Evaluation of Fatty Acid Whole Blood Concentrations in the San Cristóbal Galápagos Tortoise *(Chelonoidis chathamensis)*

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To continue releasing San Cristóbal Galápagos tortoises housed in managed-care facilities at the Giant Tortoise Breeding Center of Galápagos National Park (Galapaguera de Cerro Colorado) to the Otoy Ecological Farm, health assessments and physical examinations were conducted. As a part of these wellness examinations, blood was drawn from 11 tortoises to analyze fatty acid concentrations. Fatty acid levels can provide insight into the nutritional profiles, immune status, and reproductive health of vertebrates. To the co-author's knowledge, there is no current information about fatty acids in this species. It was hypothesized that there would be inherent differences based on the different geographic ranges, diets, sex, and age of turtles. It was noted that the ω-6/ω-3 ratio was higher for the breeding center than for the ecological farm and that overall polyunsaturated fatty acids (PUFAs) did not have any significant differences. The ω-6/ω-3 findings can contribute to a global picture of these fatty acids across taxa, as reptiles are underrepresented in this area of research. Additional results are a resourceful starting point for future investigations into how fatty acids are affected in Galápagos tortoises.

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2 **Galápagos Tortoise** *(Chelonoidis chathamensis)*

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26 **Abstract**
27 To continue releasing San Cristóbal Galápagos tortoises housed in managed-care

28 facilities at the Giant Tortoise Breeding Center of Galápagos National Park

29 (Galapaguera de Cerro Colorado) to the Otoy Ecological Farm, health assessments and

30 physical examinations were conducted. As a part of these wellness examinations, blood

31 was drawn from 11 tortoises to analyze fatty acid concentrations. Fatty acid levels can

32 provide insight into the nutritional profiles, immune status, and reproductive health of

33 vertebrates. To the co-author's knowledge, there is no current information about fatty

34 acids in this species. It was hypothesized that there would be inherent differences

35   based on the different geographic ranges, diets, sex, and age of turtles. It was noted

36   that the ω-6/ω-3 ratio was higher for the breeding center than for the ecological farm

37   and that overall polyunsaturated fatty acids (PUFAs) did not have any significant

38   differences. The ω-6/ω-3 findings can contribute to a global picture of these fatty acids

39   across taxa, as reptiles are underrepresented in this area of research. Additional results

40   are a resourceful starting point for future investigations into how fatty acids are affected

41   in Galápagos tortoises.

42   **Introduction**
43   The San Cristóbal Galápagos tortoise (SCGT), *Chelonoidis chathamensis,* is endemic

44   to one of the oldest islands in the Galápagos Archipelago (Parent et al., 2008). As the

45   human population in the Galápagos Islands continues to expand, the SCGT faces

46   growing threats and has been categorized as Endangered (EN) in the IUCN red list

47   (Caccone et al. 2017).

48

49   Recent population genetic analyses have exposed that the population is recovering

50   from a severe bottleneck that depleted its genetic diversity, with a population trend

51   increasing of 2,950 adult individuals (Garrick et al. 2014). The species is protected for

52   any commercial use and international trade under CITES Appendix 1 (Turtle Taxonomy

53   Working Group, 2017). In an effort to restore their population, the SCGT has been

54   brought into managed facilities for care, propagation, and reintroduction into the wild.

55   Maintaining proper husbandry and conducting frequent health screenings at these

56   facilities are essential components to help increase the chance of successful breeding

57   and restocking programs.

58

59   Many health assessments and annual physical examinations include blood collection

60   and analysis for standard hematology and biochemistry parameters (Geffre et al., 2009;

61   Zhang et al., 2011; Gibbons et al., 2013; Lewbart et al., 2018; Carreta et al., 2019).

62   Fatty acid (FA) profiles are not commonly included in health assessments of this or

63   other tortoise species, so reference values have not been established. This study

64   presents the fatty acid profile of *C. chathamensis* from both a captive breeding center

65   and ecological farm where SCGT has been introduced for a conservation and

66   ecotourism initiative and evaluates potential connections to nutritional status, immune

67   function, and reproductive capabilities (Cartland-Shaw et al., 1998, Rustan and Drevon,

68   2005; Samee et al., 2018).

