**Rainbow trout (*Oncorhynchus mykiss*) immune response towards a recombinant vaccine
targeting the parasitic ciliate *Ichthyophthirius multifiliis***

Louise von Gersdorff Jørgensen¹, Per Walter Kania¹, Karina Juhl Rasmussen², Andreas Holm
Mattsson³, Jacob Schmidt¹, Azmi Al-Jubury¹, Adam Sander⁴, Ali Salanti⁴ and Kurt Buchmann¹

¹ *Laboratory of Aquatic Pathobiology at the Department of Veterinary and Animal Sciences,
University of Copenhagen, Frederiksberg, Denmark*

² *Department of Cancer and Inflammation Research, University of Southern Denmark, Odense,
Denmark*

³ *Evaxion Biotech, Copenhagen, Denmark*

⁴ *Centre for Medical Parasitology at the Department of Immunology and
Microbiology, University of Copenhagen, Copenhagen, Denmark*

Corresponding author: Louise von Gersdorff Jørgensen, lvgj@sund.ku.dk, Stigbøjlen 7, DK-1870
Frederiksberg C, Frederiksberg, Denmark

Short running title: Recombinant vaccine against *Ichthyophthirius multifiliis*

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Abstract

The protective effect in rainbow trout (*Oncorhynchus mykiss*) of an experimental sub-unit vaccine
targeting antigens in the parasite *Ichthyophthirius multifiliis* has been evaluated and compared to
effects elicited by a classical parasite homogenate vaccine. Three recombinant parasite proteins
(two produced in *E. coli* and one in insect cells) were combined and injected i.p. and subsequently
protection and antibody responses were analysed. Both the experimental and the benchmark vaccine
induced partial but significant protection against *I. multifiliis* when compared to control fish.
Specific antibody responses of vaccinated trout (sub-unit vaccine) were raised against one
neurohypophysial n-terminal domain protein #10 out of three recombinant proteins, whereas the
benchmark vaccine group showed specific antibody production against all three recombinant

49 proteins. The immunogenic parasite protein #10 may be a potential vaccine candidate
50 supplementing the protective I-antigen in future vaccine trials.

51 Keywords: Sub-unit vaccine, vaccine, parasite *Ichthyophthirius multifiliis*, rainbow trout

52

53 **Background**

54 *Ichthyophthirius multifiliis* is a parasitic ciliate infecting freshwater fish worldwide. The parasite is
55 the causative agent of ichthyophthiriasis (white spot disease) inducing high morbidity and mortality
56 in fish (Matthews 2005, Findly et al. 2013, Xu et al. 2016). The parasite infects fish skin and gills in
57 a stage termed the trophont and after a temperature dependent maturation period the parasite leaves
58 the fish as a tomont, attaches to the fish tank surface and transforms into a tomocyst, which
59 subsequently releases infective theront stages (Wagner 1960, Aihua and Buchmann 2001).

60 Repetitive treatments of infected fish rearing systems are required to control the infection and
61 several auxiliary compounds comprising peracetic acid, sodium percarbonate and formaldehyde are
62 currently used in fish farms but only with partial success (Heinecke and Buchmann 2009).

63 Alternative control strategies are needed and management methods such as continuous water
64 filtration to remove parasites have been suggested (Heinecke and Buchmann 2009). An alternative
65 strategy is based on the ability of fish to raise a protective immune response against the parasite.

66 Fish surviving a natural infection can acquire a high level of immunity against re-infection (Bauer
67 1953, Hines and Spira 1974, Clark, Dickerson and Findly 1988, Jorgensen et al. 2011), which
68 suggests that vaccination against this parasitic infection will be a future control possibility.

69 Different vaccines have been experimentally tested against *I. multifiliis* but only a few have proven
70 efficient. Intraperitoneal injection of live theronts (the infective stage) confers a significant
71 protection to fish (Burkart, Clark and Dickerson 1990, Alishahi and Buchmann 2006, Jorgensen et
72 al. 2008) but even injection of parasite homogenates elicits a partial protection (Dalgaard,

