Fish feed as source of potentially allergenic peptides from the fish parasite *Anisakis simplex* (s.l.)

Running head: Carry-over of allergenic peptides from feed to fish

Fish feed as source of potentially allergenic peptides from the fish parasite *Anisakis simplex* (sl)

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Abbreviations: ELISA: enzyme-linked immunosorbent assay; LCMSMS: liquid chromatography tandem mass spectrometry; m/z: mass-to-charge ratio; LLA: lower limit of application; LOD: limit of detection; PCR: polymerase chain reaction; RFLP: restriction fragment length polymorphism

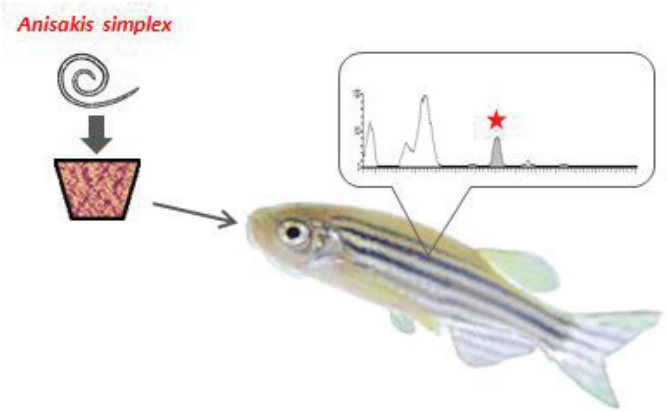
Abstract

The carry-over of certain feed components into animal products can be of concern for human health. The safety assessment of chemical contaminants including natural toxins, agrochemicals, veterinary drugs, and environmental pollutants is a key element of the "farm-to-fork" ("One Health") approach. The transmissibility of proteinaceous feed constituents such as enzymes, proteins from genetically engineered crops, and infectious prions in animal meal has also become of interest but the transfer of proteins with allergic potential is little studied. In the present study, an exploratory zebrafish feeding trial using feed containing 20 % of processed larvae of the marine fish parasite *Anisakis simplex* was performed as a proof-of-principle experiment. After a two-week exposure period, anisakid peptides were detected in zebrafish tissue by high-resolution liquid-chromatography Orbitrap mass spectrometry and immunostaining using specific polyclonal antibodies or sera from patients with confirmed allergy to *A. simplex*. Since fishmeal produced from marine pelagic fish is an important feed component in the culture of Atlantic salmon and in the poultry industry, it should be considered as a source of potentially allergenic peptides in the final products. Furthermore, the substitution of fishmeal with plant proteins would not eliminate the potential health risk by allergen carry-over since crops of high nutritional value such as legumes also contain important food allergens. If our preliminary results from the present zebrafish feeding trial should be confirmed in necessary follow-up experiments, the question of labeling information on fish and animal food products raised on feed containing potentially allergenic ingredients could arise in order to minimize the exposure risk of allergic consumers.

Keywords

Anisakis simplex; feeding trial; zebrafish (*Danio rerio*); peptide transmissibility; allergenic peptides

Graphical abstract



1. Introduction

The larvae of the marine fish parasite *Anisakis simplex*, commonly occurring in popular food fish such as mackerel, herring, wild salmon, and cod, may adversely affect consumer health through direct infection (anisakiasis) and/or by eliciting allergic reactions including urticaria, angioderma, anaphylaxis, and asthma (Deardorff et al. 1991; Pravettoni et al., 2012). Anisakiasis always assumes consumption of raw or undercooked, previously unfrozen seafood (Sakanari and McKerrow, 1989; Daschner et al., 2002; Abe and Teramoto, 2014). However, allergic reactions to *A. simplex* proteins can also be elicited in sensitized persons by the accidental consumption of dead larvae or molecular traces thereof in strongly processed fishery products and fish containing anisakid proteins (Audicana et al., 1995; Daschner et al., 2000; Audicana et al., 2002; Daschner et al., 2002; Nieuwenhuizen et al., 2006). Additionally, cases of *A. simplex* allergy due to occupational exposure by fish-based feed have been reported (Mazzucco et al., 2012).

Several food allergens have been found to be heat-stable and relatively trypsin/pepsin tolerant. At least one of the major allergens of *A. simplex* appears to be highly resistant to freezing, heating and digestion (Caballero and Moneo, 2004; Moneo et al., 2005; Vidaček et al., 2009; Rodriguez-Mahillo et al., 2010; Vidaček et al., 2011). Evidentially, allergenic peptides containing intact IgE-binding epitopes resistant to gastrointestinal hydrolysis, cytosolic and systemic peptidases can also be transported by carriers across the enterocytes into the blood circulation (Webb et al., 1992; Seal and Parker, 1992). Thus, a small portion of dietary proteins can cross the epithelium barrier (Kaminogawa et al., 1999) and unfold their biological activities, e.g. the stimulation of allergen specific effector cells. It has also been reported that allergic patients have increased antigen permeability of the gut mucosa (Majamaa and Isolauri, 1996). After systemic uptake allergenic peptides can even cross the mammalian placenta or be transported into breast milk (Frank et al., 1999; Vadas et al., 2001).