69
70   **Materials & Methods**
71   **Sampling and Study Area**

72   For the present study, 16 SCGT were sampled: eleven from the Otoy Ecological Farm

73   (Finca Voluntad de Dios, Cerro Verde, Isla San Cristóbal, Islas Galápagos, Ecuador)

74   and five from Giant Tortoise Breeding Center of Galapagos National Park (La

75   Galapaguera de Cerro Colorado, Isla San Cristóbal, Islas Galápagos, Ecuador). All but

76   one of the SCGT from the Otoy Ecological Farm were male. Alternatively, most of the

77   SCGT from the Breeding Center were juveniles and thus undetermined sex, except for

78   one that was male. All tortoises included in this work were clinically healthy as

79   determined by physical examination, carapace and plastron measurements, blood

80   collection (PCV/TS and biochemistry analysis), temperature, and fecal collection.

81   Samples were collected in the springtime (March, 2018). Typical weather conditions

82   during this time are 25-30°C with sunshine and minimal cloud coverage. This study was

83 performed as part of a population health assessment authorized by the Galapagos

84 National Park Directorate (permit Nos. PC-21-18, PC-57-19) and approved by the

85 Galápagos Science Center and North Carolina State University ethics and animal

86 handling protocol. All handling and sampling procedures were consistent with standard

87 vertebrate protocols and veterinary practices. No experiments were performed and

88 such work is rarely if ever permitted in the Galápagos. Having access to these animals

89 is a privilege and we were taking advantage of a valuable permit related to a health

90 assessment to obtain as much information as possible form these animals while

91 handling and taking blood samples. The data collected are simply a quantification of

92 the fatty acids in this species of Galápagos tortoise.

93

94 Both sampling sites are located on the Northeast side of San Cristóbal Island and share

95 some similarities. The "Galapaguera de Cerro Colorado" is one of a few sanctuaries that

96 breeds Galápagos giant tortoises in captivity. It encompasses 11 hectares (30 acres)

97 and holds 180 free-ranging SCGT that are either in the breeding program or are the

98 offspring of these breeding pairs. The SCGT are allowed to roam freely and have

99 access to a vast quantity of native vegetation, i.e. Manzanillo (*Hippomane mancinella)*.

100

101 In comparison, the Otoy farm is situated on 16 hectares of private land where SCGT

102 roam freely over an area of 1 and a half hectare with sparsely native plants and

103 introduced trees. The SCGT are fed a mixture of otoy *(Otoy yautia)* and elephant grass

104 *(Pennisetum purpureum)* at 5 kg/tortoise every other day. Additionally, there is a diverse

105 group of non-native fruits at the site including passion fruit *(Passiflora edulis),* banana

106 *(Musa paradisciaca),* guava *(Psidium guaiva),* plum *(Spondias movie)* and orange

107 *(Citrus aurantium)* that the tortoises could have access. A similarity between the diets of

108 both groups is that they have access to opuntia cactus *(Opuntia echios).*

109

110 **Blood Sample Collection and Handling**

111 The SCGT chosen to be analyzed as a part of this study were part of an annual

112 continuing health assessment performed as a part of pre-release screening. They were

113 all healthy as determined by physical examination, fecal, and bloodwork parameters.

114 Blood was collected from either the brachial sinus or dorsal coccygeal vein using a

115 heparinized (Heparin Sodium USP, 1000 units/ml; APP Pharmaceuticals, LLC,

116 Schaumburg, IL, USA) 3.0 ml syringe with a 1.0" 22-gauge needle. Two to three drops

117 of whole blood were placed onto a paper card containing anti-oxidant stabilizing agents

118 (PerkinElmer 226 Spot Saver RUO Card, PerkinElmer Health Sciences, Inc., Greenville,

119 SC, USA) and allowed to air dry. Samples on the filter paper were stored at room

120 temperature during transportation. Whole blood was collected and applied to a

121 stabilized Perkin Elmer 206 blood spot card. The card was allowed to dry and then

