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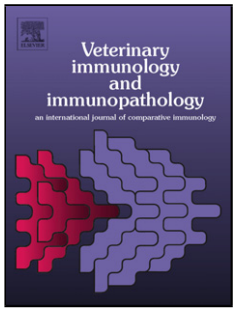

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CK-2 of rainbow trout (*Oncorhynchus mykiss*) has two differentially regulated alleles that encode a functional chemokine

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The nucleotide sequence data reported in this paper has been submitted to the GenBank nucleotide sequence databases and have been assigned the accession numbers: AY372431, AY372432 and AY372433.

Highlights

- Rainbow trout CK-2 has two differentially expressed alleles, referred to here as CK-2 and CK-2.1
- CK-2 and CK-2.1 transcripts are both present *in vitro* and *in vivo* but display tissue/cell culture dependent expression in both control and PHA stimulated samples
- A polyclonal antibody to recombinant CK-2/CK-2.1 was developed.
- CK-2/CK-2.1 protein production was only observed in the brain, liver and head kidney of PHA stimulated rainbow trout samples.
- Both peripheral blood leukocytes and the RTS-11 cell line migrated towards recombinant CK-2 in a concentration dependent matter

Abstract (At 282 words right now, max 400 words)

Rainbow trout chemokine 2 (CK-2) is currently the only known CC chemokine to have a mucin stalk. Further analysis of the mucin stalk region revealed a second, related CC chemokine sequence, denoted here as CK-2.1. This second sequence was determined to be an allele of CK-2 following genomic PCR analysis on several outbred individuals. Furthermore, in both *in vivo* and *in vitro* trials, CK-2 and CK-2.1 were both present, but appeared to have differential tissue expression in both control and PHA stimulated samples. Upon the development of a polyclonal antibody to rCK-2, CK-2 was only observed in the brain, liver and head kidney of PHA stimulated rainbow trout tissues. In comparison, when using the rainbow trout monocyte/macrophage-like cell line, RTS-11, CK-2 protein was observed in both control and PHA stimulated conditions. When studying the function of CK-2, a chemotaxis assay revealed that both peripheral blood leukocytes and RTS-11 cells migrated towards rCK-2 significantly at all concentrations studied when compared to truncated β_2m. Interestingly, this migration was lowest at both the highest concentration and the lowest concentrations of CK-2. Thus, teleostean

chemokine receptors may become desensitized when overstimulated as has been observed in mammalian models. The observed chemotactic function was indeed due to rCK-2 as cell migration was inhibited through pre-treatment of both the cells and the polyclonal antibody with rCK-2. As has been observed thus far with all other chemokines, CK-2 does appear to function through binding to a G-coupled protein receptor as chemotaxis could be inhibited through pre-treatment with pertussis toxin. Overall, the results of this study indicate that CK-2 is a functional chemokine that is encoded by two differentially expressed alleles in rainbow trout, CK-2 and CK-2.1.

Keywords: phytohemagglutinin (PHA), peripheral blood leukocytes, rainbow trout, chemotaxis, CC chemokine

1. Introduction

The immune system of vertebrates is tremendously complex, involving numerous cell types, barriers and specialized systems which are all used to prevent the entry and colonization of foreign entities throughout the body. The function of this vast network is dependent upon the ability of immune cells to migrate and interact with one another, a role fulfilled by extracellular mediators known as chemokines. Chemotactic cytokines, or chemokines, are a large family of small cytokines responsible for controlling the migratory patterns and positioning of immune cells (reviewed in Griffith et al, 2014). Chemokines were originally believed to have only pro-inflammatory functions but further study has revealed that these small proteins also play critical roles in both tissue homeostasis and development (Tachibana, et al, 1998, Gouon-Evans et al, 2002, Virgintino et al, 2013). Regardless of their specific function, all chemokines initiate their actions through binding to appropriate receptors which can be located on a variety of cell types. Of the chemokine receptors discovered thus far, all belong to the family of pertussis toxin (PTX) sensitive, G-coupled protein receptors (GCPR) (reviewed in Rossi and Zlotnik, 2000).

In the highly-studied mammalian model, 46 chemokine genes are currently known (reviewed in Zlotnik et al, 2006, Nomiyama et al, 2010) and are segregated into four families based on their differences in their structure and function. The largest family are the CC chemokines, so named because the first two of four cysteine residues are found adjacent to each other in members of this group. In comparison, a second family, called the CXC chemokines, has a single amino acid residue located between the first two cysteines. The third family of chemokines, the CX_3C family, consists of a single member, fractalkine. Like the nomenclature of the previous families, the CX_3C group has three amino acid residues separating the first two cysteines of these molecules. The fourth and final family of chemokines has a single cysteine residue and consists of one member, lymphotactin (reviewed in Charo and Ransohoff, 2006). Of these many mammalian chemokines, there are only two known to contain a mucin stalk: fractalkine and CXCL16 (Bazan et al, 1997, Matloubian et al, 2000). The mucin stalk can play an interesting role in chemokine function as it enables both membrane bound and extracellular forms of the attached chemokine depending on cleavage in this region (Haskell et al, 2000, Yoneda et al, 2003).

Recently, there has been a great deal of interest pertaining to the characterization and study of chemokines in teleost species (Laing and Secombes, 2004). As the largest group of vertebrates, consisting of almost 26,000 species (McKenzie et al, 2011), obtaining a deeper understanding of the function and diversification of teleost chemokines could provide insight into the origins and evolution of these small proteins. Since the discovery of the very first teleostean chemokine in rainbow trout (Dixon et al, 1998) several chemokines have been revealed in multiple teleost species including eighteen in rainbow trout, thirty in Atlantic salmon, and eighty-one in zebrafish (Peatman and Liu, 2007, Nomiyama et al, 2008, Bird and Tafalla, 2015). As chemokines have been recognized as one of the eight most rapidly evolving proteins (Waterston et al, 2002) clear orthologues between fish and mammalian counterparts are rarely observed. As a result, the function of each newly discovered chemokine in individual teleost species must be experimentally determined.

A unique CC chemokine in rainbow trout is CK-2. The rainbow trout CK-2 gene was discovered in 2002 and is the only CC chemokine known to have a mucin stalk (Liu et al., 2002). In the original description of this gene, northern blots gave two bands, which suggested an additional CK-2 transcript that could have arisen from

either alternative splicing or from an allele. In this study, a CK-2 allele has indeed been discovered and is designated CK-2.1. Whether these chemokines are functional is of special interest because of their mucin stalks but to date CK-2 functions have yet to be explored. Therefore, another aspect to our study has been the generation of recombinant CK-2 and a polyclonal antibody to CK-2. Together these reagents have allowed the function and tissue expression of CK-2 to be examined for the first time.

2. Materials and Methods

2.1 Fish

Rainbow trout (150-200 g) were obtained from Mimosa Springs Hatchery (Guelph, ON) and kept in 200 L freshwater flow-through tanks at the University of Waterloo. All fish were kept and handled under a permit from the University of Waterloo Animal Care Committee according to CCAC guidelines. Blood was drawn from the caudal vein after anesthesia with 0.01% MS-222 and tissue samples either had leukocytes isolated immediately or were collected in RNA Later and stored at -80ºC for later use. All procedures were performed following guidelines of the Animal Care Committee of the University of Waterloo.

