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Specific regulation of the chemokine response to viral hemorrhagic septicemia virus (VHSV) at the entry site

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Running title: Chemokines at VHSV entry site

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The fin bases constitute the main portal of rhabdovirus entry into rainbow trout (*Oncorhynchus mykiss*), and replication in this first site strongly conditions the outcome of the infection. In this context, we studied the chemokine response elicited in this area in response to viral hemorrhagic septicemia rhabdovirus (VHSV). Among all the rainbow trout chemokine genes studied, only the transcription levels of CK10 and CK12 were significantly up-regulated in response to VHSV. As the virus had previously shown to elicit a much stronger chemokine response in internal organs, we compared the effect of VHSV on the gills, another mucosal site which does not constitute the main site of viral entry nor rhabdoviral replication. In this case, a significantly stronger chemokine response was triggered, with CK1, CK3, CK9 and CK11 being up-regulated in response to VHSV and CK10 and CK12 being down-modulated by the virus. We then conducted further experiments to understand how these different chemokine responses of mucosal tissues could correlate with their capacity to support VHSV replication. No viral replication was detected in the gills, while at the fin bases, only the skin and the muscle were actively supporting viral replication. Within the skin, viral replication took place in the dermis, while the viral replication was blocked within epidermal cells at some point before the protein translation level. This different susceptibility of the different skin layers to VHSV correlated with the effect that VHSV has on their capacity to secrete chemotactic factors. Altogether these results suggest a VHSV interference mechanism on the early chemokine response at its active replication sites within mucosal tissues, a possible key process that may facilitate viral entry.

INTRODUCTION

Viral hemorrhagic septicemia virus (VHSV) belongs to the *Novirhabdovirus* genus within the *Rhabdoviridae* family, and is the etiological agent of a lethal disease for many cultivated fish species worldwide, including rainbow trout (*Oncorhynchus mykiss*). In fish, the fin bases constitute one of the main portals of pathogen entry and pathogen multiplication prior to dissemination through the host, as has been demonstrated for many different pathogens (5, 11, 33-35). This is also true for VHSV and a very closely related salmonid pathogen, the infectious hematopoietic necrosis virus (IHNV) (17, 43, 44), since bioluminescence imaging of live infected rainbow trout revealed the fin bases and not the gills as the major portal of entry (17), prior to dissemination to hematopoietic tissues, where these viruses replicate most frequently (9, 48). Upon bath exposure, viral replication was already visible as early as 8h post-infection in this area, whereas no replication was observed at this point in the gills. Moreover, when fish were exposed to a non-pathogenic recombinant IHNV, viral replication remained limited to the fin bases, suggesting that in this case the local immune response was sufficient to block further viral dissemination (17). For VHSV, viral replication in excised fin tissue has even been shown to correlate with mortality after waterborne infection (43, 44), highlighting again the importance of this early replication at fin bases in the outcome of the infection. However, whether the level of viral replication in skin tissues determines if the amount of virus that arrives to the internal organs is too high for the internal defenses to eliminate, or if the external fin tissues send the appropriate “danger” signals to the immune system enabling the systemic defenses to clear the virus in internal organs, remains unsolved.

75 Chemokines constitute one of the first secreted immune factors upon an
76 encounter with a pathogen that not only orchestrate immune cell recruitment to the area
77 of inflammation, but also condition the immune response that is mounted as they
78 regulate the immune functions of their target cells (14). Chemokines have been shown
79 to be crucial for the elimination of many different viruses (2, 7, 10, 40), but on the other
80 hand, inappropriate persistence of chemokine expression in viral infections can drive
81 tissue damage and inflammation (2, 7, 18). Although the mucosal tissues (epithelium
82 and associated immune tissue) such as the skin or the gills provide a first line of defense
83 against viral entry, early innate signaling molecules such as chemokines are crucial for
84 the protection against viral infections. Therefore, for a complete understanding of
85 VHSV pathogenesis it is of great importance to study the chemokine response to VHSV
86 at these mucosal sites. Through the comparison of the chemokine response between the
87 fin bases and the gills in which the virus replicates very differently, we may deduce
88 whether the chemokine response is a consequence of viral encounter or if viral
89 replication influences the response.

90 In rainbow trout, twenty two different chemokine genes have been identified to
91 date, even though functional studies to determine their immune role have not yet been
92 performed for most of them, and only their chemotactic capacity has been described for
93 some of them (28, 36, 41). The extensive duplication events and the fact that
94 chemokines evolve more quickly than other immune genes, being one of the eight most
95 rapidly changing proteins as a reflection of different infectious experiences (42, 53) },
96 make difficult the establishment of true orthologues between fish and mammalian
97 chemokines. Therefore, no clear inferences as to the chemokine functions can be made
98 based on their similarities to potential mammalian counterparts, and their roles have to

be experimentally addressed. However, recent very complete studies have completely changed the previous phylogenetic grouping of fish chemokines into groups or clades trying to reflect better the ascription of orthologues and homologues with their mammalian counterparts (21, 42). Phylogenetic analysis of teleost CXC chemokine sequences have identified six different CXC chemokine clades: CXCa, CXCb, CXCc, CXCd, CXCL12, and CXCL14 (reviewed in (21)), but in rainbow trout, only representatives of three clades have been identified so far, IL-8 (CXCa) (26), γIP (CXCb) (24) and CXCd1/2 (54). Concerning CC chemokines, after the identification of CK1 (13), CK2 (30) and CK3 (EMBL Accession number AJ315149), fifteen new rainbow trout CC chemokine sequences were identified within expressed sequence tag (EST) databases (25), bringing the total to eighteen. Recently, seven large groups of fish CC chemokines have been established through phylogenetic analysis: the CCL19/21/25 group, the CCL20 group, the CCL27/28 group, the CCL17/22 group, the macrophage inflammatory protein (MIP) group, the monocyte chemotactic protein (MCP) group and a fish-specific group (42).

In this work, we analyzed the chemokine response to VHSV in the fin bases and gills by choosing representatives of each of these mentioned phylogenetic groups, and in those cases in which we observed an important effect of VHSV on their expression, we then proceeded to study all the chemokines within that group. Our results revealed that while only two specific chemokines were up-regulated in response to VHSV at the fin bases, a much wider effect was observed in the gills, where we detected no viral replication. Moreover, our studies revealed that are mainly chemokines belonging to phylogenetic groups CCL19/21/25 group and CCL27/28 which play a major role in mucosal immunity. As the skin revealed as a major chemokine-producing tissue and a

123 major viral replication site within the fin bases, we studied the capacity of different skin
124 cell types to support viral replication in combination with studies concerning the effects
125 that VHSV provoked on their capacity to produce chemotactic factors. Having observed
126 that dermis cells support active replication but viral translation is interrupted within
127 epidermis cells, a correlation between these differences in their susceptibility to VHSV
128 with the effect that VHSV has on their capacity to secrete chemotactic factors might be
129 established. Our results highlight the very specific chemokine response elicited by
130 VHSV in the area of viral entry in which the skin plays a major role, and suggests a
131 viral interference effect on the chemokine response, a key mechanism to begin an
132 effective local inflammation and a correct triggering of the systemic immune response.
133
134
135 **MATERIALS AND METHODS**
136
137 **Fish.** For bath infection experiments, healthy specimens of rainbow trout
138 (*Oncorhynchus mykiss*) were obtained from Centro de Acuicultura El Molino (Madrid,
139 Spain), located in a VHSV-free zone. Fish were maintained at the Centro de
140 Investigaciones en Sanidad Animal (CISA-INIA) laboratory at 14ºC with a re-
141 circulating water system, 12:12 hours L:D photoperiod and fed daily with a commercial
142 diet (Trow, Spain).
143 Prior to any experimental procedure, fish were acclimatized to laboratory
144 conditions for 2 weeks and during this period no clinical signs were ever observed. The
145 experiments described comply with the Guidelines of the European Union Council
146 (86/609/EU) for the use of laboratory animals.

147

148 **Virus preparation.** VHSV (0771 strain) was propagated in the RTG-2 rainbow
149 trout cell line. Cells were cultured at 18 ºC in Minimal Essential Medium (MEM,
150 Invitrogen, Carlsbad CA, USA) supplemented with 10 % fetal calf serum (FCS,
151 Invitrogen), containing 100 units/ml penicillin and 100 µg/ml streptomycin. The virus
152 was inoculated on RTG-2 grown in MEM with antibiotics and 2 % FCS at 14 ºC. When
153 cytophatic effect was extensive, supernatants were harvested and centrifuged to
154 eliminate cell debris. Clarified supernatants were used for the experiments. All virus
155 stocks were titrated in 96-well plates according to Reed and Müench (45).

156

157 ***VHSV bath infection.*** For the VHSV challenge, 30 rainbow trout of
158 approximately 4-6 cm were transferred to 2 l of a viral solution containing 5 $x10^5$
159 $TCID_{50}$/ml. After 1 h of viral adsorption with strong aeration at 14ºC, the water volume
160 was restored to 50 l. A mock-infected group treated in the same way was included as a
161 control.

