The onset of piscivory in a freshwater fish species: analysis of behavioural and physiological traits

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Abstract

The onset of piscivory in fish, resulting in a shift from zooplankton or invertebrate to fish prey, was studied on pikeperch (*Sander lucioperca*) using behavioural (attack, capture and swimming activity), morphological (allometry) and digestive enzymatic (trypsin, α-amylase and pepsin) analyses between larvae displaying or not piscivorous behaviour at different ages (23, 30, 37, 44 and 52 days post-hatching). The shift from zooplanktonic food items (*Artemia* nauplii) to a piscivorous diet did not occur at the same time for all individuals within the same cohort. Predation tests, conducted under controlled conditions (20ºC; *ad libitum* feeding), showed that some larvae attacked fish prey as early as the age of three weeks (11.0 ± 1.3 mm TL), while others did not start until the age of six weeks (16.6 ± 1.9 mm TL). Piscivorous individuals were bigger, with larger heads, longer tails, higher acid protease and lower alkaline protease activities, than non-piscivorous conspecifics. In conclusion, high inter-individual variability in morphological and digestive system developments linked to predatory ability development could induce cannibalism in fish.

Keywords: predatory behaviour; behavioural tests; freshwater fish; early life stages; *Sander lucioperca*.

Introduction

Traditionally, studies on fish ontogeny analyse global changes in a species during its growth and build up a descriptive developmental table summarizing the most relevant morphological changes (Ott et al., 2012; Tsai et al., 2013; Alix et al., 2015), and/or determine some key moments in development such as hatching or onset of exogenous feeding (Yamagami, 1988; Yúfera and Darias, 2007). In fish, recent studies have highlighted that early development could play an important role in shaping individual life histories (Van Leeunwen et al., 2017; Jonsson and Jonsson, 2019).

Ontogenetic changes in predatory behaviour are not necessarily essential for generalist predators, which can exhibit different behavioural tactics of capture due to the high variability of prey and can shift to another prey type without having to learn a new tactic rapidly (Cárdenas et al., 2014). Conversely, a specialist-like species has to deal with the shift to a new prey type. In piscivorous fish, the change from a zooplanktivorous to a piscivorous diet occurs over a short period of time, and could be related to morphological changes (Hart and Ison, 1991; Buijse, 1992; Galarowicz and Wahl, 2005), physiological needs (Pedersen and Falk-Petersen, 1992) and behaviours through learning processes (Benhaïm et al., 2013).

The onset of piscivory behaviour has been documented in fish, especially for freshwater species (Mittelbach and Persson, 1998), which undergo major ontogenic shifts in their diets. Several hypotheses have been proposed to explain an early shift to piscivory, among which morphological trait changes such as mouth size (Hecht and Appelbaum, 1988; Otterå and Folkvord, 1993) or digestive functions (Kaji et al., 2002). One of the requirements for the onset of piscivory is the size difference between the predator and its prey (Dörner and Wagner, 2003). Indeed, Mittelbach and Persson (1998) observed that the largest individuals in a cohort were the first to shift to piscivory. Furthermore, in fish, particularly in Percids, the development of digestive structures and activities seems similar to that of other carnivorous species (Rønnestad

et al., 2013; Hamza et al., 2015). The ontogenetic development of digestive organs and the activity of digestive enzymes can be modified by the nature and quality of the diet (i.e. live prey vs. compound artificial diet, nutritional dietary profile among others), the nutritional condition of the individual, the circadian rhythm, as well as other biotic and abiotic factors (Rønnestad et al., 2013; Hamza et al., 2015).

Pikeperch is a freshwater species with high economic potential for inland aquaculture diversification and fisheries in Europe. Its market demand has been boosted by the decline in wild catches (FAO, 2017). Thus, its intensive farming is needed, but there have been bottlenecks in most captive-rearing attempts so far. One of the main constraints is the high cannibalism rate (between 20% and 54% - Molnár et al., 2004; Kestemont et al., 2007) occurring between 14 and 21 days post-hatching (dph) at 20°C (11.0 ± 1.3 mm TL - Colchen et al., 2019). At this larval stage, fish show a typical predatory 'S-Shape' behaviour (Houde, 2001; Turesson et al., 2002), which changes to a 'hide and chase' behaviour at the juvenile stage (Sullivan and Atchison, 1978). In pikeperch, the ontogenic development of digestive enzyme activities consists in the gradual development of the exocrine pancreas along the endogenous feeding stage that continues throughout the first weeks of exogenous feeding; in contrast, the stomach, which is involved in acid digestion, becomes fully functional several weeks after the first exogenous feeding (Mani-Ponset et al., 1994; Ostaszewska et al., 2005; Hamza et al., 2007; Rønnestad et al., 2013). For pikeperch, the shift to piscivory has been reported to occur in juveniles measuring between 35 and 100 mm (Mittelbach and Persson, 1998). Cannibalism can be considered as predatory behaviour and its onset in captive populations could be directly related to the onset of piscivorous behaviour. Together, these findings make pikeperch a good candidate for use in studying the onset of piscivory in the early life stages.

The main objective of our study was to perform a holistic analysis to highlight possible links between the onset of piscivory, morphological ontogenetic changes and digestive enzymatic development in pikeperch by comparing pikeperch larvae of five different ages, ranging from 23 to 52 dph (460 and 1040 degree days at 20°C). In addition, another objective was to determine whether early piscivorous pikeperch larvae had early morphological and digestive enzyme developmental traits.

Materials and Methods

Ethical note

During all procedures, we took care to minimize handling and stress as much as possible for the study animals. All fish treatments and procedures used in this study were in accordance with the guidelines of the Council of the European Union (2010/63/UE) and approved by the French Animal Care Guidelines (Animal approval No. APAFIS#1813-2015111618046759v2).

Rearing conditions

The experiment was carried out at the Aquaculture Experimental Platform (AEP, registration number for animal experimentation C54-547-18) belonging to the URAFPA (Unité de Recherche Animal et Fonctionnalités des Produits Animaux) laboratory, located at the Faculty of Sciences and Technologies of the University of Lorraine (France). Eggs were obtained from one mature female (2.1 kg) previously injected with sGnRHa (50 $\mu g.kg^{-1}$; ovaRH, Syndel laboratories, Ltd) and fertilized by one male (Asialor SARL, Pierrevillers, Moselle, France). Only one male and one female were used in this trial, since we wanted to minimize genetic variability between different parental origins. Upon their arrival at the AEP facilities on the 1st February 2016, the fertilized eggs were put in a 500 L tank where larvae hatched shortly afterwards and developed. Artificial lighting (50 Lx) followed a 12L/12D cycle

with lights on from 08:00 to 20:00 with 30 min dawn and dusk simulations. Water temperature was maintained between 16°C and 17°C until 23 dph and then increased by 1°C per day until reaching 20°C. Water parameters (mean ± standard deviation, SD) were measured once or twice a week: dissolved oxygen = 8.5 ± 0.6 $mg.L^{-1}$, pH = 7.8 ± 0.2, salinity = 0.25 ± 0.05 $g.L^{-1}$, ammonia (NH_4^+) = 2.3 ± 1.9 $mg.L^{-1}$ and nitrite (NO_2^-) = 0.5 ± 0.3 $mg.L^{-1}$. From 4 dph, larvae were fed *Artemia* nauplii (550-600 μm; Catvis, Hertogenbosch, Netherlands) until weaning, and then 100% artificial feed from 22 dph to the end of the experiment (Larviva and Inicio Plus, Biomar, Denmark).

Behavioural analysis: the onset of piscivory

For this experiment, pikeperch larvae were transferred 24 h prior to testing to a small aquarium (20 L) set at 20°C, where larvae were not fed. Tests were conducted on 20 larvae (17 larvae at 44 dph) randomly chosen from the 500 L tank; the following five age groups were considered: 23 dph (9.8 ± 0.6 mm total length, TL), 30 dph (10.8 ± 1.4 mm TL), 37 dph (17.11 ± 1.9 mm TL), 44 dph (20.0 ± 2.0 mm TL) and 52 dph (28.8 ± 4.9 mm TL). Each larval age group was made up of a different set of individuals. First, larvae were transferred from the aquarium to a rectangular device (20 x 7 x 4 cm with 2 cm of water), which was placed on a translucent table lit (50 Lx) from below. The device was divided into two equal parts separated by a divider. A pikeperch larva was put in one compartment and prey in the other. Two different kinds of prey were used: zebrafish (*Danio rerio*) larvae (4.1 ± 0.81 mm TL) and *Artemia* nauplii (550-600 μm; Catvis, Hertogenbosch, Netherlands). For each pikeperch larva, the two prey types were always tested in the same order: zebrafish larvae (n = 3) to start with, followed by *Artemia* nauplii (n = *ca.* 40), to control the appetite of the experimental fish. After a 30 min acclimatization period for both the pikeperch larva and zebrafish larvae, the divider between the two parts of the device was removed and the behaviours of the pikeperch larva and prey

were video recorded for 20 min. Then, the surviving zebrafish were removed, the divider was placed back in the experimental device and *Artemia* nauplii were put in the empty part of the device. Next, the divider was removed again, and the pikeperch larva was allowed 20 min to forage on this live prey. All behaviours were video recorded with a digital camera (Sony Handycam, DCR-SR72) positioned 80 cm above the device. The water in the device was the same as that in the aquarium and renewed between each test.

