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Individual variations in fatty acid composition and concentration as indicators of the nutritional condition of wild pointhead flounder larvae

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Abstract

We investigated the fatty acid compositions and concentrations of wild marine fish larvae with a highly accurate method because our knowledge of them has been seriously limited compared with cultured larvae. This study presents estimates of the fatty-acid-based nutritional condition of individual larvae in the field. Because the pointhead flounder *Cleisthenes pinetorum* displays relatively high stock size fluctuations, we investigated the developmental change in the fatty acid compositions of the body trunk, head, and eye and the annual fluctuations in the fatty acid concentrations in the trunk. We show that the process of fatty acid accumulation is not uniform across body parts and that the trunk is a better indicator of larval nutritional status than other parts because there is less time lag. Starved larvae with simultaneously high docosahexaenomic acid ratios and low total fatty acid concentrations, as observed in laboratory experiments, are rare in the wild. Thus, starved larvae must be removed rapidly by predators before they can experience a relatively long period of starvation in the wild. Fatty acid accumulation was greater in the larvae of the 2005 year class than in those of the 2006 year class in their first feeding stage, according to the optimal model derived with GLM. A previous study indicated that the 2005 year class showed stronger recruitment than the 2006 year class. We conclude that the fatty acid analysis of wild larvae is a useful index of their nutritional status and mortality, especially in the first feeding stage.

Key words: docosahexaenoic acid, fatty acid composition, pointhead flounder, starvation, wild marine fish larvae

28

29 **Introduction**

30 Annual variations in the mortality of marine fish during their early life stages have been considered the
31 main reason for fluctuations in fish stocks. To clarify the survival process in early life, various hypotheses
32 have been proposed. The growth rate determined from the otolith microstructure is used as a particularly
33 good indicator of the survival rate (Campana and Neilson 1985), and a relationship between survival and
34 growth has become apparent (Takasuka et al. 2004). However, it is necessary to introduce multilateral
35 indices to any discussion of the survival process in the early lives of marine fish. Therefore, different
36 high-performance indices of nutritional status, other than the growth rate estimated from the otolith
37 structure, are required.

38 Most marine fish are incapable of synthesizing docosahexaenic acid (DHA, 22:6n-3) from precursors,
39 so it is essential for them to ingest n-3 polyunsaturated fatty acids (PUFAs) such as DHA from prey
40 organisms (Takeuchi 2001). Therefore, they take PUFAs from their natural diet, which must contain
41 sufficient DHA and/or eicosapentaenoic acid (EPA, 20:5n-3; Sargent et al. 1999; Takeuchi 1991). It has
42 been demonstrated in laboratory experiments that the success or failure of a diet containing PUFAs can
43 affect larval growth (Sargent et al. 1997), the development of their visual capacity (Bell et al. 1995), their
44 schooling behavior (Masuda et al. 1999; Ishizaki et al. 2001), pigment deposition (Rainuzzo et al. 1994;
45 Copeman et al. 2002; Villalta et al. 2005) and tolerance of low dissolved oxygen and high water
46 temperatures (Kanazawa 1997). PUFAs tend to accumulate in the larval brain, spinal cord, and retina
47 (Tocher and Harvie 1988; Masuda et al. 1999; Mourente 2003). It has also been shown that the amount of
48 DHA distributed in the brain controls the formation of neural networks (Masuda et al. 1999) and that
49 larval mortality is affected by the quantity of DHA that is consumed in the wild (Masuda and Tsukamoto
50 1999; Masuda 2003). Accordingly, the functions of fatty acids suggest that the fatty acid compositions in
51 various body parts, such as the head and eye, should be investigated as potential indices of nutritional

52 condition.

53 Like proteins, lipids and their constituent fatty acids are major organic elements, and play major roles
54 as sources of metabolic and energy for growth and reproduction (Tocher 2003). It has been suggested that
55 a deficiency in fatty acids retards growth and reduces survival (Izquierdo 1996; Bransden et al. 2005),
56 leading to low recruitment into adult stocks (Bell and Sargent 1996). For this reason, it should be possible
57 to use fatty acid accumulation and composition as indicators of recruitment, based on the quality and
58 quantity of the fish diet. However, our knowledge of the fatty acid accumulation in wild larvae is limited
59 and few studies have investigated how it affects their survival in the wild (Paulsen et al., 2013a; 2013b).
60 Because there has been no way to analyze small samples, such as individual larvae (< 10 μg), no study
61 has effectively and accurately analyzed the fatty acid accumulation and composition of fish larvae, much
62 less in each body part of individual larvae.

63 Based on the method of fatty acid analysis proposed by Ando et al. (2007) for the wild pointhead
64 flounder *Cleisthenes pinetorum*, this study demonstrates the accumulation of fatty acids in fish larvae by
65 investigating the variability in their concentrations and compositions in each body part and at each
66 developmental stage. The annual variability in the total fatty acid concentration and the relationship
67 between fatty acid composition and concentrations were also investigated to examine the potential utility
68 of fatty acids as an indicator of mortality in the early developmental stages of marine fish. The pointhead
69 flounder is an ideal species in which to investigate the relationship between larval nutritional status and
70 recruitment because the relatively high fluctuations in stock size reflect the feeding success or failure of
71 its pelagic larval stages (Kurifuji et al. 2005; Hiraoka et al. 2005; Hiraoka et al. 2009).

72

73 **Materials and Methods**

74 The pointhead flounder larvae used for the fatty acid analysis were collected in the survey reported by
75 Hiraoka et al. (2009), conducted aboard T/S *Ushio-maru* (179 tons) of the Faculty of Fisheries, Hokkaido

University. Zooplankton samples that included fish larvae were collected with a ring net (80 cm diameter and 0.33 mm mesh size) and MTD net (Motoda horizontal net) system (56 cm diameter and 0.33 mm mesh size; Motoda 1971) with a flow meter in Funka Bay (Fig. 1) on 13–16 September and 30 September–3 October in 2005, and on 2–4 August and 24–26 September in 2006. MTD nets can be used for simultaneous -horizontal tows without contamination from other layers. The pointhead flounder larvae were picked from the zooplankton samples on board soon after the plankton nets were retrieved. They were brought back to the laboratory in individual plastic cases at –50 °C.

In the laboratory, the larvae were thawed on slide glasses and photographed individually with a digital camera mounted on a dissecting microscope. Body length (BL: the linear distance between the tip of the snout and the tip of the notochord) and body depth (BD: the linear dimension of the body trunk at the anus, without the pelvic fin, at right-angles to the body length) were measured to the nearest 0.1 mm on the digital images imported into a computer using ImageJ software. The developmental stages of the larvae without yolk sacs were determined according to Nagasawa (1990): stage A, the digestive tract beginning to coil but unlooped; stage B, the digestive tract looped and the notochord tip straight; stage C, preflexion and the hypural element beginning to form; stage D, flexion and caudal fin ray apparent; stage E, postflexion and the left eye not visible from the right side; stage F, upper edge of the left eye visible from the right side; stage G, left eye has moved to the back side of the head and the right eye is beginning to lean toward the ventral surface. Larvae in stages A–E were used and yolk-sac larvae were not used in this study. Each larva was split up the trunk of the body using a dissecting needle, and then individually transferred to a glass homogenizer. To analyze the trunk, 1–21 larvae were selected and analyzed based on the number of larvae collected by month and developmental stage (Table 1).