2.2 Maintenance of cell lines

Three cell lines were used for these studies: the gonadal fibroblast RTG2 (Wolf and Quimby, 1962), the spleen monocyte/macrophage RTS11 (Ganassin and Bols, 1998) and the spleen stromal RTS 34 ST (Ganassin and Bols, 1999). The cell lines were routinely maintained in the basal medium L15 with a supplement of fetal bovine serum (FBS) as recently reviewed (Bols et al., 2017).

2.3 Determination of CK-2 genotypes

Genomic DNA was extracted from RTS-11, RTG2, RTS 34 ST and from the whole blood of 55 individual rainbow trout as described by Sambrook et al, 1989. It was previously observed by Liu et al (2002) that there are at least two copies of CK-2 in rainbow trout. These variants differed in size, thus primers were developed to further explore the origin of this variability. The four primer sets used in this study (Table 1) flanked the mucin stalk which amplified the region containing the size difference between CK-2 and CK-2.1. The PCR parameters were as follows: 95°C for 5 min; 30 cycles of 95°C for 30 seconds (sec), the appropriate annealing temperature for the primer pair for 30 sec (see Table 1) and 72°C for 1 min followed by a final elongation of 72°C for 5 min. The amplified PCR products were run on a 1.5% agarose gel containing 0.1% ethidium bromide (EtBr) and the DNA bands were visualized with a UV transilluminator containing an EtBr filter. Based on the band sizes, rainbow trout individuals and cell lines were determined to be homozygous for CK-2, homozygous for CK-2.1 or heterozygous.

Upon genotyping of the 55 outbred rainbow trout for CK-2/CK-2.1, the data was assessed to determine whether the alleles were in Hardy-Weinberg Equilibrium using the equation of $p^2 + 2pq + q^2 = 1$ where p represented the frequency of CK-2 and q represented the frequency of CK-2.1.

2.4 Peripheral blood leukocytes (PBL) and head kidney leukocytes (HKL) isolation

Peripheral blood leukocytes (PBLs) were separated by density gradient centrifugation using Histopaque 1077 (Sigma-Aldrich) according to the manufacturer's instructions. Cells were stained with trypan blue to determine viability and counted using a hemocytometer (Hausser Scientific) to give a final concentration of $1x10^7$ live cells/ml in L-15 medium supplemented with 20% FBS, 100 U/ml of penicillin and 100 U/ml of streptomycin. Head kidney leukocytes were isolated in the same manner at a density of $1x10^7$ cells/ml in RPMI-1640 medium containing 25 mM Hepes, 10000 U/ml penicillin, 10 mg/ml streptomycin and 200 units/ml of heparin sodium salt.

2.5 Studying CK-2 and CK-2.1 transcript expression in vivo and in vitro

Reverse-Transcription Polymerase Chain Reaction (RT-PCR) was performed to study the expression of CK-2 and CK-2.1 *in vivo* and *in vitro* and in response to the plant lectin, phytohemagglutinin (PHA). To detect variations in expression of CK-2 following stimulation *in vivo*, six heterozygous rainbow trout were given an intravenous injection of PHA at a final concentration of 50 μg per 250g of body weight. Two

non-stimulated fish (PBS injected) were sacrificed at the time of injection (0hr), and two stimulated fish were sacrificed at 4hr, 8hr, and 24hr after stimulation so that head kidney (HK), spleen and brain samples could be obtained for each of these time points.

To detect *in vitro* expression of CK-2 and CK-2.1, as well as potential upregulation in response to PHA, primary PBL and HKL were isolated as described in section *2.4.* Pre-screened rainbow trout containing all three possible allelic combinations were chosen for this experiment and both blood and head kidney samples were obtained from each fish to isolate leukocytes. For each of the three possible genotypes, both PBLs and HKLs were plated in a 6-well cell culture plate at a density of $1x10^7$ cells/well. Following overnight adherence at room temperature (RT), all experimental plates were treated with 10µg/ml PHA per well while control plates were treated with an equivalent volume of 1X phosphate buffered saline (PBS) for 24 hours. RNA was extracted from both the control and stimulated plates to be used for RT-PCR expression analysis.

Transcript expression of CK-2 and CK-2.1 was also analyzed *in vitro* using the RTS-11, RTG2 and RTS 34 ST cell lines. For experimental use, RTS-11 was plated in 6-well cell culture plates at a density of $1x10^7$ cells/well while RTG2 and RTS 34 ST were both seeded at a density of $3x10^5$ cells/well. Following overnight adherence at RT for RTS-11 and 18ºC for RTG2 and RTS 34 ST, experimental wells were stimulated with 10 µg/ml PHA per well while control plates were treated with an equivalent volume of 1X phosphate buffered saline (PBS) for 24 hours. RNA was then extracted from both the control and stimulated plates as described below.

2.5.1 RNA extraction and cDNA synthesis

Depending on the sample used, either 50 mg of tissue or a cell pellet containing a maximum of 1×10^7 cells was homogenized in 1 ml of Trizol reagent and RNA was extracted according to the manufacturer's protocol (Invitrogen). To shear genomic DNA, the RNA sample was passed through a 23-gauge needle 5 times. The RNA was dissolved in 20 µl of DEPC water and stored at -80ºC until used.

A RT-PCR kit (Fermentas) was used to synthesize cDNA from RNA as per the manufacturer's protocol. This procedure was followed by a PCR reaction using forward and reverse primers spanning an intron to detect potential genomic DNA contamination (see Table 1) using the same PCR protocol as described above in section *2.3*. As an internal control, PCR was performed using primers for elongation factor alpha (EF1-α) as outlined in Table 1. For this control gene, the PCR parameters were as follows: 30 sec at 95ºC, 30 cycles of 94ºC for 40 sec, 30 sec at 54ºC, 1 min at 72ºC and a final elongation of 5 min at 72ºC.

2.6 Development of polyclonal antibodies to CK-2

2.6.1 Construction of CK-2 expression vector

The full coding region of CK-2 was amplified using PCR primers designed to contain BamHI and HindIII sites in the sense and antisense primers respectively using rainbow trout blood cDNA as the template. The amplified PCR product was ligated into pGEM T-easy (Promega) and sequenced at the center for Applied Genomics (Toronto, ON, CA) to confirm the sequence identity. The CK-2 sequence was excised and ligated into the expression vector, pRSET A, and then transformed into BL21 (DE3) *Escherichia coli* as described previously by Sever et al, 2013.

