) is a term for lipid mediators derived from EPA
94 and DHA that promote the active resolution of inflammation and SPM includes resolvins,
95 protectins, and maresins [9]. Omega-3 fatty acids may also induce anti-inflammatory effects
96 in macrophages and mature adipocytes via GPR120 signaling [10]. [11] In addition, long
97 chain n-3 PUFA enhance B cell-mediated immunity in mice fed both control and high fat diets
98 [11].

99 Most studies that have examined the inflammatory potential of dietary PUFA have
100 focused on long chain PUFA (EPA and DHA) interventions that may not be relevant to
101 modern, western populations. For example, EPA and DHA intake is estimated to account for
102 less than 0.05% of daily caloric intake compared to ~8% of daily caloric intake from plant
103 derived LA and ALA [7]. While there is a general consensus in the literature that high intakes
104 of n-6 fatty acids can result in a pro-inflammatory state, there are few studies in which both
105 n-6:n-3 ratios and total PUFA concentrations have been investigated together. Moreover,
106 most studies investigating inflammatory potential of dietary PUFA have focused on long
107 chain n-3 interventions.

108 The goals of this study were to determine if the total PUFA content of the diet and/or the
109 n-6:3 ratio has an effect on the acute inflammatory response in C57BL6/J mice.
110 Consequently, we formulated nine diets in which the fat source of the AIN-93G diet was
111 modified to produce 3 different PUFA levels (2.5%, 5% and 10% of total kcal) at three ratios
112 of n-6 to n-3 (1:1, 10:1 and 20:1) using fatty acids exclusively from plant sources. These PUFA
113 concentrations, n-6 to n-3 ratios and dietary sources are physiologically relevant in the
114 context of modern western diets, however long chain PUFA and animal fats were
115 purposefully avoided as each may have complex actions on the inflammatory response. Mice

116 were then challenged with LPS, a stressor that is well-known to induce an acute
117 inflammatory response.

119 **2. Materials and Methods**

120 *2.1 Diet Formulation*

121 Nine diets differing in total PUFA content and n-6 to n-3 ratios were formulated using the
122 AIN-93G purified rodent diet. This diet has a caloric density of 3.8 kcal/g and derives 18.8 %
123 kcal from protein, and 63.9 % kcal from carbohydrate and 17.2 % kcal from fat. Nine diets
124 were formulated with three different PUFA levels (2.5%, 5% and 10%) at three different
125 ratios (1:1, 10:1 and 20:1). To produce the different fat compositions, seven different
126 vegetable oils were blended at various proportions (Table 1). Diets were produced by Harlan
127 Laboratories (Madison, WI), and individual catalog numbers are provided in Table 1. To
128 protect against lipid oxidation, all diets contained 14 mg/kg TBHQ and were stored at 4°C.

130 *2.2 LPS Challenge*

131 All animal experimental protocols were approved by the Utah State University Institutional
132 Animal Care and Use Committee. Male 4-5 week old C57BL/6J mice (The Jackson Laboratory,
133 Bar Harbor, ME) (n=12) were fed the experimental diets ad-libitum for 8 weeks. Food intake
134 and animal weights were determined weekly. Three days prior to sacrifice, body
135 composition was determined by NMR (EcoMRI LLC, Houston, TX). After 8 weeks, mice were
136 injected intraperitoneally with 5mg/kg lipopolysaccharide (LPS, Sigma Aldrich, St. Louis,
137 MO) 24-h prior to sacrifice and six mice were injected with saline. Mice were euthanized by

138 CO₂ asphyxiation, blood was collected via cardiac puncture, and plasma was isolated by
139 centrifugation.

140

141 *2.3 Plasma Cytokine Analysis:*

142 Plasma cytokine analysis was performed using the Q-Plex™ Mouse Cytokine - Screen (16-
143 plex) array (Quansys Biosciences, Logan, UT, USA). Cytokines analyzed include: IL-1 α , IL-1 β ,
144 IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17, MCP-1, IFN γ , TNF α , MIP-1 α , GMCSF, and
145 RANTES.

146

147 *2.4 Fatty Acid Analysis*

148 Lipids were quantified in diets and red blood cells using gas chromatographic analysis of
149 fatty acid methyl ester derivatives (FAMES). The direct derivitization method of O'Fallon [12]
150 was used after adaption to smaller sample sizes. In short, diet or erythrocytes samples
151 (~100mg) were mixed with 234 μ l of 10N KOH in water and 1.77ml of methanol. Samples
152 were vortexed for 30s and then incubated at 55 °C for 1.5h in a shaking water bath. Samples
153 were removed every 20m and vortexed for 30s. Next, samples were cooled, and 193 μ l of
154 24N H₂SO₄ was added. Samples were vortexed for 30s and then incubated at 55 °C for 1.5h
155 in a shaking water bath with vortexing every 20m for 30s. Samples were cooled and 300 μ l
156 of hexane with 0.05% BHT was added. Samples were vortexed for 30s and then centrifuged
157 at 1000 \times g for 5min. The upper hexane layer was removed to a GC vial and analyzed
158 according to the method of Zhou et al [13] using a Shimadzu GC2010 gas chromatograph
159 with flame ionization detection (Shimadzu Corporation, Columbia, MD). Fatty acids were
160 separated using an HP-88 column (Agilent Corporation, Santa Clara, CA). The retention time

161 for fatty acids and response factors were determined using GLC-463, a pure standard of fatty
162 acid methyl esters (Nu-Chek Prep, Elysian, MN).

163

164 *2.5 Statistical Analysis*

165 All data were analyzed using a 2 way ANOVA with n6:n3 ratio and % dietary PUFA as the
166 main effects. Cytokine data was normalized to plasma protein concentration, and log₁₀
167 transformed to adjust for non-normal distribution. After log transformation the data was
168 normally distributed allowing for parametric testing.

169 **3. Results**

170 *3.1 Fatty Acid Composition of Diets*

171 The fat sources used to create the experimental diets are shown in Table 1. The low PUFA
172 diets contained significant proportions of low PUFA triglyceride sources, such as cocoa
173 butter (CB) and olive oil (OO). On the other hand, the n-6 and n-3 content of the diets were
174 primarily manipulated by including different proportions of corn oil (CO), cottonseed oil
175 (CSO), flaxseed oil (FSO), sunflower oil (SFO), and soybean oil (SO).

176 The measured fatty acid composition of the experimental diets is shown in Table 2. The
177 primary focus of the diet design was to produce diets that contained different concentrations
178 of PUFA at different ratios, yet at the same time to keep the saturated fatty acid (SFA) content
179 similar. Therefore, the major difference between the low PUFA diets (2.5% kcal) and the high
180 PUFA diets (10% kcal) was in the monounsaturated fatty acid content (MUFA). Despite
181 careful formulation of the diets, the target PUFA contents and n-6:n-3 ratios were not all
182 achieved. Nonetheless, the diets do segregate into low, medium and high PUFA contents with
183 low, medium and high n-6:n-3 ratios.