162 At days 1, 3, and 6 post-infection, seven trout from each group were sacrificed
163 by overexposure to MS-222. The area surrounding the base of the dorsal fin and the
164 gills were removed for RNA extraction in the case of four or five fish and for
165 immunohistochemistry in the case of the other three.

166

167 **cDNA preparation.** Total RNA was extracted using Trizol (Invitrogen)
168 following the manufacturer´s instructions. Tissues were first homogenized in 1 ml of
169 Trizol in an ice bath, 200 µl of chloroform were added and the suspension was then

centrifuged at 12 000 x g for 15 min. The clear upper phase was aspirated and placed in a clean tube. Five hundred µl of isopropanol were then added, and the samples were again centrifuged at 12 000 x g for 10 min. The RNA pellets were washed with 75% ethanol, dissolved in diethylpyrocarbonate (DEPC)-treated water and stored at -80ºC.

RNAs were treated with DNAse I to remove any genomic DNA traces that might interfere with the PCR reactions. One µg of RNA was used to obtain cDNA in each sample using the Superscript III reverse transcriptase (Invitrogen). In all cases, RNAs were incubated with 1 µl of oligo $(dT)_{12-18}$ (0.5 µg/ml) and 1 µl 10 mM dinucleoside triphosphate (dNTP) mix for 5 min at 65ºC. After the incubation, 4 µl of 5x first strand buffer, 1 µl of 0.1 M dithiothreitol (DTT) and 1 µl of Superscript III reverse transcriptase were added, mixed and incubated for 1h at 50ºC. The reaction was stopped by heating at 70ºC for 15 min, and the resulting cDNA was diluted in a 1:10 proportion with water and stored at -20ºC.

Evaluation of chemokine gene expression by real time PCR. To evaluate the levels of transcription of the different chemokine genes studied, real-time PCR was performed with an Mx3005P™ QPCR instrument (Stratagene) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures containing 10 µl of 2x SYBR Green supermix, 5 µl of primers (0.6 mM each) and 5 µl of cDNA template were incubated for 10 min at 95ºC, followed by 40 amplification cycles (30 s at 95ºC and 1 min at 60ºC) and a dissociation cycle (30 s at 95ºC, 1 min 60ºC and 30 s at 95ºC). For each mRNA, gene expression was corrected by the elongation factor 1α (EF-1α) expression in each sample and expressed as $2^{-\Delta Ct}$, where ΔCt is determined by subtracting the EF-1α Ct value from the target Ct as previously described (8). The

194 primers used were designed from sequences available in the GenBank using the Oligo
195 Perfect software tool (Invitrogen) and are shown in Table I. All amplifications were
196 performed in duplicate to confirm the results. Negative controls with no template were
197 always included in the reactions. As controls for effective viral infection in the fin
198 bases, the levels of expression of the interferon (IFN) induced Mx gene and the VHSV
199 N gene were also evaluated by real time PCR using primers previously described (8).
200
201 **Light microscopy and immunocytochemistry** Fin tissue including the fin
202 bases obtained from control and VHSV-infected fish at different times post-infection
203 were fixed in Bouin's solution for 24 h, embedded in paraffin (Paraplast Plus;
204 Sherwood Medical) and sectioned at 5 μm. After dewaxing and rehydration, some
205 sections were stained with hematoxylin–eosin in order to determine the levels of
206 infiltration or any other apparent damages or pathological changes. Other sections were
207 subjected to an indirect immunocytochemical method to detect VHSV using 1P1D11, a
208 monoclonal antibody (mAb) specific to the G protein of VHSV, obtained from Dr N.
209 Lorenzen at the Danish Institute for Food and Veterinary Research (Århus, Denmark)
210 (31). The sections were first incubated for 30 min phosphate-buffered saline (PBS, pH
211 7.2-7.4) containing 5% BSA (PBT). Then sections were incubated overnight at 4ºC with
212 the mAb at an optimal dilution of 1:100 in PBS with 1% BSA. After washing in PBT,
213 the sections were exposed to anti-mouse IgG biotin-conjugated antibody (Sigma)
214 diluted 1:100 for 1 h at room temperature. The samples were then washed in PBT and
215 incubated for 1 h with Avidin-biotin alkaline phosphatase mouse IgG, The
216 immunocytochemical reactions were then revealed by incubation with Fast-Red (Sigma)
217 diluted in Tris-HCl buffer (pH 7.6) for 15 minutes at room temperature. The specificity

of the reactions was determined by omitting the first antiserum and comparing the results obtained in control fins. Slides were examined with an Axiolab (Zeiss) light microscope.

Skin primary cultures. Complete skin cultures were established after removing round sections of 1 cm of diameter of skin with a scalpel. For each rainbow trout, four different sections were obtained and each section was then placed in 24 well plates with 1 ml of Leibovitz medium (L-15, Invitrogen) supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin and 5% FCS.

In other cases, only epidermal cells were removed from round sections of 1 cm of diameter by scratching the skin surface with a scalpel. Posterior histological examination of the area showed that only epidermal cells were removed through this technique.

To determine the susceptibility of each of these cultures to VHSV, cultures were infected with VHSV at a final concentration of 5 x10^4 $TCID_{50}$/ml in culture medium with 2%FCS or mock infected with medium alone and incubated at 14ºC for different time points depending on the experiment performed.

Isolation of peripheral blood leukocytes (PBLs). PBLs were isolated from labeled rainbow trout from which fin explants or epidermal cultures had been established following the method previously described (16). Briefly, blood was extracted with a heparinized needle from the caudal vein and diluted 10 times with L-15 supplemented with antibiotics, 10 units/ml heparin and 2% FCS. The resulting cell suspension was placed onto 51% Percoll and centrifuged at 500 x *g* for 30 min at 4ºC.

242 The interface cells were collected and washed twice at 500 x *g* for 5 min in L-15
243 containing 0.1% FCS. The viable cell concentration was determined by Trypan blue
244 exclusion. Cells were resuspended in L-15 with 2% FCS at a concentration of $1x10^6$
245 cells/ml.
246
247 **Chemotactic capacity of supernatants from primary skin cultures infected**
248 **with VHSV.** Complete skin or epidermal cell cultures were infected with VHSV at a
249 final concentration of 5 $x10^5$ $TCID_{50}$/ml in culture medium with 2%FCS or mock
250 infected with medium alone. After 3 days of incubation at 14ºC, culture supernatants
251 were collected and tested on their capacity to induce the migration of PBLs from the
252 same individual rainbow trout. The same day that supernatants were collected, PBLs
253 were extracted from each trout and the chemotaxis assay later performed.
254 The chemotaxis assays were performed chemotaxis chambers introduced in 24-
255 well plates (Costar-Corning Life Sciences). Six hundred µl of 1:2 dilutions of the different
256 supernatants in culture medium were placed in the wells. Controls with media alone and
257 media and VHSV were also included. After introducing the chemotaxis chambers in each
258 of the wells, 100 µl of the PBL cell suspensions were loaded to the upper part of the
259 chamber. The upper and lower chambers are separated by a 3 µm pore-sized polycarbonate
260 filter. After 2h of incubation at 20ºC, the number of cells that had migrated to the bottom
261 of the wells was quantified by flow cytometry (FACSCalibur, Becton Dickinson). Cell
262 number was determined at constant flow time (1 min) of the medium in the lower
263 chamber. The migrating cells were analyzed based of forward and side light scatter
264 parameters. All experiments were performed in duplicate
265

266 **Western blot analysis of viral proteins.** Cell lysates were prepared from either
267 fin cultures or epidermal cell cultures exposed to VHSV as described above.
268 Electrophoresis of cell lysates and Western blotting were performed as described
269 previously (12, 49) using the IP5B11 monoclonal antibody recognizing the N protein of
270 VHSV provided by Dr N. Lorenzen at the Danish Institute for Food and Veterinary
271 Research (Århus, Denmark).
272
273
274 **RESULTS**
275
276 **Chemokine gene expression at the fin bases in response to VHSV.** In order to
277 elucidate the chemokine role at the fin bases, the main portal of VHSV entry, we
278 studied the levels of expression of a wide selection of rainbow trout chemokine genes
279 by real-time PCR upon a VHSV bath infection. Table II shows the mean levels of
280 transcription of all the chemokine genes studied at the fin bases in response to the
281 VHSV infection at days 1, 3 and 6 post-infection. We first determined the level of
282 expression of one rainbow trout chemokine representative gene ascribed to either one of
283 the CXC clades or CC chemokine groups previously described. As this first screening
284 revealed an important effect of VHSV on CK12 regulation of transcription, we also
285 included the other rainbow trout chemokines belonging to the CCL19/21/25 group, CK9
286 and CK10. Out of the twelve genes studies, VHSV was only capable of significantly up-
287 regulating the levels of expression of CK10 and CK12 at day 3 post-infection, and only
288 CK10 at day 6 post-infection, revealing a very specific response, as VHSV had been
289 proved to significantly up-regulate the transcription of many of these other chemokine

290 genes in other organs such as spleen or head kidney (37). No significant down-
291 regulations were observed.