2.6.2 Production and purification of recombinant CK-2

Fifty milliliters of overnight culture grown at 37ºC was used to inoculate 500 ml of LB broth. The cells were grown until the OD600 reached 0.5 and then induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 mM. Following induction, the cells were grown for another 5 h and harvested by centrifugation at 10,000 rpm for 10 min. Protein was purified using Ni-NTA Agarose (Qiagen, Mississauga, ON) according to manufacturer's instruction. Briefly, one ml of Ni-NTA Agarose resin was added to 10 ml of cleared cell lysate and incubated for 1 h at 4 C. After incubation, the lysate was loaded onto a 25 ml econocolumn (Bio-Rad) and washed with binding buffer (100 mM NaH_2PO_4, 10 mM Tris, 8 M urea) equilibrated to pH 8.0, pH 6.3, and pH 5.9. Finally, recombinant CK-2 (rCK-2) was eluted at pH 4.5 and was stored at 4ºC. The elution fractions were separated on a 17% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Following SDS-PAGE, the proteins were transferred onto a nitrocellulose membrane blocked with 5% skim milk in TBS-T buffer (0.14 M NaCl, 2.7 mM KCL, 25 mM Tris, 0.5% Tween 20, pH 8) for 1 h. The membrane was washed with TBS-T for 5 min and probed with 1:5000 dilution of mouse anti-

express antiserum (Invitrogen, Carlsbad, CA) to identify the specific Xpress epitope tag on the recombinant protein.

2.6.3 Production of polyclonal antibodies to CK-2

Two New Zealand white rabbits (Charles River, ON, CA) were injected intramuscularly with an initial dose of 0.5 mg of rCK-2 with Freund's complete adjuvant (1:1). A boost using 0.5 mg rCK-2 with Freund's incomplete adjuvant (1:1) was given every three weeks. Rabbits were exsanguinated after 12 weeks with ear bleeds performed every three weeks to verify the antibody titer.

To determine the specificity of the antibody, extracts from rainbow trout tissues (as described in 2.6.3) were prepared, run on an SDS-PAGE gel and transferred to nitrocellulose membranes (Bio-Rad) for western blot analysis. The nitrocellulose membranes were incubated with or without recombinant CK-2 protein (0.5 mg/ml) added to the polyclonal antiserum. When the recombinant protein was absent, bands of 36, 98 and ~250 kDa were detected (see **Fig. 5**). No bands were detected when the rCK-2 protein was present. Thus all bands detected with the developed antibody are variants of CK-2 or CK-2.1. It is anticipated that multiple isoforms may be detected by this antibody due to its polyclonal nature. Further, blots with recombinant CK-2 were probed with pre-immune serum, crude anti-CK2 at a 1:5000 dilution and affinity purified anti-CK2 at a 1:50 dilution.

2.7 CK-2 protein levels in vivo and in vitro

Western blotting with the antibody prepared in the above section was used to study the expression of the CK-2 proteins *in vivo* with and without 24 h PHA stimulation and *in vitro* with PHA stimulated RTS11 cultures with and without FBS. Rainbow trout heterozygous for CK-2 and CK-2.1 received intravenous (i.v.) injections of PHA at a final concentration of 50μg per 250g of body weight. Control fish received an i.v. injection of 100 μl of 1X PBS. After 24 hours, fish were sacrificed and head kidney (HK), liver, peripheral blood leukocytes (PBL), brain, spleen, intestine, muscle, and heart were collected. Protein was extracted from these tissues for western blot analysis. RTS-11 cells exposed to PHA in same manner as described above for transcript expression analysis. Following 24 h stimulation, protein was extracted from control and test wells and subject to western blot analysis as described below.

For protein extraction, 300 ml of NP-40 lysis buffer (1% (v/v) NP-40, 150 mM NaCl, 50 mM Tris [pH 8.0]) supplemented with 1X protease inhibitor cocktail (Roche) was added to either 70 mg of tissue or a cell pellet containing approximately 2 x 10^7 cells. Tissues were homogenized through sonication on ice in short bursts and crude lysates were cleared by centrifuging at 4°C for 20 min at 14,500 rpm. Supernatants were collected and the protein concentrations were quantified using the bicinchoninic acid (BCA) assay (Thermo Fisher) following the manufacturer's protocol. Tissue lysates (50 mg) were mixed with 5X Laemmli sample buffer and boiled for 10 min prior to loading. Samples were separated on a 12% acrylamide gel, transferred to nitrocellulose membranes and subsequently blocked with 5% skim milk in TBS-T (0.14 M NaCl, 2.7 mM KCl, 25 mM Tris, 1% Tween 20; pH 7.5) for 1 hour. Blots were probed with primary antibody for 1 hour using purified CK-2 antisera at dilution of 1:50. The membranes were washed with TBS-T three times for 10 min each then the secondary antibody, goat anti-rabbit alkaline phosphatase (Sigma Aldrich), was applied at a 1:30000 dilution and probed for 1 hour. Bands were detected using NBT/BCIP (Roche) according to manufacturer's instructions.

2.8 Elucidating the function of rainbow trout CK-2 using a chemotaxis assay

To observe the impact of CK-2 on the chemotaxis of RTS-11 and peripheral blood leukocytes, a 48-well micro chemotaxis assembly (NeuroProbe) was used. The wells of the lower chamber were filled in triplicate with either 50 ng/ml 100 ng/ml or 500 ng/ml or 1μg/ml of CK-2 suspended in 1X PBS. For each concentration of CK-2, the same concentration of recombinant truncated β_2m protein (as developed by Kales et al, 2006) suspended in 1X PBS was used as a negative control to confirm that any cell migration was due to CK-2 and was not resulting from the chosen method of recombinant protein production. Another negative control containing 1X PBS alone was also used while either zymosan activated serum (ZAS) or complement factor C5a were used as positive controls. The wells of the top chamber were filled with 45 μl of L-15 containing 1 x 10^7 cells/ml and the two chambers were divided by a polyvinylpyrrolidone (PVP) free polycarbonate membrane with a pore size of 5 μm. The assembly was then incubated for 1 hour at room temperature (RT). Following incubation, the filter was removed and washed three times with 1X PBS.

The filter was then fixed in methanol for 1 minute and stained with 1% Giemsa (dissolved in 100% ethanol) for 1 hour. After staining, the filter was washed twice with deionized water, fixed with Permount (Thermo Fisher) and mounted on a microscope slide. Cells which had migrated to the bottom side of the filter were counted in a total of five different fields per well at a magnification of 400x. The chemotactic index was calculated by dividing the total number of migrating cells in the test wells by the number of cells that spontaneously migrated toward the PBS negative control.

To provide further validation for the results of the chemotaxis assays, RTS-11 or primary PBLs were incubated with either CK-2 (100 ng/ml) alone or CK-2 (100 ng/ml) with pertussis toxin (100 ng/ml) as PTX is known to inhibit chemotactic function. After the 1 hr incubation time at RT, the PBLs were washed twice with 1X PBS and resuspended in L-15 to a final concentration of 1 x 10^7 cells/ml for use in the chemotaxis assay as described above. To show that the observed migration towards CK-2 and CK-2.1 was not due to the mucin stalk, PBLs were incubated with both proteins alone (500 ng/ml) as well as with the mucin stalk of CK-2.1 alone (1 μg/ml) and chemotaxis was measured as described above.