73 Buchmann and Li 2002). Production of parasite material for a homogenate vaccine relies on
74 propagation of the parasite in live fish because *in vitro* cultivation of the parasite is only partly
75 feasible at present (Nielsen and Buchmann 2000). Sufficient vaccine volumes for use in the industry
76 are likely to rely on biotechnological approaches. Previously recombinant vaccine preparations,
77 based on the immobilization antigen (I-antigen), have been presented (He et al. 1997, Gaertig et al.
78 1999) but a vaccine has never reached the fish farm industry. We have, based on proteomic
79 screening of an *I. multifiliis* strain G5 (Abernathy et al. 2007), selected and tested three new
80 recombinant proteins and combined them in a sub-unit vaccine. The three proteins were constructed
81 based on conventional *in silico* analysis predicting presence of a signal peptide, transmembrane
82 regions, functional and structural domains. The proteins were recombinantly expressed, purified
83 and evaluated by injecting them intraperitoneally into rainbow trout (*Oncorhynchus mykiss*) with an
84 adjuvant. Following subsequent experimental challenge with live parasites the conferred protection
85 and specific antibody production were compared to corresponding reactions in trout vaccinated with
86 a benchmark vaccine containing an adjuvanted classical parasite homogenate.

87

88 **Materials and methods**

89 *Ethics*

90 All experiments were conducted in agreement with the Animal Experiments Inspectorate under the
91 Danish Ministry of Environment and Food (License number 2013-15-2934-00794). This authority
92 has approved the study and the methods were carried out according to the approved guidelines.
93 MS222 was used for anaesthesia and euthanasia.

94 *Fish*

95 Rainbow trout (Fousing strain) were hatched and reared in pathogen-free conditions at the
96 Bornholm Salmon Hatchery (Nexø, Bornholm) (Xueqin, Kania and Buchmann 2012) and brought

97 to our infection facility at the University of Copenhagen at an average body weight of 11.2 g (SD:
98 1.8). Fish were acclimated for 14 days prior to the experiment and fed 1% of their body weight per
99 day using pelleted dry feed (Biomar, Denmark). Each fish duplicate was kept in 200 L tanks
100 containing 160 L municipal tapwater at pH 7.6 and temperature 12-15 °C. This temperature range is
101 optimal for rainbow trout and suitable for controlled *I. multifiliis* infections. Water was
102 continuously aerated and rinsed by internal biofilters (Eheim, Germany) with a 50% water
103 exchange every second day.

104 *Vaccine groups*

105 A total of 190 fish were divided into 5 duplicated groups with 20 fish per replicate (Table 1), with
106 exception of the parasite homogenate group, which only contained 2x15 fish (due to quantitative
107 limitations of the vaccine). Group 1 consisted of naïve control fish to be challenged, Group 2
108 comprised adjuvant injected control fish to be challenged, Group 3 was injected with the
109 experimental sub-unit vaccine and subsequently challenged, Group 4 comprised trout injected with
110 parasite homogenate and subsequently challenged whereas Group 5 contained naïve control fish,
111 which were kept un-infected throughout the study.

112 *Adjuvant*

113 Each fish in the adjuvant only group received adjuvant consisting of 150 µL of equal amounts of
114 alhydrogel® (Aluminium hydroxide gel adjuvant, Brenntag, Denmark) and Freund's incomplete
115 adjuvant (FIA) (Sigma-Aldrich, Denmark).

116 *Vaccine*

117 Each fish in the recombinant vaccine group received 150 µL vaccine consisting of equal amounts of
118 alhydrogel and FIA with a mixture of the recombinant proteins bound to alhydrogel. We included
119 40 µg each of two protein fragments (#5a and #5b), 40 µg of protein #11 and 25 µg (due to
120 quantitative limitations) of protein #10 (Table 1). Group 4 was injected with homogenised parasites

121 (the tomont stage). Each of these fish received 150 μ L of a vaccine consisting of equal parts
122 alhydrogel and FIA and 100 μ g of homogenised tomonts.