Videos were analysed with The Observer XT10® software (Noldus, Netherlands). The analysis focused on the following variables: (i) attack, which was characterized by rapid movement of the pikeperch larva towards the zebrafish larvae, with its mouth open. This behaviour could easily be identified: just before the attack, the larva either stopped and took on an 'S' shape (Houde, 2001; Turesson et al., 2002) or just changed the orientation of its caudal fin without stopping; (ii) capture, which corresponded to the biting of prey by pikeperch; (iii) the distance between the prey and the pikeperch larva before the attack (when the 'S' shape was clearly observed), measured from the mouth of the predator to the middle of the body of the prey on video screenshot with ImageJ® after the calibration of scale; (iv) the swimming activity of pikeperch larvae in the presence of zebrafish larvae, defined as the displacement during 3 min of the larva of more than its body length in less than 1 second; (v) the effectiveness of pikeperch larvae in attacking zebrafish larvae calculated by the number of captures relative to the total number of attacks directed by pikeperch larvae that attacked at least once. All larvae that did not attack were removed from this analysis; (vi) for *Artemia* nauplii, it was not possible to see the capture by the pikeperch larvae because the shape of *Artemia* nauplii was not visible on the video recording. Thus, only attacks were recorded, when they were clearly identified by the 'S' shape of the pikeperch larvae.

164 At a given age, the behavioural analysis allowed us to categorize pikeperch larvae as
165 piscivores (individuals that attacked zebrafish prey = piscivores) or not (individuals that did not
166 attack zebrafish prey = non-piscivores).
167
168 Morphological traits and growth measurements
169 In order to correlate the onset of piscivory with morphometric larval changes, pikeperch
170 larvae were euthanized with an overdose (240 $mg.L^{-1}$) of tricaine methanesulfonate (MS-222)
171 anaesthetic after completion of the behavioural tests, and measured using a binocular
172 microscope (Optika equipped with a Sony camera, Microvision, Lw1235C-GTI, Japan). Body
173 morphometrics were taken from digital images using the image analysis software Archimed®
174 (Microvision Instrument, France) and ImageJ®. Nine different morphometric characters
175 associated with locomotion, vision and feeding were measured (**Fig. 1**): Total body Length (TL)
176 is the distance between the snout to the tip of the tail; Eye Diameter (ED) is the average of the
177 maximum and minimum diameters of the eye orbit; Head Length (HL) is the distance between
178 the tip of the snout and the pre-opercula edge; Head Height (HH) is the greatest height of the
179 head measured perpendicularly to the mid-section of the eye; Tail Length (TaL) is the distance
180 between the anus and the base of the caudal fin; Tail Height (TH) is the distance perpendicular
181 to the axis of the tail between the dorsal and caudal fins; Head Width (HW) is the greatest width
182 of the head behind the eyes; Mouth Perimeter (MP) is the distance between the eyes following
183 the superior jaw; and Mouth Width (MW) is the distance between the eyes and parallel to HW.
184 All characters were measured to the nearest 0.01 mm (deformed specimens were discarded).
185
186 Digestive enzyme analysis
187 All larvae were frozen and freeze-dried (INRA, Champenoux, France) before being sent
188 to IRTA (Institute of Agrifood Research and Technology, Sant Carles de la Rapita, Spain) for

digestive enzyme analyses. These analyses were conducted after larva dissection (tail and head were removed). Extracts were prepared by homogenization of a single individual in 500 µL distilled water by sonication in an ice bath with three short pulses of 2 s (Vibra-cell, Sonics, USA). The homogenates were then centrifuged for 5 min at 13,000 g at 4ºC, and the extracts were used for the analysis of enzyme activities by fluorimetry. The following enzymes were assayed: pepsin, a protease produced in the stomach and responsible for acid digestion; and two pancreatic digestive enzymes, trypsin, a protease produced in exocrine pancreas and responsible for alkaline protein digestion, and α-amylase, a carbohydrase whose higher activities during larval development may be used as a marker of a delay in the development of juvenile digestion (Cahu and Zambonino-Infante, 2001). *EnzChek® Protease Assay Kit* and *EnzChek® Ultra Amylase Assay Kit* (Thermo Fisher Scientific) were used to quantify proteases (pepsin and trypsin) and α-amylase, respectively. Analyses were conducted according to the kits' manufacturer instructions. Fluorescence was read with TECAN© Infinite 200 series (Tecan Group Ltd., Männedorf, Switzerland). Enzyme activity was expressed in specific units ($U.mg^{-1}$ of protein) and the protein content of larval extracts was measured using the Bradford method (Bradford, 1976). Bovine serum albumin was used as standard. All the assays from each larva were made in triplicate (methodological replicates). Digestive enzyme analyses were conducted in pikeperch larvae aged 30 and 52 dph. Enzyme determination could not be conducted at earlier stages (23 dph) because an insufficient number of larvae displayed piscivory behaviour.

Statistical analyses

Firstly, we tested whether the time of day when tests were conducted could influence larval behaviours (attack and capture) by means of a Generalized Linear Model [glm, package 'lme4' (Bates et al., 2014)]. According to the peculiarity of the studied variables, that were the numbers of attacks and captures, *i.e.* counts, the distribution used in GLM was Poisson

(corresponding natural link function: log). The time of day was separated into four time periods (period 1: from 8:30 to 11:30, period 2: from 11:30 to 14:30, period 3: from 14:30 to 17:30, and period 4: from 17:30 to 20:30). The time of day did not affect the number of captures (χ^2 = 3.4, *d.f.* = 3, P = 0.34) and affect the number of attacks (χ^2 = 32.5, *d.f.* = 3, $P < 0.0001$) with period 1 different from period 4 (z = 4.3, P = 0.0001), and period 2 different from period 3 (z = 3.1, P = 0.01) and 4 (z = 4.9, $P < 0.0001$). Attacks occurred more during the beginning of the day than during the afternoon.

Then, we compared the percentages of pikeperch larvae that attacked and ate zebrafish or *Artemia* nauplii between all the age groups with a χ^2 test. When the global comparison between the five tested age groups was significant ($P < 0.05$), we compared the percentages of attacking larvae of different age groups two by two. After this comparison, for other analyses, we excluded the data obtained for the first age group (23 dph), because there was only one attack with no capture of zebrafish. The normality of the data was tested for attack effectiveness and morphological analyses with Shapiro-Wilk test (shapiro.test (R Core Team, 2017)) and the homogeneity of variance was tested with Levene test (leveneTest package 'car' (Fox and Weisberg, 2016)). When data did not fit with normality or homogeneity, we used a non-parametric test. For comparison between age groups, we used a Kruskall-Wallis test due to the non-homogeneity of data.

Secondly, we compared the distances of attack and the swimming activity levels between four age groups (the 23 dph-group was also excluded) to evaluate pikeperch larval predatory abilities. For attack distances, pikeperch larvae were divided into three groups for each studied age group: (i) a larva could make a successful attack with prey capture (AC), (ii) unsuccessful attacks, but larva had already captured zebrafish larvae previously (AnC1) or (iii) no successful attacks throughout the whole duration of the test (AnC2). We tested the effect of the age and of a previous success (AC *vs.* AnC1) of capture on the distance of attack, by using

a Wald χ^2 test applied on a Linear Mixed Model [lmer, package 'lme4' (Bates et al., 2014)] including the age as covariate, the previous success as fixed factor, and the interaction between age and experience, and individual as random factor. To compare AnC1 and AnC2, the effect of the age and the previous success of capture on the distance was tested using an ANCOVA [Anova, 'package 'car' (Fox and Weisberg, 2016)] including the age as covariate, the previous success as fixed factor and the interaction between age and experience.

Swimming activity was tested using F-tests (Anova, package 'car' (Fox and Weisberg, 2016)) applied on a Linear Model [lm, package 'lme4' (Bates et al., 2014)] with age as a fixed factor and number of attacks (transformation square root) as a covariate. Then, a Tukey method was used as post-hoc test with *P*-value adjustment (fdr) considering the mean of attacks [contrast, package 'emmeans' (Lenth et al., 2019)].