292 One noticeable result was the level of constitutive transcription that we observed
293 in fin bases for CK9 and CK11. The mean constitutive expression levels for these two
294 chemokines varied throughout the sampling time points from 52 to 235 times the level
295 of expression of the EF-1α house-keeping gene for CK9, and from 7 to 72 times for
296 CK11, suggesting an important role of these chemokines in this fin base area. In
297 response to VHSV, the levels of expression of these highly expressed chemokines
298 decreased moderately, but differences were not significant.
299
300 **Chemokine gene expression at the gills in response to VHSV.** Since the
301 chemokine induction pattern in response to the virus at the fin bases was much more
302 specific in comparison to what we had previously observed in immune organs (37), we
303 studied in parallel the chemokine response to another mucosal tissue, the gills. Previous
304 studies had revealed that although some viral particles could be found in the gills upon
305 bath infection, this is not the main site of viral entry for fish rhabdoviruses (17) and
306 viral replication can only be detected in a few specific cells (4). In this case, the
307 chemokine response to the virus was very different than that observed in the fin bases
308 (Table III). At day 1 post-infection, VHSV provoked a significant up-regulation of the
309 levels of transcription of CK1, CK3, CK9 and CK11. At day 3, the only up-regulation
310 that is maintained is that of CK3, whereas at this point we found an unexpected down-
311 regulation of CK10 and CK12 transcription in response to the virus that is maintained
312 for CK12 at day 6. These results show that the response to a viral infection at mucosal
313 surfaces is highly specific, and most probably is dependant on both the cell composition

314 of the mucosal tissue and the degree of viral susceptibility of the different cell types
315 present.
316
317 **Viral gene and Mx transcription in response to VHSV in the fin bases and**
318 **gills.** To further understand the distinct chemokine response to VHSV of these two
319 mucosal tissues, we also evaluated the levels of transcription of the N viral gene as well
320 as the transcription of the IFN-induced Mx gene, since the levels of expression of Mx in
321 fish have been shown to correlate well with an efficient immune response to viral
322 replication (32).
323 Fig. 1 shows the Mx transcription in response to VHSV at the fin bases (Fig. 1A)
324 and gills (Fig. 1B). While the levels of Mx were significantly induced in the fin bases in
325 response to VHSV at day 3 post-infection, the levels of Mx transcription in the gills
326 were not significantly modulated by the viral encounter. These results are in correlation
327 with those obtained for the transcription of the N VHSV gene, since while a significant
328 viral transcription was detected at both days 1 and 3 post-infection at the fin bases (Fig.
329 2), no viral transcription was ever detected in our gill samples.
330
331 **VHSV replication at the fin bases.** To further understand the relation between
332 chemokine production and viral replication and confirm in which specific tissues the
333 virus was replicating at the fin bases, we next performed immunohistochemical studies
334 to detect VHSV G gene expression in this area (Fig. 3). The fin base section in which
335 we studied the chemokine response is represented in Fig. 3A showing the presence of
336 the different skin layers, muscle and cartilages that may support viral replication
337 differently upon viral entry. Among these different tissues, we detected the G viral

protein in the dermis and muscle but never in the epidermis layer of the skin (Fig. 3B-F).

VHSV actively replicates only in dermis cells but not in skin epidermis. To verify the results obtained through immunohistochemical studies of complete fin bases in which we had seen different susceptibility to VHSV in the different skin layers, we conducted *in vitro* experiments comparing the response of the complete dissectioned skin to epidermis cell cultures in which only this outermost layer was present as verified by histology. When studying viral transcription through real time PCR analysis of N gene expression, we detected active transcription in both explants and epidermal primary cultures (Fig. 4A) indicating that the virus was capable of entering both endodermis and epidermis cells thus transcribing its viral genome. Lower levels of transcription were observed in epidermis cells than in dermis cells, but the differences were not significant. No significant differences in the levels of Mx induction in response to VHSV were observed either between epidermis and complete skin. However, and in correlation to what we had previously observed through immunohistochemistry, viral N protein expression was strongly detected in fin explants in which dermis tissue was also present, but was only weakly detected in epidermis cultures (Fig. 4B). Finally, the titration of viral yields in these cultures further confirmed these results (Fig. 4C), since the viral titers were significantly higher after 7 days of infection in complete skin cultures than in epidermis cultures in which the viral titer obtained was even lower that the viral input. Therefore, it seems as VHSV is capable of entering both the dermis and the epidermis, but this outermost layer is

361 capable of interrupting the viral cycle to abort the infection at some point before the
362 translation of the viral genes.
363
364 **Epidermis cells are the main contributors to chemokine production.** As a
365 step towards the understanding of the contribution of each skin layer to the chemokine
366 response observed at the fin bases, we compared their chemokine production capacity of
367 the different skin layers through the primary skin cultures established. Among the CXC
368 chemokines studied, the skin only transcribed IL-8, at similar relative levels in
369 epidermis cells than in complete skin indicating that both dermis and epidermis
370 contribute to its mRNA expression (Fig. 5). Among CC chemokines, there were
371 specially the chemokines previously catalogued within the CCL19/21/25 and CCL27/28
372 groups, CK9, CK10, CK11 and CK12 that were being mainly expressed the different
373 skin layers. In the case of CK9, the epidermis does not seem as a major source for its
374 transcription. Apart from chemokines in these phylogenetic groups, the epidermis cells
375 strongly expressed CK6 and both types of skin cultures expressed CK3. .
376
377 **VHSV infection affects the chemotactic capacity of dermis and epidermal**
378 **skin cells differently**. Since VHSV replicates differently in dermis and epidermal cells,
379 we next wanted to determine the effect that VHSV had on their capacity to produce
380 chemotactic factors, and then compare these responses to the effects observed in the
381 original samples in which the chemokine transcription levels were determined. For this,
382 we obtained supernatants from either skin sections or epidermal primary cultures
383 infected or not with VHSV and compared their ability to attract autologous PBLs.

Supernatants derived either from fin explants or from epidermal cell cultures strongly attracted PBLs (Fig. 6). However, when these cultures were infected with VHSV prior to the supernatant generation, the effects differed in both types of cultures. When fin explants were infected with VHSV, the chemotactic capacity of the supernatants significantly decreased, whereas when epidermal cell cultures were infected with VHSV, the chemotactic capacity of their supernatants significantly increased. A clear ascription of these viral effects to a specific chemokine was not possible, as it may be an overall effect in which many of these skin chemokines cooperate. What seems clear is that epidermis and dermis cells produce a very different secretion of chemotactic factors in response to VHSV.

DISCUSSION

A complete knowledge of the early immune mechanisms triggered at the site of viral entry into the host, provides us with important information for the understanding of viral pathogenesis. It has been recently demonstrated that rhabdovirus enter the fish through the fin bases and moreover that early replication in this first site strongly conditions the outcome of the infection (17). While a virulent IHNV replicates in this area as a first step to then distribute itself through the organism, a low virulence IHNV remains confined to this area, highlighting the importance of the early local immune mechanisms for controlling rhabdoviral infections.

In this context and given the central role of chemokines in antiviral defense, we have determined which rainbow trout chemokines are modulated by a viral encounter in

this fin base area in comparison to the viral effect in a further mucosal tissue, the gills, in which the virus replicates poorly. Our results revealed that the local chemokine response is much stronger in a low replication tissue such as the gills that in one in which the virus efficiently replicates such as the fin bases. More in depth studies in this fin base area demonstrated that epidermal cells and dermal cells support differently VHSV replication, and again produce chemotactic factors differently in response to the virus, pointing again to an interference of viral replication with the chemokine response. This viral interference may be an important pathogenicity factor that may explain why the virus enters the body through the fin base in which the epidermis layer is thinner and even interrupted, highlighting as well the importance of the epidermis in the fish antiviral defense.

Although IL-8 is strongly expressed constitutively in the skin, when studying the effect of VHSV on CXC chemokines of mucosal tissues, we included all the genes that have been characterized in rainbow trout to date, and found no significant effect of the virus on their levels of transcription. This suggests that these chemokines, which are known to act preferentially on neutrophils and some lymphocyte subsets (6, 39), do not have a preferential role in mucosal antiviral immunity. CC chemokines, on the other hand, act primarily on monocytes instead of neutrophils, as well as on other specific lymphocyte subsets (50). In this case, we selected a group of chemokine genes belonging to different phylogenetic groups that had been proven to be strongly regulated in response to VHSV in lymphoid organs such as spleen and head kidney (37). In fact, VHSV strongly up-regulates γIP, CXCd, CK3, CK5B, CK6 and CK12 in spleen and γIP, CXCd, CK3 and CK12 in head kidney. In our current study, only CK10

and CK12 were significantly up-regulated in response to VHSV infection in the fin bases, whereas CK1, CK3, CK9 and CK11 were strongly up-regulated in the gills.