122 mailed to Lipid Technologies (1600 19th Avenue SW, Austin MN 55912) for analysis

123 (Marangoni et al. 2004). Dried blood spots were then punched out into a 16x100mm

124 screw-top test and subjected to direct derivatization with acidic methanol. The resulting

125 fatty acid methyl esters were extracted with hexane and separated by capillary column

126 gas chromatography on a Shimadzu GC2010 gas chromatograph equipped with a 30 m

127 FAMEWAX (Restek, State College, PA) column. Fatty acid methyl esters were

128   identified by flame ionization detection. Retention times were compared to a mixed fatty

129   acid methyl ester standard from NuChek Prep (Elysian, MN).

130

131   **Statistical analysis**

132   Differences in fatty acids levels between the ω-6/ω-3 ratio and PUFA were

133   analyzed. Sampling sites were determined using a Wilcoxon test to determine if

134   the pairs were statistically the same or not based on current literature. The Holm-

135   Bonferroni correction was applied to set an acceptable p-value cut off, given the

136   high number of comparisons performed and decrease false-positive results. All

137   statistical analysis was performed in R (version 3.4.3, The R Foundation for

138   Statistical Computing).

139
140   **Results**
141   The present data shows the median values for the full fatty acid profile from both

142   environments, the Ecological Farm and Breeding Center *(Table I).* The ω-6/ω-3 ratio

143   showed a significant difference *(p-value = 0.014)* with the group from the breeding

144   center being higher than the ranch (2.023 vs 1.133, respectively) *(Figure I).* Comparison

145   of PUFA between the center and the ranch (15.958 vs 13.954, respectively) was not

146   significantly different *(p-value = 0.563) (Figure 2)*.

147

148   **Discussion**

149   There is no information regarding normal circulating levels of fatty acids in Galapagos

150   tortoises. Many factors influence the levels of fatty acids including diet, age,

151   physiological status, and stress (Clauss et al., 2007, Joseph et al., 1985; Cartland-Shaw

152   et al., 1998; Samee et al., 2018), which make it difficult for inter-species extrapolation.

153   These results can provide answers for improving husbandry of animals kept in managed

154   care facilities. Reptiles have unique fatty acids that are important to their biology.

155   Current information in this area has shown a trend of higher ω-6/ω-3 ratio in-managed

156   care settings or higher PUFA in wild reptiles (Cartland-Shaw et al., 1998; Clauss et al.,

157   2007, Joseph et al., 1985).

158

159   Increasing research in FA effects in human and companion pet nutrition has led to an

160   awareness of some potential adverse effects with dysregulation of ω-6/ω-3 ratio.

161   Studies in human nutrition have shown how the shift in the ω-6/ω-3 ratio from 1/1 to 5/1-

162   50/1 has negative impacts on the function of the neural and cardiovascular system and

163   diseases including cancer and arthritis (Simopolous and Cleland, 2003). One study of

164   companion animals examined FA levels in commercially prepared diets for cats and

165   dogs (Lenox and Bauer, 2013). An increased ω-6/ω-3 ratio in cats showed altered

166   platelet functions and improved glucose control via decreased serum insulin. While in

167   dogs, a decreased ω-6/ω-3 ratio showed delayed wound epithelialization of wounds,

168   decreased number of neutrophils and leukotrienes B4, decreased CD4+ T lymphocytes,

169   and lower delayed-type hypersensitivity response (Lenox and Bauer, 2013). Our study

170   shows significantly higher values of ω-6/ω-3 in the breeding center compared to farm.

171   This could be due to higher levels of ω-6 or lower levels of ω-3 in the diet. While these

172   trends and adverse effects of ω-6/ω-3 have been observed in mammals, it is not

173   guaranteed to translate to reptiles. The animals in our study were all healthy and were

174   not likely in an inflammatory state and thus this was not a confounding factor for the ω-

175   6/ω-3.