2.9 Statistical analysis

Statistical analysis was performed using a one-way ANOVA followed by Fischer's Least Significant Difference (LSD) post hoc test to determine if there was a significant change of CK-2 or CK-2.1 between groups. For the chemotaxis assays, after the data was transformed by taking the natural logarithm to achieve equal variance and normality, a one-way ANOVA was performed followed by the LSD post hoc test to determine whether there was a significant change in cell migration. A probability of $P<0.05$ was considered statistically significant.

3. Results

3.1 Isolation of the CK-2.1 sequence and its prevalence in rainbow trout

When cDNA primers that spanned the mucin stalk region of rainbow trout CK-2 were employed, a doublet was observed. To examine the nature of this possible second chemokine gene, a rainbow trout phytohemagglutinin (PHA) stimulated head kidney cDNA library was screened with a full-length clone of CK-2. This analysis revealed a new cDNA clone that was isolated and sequenced. The 702 nucleotide (nt) open reading frame encoded a putative 233 amino acid (aa) protein that shared sequence similarity with known chemokines in both mammals and fish, but most notably it shared 99% amino acid sequence similarity with rainbow trout CK-2 in overlapping regions. When this newly discovered clone, referred to as CK-2.1, was aligned with CK-2, the differences between the two amino acid sequences resided in the mucin stalk regions where the CK-2.1 sequence encoded an extra 42 amino acids with 53 possible O-glycosylation sites (**Fig. 1A**).

When 55 rainbow trout individuals were screened using the CK-2 primers that spanned the mucin stalk region, both the smaller CK-2 band (600 bp) and the larger band of CK-2.1 (726 bp) are visible, with some individuals expressing both of these bands (**Fig. 1B**) indicating that these may be two separate alleles of the same gene. Using the Hardy-Weinberg equation with the observed data, the two alleles appear to be in equilibrium within the studied rainbow trout population [$(0.42)^2+2(0.42)(0.58)+(0.58)^2=1$] but a larger sample size of wild outbred fish may be required to confirm this.

3.2 Induction of CK-2 and CK-2.1 transcripts in cell cultures by PHA

When added to HKL primary cell cultures at a concentration of 10 μg/ml, the plant lectin, phytohemagglutinin, was able to significantly increase the gene expression of CK-2 and CK-2.1 alone, or both alleles in the case of heterozygotes ($p = 0.016$) as detected by RT-PCR (**Fig. 2A**). In contrast, when receiving the same treatment, the PBL primary cell cultures exhibit equal expression of both of these alleles before and after stimulation (**Fig. 2B**). This pattern was observed in both homozygotes and heterozygotes.

To detect the transcript expression of the CK-2 allele in different rainbow trout cell lines, it was important to first determine which alleles were present within each cell line. After PCR amplification using genomic DNA, it was shown that RTS-11 contains CK-2 alone, while RTG2 only has the CK-2.1 allele. In comparison, RTS 34 ST was heterozygous, containing both CK-2 and CK-2.1 alleles (**Fig. 3A**). To further study the induction of the CK-2 allele, RTS-11 was stimulated with 10 μg/ml of PHA. When 20% FBS was present within the culture media, expression of CK-2 did not appear to be significantly influenced by exposure to PHA over a period of 24 hours ($p = 0.726$). Despite this lack of significance, there did appear to

be a trend towards reduced expression of CK-2 following stimulation with PHA (**Fig. 3B**). In comparison, when RTS-11 was stimulated with PHA in media lacking FBS, a significant decrease in CK-2 transcript expression was observed at 24-hours post-stimulation as detected by RT-PCR ($p = 0.046$) (**Fig. 3C**). Interestingly, despite RTG2 having the CK-2.1 allele alone, and RTS 34 ST being heterozygous for both alleles, nether of these cell lines displayed an increase in transcript expression of either gene when exposed to 10 μg/ml of PHA (**Fig. 3D** and **E**).

3.3 Induction of CK-2 and CK-2.1 transcripts in fish by PHA

Following injection with PHA (50 μg/250 g body weight), CK-2 gene expression in heterozygous rainbow trout had variable results depending on the tissue studied. In both head kidney and brain, there was a strong and relatively even expression of both CK-2 and CK-2.1 in the unstimulated fish (0 hr) that declined steadily after stimulation (**Fig. 4A** and **4B** respectively). In stimulated spleen tissues, CK-2 expression alone appeared to be induced 4 hours after injection with a continuous decline thereafter. In these same spleen samples, CK-2.1 appeared to have consistent expression levels throughout the entire time course observed in both groups studied (**Fig. 4C** and **4D**). Thus, it appears that PHA stimulation induces differential expression of CK-2 over CK-2.1 in the spleens of heterozygous rainbow trout.

3.4 Induction of CK-2 protein in fish and in RTS-11 by PHA

The induction of CK-2/CK-2.1 protein was observed in only head kidney, liver and brain of rainbow trout following stimulation with PHA, with each tissue producing a different size of CK-2/CK-2.1 (36 kDA band in liver, 98 kDa band in brain and a ~250 kDa band in head kidney) (**Fig. 5Ai**). Surprisingly, all of the bands observed indicate proteins larger than the sizes predicted by the amino acid sequences (~30 kDa for CK-2 and ~35 kDa for CK-2.1). In comparison, no protein production was detectable in the stimulated PBLs, gill or spleen tissues (**Fig. 5Ai**) nor in any tissues of the PBS injected control fish (**Fig. 5Aii**). Regardless of stimulation with PHA and the presence or absence of 20% FBS, RTS-11 cells produced protein bands of various sizes including a strong band at 30 kDa, the anticipated size of unglycosylated CK-2 (**Fig. 5B**). In both *in vitro* and *in vivo* samples, the bands observed with a larger molecular mass may be a result of glycosylation.

Because bands of various sizes were observed by the developed CK-2/CK-2.1 antibody in **Fig. 5Ai and 5B**, it was necessary to validate that the detected signal was indeed the protein of interest. To do this, the CK-2/CK-2.1 antibody was first blocked using rCK-2 prior to probing a membrane containing samples known to contain CK-2/CK-2.1 protein bands. The resulting lack of bands following blocking verified that any bands detected by the antibody (when not blocked by rCK-2), are variants of CK-2 and CK-2.1 (**Fig. 5Aiii**). Recombinant CK-2 was also successfully detected when probed with pre-immune serum (**Fig. 5Ci**), crude anti-CK2 (**Fig. 5Cii**) and affinity purified anti-CK2 (**Fig 5Ciii**). Furthermore, the final, purified anti-CK2 antibody was also able to successfully detect both rCK-2 and rCK-2.1 individually (**Fig. 5Civ**).

3.5 Recombinant CK-2 induces migration of RTS-11 and rainbow trout PBLs

After exposure to various concentrations of rCK-2 (50 ng/ml, 100 ng/ml and 500 ng/ml, 1 μg/ml,), both rainbow trout PBLs and the RTS-11 cell line were observed to display significant migration towards the recombinant CK-2 protein when compared to the non-chemokine recombinant protein, truncated β_2m (**Fig. 6A and 6B** respectively). Regardless of the cell type stimulated, the highest level of migration was seen when a concentration of 100 ng/ml of rCK-2 was used but this was not significantly different from 500 ng/ml or the C5a positive control. All rCK-2 concentrations analyzed were significantly different from the truncated β_2m negative control tested at identical concentrations. Interestingly, chemotaxis was observed to increase from 50 ng/ml to 100 ng/ml but did not continue in a concentration dependent manner as the highest concentration, 1 μg/ml, displayed a chemotaxis index comparable to the lowest concentration of 50 ng/ml. As expected, significant migration was observed when cells were exposed to the C5a positive control while negligible chemotaxis was detected towards the PBS negative control.