The great differences that were observed in the chemokine response to VHSV in fin bases and gills do not seem to be only a consequence of different chemokines being secreted by different cell types as the constitutive chemokine profile for both mucosal tissues is very similar, thus it seems that the key difference is whether an active viral replication is taking place or not. Having seen in our experiments that the infected fish suffered strong symptomatology from viral infection and began to die as early as 6 days post-infection (data not shown), we further studied viral replication in these locations. Viral replication was confirmed by analysis of viral gene transcription and immunohistochemistry in the fin bases, and an efficient IFN response was mounted as determined through the study of the IFN-induced Mx gene. On the contrary, no viral N protein expression was detected in the gills despite the fact that other authors had described a low-moderate VHSV replication in the gills focused in the cells lining the vessels of the primary gill arch (4). Therefore it seems as an efficient viral replication is not needed for the induction of an effective chemokine response, but on the other hand, the infected tissues have a suppressed reaction.

Furthermore, through the development of fin explants in which both epidermis and dermis cells are present and epidermis cell cultures in which no dermis cells were present in combination with immunohistochemistry studies, we could conclude that both epidermis and dermis cells supported viral transcription, although the levels of transcription were slightly lower in epidermis cells. However viral N protein expression was only observed in cultures in which dermal cells were present, indicating that epidermal cells were able to block the viral cycle at some point before viral protein

455 expression, in concordance to what is observed by immunohistochemistry. This
456 blockage of viral protein expression was also consistent with viral titration of primary
457 skin cultures, as the virus produced a significantly higher viral yield in complete skin
458 cultures than in epidermis cell cultures in which the viral yield did not increase
459 throughout the infection period. Similarly, RTS11 rainbow trout monocyte-
460 macrophages have also been shown to block VHSV replication at some point of the
461 viral cycle before the translation of viral proteins (49). Consequently, the effect that the
462 virus had on the capacity of these cells to produce chemotactic factors also differed
463 between dermis and epidermis cells, since while VHSV provokes an up-regulation of
464 the chemotactic factors produced by the epidermis, it provokes a down-regulation of the
465 chemotactic factors produced by epidermis and dermis cells together. It has been very
466 difficult to ascribe the chemokine responsible for these viral effects of the different
467 layers to a specific chemokine as it may be an overall effect observed due to the
468 combined effects of different chemokines. What seems clear however is that in cells that
469 can control the viral infection, an induced chemokine response is observed, whereas in
470 cells in which there is active viral replication, this defense mechanism is impaired. It
471 may be possible that this limitation is a consequence of a general shut-off mechanism
472 induced by VHSV upon translation as widely demonstrated for rhabdovirus (22), but
473 although this may explain the reduction of the chemotactic activity, it would not explain
474 the absence of chemokine transcription up-regulation, as the shut-off does not affect
475 mRNA synthesis (23). Some viruses such as poliovirus can block secretion of proteins
476 in infected cells thus blocking chemokine release, but this again would not explain the
477 direct effect of gene transcription.

478 On the other hand, it is well known that many viruses have developed strategies
479 to either exploit or avoid chemokine networks and thus replicate more efficiently (27).
480 For large DNA viruses, the most common strategy is the encoding of chemokine
481 homologs, chemokine receptor decoy homologs or soluble chemokine binding proteins
482 (1, 27, 47), but some RNA viruses have also developed strategies to directly interfere
483 with chemokine synthesis. For example, the non-structural (NS) 3/4A protein complex
484 of hepatitis C virus down-regulates the transcription of CCL5, IL-8 and γIP through the
485 inhibition of the retinoic acid inducible gene I (RIG-I) pathway (46). On the other hand,
486 many viruses interfere with the NF-κB pathway (20), which is know to be directly
487 responsible for the transcription of many chemokine genes (29, 52).
488 Finally, in a context in which the exact immune function is unknown for most
489 rainbow trout chemokines, our results point to an important role in mucosal immunity
490 of chemokines CK9, CK10, CK11 and CK12, ascribed to phylogenetic groups
491 CCL19/21/25 and CCL27/28 by Peatman and Liu (42). All of these chemokines were
492 either modulated by the virus at mucosal sites or were produced in very high
493 constitutive levels. Interestingly, major roles in mucosal immune responses have been
494 demonstrated for mammalian chemokines belonging to these two groups, CCL19/21/25
495 and CCL27/28 (3, 15, 19, 38, 51), therefore, although much more work should be done
496 to determine if these rainbow trout chemokines are the true orthologues of these
497 mammalian chemokines, it seems that some functional equivalence is observed.
498 In conclusion, we have demonstrated that a very restricted chemokine response
499 is observed to VHSV in the area of primary replication, the fin bases, where the virus
500 actively replicates in the dermis and muscle cells, while a much stronger chemokine
501 response is observed in the gills. Within the fin bases, epidermis cells are capable of

blocking viral replication before viral translation while the virus replicates in the dermis, in which the virus is capable of limiting the production of chemotactic factors. More work should be done to determine the exact mechanism through which the virus is capable of limiting the chemokine response upon its active replication.

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REFERENCES

1. **Alcami, A.** 2003. Viral mimicry of cytokines, chemokines and their receptors. Nat. Rev. Immunol. **3:**36-50.
2. **Alfano, M., and G. Poli.** 2005. Role of cytokines and chemokines in the regulation of innate immunity and HIV infection. Mol. Immunol. **42:**161-82.
3. **Baird, J. W., R. J. Nibbs, M. Komai-Koma, J. A. Connolly, K. Ottersbach, I. Clark-Lewis, F. Y. Liew, and G. J. Graham.** 1999. ESkine, a novel beta-chemokine, is differentially spliced to produce secretable and nuclear targeted isoforms. J. Biol. Chem. **274:**33496-503.
4. **Brudeseth, B. E., J. Castric, and O. Evensen.** 2002. Studies on pathogenesis following single and double infection with viral hemorrhagic septicemia virus and infectious hematopoietic necrosis virus in rainbow trout (*Oncorhynchus mykiss*). Vet. Pathol. **39:**180-9.

5. **Buchmann, K.** 1998. Histochemical characteristics of *Gyrodactylus derjavini* parasitizing the fins of rainbow trout (*Oncorhynchus mykiss*). Folia Parasitol. (Praha) **45:**312-8.
6. **Clark-Lewis, I., B. Dewald, T. Geiser, B. Moser, and M. Baggiolini.** 1993. Platelet factor 4 binds to interleukin 8 receptors and activates neutrophils when its N terminus is modified with Glu-Leu-Arg. Proc. Natl. Acad. Sci. U S A **90:**3574-7.
7. **Cook, W. J., M. F. Kramer, R. M. Walker, T. J. Burwell, H. A. Holman, D. M. Coen, and D. M. Knipe.** 2004. Persistent expression of chemokine and chemokine receptor RNAs at primary and latent sites of herpes simplex virus 1 infection. Virol. J. **1:**5.
8. **Cuesta, A., and C. Tafalla.** 2009. Transcription of immune genes upon challenge with viral hemorrhagic septicemia virus (VHSV) in DNA vaccinated rainbow trout (*Oncorhynchus mykiss*). Vaccine **27:**280-9.
9. **Chilmonczyk, S., I. Voccia, and D. Monge.** 1995. Pathogenesis of viral haemorrhagic septicaemia virus: cellular aspects. Vet. Res. **26:**505-11.
10. **Christensen, J. E., C. de Lemos, T. Moos, J. P. Christensen, and A. R. Thomsen.** 2006. CXCL10 is the key ligand for CXCR3 on CD8+ effector T cells involved in immune surveillance of the lymphocytic choriomeningitis virus-infected central nervous system. J. Immunol. **176:**4235-43.
11. **de Kinkelin, P., P. Besse, and G. Tuffery.** 1968. A recent necrosing disease of fish teguments and fins: larval bucephalosis due to *Bucephalus polymorphus* (Baer, 1827). Bull. Off. Int. Epizoot. **69:**1207-30.
12. **Dewitte-Orr, S. J., J. A. Leong, and N. C. Bols.** 2007. Induction of antiviral genes, Mx and vig-1, by dsRNA and Chum salmon reovirus in rainbow trout monocyte/macrophage and fibroblast cell lines. Fish Shellfish Immunol. **3**: 670-82.
13. **Dixon, B., B. Shum, E. J. Adams, K. E. Magor, R. P. Hedrick, D. G. Muir, and P. Parham.** 1998. CK-1, a putative chemokine of rainbow trout (*Oncorhynchus mykiss*). Immunol. Rev. **166:**341-8.
14. **Esche, C., C. Stellato, and L. A. Beck.** 2005. Chemokines: key players in innate and adaptive immunity. J. Invest. Dermatol. **125:**615-28.
15. **Gao, J. Q., Y. Tsuda, M. Han, D. H. Xu, N. Kanagawa, Y. Hatanaka, Y. Tani, H. Mizuguchi, Y. Tsutsumi, T. Mayumi, N. Okada, and S. Nakagawa.** 2009. NK cells are migrated and indispensable in the anti-tumor activity induced by CCL27 gene therapy. Cancer Immunol. Immunother. **58:**291-9.
16. **Graham, S., and C. J. Secombes.** 1988. The production of macrophage-activating factor from rainbow trout *Salmo gairdneri* leucocytes. Immunology **65:**293-297.
17. **Harmache, A., M. Leberre, S. Droineau, M. Giovannini, and M. Bremont.** 2006. Bioluminescence imaging of live infected salmonids reveals that the fin bases are the major portal of entry for Novirhabdovirus. J. Virol. **103:**3655-9.
18. **Heydtmann, M., and D. H. Adams.** 2009. Chemokines in the immunopathogenesis of hepatitis C infection. Hepatology **49:**676-88.
19. **Hieshima, K., Y. Kawasaki, H. Hanamoto, T. Nakayama, D. Nagakubo, A. Kanamaru, and O. Yoshie.** 2004. CC chemokine ligands 25 and 28 play essential roles in intestinal extravasation of IgA antibody-secreting cells. J. Immunol. **173:**3668-75.
20. **Hiscott, J., H. Kwon, and P. Genin.** 2001. Hostile takeovers: viral appropriation of the NF-kappaB pathway. J. Clin. Invest. **107:**143-51.