For the fatty acid analysis of the different body parts, the pointhead flounder larvae were divided into four parts: the head, two individual eyes, and the body trunk, excluding the digestive tract to prevent contamination by undigested prey (Fig. 2). They were then transferred individually to a glass

100 homogenizer. For this analysis, approximately 10 individuals in each developmental stage were randomly
101 selected from the larvae collected in September and October 2005 (Table 1). When the data for both eyes
102 were available, the mean value was used as the representative value.
103 Fatty acid composition (%) and content (μg/larva) were analyzed with the method of Ando et al.
104 (2007). Fluorescent 9-anthrylmethyl esters (Nimura and Kinoshita 1980) were prepared from the samples
105 and the fatty acid peaks were detected with reversed-phase high-performance liquid chromatography. The
106 classified fatty acids were first categorized into three groups: saturated, monounsaturated, and
107 polyunsaturated fatty acids. The polyunsaturated fatty acids were then classified into four categories:
108 DHA (22:6n-3), EPA (20:5n-3), and arachidonic acid (AA, 20:4n-6), which are considered the essential
109 fatty acids (Sargent et al. 1999), and other polyunsaturated fatty acids. The compositions by weight of
110 these six categories (saturated, monounsaturated, and four polyunsaturated classes) were then estimated.
111 The fatty acid concentration (μg/mg) was calculated as the amount of total fatty acids in the trunk
112 (μg) in each sample divided by the estimated dry body weight without the digestive tract (mg). The dry
113 body weight was converted with the following allometric equation based on the measurement data for TL,
114 BD, and dry weight (DW) of 50 individuals (10 individuals at each developmental stage) collected in
115 September and October 2005, because DW could not be measured directly when the samples were used
116 for fatty acid analysis.
117 $DW = 0.39 \times BL \times BD^{1.27}$ ($r^2 = 0.98$, N = 50, $P < 0.01$)
118 where *DW* is the dry whole body weight, including trunk, head and eye, without the digestive tract (mg),
119 *BL* is the body length (mm), and *BD* is the body depth (mm). The dry body weight was measured using an
120 electric balance (Mettler-Toledo) to the nearest 1 μg after the larva had been dried for 1 h at 65 °C in an
121 electric oven. Body length and body depth were measured to the nearest 0.1 mm under a binocular
122 dissecting microscope using an ocular micrometer. The relationship among DW, BL and BD indicated
123 that DW increased with the 1.27th power of body depth than body length. Moreover, body length tends to

124 shorten during notochord flexion whereas body depth increases a linear fashion. Thus, body depth
125 continues to grow during notochord flexion. In the present study, we therefore used depth as an index of
126 body size.

127 To examine the annual changes in the fatty acid concentrations, a generalized linear model (GLM)
128 was used to estimate the influence of the explanatory variables (sampling year, sampling month, and
129 developmental stage), set on the log-transformed fatty acid concentration in the trunk. All the main effects
130 and two-way interactions were introduced into the model and body weight was included as an offset term.
131 A normal distribution was assumed as the error distribution. The full model was:

$$Concentration_{ijk} = BW_{ijk} \times \exp\left(\alpha + \beta_1 \times Month_i + \beta_2 \times Year_j + \beta_3 \times Stage_k + \beta_4 \times (Month \times Year)_{ij} + \beta_5 \times (Month \times Stage)_{ik} + \beta_6 \times (Year \times Stage)_{jk} + \varepsilon_{ijk}\right), \quad \varepsilon_{ijk} \sim N\left(0, \vartheta^2\right)$$

132
133

134 where $Concentration_{ijk}$ is the total fatty acids of an individual larva (μg), BW_{ijk} is the estimated dry weight
135 (mg), $Month_i$ is the effect of month (August–October), $Year_j$ is the effect of year (2005–2006), $Stage_k$ is
136 the effect of developmental stage (stages A–E), *Month* × *Year*, *Month* × *Stage*, and *Year* × *Stage* are the
137 interaction terms among the explanatory variables, and ε_{ijk} is the residual. The model selection process
138 was conducted with a stepwise method using the Akaike information criterion (AIC; Akaike 1974). Three
139 models with the lowest AIC values were then compared with an analysis of variance (ANOVA) table. The
140 GLM calculations were made with R2.15.1 (R Development Core Team,
141 http://cran.r-project.org/bin/windows/base/).

142

143 **Results**

144 **Differences in fatty acid composition in the trunk, head, and eye with development**

145 Small larvae of the wild pointhead flounder in stage A, about 0.2 mm BH (ca. 2.2 mm BL), showed a

high proportion of saturated fatty acids (38.3%–61.2%) and a low %DHA (8.7%–14.6%; Fig. 3). As the larvae grew to about 0.5 mm BH in stages B-C, the saturated fatty acids decreased by about 40% in the trunk, 35% in the head, and 30% in the eye, whereas %DHAs increased and became relatively stable at about 25% for the trunk and head after stage B and 40% for the eye after stage C, indicating that the accumulation of DHA in the eye was delayed relative to that in the other body parts. The total saturated fatty acids constituted a greater proportion than the other fatty acid classes in the trunk and head throughout the developmental stages (trunk: 37.7%–42.6%; head: 35.0%–39.6%), followed by %DHA (trunk: 24.6%–28.7%; head: 25.8%–29.7%; Table 2). The %DHA in the eye increased by more than 40% as the larvae developed and exceeded the percentage of saturated fatty acids after stage B.

As the larvae developed from stage A to stage B, the gradients between %DHA in the trunk and %DHA in the head or eye decreased then increased in stage C, and finally it flattens out in stage D (Fig. 4). The value of %DHA was not clearly different between the head and eye in stage A, whereas %DHA in the eye was constantly higher than that in the head after stage B.

Relationships between total fatty acid concentrations and %DHA in larvae

The relationships between %DHA and the total fatty acid concentrations in the body trunks of larvae collected in each month in 2005 and 2006 are shown in Fig. 5. The larvae with high concentrations of fatty acids (> 150 μg/mg) only showed %DHA within a narrow range of 22.9%–32.8% throughout all stages. The coefficients of variance for %DHA in stages A, B, C, D, and E were 23.5%, 12.0%, 8.6%, 13.7%, and 12.5%, respectively. A remarkably large variation in %DHA was observed in stage A, especially in larvae with fatty acid concentrations < 50 μg/mg. The relationship indicated that larvae with relatively high concentrations of fatty acids kept narrow %DHA whereas larvae with low fatty acid concentration showed varied %DHA.