Thirdly, growth during development was described with regressions estimated from each morphometric parameter divided by the total length (TL) according to the allometric growth model described by Fuiman (Fuiman, 1983). In this allometric model, the inflexion points designated the value of the body characters where the regression slopes (allometric growth coefficient) changed. To initiate the model, a Principal Component Analysis (PCA) (vegan 2.0-9 packages, R software version 3.2.4) was carried out using the covariance matrix of the measured characters divided by TL. It is generally accepted that when individuals within different growth patterns are included in the PCA, PC1 summarizes the shape variation resulting from growth allometry, while PC2 summarizes the variation of divergent growth trajectories (Nikolioudakis et al., 2010). Hence, growth patterns among different stanzas are reflected as divergent PC2 trajectories when plotted against PC1 or TL. A piecewise linear regression, fitted with a non-linear procedure, was used to estimate change in PC2 orientation [for more details on analysis procedures, see Gisbert et al. (2002) and Réalis-Doyelle et al. (2017)]. Furthermore, all morphological parameters were compared between piscivorous and

non-piscivorous larvae (larvae that attacked zebrafish or not) with Welch t-test for each age group (23, 30, 37, 44 and 52 dph) when possible (at 23 dph only one larva attacked, and at 44 dph only one larva did not attack; thus, for these two age groups, statistical comparison was not possible).

Finally, to analyse enzyme activities, interaction between status (piscivore or non-piscivore) and age (30 or 52 dph) was tested by an analysis of variance (ANOVA) using the lm function (R Core Team, 2017) with status and age as fixed effects [model = lm (enzyme~status*age)] and enzyme activity as a dependent variable (trypsin, α-amylase or pepsin). For ANOVA validation, residuals were tested for homogeneity and normality using residual *vs*. fitted value and sample *vs.* theoretical quantile (Q-Q) plots, respectively [plot (R Core Team, 2017)] followed by Shapiro-Wilk test for normality and Levene test for homogeneity of variance. As the data met the ANOVA assumptions, an ANOVA Type I was performed to calculate F-tests [ANOVA (R Core Team, 2017)]. When interactions between status and age were not significant, enzyme quantities between piscivorous and non-piscivorous larvae were compared with Student t-test for each age group tested (30 and 52 dph). Furthermore, a correlation between the number of attacks and enzyme quantities was tested at 30 and 52 dph with Pearson correlation test.

All statistical analyses were performed using the free software R version 3.6.2 (R Core Team, 2017) except for χ^2 tests, which were performed with StatView software (version 5.0). For model validations, residuals were tested for homogeneity and normality using residual *vs*. fitted value and sample *vs.* theoretical quantile (Q-Q) plots, respectively [plotresid, package 'RVAideMemoire' (Hervé, 2017)]. The level of significance used in all tests was $P < 0.05$.

Results

The onset of piscivory

289 The percentage of pikeperch larvae attacking zebrafish increased with age (χ^2_4 = 36.9,
290 P < 0.0001; **Fig. 2**), particularly between 23 and 30 dph (χ^2_1 = 13.8, P = 0.0002; **Fig. 2**).
291 Regarding *Artemia* nauplii as a live prey, this percentage showed a different trend (χ^2_4 = 26.7,
292 P < 0.0001; **Fig. 2**); it increased significantly between 23 and 30 dph (χ^2_1 = 5.2, P < 0.02),
293 stabilized to some extent between 30 and 44 dph, and decreased between 44 and 52 dph (χ^2_1 =
294 10.1, P < 0.001; **Fig. 2**).

295 Furthermore, there was a significant interaction between age and the type of food item
296 consumed (χ^2_8 = 391.5, P < 0.0001; **Fig. 3**). At 30 dph, pikeperch larvae attacked fewer *Artemia*
297 nauplii and more zebrafish larvae than they did at 23 dph (χ^2_2 = 77.5, P < 0.0001; **Fig. 3**). At
298 37 dph, their attack pattern was similar to that of 30 dph (χ^2_8 = 3.0, P = 0.22). At 44 dph,
299 pikeperch larvae, which attacked *Artemia* nauplii, attacked also zebrafish (χ^2_2 = 43.2, P <
300 0.0001; **Fig. 3**). Indeed, after 30 dph, most pikeperch larvae were able to attack fish (**Fig. 3**).
301 When excluding 23 dph from the analyses, attack effectiveness did not significantly vary with
302 age (30 dph: 0.14 ± 0.18; 37 dph: 0.06 ± 0.19; 44 dph: 0.27 ± 0.35; 52 dph: 0.27 ± 0.33;
303 Kruskall-Wallis test, H_3 = 7.5, P = 0.06; **Fig. 4**).

304 Comparison of attack distances as a function of previously successful captures (AC and
305 AnC1) showed that there was no significant interaction between age and previous success or
306 failure of prey capture (χ^2 = 0.003, *d.f.* = 1, P = 0.9; **Fig. 5**). However, there was a simple effect
307 of age on attack distances (χ^2 = 10.36, *d.f.* = 1, P = 0.001; **Fig. 5**) and of a previous success or
308 failure of prey capture (χ^2 = 14.86, *d.f.* = 1, P = 0.0001; **Fig. 5**). Comparison of attack distances
309 between unsuccessful, but previously successful, larvae (AnC1) and totally unsuccessful larvae
310 (AnC2) showed that there was no significant interaction between age and capture failure (F =
311 0.10, *d.f.* = 1, P = 0.7; **Fig. 5**). However, there was a simple effect of age (F = 9.85, *d.f.* = 1, P
312 = 0.003; **Fig. 5**) and no effect of the success of capture (AnC1 and AnC2) (F = 0.21, *d.f.* = 1, P
313 = 0.6; **Fig. 5**) on attack distances.

Regarding the swimming activity, there was no significant interaction between age and the number of attacks (F = 0.17, $d.f.$ = 3, P = 0.9). However, it was markedly affected by the number of attacks (F = 20.25, $d.f.$ = 1, P < 0.0001, coefficient = 27.9; **Fig.6**). Furthermore, swimming activity was also markedly impacted by age (30 dph: 168.2 ± 19.1 s; 37 dph: 118.0 ± 13.5 s; 44 dph: 107.3 ± 14.6; 52 dph: 126.0 ± 17.1; F = 2.82, $d.f.$ = 3, P = 0.04). Although Tukey post-hoc analysis did not reveal any notable results from age group comparison, such an effect could be explained by the difference in statistical significance limit between 30 and 44 dph (t = 2.53, $d.f.$ = 69, P = 0.08).

Concerning morphological parameters, significant differences were observed at 30 dph between pikeperch larvae that attacked zebrafish larvae and those that did not (**Table 1**). Indeed, piscivores were larger, with larger eye diameters, and longer and higher tails, than non-piscivores (**Table 1**). For all the other ages, there was no significant difference between the two statuses. Furthermore, PCA results did not show any marked changes in the oblique orientation of PC2 scores when plotted against TL (**Fig. 7**), indicating no shift in the allometric growth of pikeperch larvae between 30 and 52 dph.

A link between piscivory behaviour and digestive enzymes

There was no significant interaction between pikeperch larva status (piscivore or non-piscivore) and age (30 or 52 dph) for any of the digestive enzymes measured (**Table 2**). However, there was a significant effect of age and status considered separately for all assayed enzymes (**Table 2**). At 30 dph, trypsin and α-amylase specific activity values were higher in larvae displaying non-piscivorous behaviour than they were in those categorized as piscivores (**Fig. 8**, **Table 3**). Furthermore, pepsin specific activity values were lower in non-piscivorous than in piscivorous larvae (**Fig. 8**, **Table 3**). At 52 dph, α-amylase specific activity values were lower and pepsin activity values higher in piscivorous than in non-piscivorous larvae (**Fig. 8**,

Table 3). Moreover, when taking into account all larvae (piscivorous and non-piscivorous) of a given age, we found that i) at 30 dph, the number of attacks tended to be negatively correlated with trypsin and amylase activities (trypsin: t = -1.8, $d.f$ = 17, P = 0.09; r^2 = -0.4; amylase: t = -2.1, df = 17, P = 0.05; r^2 = -0.45), but it was not correlated with pepsin activity (t = 0.8, $d.f.$ = 17, P = 0.4; r^2 = 0.2); and ii) at 52 dph, the number of attacks was not correlated with trypsin and amylase activities (trypsin: t = -0.1, $d.f.$ = 18, P = 0.8; r^2 = -0.03; α-amylase: t = -0.7, $d.f.$ = 18, P = 0.5; r^2 = -0.16), but it was positively correlated with pepsin activity (t = 2.2, $d.f.$ = 18, P = 0.04; r^2 = 0.46).

