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Dietary amino acids impact sperm performance traits for a catadromous fish, *Anguilla anguilla* reared in captivity

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Abstract

Little is known about the role of dietary amino acids on male reproductive performance and gamete quality in fishes. Thus, the objective of this study was to investigate how "enhanced" feeds (EH-4, EH-5, EH-6), with modified amino acid composition, and the standard on-growing diet (DAN-EX) impact body composition, milt biochemistry, and sperm performance in male European eel, *Anguilla anguilla*. The fatty acid composition of EH-4, EH-5, and EH-6 was similar but differed to that in DAN-EX, while amino acid composition varied between all four diets. Diet did not influence organ-somatic indices (e.g. HSI, GSI), while males fed EH-4 were heavier than other groups. Arginine, alanine, and lysine were the most abundant amino acids in milt (>11%), followed by glycine, aspartic acid, valine, glutamic acid, and leucine (>5.66%). Diet impacted milt arginine, serine, proline, methionine, and histidine levels. Specifically, males fed DAN-EX, EH-4, and EH-5 had the highest percentages of arginine, while males fed EH-4 to EH-6 had higher percentages of serine. Proline was most abundant in males fed DAN-EX, EH-5, and EH-6. Both methionine and histidine were detected at low percentages (<2%), and were impacted by diet, where males fed EH-4 and EH-5 had higher percentages of methionine, and males fed DAN-EX, EH-4, and EH-6 had the highest percentage of histidine. Milt production increased over time, where eels fed EH-4 and EH-6 showed the highest probability of producing suited milt volumes (>0.5 mL) for fertilization procedures. Spermatocrit (43.1 ± 1.80%) did not differ between the diets (ranged from 37.57 to 47.21%). Dietary regime had an impact on sperm motility, such that eels fed EH-5 and EH-6 had the greatest percentage of motile cells. In addition, fish fed EH-5 and EH-6 (or DAN-EX) had the fastest swimming sperm. Spermatogenic maturity index of hormonally treated eels varied within groups but did not differ between dietary treatment groups after 9 weeks of injections

(ranged from 0.54 to 0.80). The most interesting amino acids to scrutinize from PCA plots were proline, histidine, and valine as well as lysine and arginine. Here, eels with highly motile sperm had milt with high relative proportions of proline, histidine, and valine, but were particularly low in lysine and arginine. Together, our findings add evidence that certain amino acids regulate milt biochemistry, and that male ejaculate traits may be promoted by amino acid intake. Further studies to evaluate effects of supplemented amino acid diets on fertilization ability and inter-linked early developmental stages are required.

Keywords: Aquaculture; Broodstock diet; Assisted Reproduction; Gamete quality; European eel

1. Introduction

When reared in captivity, fishes may exhibit different degrees of reproductive dysfunction, such as spermiation failure, spontaneous ovulation, or decreases in gamete quality (Mylonas et al., 2017). Poor gamete quality may jeopardize the survival of offspring, especially during the "critical" early life history stages, which can ultimately impact aquaculture production and limit possibilities for selective breeding (Bromage et al., 1992; Kjørsvik et al., 2003). Therefore, a stable supply of "high-quality" gametes is required for establishment of sustainable culture for any given species. In this scenario, controlling sperm quality can be a major issue for the aquaculture industry, since it is affected by a variety of factors including broodstock nutrition, epigenetics, and/or sperm handling (Cabrita et al., 2014).

It is widely accepted that broodstock nutrition or enriched diets with certain compounds greatly modulate sperm physiology and functionality (Labbé et al., 1995; Lahnsteiner et al., 2009;

70 Beirão et al., 2015) as well as male reproductive success (Asturiano et al., 2001). In this regard,
71 most studies have supplemented diets with modified lipid and fatty acid profiles (Asturiano et al.,
72 2001; Vassallo-Agius et al., 2001; Nandi et al., 2007; Cabrita et al., 2014; Beirão et al., 2015).
73 While manipulation of lipids in broodstock diets offers excellent opportunities to improve gamete
74 quality, the protein and amino acids should similarly receive consideration. The biological
75 functionality of amino acids is diverse, as they are involved in feed intake, nutrient utilization, and
76 reproduction (Izquierdo et al., 2001; Li et al., 2009; Wu, 2009). Additionally, they are precursors
77 for a wide variety of macromolecules, such as nucleotides, lipids, glycogen, steroids (Finn and
78 Fyhn, 2010), and serve as oxidizable substrates for sperm (Mann and Lutwak-Mann, 1981).
79 Evidence has accumulated that amino acids are highly beneficial for improving reproduction
80 and directly affect fertilization success and the number of viable embryos (Amirkhanov, 1980;
81 Dabrowski et al., 1985; Kwasek et al., 2014a). For example, certain amino acids (e.g. arginine) are
82 abundant in physiological fluids (Wu et al., 2009) and play a crucial role in regulating reproductive
83 functions (Li et al., 2009), survival of juvenile fish (Buentello and Gatlin, 2001), as well as
84 neuronal development and neurotransmission (Jobgen et al., 2006; Yao et al., 2008). Additionally,
85 in fish, amino acid-rich proteins called protamines within sperm nuclei are involved in sperm cell
86 growth and differentiation (Martinage et al., 1985), thus underlining a critical role for amino acids
87 in spermatogenesis (Wu et al., 2009). Previous studies reported that taurine (derived from cysteine)
88 at levels of 10 g/kg improved spawning in some marine fish species (Matsunari et al., 2006). In
89 Japanese eel, Higuchi et al. (2012) determined that taurine is essential for spermatogenesis,
90 although it can be synthetized from cysteine in the testis under the action of dihydroxyprogesterone
91 (DHP). The consequences of dietary supplementation of amino acids on male gamete quality and
92 performance are fascinating, due to the fact that amino acids are a key component of seminal

93 plasma and sperm (Lahnsteiner 2009, 2010; Kwasek et al., 2014b), impacting sperm metabolism
94 and sperm motility and/or fertility (Patel et al., 1998; He and Woods, 2003). In addition, a complex
95 antioxidant defense system exists in milt (e.g. superoxide dismutase, catalase, and the glutathione
96 peroxidase-glutathione reductase system), which is highly influenced by nutrition (Mansour et al.,
97 2006). Thus, dietary supplementation of specific amino acids may provide a novel approach to
98 improve fertility in males.

99 Sperm performance and composition in vertebrates are clearly affected by dietary
100 supplementation of amino acids (Wu et al., 2009; Kwasek et al., 2014b; Pourkhazaei et al., 2017).
101 For example, in mammals, either supplementation of diets with amino acids or direct injection
102 improved sperm traits (e.g. motility, velocity, morphologically normal sperm, and acrosome
103 integrity) and subsequent fertilization success (Dong et al., 2016; Abd-Elrazek and Ahmed-Farid,
104 2018). In teleosts, only a few studies have investigated incorporation of dietary amino acids on
105 male gonadal development and gamete quality (Akiyama et al., 1996; Kwasek et al., 2014b;
106 Pourkhazaei et al., 2017), and without directly linking the diet to the kinetic characteristics of
107 sperm. As such, knowledge regarding the role of dietary amino acids on gamete quality and
108 reproductive performance of male fish remains incomplete.

109 Here, we use European eel, *Anguilla anguilla* as our model organism. Despite increasing
110 efforts (Asturiano et al., 2005; Gallego et al., 2012; Tomkiewicz, 2012; da Silva et al., 2018; Politis
111 et al., 2018a; Benini et al., 2018), variability in gamete quality is still an issue affecting larval
112 production for this species. During the last decade, different strategies have been employed to
113 improve European eel gamete quality, especially by conducting studies on reproductive
114 performance and broodstock diets at specific developmental windows (Støttrup et al., 2013; Baeza
115 et al., 2014, 2015a,b; Butts et al., 2015; da Silva et al., 2016). For example, supplementation of

116 broodstock diets with lipids [mainly polyunsaturated fatty acid (PUFA)], improved spermiation
117 and milt quality in males (Butts et al., 2015), and oocyte growth and ovarian development in
118 females (Støttrup et al., 2016; da Silva et al., 2016). Therefore, further information on the effects
119 of broodstock diets on European eel gamete performance could enhance the process of
120 domestication for mass production of larvae. Nevertheless, the effect of supplementation of the
121 broodstock diet with amino acids on gamete quality has not yet been investigated for this species.
122 As such, we hypothesize that dietary supplementation of specific amino acids will be beneficial
123 for male gamete performance.

124 The objective of this study was to investigate how "enhanced" feeds (EH-4, EH-5, EH-6),
125 with modified amino acid composition, and the standard on-growing diet (DAN-EX) impact body
126 composition, milt biochemistry, and sperm performance in male European eel. Together, these
127 data may be used to improve broodstock diets for this catadromous fish and increase our
128 understanding on reproductive physiology in fishes.

129

130 **2. Materials and methods**

131

132 All fish were handled in accordance with the European Union regulations concerning the
133 protection of experimental animals (Dir 86/609/EEC). Eel experimental protocols were approved
134 by the Animal Experiments Inspectorate (AEI), Danish Ministry of Food, Agriculture and
135 Fisheries (permit number: 2015-15-0201-00696). All efforts were made to minimize animal
136 handling and stress.

137

138

139 *2.1. Eel broodstock husbandry*

140

141 Eels were raised from wild-caught glass eels in a commercial eel farm, Stensgården Eel Farm
142 A/S in Jutland, Denmark. Eels were grown on commercial feed (DAN-EX; BioMar A/S, Brande,
143 Denmark) in concrete tanks (18-30 m^3) at stocking density of ~50 kg/m^2. Rearing tanks were
144 equipped with recirculating aquaculture system (RAS) technology, which consisted of a rotating
145 drum filter, biofilter, trickle-filters, UV system, and oxygen cones. Water was salted to 1-2 PSU
146 and heated to 23 ± 2°C. Upon start of experimental trials, the eels were sorted based on size and
147 then transferred to 4 fiberglass tanks (2 × 2 m, volume of 2.5 m^3, flowrate 1.5 to 2 m^3/h) at an
148 initial stocking density of 50 kg/m^2. Males were fed with automatic feeders six times per day at
149 0.5 to 0.8% body weight from 1 August 2015 to 2 February 2016 on three "enhanced" feeds (EH-4,
150 EH-5, EH-6; Table 1) and the standard on-growing diet (DAN-EX) varying in fatty acid (Table 2)
151 and amino acid composition (Table 3). Final stocking density was ~80 kg/m^2.