Annual changes in larval fatty acid compositions and concentrations

Saturated fatty acids and DHA comprised the highest proportions of the fatty acids in the body trunks of

170 larvae collected in all survey months (Fig. 6). Larvae in an early developmental stage, such as at 0.2 mm
171 BH (ca. 2.2 mm BL), showed relatively high proportions of saturated fatty acids, but low proportions of
172 polyunsaturated fatty acids, such as DHA, in all months. As the larvae developed, the proportions of
173 saturated fatty acids decreased and those of polyunsaturated fatty acids increased, as in the other parts
174 (head and eye) sampled in September 2005 (Fig. 3). This trend was also apparent in all months.
175 Figure 7 shows the developmental changes in the mean values for the total fatty acid concentrations
176 in the trunk for the four survey groups (September 2005, September–October 2005, August 2006, and
177 September 2006). These results indicate an increasing trend in the total fatty acid concentrations as the
178 larvae grew in stages A–D. The total fatty acid concentrations in 2005 were higher than those in 2006 in
179 stages A and B, but this difference disappeared after stage C.
180 The total fatty acid concentrations for four survey groups and four stages described in Fig. 7 were
181 examined by GLM. The minimum to the third smallest AIC values and the ANOVA table of the three
182 models are shown in Table 3 and the difference in the AIC values between the optimal model (model 1)
183 and the second model (model 2) is less than two. The effects of *Year*, *Stage*, and *Year* × *Stage* were
184 significant for model 1, and the effects of *Stage* and *Year* × *Stage* were significant for model 2. Only the
185 effect of *Stage* was significant for model 3.
186
187 **Discussion**
188 Three parts (trunk, head and eye) of the pointhead flounder larvae might reflect a gradual accumulation of
189 DHA derived from their food, copepod nauplii and bivalve larvae, as they developed. Most marine fish
190 are incapable of synthesizing DHA from precursors, so they take PUFAs from their natural diet, which
191 must contain plenty of DHA and/or EPA (Sargent et al. 1999; Takeuchi 1991). Wild pointhead flounder
192 larvae mainly feed on copepod nauplii and bivalve larvae (Hiraoka et al. 2005), and these prey organisms
193 contain higher DHA than other organisms, such as phytoplankton, or detritus (Holland 1978; Sargent and

194 Falk-Peterson 1988; Fraser et al. 1989; Rossi et al. 2006). Therefore, larval fatty acids should reflect the
195 fatty acid composition of their diet (e.g., Copeman et al. 2002).

196 Rainuzzo et al. (1992) reported the fatty acid compositions of four marine species (Atlantic halibut
197 *Hippoglossus hippoglossus*, plaice *Pleuronectes platessa*, turbot *Scophthalmus maximum*, and cod *Gadus*
198 *morhua*) in their larval stages. These four species showed the same trends, with 16:0 accounting for the
199 highest proportion of the saturated fatty acids, 18:1 the highest proportion of the monounsaturated fatty
200 acids, and DHA the highest proportion of the polyunsaturated fatty acids. The pointhead flounder showed
201 a similar trend in its fatty acid composition (Table 2), but the process of fatty acid accumulation might not
202 be uniform across the different body parts of fish larvae (Fig. 3). Therefore, the fatty acid composition
203 and concentration in the body trunk might be a better indicator of the larval nutritional status than those in
204 other parts of the body.

205 PUFAs, such as DHA, accumulate in the brain and eyes at relatively high concentrations (Tocher and
206 Harvie 1988; Masuda et al. 1999; Mourente 2003). If larval fishes are deficient in PUFA, their abilities to
207 feed (Bell and Sargent 1995), escape predation (Nakayama et al. 2003), and school (Masuda and
208 Tsukamoto 1999; Masuda et al. 1998, 1999) decrease, and it is suggested that a deficiency of PUFA could
209 lead to high mortality during the larval stage (Bell and Sargent 1996). Saturated and monounsaturated
210 fatty acids are used as sources of metabolic energy. When fatty acids are catabolized, saturated and
211 monounsaturated fatty acids are generally preferentially consumed and PUFAs are selectively retained
212 (Rainuzzo et al. 1994; Tocher 2003). When we examined the %DHA in the pointhead flounder by body
213 part in stage D, the variation was larger in the trunk than in the head or eyes (Fig. 4). This indicates that,
214 after stage C, the wild pointhead flounder larvae retain essential fatty acids in definite proportions in their
215 neuronal cells, whereas saturated and monounsaturated fatty acids in the body trunk are rapidly
216 catabolized, as a source of energy. This means that, for pointhead flounder to reach a fully functional state,
217 it necessary for the neural cells in the head and eye (from stage D onwards) to incorporate a certain

218 amount of essential fatty acid, in preference to such accumulation in other body parts. The fatty acid
219 content in the trunk thus provides an indication of the recent nutritional status of the larvae. This has
220 proved to be more reliable indicator than that obtained when using the head or eye as indicators.

221 The use of fatty acid accumulation and composition in the body trunk as an indicator for evaluating
222 the larval condition has two main advantages. Firstly, the measurement error is reduced, because the body
223 trunk is relatively larger than the other parts of the body. Secondly, it is now possible research workers to
224 obtain otoliths from beheaded specimens and thus obtain two measures of growth: from otolith analysis,
225 and from an analysis of the somatic condition based on the fatty acid content in the larval body trunks.

226 No wild pointhead flounder larvae considered to be starving were caught for our fatty acid analysis.
227 Starving larvae of various fish species show a relatively higher proportion of DHA in laboratory
228 experiments in controlled feeding environments (turbot larvae: Rainuzzo et al. 1994; striped bass *Morone*
229 *saxatilis* larvae: Martin et al. 1984; black sea bream *Acanthopagrus schlegeli* juvenile: Om et al. 2003;
230 common carp *Cyprinus carpio* and rainbow trout *Salmo gairdneri*: Takeuchi and Watanabe 1982; rainbow
231 trout: Jezierska et al. 1982; and red spotted grouper *Epinephelus akara*: Jeong et al. 2003). This is
232 because saturated and monounsaturated fatty acids are readily catabolized, so larvae that show high DHA
233 ratios and low fatty acid concentrations would be assessed as “starving”. According to Rainuzzo et al.
234 (1994), turbot larvae reared for six days with enriched rotifers contained 18.75% DHA and a total fatty
235 acid concentration of 92.23 mg/g DW, whereas larvae reared for the same period with no prey contained
236 35.17% DHA and a total fatty acid concentration of 78.95 mg/g DW. The total fatty acid concentration of
237 striped bass larvae decreased sharply after starvation for two days (Martin et al. 1984) and the total fatty
238 acid concentration of northern anchovy *Engraulis mordax* larvae declined after four days (Håkanson
239 1989). It seems that starving pointhead flounder larvae show DHA ratio > ca. 32.5% and a lower total
240 fatty acid concentration simultaneously because larvae that contain enough (nonstarving) fatty acids have
241 about 22.5%–32.5% DHA in their body trunks (Fig. 5). Larvae with > 32.5% DHA were rare in our study