21. **Huising, M. O., R. J. Stet, C. P. Kruiswijk, H. F. Savelkoul, and B. M. Lidy Verburg-van Kemenade.** 2003. Molecular evolution of CXC chemokines: extant CXC chemokines originate from the CNS. Trends Immunol. **24:**307-13.
22. **Jayakar, H. R., E. Jeetendra, and M. A. Whitt.** 2004. Rhabdovirus assembly and budding. Virus Res. **106:**117-32.
23. **Kaariainen, L., and M. Ranki.** 1984. Inhibition of cell functions by RNA-virus infections. Annu Rev Microbiol **38:**91-109.
24. **Laing, K. J., N. Bols, and C. J. Secombes.** 2002. A CXC chemokine sequence isolated from the rainbow trout *Oncorhynchus mykiss* resembles the closely related interferon-gamma-inducible chemokines CXCL9, CXCL10 and CXCL11. Eur. Cytokine Netw. **13:**462-73.
25. **Laing, K. J., and C. J. Secombes.** 2004. Trout CC chemokines: comparison of their sequences and expression patterns. Mol. Immunol. **41:**793-808.
26. **Laing, K. J., J. J. Zou, T. Wang, N. Bols, I. Hirono, T. Aoki, and C. J. Secombes.** 2002. Identification and analysis of an interleukin 8-like molecule in rainbow trout *Oncorhynchus mykiss*. Dev. Comp. Immunol. **26:**433-44.
27. **Lalani, A. S., and G. McFadden.** 1999. Evasion and exploitation of chemokines by viruses. Cytokine Growth Factor Rev. **10:**219-33.
28. **Lally, J., F. Al-Anouti, N. Bols, and B. Dixon.** 2003. The functional characterisation of CK-1, a putative CC chemokine from rainbow trout (*Oncorhynchus mykiss*). Fish Shellfish Immunol. **15:**411-24.
29. **Le Page, C., O. Popescu, P. Genin, J. Lian, A. Paquin, J. Galipeau, and J. Hiscott.** 2001. Disruption of NF-kappa B signaling and chemokine gene activation by retroviral mediated expression of IKK gamma/NEMO mutants. Virology **286:**422-33.
30. **Liu, L., K. Fujiki, B. Dixon, and R. S. Sundick.** 2002. Cloning of a novel rainbow trout (*Oncorhynchus mykiss*) CC chemokine with a fractalkine-like stalk and a TNF decoy receptor using cDNA fragments containing AU-rich elements. Cytokine **17:**71-81.
31. **Lorenzen, N., N. J. Olesen, and P. E. V. Jorgensen.** 1988. Production and characterization of monoclonal antibodies to four Egtved virus structural proteins. Dis. Aquat. Org. **4:**35-42.
32. **McLauchlan, P. E., B. Collet, E. Ingerslev, C. J. Secombes, N. Lorenzen, and A. E. Ellis.** 2003. DNA vaccination against viral haemorrhagic septicaemia (VHS) in rainbow trout: size, dose, route of injection and duration of protection-early protection correlates with Mx expression. Fish Shellfish Immunol. **15:**39-50.
33. **Molnar, K.** 2002. Site preference of myxosporean spp. on the fins of some Hungarian fish species. Dis. Aquat. Org. **52:**123-8.
34. **Molnar, K., and C. Szekely.** 2003. Infection in the fins of the goldfish Carassius auratus caused by *Myxobolus diversus* (Myxosporea). Folia Parasitol. (Praha) **50:**31-6.
35. **Molnar, K., and C. Szekely.** 1997. An unusual location for *Ergasilus sieboldi* Nordmann (Copepoda, Ergasilidae) on the operculum and base of pectoral fins of the pikeperch (*Stizostedion lucioperca* L.). Acta Vet. Hung. **45:**165-75.
36. **Montero, J., J. Coll, N. Sevilla, A. Cuesta, N. C. Bols, and C. Tafalla.** 2008. Interleukin 8 and CK-6 chemokines specifically attract rainbow trout (*Oncorhynchus mykiss*) RTS11 monocyte-macrophage cells and have variable effects on their immune functions. Dev. Comp. Immunol. **32:**1374-84.

627 37. **Montero, J., E. Chaves-Pozo, A. Cuesta, and C. Tafalla.** 2009. Chemokine
628 transcription in rainbow trout (*Oncorhynchus mykiss*) is differently modulated in
629 response to viral hemorrhagic septicaemia virus (VHSV) or infectious pancreatic
630 necrosis virus (IPNV). Fish Shellfish Immunol. **27:**661-9.
631 38. **Morales, J., B. Homey, A. P. Vicari, S. Hudak, E. Oldham, J. Hedrick, R.**
632 **Orozco, N. G. Copeland, N. A. Jenkins, L. M. McEvoy, and A. Zlotnik.** 1999.
633 CTACK, a skin-associated chemokine that preferentially attracts skin-homing
634 memory T cells. Proc. Natl. Acad. Sci. U S A **96:**14470-5.
635 39. **Morohashi, H., T. Miyawaki, H. Nomura, K. Kuno, S. Murakami, K.**
636 **Matsushima, and N. Mukaida.** 1995. Expression of both types of human
637 interleukin-8 receptors on mature neutrophils, monocytes, and natural killer cells. J.
638 Leuk. Biol. **57:**180-7.
639 40. **Nansen, A., O. Marker, C. Bartholdy, and A. R. Thomsen.** 2000. CCR2+ and
640 CCR5+ CD8+ T cells increase during viral infection and migrate to sites of
641 infection. Eur. J. Immunol. **30:**1797-806.
642 41. **Omaima Harun, N., J. Zou, Y. A. Zhang, P. Nie, and C. J. Secombes.** 2008. The
643 biological effects of rainbow trout (*Oncorhynchus mykiss*) recombinant interleukin-
644 8. Dev. Comp. Immunol. **32**:673-81.
645 42. **Peatman, E., and Z. Liu.** 2007. Evolution of CC chemokines in teleost fish: a case
646 study in gene duplication and implications for immune diversity. Immunogenetics
647 **59:**613-23.
648 43. **Quillet, E., M. Dorson, G. Aubard, and C. Torhy.** 2007. In vitro assay to select
649 rainbow trout with variable resistance/susceptibility to viral haemorrhagic
650 septicaemia virus. Dis. Aquat. Org. **76:**7-16.
651 44. **Quillet, E., M. Dorson, G. Aubard, and C. Torhy.** 2001. In vitro viral
652 haemorrhagic septicaemia virus replication in excised fins of rainbow trout:
653 correlation with resistance to waterborne challenge and genetic variation. Dis.
654 Aquat. Org. **45:**171-182.
655 45. **Reed, L. J., and A. Muench.** 1938. A simple method of stimating fifty per cent end
656 points. J. Hyg. **27:**493-497.
657 46. **Sillanpaa, M., P. Kaukinen, K. Melen, and I. Julkunen.** 2008. Hepatitis C virus
658 proteins interfere with the activation of chemokine gene promoters and
659 downregulate chemokine gene expression. J. Gen. Virol. **89:**432-43.
660 47. **Smith, V. P., and A. Alcami.** 2000. Expression of secreted cytokine and chemokine
661 inhibitors by ectromelia virus. J. Virol. **74:**8460-71.
662 48. **Tafalla, C., A. Figueras, and B. Novoa.** 1998. In vitro interaction of viral
663 haemorrhagic septicaemia virus and leukocytes from trout (*Oncorhynchus mykiss*)
664 and turbot (*Scophthalmus maximus*). Vet. Immunol. Immunopathol. **62:**359-66.
665 49. **Tafalla, C., E. Sanchez, N. Lorenzen, S. J. DeWitte-Orr, and N. C. Bols.** 2008.
666 Effects of viral hemorrhagic septicemia virus (VHSV) on the rainbow trout
667 (*Oncorhynchus mykiss*) monocyte cell line RTS-11. Mol. Immunol. **45:**1439-48.
668 50. **Uguccioni, M., M. D'Apuzzo, M. Loetscher, B. Dewald, and M. Baggiolini.**
669 1995. Actions of the chemotactic cytokines MCP-1, MCP-2, MCP-3, RANTES,
670 MIP-1 alpha and MIP-1 beta on human monocytes. Eur. J. Immunol. **25:**64-8.
671 51. **Unsoeld, H., K. Mueller, U. Schleicher, C. Bogdan, J. Zwirner, D. Voehringer,**
672 **and H. Pircher.** 2007. Abrogation of CCL21 chemokine function by transgenic
673 over-expression impairs T cell immunity to local infections. Int. Immunol. **19:**1281-
674 9.