152 Upon completion of the commercial feeding trail, ~35 males from each dietary regime were
153 randomly selected and transported to a Technical University of Denmark (DTU Aqua) research
154 facility in Hirtshals, Denmark (57.585971 N, 9.985036 E). The eels were housed in 4 × 500 L
155 tanks equipped with RAS technology at a flowrate of 600 L/h. The RAS system consisted of a 350
156 L gravel filter, 0.3 m^3 trickle filter, and a 300 L temperature regulated sump. Temperature ranged
157 between 19-21°C, salinity ranged from 36-37 PSU, and photoperiod was kept at 12h light/12 h
158 dark at ~20 lux. Natural seawater salinity of ~32.5 PSU from the North Sea was adjusted using
159 Tropic Marin Sea Salt (Dr. Biener GmbH, Wartenberg, Germany) and verified using a
160 conductivity meter (WTW multi3410, Wissenschaftlich-Technische Werkstätten GmbH,
161 Weilheim, Germany). Acclimatization to sea water took place over one week before entering the

facility. No feed was provided during experimentation as eels cease feeding during the silvering process and are not anticipated to feed during spawning migration (Tesch, 2003).

After acclimatization, 32 randomly selected males (8 per diet, >100 g) were euthanized by submergence in an aqueous solution of benzocaine and their body weight and length were recorded to get an indication of their morphological status after receiving the experimental diets. These 32 eels were also dissected and liver weight was obtained. The remaining eels (n = 97) were anaesthetized by short-term submergence in benzocaine and tagged with a passive integrated transponder (PIT tag). The PIT tag was placed in the dorsal muscle. Male eels from each diet (DAN-EX = 27, EH-4 = 25, EH-5 = 25, EH-6 = 20) received weekly injections of human chorionic gonadotropin (hCG, Sigma Aldrich Denmark A/S) at 1.5 IU/g fish in order to induce milt production (Pérez et al., 2000). Body weight was recorded at the time of first injection and then at injection Week 4, Week 7, and Week 9. In addition, ~24 h after the Week 9 injection, 8-9 males were randomly selected per diet. Among these, the spermiating males ranged between 94-126 g. Again, after eels were euthanized, body weight, testes weight, and liver weight were recorded. Gonadosomatic index (GSI; 100 × testes weight/body weight) and hepatosomatic index (HSI; 100 × liver weight/body weight) were later calculated.

2.2. *Broodstock feed*

Previous studies have investigated dietary impacts on egg quality (Heinsbroek et al., 2013; Støttrup et al., 2013, 2016). Based on these findings, fatty acid composition of the best performing diet for females was used as basis for the fatty acid composition of these tested diets (hereafter EH-4, EH-5, and EH-6). In terms of fatty acid composition, EH-4, EH-5, and EH-6 differed

185 significantly from the commercial DAN-EX feed (Table 2). Amino acid composition varied
186 between all four diets, where the enhanced feeds were originally modified with respect to arginine
187 content (Table 1, Table 3). Here, arginine was lowest in EH-5 and highest in EH-6, while EH-4
188 showed intermediate levels.
189
190 *2.3. Amino acid analysis of milt*
191
192 Amino acid composition was analyzed using a EZ:fast™ amino acid analysis kit
193 (Phenomenex Inc., Torrance, CA, USA). Between ~300 to 2000 mg of sperm samples were used
194 for the analysis depending on how much sample was available. Separation and detection of amino
195 acids occurred by liquid chromatography using Agilent G6120BA single-quadrupol LC-MS in the
196 ESI ionization mode (Agilent Technologies, Hørsholm, Denmark). The LC was equipped with an
197 EZ:fast™ Liquid chromatography-Mass spectroscopy (LC-MS) column (250 mm × 3.0 mm,
198 Phenomenex). Amino acids were analyzed in duplicate or triplicate depending on milt volume.
199 Further details for amino acid analyses are described in Safafar et al. (2016).
200
201 *2.4. Milt production*
202
203 Males (n = 97 eels at start; n = 27 for DAN-EX; n = 25 for EH-4; n = 25 for EH-5; n = 20
204 for EH-6) were assessed weekly for spermiation one day after injection from Week 5 until Week
205 9. Fish ejaculate sperm spontaneously during natural spawning, but in captivity, it is typically
206 expressed from the sperm ducts by "stripping", where gentle abdominal pressure is applied. Thus,
207 slight pressure was applied to the abdomen of each male to assess their degree of maturity. Milt

was then classified between 0-5, where 0 = no milt, 1 = few drops with gentle pressure, 2 = low volume of milt with gentle pressure (<0.5 mL), 3 = medium volume of milt with gentle pressure (<1.0 mL), 4 = flowing milt with gentle pressure (>1.0 mL), and 5 = freely flowing. Approximately 24 h after injection on Week 9, the total weight of milt per male was recorded and standardized (g/100 g eel).

2.5. Spermatozoa kinetic traits

Milt samples from 83 males (n = 22 for DAN-EX; n = 22 for EH-4; n = 20 for EH-5; n = 19 for EH-6; one male was excluded from further analyses due to possible milt contamination) were collected 24 h after the Week 9 injection, as the highest sperm quality is obtained at this time (Pérez et al., 2000). Males were anesthetized with benzocaine (60 mg/L) to minimize any adverse effects during stripping. The urogenital pore was cleaned with ionized water and wiped dry to avoid contamination with feces, urine, or blood. To avoid any bias associated with time during sampling, the milt was collected into weight boats from two males per diet, and this was repeated until all males were sampled.

Immediately after stripping, a milt sample (100 μL) from each male was diluted in 900 μL of P1-extender medium (Peñaranda et al., 2010) and then kept in a cooler with frozen icepacks to maintain viability. Before activation, the samples were inverted for ~5 s for homogenization. The immobilized sperm suspension (0.2 μL) was then pipetted into a chamber of an 80 μm 2X-CEL glass slide (Hamilton Thorne, MA, USA) and covered with a 22 × 22 mm coverslip. The cells were then activated with 15 μL of modified seawater (36 ppt) with 1% bovine serum albumin, BSA (w/v). The BSA was added to the activation media as it prevents the sperm from sticking to

231 the glass slide.

232 Sperm activity was captured at 10, 20, and 30 s (± 1) post-activation using a compound
233 microscope (Nikon Eclipse 55i microscope, Nikon Corporation, Tokyo, Japan) equipped with a
234 Nikon DS-Fi1 camera head and negative phase objective (PL 40 x /0.66, ∞/0.17). The digital video
235 camera was attached to a personal computer and images were captured using a frame grabber at
236 50 frames/s (Procadi, PROiSER 1.4, Paterna, Spain). Three replicate activations were performed
237 for each male. Two observers continuously did all activations (752 video recordings) to avoid any
238 subjective deviation. Total motility (MOT, total number of motile spermatozoa/total number of
239 cells × 100) and curvilinear velocity (VCL, defined as velocity of sperm along its actual curvilinear
240 path) were then assessed using a computer-assisted sperm analysis (CASA; ISAS v1; PROiSER
241 R+D, S.L., Paterna, Spain).

242

243 *2.6. Spermatocrit*

244

245 Spermatocrit, defined as the ratio of packed sperm cells to the total volume of milt × 100,
246 was used to estimate sperm concentration on Week 9 (Sørensen et al., 2013). For each male (n =
247 84 eels), samples of milt were drawn into three microhematocrit capillary tubes (75 mm length
248 and 1.1-1.2 mm opening) and sealed at the end with Vitrex Sigillum wax. The tube was then
249 centrifuged for 10 min at 6000 × g (Haematokrit 210, Andreas Hettich GmbH & Co. KG,
250 Tuttlingen, Germany) and the average of three replicate tubes was used for statistical analysis.

251

252

253

2.7. Histological analyses

To assess testes development, testicular lobes were sampled after 9 weeks of hormonal treatment from the middle of testes and preserved (n = 8 per diet) in a 4% solution of formalin buffered by NaH_2PO_4-H_2O and Na_2HPO_4-$2H_2O$. Subsequently, the tissue samples were dehydrated, embedded in paraffin, and sectioned at 5 µm. The sections were stained with haematoxylin and eosin (H & E, VWR-Bie & Berntsen A/S, Denmark). The histological sections were photographed (Olympus BX53 digital camera) at 200× magnification for identification of gamete development stages and tissue, i.e. spermatogonia (Sg), spermatocytes (Sc), spermatids (St), and spermatozoa (Sz). Testes tissues of five micrographs per male were categorized according to gamete stages (i.e. Sg, Sc, St, and Sz) and their relative area fraction (*F*) and progression of spermatogenesis was assessed using a spermatogenic maturity index (SMI) (see Tomkiewicz et al., 2011). SMI was estimated for each of the testes images in order to compare the morphological development of the testes tissue in males receiving different amino acid diets (n = 8-9 fish per diet).

2.8. Statistical analysis

Data were analysed using SAS statistical analysis software (v.9.1; SAS Institute Inc., Cary, NC, USA) and R programming language (Venables and Ripley, 2002). Residuals were tested for normality (Shapiro-Wilk test) and homogenous of variances (Levene's test). The significance level was set at $\alpha = 0.05$. Treatment means were determined using the honest significant difference Tukey's test. Prior to or at the end of the experiment (Week 9), length, weight, liver weight, testes

277 weight, GSI, and HSI were compared across the diets using a series of one-way ANOVAs. During
278 injections, eel body weights were compared using a repeated measures ANOVA model that
279 contained the factors: Time, Diet, and Time × Diet. One-way ANOVAs were used to compare
280 amino acids between the dietary groups. For statistical comparisons the amino acid composition
281 was compared as a percentage.