Discussion

By combining the analyses of several traits (behaviour, morphology and physiology), we highlighted the complex ontogenetic shift leading to piscivory in pikeperch larvae. This behavioural specialization on fish prey was associated with morphological and physiological traits. This shift from a zooplanktophagous feeding behaviour to piscivory occurred at about three weeks post-hatching at 20ºC, when larvae were approximately 11.0 (± 1.3) mm in TL. Moreover, we demonstrated that the shift to piscivory did not occur at the same time among all individuals, and it can therefore be described as an individual characteristic/trait. At 23 dph, only one larva was able to attack fish prey, compared to 60% at 30 dph and more than 90% at 44 dph. In our study, morphological differences were found at 30 dph between larvae of the two statuses (11.29 ± 1.39 mm TL in piscivores vs. 9.89 ± 0.94 mm TL in non-piscivores), regardless of the timing of the shift to piscivory. Such a shift seemed to be linked to TL, eye diameter, and tail length and height changes. Finally, digestive enzymes exhibited different activity levels in piscivores and non-piscivores. Indeed, trypsin and α-amylase activity values were higher in non-piscivores than they were in piscivores, whereas pepsin activity values were lower in non-piscivores than they were in piscivores.

In fish, when predatory shift occurred, individuals have limited time to learn effective capture behaviours, as morphogenesis in fish larvae occurs very rapidly compared to that in other vertebrates (Osse and Van den Boogaart, 1995). Even though the shift to piscivory requires morphological and physical aptitudes (Hecht and Appelbaum, 1988), the effectiveness of predatory behaviour depends on the development of cognitive abilities, such as learning. The acquisition of these aptitudes throughout ontogenic development could explain the shift to piscivory. Dietary changes during development could result from the development of some morphological and physiological traits (Hecht and Appelbaum, 1988; Otterå and Folkvord, 1993; Kaji et al., 2002). For example, mouth gape differs between the larval and adult stages and, as such, is a major factor in determining dietary changes (Bellwood et al., 2015). Changes in mouth morphology could be related to the shift to piscivory (Hellig et al., 2010) and could be a limiting factor to catch larger prey (Nilsson and Brönmark, 2000). In their review, Mittelbach and Persson (1998) concluded that the variation found in the sizes of prey eaten by piscivores was due to differences in their body sizes rather than to other factors. In the case of pikeperch larvae, our study showed some morphological differences at 30 dph between piscivores and non-piscivores, *i.e*. total length, eye diameter, and tail length and height. Differences in tail size seemed to indicate that this body part might be stronger in piscivores than in non-piscivores. Such differences may be correlated with the greater physical abilities of fish to attack thanks to the propulsive role of their tails in burst swimming used in prey capture (Osse and Van den Boogaart, 1995). Furthermore, eye diameter was larger in piscivorous than in non-piscivorous pikeperch larvae. Under the present experimental conditions, an increase in eye diameter was linked to aggressive behaviour and cannibalism (Miyashita et al., 2001). In addition, head length and width tended to be significantly different between piscivores and non-piscivores. Larvae with longer heads had higher branchial arches, which resulted in their greater capacity for gas exchange and, consequently, potential oxygen supply (Gisbert et al., 2002) for

increased locomotor activity and faster attacks (Osse and Van den Boogaart, 1995). A larger head might be correlated with the development of nervous (neurocranium) and feeding (splanchnocranium) systems (Gisbert et al., 2002; Eshaghzadeh et al., 2017). This development of feeding (functional jaw) and sensory (eye) structures may improve considerably prey capture and thus, increase larval growth and survival chances (Herbing, 2001). Our study highlighted that the shift to a piscivory diet required some morphological changes, which were associated to a higher number of attacks. The fact that we were not able to find significant differences between piscivores and non-piscivores in the other age groups (37, 44 and 52 dph) further stressed the importance of morphological changes for the shift to piscivory. Consequently, the larvae undergoing early morphological differentiation could shift to a piscivore diet more rapidly, which provided them with an adaptive advantage over their congeners less morphologically developed. An early shift to piscivory must lead to organ differentiation, particularly for the gut to optimize the digestion of fish prey. A previous histological study revealed that the onset of differentiation of all digestive structures in pikeperch larvae, except for the stomach, occurred at first feeding (Hamza et al., 2007). The development of the stomach and functionality of the gastric glands with pepsin secretion was found to indicate the end of the larval stage (Hamza et al., 2007) and the acquisition of an adult mode of digestion at approximately 29 dph (19-20°C) in pikeperch larvae. In our study, we demonstrated that piscivorous pikeperch larvae had a more developed digestive system than non-piscivorous pikeperch larvae. Therefore, there was a direct relationship between the onset of piscivory and the digestive system development. Comparison between piscivores and non-piscivores showed that the former had higher levels of acid proteases (pepsin) than of alkaline proteases (trypsin) (Solovyev et al., 2014). This observation highlighted inter-individual variability in digestive system development and supported the idea that acid protease-based digestion in juveniles was consistent with piscivory feeding habits, acid proteases being more effective than alkaline

proteases at digesting proteins (Rønnestad et al., 2013). In addition, non-piscivorous larvae exhibited higher α-amylase activity, which corroborated the conclusion that they had less developed digestive systems than their piscivorous counterparts. The activity of α-amylase generally tends to decrease with ontogeny in carnivorous species (Cahu and Zambonino-Infante, 2001), a pattern that was not observed in non-piscivorous fish in comparison to piscivorous specimens. Such variability in growth rate and enzyme activity level in fish of the same age had already been observed (Kuz'mina, 1996) and attributed to their genetic potential rather than differences in their nutrition.

Differences between piscivores and non-piscivores led us to investigate differences in energy use and growth. Juveniles that shifted earlier from a zooplankton or invertebrate-based diet to a piscivorous diet tended to gain a lot in their use of energy when shifting to piscivory (Graeb et al., 2006). For example, when age-0 yellow perch shifted from zooplankton to benthic invertebrates and then to fish prey items, their size increased following an increased energetic gain and decreased foraging costs (Graeb et al., 2006). This growth acceleration exists for a large number of marine and freshwater species (Keast, 1985; Wicker and Johnson, 1987; Juanes et al., 1994; Galarowicz et al., 2006; Scharf et al., 2009). In short, the shifting of individuals to piscivory may lead to population size heterogeneity. Under natural conditions, this can be regulated by the presence of a wide range of prey types and sizes: fast-developing individuals may benefit from faster prey, while reactive individuals may benefit from the presence of other trophic resources such as plankton or invertebrates. In conclusion, both populations could maintain themselves over long-term periods and adapt to resources available in the short term. Our results showed two types of adaptation to environmental features allowing this species in its early life stages to provide an adaptive response to environmental variability.

Such adaptive advantages in field populations resulting in high size heterogeneity could be a problem for fish farming. Most often, such size heterogeneity for predatory species results

in a high rate of intra-cohort cannibalism (type II) under farm conditions (Colchen et al., 2019). As cannibalism is intraspecific predation, it requires piscivorous behaviour and could be linked to the same behavioural, morphological and physiological changes as those leading to the onset of piscivory. Under intensive farming conditions, fish live with conspecific individuals belonging most of the time to the same cohort. In aquaculture, cannibalism is a major bottleneck for the domestication of emergent predatory species, mostly in larviculture (Teletchea et al., 2011). In this context, studying the onset of piscivory is essential to better understand fish cannibalism. Intra-cohort cannibalism is mainly observed in farmed predatory species especially during the larval and juvenile stages (Baras, 2012; Pereira et al., 2017). This behaviour generally matches with the shift from a planktonic to a carnivorous diet. Under aquaculture-controlled conditions, a carnivorous diet is generally represented by compound diets (pellets). We could interpret the high level of pikeperch cannibalism under farming conditions as the consequence of an early onset of piscivory for several individuals. In this case, the only prey they have at their disposal are conspecifics and the first meal gives them a growth advantage over the other fish (Cortay et al., 2019). Moreover, our results suggested that these individuals were more active and displayed more foraging behaviour than the other fish.

Summarizing, the shift from a zooplanktophagous feeding behaviour to piscivory was observed to occur very early (at three weeks after hatching: 11.0 ± 1.3 mm TL at 20°C) in pikeperch. Furthermore, all pikeperch larvae were able to hunt fish prey when they were six weeks old (16.58 ± 1.90 mm TL, 20°C). The shift to piscivory seemed to be linked to morphological and physiological changes. Indeed, piscivorous pikeperch larvae had more developed digestive systems and larger heads and tails than their non-piscivorous counterparts. Consequently, this early onset of piscivory for some individuals could account for the early emergence of cannibalism in reared populations. To explain cannibalism, it seems important to

look for inter-individual differences in ontogenetic development particularly in morphological, physiological and behavioural parameters that might be linked to genetic factors.