To confirm whether the observed chemotaxis was due to rCK-2, various blocking experiments were conducted. After pre-treatment with an excess of rCK-2 to block the appropriate chemokine receptors, PBL chemotaxis to rCK-2 was significantly inhibited ($p = 0.001$, data not shown). Furthermore, if the rCK-2 was blocked with anti-CK2 prior to use in the chemotaxis assay, the rCK-2 was unable to induce chemotaxis of rainbow trout PBLs ($p = 0.006$, data not shown). Chemokines are known to act through G-protein coupled

receptors, thus to test whether CK-2 used this mechanism, pertussis toxin (PTX), a known inhibitor of these receptors was used as a treatment. When RTS-11 and PBLs were exposed to both PTX and rCK-2, the ability of both cell types to migrate towards rCK-2 was significantly reduced to background levels ($p = 0.006$) (**Fig. 6C** and **D**), indicating that CK-2 does act through G-protein coupled receptors. Lastly, to determine whether the mucin stalk of CK-2 influenced the chemotactic ability of the chemokine, PBLs were exposed to a recombinant fragment containing only the mucin domain of CK-2. When compared to the chemotaxis induced by CK-2 and CK-2.1 recombinant proteins alone, the mucin stalk alone failed to elicit significant cell migration as observed by the positive control and CK-2 or CK-2.1 alone (**Fig. 6E**). Furthermore, there was no significant difference in the number of migrating cells responding to CK-2 and CK-2.1 at a concentration of 500 ng/ml (**Fig. 6E**).

4. Discussion

4.1 The discovery of CK-2.1, an allele of CK-2

When attempting to characterize the rainbow trout CK-2 gene, northern blot analysis revealed two separate bands that suggested the presence of another transcript (Liu et al, 2002). It was hypothesized that this unexpected mRNA transcript could be derived from either alternative splicing of the CK-2 gene or from a separate, yet closely related gene. To further explore the origin of this alternative mRNA band, a cDNA library was probed and a novel chemokine was isolated and sequenced. The newly discovered chemokine sequence, referred to as CK-2.1, was highly similar to the known sequence of the CC chemokine, CK-2 (Liu et al, 2002), but it encoded an additional 42 amino acids of mucin stalk. This variation in the length of the mucin domain is not unusual as many mucins have been shown to exhibit this feature due to alternative splicing of their variable repeat region (Debailleul et al, 1998, Williams et al, 1999, Zhang et al, 2013), yet after further analysis splicing was not occurring in this situation. After amplifying the region surrounding the mucin stalk of CK-2.1, rainbow trout individuals either had CK-2 alone, CK-2.1 alone or both in proportions that were in Hardy-Weinberg equilibrium suggesting that CK-2.1 is an allele of CK-2 and not a separate gene.

4.2 Expression of CK-2 and CK-2.1 in rainbow trout tissues and cell culture

To determine whether CK-2 and CK-2.1 transcripts were regulated in either a coordinated manner or differentially, transcript expression was observed in both homozygous and heterozygous individuals using RT-PCR. Northern blot analysis was not possible due to the high degree of sequence similarity between these two alleles that would render individual probe design and the recognition of separate bands extremely difficult. To investigate whether the level of expression of both CK-2 and CK-2.1 were influenced by immune challenge, primary HKLs and PBLs were stimulated with phytohemagglutinin, a mitogen commonly used for the stimulation of cell division in T lymphocytes (Movafagh et al, 2011). Regardless of whether the individual was homozygous for CK-2, homozygous for CK-2.1 or heterozygous, transcript expression of the chemokine significantly increased in HKLs but not in PBLs. This may indicate the activation status of the cell mixture in question, with PBLs not being fully mature while HKLs are primed and responding to the immune stimuli. After validating that long-term cell cultures are also capable of being homozygous or heterozygous for the CK-2/CK-2.1 alleles, the influence of immune stimulation on transcript expression was observed. Similar to what was observed with PBLs, PHA stimulation did not induce expression of CK-2 in RTS-11. Instead, there was a continuous level of CK-2 expression over 24 hours. As RTS-11 has both monocytic and macrophage-like cells (Montero et al, 2008), it may be that monocytes and/or macrophages are either not responsive to stimuli due to their maturation state or need other cytokine signals to prime them into a state where they can produce further CK-2/CK-2.1. The steady level of CK-2/CK-2.1 transcript expression in RTS-11 was seen to decrease after removing FBS from the media. This is not surprising as FBS-supplementation is often required to observe chemotactic activity in mammalian cell lines thus, media lacking FBS is frequently used as a negative control in these studies (Alberson et al, 2013, Fang et al, 2017). This could explain the observed decrease in CK-2/CK-2.1 transcript production in the absence of FBS despite the RTS-11 cells undergoing 24 hours of stimulation with PHA.

Interestingly, an entirely different trend was observed when looking at transcript levels of CK-2 and CK-2.1 in whole fish tissues after the animals received i.p. injections of PHA. The head kidney had a decrease in gene expression over 24 hours while spleen tissues had an initial increase at 4 hours, followed by a rapid

reduction in expression over the following 20 hours. In both mammalian and teleostean models, PHA is known to primarily stimulate T lymphocytes (Mier and Gallo, 1980, Grondel and Harmsen, 1984, Siegl et al, 1993). From there, lymphokines and growth factors important for hematopoiesis are released from the replicating cells (Mier and Gallo, 1980). Following injection with PHA, perhaps T lymphocyte replication occurred at a rapid rate within the head kidney thus chemokines are not required to attract cells to that location. Upon production of an adequate T lymphocyte arsenal, these cells may have been released into the periphery to travel to the spleen as seen by the short-lived increase in CK-2 transcript expression at 4 hours. Based on this explanation, one would expect PBLs to display an increase in transcript expression of CK-2 but perhaps this would only occur between 0 and 4 hours and was therefore not observed during the time points selected for this trial. This theorized pattern displays that when *in vivo*, the cells will be receiving all of the appropriate priming signals that cell cultures would not, possibly explaining some of the differences when comparing transcript expression in the *in vitro* models.