The transmissibility of peptides and small extremely resistant proteinaceous infectious particles (prions) from feed or food to various tissues of the final host organisms in a still bioactive stage can sometimes have devastating effects, e.g. in bovine spongiform encephalopathy (BSE) (Colchester and Colchester, 2005). Several animal models have been established to assess prion transmissibility and convertibility and zebrafish are frequently used as a model for prion pathobiology (Málaga-Trillo et al., 2011). There is also evidence that allergenic peptides can carry-over from animal feed into food products causing symptoms in sensitized consumers (Armentia et al., 2006). Comparably, fragments of plant DNA have been detected in pig and poultry organs and meat (Klotz et al., 2002; Chesson and Flachowsky, 2003).

Increasing attention has been paid to feed quality in food production. The safety assessment of feed components is a key element of the "farm-to-fork" ("One Health") approach (Mantovani et al., 2009). Commonly, this evaluation considers chemical residues in feed including natural toxins, agrochemicals, veterinary drugs, and environmental contaminants. However, the experiences with the BSE epidemic, the addition of enzymes to animal feed (Pariza and Cook, 2010), and the introduction of genetically engineered crops into feed and food (Goodman et al., 2005) have led to the inclusion of peptides into the list of transmissible compounds of possible health concern.

In this context, *A. simplex* is an interesting source for the study of peptides with carry-over potential. The detection of *A. simplex* peptides in the sera of chickens that had been fed with fishmeal-containing feed indicates considerable peptide transmissibility (Armentia et al., 2006). Furthermore, eight patients with high sensitization to *A. simplex* experienced allergic symptoms after having consumed raw meat from those chickens suggesting that allergenic *A. simplex* peptides had passed over from the feed and had at least partly retained their biological activity.

In a recent study the presence of *A. simplex* -related peptides in the belly flap musculature of freshly harvested, net pen-reared Atlantic salmon was demonstrated (Fæste et al., 2014a). Since there was no concurrent infection with *A. simplex* larvae, or any sign of previous infections, the parasite-related peptides may have reached the muscle tissue, or its vascular network, through the fish feed. Generally, farmed fish are fed processed feed only and considered to be free of parasites (EFSA, 2010). However, products of pelagic fish (fishmeal, fish oil, silage) are important components in feed for domestic animals (including farmed fish), and e.g. feeding stuffs for chicken, turkey or suckling piglets contain up to 4, 6 or 12 % fishmeal, respectively (data from the Norwegian Food Safety Authority and Norwegian feed manufacturers). Our analysis of commercial feed samples for salmon and poultry farming using a specific ELISA method for the detection of *A. simplex* (Werner et al., 2011) resulted in maximum contents of 40 and 60 mg/kg, respectively (unpublished data).

Based on these findings we have therefore conducted a pilot feeding trial using laboratory-raised zebrafish (*Danio rerio*) and fish feed containing processed *A. simplex* larvae, in order to investigate if or to what extent, *A. simplex*-related peptides may be transferred from the feed into the zebrafish tissue or its percolating blood.

2. Materials and Methods

2.1. Preparation of feed for the zebrafish trial.

Four days prior to trial onset, three types of feed were prepared (Table 1), composed of basic commercial zebrafish feed (Aqua Schwarz GmbH, Göttingen, Germany) and 12% gelatin, and in addition either freeze-dried *A. simplex* larvae (F1), fish meal (F2) that had been exclusively produced from Atlantic herring (*Clupea harengus*) for research purposes (NOFIMA AS, Bergen, Norway), or without further supplements (F3).

The *A. simplex* larvae used in trial feed preparation (F1) were collected fresh from the visceral organs of Blue Whiting (*Micromesistius poutassou*) caught eight months pre-trial in northeastern Atlantic waters (N58º16'W09º36'). After removing the host-induced capsule each larva was morphologically identified to genus-level (*Anisakis* ssp.) based on *in-situ* appearance (coil-shaped), and the presence of both a caudal mucron and an esophageal ventricle without caeci. After repeated washing in physiological saltwater (0.9%), the larvae were deep-frozen (-20 ºC) in bulk before further use. Subsamples of larvae were molecularly identified to species level (*A. simplex* s.l.) by RFLP-PCR of the rDNA ITS region (ITS-1, 5.8S and ITS-2) using the nucleases Hha I and Hin fI (D'Amelio et al., 2000; Farjallah et al., 2008).

The different feed types were prepared as follows: commercial gelatin powder was weighed as designed for each group (Table 1) and dissolved 1:9 w/v in heated tap water (~ 80 ºC). Three days prior to trial onset, frozen *A. simplex* larvae of the above lot were thawed and weighed (total wet-weight) before freeze-drying and subsequent weighing (dry-weight). The different components per feed group, i.e. dried *A. simplex* larvae (F1), fish meal (F2) or basic zebrafish feed (F3), were blended and fine-grinded in a ceramic mortar, separately for each group, before transfer into 100 ml glass beakers. After adding the respective volumes of gelatin solution, each mixture was thoroughly stirred and then placed overnight in an