184

185 *3.2 Food Consumption, Weight Gain and Body Composition*

186 The experimental diets did not significantly affect total food intake or weight gain over
187 the course of the study (Table 3). MRI data of body composition indicated there were no
188 differences in lean mass, water content, but there were differences in both the absolute
189 amount of fat (data not shown) and the relative amount (% body fat). The differences in
190 body composition were the result of fat mass, as there were no differences in the absolute
191 amount of lean tissue (data not shown). In addition, there was an effect of PUFA intake on
192 feed efficiency, which is the ratio of weight gained to food intake (Table 3; Figure 1A).

193 When expressed as % body fat, there was an effect of the %PUFA, the ratio and a PUFA ×
194 ratio interaction for the percent body fat of the mice after consuming the diets for 8 weeks
195 (Table 3; Figure 1B). Mice fed the 10% PUFA diet at the 20:1 n-6:n-3 ratio had significantly
196 less body fat compared to those fed the same amount of PUFA but at the 1:1 n-6:n-3 ratio (P
197 < 0.05).

198

199 *3.3 Effects of Diets on Red Blood Cell Fatty Acid Composition*

200 The effects of PUFA concentration and the n-6:n-3 ratio on RBC fatty acid content are
201 shown in Table 4. Of the 19 fatty acids measured, 10 were significantly affected by the total
202 percent PUFA in the diets, whereas 12 were affected by the n-6:n-3 ratio. According to the
203 two-way ANOVA analysis, there was a PUFA × ratio interaction for four long chain
204 polyunsaturates (20:4n-3, 22:5n-6, 22:5n-3 and 22:6n-3).

205 The five most prominent fatty acids in the diets (16:0, 18:0, 18:1n9, 18:2n6 and 18:3n3;
206 Table 2) made up between 97-98% of the dietary fat and were variable across the diets.

207 Thus, it is not surprising all five were significantly different in the RBCs due to the PUFA
208 percentage of the diets. One fatty acid of interest that decreased as a percentage of total
209 RBC fatty acids with increased dietary PUFA was 20:3n9, Mead's acid. This fatty acid is
210 produced via elongation of oleic acid at low PUFA intakes and has been suggested as a
211 potential biomarker for low PUFA intakes [8].

212 As virtually all the fatty acids in the diets were <18 carbons, those in the RBCs with >18
213 carbons were primarily synthesized via elongation and desaturation of dietary precursors.
214 Of the thirteen fatty acids with >18 carbons, five were significantly different according to
215 the %PUFA whereas eleven of the thirteen were different according to the n-6:n-3 ratio
216 (Table 4).

217 A post-hoc analysis was conducted on those fatty acids for which there was a %PUFA ×
218 ratio interaction and the results are shown in Figure 2. For EPA (20:5n-3) there were
219 significant differences between the mice fed the 1:1 diets and those fed the 10:1 and 20:1
220 diets. In addition, the EPA percentage of the RBCs increased at the 1:1 ratio when the mice
221 were diets contained 5% PUFA (2.5% n-3) compared to the 2.5% PUFA(1.25% n-3), but did
222 not increase further when the PUFA content was raised to 10% (5% n-3). The RBC
223 percentage of Osbond acid (22:5n-6) increased with the n-6:n-3 ratio. Similar to EPA, there
224 was an increase in the concentration when the PUFA content of the diet was increased from
225 2.5% to 5%, but no further increase when the PUFA was at 10%. The RBC percentage of
226 clupanodonic acid (22:5n-3) was higher in the low ratio diets (1:1) but increased as the
227 dietary PUFA went from 2.5% of kcal to 10%. Lastly, the RBC DHA content (22:6n-3) did
228 not differ at the low ratio (1:1) regardless of the %kcal from PUFA, but was lower at higher
229 n-6:n-3 ratios.

230 The omega-3 index is the sum of EPA and DHA in red blood cells, and the average values
231 for mice in each diet group are shown at the bottom of Table 4. s According to the two way
232 ANOVA, there is both a %PUFA and a ratio effect, but no interaction. Mice fed the 1:1 ratio
233 of n-6:n-3 had omega-3 indices over 10, while those fed the 10:1 ratio were between 4.7
234 and 6.2. Mice fed the high n-6:n-3 ratio diets had the lowest omega-3 indices between 3.3
235 and 4.6.

236

237 *3.4 Effect of Diets on LPS Induced Systemic Inflammation*

238 Plasma cytokine data from mice given the sham injections is not shown, but there were few
239 statistical differences between groups. Conversely, levels of all cytokines were significantly
240 higher in mice treated with the 5 mg/kg LPS. This dose was selected to induce a vigorous,
241 yet non-lethal inflammatory response. In a previous study in our lab, 10 mg/kg was lethal to
242 mice in some diet groups but not others [14], despite the fact that it is regularly used to
243 induce non-lethal inflammation [15]. Due to the distribution of the raw data, all cytokine data
244 was log transformed prior to statistical analysis. For presentation in the figures, the data was
245 back transformed, and is shown in mass per mg protein. Of note, IL-6, RANTES and MCP-1
246 are all in ng/mg protein, whereas the rest are in pg/mg protein.

247 Cytokines significantly affected by the dietary PUFA modulations are shown in Table 5
248 and Figure 2. According to the data, 11 of the 16 cytokines measured were significantly
249 affected by the %PUFA content of the diet, whereas 5 of 16 responded to changes in the n-
250 6:n-3 ratio. Furthermore, with 7 cytokines there was a ratio \times concentration effect. The first
251 five cytokines shown in Figure 2 (INF γ , IL-5, IL-6, IL-10 and IL-12p70) were affected by the
252 %PUFA content of the diet, but not the ratio. For these cytokines, comparison is made at each

253 ratio, and significant differences are designated by lower case letter, and boxes are placed
254 around groups within each ratio. For example, for INF γ , increasing the %PUFA of the diets
255 led to significant differences at each ratio. Thus, there was a greater INF γ response at ~10%
256 PUFA than at 2.5% regardless of ratio. For IL-5, the response was greater in mice fed
257 10%PUFA at the 1:1 ratio compared to mice fed 2.8% PUFA. At the 10:1 ratio mice fed the
258 10% PUFA diets had a greater IL-5 response than mice fed the 2.5 and 5% PUFA diets. Lastly,
259 for the high ratio, increasing the %PUFA content of the diet led to a more robust IL-5
260 response at each level.