242 (Fig. 5), so it is unlikely that any larvae that had starved for more than six days were present. Starving
243 larvae may be removed by predators before they can experience such a long period of starvation in the
244 wild (Jørgensen et al., 2013). However, large numbers of pointhead flounder larvae in poor nutritional
245 condition might be present in the initial feeding period in the wild, because a remarkably high variation
246 in %DHA was observed in stage A, with fatty acid concentrations below 50 μg/mg (Fig. 5). We consider
247 that some larvae were in the process of accumulating fatty acids, but large numbers of larvae could not
248 take in enough nutrition, unlike larvae in the more developed stages. The first-feeding larvae generally
249 have low feeding ability, and their digestive tracts are very incompletely filled. Furthermore, individual
250 feeding abilities are likely to be extremely diverse at this stage. Considerable numbers of pointhead
251 flounder larvae would be poor nutritional status in their initial feeding stage.

252 According to our results, the larvae with high fatty acid concentrations were in good nutritional
253 condition. In the laboratory experiments, the larvae with higher fatty acid concentrations showed the
254 highest growth rates and survival rates (yellowtail flounder *Limanda ferruginea*: Copeman et al. 2002;
255 striped trumpeter *Latris lineata*: Bransden et al. 2005). The pointhead flounder larvae with high fatty acid
256 concentrations in their trunks showed a narrow range of %DHA fluctuations (Fig. 5). Larvae with good
257 feeding ability might show large amounts of fatty acids in their body trunks, obtained from prey
258 organisms, and might be able to maintain an adequate fatty acid composition (ca. 25% DHA). The lipids
259 making up the larval body are predominantly phospholipids and triacylglycerols. Phospholipids play a
260 role in maintaining the cell structure and in the functions of biological membranes, whereas
261 triacylglycerols act an energy source. The concentration of triacylglycerols has been used as an index of
262 nutritional status (Fraser and Sargent 1987; Zenitani 1999) because the absolute quantity of
263 triacylglycerols increases, whereas phospholipids remain steady, as the total lipids increase.

264 A fatty acid analysis of wild larvae is a useful criterion with which to assess their nutritional
265 condition. In our previous study, we concluded that the recruitment of the pointhead flounder is affected

266 by the success or failure of the first feeding stages (mainly stages A–B), because the 2005 year class
267 showed a high abundance of early-stage larvae in September (stages A–D) and a variety of different-stage
268 larvae in late September and early October (stages A–G) whereas the 2006 year class showed a narrow
269 range of developmental stages (stage A–B) in September (Fig. 1, Hiraoka et al. 2009). Two- and
270 three-year-old pointhead flounder were abundant in the 2005 year class, but low in the 2006 year class
271 (Hiraoka et al. 2009). A significant difference in the fatty acid accumulation of the 2005 and 2006 year
272 classes was identified with the optimal model in a GLM analysis (Table 3), and indicates that the larvae in
273 2005 obtained more energy from prey than those in 2006. According to Masuda and Tsukamoto (1999),
274 however, carangid fish larvae that cannot ingest sufficient DHA, will develop into juveniles that are
275 extremely vulnerable to predation because of their incapacity to school. In the pointhead flounder larvae,
276 as previously indicated, the retention of essential fatty acids in the nerve cells begin during stage D (Fig.
277 4). Thus the role of essential fatty acids in neural cells, such as the capability for visual perception, will
278 become a critical factor for survival. There is a lack of data on larval and juvenile schooling behavior in
279 this species, but the development of visual function would clearly be an important factor for escaping
280 predation. For some marine fishes including pointhead flounder, it is generally known that mortality in
281 the pre-juvenile stage regulates the year-class size (Leggett and Deblois 1994). In conclusion, we suggest
282 that the accumulation of total fatty acids in the larval body trunk is a more appropriate indication of larval
283 mortality than the level of essential fatty acid accumulation in the nerve cells during the first feeding
284 stages.

285 Lipids and fatty acids have been considered difficult to use as indices of nutritional status because
286 differences in their accumulation are generally offset by their utilization as energy sources for growth
287 (Ferron and Leggett 1994; Suthers et al. 1992). In laboratory experiments, larvae with high fatty acid
288 concentrations have shown high growth rates and survival rates, but several contaminated individuals
289 were analyzed in those studies (Copeman et al. 2002; Bransden et al. 2005). This study provides estimates

290 of larval nutritional status based on the fatty acids of individual wild fish larvae using the methods
291 developed by Ando et al. (2007). These have made it possible to evaluate the variability in fatty acid
292 compositions and concentrations in individual larvae of < 12 μg DW. It is also possible to identify larval
293 starvation with the combined analysis of fatty acid accumulation and the proportion of DHA. Growth
294 analyses using otoliths and fatty acid analyses should be conducted in the same individuals in future
295 studies to clarify the relationship between growth rate and fatty acid concentrations.
296
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302
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Figure 1. Location of Funka Bay (A); horizontal distribution of *Cleisthenes pinetorum* larvae (individuals [ind.]/m^2) collected with a ring net using oblique hauls from the 60 m depth layer to the sea surface in 2005 and 2006 (B–E:after Hiraoka et al. 2009). The area of each circle in B–E is proportional to the density of the larvae. Mean densities during September 13–16 and September 30–October 3 in 2005 were 21.4 ind/m^2 and 1.20 ind/m^2, respectively (B–C). Mean densities during August 2–4 and September 24–26 in 2006 were 0.47 ind/m^2 and 1.09 ind/m^2, respectively (D–E). Developmental stage ranges for stages A–D and A–G during September 13–16 and September 30–October 3 in 2005, and for stages A–B during August 2–4 and September 24–26 in 2006.

Figure 2. Schematic diagram of the body parts used for the fatty acid analysis.