147 incubator at 40 ºC in order to allow evaporation of the excess water. Feed rations per
148 experimental zebrafish group/tank and trial day were weighed out prior to transfer into
149 separate 12.5 ml sealed plastic vials, which were cool-stored before use.
150 *2.2. Design of the zebrafish feeding trial.*
151 The zebrafish used in the present trial (n=90) were young adults of a F4 generation of the
152 "Tupfel long-fin" wild-type strain line (ZFIN ID: ZDB-GENO-990623-2;
153 http://zfin.org/action/genotype/genotype-detail?zdbID=ZDB-GENO-990623-2). The trial set-
154 up consisted of six coarsely transparent 3.0 L plastic tanks with continuous slow water
155 exchange (Figure 1). At trial onset (day 1), each tank was stocked with 15 randomly chosen
156 zebrafish, which during adolescence and pre-trial periods were exclusively given commercial
157 zebrafish feed (see above). For each of the three study groups (Z1-Z3) two tanks were placed
158 next to each other. The fish were fed twice a day (early noon and late afternoon) throughout
159 the trial period, corresponding to a daily feed ration of about 10 mg per fish (2.5 % of body
160 weight), with the water exchange shut off during feeding. Excess feed (F1-F3) at trial end was
161 analyzed with respect to *Anisakis* content separately for each trial group using ELISA, PCR,
162 LCMSMS and Immunostaining.
163 At each sampling, i.e. on the trial days 3, 7 and 14, five zebrafish were randomly removed
164 from each tank with a hand-net and instantly killed by submerging them in crushed ice.
165 Freshly-killed fish were kept cool in sealed plastic tubes, separately for each tank, and then
166 transferred to the laboratory for immediate sample extraction. The visceral organs including
167 the intestinal tract and the gonads were removed and the remaining carcasses were thoroughly
168 washed in tap water before storage in small sealed plastic bags, separately for each tank, in a
169 freezer at -20 ºC. The five zebrafish of each group (Z1-Z3, in duplicate, days 3, 7, and 14)
170 were extracted together and subsequently analyzed for the presence of *A. simplex* protein
171 traces by ELISA, LCMSMS, and Immunostaining.

172 *2.3. Real-time polymerase chain reaction (PCR) assay for the detection of Anisakis simplex.*

173 DNA was isolated from *A. simplex* larvae for the preparation of standard DNA and from
174 feed samples using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI,
175 USA). The extracted DNA was purified using the Wizard DNA Clean-Up System and eluted
176 with sterile water. The final DNA concentration was measured by absorbance at 260 nm.
177 Positive controls of 5 and 10 ng/µL *A. simplex* DNA, negative extraction controls and water
178 controls were included in all assays. In total 100 ng purified DNA per feed sample were
179 analyzed in duplicates in multiple rtPCR assays.

180 RtPCR was performed in accordance with a published method (Lopez and Pardo, 2010).
181 Briefly, a 260 bp fragment of the mitochondrial cytochrome c oxidase II gene (COII) was
182 amplified with two specific primers and a fluorescent-labelled Taq DNA polymerase probe
183 (Amersham Biosciences, Chalfont St Giles, UK) in a TagMan Universal Master Mix (Applied
184 Biosystems, Foster City, CA, USA). Reactions were run by using the ABI Prism sequence
185 detection system (Applied Biosystems) with 40 reaction cycles.

186 *2.4. Polyclonal sandwich ELISA for the detection of Anisakis simplex protein.*

187 Fish and feed samples (2 g) were homogenized and extracted under shaking with
188 phosphate-buffered saline pH 7.4 (PBS) (Oxoid, Basingstoke, UK) at room temperature for
189 1h as described earlier (Werner et al., 2011; Fæste et al., 2014a). Extracts were diluted at least
190 1:20 in PBS before analysis. Further dilution was performed if required to reach the working
191 range of the ELISA.

192 Samples were analyzed using a previously developed polyclonal sandwich ELISA
193 (Werner et al., 2011) that specifically detects *A. simplex* proteins. The standard curve of the
194 ELISA was constructed with 12 concentrations of PBS-extracted total *A. simplex* protein
195 ranging from 0 to 1000 µg/L. Three control samples (naturally-contaminated cod liver,
196 naturally-contaminated salmon muscle, and cod muscle spiked with a definite amount of *A.*

197 *simplex* protein) were included in all assays to confirm the performance of the ELISA by
198 assessing intra- and interday precision and recovery (Fæste et al., 2014a).
199 *2.5. Gel electrophoresis and Immunostaining with polyclonal antibodies and patient sera.*
200 *A. simplex* protein extract, identical to the ELISA standard protein, (10 µg per lane) and
201 zebrafish or feed samples (30 µg per lane) were analyzed by gradient gel electrophoresis and
202 subsequent immunostaining with either self-produced specific polyclonal anti-*A. simplex*
203 antibodies (IgG_1) (Werner et al., 2011) or serum of a patient with *A. simplex* allergy (IgE_1)
204 (Fæste et al., 2014a). The patient, a 60 year-old Spanish man with gastro-allergic anisakiasis,
205 had a class 4 IgE-serum level (18.1 kU_A/l) to *A. simplex* proteins, was positive in skin-prick
206 testing and showed no cross-reactivity to arthropod proteins (shrimp, mite). The experiments
207 were performed as described before (Fæste et al., 2014) with 5 % horse serum in Tris-
208 buffered saline pH 7.6 containing 0.1% Tween 20 as blocking buffer. The polyclonal antibody
209 was diluted 1:250,000 and patient serum was diluted 1:20.
210 In a second immunostaining experiment a pool of sera (IgE_2) from ten Spanish patients
211 with gastro-allergic anisakiasis, positive skin prick tests, and high anti- *A. simplex* IgE-levels
212 (12.4 - 437.5 kU_A/L) or rabbit polyclonal antibodies against *A. simplex* (IgG_2) (Charles River
213 Laboratories, Sulzfeld, Germany) were used as described before (Lin et al., 2012; Lin et al.,
214 2014). *A. simplex* extract was produced by extracting larvae with PBS at 4°C overnight (ON);
215 zebrafish and feed samples were identical to those used in the first immunostaining
216 experiment. Protein extracts (20 µg per lane) were separated by gel electrophoresis,
217 transferred to nitrocellulose membrane, and analyzed with 1:5000 diluted rabbit or the 1:4
218 diluted patient sera.
219 *2.6. LCMSMS for the detection of Anisakis simplex protein in feed and fish.*
220 The samples were prepared and analyzed by liquid chromatography coupled high-
221 resolution mass spectrometry (LCMSMS) as described earlier (Fæste et al., 2014a). Protein