261 The IL-6 response to LPS was primarily driven by the %PUFA as well. At each ratio,
262 increasing the PUFA of the diet resulted in a more robust IL-6 response. However, there was
263 no ratio effect and thus, the IL-6 response in the high PUFA diets was not different between
264 the mice fed the 1:1 and 10:1 ratios at any PUFA level. For IL-10, the only significant
265 differences were at the high ratio. Mice fed the 2.5% PUFA diet had a less robust IL-10
266 response compared to mice fed the 5% and 10% PUFA diets. For IL-12p70 the results are
267 similar to IL-5 and IL-6. Increasing the %PUFA content of the diet resulted in more IL-12p70,
268 but only at the ~10% PUFA intakes.

269 For the remaining seven cytokines measured, there was a %PUFA \times ratio interaction and
270 the post-hoc statistical analysis compared all diet groups. Consequently, in Figure 2, the
271 arrangement of diet groups for the last seven cytokines is by %PUFA along the bottom axis.
272 For IL-1 β , there was a diminished response at the low PUFA level. Interestingly, mice fed the
273 1:1 ratio at 10% PUFA had a greater response than mice fed the 2.5% or 5% PUFA. For IL-
274 17, the only differences were between the 2.5% PUFA and 16:1 ratio and the 10:1 and 24:1
275 ratios at 10% PUFA. The IL-3 response pattern among diets was unique. Mice fed the 1:1

276 ratio at 10% PUFA had a more robust response than all other diet groups. Conversely, for
277 MIP1 α , mice fed the 1:1 ratio at 10% PUFA had a lower response compared to mice fed the
278 10% PUFA at the 10:1 and 24:1 ratio. The response of TNF α was qualitatively similar to
279 MIP1 α , in mice fed the 1:1 ratio at 10% PUFA had a lower response than mice fed the 10:1
280 and 24:1 ratios. The other group that had a low expression of TNF α was for mice fed the low
281 2.5% PUFA diet at a 16:1 ratio.

282 The RANTES response to the LPS challenge was similar across all diet groups, except mice
283 fed the low ratio, high PUFA diet. Lastly, for MCP-1, there were no differences at the low and
284 medium PUFA intakes, but dramatic effects at the high PUFA level. At ~10% PUFA, a 10:1
285 ratio resulted in the greatest response, followed by the 24:1 and 1:1 ratios.

286

287 **4. Discussion and Conclusions**

288 The goal of this study was to determine how the %kcal from LA and ALA PUFA and the n-
289 6:n-3 ratio affect the acute inflammatory response to intraperitoneal LPS injection in mice
290 when provided on the background of the AIN-93G diet. The fat composition of the diets had
291 a significant impact on feed efficiency and body composition despite the short feeding time.
292 The fat composition of the diets significantly affected the RBC composition, with the 1:1 ratio
293 diets resulting in the largest values for the omega-3 index. LPS administration caused a
294 significant increase in all 12 cytokines measured, compared to the sham injected mice, and
295 there were significant differences between mice fed different fat formulas. Some cytokines
296 responded only to the %PUFA level, some to the ratio, and for some there was a %PUFA \times
297 ratio interaction.

298 The higher level of body fat in mice fed the 10% PUFA diet at the 1:1 ratio, compared to
299 the 24:1 ratio was a surprise. This effect was present on both an absolute and percent body
300 composition basis. In addition, according to the MRI data, there were no differences between
301 groups in either lean mass, or water content, which indicates the diets did not restrict
302 growth. One reason this result was unexpected is that it has previously been shown in
303 C57BL/6J mice that high LA diets induce adipocyte differentiation via prostacyclin signaling
304 of AA metabolites [16]. Yet, according to Table 4, the mice with more adipose (10%PUFA, 1:1
305 ratio) had substantially less AA in their RBC membranes than mice fed the 10% PUFA diets
306 with a 24:1 n6:n3 ratio. One significant difference between their design, and ours, is that in
307 the present study the diets only provided 17% of energy as fat, whereas in the study cited
308 above it was 40%.

309 Hanbauer et al. showed that a high n-6:n-3 ratio fed over three generations will induce
310 obesity [17]. The low n-6:n-3 ratio diet contained 9.68% LA and 1.02% ALA, whereas the
311 high ratio diet was 12.31% LA and 0.16% ALA. Thus, the n-6:n3 ratios were 9.5:1 and 77:1.
312 Interestingly, the diets were less energy dense than ours, as their diets were 3.1 and 3.2
313 kcal/g wherease ours were 3.9 kcal/g. One other notable difference is that they used B6C3Fe
314 mice. Massiera et al. also showed that mice fed a high n-6:n-3 ratio diet became gradually
315 more obese over four generations [18]. However, one major issue with this study is that the
316 high n-6 diet had 3X the amount of fat as the control diet. Thus, it is not clear what the
317 primary driver of fat mass was over successive generations.

318 Alvheim et al. also found that increasing the dietary intake of LA was associated with more
319 adipose gain [19]. C57BL/6j mice were fed either moderate (35% kcal) or high-fat (60%
320 kcal) diets for 14 weeks. The diets contained either 1% or 8% of calories as LA, and ALA was

321 held constant at 1% of calories. In addition, a third group was fed 8% LA and had an
322 additional 1% of a mixture of EPA and DHA. Their results indicated that high LA induced
323 obesity via increases in the proportion of AA in liver and erythrocyte phospholipids and
324 associated endocannabinoid signaling. In a separate study using similar LA and ALA
325 treatments on a low fat diet, Alvheim et al found that 8% LA caused greater weight gain over
326 1% ALA. However, there were no differences when the same PUFA treatments were
327 presented in medium fat diets (35% kcal from fat). In both the studies by Alvheim, higher
328 intakes of LA increased feed efficiency. We found a %PUFA effect on feed efficiency, but no
329 ratio effect (Table 3; Figure 2). Other groups have not found an effect of high LA intakes and
330 high n-6:n-3 ratios on obesity and weight gain. For example, Enos et al. fed mice diets
331 composed of 40% kcal by fat where n-6 fatty acids comprised 4.7%, 7.8%, 8.6% and 9% kcal
332 [20]. By also changing the n-3 content the diets had 1:1, 5:1, 10:1 and 20:1 ratios of n-6 to n-
333 3. In contrast to the other studies described above, there was no effect of the different fat
334 formulas on weight gain, visceral adipose mass, or adipocyte size, despite significant effects
335 on the ratios of AA and both EPA and DHA in adipose phospholipids. While there may be an
336 effect of higher LA intakes on weight gain and adiposity in some animals and on some diet
337 backgrounds, the results are not consistent.