4.3 Induction of CK-2 protein in fish and in RTS-11 by PHA

The developed polyclonal antibody, raised against recombinant rainbow trout CK-2, was shown to cross-react with both CK-2 and CK-2.1 recombinant protein. Given the observed differential expression of CK-2 and CK-2.1, heterozygous fish were chosen so that the expression pattern of both CK-2 and CK-2.1 could be detected. Despite the presence of both CK-2 and CK2.1 transcripts in unstimulated tissues, no detectable protein was observed in western blot analysis of unstimulated tissues, indicating potential post-transcriptional control of protein production. However, following stimulation, cross-reactive bands were seen in the head kidney, liver and brain while neither spleen nor PBLs showed any detectable protein production. The low levels of protein in unstimulated tissues corresponds to our previous work wherein there was no detectable transcript expression of CK-2 in unstimulated HKL and PBL by northern blot analysis (Liu et al, 2002). The fact that PHA stimulated PBLs express CK-2 mRNA but not the protein suggests that the cells that produce this protein are circulating lymphocytes. The lack of protein expression in stimulated PBLs supports the idea that these cells are not mature or activated and thus do not respond to stimuli as seen in the mRNA expression. Taken together, these results suggest that perhaps CK-2/CK-2.1 plays a pro-inflammatory role *in vivo*, as the CK-2/CK-2.1 protein appears to only be produced in response to the PHA stimulus. Contrary to what was observed *in vivo*, it appears that the *in vitro* model, RTS-11, was capable of producing CK-2 protein regardless of whether the cells were stimulated or not. Furthermore, protein levels were consistent whether FBS was present in the media or absent, a factor that was able to impact CK-2 transcript expression.

When analyzing the molecular weight of the protein produced in different tissues, although heterozygous animals were used for all *in vitro* western blot analyses, only a single band was detected in the head kidney and brain, while the liver showed a single prominent band as well as several faint bands that might correspond to other allelic forms of CK-2/CK-2.1. The expected molecular weights of CK-2/CK-2.1 based on amino acid sequences were 30 kDa for CK-2 and 35 kDa for CK-2.1. However, there are 42 possible *O*-glycosylation sites in the mucin stalk of CK-2 (Liu et al, 2002) and 53 in that of CK-2.1, which may influence the multiple band sizes observed in some tissues. As expected, a 35 kDa band was detected in liver tissue, likely representing CK-2.1, while a 98 kDa band and a larger (~250 kDa) band was also detected in the brain and head kidney respectively. In contrast, the protein production observed in both stimulated and unstimulated RTS-11 cells revealed four prominent bands, the smallest corresponded to unglycosylated CK-2, with a faint band at 35 kDa that could be CK2.1, while the other three were larger than 64 kDa. This variability in protein size could be attributed to typical characteristics of *O*-linked sugars, in which it has been well established that the terminal glycosylation sequence differs from tissue to tissue between cell types (Bennett et al, 1996, Hagen et al, 1997 and reviewed in Rademacher et al, 1988). It has also been shown in mammals that different tissues contain different transferase activities (Sorensen et al, 1995) thus CK-2 could be differentially glycosylated according to tissue-specific expression of the different glycosyltransferases. Because the RTS-11 cell line is heterogeneous, containing both monocyte/macrophage-like cells (Montero et al, 2008), it may be that different cell types within the culture are producing the different size variants of CK-2/CK-2.1 based on the maturation stage of the cells.

A high level of protein production was observed in the brain of stimulated rainbow trout similarly to what has been observed in the murine mammalian model, wherein fractalkine, the sole member of the CX_3C chemokine group, plays an important role in controlling normal interactions between microglial cells and

neurons as well as modulating microglial activation (Ransohoff et al, 2009). When constitutively expressed, it appears that fractalkine plays an immunosuppressive role resulting in the inhibition of microglial activation (Biber et al, 2007). This is further supported through analysis of animal models with induced neuropathologies wherein a deficiency of fractalkine leads to an increased production of proinflammatory molecules, and thus a massive increase in cell death (Cardona et al, 2006, Morganti et al, 2012). At the structural level, much like CK-2/CK-2.1, fractalkine also has a mucin stalk enabling the chemokine to have both membrane-bound and secreted forms (Fong et al, 2000). Further comparison of these chemokines reveals that the C terminal end (amino acids 101-191) of CK-2 is highly similar to fractalkine, yet when the full lengths of the chemokines are aligned, CK-2 is only 25% identical and possesses a typical CC chemokine structure. When taking all this information together, perhaps rainbow trout CK-2 plays a similar role in suppressing inflammatory responses as evidenced by its high protein levels in rainbow trout brain following stimulation.

4.4 The impact of CK-2 on cell chemotaxis

Regardless of whether CK-2 is playing a stimulatory or regulatory role in immune responses, the defining feature of any chemokine is the capacity to coordinate the movement and positioning of immune cells (Griffith et al, 2014). At varying concentrations, rCK-2 was able to significantly attract both PBLs and the RTS-11 cell line when compared to the truncated β_2m negative control. Interestingly, this chemotaxis did not occur in a concentration dependent matter with the highest concentration (1 μg/ml) inducing less chemotaxis than 100 ng/ml and 500 ng/ml. This phenomenon has been observed in the mammalian model wherein prolonged exposure to its ligand can result in the desensitization of associated chemokine receptors. In these situations, chemotaxis will be observed at low chemokine concentrations and will increase in a concentration-dependent manner. However, when the concentration becomes too high, the cells will no longer migrate towards the same chemoattractant. This is a believed to be a regulatory process meant to diminish the responsiveness of chemokine receptors as a method of protecting the cell from overstimulation (Lefkowitz, 1998, Bennett et al, 2011). The mechanism responsible for abolishing chemotaxis at high chemokine concentrations is currently unclear but is believed to be involved with agonist-dependent desensitization and endocytosis mediated by G protein-coupled receptor kinases and β-arrestins (Lefkowitz, 1998, Fong et al, 2002). As a similar pattern has been observed in this study using the rainbow trout chemokine, CK-2, perhaps a similar regulatory mechanism occurs for teleostean immune cells.

Although CK-2 did induce chemotaxis in rainbow trout leukocytes, it was important to validate whether the different alleles and protein domains of CK-2 would play individual roles in this observed cell migration. When measuring the chemotactic ability of CK-2 and CK-2.1 proteins separately, there is no significant difference between the proteins regarding their impact on cell migration. This further validates the concept that although the CK-2 and CK-2.1 gene transcripts can display differential expression in rainbow trout individuals, the resulting function, regardless of which protein is produced, does not appear to be affected. Furthermore, the mucin stalk region alone was not enough to induce chemotaxis in rainbow trout leukocytes similarly to what has been previously observed in mammalian models where the mucin stalk of fractalkine, did not induce chemotaxis (Haskell et al, 2000, Volin et al, 2001).

Given that RTS-11 was shown to produce CK-2 protein both constitutively and in response to stimulation, it was surprising that these cells were also shown to react and migrate towards CK-2. Due to the heterogeneous nature of RTS-11, containing a mixture of both immature monocytes and mature macrophages (Montero et al, 2008), it is possible that one population is attracted to the CK-2 that is produced by the other. This would be very similar to what has been observed with lymphotactin, wherein NK cells produce this chemokine in an effort to attract both NK cells and T lymphocytes to sites of injury (Hedrick et al, 1997). Thus, it is not unheard of for leukocytes to both produce a specific chemokine, as well as react to that same chemokine. However, before any definitive conclusions can be made regarding which cell types produce and are attracted to CK-2, further investigation is required.