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644

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652

653 **Author Contributions**

654 All authors have given their approval to the final version of the manuscript. TC FT PF AP:
655 conceived and designed the experiments. TC AD: performed the experiments. TC AD: analysed

the behavioural video recordings. TC AD AP: analysed the behavioural and morphological data. EG: performed and analysed the digestive enzyme data. TC EG FT PF AP: wrote the paper.

Competing Interests

The authors declare no competing interests.

Table 1: Mean and Standard Deviation (SD) of morphological parameters of piscivorous and non-piscivorous pikeperch larvae and results of unpaired Student t-test (*t* and *P*-value) comparing the morphological parameters (in mm) of piscivorous and non-piscivorous pikeperch larvae: Total Length (TL), Eye Diameter (ED), Head Length (HL), Head Height (HH), Tail Length (TaL), Tail Height (TH), Head Width (HW), Mouth Perimeter (MP) and Mouth Width (MW). [For 23 and 44 dph age groups, only one larva attacked and only one larva did not attack, respectively, so statistical comparison was not possible. Significant results ($P < 0.05$) are in bold. Nd means no data.

Age (dph)	Morphological parameters	Piscivores	Non-piscivores	*t*	*P*-value
23	TL	8.85	9.82 ± 0.64	nd	nd
30	TL	11.29 ± 1.39	9.89 ± 0.94	**2.67**	**0.02**
	ED	0.80 ± 0.08	0.71 ± 0.06	**3.00**	**0.01**
	HL	1.98 ± 0.25	1.81 ± 0.08	2.05	0.06
	HH	1.79 ± 0.23	1.65 ± 0.20	1.35	0.20
	TaL	4.18 ± 0.54	3.59 ± 0.23	**3.19**	**0.006**
	TH	0.81 ± 0.13	0.66 ± 0.07	**3.14**	**0.006**
	HW	3.04 ± 1.05	2.03 ± 1.13	1.92	0.08
	MP	3.96 ± 1.36	2.91 ± 1.63	1.44	0.18
	MW	2.30 ± 0.83	1.61 ± 0.88	1.68	0.12
37	TL	16.58 ± 1.90	18.10 ± 1.74	-1.80	0.09
	ED	1.18 ± 0.15	1.30 ± 0.13	-1.80	0.09
	HL	3.09 ± 0.47	3.48 ± 0.55	-1.61	0.14
	HH	2.71 ± 0.35	2.89 ± 0.35	-1.07	0.30
	TaL	5.90 ± 0.66	6.41 ± 0.54	-1.88	0.08

	TH	1.23 ± 0.18	1.35 ± 0.14	-1.58	0.13
	HW	3.64 ± 0.52	3.95 ± 0.44	-1.42	0.18
	MP	4.83 ± 0.70	5.39 ± 0.80	-1.54	0.15
	MW	2.95 ± 0.42	3.20 ± 0.25	-1.71	0.10
44	TL	19.93 ± 2.09	21.25	nd	nd
	ED	1.43 ± 0.14	1.48	nd	nd
	HL	4.07 ± 0.68	3.88	nd	nd
	HH	3.08 ± 0.38	3.26	nd	nd
	TaL	7.01 ± 0.77	7.59	nd	nd
	TH	1.52 ± 0.22	1.64	nd	nd
	HW	4.38 ± 0.69	4.75	nd	nd
	MP	5.92 ± 1.23	6.54	nd	nd
	MW	3.46 ± 0.58	3.66	nd	nd
52	TL	27.82 ± 3.54	32.93 ± 7.95	-1.35	0.29
	ED	1.91 ± 0.17	2.13 ± 0.27	-1.57	0.20
	HL	5.42 ± 0.85	6.02 ± 0.99	-1.10	0.32
	HH	4.14 ± 0.44	4.70 ± 0.70	-1.51	0.21
	TaL	9.47 ± 1.15	10.93 ± 1.62	-1.69	0.17
	TH	2.13 ± 0.29	2.42 ± 0.27	-1.87	0.12
	HW	5.96 ± 0.64	6.97 ± 0.86	-2.20	0.09
	MP	8.54 ± 1.21	10.19 ± 2.35	-1.36	0.26
	MW	4.76 ± 0.50	5.59 ± 1.17	-1.38	0.25

Table 2. Effects of age and piscivory status (piscivores and non-piscivores) (ANOVA table) on trypsin, α-amylase and pepsin activities with F-value (F), degree of freedom (*d.f.*) and p-value (p). Bold values indicate significant effects ($p < 0.05$).

Factors	**Trypsin**	**α-Amylase**	**Pepsin**
Age * Status	$F = 0.8$; *d.f.* = 1 ; $p = 0.37$	$F = 2.4$; *d.f.* = 1 ; $p = 0.1$	$F = 0.004$; *d.f.* = 1 ; $p = 0.9$
Age (30 and 52 dph)	$F = 24.7$; *d.f.* = 1 ; **$p < 0.0001$**	$F = 22.7$; *d.f.* = 1 ; **$p < 0.0001$**	$F = 46.2$; *d.f.* = 1 ; **$p < 0.001$**
Status (Piscivores and Non-piscivores)	$F = 9.3$; *d.f.* = 1 ; **$p = 0.004$**	$F = 12.9$; *d.f.* = 1 ; **$p = 0.001$**	F = 13.3 ; *d.f.* = 1 ; **$p < 0.001$**

Table 3. Comparison (Student t-test; *t*) of trypsin, α-amylase and pepsin activities between piscivorous and non-piscivorous pikeperch larvae at 30 and 52 Abbreviations: degree of freedom (*d.f.*) and p-value (*p*). Bold values indicate significant effects ($p < 0.05$).

Age	Trypsin	α-Amylase	Pepsin
30 dph	*t* = 2.46; *d.f.* = 17; ***p* = 0.02**	*t* = 2.83 ; *d.f.* = 17; ***p* = 0.01**	*t* = -3.14; *d.f.* = 17; ***p* = 0.006**
52 dph	*t* =1.61; *d.f.* = 18; *p* = 0.12	*t* = 2.10; *d.f.* = 18; ***p* = 0.05**	t = -2.17; *d.f.* = 17; ***p* = 0.04**

Figure legends

Figure 1: Morphological parameters measured (in mm) on pikeperch larvae at five ages (23, 30, 37, 44 and 52 dph). Abbreviatons: Total Length (TL), Eye Diameter (ED), Head Length (HL), Head Height (HH), Tail Length (TaL), Tail Height (TH), Head Width (HW), Mouth Perimeter (MP) and Mouth Width (MW).

Figure 2: Percentage of pikeperch larvae attacking zebrafish larvae (A) and *Artemia* nauplii (B) as a function of their age. Different letters mean a significant difference at $p < 0.05$. The numbers above the histograms represent the total number of larvae that attacked prey.

Figure 3: Percentage of pikeperch larvae attacking zebrafish larvae (grey bars) or *Artemia* nauplii (black bars) or both zebrafish and *Artemia* nauplii (white bars) as a function of their age. The numbers in the histograms represent the total number of larvae that attacked each type or both types of prey.

Figure 4: Box-plot representation of attack effectiveness (ratio of the number of captures to the total number of attacks on zebrafish larvae) of pikeperch larvae as a function of age (30, 37, 44 and 52 dph). The black line is the median, the black triangle is the mean, the white dots are outsiders and the top lines are first quartiles.

Figure 5: Distance of attack (mm) in function of age (30, 37, 44, 52 dph). Each circle represents one individual tested distinguish by their previous success or not of capture: white (a successful attack with a capture: AC), light grey (an unsuccessful attacks but larva had already capturing zebrafish larvae previously: AnC1) and dark grey (no successful attacks: AnC2). Relation line, adjusted by the model, is represent for each type of capture (dark: AC, light grey: AnC1, dark

grey: AnC2). *** represent a significative difference ($p < 0.0001$) and NS, non-significative difference ($p > 0.05$).

Figure 6: Relation between swimming activity and the number of attacks. Each line represents this relation for each age: solid: 30 dph, dashed: 37 dph, dotted: 44 dph, dotdash: 52 dph.

Figure 7: Piecewise regression of PC2 scores on total length (TL in mm) in pikeperch larvae for all individuals of the 30, 37, 44 and 52 dph age groups. All morphometrical parameters were divided by the total length of each age group. PC2 scores summarize the variation of divergent growth trajectories. Piscivorous larvae are represented with black circles and non-piscivorous larvae with black triangles.

Figure 8: Box-plot representation of digestive enzymatic activity of piscivorous and non-piscivorous pikeperch larvae for two age groups (30 and 52 dph). The black line is the median, the black triangle is the mean, the white dots are outsiders, and the top and bottom lines are the first and third quartiles.