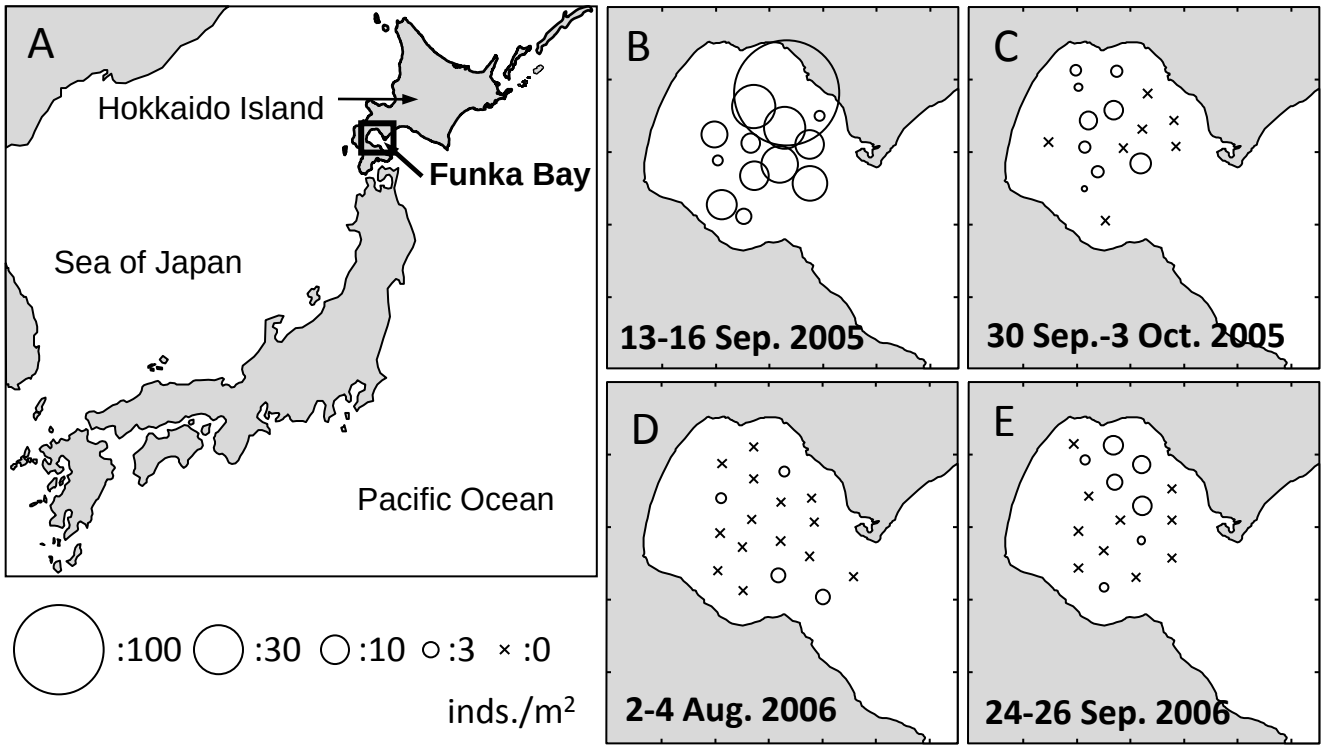
Figure 3. Relationship between the percentage of total saturated fats and %DHA by body part and body depth of *C. pinetorum* larvae collected in September and October 2005.

Figure 4. Matrix of %DHA in trunk, head, and eyes derived from the same individual *C. pinetorum* larva in stages A–E collected in September and October 2005.

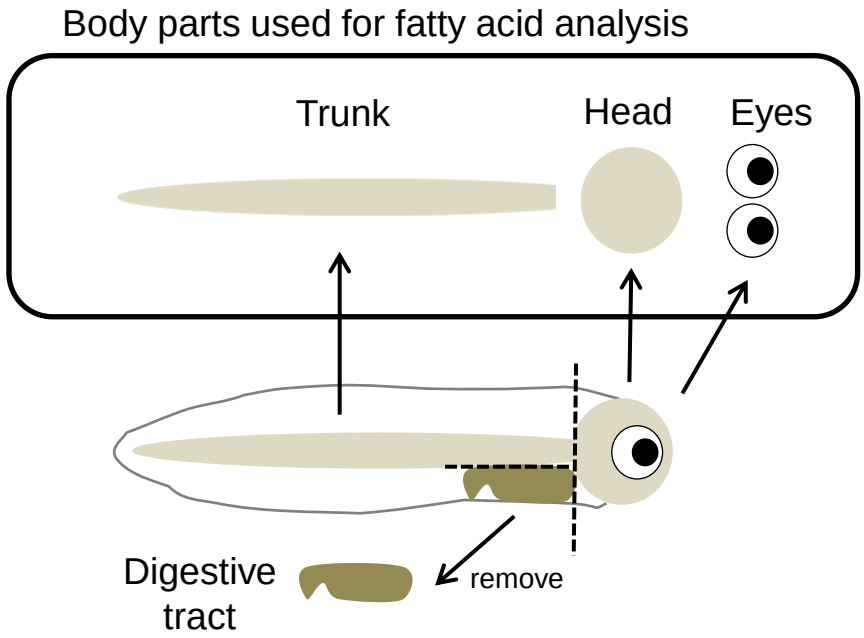
Figure 5. Relationship between %DHA and total fatty acid concentrations in *C. pinetorum* larvae in stages A–E collected in 2005 and 2006.

Figure 6. Changes in the gravimetric fatty acid composition of the body trunks of *C. pinetorum* larvae collected in 2005 (upper) and 2006 (lower) according to body depth.

Figure 7. Box plot of total fatty acid concentrations by developmental stage in the trunks of *C. pinetorum* larvae collected in 2005 and 2006. Each box, bar, and numeral show first quartile-median-third quartile, the lowest datum still within 1.5 interquartile range of the first quartile or the highest datum still within 1.5 interquartile range of the third quartile, and sample size, respectively. Outliers are plotted as individual points.