extracts (50 µL, 1 mg/mL) were digested with trypsin over night at 37 °C on ultrafiltration filters and peptides were eluted, dried and re-dissolved in 20 µL 0.1 % formic acid.

Peptides (3µl per sample) were injected with 10 µL/min onto a 5 x 0.3-mm 5 µm Zorbax 300 SB-C18 pre-column, separated with 0.2 µL/min on a 150 × 0.075-mm 3 µm GlycproSIL C18–80Å column using a gradient from 5 to 55% acetonitrile in water/0.1 % formic acid in 68 min, and analyzed on a nano-electrospray LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Mass spectra were acquired in the positive ion mode in the mass range of m/z 200–2000, followed by MS/MS using collision-induced dissociation of the most intense parent ions with 10 ppm accuracy and 3 m/z isolation width. Data analysis was performed by Xcalibur V2.0. Previously identified marker peptides of *A. simplex* hemoglobin (Fæste et al., 2014a) were extracted with 10 ppm accuracy and spectra were manually verified. Zebrafish and feed samples with and without *A. simplex* contamination were analyzed and compared. Standard *A. simplex* protein in buffer was used for semi-quantitatively external calibration.

236 **3. Results**

237 *3.1. Determination of Anisakis simplex by rtPCR and ELISA.*

238 The quantitative PCR and ELISA assays used to analyze the fish feed and zebrafish
239 carcasses have both been validated in previous studies for their sensitivity, specificity,
240 precision, and recovery (Lopez and Pardo, 2010; Werner et al., 2011; Fæste et al., 2014a).

241 The real-time PCR had been optimized for the detection of *A. simplex* DNA in fish and
242 food products and was in the present study successfully applied to complex feed samples.
243 High-quality DNA was isolated by using the commercial clean-up and purification kits and
244 the positive control sample delivered highly reproducible results in all assays. The working
245 range of the rtPCR assay ranged from 10^{-5} to 10 ng DNA (Ct 35 to Ct 7.78) using logarithmic
246 regression for the standard curve of *A. simplex* DNA (R^2=0.9953). The lower limit of
247 application (LLA) in fish feed was set to $4*10^{-4}$ ng/100 ng extracted DNA (Ct 28.6)
248 considering signal noise from the matrix.

249 The ELISA was based on polyclonal rabbit antibodies with high specificity to *Anisakis*
250 *simplex*. The working range of the ELISA ranged from 1 to 250 ng/mL using polynomial
251 regression for the standard curve of *A. simplex* standard protein (R^2 = 9998). The assay
252 showed high sensitivity with a limit of detection at 0.3 mg/kg. However, the LLA was set to 2
253 mg/kg in zebrafish and 5 mg/kg in fish feed considering signal noise from the respective
254 matrices.

255 *3.2. Specific LCMSMS detection of anisakid proteins by typical marker peptides.*

256 High-resolution liquid chromatography tandem mass spectrometry (LCMSMS) analysis of
257 trypsinated *A. simplex* proteins resulted in the detection of specific peptides originating from
258 definite proteins. The peptides were recognized by their typical mass patterns (precursor mass
259 spectra; MS) and mass fragments (product ion spectra; MSMS). The mass patterns allowed
260 protein recognition by comparison with protein databases, whereas the fragmentation

261 determined the amino acid sequence of a peptide. The detection of *A. simplex* proteins by
262 mass spectrometry has been previously described and two anisakid hemoglobin peptides with
263 mass-to-charge ratios of m/z=615.27 and m/z=563.79, respectively, had been identified as
264 suitable marker peptides for the specific analysis of *A. simplex* in fish and food products
265 (Fæste et al., 2014a). The comparison of hemoglobin from *Anisakis pegreffii* (K9USK2 in
266 UniProt database) and identified *Anisakis simplex* peptides to zebrafish hemoglobins (*Danio*
267 *rerio*) by amino acid sequence alignment did not show any homologies (Figure 2). Thus,
268 matrix interferences were not to be expected for the analysis of anisakid hemoglobin in
269 zebrafish samples.