338 Populations that consume increased amounts of n-3 fatty acids appear to receive
339 protection from some pathologies associated with inflammation, advice given to individuals
340 is to change the n-6 to n-3 ratio by eating more oily fish, and other foods rich in n-3s like flax
341 seed oil. In the diet of early humans, the estimated ratio of n-6 to n-3 fatty acids was 0.8:1,
342 yet in some contemporary populations the ratio has increased to ~17:1 [21], presumably
343 due to the increased consumption n-6 rich vegetable oils and decreased fish consumption

344 [2]. In our LPS challenge model, increased dietary PUFA concentration had a strong
345 stimulatory effect on several plasma cytokines regardless of n-6 to n-3 ratio including IL-1 β ,
346 IL-5, IL-6, IL-10, IL-12p70, IL-17 and INF- γ and the dietary n-6 to n-3 PUFA ratios affected
347 MCP-1, TNF- α , MIP-1 α and RANTES. Our data suggests that the total amount of dietary PUFA
348 may be an important determinant in the acute LPS inflammatory response and the n-6 to n-
349 3 ratio may only be important at higher concentrations of total dietary PUFA.

350 In recent years it has been suggested that a high n-6 intake and high n-6:n-3 ratio promote
351 the pathogenesis of many diseases [20]. The link between increased n-6 intake, inflammation
352 and disease progression is based on the idea that higher dietary intakes of LA will increase
353 cellular pools of AA, and will promote a more inflammatory cytokine response [21]. Yet,
354 despite this supposition, in humans consuming Western-type diets, plasma AA is resistant to
355 large changes in dietary LA [22]. In addition, while human peripheral blood mononuclear
356 cell PUFA composition does affect immune function, the LA, ALA, AA, EPA and DHA levels do
357 not necessarily correlate with dietary intake [23]. Furthermore, in a cross-sectional analysis
358 of plasma PUFA levels and inflammatory markers, the lowest quartile of n-6 PUFA was
359 associated with the highest concentration of TNF α and IL-6 [24]. Because the n-6:n-3 ratio is
360 driven by both n-6 and n-3 consumption, effects will be affected by both the total and relative
361 intakes.

362 Several studies have investigated the effects of dietary n-3 on systemic inflammation but
363 the focus of the majority of these studies has been on long chain n-3 fatty acids, primarily
364 EPA and DHA. Our data suggest that dietary intake and relative ratios of plant derived n-6
365 and n-3 fatty acids have profound effects on the LPS induced inflammatory response. Very
366 few studies have investigated the effects of dietary n-6 and n-3 fatty acids on acute, LPS

367 initiated inflammation and to our knowledge, no studies have investigated the relative
368 contributions of both total PUFA concentration and ratios of n-6 to n-3. Chavali et al. [22]
369 injected Balb/c mice with a lethal dose (20 mg/kg) of LPS. Mice were fed either safflower oil
370 diets or safflower + linseed oil diets. The fat content of the safflower oil diets was 79.4% LA
371 or ~24% of total energy with negligible ALA. The safflower + linseed oil diet contained
372 approximately equal LA and ALA, approximately 12% of total dietary energy for each fatty
373 acid. Plasma levels of IL-6 were higher in the LA fed animals compared to the LA + ALA fed
374 mice while TNF- α levels were higher in the LA + ALA animals. Although in our study much
375 lower levels of dietary PUFA were fed, we found that that decreasing the n-6 to n-3 ratio by
376 incorporating dietary ALA decreases LPS induction of plasma TNF- α . In a previous LPS
377 challenge study by our group [23], mice were fed either the AIN-76 diet or a modified AIN-
378 76 diet that replaced the corn oil fat source with dairy lipids. The dairy lipid diet contained
379 only 0.6% of calories from PUFA and had had an n-6 to n-3 ratio of 9.4. Conversely, the corn
380 oil fat sourced AIN-76 diet contained 6.9% of calories from PUFA and n-6 to n-3 ratio of 56.4.
381 Similar to the current study, mice fed the lower PUFA, lower n-6 to n-3 ratio diet had
382 significantly lowered levels of plasma IL-6, IL-3, IL-10, IL-17, MCP-1, IFN- γ , TNF- α and IL-
383 12p70.

384 The effects of dietary LA and ALA have been investigated in other models of acute
385 inflammation, including chemically induced colitis. In a recent study [24], colitis was
386 induced in rats fed either corn oil or an ALA rich camelina oil diet. Rats fed the ALA rich diet
387 had lowered colonic expression of IL-1 β and TNF- α . Similarly, in a study by Tyagi and
388 coworkers [25] rats were fed differing n-6 to n-3 ratios by manipulating dietary LA and ALA
389 levels varying from 215:1, 50:1, 10:1 and 2:1. Inflammatory colitis was induced by dextran

390 sodium sulfate treatment. Colonic expression of both IL-1 β and TNF- α was lowest at the 2:1
391 ratio. In our study, the n-6 to n-3 ratio significantly affected plasma levels of TNF- α however,
392 plasma IL-1 β were not responsive to the PUFA ratio and increased as a function of total
393 dietary PUFA.

394 The inflammatory response to LPS is mediated through the LPS receptor, Toll-like
395 receptor 4 (TLR4). Circulating LPS is bound by the secreted LPS binding protein (LBP) and
396 then can form a complex with CD14 and interact TLR4 to initiate a signaling cascade that
397 results in the degradation of I κ B and binding of NF- κ B to the promoters of immune response
398 genes [26]. NF- κ B is a ubiquitously expressed transcription factor that mediates the
399 inflammatory response to a diverse set of signals including LPS. Activation of NF- κ B involves
400 translocation from the cytosol to the nucleus after disassociation from the inhibitory subunit
401 I κ B. The resulting signaling causes increased expression of several inflammatory cytokines
402 including TNF- α , IL-1 β , IL-6 and INF- γ . Fatty acids are known to play a role in the NF- κ B
403 signaling pathway. Zhao et al (2004) demonstrated that EPA inhibited NF- κ B inflammatory
404 activation by preventing the phosphorylation of I κ B in THP-1 cells and inhibited NF- κ B
405 activation similar to DHA in LPS induced THP-1 cells [27, 28]. Inhibition of NF- κ B activation
406 by fish oil has also been reported in RAW 267.4 macrophages [28]. Lee and coworkers [29]
407 demonstrated that DHA and EPA are the most potent inhibitors of LPS induced NF- κ B
408 activation in RAW 264.7 mouse monocytes. However, arachidonic, linoleic and oleic acid
409 also inhibited NF- κ B activation compared to cells treated with lauric acid. Fatty acids are also
410 thought to influence inflammatory pathways by acting as ligands for PPAR γ . PPAR γ is
411 expressed in many tissues including adipose, muscle, and vascular cells. Activated PPAR γ
412 induces lipoprotein lipase and fatty acid transporters and enhances adipocyte differentiation

413 as well as inhibiting NF- κ B function, cytokine and COX-2 expression. Acting as a PPAR γ
414 ligand, EPA has been demonstrated to attenuate expression of IL-6 in C6 glioma cells [30]
415 and Caco-2 intestinal cells [31].