123 *Protein expression and purification*

124 We used M15 competent *E. coli* (Quiagen, Denmark) as our expression system for #5a, #5b and
125 #11. Approximately 80 ng of the plasmids were transferred to 100 μ L of competent M15 cells.
126 Cells were incubated 20 min on ice and subsequently heat shocked at 42 °C for 90 seconds and
127 immediately placed back on ice. 500 μ L Luria-Bertani (LB) (Becton Dickinson a/s, Denmark) were
128 added to the Eppendorf tubes and incubated for 60 min at 37 °C with vigorous shaking (200 rpm).
129 The bacteria were subsequently plated out on LB agar-plates containing ampicillin (Amp) and
130 kanamycin (Kan) in concentrations of 50 μ g/mL and 25 μ g/mL respectively and incubated at 37 °C
131 over night (ON). Up to 8 colonies were subsequently cultured for each transfection. For expression
132 analyses, the cultures were grown until the optical density (OD) reached 1 and induced for 4 hours
133 with Isopropyl-B-D-thiogalactopyranoside (IPTG; VWR, Denmark), to a final concentration of 1
134 mM. Expression of the peptides were verified by standard SDS page and visualized by Coomassie
135 blue (Simply Blue SafeStain, Invitrogen, Denmark) staining. Following successful expression,
136 cultures were grown in 2 L of LB, induced for 4 h with IPTG and centrifuged at 5000 rpm (Sorvall®
137 RC-5B; Thermo Scientific, Denmark) for 20 min at room temperature. The pellet was subsequently
138 disrupted in 50 mL binding buffer (8 M urea, 100 mM NaH₂(PO)₄, 100 mM tris (base), 300 mM
139 NaCl, 5 mM imidazole, pH 8.0) and placed at 4 °C ON.

140 *Protein purification*

141 The disrupted pellet was rotated end-over-end for 60 min at room temperature (lysis) and
142 subsequently centrifuged at 1500 rpm for 20 min at room temperature. The supernatant was filtered
143 with a 0.45 μ m filter and 8 mL (4 mL Ni-resin beads) of prewashed Ni-resin in binding buffer
144 (Thermofischer Scientific, Denmark) was added to the supernatant and subsequently rotated end-

over-end for one hour at room temperature. The recombinant proteins bound to the Ni-resin with a c-terminal His-tag. The resin was washed three times in binding buffer prior to elution of the protein in elution buffer (8 M urea, 100 mM NaH₂PO₄, 100 mM tris (base), 300 mM NaCl, 300 mM imidazole, pH 8.0). The eluates were stored at 4 °C. The resulting purity of the peptides was evaluated by means of SDS–PAGE and Coomassie Brilliant Blue staining and the concentrations were estimated by measuring OD_{280nm}. The eluates were dialysed twice ON in 5 L of 6 M urea in 0.9 % NaCl to remove the imidazole without precipitating the peptides and stored at -20 °C.

Protein expression and purification using a baculovirus system

Proteins #3 and #10, where #10 contained 44 cysteines, were expressed in a baculovirus system. The fragment was cloned into the Baculovirus vector, pAcGP67-A (BD Biosciences, <http://www.bdbiosciences.com>), which was modified to contain the V5 epitope upstream of a histidine tag in the C-terminal end of the constructs. Linearised Bakpak6 Baculovirus DNA (BD Biosciences) was co-transfected with pAcGP67-A into Sf9 insect cells for generation of recombinant virus particles. Recombinant protein was purified on Ni⁺⁺ metal-chelate agarose columns as secreted histidine-tagged proteins from the supernatant of infected High-Five insect cells.

Sub-unit vaccine preparation

Two fragments of #5 (#5a and #5b) and two full-length proteins (#10, #11) were included in the vaccine. All four recombinant fragments/proteins were mixed in a 50 mL NUNC tube with alhydrogel in a ratio of 100 µL alhydrogel to 150 µg protein and mixed end-over-end at room temperature for one hour. The mixture was subsequently spun at 100 x g and washed twice in 0.9 % NaCl. An equal volume of FIA was added and it was vigorously shaken at maximum speed for one hour at room temperature until a high viscosity emulsion was reached. The procedure for preparing

168 the control adjuvant and for including adjuvant into the parasite homogenate vaccine followed this
169 protocol.

170 *Production of the parasite homogenate vaccine*

171 The benchmark vaccine was formulated with homogenised parasites as antigen source. Fish
172 infected with mature trophonts (feeding stage of the parasite) were killed with an overdose of
173 MS222 (Sigma-Aldrich, Denmark) in a concentration of 300 mg/L whereby parasites were released
174 from the skin and collected. The parasites were washed three times in phosphate buffered saline
175 (PBS) and stored at -80 °C until use. Parasites were homogenised by sonication (285 W, Heat
176 Systems XL 2020, New York, USA) on ice and protein measurement was conducted with Pierce
177 BCA protein assay kit (Thermo Fisher Scientific, Denmark).