176

177   Highly unsaturated fatty acids (HUFA) are those that contain multiple double bonds in

178   their carbon chains and therefore are a specific type of PUFA. It has been established

179   that plants and phytoplankton are among the only organisms that can synthesis these

180   PUFAs, making them an essential dietary requirement for most animals, including

181   reptiles (Cartland-Shaw et al., 1998; Rustan and Drevon, 2005). In the present study,

182   there were no significant differences between the two groups for PUFAs, although the

183   breeding center did numerically have higher concentrations than the farm. This finding

184   is in contrast to a study that examines dietary and plasma concentrations of fatty acids

185   between wild and managed tuatara (*Sphenodon punctatus)* and showed higher plasma

186   concentrations of ω -3 PUFAs of the wild group than the managed group (Cartland-

187   Shaw et al., 1998). It is interesting to hypothesis why the ω -3 HUFAs of the SCGTs at

188   the breeding center were at such high levels and whether this impacted any of their

189   membrane functionality in terms of fluid balance, immune system, or reproductive

190   capability.

191

192   Since several tortoises in the present study came from a breeding center, it is essential

193   to discuss the role of FA in reproduction. There have been several reproductive studies

194   conducted to examine the role of fatty acid concentrations on fertility in vertebrates

195   (Cartland-Shaw et al., 1998; Lin et al., 2016; Len Van Tran et al., 2017; Samee et al.,

196   2018). In one study using zebrafish, it was concluded that the addition of extra virgin

197   olive oil extracted from Koroneiki that was rich in oleic acid (18:1 ω-9) and linoleic acid

198   (18:2 ω-6) improved the reproductive capability of both males and females in terms of

199   sperm quality (motility, fertility, and hatching), frequency of follicular development, and

200   accelerated hatching (Samee et al. 2018). Another study looked at the importance of ω-

201   3 and ω-6 PUFA for male reproductive health in ruminants, including parameters of

202   sperm membrane integrity, maintaining viable sperm during the chilling and freezing

203   process, and testicular development (Len Van Tran et al., 2017). The ω-3/ω-6 ratio in

204   boar's was examined and determined to improve testicular development and possibly

205   chances for breeding selection overall libido in animals fed a 1:1 ω-3/ω-6 ratio (Yan Lin

206   et al., 2016). While these references are promising developments to be correlated to

207   reproductive success, the SCGT values in the present study were not examined for

208   fecundity. This may be a future avenue to explore once normal FA have been

209   established for this species.

210
211   **Conclusions**

212   A unique opportunity to analyze fatty acids vales for healthy Galapagos Tortoises

213   endemic to to San Cristóbal Island was incorporated into the annual health

214   examinations of this population. This novel technique is the first time such a study has

215   been performed in this species. This study supported this finding for the ω-6/ω-3 ratio

216   being higher in-managed care animals compared to wild animals. The results did not

217   support the findings of overall PUFAs higher for in-managed care animals. Additionally,

218   parameters influencing the differences of these fatty acids such as diet, sex, and

219   physiologic status, could be examined.

220

221 **Acknowledgements**
222 We wish to thank the following people for their support and assistance: Sofía Tacle,

223 Carlos Mena, Stephen Walsh, Philip Page, Kent Passingham, Soledad Sarzosa, Ana

224 Carrión, Sylvia Sotamba and Doug Bibus. Also, we thank the Galapagos National Park

225 Directorate (GNPD) for the permits granted for sampling, and Galapagos Science

226 Center (GSC) for the logistic support during the study. Finally, we thank the GNPD

227 rangers of San Cristobal Island Technical Office, especially Rafael Díaz and Jeffrey

228 Málaga, for their help and assistance in collecting data.

229

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307

Figure 1

Fatty acid ω-6/ω-3 ratio in Galapagos Tortoise

Fatty acid concentrations of ω-6/ω-3 ratio in San Cristóbal Galapagos Tortoise, *Chelonoidis chathamensis*