282 Logistic regression analysis was applied to estimate the parameters of a logistic model
283 consisting of a dependent variable (*milt production*) with two possible values, "unsuited"
284 (representing categories 0-2) or "suited" (categories 3-5) for further fertilization procedures (see
285 Section 2.4). In the logistic model, the log-odds for the value labeled "suited" is a linear
286 combination of two independent variables; a binary variable (*diet*) and a continuous variable
287 (*time*). The corresponding probability varied between 0 ("unsuited") and 1 ("suited"). At the end
288 of the experiment, the total weight of milt per male was recorded and standardized across the diets
289 using one-way ANOVA model.

290 Spermatocrit was compared across the diets with a one-way ANOVA model. A one-way
291 ANOVA model was also used to compare SMI between the diets on Week 9.Total motility and
292 curvilinear velocity were compared using a repeated measures ANOVA model that contained the
293 factors: Time (10, 20 and 30 s), Diet, and Time × Diet.

294 Additionally, two principal components analyses (PCA) were performed to study the 1)
295 correlation between amino acid composition (in % of total amino acids) in the diet and in the milt
296 samples, and 2) the correlation between amino acid composition (in % of total amino acids) in the
297 milt samples and sperm motility and velocity. In both PCAs, the diets (DAN-EX, EH-4, EH-5, and
298 EH-6) were used as category variables. All parameters were weighted by 1/SD and full cross
299 validation was used. For the first PCA, the mean value for each amino acid in each diet was used

for all milt samples receiving the same diet.

3. Results

3.1. Body morphometric measures

Prior to hormonal treatment, total body weight, length, and liver weight of eels ranged from 72 to 163 g, 34 to 44 cm, and 0.72 to 1.52 g respectively. There were no significant differences detected between diets for these three morphometric traits ($P > 0.05$).

For the hormonally treated males, total body weight ranged from 104 to 167 g for males fed DAN-EX, 101 to 179 g for males fed EH-4, 100 to 150 g for males fed EH-5, and 98 to 130 g for males fed EH-6. For the repeated measures ANOVA, the Time × Diet interaction and Time effect were both non-significant ($P > 0.05$). On the contrary, dietary regime impacted total weight of the males ($P < 0.0001$; Fig. 1A), such that males fed EH-4 were the heaviest, while males fed EH-5 and EH-6 were the lightest (Fig. 1B).

At the end of the experiment (i.e. after Week 9 injection) liver weight, testes weight, GSI, and HSI were compared across the diets. Here, liver weight and testes weight ranged from 1.04 to 1.09 and 9.42 to 11.41 g, respectively, and there were no significant differences between the diets ($P > 0.05$). Additionally, GSI (ranged from 7.05 to 9.44) and HSI (ranged from 0.94 to 1.03) were not significantly influenced by dietary regime ($P > 0.05$).

323 *3.2. Amino acid analysis of milt*

324

325 The amino acids detected in eel milt are displayed in Table 4. Arginine, alanine, and lysine
326 were the most abundant amino acids in eel milt (all >11%), followed by glycine, aspartic acid,
327 valine, glutamic acid, and leucine (all > 5.66%). Dietary regime significantly impacted arginine (P
328 = 0.01), serine (P < 0.0001), proline (P = 0.02), methionine (P = 0.01), and histidine (P < 0.0001)
329 levels. Specifically, males fed DAN-EX, EH-4, and EH-5 had the highest percentages of arginine,
330 while males fed EH-4 to EH-6 had highest percentages of serine. Proline was most abundant in
331 males fed DAN-EX, EH-5, and EH-6. Both methionine and histidine were detected at low levels
332 (<2%), but were still impacted by dietary regime, where males fed EH-4 and EH-5 had the highest
333 percentages of methionine, and males fed DAN-EX, EH-4, and EH-6 had highest percentages of
334 histidine.

335

336 *3.3. Milt production and Spermatocrit*

337

338 Milt production was initially graded from 0 (no milt release) to 5 (flowing milt) and then
339 grouped as "unsuited" (categories 0-2) or "suited" (categories 3-5; Fig. 2A-F). Logistic regression
340 showed that milt production was significantly influenced by the time of hormonal treatment (p <
341 0.001) and dietary regime (p = 0.020). Generally, milt production increased over time from almost
342 no milt on Week 5 to reach highest values on Week 9, with 65% and 68% probability of milt
343 "suited" for fertilization procedures when males were fed EH-4 or EH-6 respectively, compared
344 to 18% or 28% when fed DAN-EX or EH-5, respectively (Fig A-F). Mean ± SEM spermatocrit
345 for the males was 43.1 ± 1.80% and it did not differ between the diets (P > 0.05, ranged from

346 37.57% for males fed EH-6 to 47.21% for males fed EH-4; Fig. 2G). The one-way ANOVA model
347 showed no impact of dietary regime on total milt weight, where it ranged from 1.91 g/100 g eel
348 for males fed EH-6 to 2.32 g/100 g eel for males fed EH-4 ($P > 0.05$; Fig. 2H).
349
350 *3.4. Spermatozoa kinetic traits*
351
352 For sperm motility, the Time × Diet interaction ($P > 0.05$; Fig. 3A) and Time effect were
353 both non-significant ($P > 0.05$). Dietary regime had an impact on sperm motility ($P = 0.007$; Fig.
354 3B), such that eels fed EH-5 or EH-6 had the greatest percentage of motile cells. The Time × Diet
355 interaction ($P > 0.05$; Fig. 3C) and Time effect were also not significant for sperm velocity ($P >$
356 0.05), while the dietary regime had an impact ($P = 0.003$; Fig. 3D). Here, fish fed the DAN-EX
357 diet or EH-5 and EH-6 had the fastest swimming sperm.
358
359 *3.5. Histological analyses*
360
361 The SMI of hormonally treated eels ranged from 0.54 to 0.80 after 9 weeks of hormonal
362 injections and did not differ between dietary treatment groups ($P > 0.05$). All hormonally treated
363 males responded to treatment with testes showing progressed development, including
364 spermatocytes (Sc), spermatids (St) and spermatozoa (Sz) (Fig. 4). Sc and St dominated the least
365 developed males, but also tubules with attached developing Sz were observed (Fig. 4). In contrast,
366 free Sz in enlarged tubules dominated the most developed males, but still with prevalent Sc and
367 St. The continuous presence of Sg and Sc showed ongoing spermatogenesis in all males
368 independently of the diet they received (Fig. 4).

369

370 *3.6. Principal components analysis of amino acid composition in diet and milt samples*

371

372 PCA was performed to study the correlation between the amino acid composition (% of total
373 amino acids) in the diet and milt, where diets were used as category variables. From this PCA, it
374 was evident that three samples (two receiving EH-6 and one receiving EH-4) behaved as outliers.
375 A new PCA without these samples was therefore performed (Fig. 5A and B). PC1 and PC2
376 explained 43 % and 15 % of the variation in the data, respectively (Supplementary Table 1). Both
377 the scores and the loadings plot showed that PC1 explained the difference between the DAN-EX
378 diet/milt and the other samples, whereas PC2 mainly explained differences between EH-5 and EH-
379 6 diet/milt. When taking these findings into consideration, the most interesting amino acids to
380 scrutinize further in the second PCA were proline, histidine and valine as well as lysine and
381 arginine (Fig. 5A and 5B). In Fig. 5B, proline, histidine and valine in the milt samples were located
382 close to EH-6 (in the first quadrant). These three amino acids in the diets (D-PRO, D-HIS, and D-
383 VAL) were all located to the far right indicating that the DAN-EX diet had a low content of these
384 three amino acids and that there was a positive correlation between the content of these amino
385 acids in the diet and in the milt samples. The same was also the case for glutamic acid and D-GLU,
386 which were both located to the left. Interestingly, lysine and arginine in the milt were located in
387 the 3rd quadrant, whereas the variables for these two amino acids in the diet (D-LYS and D-ARG)
388 were located directly opposite in the 1st quadrant, suggesting a negative correlation between the
389 presence of these amino acids in the diet and in the milt samples. The same was also the case for
390 other amino acids such as hydroxyproline (HYP to the left) and D-HYP (to the right) and for
391 phenylalanine (PHE to the right) and D-PHE (to the left).

392 *3.7. Principal components analysis of sperm motility and amino acid composition in milt*

393

394 In order to study the correlation between sperm motility and amino acid composition (in %
395 of total amino acids) a PCA including all samples and using diets as category variables was
396 performed. All samples excluding the three outliers mentioned above were used for the analysis
397 (Fig. 6A and B). PC1 and PC2 together explained 43% of the variation in the data (Supplementary
398 Table 2). The scores plot (Fig. 6A) showed that PC1 mainly explained differences between DAN-
399 EX samples to the left and the samples obtained from eel receiving the other diets (EH-4 to EH-
400 6), which were mainly located to the right. PC2 mainly explained differences between eels
401 receiving diet EH-4 in the top of the plot and EH-6 in the bottom of the plot. This interpretation of
402 the scores plot was also confirmed by the location of the diet category variables in the correlation
403 loadings plot (Fig. 6B). The correlation loadings plot showed a clear positive correlation between
404 motility parameters in the lower right corner and eel receiving diet EH-6. However, this
405 interpretation of the model was only partly confirmed by the original data in Figure 3, which did
406 not show a significant difference between EH-5 and EH-6. Further inspection of the scores plot in
407 Fig. 6A showed some overlap between the locations of the samples receiving different diets.
408 Particularly EH-4 and EH-5 samples were scattered in the plot. It was, however, clear that all
409 DAN-EX samples were located to the left in the scores plot and all EH-6 samples were located in
410 the lower part of the plot and mainly to the right. These findings thus suggest that eels fed EH-6
411 to a higher degree than eels fed DAN-EX had high motility sperm. Eels with high motility sperm
412 had milt with high relative proportions of proline, histidine and valine, but were particularly low
413 in lysine and arginine. They were also to some extent high in the amino acids located in the lower
414 part of the 1st quadrant (threonine, isoleucine, serine, glycine).