52. **Viatour, P., M. P. Merville, V. Bours, and A. Chariot.** 2005. Phosphorylation of NF-kappaB and IkappaB proteins: implications in cancer and inflammation. Trends Biochem. Sci. **30:**43-52.
53. **Waterston, R. H., K. Lindblad-Toh, E. Birney, J. Rogers, J. F. Abril, P. Agarwal, R. Agarwala, R. Ainscough, M. Alexandersson, P. An, S. E. Antonarakis, et al.** 2002. Initial sequencing and comparative analysis of the mouse genome. Nature **420:**520-62.
54. **Wiens, G. D., G. W. Glenney, S. E. Lapatra, and T. J. Welch.** 2006. Identification of novel rainbow trout (*Onchorynchus mykiss*) chemokines, CXCd1 and CXCd2: mRNA expression after *Yersinia ruckeri* vaccination and challenge. Immunogenetics **58:**308-23.

689

690 **Figure legends**

691

692 FIG. 1. Levels of Mx gene transcription in response to VHSV bath infection in fin bases
693 (A) and gills (B). Samples were collected from both infected and mock-infected
694 controls after 1 or 3 days of VHSV infection, RNA obtained and the levels of
695 transcription of the Mx gene determined by real time PCR. Individual data (black
696 circles) were analyzed in triplicate and are shown as the mean gene expression relative
697 to the expression of endogenous control EF1-α . *Relative mean expression (black bar)
698 significantly higher than the relative mean expression in the respective control ($p<0.05$).

699

700 FIG. 2. Levels of VHSV N gene transcription in the fin bases in response to VHSV. Fin
701 bases were collected from both infected and mock-infected controls after 1 or 3 days of
702 VHSV infection, RNA obtained and the levels of transcription of the N viral gene
703 determined by real time PCR. Individual data (black circles) were analyzed in triplicate
704 and are shown as the mean gene expression relative to the expression of endogenous
705 control EF1-α . *Relative mean expression (black bar) significantly higher than the
706 relative mean expression in the respective control ($p<0.05$).

707

708 FIG. 3. Immunohistochemical staining of fin bases infected with VHSV. Although the
709 immunohistochemical staining was performed with both control and infected fish
710 obtained at days 1, 3 and 6 post-infection, no significant differences in the level or
711 distribution of staining was observed in infected fish at different days post-infection.
712 Therefore, samples taken at day 1 post-infection are shown. A shows a control fin base

713 area stained with hematoxilin eosin corresponding to the area sampled in chemokine
714 expression studies (10X magnification). B, D and F constitute images from control fish
715 showing dermis, epidermis and muscle respectively, in which no staining is observed
716 (20x magnification). C, E and G constitute images from infected fish showing dermis,
717 epidermis and muscle respectively (20x magnification). Specific staining for VHSV in
718 red was always observed in muscle and dermis, but not in epidermis.
719
720 FIG. 4. Cultures were established from either from complete skin in which both
721 epidermis cells and dermis cells were present, or exclusively from epidermal cells.
722 These two types of skin cultures were infected *in vitro* with VHSV or mock-infected to
723 determine their susceptibility to VHSV. A, Levels of transcription of the viral N gene
724 and Mx gene transcription determined through real time PCR in complete skin or
725 epidermis cultures after 3 days of incubation with the virus *in vitro*. Data are shown as
726 the gene expression relative to the expression of endogenous control EF1-α obtained in
727 individual cultures or as stimulation indexes in the case of Mx obtained by dividing the
728 levels of Mx expression observed in response to VHSV to those observed in the
729 respective mock-infected culture. B, Detection of N viral protein through Western blot
730 in fin explants or epidermis cell cultures infected with VHSV (V) or mock-infected (C)
731 *in vitro* at day 3 post-infection. C, Viral titer obtained in four independent cultures after
732 7 days of infection with VHSV at a final concentration of 5 x10^4 $TCID_{50}$/ml.
733
734 FIG. 5. Levels of transcription of the different chemokine genes in the different skin
735 cultures established. RNA was extracted either from complete skin cultures or
736 epidermis cell cultures from which supernatants were collected and the levels of

737 transcription of these chemokines studied through real time PCR. Data are shown as the
738 mean chemokine gene expression relative to the expression of endogenous control EF1-
739 α ± SD of four independent cultures.
740
741 FIG. 5. Effect of VHSV on the capacity of the different skin cultures to secret
742 chemotactic factors. Cultures were established from either from complete skin in which
743 both epidermis cells and dermis cells were present, or exclusively from epidermal cells.
744 These two types of skin cultures were infected *in vitro* with VHSV or mock-infected
745 and incubated for 3 days at 14ºC. At this point, supernatants from VHSV-infected
746 cultures (VHSV SN) as well as from mock-infected cultures (Control SN) were
747 collected to determine the capacity of these supernatants to induce migration of
748 autologous PBLs comparing the migration to the migration observed towards media
749 alone (Control) or media with VHSV (VHSV). The chemotaxis assay was performed as
750 described in the Materials and Methods and FACS analysis used to enumerate the
751 number of migrating cells. Experiments were performed in triplicate and data are shown
752 as the mean number of migrating cells obtained in individual rainbow trout (RT) ± SD.
753 *Migration levels towards supernatants from infected cultures significantly different
754 than migration levels obtained with supernatants from mock-infected cultures ($p<0.05$).
755
756
757
758

TABLE I. Chemokine genes analyzed in this study with accession numbers, primer sequences and phylogenetic clade or group to which each of these chemokines is assigned.

GENE	ACC. NUMBER	PRIMER SEQUENCE (5'→3')	PHYLOGENETIC CLADE / GROUP
EF1-α	AF498320	F: GATCCAGAAGGAGGTCACCA R: TTACGTTCGACCTTCCATCC	
IL-8	AJ279069	F: ATTGAGACGGAAAGCAGACG R: CTTGCTCAGAGTGGCAATGA	CXCa
γIP	AJ417078	F: TGGACTGGTGAACCGTGTTA R: TCTTGGCAAATGGAGCTTCT	CXCb
CXCd	DQ191448 (1) DQ191448 (2)	F: GCTCACACTGCTCTAAGGAAGAA R: GGAGAGAGTCTCAATGGAACGT	CXCd
CK1	AF093802	F: GATGGCTGAAAGGCTACACC R: TGGGATTTGTTCTCCTGACG	CCL20
CK3	AJ315149	F: AGATCACCGTTCCCATCATC R: GTGACTTTCTGGCCATCTCC	Fish CC group
CK5B	CA374135	F: TTTGCTGATCGTCAGATACCC R: GTGTCTGCTCCCCAGACTTC	MIP group
CK6	CA355962 (A) CA355812 (B)	F: TGAAAGGCCTACGAATCTGC R: GTTGTTGTTGGCTGGTTGTG	CCL17/22
CK7A	CA343117	F: CCGAGAATCCCTCTTCAACA R: TCATCGTCGTCTTGGCAGTA	MCP group
CK9	CA378686	F: GGCTCTTATGGGAACTGCTG R: CTGGGATTGGCACAAACAG	CCL19/21/25
CK10	CA361535	F: ATTGCCAAGATCCTCTTCTGTGTTC R: CCTGAGGCTGGTAACCTATGACAAC	CCL19/21/25
CK11	BX072681	F: CCTTTGAGCATACTAATGCGAGTGG R: GTCTGCACAATACTTCCTCCCATTG	CCL27/28
CK12	CA358073 (A) CA346383 (B)	F: GACATCGATGCCACTGTGTT R: GGAGATGGTTCGCTCCAGAC	CCL19/21/25
CCR7	CX721232 CU065128	F: TTCACTGATTACCCCACAGACAATA R: AAGCAGATGAGGGAGTAAAAGGTG	
CCR9	AJ003159.1	F: TCAATCCCTTCCTGTATGTGTTTGT R: GTCCGTGTCTGACATAACTGAGGAG	

TABLE II. Levels of transcription of a selection of rainbow trout CXC and CC chemokines in the fin bases in response to VHSV bath infection. At days 1, 3 and 6 post-injection four trout from both the infected and the mock-infected group were sacrificed, RNA extracted and the levels of expression of the different chemokines studied through real-time PCR in triplicate. Data are shown as the mean chemokine gene expression relative to the expression of endogenous control EF1-α ± SD. *Values in bold constitute relative expression levels significantly higher than the relative expression in respective control ($p<0.05$). ND= levels of expression not detected.