178 *Vaccination procedure*

179 Fish, anesthetized in MS222 (90 mg/L), were intraperitoneally injected (volume 0.15 ml/fish) either
180 with adjuvant alone, adjuvanted recombinant vaccine or adjuvanted homogenate vaccine,
181 whereafter fish were transferred to aerated water for recovery.

182 *Challenge procedure*

183 Infective theront stages for challenge were produced according to standard procedures. In brief,
184 rainbow trout carrying visible trophonts (with diameters of 300-700 µm) were euthanized (300
185 mg/L MS222) and left in clean water for 4 hours to shed parasites. Released parasites (tomonts)
186 were subsequently left for one-two days for tomocyst development, which subsequently released
187 the infective parasite stage (theronts). These were counted and used for challenge, which was
188 conducted by adding a suspension of theronts (10,000 theronts per fish) to the fish tank. Filtration
189 was removed for the first 4 hours during challenge.

190 *Blood sampling*

191 Blood sampling of 4 fish per tank was conducted at 4 weeks post vaccination (pv) and at 25 days
192 post challenge (dpch) corresponding to 13 weeks pv. Fish were euthanized in an overdose of
193 MS222 (300 mg/L) and blood samples were taken by caudal vein puncture using heparinized
194 syringes. Plasma was separated from blood by centrifugation (4000 g for 10 min) and kept at -80 °C
195 until use.

196 *Parasite counting on fish*

197 Twenty-five days post challenge 4 fish per replicate were sampled and a subpopulation of the
198 parasites on the host counted on the body (one side of the fish). Weight and length parameters were
199 recorded and the parasite burden was calculated taking the size of the fish into account (number of
200 parasites per unit weight and length). Statistical analyses were based on a one-way Anova test
201 between groups (Graphpad Prism).

202 *Immobilization assay*

203 Blood samples (13 weeks pv) from 4 fish per replicate group was pooled and diluted 1:10 in PBS.
204 Volumes of 100 µL were then distributed in duplicate on a 96 well non-absorbing round bottomed
205 microplate (manufacturer, country). Plasma from a naturally immunized rainbow trout (a survivor
206 of several *I. multifiliis* infections) was included as a positive control. A total of 75 theronts in 100
207 µL water were distributed in each well with diluted plasma and mobility was then recorded 1, 30,
208 60, 90 and 120 min later. Mobility was ranked as: 0 = no mobility, 1 = mobility severely decreased,
209 2 = mobility slightly decreased and 3 = 100% mobility.

210 *Enzyme-linked immunosorbent assay (ELISA)*

211 Specific trout antibody production was determined by using ELISA standard procedures. Microtiter
212 plates were either coated with 100 µL parasite antigen in a concentration of 5 µg protein/mL or 50
213 µL of each of the peptide antigens in a concentration of 1 µg/L in coating buffer (C3041, Sigma-
214 Aldrich, Denmark) ON at 4 °C. Non-specific binding sites were blocked 1 h at room temperature

215 using 1 % BSA and 0.2 % Tween 20 in PBS. Plasma samples were diluted 1:10, 1:100, 1:1,000 and
216 1:10,000 and analysed in duplicate. Mouse anti-salmonid Ig (MCA2182, AbD Serotec, UK) was
217 used as a secondary antibody (1:500) and HRP conjugated rabbit anti-mouse IgG (STAR13B, AbD
218 Serotec, UK) used as the tertiary antibody (1:500). At least 4 wells were dedicated to background
219 readings that were subtracted from the readouts before analysis (t-test, Graphpad Prism).

220 *Protein characteristics*

221 The proteins, which were used in the sub-unit vaccine, have different characteristics, which are
222 described in Table 1.

223 Table 1. Characteristics of the proteins (or fragments) used in the sub-unit vaccine.

224 Proteins/fragments, the length of the sequence, the number of cysteines, proteinID, a description of
225 the proteins/fragments, subcellular location, function and their domains are indicated. Originally the
226 proteinID #5b sequence had 4 cysteines but 2 cysteines were converted to serines in order to have
227 only one disulphide bridge, increasing the probability of successful expression in *E. coli*.

228

229 **Results**

230 *Protein selection and expression*

231 Three proteins were selected but protein #5 was split into two fragments (5a and 5b) in order to
232 increase the chance of expression. Protein #5a, #5b and #11 were successfully expressed in M15 *E.*
233 *coli* cells and protein #10 was expressed in insect cells. A mixture of these proteins was used for the
234 sub-unit vaccine. Attempts to refold the *E. coli* derived proteins by stepwise decreasing the
235 concentration of urea before binding resulted in precipitation; hence the proteins were not refolded
236 by urea removal but may have refolded when binding to alhydrogel during the washout of urea. The
237 maximum yield for these proteins was reached with 43 mg from a 2 L culture for #5a and the
238 minimum yield reached 2 mg for #11.