4. Discussion

In teleosts, studies have focused on amino acid requirements for growth and metabolism (Li et al., 2009), however, their physiological significance in relation to reproduction and/or gamete performance has not been well elucidated. Proteins and amino acids are the most abundant constituents in fish gametes [e.g. free amino acids (FAAs) constitute up to 50% of the total amino acid pool in marine pelagic fish eggs]. Here, they serve as an energy source during embryonic development (Rønnestad et al., 1992; Syama Dayal et al., 2003), are important osmotic effectors during oocyte hydration (Cerdà et al., 2007), and can even impact fertilization success (Kwasek et al., 2014a). Moreover, dietary protein/amino acids modulate the time of puberty and rate of maturation indirectly by impacting growth (Gunasekera et al., 1995). Therefore, dietary supplementation of amino acids can provide quantitative evidence on whether the reproductive performance or gamete quality in fish can be modified, particularly under captive conditions.

Amino acids or short peptides are produced as hydrolysates of intercellular matrix proteins, to act as signals for maturation and timing of spermiation (Kawabata et al., 1992). In the present study, diet did not influence organ-somatic indices (e.g. HSI, GSI) and testes histology, while it impacted total weight of the males, where males fed EH-4 were the heaviest in comparison to other groups. Moreover, induction of spermiation was significantly impacted by diet and time of hormonal treatment, such that eels fed EH-4 and EH-6, on each week, showed the highest probability of producing "suited" milt for further fertilization procedures, reaching values of 65% and 68% on week 9, respectively. This observation is in line with several other studies in which certain amino acids positively impacted spermiation and male or female reproductive success. For example, in ayu, *Plecoglossus altivelis*, additional tryptophan in the broodstock diet advanced the

peak of serum testosterone levels and spermiation time in males and final maturation in females (Akiyama et al., 1996). In the male rose bitterling *Rhodeus ocellatus*, Kawabata et al. (1992) indicated that spermiation and sexual behavior was induced by several amino acids, such as cysteine, serine, alanine, glycine, and lysine. In addition, supplementation of a diet with higher levels of lysine significantly increased milt volume in silver catfish, *Rhamdia voulezi* (Diemer et al., 2014), indicating relationships between levels of certain amino acids in the diet and spermiation. Therefore, in the present study, it is quite likely that well-balanced amino acids or specific amino acids in diets EH-4 and EH-6 were more favorable for European eel spermatogenesis in comparison to the diets EH-5 and DAN-EX. Moreover, certain amino acids (e.g. tyrosine, phenylalanine, glutamine, and leucine) are precursors for the synthesis and secretion of hormones such as thyroid hormones, insulin hormones, growth hormones, prolactin, and progesterone (Wu, 2009). Presumably, in the present study, elevated levels of these hormones in males fed EH-4 and EH-6 may have partly mediated induction of spermatogenesis, which warrants further investigation also in relation to transmission of effects to offspring as these hormones play a key role during early life development (Politis et al., 2017, 2018b, 2018c).

Sperm motility and velocity are regarded as primary determinants of reproductive success and are commonly used to assess male gamete quality and fertilization potential (Gage et al., 2004; Rurangwa et al., 2004; Gallego and Asturiano, 2018a,b; Zadmajid et al., 2019). Typically, sperm with higher velocity and motility have the advantage of reaching the micropyle within a shorter window of time during a fertilization event, while correlations have been found between sperm motility parameters and fertilization rates in several fish species (reviewed by Gallego and Asturiano, 2018a). Sperm motility parameters have been positively impacted by broodstock nutrition for various fish species, such as barbel, *Barbus barbus* (Alavi et al., 2009), Senegalese

sole, *Solea senegalensis* (Beirão et al., 2015), and European eel (Butts et al., 2015). Contrary to fish, in mammals, the role of amino acids on sperm quality/function has received great attention. For instance, incubation of goat sperm with specific amino acids (e.g. arginine), not only enhances the pH and metabolic activity of sperm, but also the synthesis of ATP, which is essential for sperm motility (Patel et al., 1998). In addition, supplementation of diets with amino acids improved sperm motility and subsequent fertilization success in mice (Bahadorani et al., 2019), while for humans, amino acid-deficient diets resulted in a ~10-fold increase in the percentage of non-motile sperm (Wu et al., 2000). This striking observation underlines a critical role of amino acids for male gamete performance.

We observed that dietary amino acids impacted sperm quality, where eels fed EH-5 or EH-6 presented an improvement in sperm motility parameters, which most likely increases chances for sperm to achieve fertilization. The underlying mechanism(s) may be related to enhanced synthesis of polyamines and amino acid-rich basic proteins in the sperm cells (Wu et al., 2009) or activation of signaling molecules such as nitric oxide, which acts as a stimulator of sperm motility/velocity (Creech et al., 1998; Barman et al., 2013). On the other hand, protein phosphorylation processes, which trigger further cell signaling processes such as cyclic adenosine monophosphate (cAMP) and hydrolysis of ATP catalyzed by dynein ATPase, are important regulatory components for sperm swimming trajectories (Dzyuba and Cosson, 2014; Zilli et al., 2008,2017). Therefore, balanced amino acids in the diets EH-5 or EH-6 may have increased intracellular ATP stores (Perchec Poupard et al., 1998), or changed the protein phosphorylation state via production of proteins, which are involved in sperm motility activation, such as motor proteins [e.g. A-kinase anchor protein (AKAP), axonemal dynein; Zilli et al., 2017], signaling proteins [e.g. protein kinase A (PKA), Caspase 3, cleavage of PARP; Silva et al., 2015], and

proteins involved in cell metabolism, including metabolism of reactive oxygen species (ROS) [e.g. Acetyl-CoA synthetase, Cu/Zn superoxide dismutase (Cu/Zn SOD); Zilli et al., 2017]. Interestingly, in our study, PCA analysis showed that eels with high motility sperm had milt with high relative proportions of proline, histidine, and valine, but were particularly low in lysine and arginine. However, when comparing to other studies, the impact of amino acids on teleosts sperm traits shows high species-specific variability. For example, *in vitro* incubation of rainbow trout, *Oncorhynchus mykiss* spermatozoa with proline, isoleucine, and methionine had a positive effect on sperm traits (e.g. motility, velocity and viability), while, proline, glutamine, cysteine, asparagine, isoleucine, phenylalanine, serine, and histidine had a negative impact on common carp, *Cyprinus carpio* sperm viability (Lahnsteiner, 2009). In both perch, *Perca fluviatilis* and gilthead sea bream, *Sparus aurata,* glycine, lysine, methionine, and serine had a positive effect on sperm motility *in vitro* (Lahnsteiner, 2010). In male yellow perch, *Perca flavescens* sperm motility and fertilization rate were significantly decreased in the lysine deficient group (Kwasek et al., 2014a,b). In addition, there is some evidence that *in vitro* supplementation of arginine with sperm cells positively impacts sperm swimming behaviors in fish (Lahnsteiner, 2010), human (Keller and Polakoski, 1975), rabbit (Radany and Atherton, 1981), and rat (Abd-Elrazek and Ahmed-Farid, 2018). The impact of amino acids on sperm motility and velocity has also been highlighted during the freeze-thawing processes by protecting sperm cells against free-radical-induced damage (Cabrita et al., 2011; Sangeeta et al., 2015). In ram semen, supplementation of freezing media with proline led to a significant improvement in sperm motility, velocity, and structural and functional integrity of biological membranes during the freezing and post-thawing process (Sangeeta et al., 2015). From the above reports, it clearly emerges that protein and amino acids are highly involved in sperm motility initiation by different mechanisms, but with specific amino acid preference

among species. Generally, it is not surprising that amino acids have species-specific impacts on teleosts sperm performance, as amino acid composition and metabolism in general differs greatly between fish.

In teleosts, it is well documented that dietary amino acid profiles are influencing post-feeding levels of amino acids in the body, tissues, liver, and muscles (Kaushik et al., 1988; Mai et al., 2006; Mozanzadeh et al., 2018). In addition, several authors have suggested that profiles of amino acids present in the plasma are directly related to dietary composition. For example, dietary supplementation of amino acids, enhanced the concentration of several amino acids in either blood plasma or seminal plasma in different fish or mammal species (Tantikitti and March, 1995; Wu et al., 2007; Dong et al., 2016). Nevertheless, relatively little information is available about the amino acid composition of male germ cells and whether their accumulation impacts sperm functionality. Available evidence shows that amino acid profiles in fish gametes are directly related to sperm quality and fertilization success (He and Woods, 2003; Kwasek et al., 2014b). In support of this notion, quantifying the free amino acid composition of sperm from several fish species revealed that amino acids play a significant role in stimulation of sperm metabolic activity and viability, and participates in various detoxifying functions (Lahnsteiner, 2009, 2010). Interestingly, in our study, even on a quantitative basis, there was high accumulation (>35% of total) of arginine, alanine, and lysine in milt, however, their incorporation at higher concentrations could not impact sperm function in eel compared to other amino acids such as proline, histidine, and valine as validated by PCA analysis. Similarly, previous studies on male European eel (Baeza et al., 2014, 2015a) reported that the specific use of every type of fatty acid depends on the tissue and phase of spermatogenesis. Overall, the physiological roles of specific fatty acids and amino acids must be clarified. In addition, the concentration of amino acids in male gonads or germ cells varies among

and even within species, which makes it difficult to discern a particular pattern in amino acid profiles. For example, methionine, arginine, and cysteine were found to be the main amino acids in milt of rainbow trout (Lahnsteiner, 2009); leucine, arginine, glutamic acid, histidine, and lysine in common carp, *Cyprinus carpio* (Lahnsteiner, 2009); arginine, alanine, isoleucine, tyrosine, asparagine, methionine, tryptophan, glutamic acid, and lysine in perch (Lahnsteiner, 2010); and leucine, arginine, methionine, glycine, hydroxyproline, cysteine, isoleucine, serine, glutamic acid, lysine, phenylalanine, and asparagine in gilthead sea bream (Lahnsteiner, 2010). This variation is largely due to differences in dietary protein sources (Forster and Ogata, 1998), diet formulation, size, age of species, genetic differences, rearing conditions, and feeding practices (Ruchimat et al., 1997). Furthermore, a considerable amount of amino acids are produced from the spermatic duct epithelium (Lahnsteiner et al., 1993, 1994) or by proteolysis of seminal plasma (Ciereszko et al., 1998), which could both change amino acid profiles in male gametes.