Hiraoka et al. Figure 1



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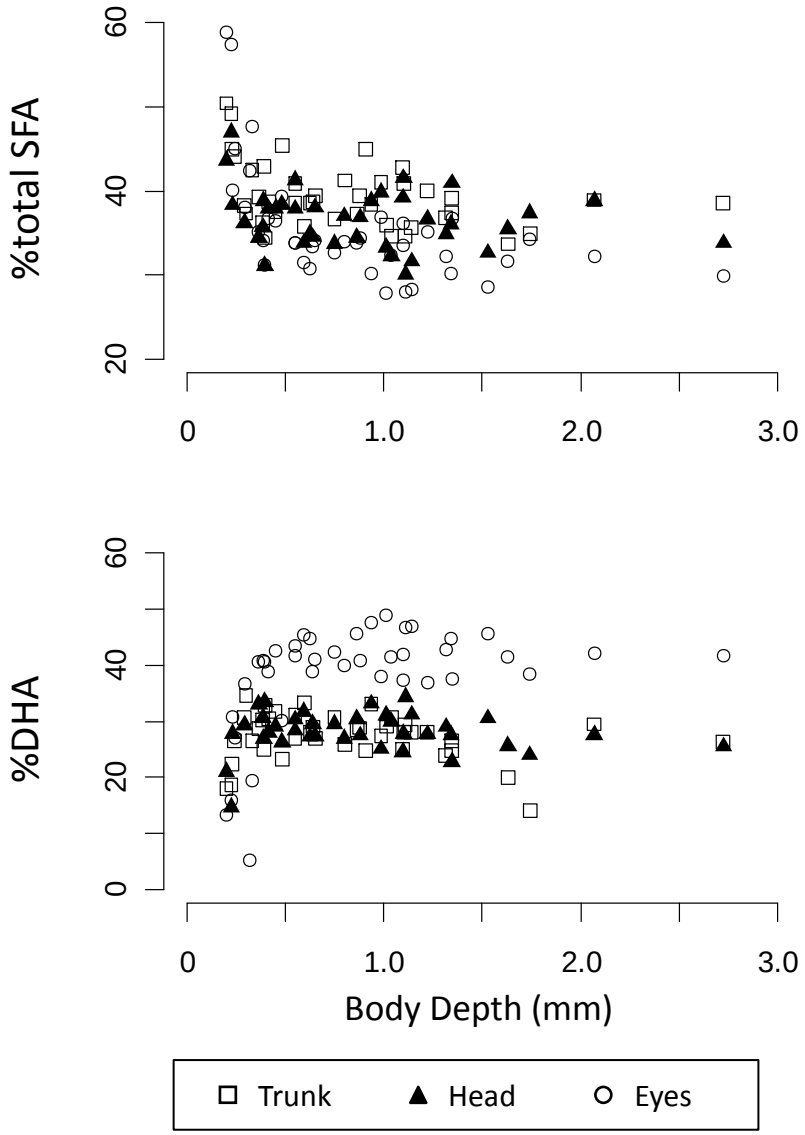
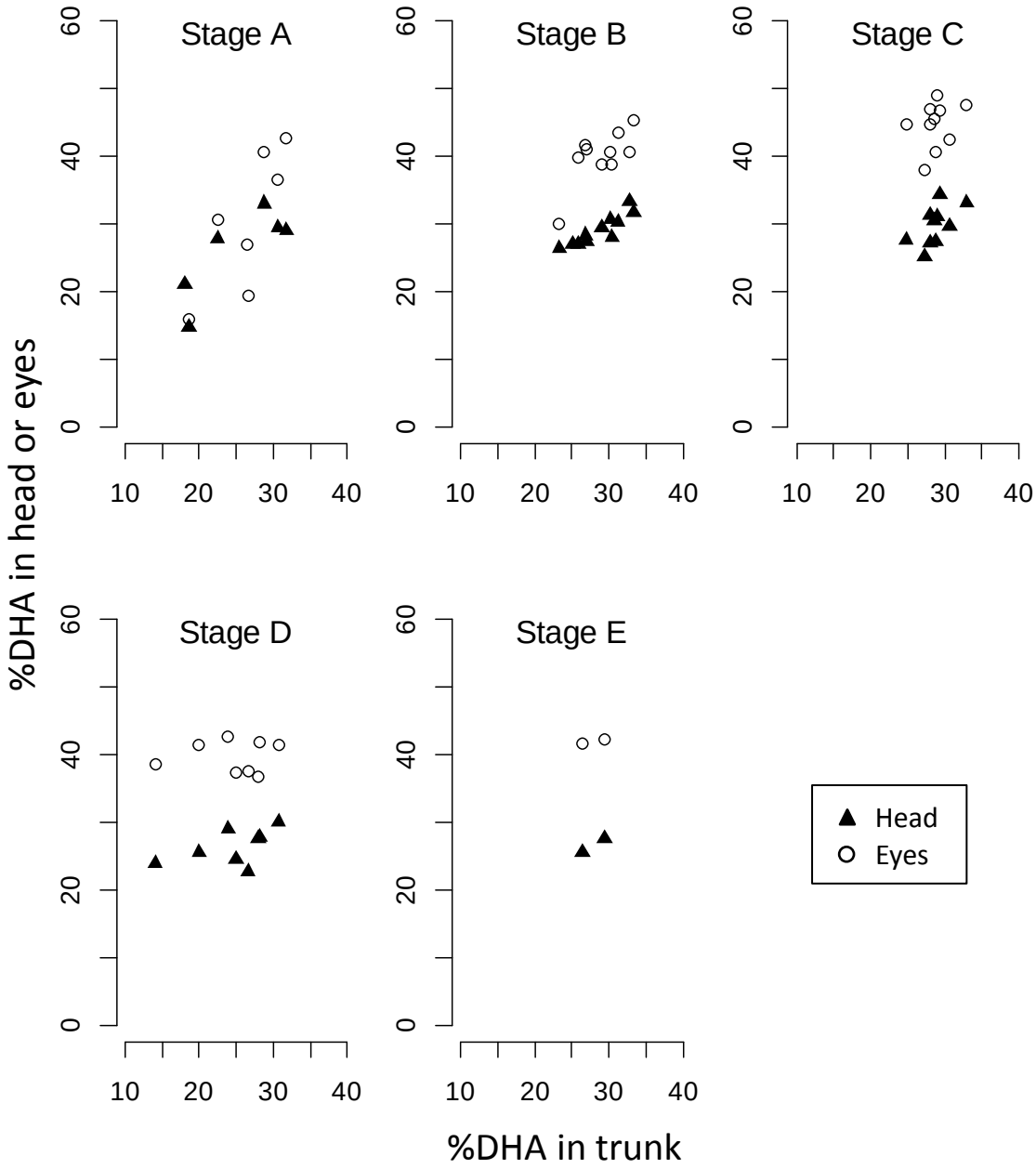