270 *3.3. Characterization of the zebrafish feed.*

271 The three feed preparations (F1-F3; Table 1) used in the zebrafish trial were analyzed at
272 study end by quantitative rtPCR and ELISA assays, semi-quantitative LCMSMS method and
273 qualitative immunostaining method. The different experiments were consistent and mutually
274 corroborative detecting a high level of *A. simplex* protein in F1, and none above the respective
275 method LLAs in F2 and F3 (Table 2). The ELISA measured >10000 mg anisakid protein/kg
276 feed in F1 whereas the rtPCR found 63 pg anisakid DNA/100ng feed.

277 The immunostaining of the feed samples showed coherent results with the four antibody
278 preparations used although different anisakid protein bands were detected by the individual
279 fractions (Figure 3a). The *A. simplex* protein extracts A (PBS, 1h, RT), identical to the ELISA
280 standard protein, and Ae (PBS, overnight (ON), 4°C) showed little differences on gel,
281 although the bands appeared to be slightly diffused in Ae. The comparison of the
282 immunostaining band pattern of F1 to the *A. simplex* protein extracts demonstrated that
283 especially proteins with molecular weights of about 70 kDa, 64 kDa, 38 kDa, 33 kDa, 28 kDa,
284 20 kDa, and 15 kDa were detected in the feed.

285 The LCMSMS feed analysis resulted in the detection of the two hemoglobin marker
286 peptides HSWTTIGEEFGHEADK (m/z=615.27) and LFAEYLDQK (m/z=563.79) with
287 relative strong intensities ($2.3*10^6$ and $6.7*10^5$, respectively) in F1, whereas they were not
288 detected ($< 10^2$) in F2 and F3 (Figure 3b).
289 *3.4. Detection of Anisakis simplex proteins in exposed zebrafish.*
290 Zebrafish from the three different trial groups (Z1-Z3) were analyzed with quantitative
291 ELISA, semi-quantitative LCMSMS and qualitative immunostaining for contents of *A.*
292 *simplex* proteins with correlating results. The results for fish sampled on trial days 3 and 7
293 were all negative (data not shown), whereas differences between groups were observed for
294 day 14 (Table 3). The ELISA could not differentiate between the samples because the method
295 with an LLA of 2 mg/kg was apparently not sensitive enough. However, both the
296 immunostaining and the LCMSMS gave positive read-outs for Z1 and negative for Z2 and Z3
297 in fish fed for the full two-week trial period.
298 In the immunostaining experiments with zebrafish samples (Figure 4a) the background
299 noise was considerable higher than with the feed samples. Nevertheless, the four antibody
300 preparations (IgG_1, IgE_1, IgG_2, IgE_2) all detected weak binding signals (marked with
301 asterisks) for Z1 that were not present in Z2 and Z3. The signals in Z1 were observed at about
302 85 kDa and 20 kDa for IgG_1, at 64 kDa, 25 kDa and 18 kDa for IgE_1, at 33 kDa for IgG_2, and
303 105 kDa for IgE_2. The LCMSMS analysis of the zebrafish tissue samples (Figure 4b) detected
304 the most sensitive anisakid hemoglobin marker LFAEYLDQK (m/z=563.79) (Fæste et al.,
305 2014a) with an intensity of $5*10^3$ in Z1, whereas the second marker was not identified. The
306 marker peptides were not found in Z2 and Z3.

4. Discussion

The transmissibility of *A. simplex* peptides was examined in the present study in a zebrafish feeding trial by determining the presence of the exogenous proteins in the fish tissue. The customized feed contained an artificially high amount of *A. simplex* larvae that had been deep-frozen, freeze-dried, fine-grinded, and heated to 40°C for several hours. Measurable peptides or DNA fragments were detectable in the feed by all four specific detection methods used. The results from both immunological techniques used, ELISA and immunostaining, indicated that a considerable number of antigenic and allergenic epitopes had been retained throughout feed processing, confirming the presence of active, heat-stable and degradation-resistant immunoglobulin-binding sites on *A. simplex* peptides, which is in accordance with previous findings (Caballero and Moneo, 2004; Moneo et al., 2005; Vidaček et al., 2009; Pariza and Cook, 2010; Vidaček et al., 2011). Furthermore, peptides of considerable length (containing up to 20 amino acids) were detected by LCMSMS, including several fragments of the chosen marker protein anisakid hemoglobin, and additionally, other characteristic proteins (Fæste et al., 2014). The rtPCR feed analysis showed that also *A. simplex* DNA fragments of relevant sizes had withstood the feed manufacturing procedures.

The zebrafish trial was designed in the described manner to allow a basic proof-of-principle investigation of the potential carry-over of *A. simplex* peptides from feed to fish. In total 90 fish were kept in six fish tanks with separate circulation systems, which ensured the separation of the different study populations, their feed and wastewater. Zebrafish were chosen as the study object due to their rapid maturation, growth rate, and favorable small size affording less space and feed than edible fish, e.g. salmon or trout. The trial feed contained 20 % *A. simplex* larvae leading to high exposure, which was intended to compensate for the short duration of the study. However, this percentage was much higher than the weight-to-weight ratio of naturally infested fish that is used as fish meal in commercial feed for farmed fish and

domestic animals. Therefore, the present short-term model study should be repeated with relevant fish species such as Atlantic salmon for an extended time of exposure while using feed with a much lower *A. simplex* content in order to better reflect authentic feeding conditions in the marine aquaculture industry.