416 It is not clear why the diets lowest in PUFA generally resulted in less inflammation in the
417 current study. Animals fed the 2.5% PUFA diets at the 10:1 and 24:1 rations did not have
418 lower levels of AA or higher levels of EPA or DHA as RBC fatty acids compared to the 10%
419 PUFA diets. Therefore, our data suggests that the reduced inflammation observed in the low
420 PUFA compared to the high PUFA diets may be independent from conversion of LA to AA or
421 ALA to EPA and DHA.

422 Taken together, our data suggest that the most commonly consumed n-3 and n-6 fatty
423 acids in modern diets, LA and ALA, play an important role in the susceptibility to acute
424 inflammatory stressors. Current public health recommendations have stressed the
425 importance of lowering the n-6 to n-3 ratio and incorporating long chain n-3 fatty acids such
426 as EPA and DHA into diets. By designing a study to determine the inflammatory potential of
427 both dietary PUFA concentration and n-6 to n-3 ratio using PUFA sources relevant to modern
428 diets; we have found that total dietary PUFA concentration must be considered as an
429 important factor in acute inflammation. This finding may have implications for conditions
430 such as sepsis and bacterial infections. However, the molecular mechanisms as to how
431 different concentrations and ratios of dietary LA and ALA influence LPS mediated
432 inflammation are not known. Future work examining the effects of these diets on candidate
433 pathways such as expression of toll like receptors, NF- κ B and PPAR- γ , and on PUFA-derived
434 lipid mediators is warranted.

435

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441

442 REW and KJH were involved in the study design. REW designed the diets and KJH and JT
443 conducted experiment 1. BBG and JH conducted experiment 2. REW, KJH and ML performed
444 the data analysis and interpretation. KJH and REW wrote the manuscript and all authors
445 contributed to producing the final manuscript.

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Table 1: Fat formulas for experimental diets. Values are expressed as a percentage of total dietary fat. Abbreviations are: Cocoa butter (CB), corn oil (CO), cottonseed oil (CSO), flaxseed oil (FSO), olive oil (OO), sunflower oil (SFO) and soybean oil (SO). The diets were prepared by Harlan Laboratories (Madison, WI) and the catalog numbers are given in the table.

Diet	Target %PUFA	n-6:n-3 Ratio	CB	CO	CSO	FSO	OO	SFO	SO	Catalog #
1	2.5	1:1	45.5	--	--	12.5	42.0	--	--	TD.10148
2	2.5	10:1	29.5	11.0	--	15.5	58.0	--	--	TD.10149
3	2.5	20:1	23.0	9.5	--	--	65.0	--	2.5	TD.10150
4	5	1:1	45.0	--	--	23.0	15.5	--	16.5	TD.10151
5	5	10:1	26.2	39.0	--	3.8	31.0	--	--	TD.10152
6	5	20:1	15	--	30.0	--	42.0	--	13.0	TD.10153
7	10	1:1	8.0	--	40.0	52.0	--	--	--	TD.10154
8	10	10:1	--	--	79.4	9.1	--	11.5	--	TD.10155
9	10	20:1	--	--	65.5	4.5	5.0	25.0	--	TD.10156

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Table 2. Fatty acid composition of diets. Values are expressed as a percentage of total dietary energy.

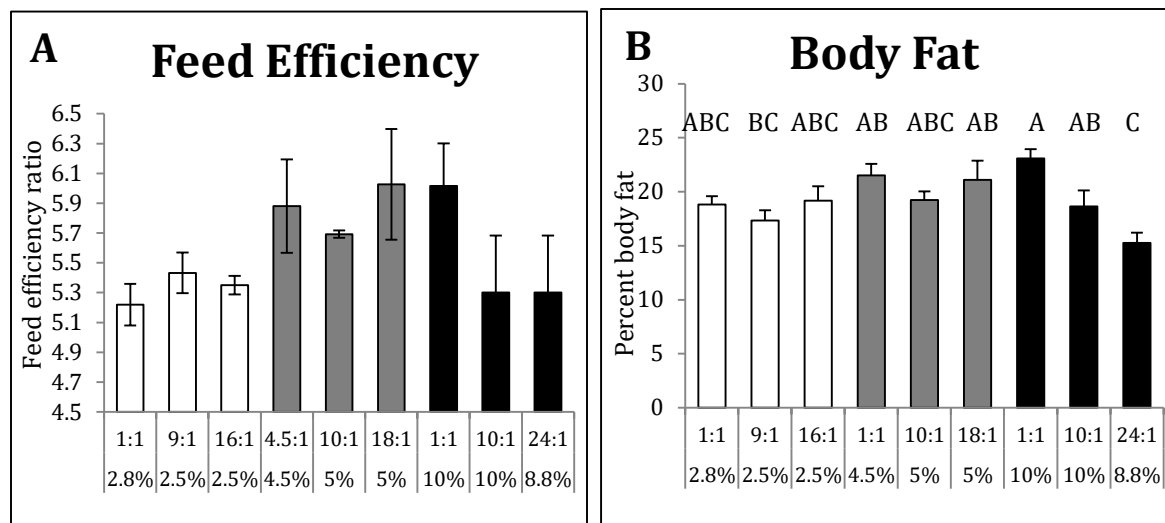
Diet	<i>Target Values</i>		<i>Measured Values</i>								
	PUFA%	Ratio	16:0	18:0	18:1n9	18:2n6	18:3n3	SFA	MUFA	PUFA	ratio
1	2.5	1:1	2.8±.1	2.8±.1	8.4±.1	1.5±.1	1.4±.1	5.6	8.4	2.8	1:1
2	2.5	10:1	2.5±.1	2.0±.2	9.8±.1	2.3±.1	0.3±.1	4.5	9.8	2.5	9:1
3	2.5	20:1	2.4±.1	1.7±.1	10.2±.1	2.4±.1	0.2±.1	4.1	10.2	2.5	16:1
4	5	1:1	2.6±.1	3.5±.2	6.2±.4	2.6±.4	2.0±.7	6.1	6.2	4.5	1:1
5	5	10:1	2.4±.1	2.0±.3	7.5±.2	4.5±.3	0.5±.1	4.4	7.5	5	10:1
6	5	20:1	2.6±.1	1.4±.2	7.9±.2	4.8±.3	0.3±.1	3.9	7.8	5	18:1
7	10	1:1	2.2±.1	1.1±.3	3.7±.4	5.3±.2	4.8±.7	3.3	3.7	10	1:1
8	10	10:1	2.8±.1	0.5±.1	3.4±.1	9.2±.2	0.9±.1	3.3	3.4	10	10:1
9	10	20:1	2.7±.2	0.8±.2	4.5±.5	8.5±.8	0.4±.1	3.5	4.5	8.8	24:1

Table 3: Results of two way ANOVA for food intake, weight gain, and body composition.