Overall, these findings not only add evidence that certain amino acids are essential for regulating milt biochemistry, but also show that some ejaculate traits may be promoted by amino acid intake (e.g. proline, histidine, and valine in the present study). Thus, further studies to evaluate the effect of these supplemented diets on sperm fertilization ability and interlinked early developmental stages (i.e., egg/embryo to early juveniles) are required. Moreover, new approaches with high-throughput functional genomics, metabolomics, and proteomics may help to uncover regulatory roles of these amino acids for gene and protein function. Thus, it would be interesting to highlight these innovative methods in future attempts in order to expand our knowledge of amino acid function for fish reproduction, especially for a critically endangered and economically important catadromous fish species such as European eel.

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Figure captions:

Fig. 1. Total weight of male European eel, *Anguilla anguilla* fed with four diets (DAN-EX, EH-4, EH-5, EH-6). Box-and-whisker plot represent the diets for total weight of eels from Week 1 to Week 9 (A) and the bar chart represents recorded total weight of fish between the diets (B). Values with different subscripts differ ($P < 0.05$). Results are expressed as mean values ± SEM.

Fig. 2. European eel males, *Anguilla anguilla* fed four diets (DAN-EX, EH-4, EH-5, EH-6) were assessed for milt production from injection Week 5 until Week 9. Milt production was initially graded from 0 (no milt release) to 5 (flowing milt) and then grouped as "unsuited" (categories 0-2) or "suited" (categories 3-5; A-E). Regression analysis was then applied to estimate parameters of a logistic model, where corresponding probabilities varied between 0 for "unsuited" and 1 for "suited" milt for fertilization procedures (F). Spermatocrit (G) and standardized milt production [g /100 g eel, (H)] for males fed the four diets was also determined. Values with different subscripts are significantly different ($P < 0.05$). Results are expressed as mean values ± SEM.

Fig. 3. Spermatozoa kinetic traits (A-D) of male European eel, *Anguilla anguilla* fed four diets (DAN-EX, EH-4, EH-5, EH-6). For sperm motility and velocity, the Time × Diet interaction was non-significant (A, C), therefore the Diet main effect was interpreted for each trait (B, D). Values with different subscripts are significantly different ($P < 0.05$). Results are expressed as mean values ± SEM.

Fig. 4. Photomicrographs of histological sections of testes from selected male European eel, *Anguilla anguilla*, in different developmental stages. Testis tissues were categorized according to

841 prevalence of different cell types using the spermatogenic maturity index (SMI). Examples include
842 (A) SMI = 0.54, *male ID 39CD*; (B) SMI = 0.56, *male ID 2CBC*; (C) SMI = 0.71, *male ID 7DE6*;
843 (D) SMI = 0.80, *male ID DCBA*. Symbols indicate germ cells: Sg = spermatogonia; Sc =
844 spermatocytes; St = spermatids; Sz = spermatozoa; as well as Ad = adipocytes.
845
846 **Fig. 5.** Score plot (A) and correlation loadings (B) from principal component analysis to study the
847 correlation between amino acid composition (in % of total amino acids) in the diet and milt of
848 European eel, *Anguilla anguilla*. The diets (DAN-EX, EH-4, EH-5 and EH-6) were used as
849 category variables and the locations of the category variables are shown in the loadings plot. All
850 parameters were weighted by 1/SD and full cross validation was used. In the top panel (A), the
851 blue squares correspond to milt samples from fish receiving the DAN-EX diet, red circles to milt
852 from fish receiving the EH-4 diet, green triangles to milt from fish receiving the EH-5 diet, pink
853 diamonds to milt from fish receiving the EH-6 diet. The mean value for each amino acid in each
854 diet was used for all milt samples receiving the same diet. These amino acids are marked with "D"
855 before the amino acid to show that these are the values from the diets.
856
857 **Fig. 6.** Score plot (A) and correlation loadings (B) from principal component analysis to study the
858 correlation between amino acid composition (in % of total amino acids) in milt samples, sperm
859 motility [MOT(10), MOT(20), and MOT(30)], and velocity [VLC(10) and VCL(20)] of European
860 eel, *Anguilla anguilla*. In the top panel (A), the blue squares correspond to milt samples from fish
861 receiving the DAN-EX diet, red circles to milt from fish receiving the EH-4 diet, green triangles
862 to milt from fish receiving the EH-5 diet, pink diamonds to milt from fish receiving the EH-6 diet.
863 The diets (DAN-EX, EH-4, EH-5 and EH-6) were used as category variables and the locations of

the category variables are shown in the loadings plot. All parameters were weighted by 1/SD and full cross validation was used.

Table captions:

Table 1. Dietary formulation for the "enhanced" feeds (EH-4, EH-5, EH-6) that were fed to male European eel, *Anguilla anguilla.*

Table 2. Composition of fatty acids in the diets (EH-4, EH-5, EH-6, n = 2 samples analyzed per diet) that were fed to male European eel, *Anguilla anguilla*. The commercial feed was DAN-EX 2848 (n = 2 samples analyzed). Diets are presented as average (% of total fatty acids in the feed) ± standard deviation (SD).

Table 3. Composition of amino acids in the diets (EH-4, EH-5, EH-6; n = 3 samples analyzed per diet) that were fed to male European eel, *Anguilla anguilla*. The commercial feed was DAN-EX 2848 (n = 2 samples analyzed). Diets are presented as average (% of total amino acids in the feed) ± standard deviation (SD).

Table 4. Composition of amino acids (percentage of total amino acids) in milt of male European eel, *Anguilla anguilla* fed different diets (EH-4, EH-5, EH-6, DAN-EX). Commercial feed used was DAN-EX 2848. Results are presented as average (% of total amino acids in the feed) ± standard deviation (SEM). Small letters show significant differences ($P < 0.05$) in each amino acid over the dietary regimes.

Fig. 1

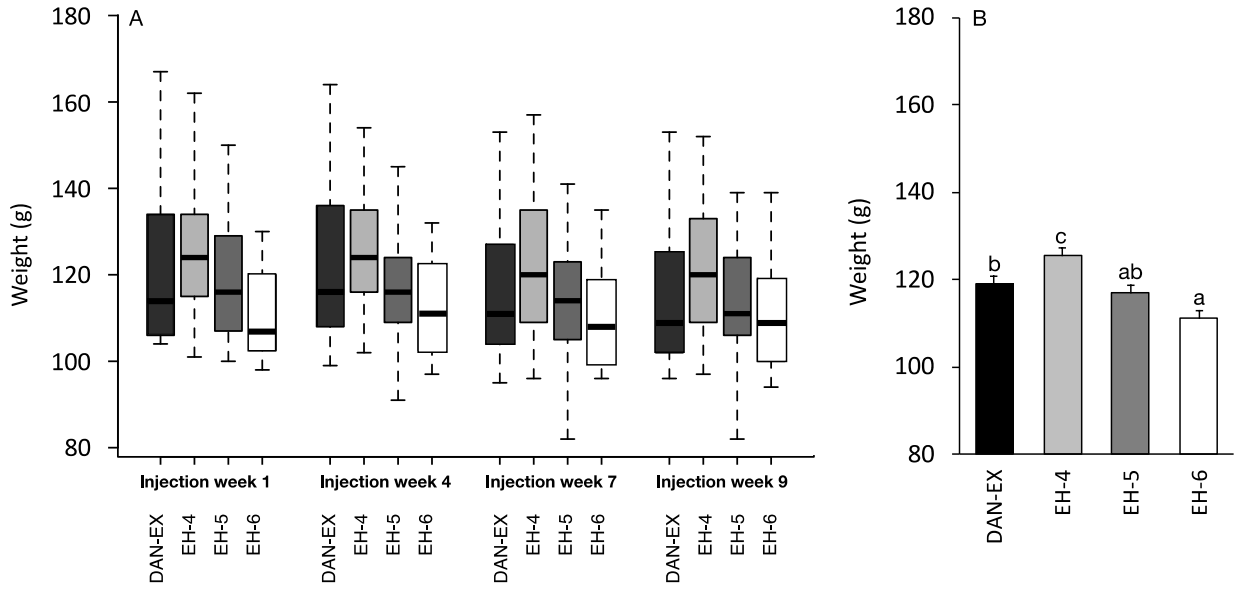

A
B
Weight (g)
180
160
140
120
100
80
Injection week 1
Injection week 4
Injection week 7
Injection week 9
DAN-EX
EH-4
EH-5
EH-6
b
c
ab
a