	Day1		**Day 3**		**Day 6**	
	Control	**VHSV**	**Control**	**VHSV**	**Control**	**VHSV**
IL-8	0.0152 ± 0.02	0.0022 ± 0.00	0.0274 ± 0.05	0.0060 ± 0.00	0.0359 ± 0.02	0.0396 ± 0.05
γIP	0.0272 ± 0.01	0.0284 ± 0.02	0.0699 ± 0.13	1.1948 ± 0.73	0.0130 ± 0.01	0.0548± 0.07
CXCd	0.2902 ± 0.30	0.0279 ± 0.01	0.0225 ± 0.01	0.0431 ± 0.07	0.0350 ± 0.04	0.0064 ± 0.00
CK1	ND	ND	ND	ND	ND	ND
CK3	0.6826 ± 0.76	0.2642 ± 0.18	0.3118 ± 0.44	2.7193 ± 2.64	1.1951 ± 1.16	1.2815 ± 0.60
CK5B	0.0245 ± 0.01	0.0108 ± 0.00	0.0182 ± 0.02	0.0243 ± 0.02	0.0146 ± 0.01	0.0057 ± 0.00
CK6	0.3394 ± 0.41	0.0443 ± 0.04	0.2033 ± 0.38	0.0305 ± 0.02	0.0309 ± 0.02	0.2558 ± 0.47
CK7A	ND	ND	ND	ND	ND	ND
CK9	235.0912 ± 198.07	58.8618 ± 68.73	54.0372 ± 77.60	38.4374 ± 36.95	52.7418 ± 46.44	10.7460 ± 13.26
CK10	0.0309 ± 0.02	0.0168 ± 0.01	0.0146 ± 0.01	**2.0167 ± 1.10**	0.0158 ± 0.00	**0.9253 ± 0.45**
CK11	72.8666 ± 123.19	4.0194 ± 3.08	36.9005 ± 68.64	7.1217 ± 7.58	7.8178 ± 6.53	1.0905 ± 1.25
CK12	1.1877 ± 0.91	1.2991 ± 0.93	0.9388 ± 1.04	**33.4860± 30.00**	3.6679 ± 2.54	1.0162 ± 0.66

TABLE III. Levels of transcription of a selection of rainbow trout CXC and CC chemokines in gills in response to VHSV bath infection. At days 1, 3 and 6 post-injection five trout from both the infected and the mock-infected group were sacrificed, RNA extracted and the levels of expression of the different chemokines studied through real-time PCR in triplicate. Data are shown as the mean chemokine gene expression relative to the expression of endogenous control EF1-α ± SD. *Values in bold constitute relative expression levels significantly higher than the relative expression in respective control, whereas values underlined constitute expression levels significantly lower than relative expression in the respective control ($p<0.05$). ND= levels of expression not detected.

	Day1		**Day 3**		**Day 6**	
	Control	**VHSV**	**Control**	**VHSV**	**Control**	**VHSV**
IL-8	0.0005 ± 0.00	ND	0.0004 ± 0.00	0.0002 ± 0.00	0.0046 ± 0.00	0.0041 ± 0.01
γIP	0.0006 ± 0.00	0.0006 ± 0.00	0.0004 ± 0.00	ND	1.2317 ± 1.66	1.3387 ± 1.49
CXCd	0.0629 ± 0.09	0.0049 ± 0.01	0.1149 ± 0.15	0.04 ± 0.03	0.0275 ± 0.06	1.1964 ± 1.31
CK1	0.0036±0.00	**0.0536±0.03**	ND	ND	ND	ND
CK3	0.0341 ± 0.03	**5.7203 ± 5.25**	0.2413 ± 0.31	**7.380 ± 4.79**	0.0041 ± 0.01	0.0039 ± 0.01
CK5B	ND	ND	0.0004 ± 0.00	0.0002 ± 0.00	0.0680 ± 0.06	0.0309 ± 0.03
CK6	0.4987 ± 1.00	0.0137 ± 0.01	0.05108 ± 0.07	0.0925 ± 0.12	0.0986 ± 0.18	0.1102 ± 0.19
CK7A	ND	ND	ND	ND	ND	ND
CK9	0.0611 ± 0.03	**27.4551 ± 18.66**	0.1843 ± 0.22	0.2556 ± 0.25	0.7172 ± 1.09	3.8930 ± 0.02
CK10	0.0117 ± 0.01	0.0120 ± 0.02	1.6597 ± 1.97	ND	0.5842 ± 1.09	0.01601 ± 0.02
CK11	0.0117 ± 0.01	**21.9521 ± 18.15**	0.5777 ± 0.90	281.5384 ± 304.76	1.0340 ± 1.26	1.5975 ± 2.23
CK12	0.2628 ± 0.25	0.1809 ± 0.17	1.766 ± 1.20	0.1714 ± 0.09	279.6089 ± 168.53	12.7088 ± 8.22