	% PUFA	Ratio	Interaction
	<i>p value</i>		
Food intake	0.46	0.11	0.34
Weight gain	0.08	0.36	0.12
Feed efficiency	0.04	0.43	0.13
Percent body fat	0.06	<0.01	<0.01

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595 **Figure 1: Feed efficiency ratio (A) and percent body fat (B) of mice fed nine experimental diets. There**
 596 **was a significant effect of PUFA on the feed efficiency ratio ($p=0.4$), but no n-6:n-3 ratio effect or**
 597 **interaction in the two way ANOVA (A). For body fat there was both a ratio effect ($p<0.01$) and a PUFA \times**
 598 **ratio interaction ($p<0.01$) as determined by the two way ANOVA. Groups with the same letter are not**
 599 **significantly different according to the Tukey HSD test.**

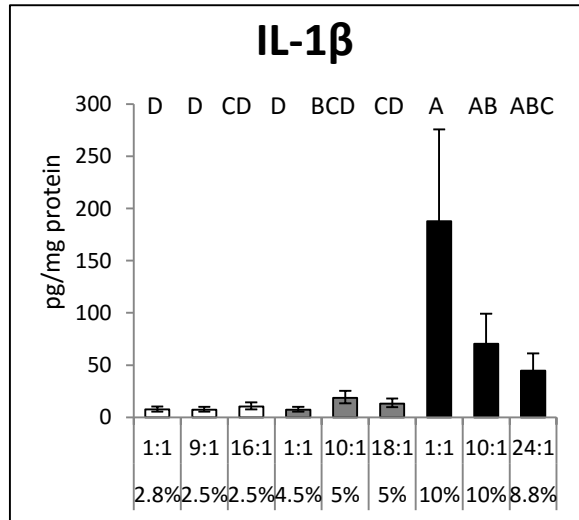
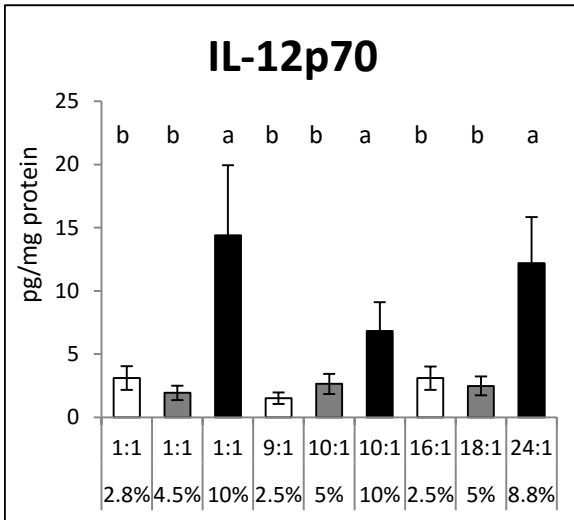
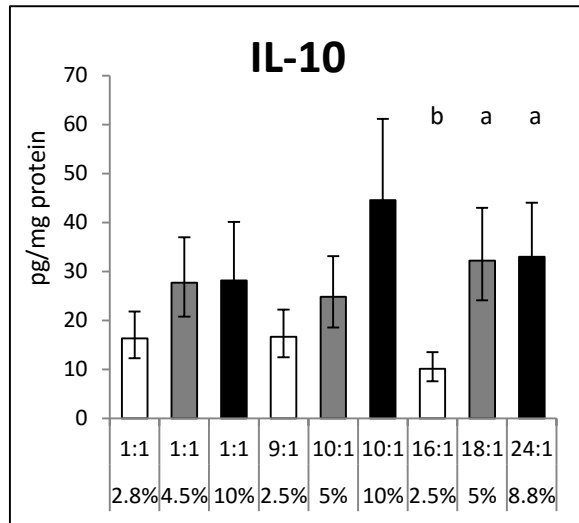
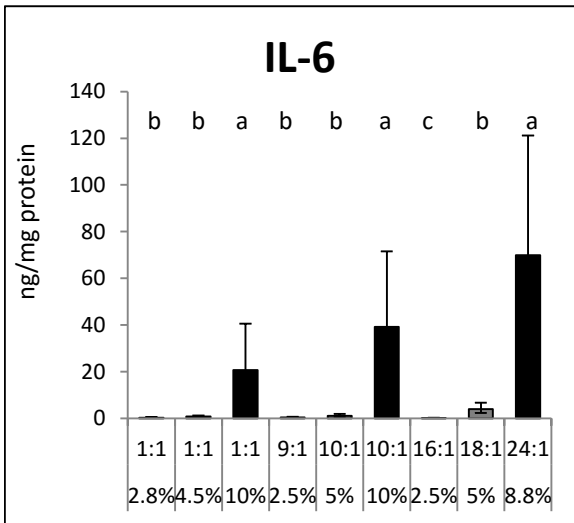
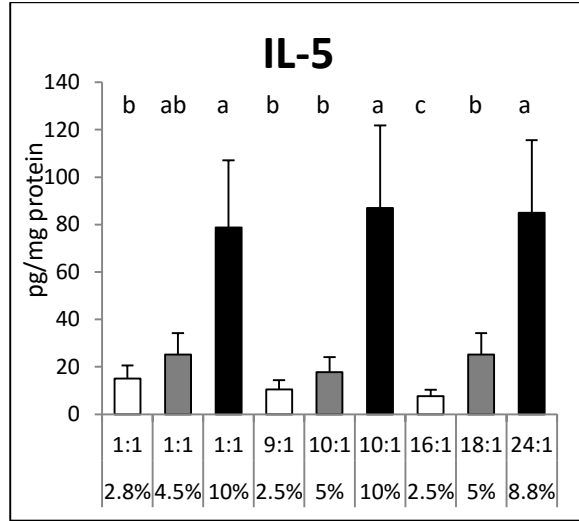
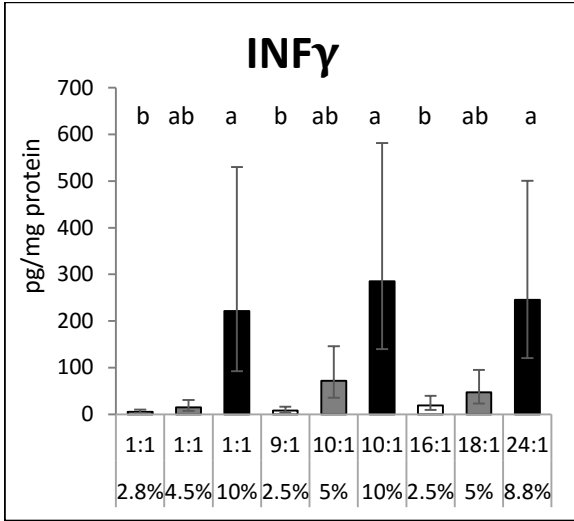
Table 4: Fatty acid composition of erythrocytes (in percent) from mice fed 9 diets differing in %PUFA and the n-6:n-3 ratio.