Conclusions

This study determined that both CK-2 and CK2.1 are alleles of the same gene and that they encode a protein that functions as a typical chemokine. When observed together, both transcript expression and protein production showed that this gene is not constitutively expressed, but rather is regulated in a complicated manner,

including differential expression of alleles in some tissues of heterozygous fish. Future studies will focus on isolating the receptor for this chemokine, as well as determining which cell types produce and respond to it. This will lead to a deeper understanding of teleost immunity as well as the evolution of the vertebrate immune system with a particular focus on chemotactic cytokines.

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Figure Legends

Figure 1: The relationship between CK-2 and CK-2.1 sequences. (**A**) The amino acid sequence alignment of rainbow trout CK-2 and CK-2.1 (**B**) PCR bands of CK-2 and CK-2.1 obtained from the genomic DNA of 10 randomly selected rainbow trout individuals. The 700bp band represents CK-2.1 whereas the 600bp represents CK-2.

Figure 2: Transcript expression of CK-2 and CK-2.1 in HKLs and PBLs primary cultures. RT-PCR analysis displaying transcript levels of CK-2 and CK-2.1 after stimulation with either 10 μg/ml of PHA (P) or the PBS control (C) for 24 hours in rainbow trout HKLs (**A**) and in rainbow trout PBLs (**B**). The term "heterozygotes" describes individuals that contain both the CK-2 and the CK-2.1 allele. This analysis was repeated with three individual fish for each genotype (n=3) with the presented gel image representing one replicate. The upper panel in each case shows amplification of CK-2/CK-2.1 while the lower panel presents the amplification of the control, EF1-α. The bar graphs below each gel image show the average ratio of expression between either CK-2 (grey bars) or CK-2.1 (white bars) and the EF1-α amplicons that were used as an internal standard for each time point. Significant difference between samples are indicated by * (P<0.05). Vertical error bars represent the standard error.

Figure 3: RT-PCR Analysis of CK-2 and CK-2.1 expression in rainbow trout cell lines. (**A**) PCR amplification of genomic DNA displaying the allelic distribution of CK-2 and CK-2.1 in three rainbow trout cell lines, RTS-11, RTS 34 ST, and RTG2. Rainbow trout previously observed to be homozygous for CK-2, CK-2.1 as well as heterozygous were used for the positive control lanes. (**B**) RT-PCR analysis showing transcript levels of CK-2 in RTS-11 after stimulation with 10 μg/ml of PHA during a 24-hour time course challenge when 20% FBS was either present in the culture media (**B**) or absent (**C**). For **B**, **C**, **D** and **E**, the upper panels represent the CK-2 mRNA bands while the bottom panel is the control, EF1-α. The bar graphs below each gel image (**B** and **C**) show the ratio of expression between CK-2 and the EF1-α amplicons that were used as an internal standard for each time point. All data was repeated a minimum of three times (n=3). All data was transformed into natural logarithm with significant differences indicated by the * (P<0.05). Vertical error bars represent the standard error.

Figure 4: RT-PCR analysis of CK-2/CK2.1 in PHA stimulated heterozygous rainbow trout tissues. RT PCR analysis showing transcript levels of CK-2 and CK-2.1 in heterozygous rainbow trout tissues after injection with 50 μg of PHA per 250 g of body weight during a 24-hour time course challenge. The tissues studied include head kidney (**A**), brains (**B**) and spleen samples from duplicate groups of individuals (**C** and **D**). The upper panels represent the expression of CK-2/CK-2.1 mRNA while the middle panel is the control, EF1-α. The bar graphs below each gel image show the ratio of expression between either CK-2 (grey bars) or CK-2.1 (white bars) and the EF1-α amplicons that were used as an internal standard for each time point.

Figure 5: Protein production of CK-2 in stimulated rainbow trout tissues/RTS-11 and validation of the anti-CK2 antibody. Protein production of CK-2/CK-2.1 in rainbow trout tissues (**A**) following 24 h stimulation with PHA (i) or PBS control (ii). Anti-CK2 was also incubated with rCK-2 prior to probing the PHA stimulated rainbow trout tissues to ensure that any bands were lost when the antibody was effectively blocked (iii). RTS-11 cultures following 24 h PHA stimulation or PBS control in L15 either with or without FBS (**B**). Further validation regarding the specificity of the anti-CK2 antibody (**C**) with rCK-2 being probed with (i) pre-immune serum, (ii) crude anti-CK2 at a 1:5000 dilution, (iii) affinity purified anti-CK2 at a 1:50 dilution. The final, purified anti-CK2 antibody was also shown to detect both CK-2 and CK-2.1 recombinant protein individually (iv). Molecular weight markers from high to low were 98, 64, 50, 36, 30 and 16 kDa and are indicated by the – on the left-hand side of the blots.

Figure 6: The impact of recombinant CK-2 on rainbow trout cell chemotaxis. After exposure to recombinant CK-2 protein for 1 hour at concentrations of 1 μg/ml, 50 ng/ml, 100 ng/ml and 500 ng/ml, the chemotaxis of both the RTS-11 cell line (**A**) and primary culture PBLs (**B**) was observed. Significant differences in chemotaxis between CK-2 and truncated β_2m of the same concentration are indicated by * ($P<0.05$). The chemotaxis of RTS-11 (**C**) and primary culture PBLs (**D**) following treatment with pertussis toxin (PTX) at 100 ng/ml prior to stimulation with 100 ng/ml of CK-2 for 1 hour. (**E**) The chemotaxis of rainbow trout PBLs in response to 1 hour of stimulation with rCK-2 (500 ng/ml), rCK-2.1 (500 ng/ml) or the mucin stalk alone (1 μg/ml). For all panels, truncated β_2m (a non-chemokine recombinant protein) and PBS were used as negative controls that would not induce chemotaxis, while zymosan activated serum (ZAS) and the complement factor C5a were used as positive controls known to induce chemotaxis. For all chemotaxis assays, experiments were repeated a minimum of three times (n=3). Vertical error bars represent the standard error.

A

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CK-2      MVTCGTLVKIWTLAVVIAALGWTGTVDAEKLVSCCKTVSRTEVNDPITGYWIQNYNAPCVRAVIFETKKGLFCSYHKQPWVRRKI
CK-2.1    ----------------------------------------------------------------------------------------

CK-2      HQFEMARLSSTFLSLSIPNSLTSTSTPTTTSLPSSPPS****************************************SLFPL
CK-2.1    -------------------------------------INSSPPSLTSTSTSLTSSPSSSPPSLTSTPTFLPSSPLSVFS-----