239 *Parasite burden*

240 Protection of fish in the different vaccination groups were reflected by the parasite burden achieved
241 after challenge. The control group carried a mean of 2.8 parasites per g/cm fish, whereas the
242 adjuvant control group had a significantly higher mean of 4.1. The sub-unit vaccine group and the
243 parasite homogenate vaccine group were significantly less infected with mean intensities of 1.3 and
244 0.8, respectively when compared to the adjuvant control group (Fig. 1). No differences between the
245 recombinant and homogenate vaccine groups were recorded.

246 *Immobilisation assay*

247 The ability of plasma from vaccinated fish to immobilize theronts was tested in an immobilisation
248 assay. Plasma from a trout surviving several exposures to the parasite immobilised all the theronts
249 within 30 min, whereas plasma from the uninfected controls did not immobilise the theronts at all
250 (Fig. 2). Plasma from fish injected with parasite homogenate showed a clear inhibition of theront
251 movement, whereas the sub-unit vaccine group inhibited theront mobility slightly. Fish plasma
252 from the adjuvant only group showed a slight and weaker immobilizing activity compared to the
253 vaccinated groups.

254 *ELISA*

255 Production of specific antibodies against the recombinant parasite proteins following vaccination
256 was measured by ELISA analyses. No significant differences between any of the replicates of the
257 vaccine groups were found (t-test, $p < 0.05$) and replicates were pooled for analysis at a plasma
258 dilution of 1:100. Fish immunized by the recombinant subunit vaccine and by the homogenate
259 vaccine preparation showed a significantly higher antibody reaction compared to both naïve control
260 and adjuvant injected fish. The former group reacted strongly against protein #10 and slightly
261 against #11, whereas the latter group showed elevated responses against all three proteins (#5, #10
262 and #11) (Fig. 3).

263

264 **Discussion**

265 Rainbow trout injected with an experimental sub-unit vaccine containing three recombinant parasite
266 proteins showed a partial protective response, which did not differ significantly from responses in
267 fish receiving a classical parasite homogenate vaccine. The parasite burden was higher in the
268 control and the adjuvant control group when compared to both sub-unit and homogenate vaccinated
269 fish. Previous studies have described some protection obtained following injection with an I-antigen
270 (a surface immobilisation antigen of the parasite) in channel catfish (*Ictalurus punctatus*) (Wang et
271 al. 2002), injection of live theronts (Jorgensen et al. 2008) or injection of sonicated tomonts in
272 rainbow trout (*Oncorhynchus mykiss*) (Dalgaard et al. 2002), goldfish (*Carassius auratus*) (Osman
273 et al. 2009), channel catfish (Xu, Klesius and Shelby 2004) and Nile tilapia (*Oreochromis niloticus*)
274 (Xu, Klesius and Shoemaker 2008). The I-antigen is without doubt an important, but serotype
275 specific protein, with respect to interactions between the fish host and *I. multifiliis* but other
276 antigens common to all serotypes must have protective functions as well since natural infections
277 induce protection across serotypes (Wang et al. 2002). In this study rainbow trout, immunized
278 against three recombinant proteins (#5, #10 and #11), raised a significantly elevated antibody
279 response towards protein #10, which suggests that this antigen is immunogenic and has protective
280 potential. In light of the successful production of the three new antigens described in this work, we
281 suggest that further studies should be conducted for protein #10 in order to elucidate its potential as
282 a vaccine candidate combined with the I-antigen for future sub-unit vaccines, as a part of the
283 immune-prophylactic solution of white spot disease in freshwater fish farms. Even though
284 protection against *I. multifiliis* in rainbow trout involves a mucosal response (Xu et al. 2013, Xu et
285 al. 2016, Jorgensen et al. 2011) it is clear that a systemic response is included (Buchmann et al.
286 2001, Jorgensen et al. 2008). Plasma from fish surviving several natural infections with the parasite