The outcome of the exploratory zebrafish trial showing low amounts of anisakid peptides in the exposed group after two weeks was rather unexpected. Whereas the ELISA method with an LLA of 2 mg/kg was not sensitive enough for the detection of the trace amounts, both immunostaining and LCMSMS indicated the presence of *A. simplex* peptides in the fish tissue. The four different immunoglobulin fractions including polyclonal antibodies from rabbits and sera from patients with allergy to *A. simplex* all detected weak but distinct binding signals in the zebrafish extracts. The observed bands were specific for the different antibodies, but at typical molecular weights coinciding with results from previous studies (Baeza et al., 2004; Fæste et al., 2014). The LCMSMS measurement delivered confirmative evidence for the contamination of the zebrafish with *A. simplex* peptides. The most sensitive anisakid hemoglobin marker peptide was detected with a relative intensity that was clearly different from the background noise, and corresponding analyses of unexposed fish were negative. Considering the great specificity of the used high-resolution LCMSMS method this result could be regarded as a positive proof for the transmissibility of *A. simplex* peptides from feed to fish.

Since the zebrafish were not bled immediately after sampling, small amounts of blood may still have been present in the tissue during the analyses of the zebrafish carcasses for traces of anisakid proteins. Thus, the positive findings could actually be due to the presence of *A. simplex*-related peptides in the remaining blood. However, this would still be relevant since small amounts of blood are always retained in the tissue of fresh fishery products including fillets from farmed Atlantic salmon.

The marine aquaculture industry still largely depends on the nutrient input from industrially produced aquafeed that contains fishmeal and fish oil originating from wild fisheries resources (Tacon and Metian, 2008). Especially carnivorous finfish and crustaceans require a certain ratio of fish protein in their diet and the estimated global use of fishmeal in aquafeed was in 2007 as high as 17 % for Atlantic salmon and 24 % for marine shrimp. Nevertheless, efforts have been made to reduce the overall fish-in to fish-out ratio (FI/FO) due to finite resources, increasing costs, and chemical contamination of marine forage fish. Thus, the FI/FO has fallen by more than one-third from 1.04 in 1995 to 0.63 in 2006 as a whole, but has remained at 5.0 for Atlantic salmon (Naylor et al., 2009). Where applicable, plant-, animal, or microorganism-based alternatives have been introduced as protein and oil sources in fish feed. However, the substitution of fish by plant proteins leads to new challenges, whether regarding fish growth rates, feed efficiency values, consumer acceptance or food safety concerns (Hardy, 2010). Plants with high nutritional value including legumes such as soy, peanut and lupine are also known for their content of important food allergens, and thus the problem of peptide transmissibility from feed to food remains relevant. If the carry-over observed in the present study was confirmed as a general phenomenon in necessary follow-up experiments, the question of labeling fish and animal products with the used feed ingredients could arise for the protection of allergic consumers.

In conclusion, the detection of immunoreactive anisakid peptides in the tissue of zebrafish exposed to high amounts of *A. simplex* in the feed can be regarded as a proof-of-principle that allergenic peptides may be transferred from animal feed into the final food products.

Acknowledgements

The authors greatly appreciate Dr. Alvaro Daschner from the Servicio de Alergia, Instituto de Investigación, Sanitaria-Hospital Universitario de La Princesa, Madrid, Spain, for his kind supply of well-characterized sera from Spanish patients with allergy to *Anisakis simplex*. We also would like to thank Prof. em. Erik Florvaag from the Department of Clinical Science, University of Bergen and the Laboratory of Clinical Biochemistry, Haukeland University Hospital, Bergen, Norway, for his support of the project. This study was funded by the Orkla Foundation, Norway.

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501 **Figure legends**

502 **Figure 1.** Zebrafish trial set-up consisting of six coarsely transparent 3.0 l plastic tanks with
503 continuous slow water exchange via separate circulation systems. The three trial groups (Z1-
504 Z3) were examined in duplicate in tanks placed next to each other.

505 **Figure 2.** Alignment of six *Anisakis simplex* hemoglobin peptides identified by LCMSMS
506 and hemoglobin from *Anisakis peregreffi* (Uniprot database accession number: K9USK2) and
507 hemoglobin forms of zebrafish (*Danio rerio*) using T-Coffee (Version_9.03.r1318; Swiss
508 Institute of Bioinformatics).

509 **Figure 3a.** Fish feed (F1-F3) analysis by immunostaining. Gel electrophoresis of *Anisakis*
510 *simplex* proteins (left panels) and immunostaining with polyclonal rabbit antibodies (IgG_1 and
511 IgG_2), and with sera (right panels) from one patient (IgE_1) or with a serum pool (IgE_2). M1
512 (SeeBluePlus2, Invitrogen) and M2 (Low-Range pre-stained Natural Standard, Bio-Rad):
513 molecular weight markers [kDa] (indicated on the left side of the gels); A: *A. simplex* extract
514 (ELISA standard protein); Ae: *A. simplex* extract (ON); F1: basic feed with *A. simplex* (Table
515 1); F2: basic feed with fish meal; F3: basic feed.