Hiraoka et al. Figure 3



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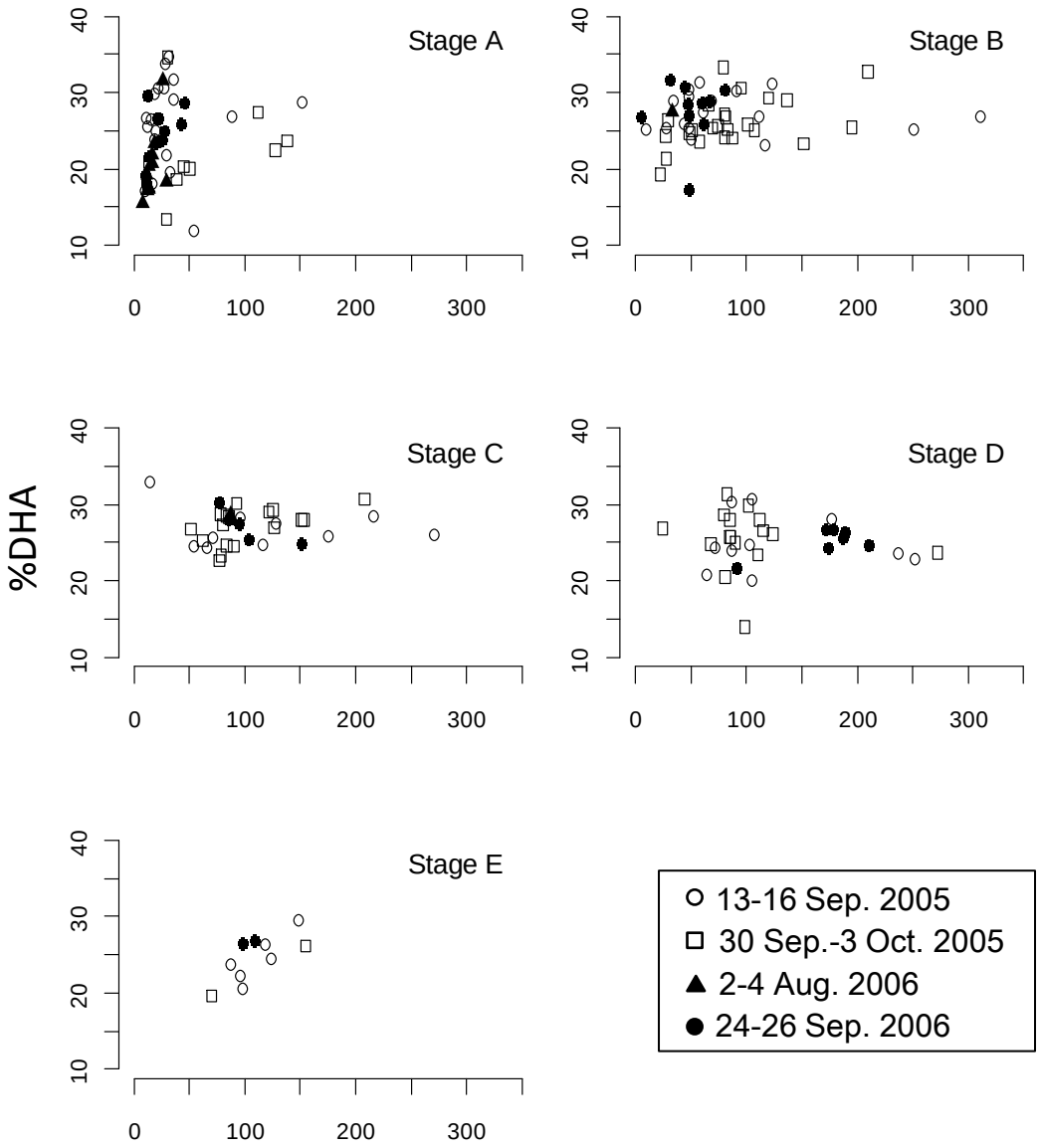
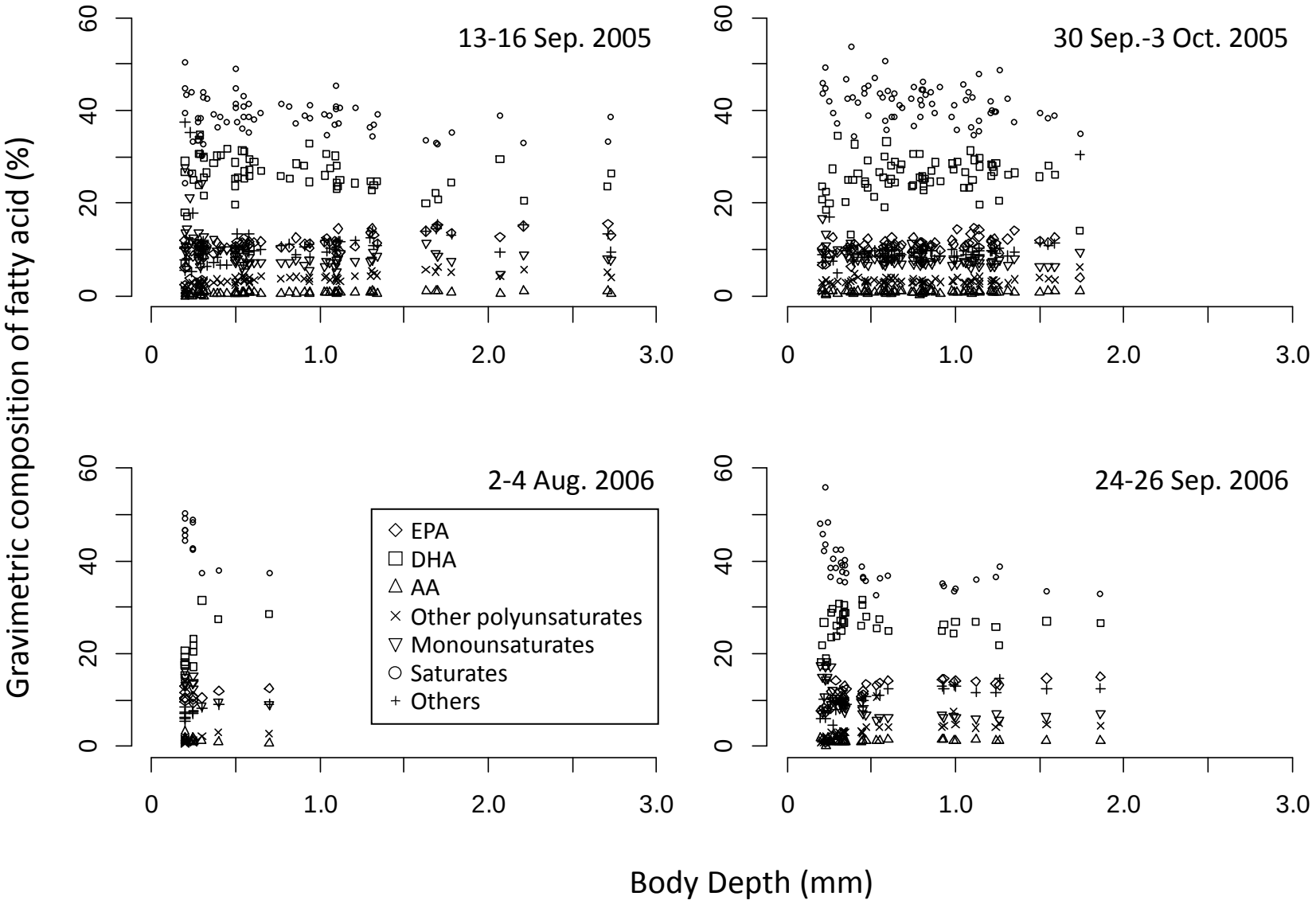