Fig. 2

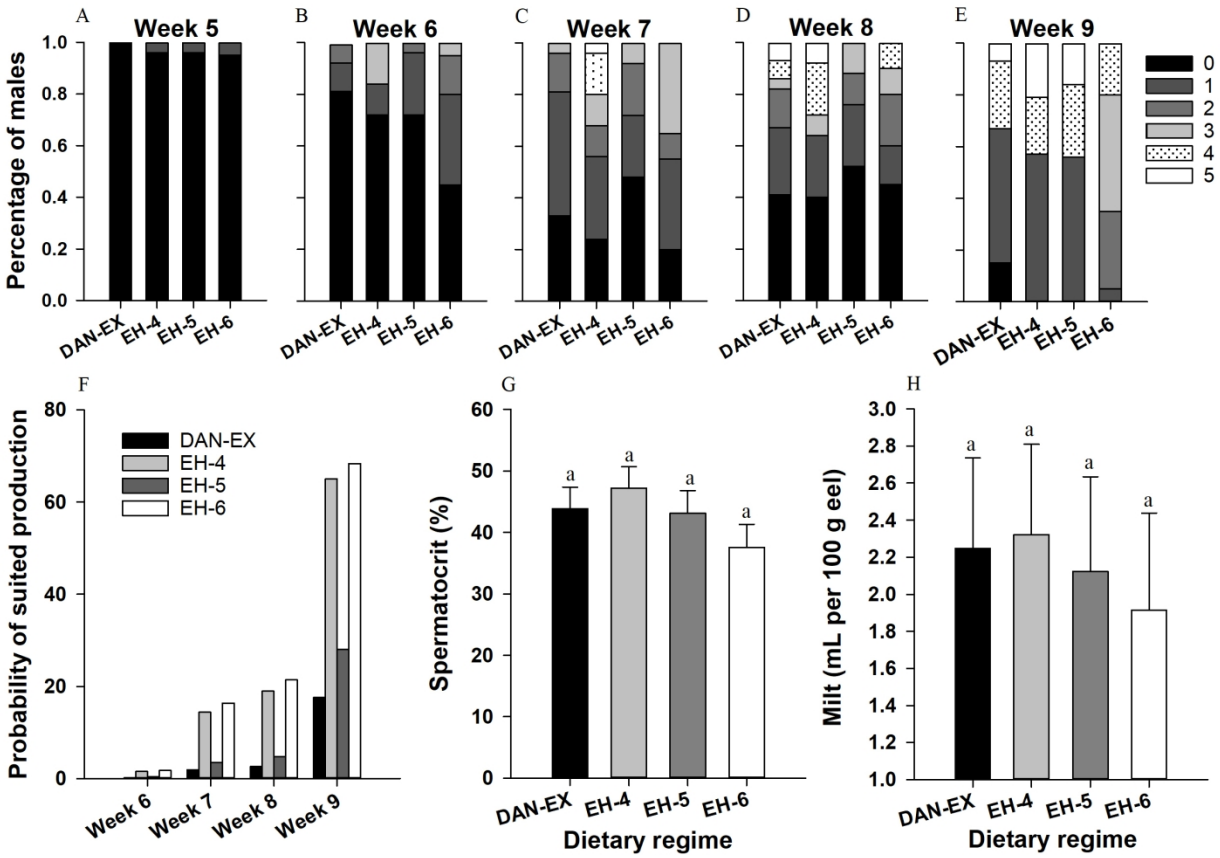
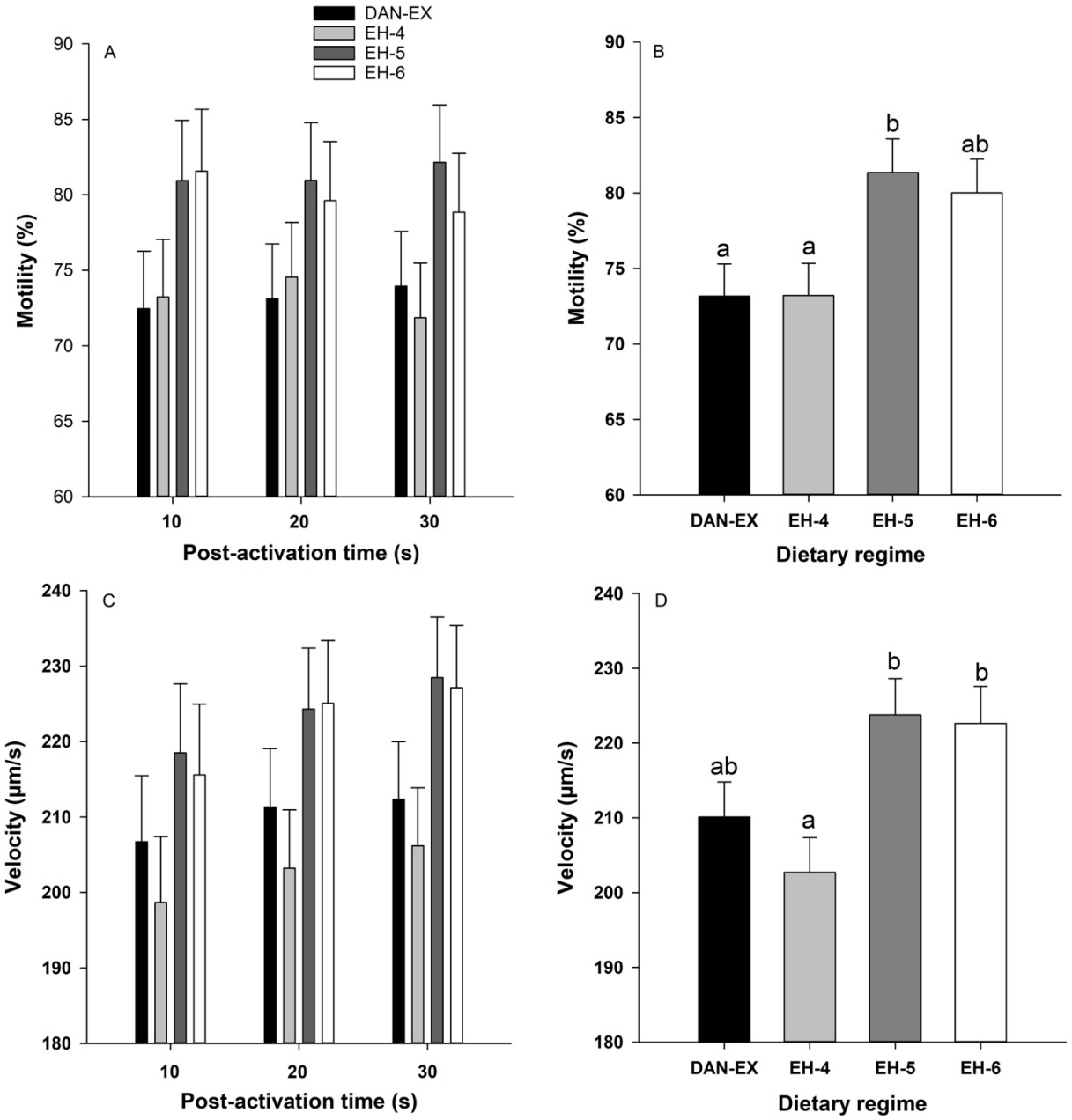

A
Week 5
B
Week 6
C
Week 7
D
Week 8
E
Week 9
Percentage of males
1.0
0.8
0.6
0.4
0.2
0.0
DAN-EX
EH-4
EH-5
EH-6
0
1
2
3
4
5
F
Probability of suited production
80
60
40
20
0
DAN-EX
EH-4
EH-5
EH-6
Week 6
Week 7
Week 8
Week 9
G
Spermatocrit (%)
60
50
40
30
20
10
0
a
a
a
a
DAN-EX
EH-4
EH-5
EH-6
Dietary regime
H
Milt (mL per 100 g eel)
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
a
a
a
a
DAN-EX
EH-4
EH-5
EH-6
Dietary regime

Fig. 3


A
DAN-EX
EH-4
EH-5
EH-6
Motility (%)
Post-activation time (s)
B
Motility (%)
a
a
b
ab
DAN-EX
EH-4
EH-5
EH-6
Dietary regime
C
Velocity (µm/s)
Post-activation time (s)
D
Velocity (µm/s)
ab
a
b
b
DAN-EX
EH-4
EH-5
EH-6
Dietary regime

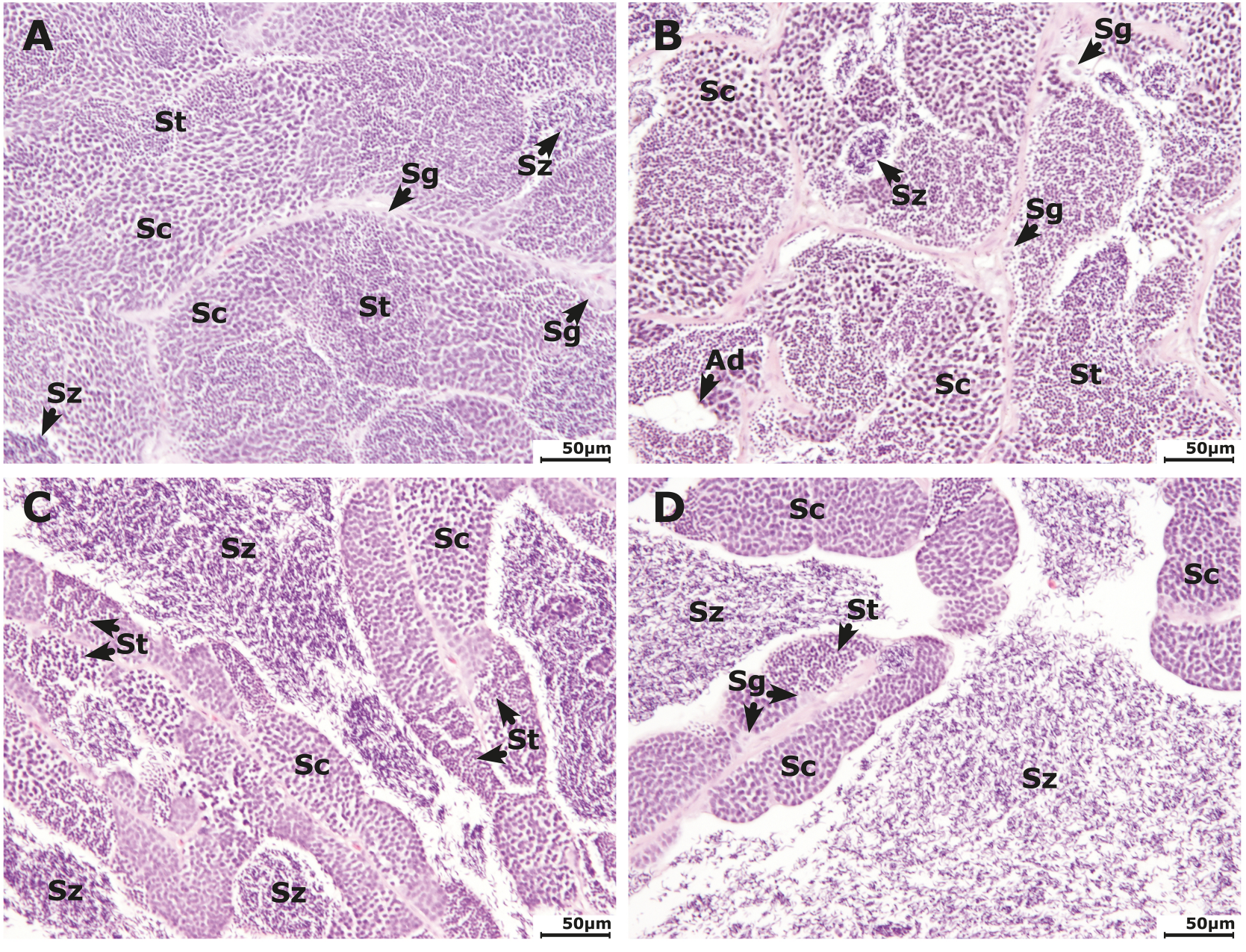

A
St
Sg
Sz
Sc
Sc
St
Sg
Sz
50μm
B
Sg
Sc
Sz
Sg
Ad
Sc
St
50μm
C
Sz
Sc
St
St
Sc
Sz
Sz
50μm
D
Sc
Sc
Sz
St
Sg
Sc
Sz
50μm