516 **Figure 3b.** LCMSMS analysis of fish feed (F1-F3) by detection of two typical marker
517 peptides of *Anisakis simplex* hemoglobin. Total ion count spectrum (retention time 0-68 min),
518 spectrum of m/z 615.28 (peptide: HSWTTIGEEFGHEADK), spectrum of m/z 563.79
519 (peptide LFAEYLDQK). Relative ion abundances are shown; absolute intensities (NL) are
520 indicated on the right side of each spectrum.

521 **Figure 4a.** Zebrafish (Z1-Z3) analysis by immunostaining. Gel electrophoresis of *Anisakis*
522 *simplex* proteins (left panels) and immunostaining with polyclonal rabbit antibodies (IgG_1 and
523 IgG_2), and with sera (right panels) from one patient (IgE_1) or with a serum pool (IgE_2). M1
524 and M2: molecular weight markers [kDa] (indicated on the left side of the gels); A: A.

525 simplex extract (ELISA standard protein); Ae: A. simplex extract (ON); Z1: zebrafish fed
526 with F1; Z2: zebrafish fed with F2; Z3: zebrafish fed with F3. Binding signals of interest in
527 Z1 are marked with asterisks (*).

528 **Figure 4b.** LCMSMS analysis of zebrafish (Z1-Z3) by detection of two typical marker
529 peptides of *Anisakis simplex* hemoglobin. Total ion count spectrum (retention time 0-68 min),
530 spectrum of m/z 615.28 (peptide: HSWTTIGEEFGHEADK), spectrum of m/z 563.79
531 (peptide LFAEYLDQK). Relative ion abundances are shown; absolute intensities (NL) are
532 indicated on the right side of each spectrum.

533

534 **Tables**

535 **Table 1.** Feed compositions for the zebrafish feeding study.

Feed components	***A. simplex* feed F1**		**Fish meal F2**		**Control F3**	
	[mg]	[%]	[mg]	[%]	[mg]	[%]
Basic zebrafish feed[a]	2750	68	2300	38	5300	88
Gelatin	460	12	700	12	700	12
A. simplex larvae	790	20	-	-	-	-
Fish meal	-	-	3000	50	-	-
Total (sum)	4000	100	6000	100	6000	100

536 [a]We were unable to obtain any details on the specific ingredients of the basic zebrafish feed.

537

538

539 **Table 2.** Content of *A. simplex* protein in the different feed types.

Feed type	ELISA [mg/kg]	PCR[a] [pg/100ng]	Immunostaining	LCMSMS [µg/ml]
F1	> 10000	63	positive	10
F2	< 5	< 0.4	negative	< 0.1
F3	< 5	< 0.4	negative	< 0.1

540 [a]DNA-content as measured by PCR.

541

542 **Table 3.** Content of *A. simplex* protein in zebrafish fed with different feed types

Zebrafish group	**ELISA [mg/kg]**	**Immunostaining**	**LCMSMS [µg/ml]**
Z1	< 2	positive	0.2
Z2	< 2	negative	< 0.1
Z3	< 2	negative	< 0.1

543

Human and animal rights

The use of patient sera was approved by the Spanish study centre's institutional review board and all patients had given their written informed consent.

The zebrafish trial was performed in the zebrafish research laboratory of the National Institute of Nutrition and Seafood Research, Bergen, Norway, after approval by the institutional review board and with regard to the Norwegian legislation for ethics in animal research.

Figure 1.

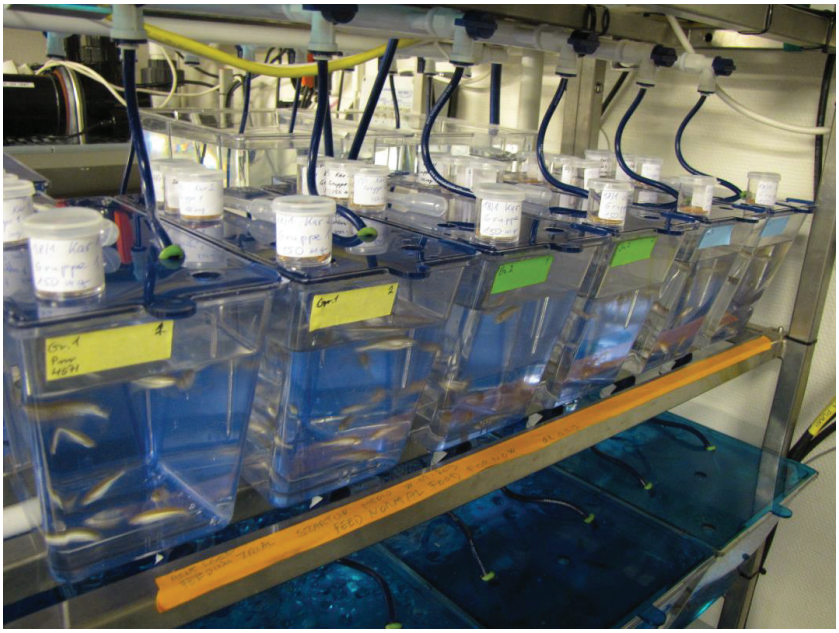

Figure 2.