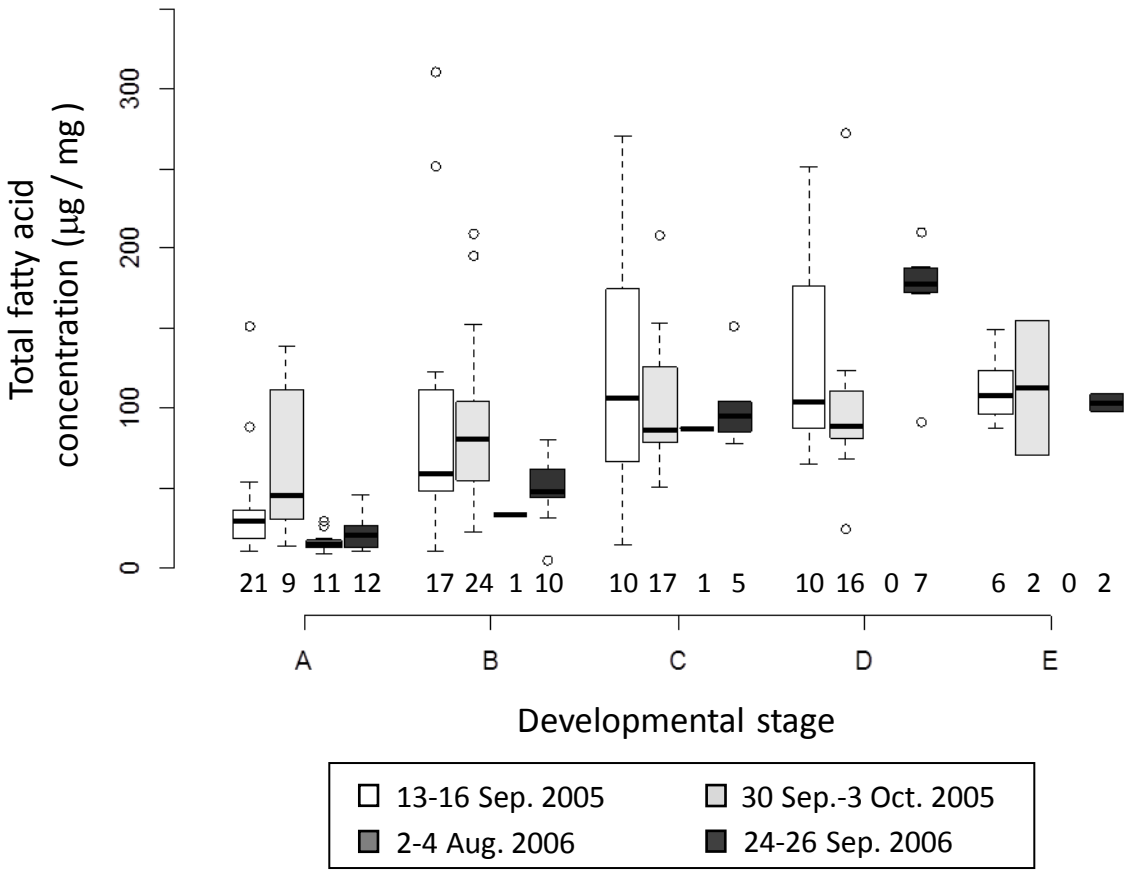
Hiraoka et al. Figure 5

Figure6



Hiraoka et al. Figure 6

Figure7



Hiraoka et al. Figure 7

Table

Table 1. Size range and numbers of *Cleisthenes pinetorum* larvae by developmental stage. Figures in parentheses indicate the numbers of individuals used for fatty acid analysis by body part

	A	B	C	D	E	Total
Size range (min-max)						
Body Length (mm)	2.3-4.6	3.1-4.8	4.8-8.4	6.0-8.4	6.6-8.8	-
Body Depth (mm)	0.2-0.5	0.2-1.1	0.5-1.3	0.8-1.7	1.1-2.7	-
Number of larvae analyzed						
13-16 Sep. 2005	21	17	10	10	6	64
	(6)	(5)	(3)	(4)	(2)	
30 Sep.-3 Oct. 2005	9	24	17	16	2	68
	(3)	(6)	(7)	(5)	(0)	
2-4 Aug. 2006	11	1	1	0	0	13
24-26 Sep. 2006	12	10	5	7	2	36

Table 2. Mean fatty acid composition of the body trunk, head, and eye of *C. pinetorum* larvae collected in September and October 2005

Body part	Trunk					Head					Eyes				
Developmental stage	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
Number of samples	9	11	10	9	2	6	11	10	9	2	16	22	20	18	4
Total fatty acids (µg/part)	1.6	11.3	31.4	37.4	126.3	1.9	9.2	20.1	21.3	67.5	0.2	0.8	1.6	1.9	4.8
Composition (%)															
14:0	7.3	5.9	5.3	5.7	4.9	5.7	4.4	3.9	3.9	4.2	7.0	3.8	2.8	2.8	2.6
16:0	26.1	23.9	23.9	23.6	16.9	25.3	22.7	22.0	23.5	16.6	24.7	20.2	19.0	20.6	14.0
18:0	6.2	3.9	3.4	3.5	1.8	5.5	4.5	4.3	4.3	2.4	9.3	6.2	5.6	5.7	3.0
18:1	9.6	5.8	5.5	6.1	3.2	11.2	8.6	9.1	9.7	5.3	9.9	6.7	6.1	7.0	4.2
16:1+18:2n-6+ 20:3+22:4n-6	4.3	4.6	5.0	6.2	6.0	4.3	4.5	4.9	5.8	6.4	3.5	3.2	2.9	3.7	4.0
AA (20:4n-6)	0.4	0.6	0.7	0.8	0.6	0.3	0.5	0.6	0.5	0.5	0.2	0.4	0.4	0.3	0.3
EPA (20:5n-3)	9.2	11.2	12.7	11.3	12.9	9.0	10.4	10.9	10.1	12.4	5.8	7.2	6.6	6.8	7.9
DHA (22:6n-3)	26.4	28.6	28.7	24.6	27.9	25.8	29.1	29.7	26.9	26.6	28.1	39.6	44.7	40.4	41.9
others	10.2	15.5	14.9	18.4	25.8	12.3	15.3	14.6	15.0	25.6	11.3	12.7	11.9	12.5	22.0
SFA	42.6	39.3	37.7	38.5	38.7	39.6	36.8	35.0	36.8	36.3	44.0	34.5	31.1	33.4	31.1
MUFA	11.4	8.5	7.5	8.2	6.2	13.3	11.0	11.3	12.3	9.2	12.4	9.8	9.7	10.5	8.8
PUFA	38.6	44.1	45.9	40.8	45.6	37.6	43.5	44.8	41.2	44.4	35.2	49.2	53.8	49.8	53.1

Table 3. AIC values and analysis of variance for three nested models with the lowest AIC values by GLM

	AIC	Effect	d.f.	SS	MS	F-value	Pr>F
Model 1	338.22	*Year*	1	3.29	3.29	9.26	0.0002
		Stage	4	54.67	13.67	38.43	<0.0001
		Year×Stage	4	6.44	1.61	4.53	0.0017
Model 2	339.82	*Year*	1	0.68	0.68	1.93	0.17
		Month	2	0.80	0.40	1.13	0.33
		Stage	4	44.39	11.10	31.26	<0.0001
		Year×Stage	4	5.48	1.37	3.86	0.005
Model 3	344.94	*Year*	1	0.73	0.73	2.07	0.15
		Month	2	0.80	0.40	1.13	0.32
		Stage	4	44.39	11.10	31.32	<0.0001
		Year×Stage	4	2.21	0.55	1.56	0.18
		Month×Stage	6	2.24	0.37	1.05	0.39