Fig. 5

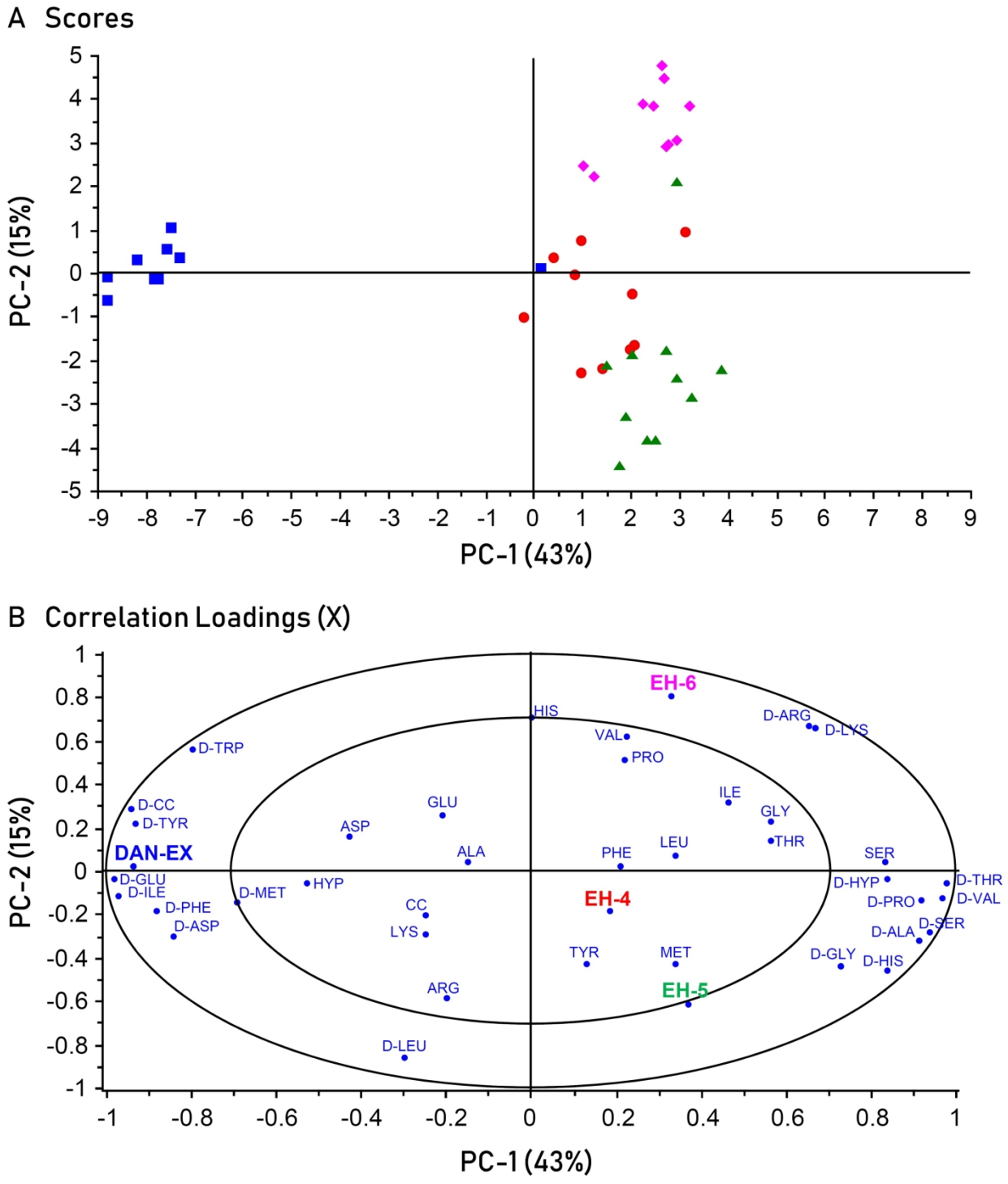

A Scores
PC-2 (15%)
PC-1 (43%)
B Correlation Loadings (X)
PC-2 (15%)
PC-1 (43%)
EH-6
EH-4
EH-5
DAN-EX
HIS
VAL
PRO
D-ARG
D-LYS
D-TRP
ILE
GLY
THR
SER
GLU
ASP
ALA
PHE
LEU
D-CC
D-TYR
D-GLU
D-ILE
D-PHE
D-ASP
D-MET
HYP
CC
LYS
ARG
D-LEU
TYR
MET
D-HYP
D-PRO
D-ALA
D-GLY
D-HIS
D-SER
D-THR
D-VAL

Fig. 6

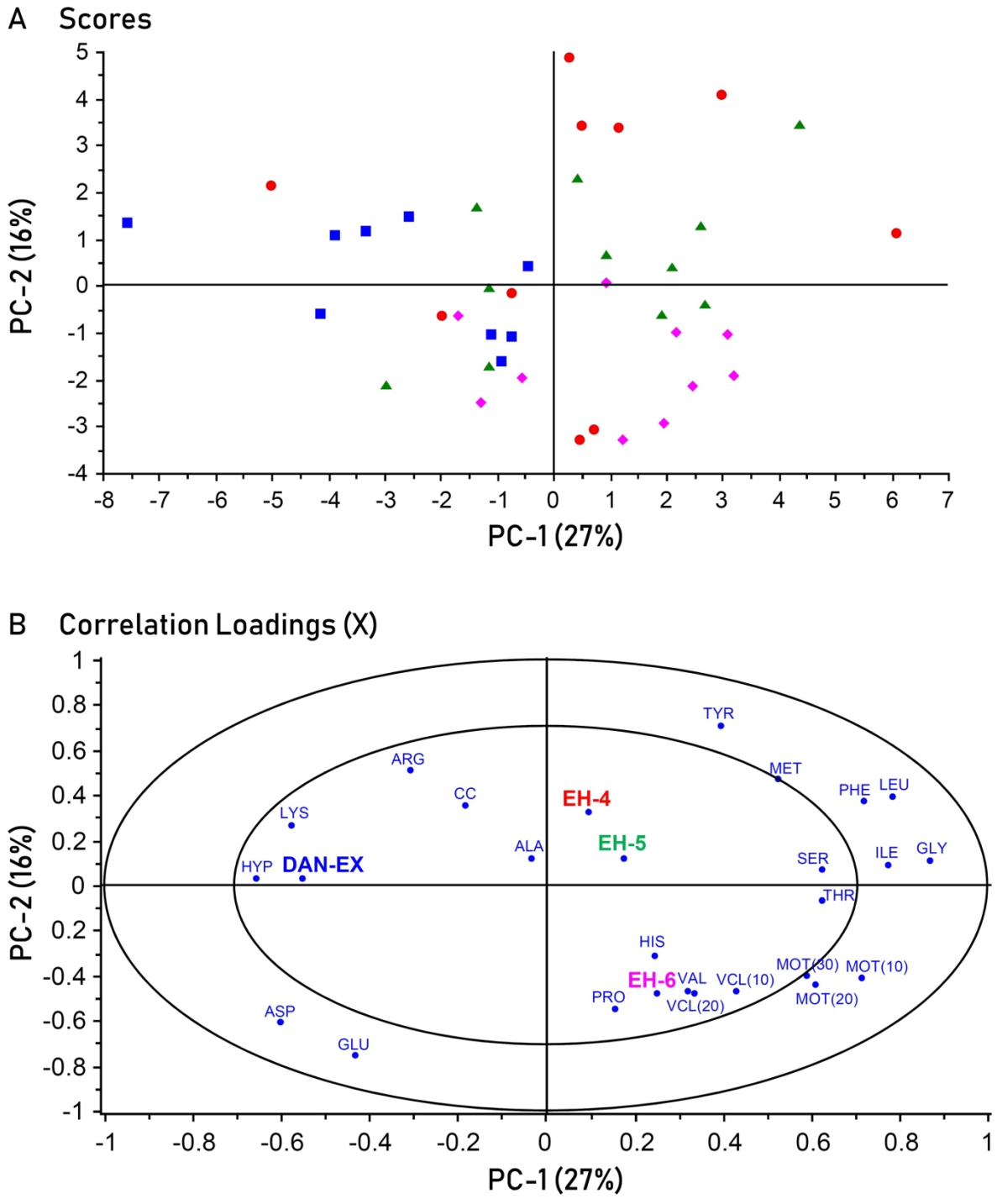

A Scores
PC-2 (16%)
PC-1 (27%)
B Correlation Loadings (X)
PC-2 (16%)
PC-1 (27%)
TYR
MET
PHE
LEU
ARG
CC
EH-4
LYS
ALA
EH-5
ILE
GLY
HYP
DAN-EX
SER
THR
HIS
MOT(30)
MOT(10)
EH-6
VAL
VCL(10)
PRO
VCL(20)
MOT(20)
ASP
GLU

Table 1

Dietary formulation	**EH-4**	**EH-5**	**EH-6**
Fish Meal LT	53.275	55.136	53.727
Wheat Gluten	9	9	9
Wheat	22	20	20
Fish Oil	6.03	5.79	5.95
Rapeseed Oil	5	5	5
Vevodar	2.35	2.39	2.37
Premix	2.03	1.82	1.93
L-Lysine HCI (78%)	.	0.17	0.28
DL-Methionine (99%)	0.22	0.19	0.22
L-Threonine (98%)	.	0.19	0.23
L-Histidine (74%)	.	.	0.03
Water change	-3.423	-3.204	-3.083
Lecithin, liquid	0.5	0.5	0.5
DHA Liqiud	3	3	3
L-argenin	.	.	0.828
Total	*100*	*100*	*100*
Moisture (%)	5.47	5.528	5.529
Protein - crude (%)	48.29	49.783	49.802
Fat - crude (%)	23.032	22.979	22.976
Ash (%)	8.061	8.065	7.992
Cellulose - crude (%)	0.579	0.535	0.535
Crude fiber (%)	0.579	0.535	0.535

Table 2.