Figure 1

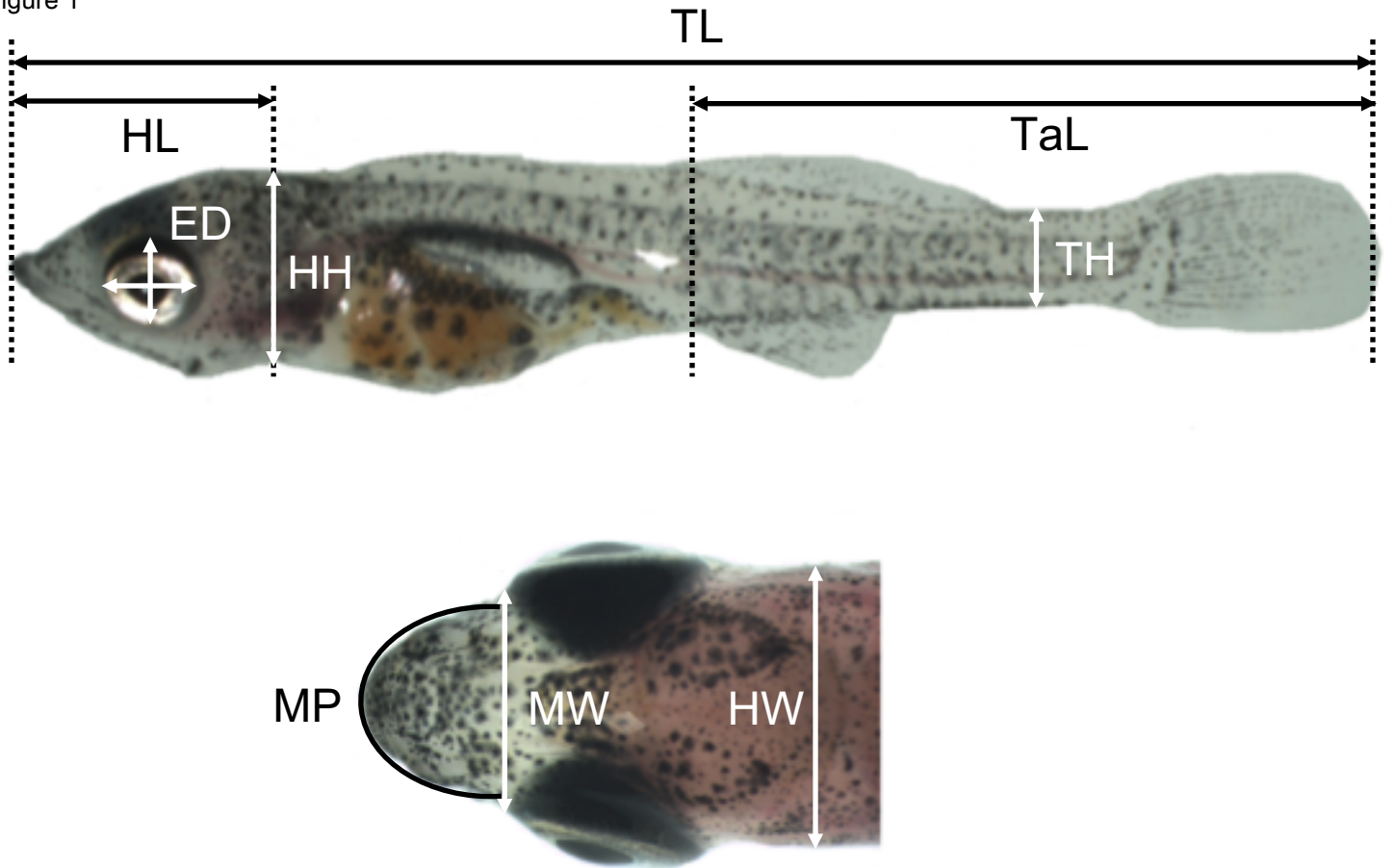


Figure 2

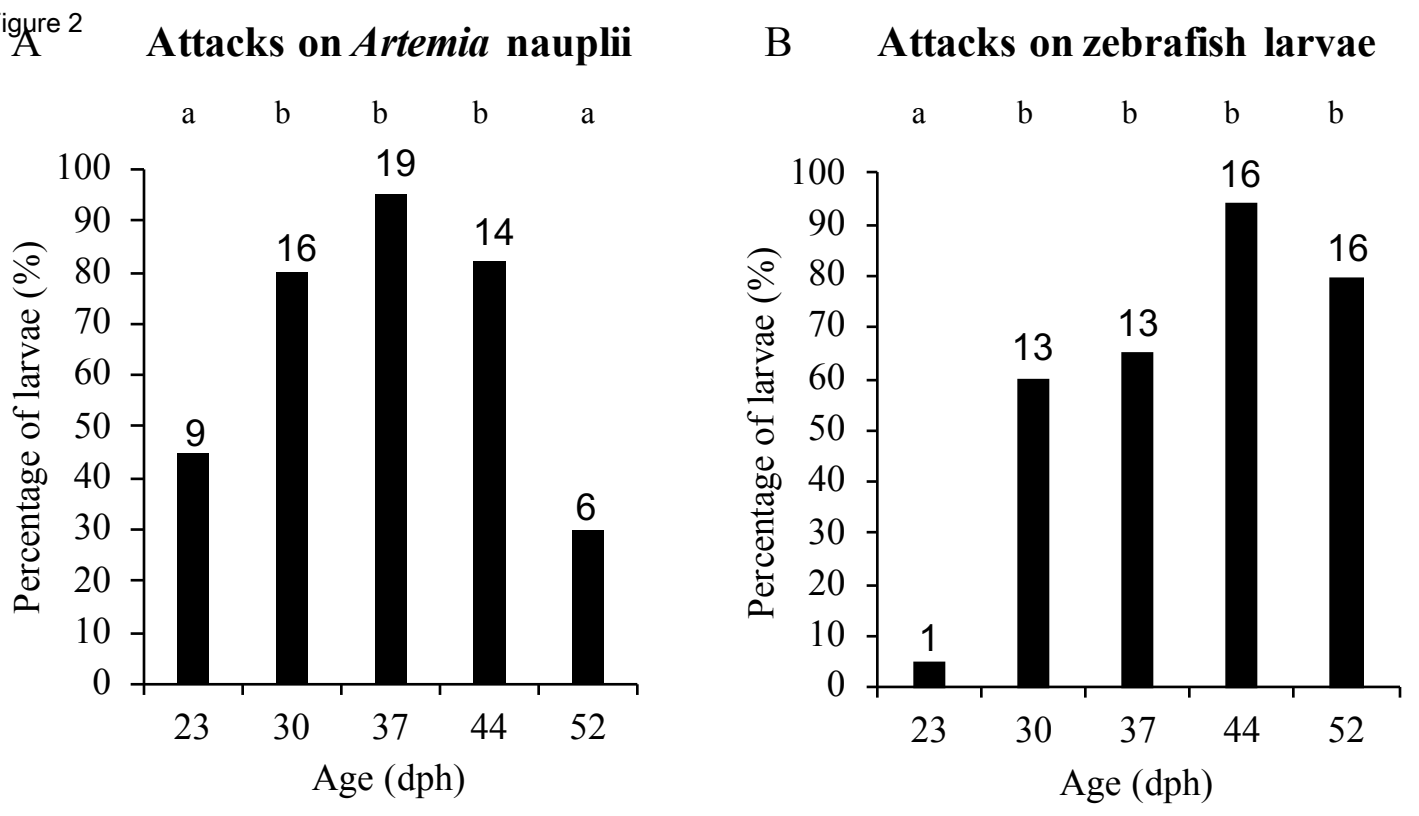

A
Attacks on *Artemia* nauplii
a b b b a
100 90 80 70 60 50 40 30 20 10 0
Percentage of larvae (%)
9 16 19 14 6
23 30 37 44 52
Age (dph)
B
Attacks on zebrafish larvae
a b b b b
100 90 80 70 60 50 40 30 20 10 0
Percentage of larvae (%)
1 13 13 16 16
23 30 37 44 52
Age (dph)