has previously been shown to exhibit a superior ability to immobilize theronts and this was confirmed in the present study. It is noteworthy that plasma from the sub-unit vaccine exposed fish was capable of immobilising the parasite *in vitro*, which indicates that the recombinant proteins and not only the classical I-antigen are involved in immobilization. Trout from the homogenate vaccine injected group reacted against the zinc-finger like protein and the hypothetical protein (fragments #5a-b and protein #11) with an elevated antibody production. However, these proteins did not induce a high level of antibodies when injected as part of the experimental vaccine into naïve fish, which suggests that they are less immunogenic. Fish from the homogenised parasite vaccine group did erected antibody reactions against all three proteins and one of those (#10) induced a specific antibody response when injected into naïve fish. This correspondence between protective responses and antibody production indicates that this recombinant protein should be considered as immunogenic and a potential vaccine candidate. However, the level of protection induced was still moderate and additional work is needed before a subunit vaccine with a potential to induce a high level of protection against *I. multifiliis* can be produced. First of all, it may be relevant to include other immunogenic recombinant proteins common for all serotypes together with #10. Secondly the antigen concentration in fish vaccine preparations is known to play a significant role for protection (Marana et al. 2017), which calls for additional dose-response studies with regard to this sub-unit vaccine. In conclusion, the parasite protein #10, which was tested in the present study was shown to confer protection and elicit an antibody response. This suggests that the protein may be a future vaccine candidate supplementing the already described I-antigen. Therefore, further studies on its use in a vaccine against white spot disease should be performed. In this context, it would be important to perform dose-response assays on single proteins and booster effect studies.

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387

388 Tables

389

390 Table 1

Protein	Fragment region	Fragment Length	Full Length	Cysteines	ProteinID ^{***}	Description ^{***}	Subcellular Location ^{**}	Function [*]	Domain [*]
#5a	1-230aa	230 aa	925 aa	2	XP_004035116	hypothetical protein	cytoplasm/nucleus	nucleic acid binding, zinc ion binding	Zinc finger, DnaJ
#5b	232-925aa	694 aa	925 aa	2					
#10	1-329aa	329 aa	329 aa	44	XP_004034776	neurohypophyseal n-terminal domain protein	extracellular	protein binding	growth factor receptor, EGF-like, Furin-like
#11	1-431aa	431 aa	431 aa	1	XP_004031223	hypothetical protein	extracellular/cytoplasm/nucleus	-	-
[*]: by InterPro Server									
[**]: by Wolf PSORT Server									
[***]: by NCBI									

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393 Figures

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395

396 **Fig. 1.** Parasite burden following challenge with *I. multifiliis*. Rainbow trout were vaccinated
 397 against *I. multifiliis* using one experimental sub-unit vaccine and a benchmark vaccine consisting of
 398 parasite homogenate. Challenge was conducted 9.5 weeks post vaccination, whereafter the parasite
 399 burden was recorded. Number of parasites per length/weight of the fish in each vaccine group is
 400 shown. Asterisks * indicate statistical significant differences ($P < 0.05$) between the vaccine groups
 401 and the adjuvant control group.

402

403 **Fig. 2.** Immobilisation of *I. multifiliis* theronts following incubation with plasma from rainbow trout
 404 1) vaccinated against *I. multifiliis* using an experimental sub-unit vaccine, 2) vaccinated with a

405 benchmark vaccine consisting of parasite homogenate, 3) adjuvant injected, 4) naïve non-injected.
 406 The Y-axis shows theront mobility with 3 being maximum mobility and 0 being complete
 407 immobilisation.

408
 409 **Fig. 3.** ELISA data from rainbow trout following different vaccine treatments against the parasite *I.*
 410 *multifiliis*. Plasma samples 4 and 13 weeks post injection were analysed in a dilution of 1:100. The
 411 specific antibody response against a zinc-finger-like protein (fragment #5a and fragment #5b), a
 412 neurohypophysial n-terminal domain protein (#10), a hypothetical protein (#11) and parasite
 413 homogenate is shown for the different vaccine groups (uninfected control, infected control,
 414 adjuvant control, sub-unit vaccine and parasite homogenate). In the sub-unit vaccine group, specific
 415 antibodies against protein #10 were produced and in the parasite homogenate group specific
 416 antibodies against all recombinant proteins were produced. a = significantly different from
 417 uninfected control, b = significantly different from uninfected control and control, c = significantly
 418 different from uninfected control, control and adjuvant control, d = significantly different from all
 419 other groups. Homog. = homogenate.