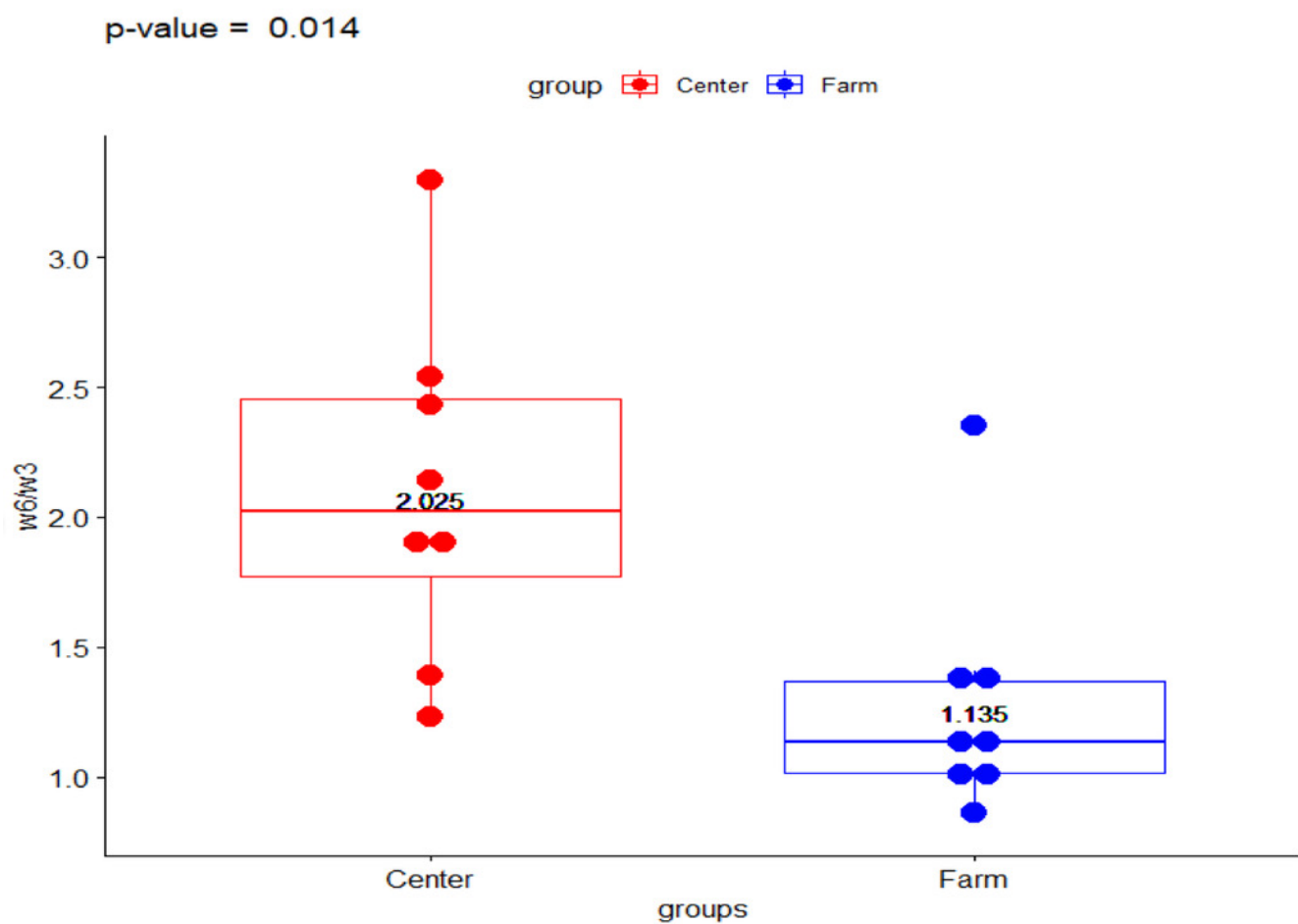



Figure 2

Polyunsaturated fatty acids in Galapagos tortoises

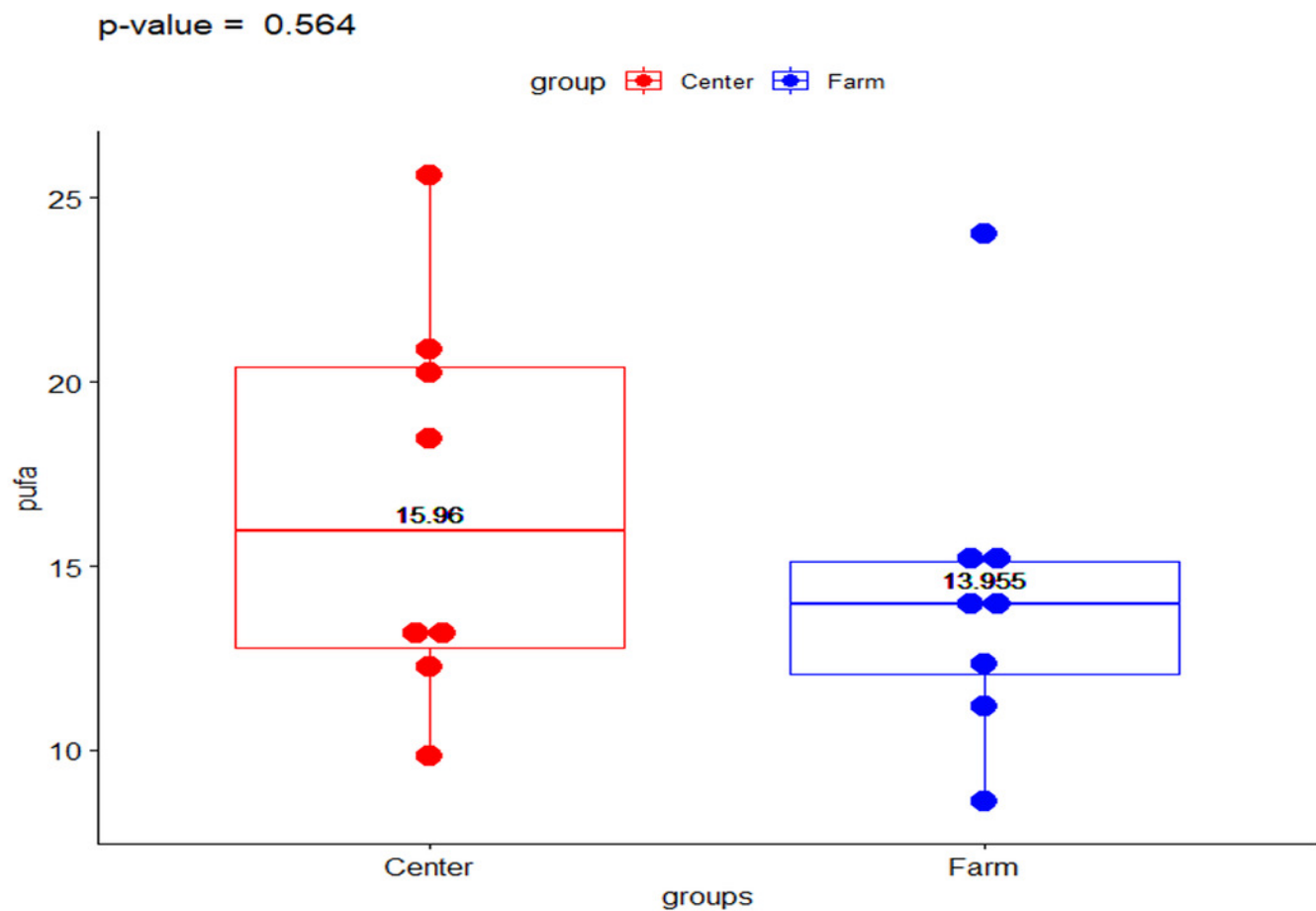
Polyunsaturated fatty acids concentrations in San Cristóbal Galapagos Tortoise, *Chelonoidis chathamensis*

Fatty Acid Profile As Percent (%).

Table I. Total Fatty Acid Profile (Percent, %) of San Cristóbal Galápagos Tortoises, *Chelonoidis chathamensis*.