Figure 3

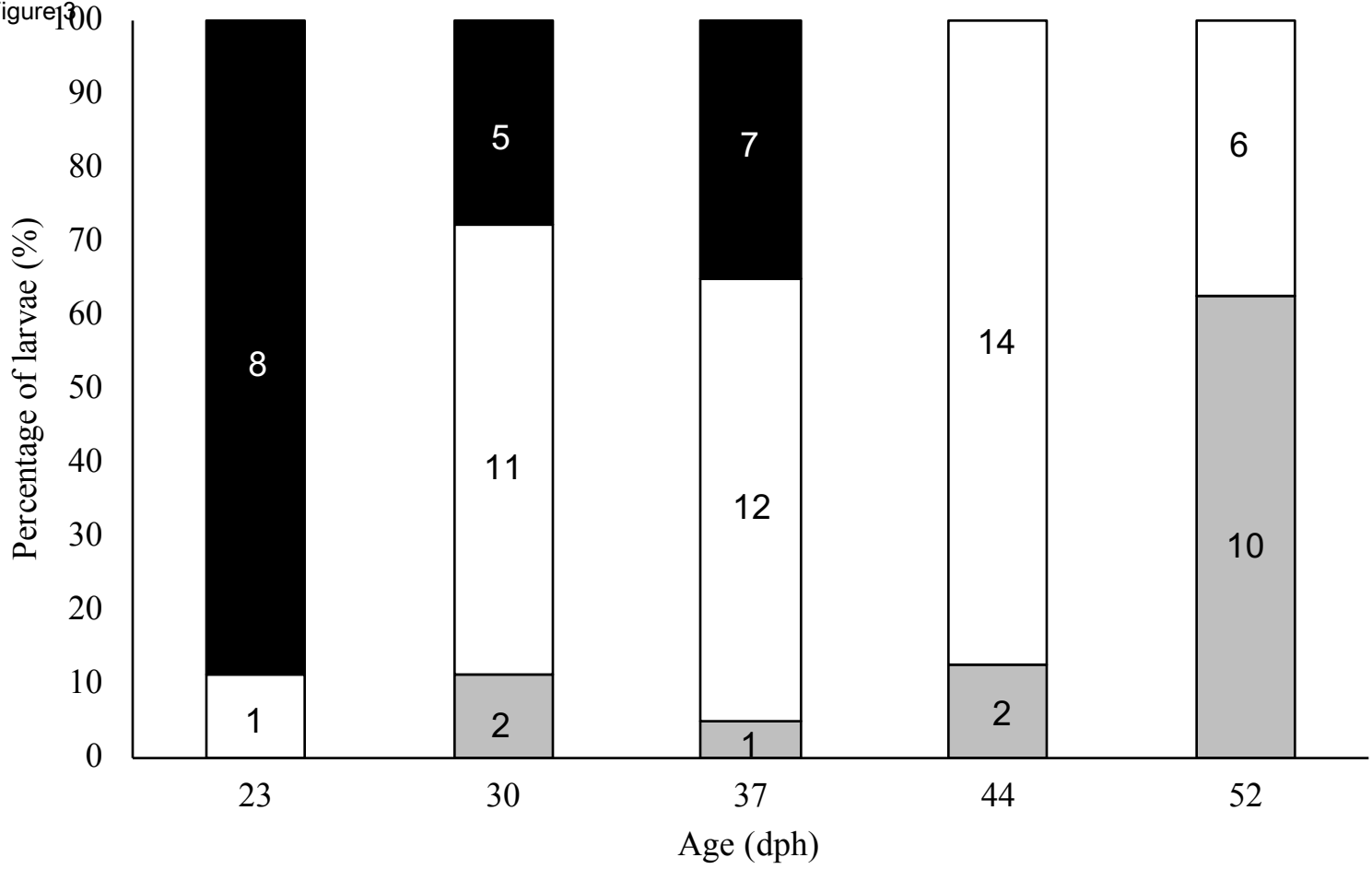

Percentage of larvae (%)
100
90
80
70
60
50
40
30
20
10
0
8
1
5
11
2
7
12
1
14
2
6
10
23
30
37
44
52
Age (dph)

Figure 4

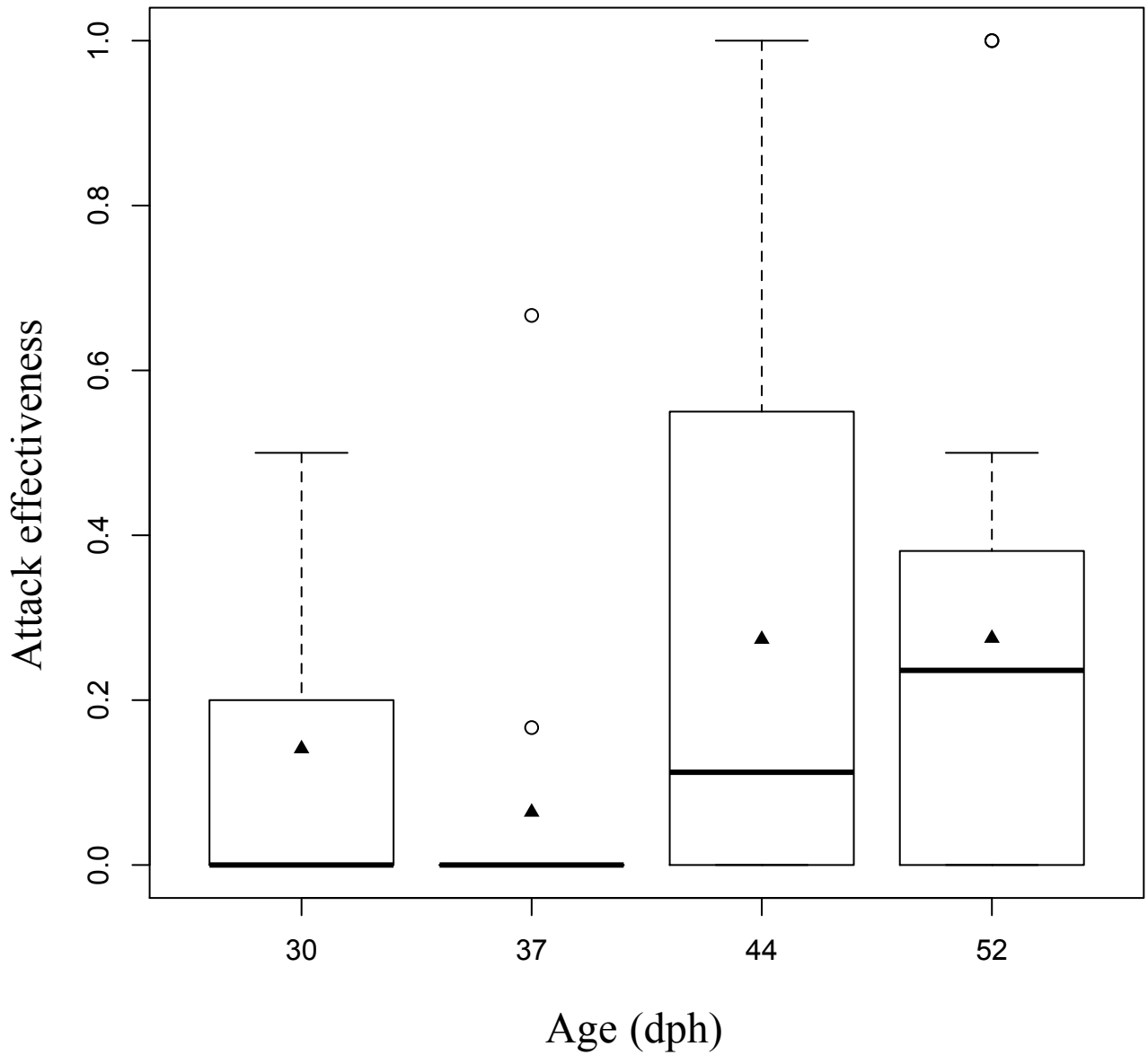


Figure 5

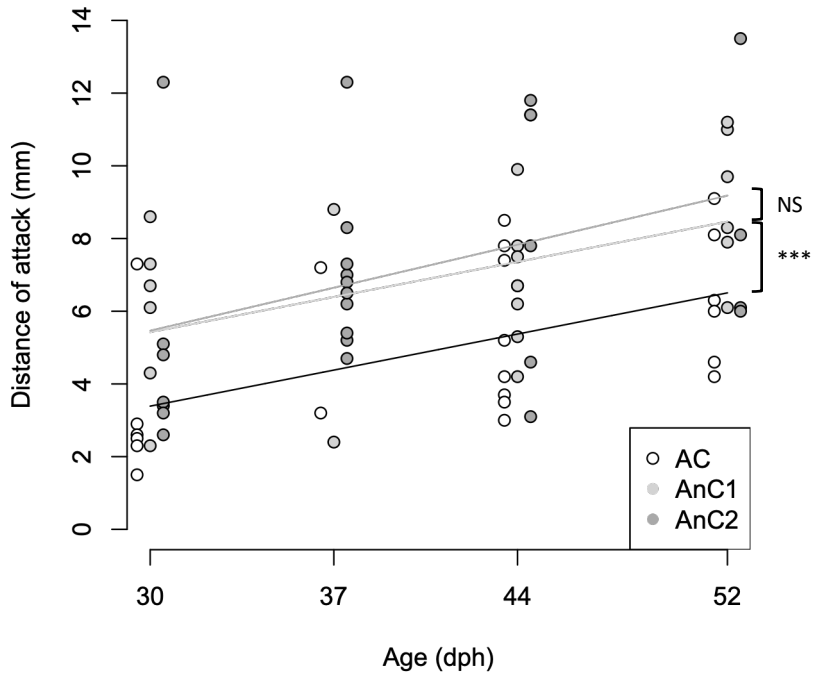

Distance of attack (mm)
0
2
4
6
8
10
12
14
30
37
44
52
Age (dph)
AC
AnC1
AnC2
NS
***