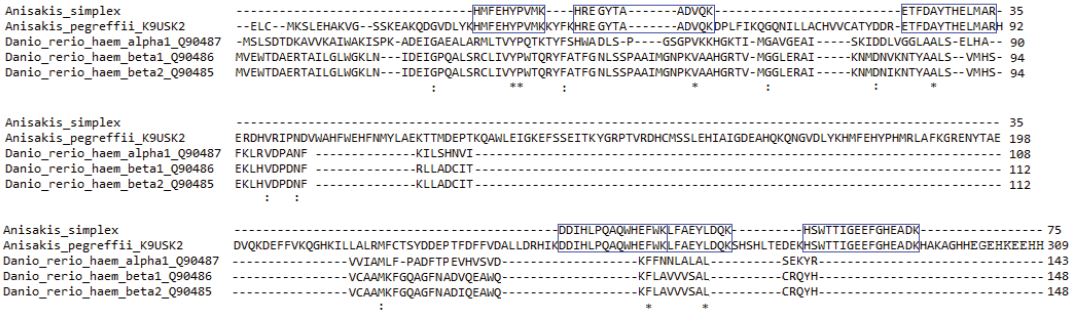
Anisakis_simplex
Anisakis_pegreffii_K9USK2
Danio_rerio_haem_alpha1_Q90487
Danio_rerio_haem_beta1_Q90486
Danio_rerio_haem_beta2_Q90485

Figure 3a.

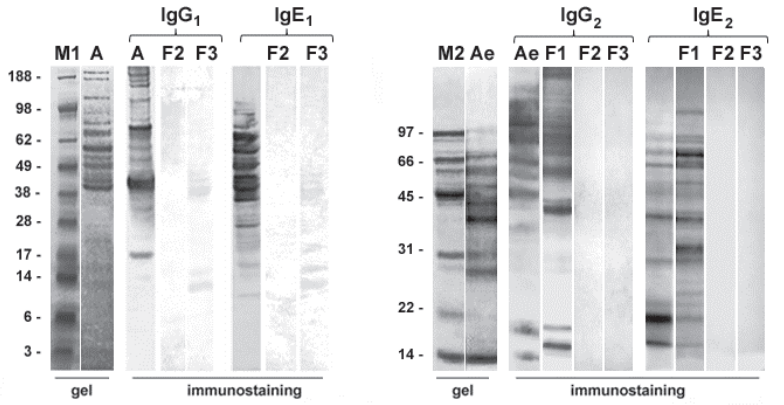

Figure 3b.

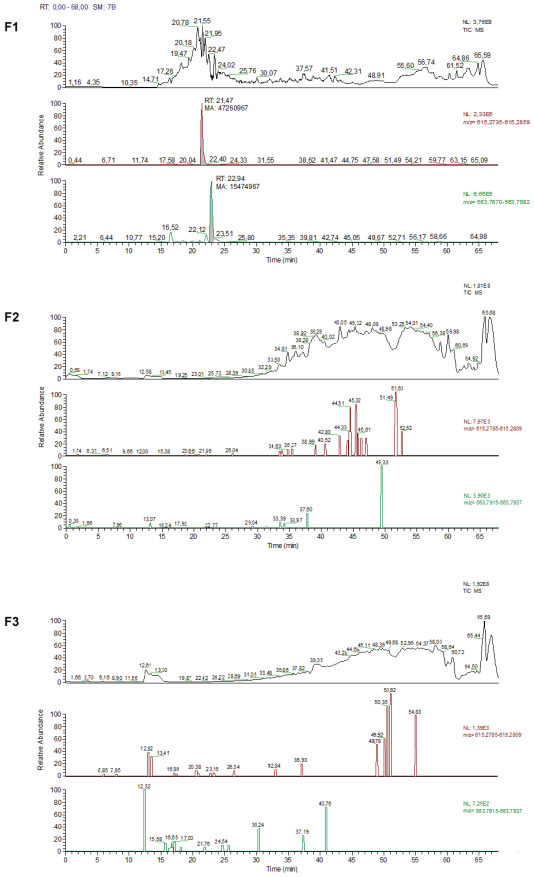

Figure 4a.

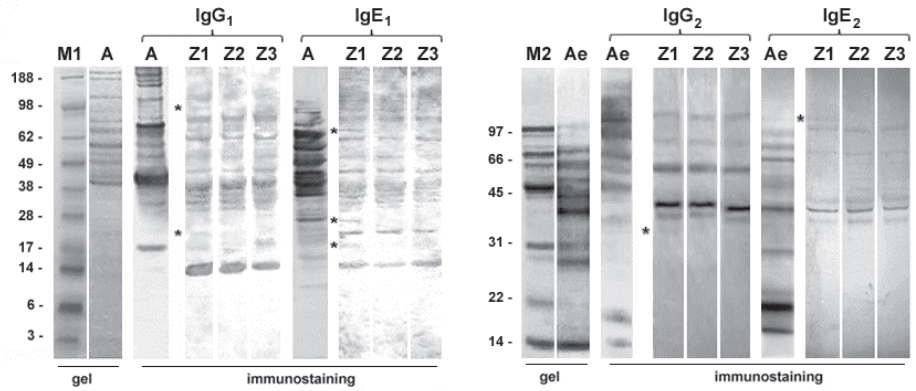

Figure 4b.