1 Table I. Total Fatty Acid Profile (Percent, %) of San Cristóbal Galápagos Tortoises, *Chelonoidis chathamensis*.

Fatty Acid	Median (Breeding Center)	Mean (Breeding Center)	Median (Ecological Farm)	Mean (Ecological Farm)
14:0	2.16	2.10 ± 0.31	1.72	1.86 ± 0.38
14:1	1.04	1.15 ± 0.85	1.15	1.05 ± 1.00
15:0	0.93	1.03 ± 0.57	0.59	0.80 ± 0.54
16:0	17.35	16.96 ± 1.61	16.38	16.52 ± 1.03
16:1 ω-7	8.18	7.66 ± 1.07	9.50	8.98 ± 1.84
17:0	0.05	0.13 ± 0.17	0.04	0.07 ± 0.08
17:1	0.24	0.26 ± 0.21	0.18	0.13 ± 0.11
18:0	9.03	8.95 ± 1.31	8.36	8.72 ± 1.16
18:1 ω-9	45.13	44.52 ± 7.68	45.52	44.96 ± 6.37
18:2 ω-6	3.82	4.54 ± 2.42	3.37	4.00 ± 1.84
18:3 ω-6	0.06	0.10 ± 0.12	0.06	0.08 ± 0.08
18:3 ω-3	1.59	1.95 ± 1.05	1.38	1.45 ± 0.87
20:0	0.06	0.08 ± 0.06	0.15	0.13 ± 0.08
20:1 ω-9	0.00	0.01 ± 0.01	0.04	0.03 ± 0.03
20:1 ω-7	0.53	0.58 ± 0.36	0.80	0.82 ± 0.30
20:2 ω-6	0.05	0.06 ± 0.03	0.04	0.04 ± 0.01
20:3 ω-9	0.23	0.23 ± 0.05	0.26	0.26 ± 0.10
20:3 ω-6	0.51	0.55 ± 0.23	0.44	0.51 ± 0.16
20:4 ω-6	2.81	3.23 ± 1.02	2.97	4.29 ± 2.35
20:3 ω-3	0.04	0.04 ± 0.02	0.02	0.06 ± 0.08
20:4 ω-3	0.27	0.23 ± 0.10	0.12	0.13 ± 0.12
20:5 ω-3	2.33	2.12 ± 0.79	1.64	1.74 ± 0.61
22:0	0.18	0.20 ± 0.11	0.24	0.21 ± 0.08
22:1 ω-9	0.08	0.09 ± 0.02	0.11	0.08 ± 0.06
22:4 ω-6	0.39	0.46 ± 0.17	0.54	0.57 ± 0.23
22:5 ω-6	0.13	0.14 ± 0.06	0.08	0.09 ± 0.04
22:5 ω-3	2.04	2.19 ± 0.82	1.89	1.85 ± 0.41

24:0	0.04	0.04 ± 0.04	0.08	0.08 ± 0.07
22:6 ω-3	0.13	0.16 ± 0.08	0.15	0.21 ± 0.18
24:1	0.257	0.22 ± 0.13	0.26	0.26 ± 0.07
Saturates	29.68	29.37 ± 3.16	28.56	28.32 ± 1.96
Monoenes	47.43	46.57 ± 7.75	46.59	47.21 ± 5.86
PUFA	15.05	16.01 ± 5.71	13.44	15.28 ± 4.87
HUFA	9.49	9.36 ± 2.44	8.45	9.71 ± 3.33
T/T	0.08	0.08 ± 0.03	0.06	0.07 ± 0.03
Tot. w ω-3	7.82	6.69 ± 2.23	5.01	5.44 ± 1.57
Tot. w ω-6	7.82	9.08 ± 3.76	7.72	9.58 ± 4.22
Tot. ω-9	47.06	45.99 ± 7.44	45.91	46.38 ±5.58
ω-6/ ω-3	1.35	1.36 ± 0.35	1.90	1.84 ± 0.78
AA/EPA	1.71	1.58 ± 0.26	2.80	2.59 ± 1.31
ω-3 HUFA	49.86	50.45 ± 4.68	39.61	43.06 ± 9.69
ω-6 HUFA	50.14	49.55 ± 4.68	60.39	59.94 ± 9.69
EPA + DHA	2.44	2.28 ± 0.86	1.78	1.95 ± 0.65