Figure 6

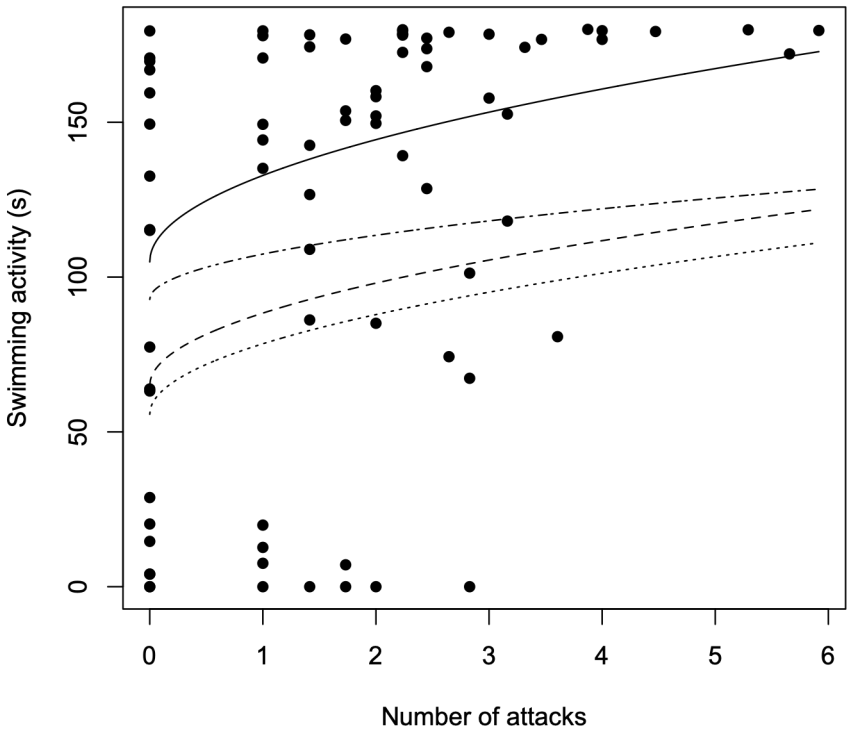


Figure 7

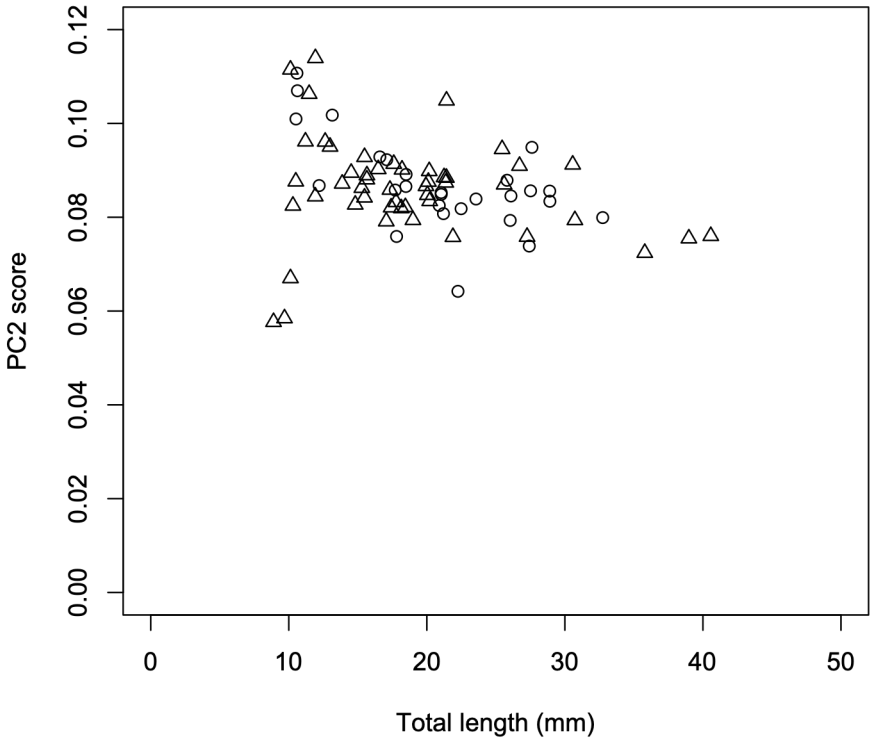
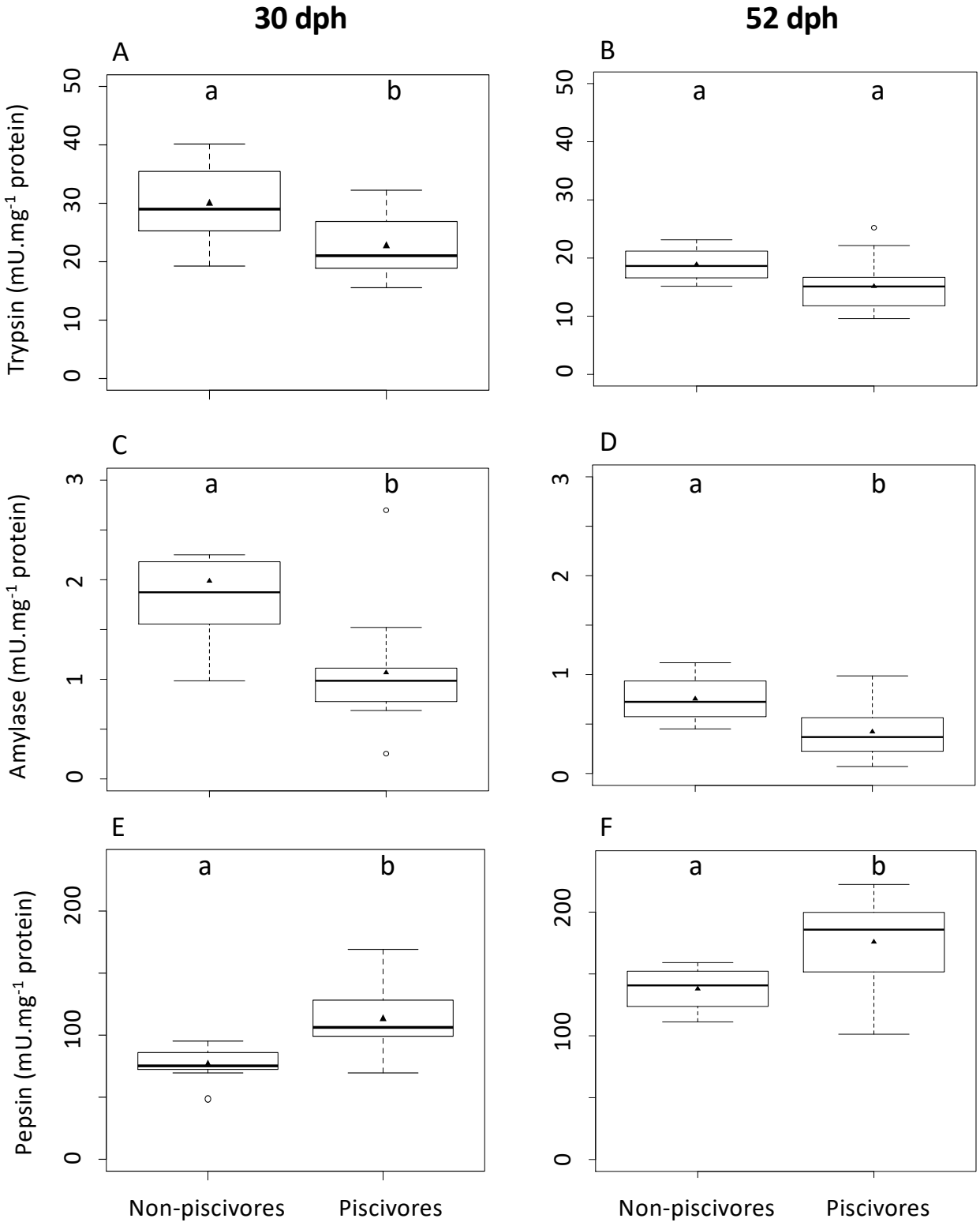

PC2 score
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0
10
20
30
40
50
Total length (mm)

Figure 8


30 dph
52 dph
A
B
C
D
E
F
Trypsin (mU.mg-1 protein)
Amylase (mU.mg-1 protein)
Pepsin (mU.mg-1 protein)
a
b
0
10
20
30
40
50
1
2
3
100
200
Non-piscivores
Piscivores