Fatty	DAN-EX (n=2)		EH-4 (n=2)		EH-5 (n=2)		EH-6 (n=2)	
Acids	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**MEAN**	**SD**
C14:00	5.19	0.01	3.44	0.04	3.39	0.02	3.48	0.01
C16:00	10.79	0.02	11.77	0.04	11.72	0.03	11.85	0.04
C16:1(n – 7)	7.35	0.01	4.31	0.02	4.16	0.03	4.22	0.02
C18:00	1.32	0.03	3.04	0.03	3.22	0.00	3.15	0.06
C18:1(n – 9)	16.07	0.02	19.28	0.19	19.02	0.12	19.02	0.03
C18:1(n – 7)	2.71	0.01	2.00	0.02	2.14	0.00	2.13	0.04
C18:2(n – 6)	6.02	0.04	12.90	0.13	12.91	0.05	13.00	0.04
C18:3(n – 3)	1.62	0.01	1.05	0.02	1.11	0.05	1.06	0.00
C20:1(n – 7)	0.74	0.00	0.37	0.00	0.38	0.01	0.38	0.00
C20:4(n – 6)	0.28	0.00	4.33	0.04	4.41	0.04	4.39	0.02
C20:5(n – 3)	5.49	0.02	3.90	0.05	3.96	0.02	4.01±	0.02
C22:1(n – 11)	13.36	0.03	8.29	0.08	8.13	0.04	8.30	0.05
C22:5(n – 3)	0.49	0.00	0.56	0.00	0.56	0.00	0.57	0.00
C22:6(n – 3)	5.32	0.04	6.61	0.09	6.79	0.02	6.91	0.02
total n – 3	14.07	0.12	13.01	0.10	13.33	0.12	13.59	0.22
total n – 6	6.74	0.05	17.96	0.17	18.12	0.17	18.13	0.07
n – 3/n – 6	2.09		0.72		0.74		0.75	
total SFA	17.98	0.07	19.18	0.23	19.18	0.07	19.34	0.13
total MUFA	54.22	0.16	42.64	0.39	42.41	0.33	42.66	0.20
total PUFA	23.74	0.20	34.44	0.53	35.24	0.49	35.29	0.30

Table 3.

Amino	DAN-EX (n=2)		EH-4 (n=3)		EH-5 (n=3)		EH-6 (n=3)	
Acids	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**MEAN**	**SD**
Alanine	4.47	0.01	7.01	0.66	7.21	1.04	6.37	0.70
Arginine	4.83	0.28	7.20	0.11	6.42	1.71	10.26	1.29
Aspartic acid	11.27	0.52	10.02	1.07	8.91	0.37	8.02	1.15
Cysteine	0.96	0.04	0.72	0.04	0.67	0.08	0.75	0.09
Glutamic acid	30.22	0.12	20.38	2.40	19.71	2.10	18.91	1.28
Glycine	4.79	.	5.23	0.53	6.15	0.67	5.43	0.35
Histidine	1.54	0.01	1.81	0.31	2.00	0.36	1.78	0.39
Hydroxyproline	0.64	0.04	0.93	0.03	1.00	0.06	0.98	0.10
Isoleucine	3.80	0.23	3.08	0.10	2.93	0.37	2.80	0.32
Leucine	6.97	0.04	6.92	0.79	7.03	0.73	6.57	0.63
Lysine	7.13	0.18	8.08	0.59	7.81	1.88	9.30	1.45
Methionine	3.56	0.21	3.03	0.43	3.35	0.25	3.20	0.40
Phenylalanine	5.22	0.17	4.17	0.72	4.53	0.56	4.23	0.62
Proline	0.34	0.03	5.06	0.07	5.27	0.48	4.79	0.17
Serine	3.64	0.10	4.47	0.21	4.77	0.26	4.45	0.24
Threonine	2.99	0.13	3.35	0.05	3.96	0.35	3.85	0.13
Tryptophan	0.32	0.01	0.27	0.02	0.25	0.05	0.29	0.07
Tyrosine	3.43	0.22	2.65	0.11	2.69	0.23	2.83	0.22
Valine	3.87	0.01	5.61	0.29	5.33	1.14	5.20	0.67

Table 4.

Amino Acids	DAN-EX		EH-4		EH-5		EH-6		P-value
	Mean	SEM	Mean	SEM	Mean	SEM	MEAN	SEM	
Alanine	11.54	1.20	13.30	1.09	10.65	1.09	13.02	1.04	0.28
Arginine	12.83[b]	0.75	11.51[ab]	0.68	13.00[b]	0.68	10.12[a]	0.65	0.01
Aspartic acid	6.91	0.45	5.76	0.40	6.09	0.40	6.59	0.39	0.23
C-C	0.81	0.06	0.79	0.06	0.77	0.06	0.85	0.05	0.75
Glutamic acid	8.29	0.63	7.42	0.57	7.67	0.57	8.87	0.55	0.28
Glycine	5.66	0.21	6.13	0.19	6.21	0.19	6.17	0.18	0.2
Histidine	1.67[b]	0.12	1.92[b]	0.11	1.26[a]	0.11	1.91[b]	0.10	<0.0001
Hydroxyproline	0.09	0.02	0.04	0.02	0.01	0.02	0.01	0.02	0.05
Isoleucine	3.83	0.19	4.03	0.18	4.18	0.18	4.10	0.17	0.6
Leucine	7.06	0.29	7.49	0.26	7.29	0.26	7.04	0.25	0.6
Lysine	13.80	0.67	12.89	0.60	13.33	0.60	12.42	0.58	0.44
Methionine	1.31[a]	0.10	1.57[ab]	0.09	1.70[b]	0.09	1.36[a]	0.08	0.01
Phenylalanine	3.12	0.15	3.37	0.13	3.18	0.13	3.27	0.13	0.6
Proline	3.87[ab]	0.11	3.79[a]	0.10	3.96[ab]	0.10	4.21[b]	0.09	0.02
Serine	3.28[a]	0.11	3.95[b]	0.10	4.16[b]	0.10	4.05[b]	0.09	<.0001
Threonine	4.87	0.15	4.95	0.13	5.27	0.13	4.99	0.13	0.18
Tyrosine	4.47	0.21	4.70	0.19	4.77	0.19	4.35	0.18	0.35
Valine	6.59	0.27	6.37	0.25	6.51	0.25	6.68	0.24	0.84

Supplementary Table 1. PCA was performed to study the correlation between amino acid composition (% of total amino acids) in the diet and milt of European eel, *Anguilla anguilla*, where diets (DAN-EX, EH-4, EH-5, EH-6) were used as category variables. The cumulative proportion of variance explained and factor loadings for two principle components are displayed.

	PC1		**PC2**	
Cumulative proportion	0.430		0.580	
DAN-EX	-0.225		0.013	
EH-4	0.045		-0.074	
EH-5	0.090		-0.251	
EH-6	0.080		0.320	
	Milt	*Diet*	*Milt*	*Diet*
Alanine	-0.035	0.219	0.017	-0.127
Arginine	-0.048	0.157	-0.236	0.273
Aspartic acid	-0.103	-0.201	0.063	-0.126
C-C	-0.059	-0.225	-0.082	0.111
Glutamic acid	-0.050	-0.235	0.105	-0.019
Glycine	0.137	0.175	0.088	-0.175
Histidine	0.001	0.201	0.283	-0.182
Hydroxyproline	-0.127	0.235	-0.023	-0.020
Isoleucine	0.112	-0.232	0.124	-0.051
Leucine	0.083	-0.071	0.027	-0.352
Lysine	-0.059	0.160	-0.116	0.268
Methionine	0.082	-0.165	-0.176	-0.064
Phenylalanine	0.052	-0.210	0.008	-0.078
Proline	0.054	0.232	0.205	-0.046
Serine	0.201	0.225	0.013	-0.110
Threonine	0.137	0.202	0.054	-0.011
Tyrosine	0.032	-0.222	-0.173	0.085
Valine	0.054	0.220	0.249	-0.053

Supplementary Table 2. PCA was performed to study the correlation between sperm motility/velocity and amino acid composition (% of total amino acids) in the milt of European eel, *Anguilla anguilla*, where diets (DAN-EX, EH-4, EH-5, EH-6) were used as category variables. The cumulative proportion of variance explained and factor loadings for two principle components are displayed.

	PC1	PC2
Cumulative proportion	0.27	0.43
DAN-EX	-0.205	0.014
EH-4	0.036	0.156
EH-5	0.066	0.058
EH-6	0.093	-0.230
Alanine	-0.012	0.058
Arginine	-0.114	0.244
Aspartic acid	-0.223	-0.291
C-C	-0.067	0.169
Glutamic acid	-0.159	-0.360
Glycine	0.323	0.054
Histidine	0.091	-0.148
Hydroxyproline	-0.243	0.015
Isoleucine	0.287	0.044
Leucine	0.291	0.186
Lysine	-0.213	0.127
Methionine	0.196	0.224
Phenylalanine	0.267	0.176
Proline	0.059	-0.260
Serine	0.232	0.030
Threonine	0.232	-0.034
Tyrosine	0.148	0.334
Valine	0.118	-0.225
Motility 10 s (MOT10)	0.266	-0.194
Curvilinear velocity 10 s (VCL10)	0.125	-0.230
Motility 20 s (MOT20)	0.226	-0.208
Curvilinear velocity 20 s (VCL10)	0.160	-0.224
Motility 30 s (MOT30)	0.218	-0.193