PUFA %kcal n-6:n-3 ratio	2.8%	2.5%	2.5%	4.5%	5%	5%	10%	10%	8.8%	<i>p</i> values for 2 × 2 ANOVA		
	1:1	9:1	16:1	1:1	10:1	18:1	1:1	10:1	24:1	PUFA	Ratio	Interaction [‡]
16:0	25.2±0.6	24.6±1.2	24.1±0.2	26.3±0.5	24.4±0.8	24.8±0.5	26.1±0.7	27.3±2.1	26.5±1.4	<0.01	NS	NS
16:1n-7	2.6±0.7	2.0±1.0	2.5±0.4	1.7±0.3	2.3±0.3	2.2±0.1	2.3±0.6	2.4±0.5	2.0±0.8	NS	NS	NS
18:0	12.6±0.3	12.6±0.3	12.3±0.3	13.7±0.1	13.6±0.6	13.4±0.2	13.6±0.7	12.6±0.8	13.0±0.1	<0.01	NS	NS
18:1n-9	22.3±1.4	21.6±1.4	21.3±0.7	17.4±0.1	17.3±0.5	18.0±0.4	14.3±0.6	12.3±0.8	11.9±0.4	<0.01	0.04	NS
18:2n-6	6.1±0.3	6.2±0.4	6.5±0.3	8.8±0.2	7.8±0.6	8.0±0.1	11.7±0.4	11.6±0.6	12.0±0.4	<0.01	NS	NS
18:3n-3	1.4±0.2	1.1±0.6	1.0±0.1	1.6±0.2	1.6±0.2	1.4±0.3	2.0±0.5	1.8±0.7	1.8±0.6	<0.01	NS	NS
20:0	0.4±0.1	0.5±0.2	0.7±0.4	0.2±0.1	0.4±0.1	0.4±0.1	0.4±0.1	0.4±0.1	0.4±0.2	NS	NS	NS
20:1n-9	0.6±0.2	0.8±0.2	0.7±0.1	0.4±0.2	0.8±0.3	0.5±0.1	0.4±0.1	0.8±0.3	0.7±0.4	NS	0.03	NS
20:3n-9	0.8±0.1	1.2±0.1	1.1±0.1	0.2±0.1	0.3±0.01	0.3±0.1	0.2±0.1	0.3±0.1	0.2±0.2	<0.01	<0.01	NS
20:3n-6	1.4±0.2	1.7±0.1	1.8±0.3	1.3±0.2	1.6±0.1	1.5±0.1	1.4±0.1	1.7±0.1	1.6±0.3	NS	<0.01	NS
20:4n-6	12.1±0.8	18.3±1.0	19.6±0.5	12.5±0.3	18.9±0.6	19.8±0.4	11.8±0.4	17.7±0.5	18.8±0.8	0.02	<0.01	NS
20:5n-3	2.3±0.3 ^B	0.3±0.1 ^C	0.2±0.1 ^C	2.9±0.1 ^A	0.5±0.5 ^C	0.1±0.02 ^C	3.0±0.2 ^A	0.3±0.1 ^C	0.1±0.02 ^C	0.03	<0.01	0.01
22:0	0.2±0.1	0.2±0.1	0.1±0.1	0.1±0.01	0.4±0.4	0.1±0.01	0.05±0.06	0.3±0.2	0.1±0.1	NS	0.04	NS
22:4n-6	0.7±0.1	2.1±0.2	2.2±0.1	0.6±0.4	2.2±0.1	2.4±0.1	0.6±0.1	2.0±0.2	2.7±0.4	NS	<0.01	NS
22:5n-6	0.2±0.02 ^D	1.7±0.1 ^C	2.2±0.2 ^B	0.2±0.03 ^D	1.5±0.1 ^C	2.7±0.2 ^A	0.1±0.04 ^D	1.5±0.1 ^C	2.7±0.2 ^A	NS	<0.01	<0.01
22:5n-3	1.7±0.1 ^C	0.4±0.1 ^{D,E}	0.2±0.1 ^E	2.2±0.1 ^B	0.5±0.4 ^{D,E}	0.3±0.03 ^{D,E}	2.7±0.1 ^A	0.7±0.02 ^D	0.5±0.1 ^{D,E}	<0.01	<0.01	<0.01
22:6n-3	8.9±0.8 ^A	4.4±0.4 ^C	3.2±0.1 ^D	9.6±0.1 ^A	5.4±0.5 ^{B,C}	3.8±0.2 ^{C,D}	9.1±0.3 ^A	6.0±0.3 ^B	4.5±0.4 ^C	<0.01	<0.01	0.02
24:0	0.2±0.03	0.2±0.1	0.1±0.03	0.2±0.1	0.2±0.03	0.2±0.03	0.2±0.01	0.2±0.03	0.2±0.04	NS	0.01	NS
24:1n-9	0.3±0.1	0.2±0.1	0.2±0.2	0.2±0.1	0.2±0.1	0.1±0.02	0.1±0.02	0.2±0.1	0.1±0.03	NS	NS	NS
Ω-3 index	11.3±1.0	4.7±0.4	3.3±0.1	12.5±0.1	5.9±0.9	3.8±0.2	12.0±0.4	6.2±0.4	4.6±0.4	<0.01	<0.01	NS