Fig. 1

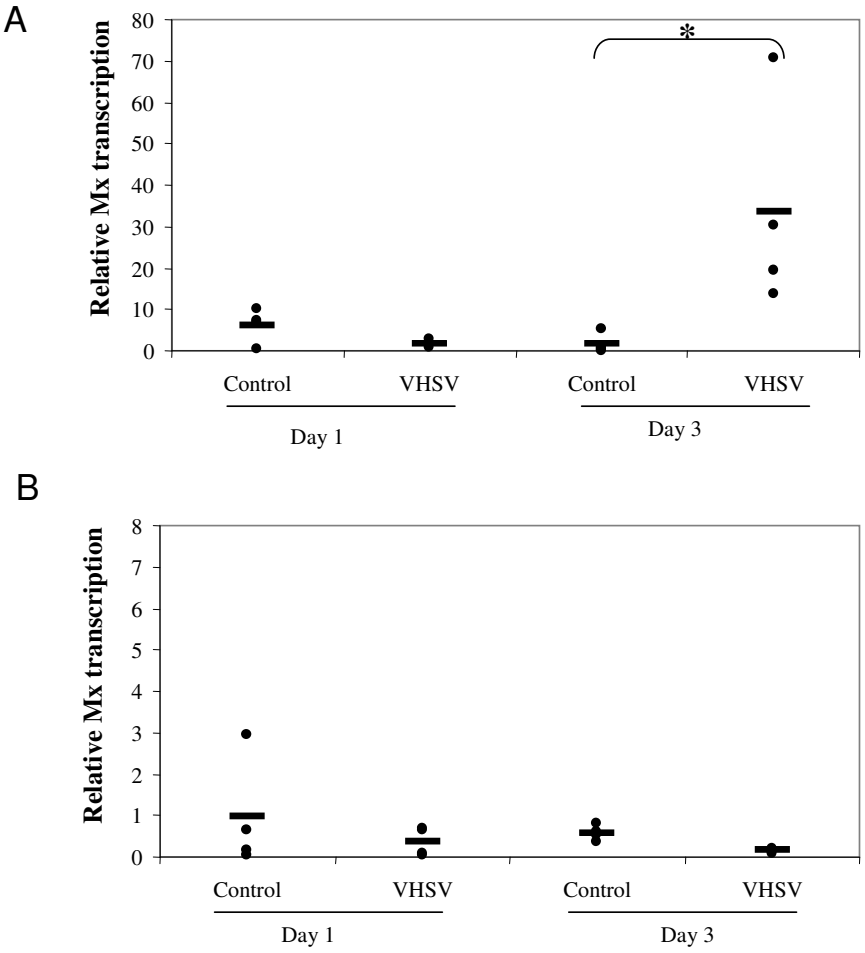


Fig. 2

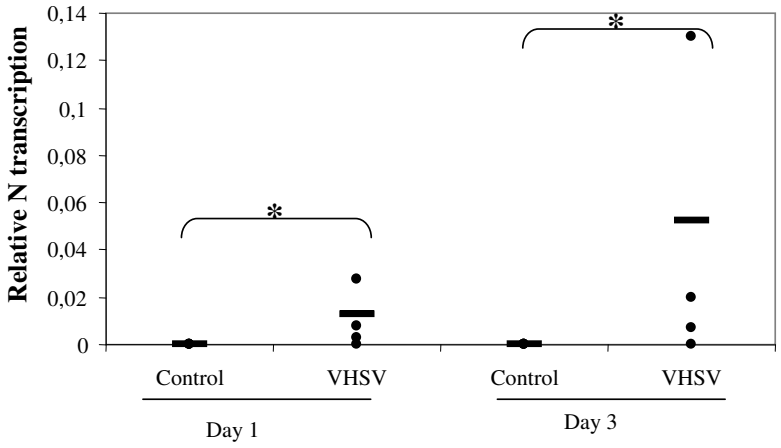


Fig. 3

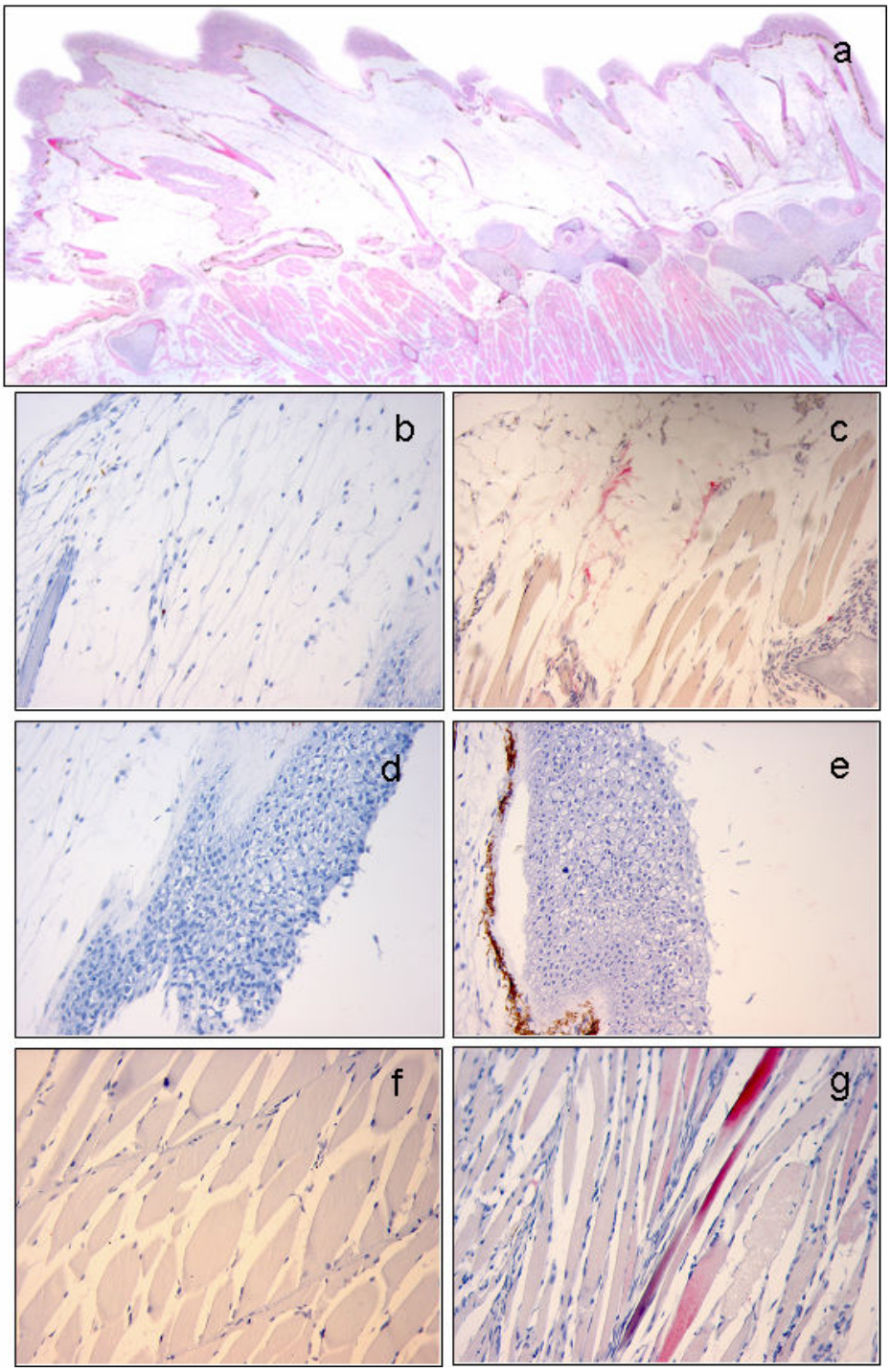
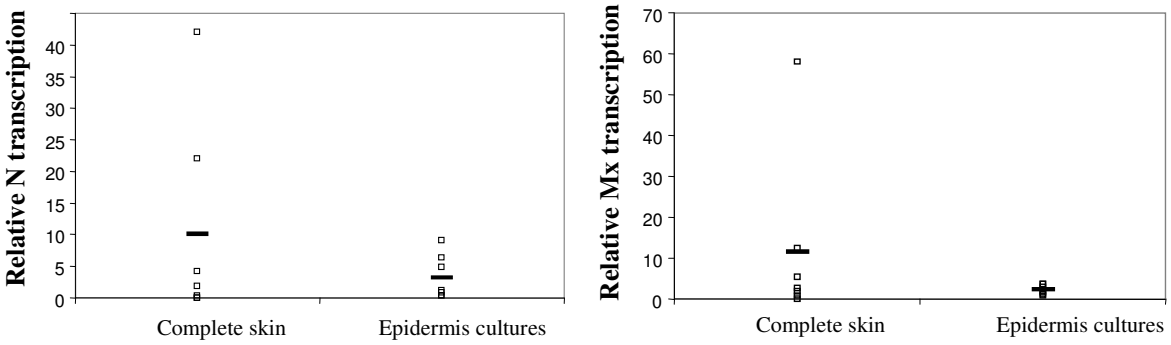
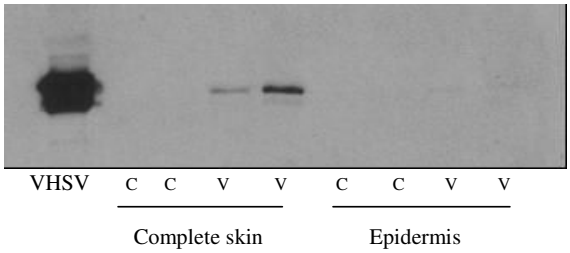


Fig. 4

A



B



C

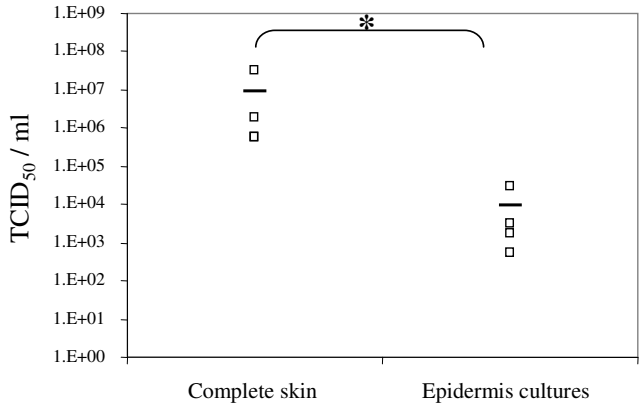


Fig. 5

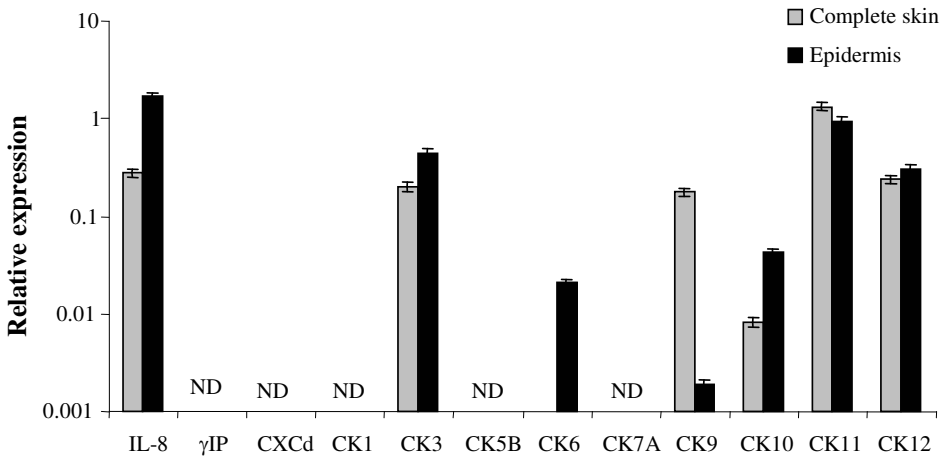


Fig. 6