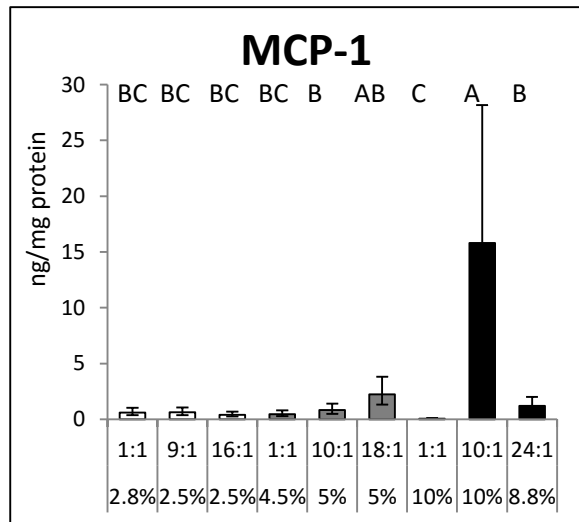
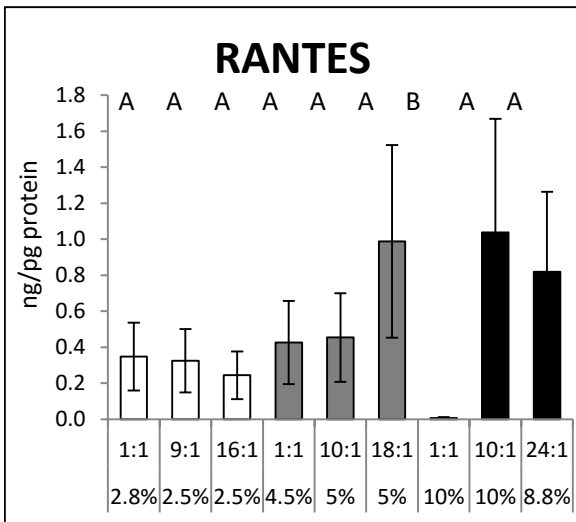
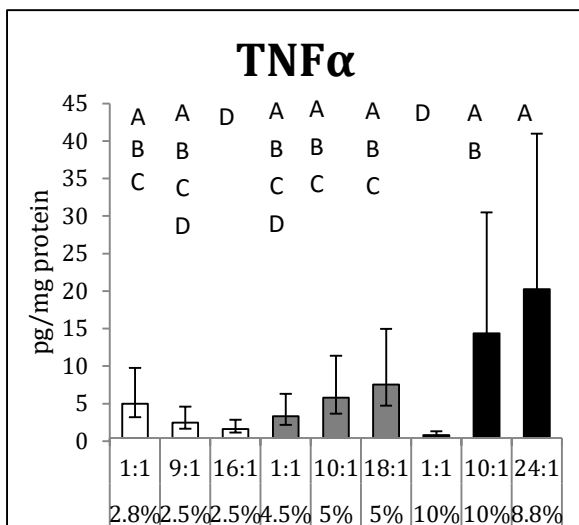
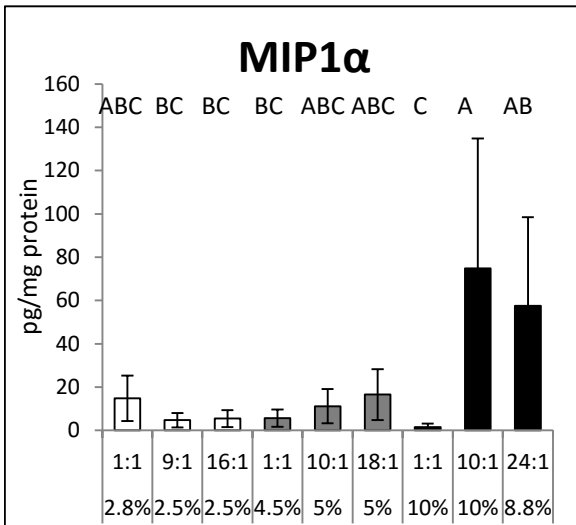
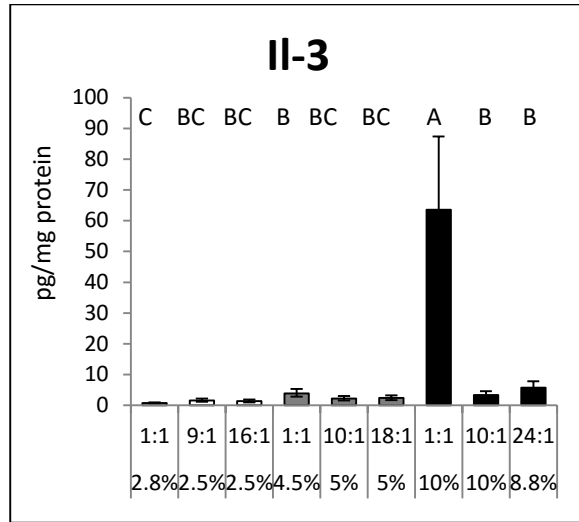
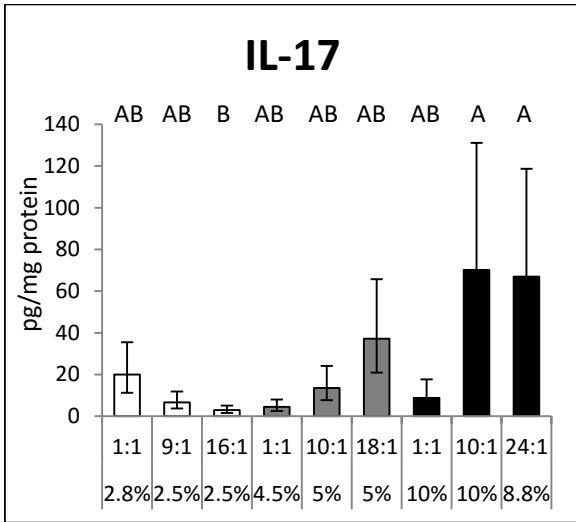
[‡]The 2 × 2 ANOVA indicated there were four fatty acids that had a %PUFA × ratio interaction (20:5n3, 22:5n6, 22:5n3, and 22:6n3). Superscripts indicated fatty acids that are significantly different according to the Tukey HSD test.

Table 5: Two-way ANOVA analysis of effects of dietary PUFA concentration and n-6:n-3 ratios on plasma cytokines in mice treated with LPS.

Cytokine	% PUFA	n-6:n-3 ratio	Interaction
INF γ	<0.01	NS	NS
Il-5	<0.01	NS	NS
Il-6	<0.01	NS	NS
Il-10	<0.01	NS	NS
Il-12p70	<0.01	NS	NS
IL-1 β	<0.01	NS	0.02
Il-17	<0.01	NS	<0.01
Il-3	<0.01	<0.01	<0.01
MIP1- α	0.03	0.05	<0.01
TNF α	0.05	<0.01	<0.01
RANTES	<0.01	<0.01	<0.01
MCP-1	NS	<0.01	<0.01



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2



3 Figure 2: Effect of diets on plasma cytokines 24h after acute LPS administration. Cytokines
4 were analyzed using a two way ANOVA and significance differences further explored using
5 the Tukey HSD test. For cytokines A-E there is an effect of PUFA, but no ratio effect nor an
6 interaction. Lower case letters for these cytokines are only comparable within a PUFA level
7 (2.5%, 5%, 10%). For cytokines F-L there was a PUFA \times ratio interaction, and upper case
8 letter indicate differences between treatments.

9