CK-2      SSSSPSVPSSPPSLSSSPSHLLSSLFPASSSPPSISSSPPSFSSPRHWESTKNASTQQSTSNQ
CK-2.1    ------------------------------------------------R---------------
```

B

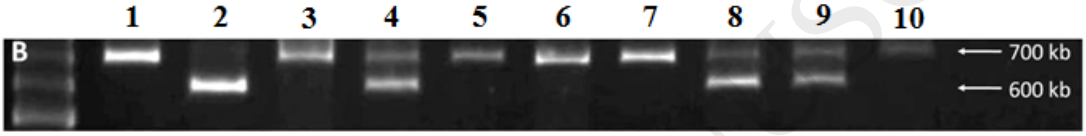

Figure 1

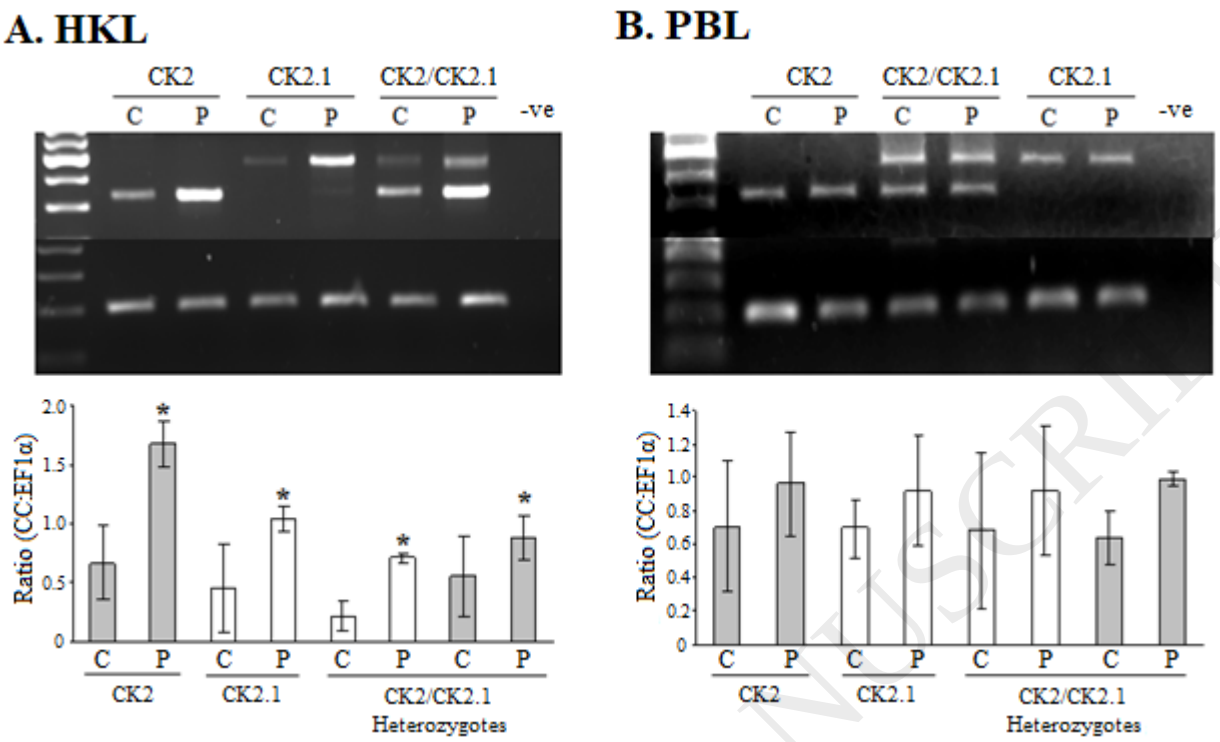

Figure 2

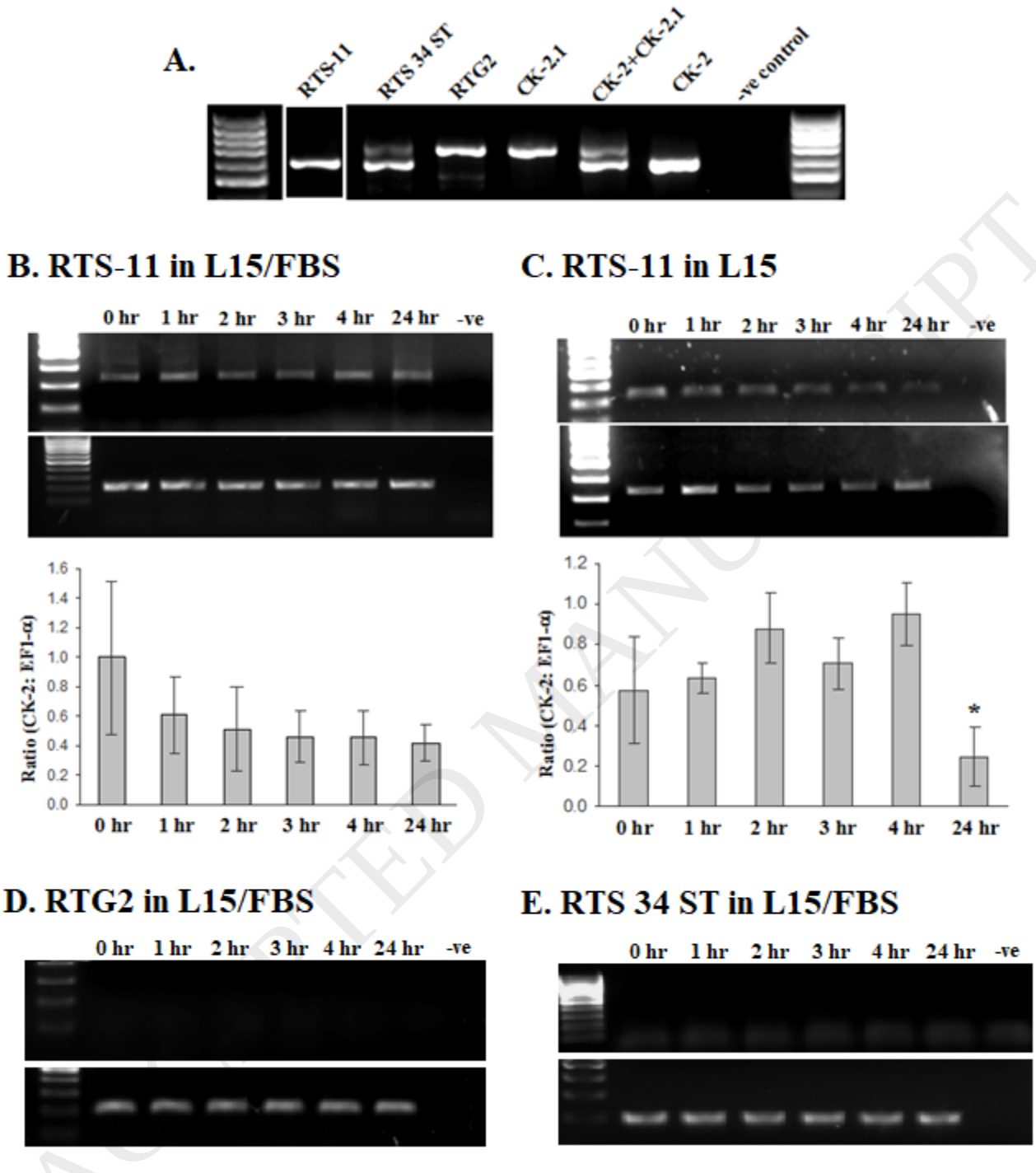

Figure 3

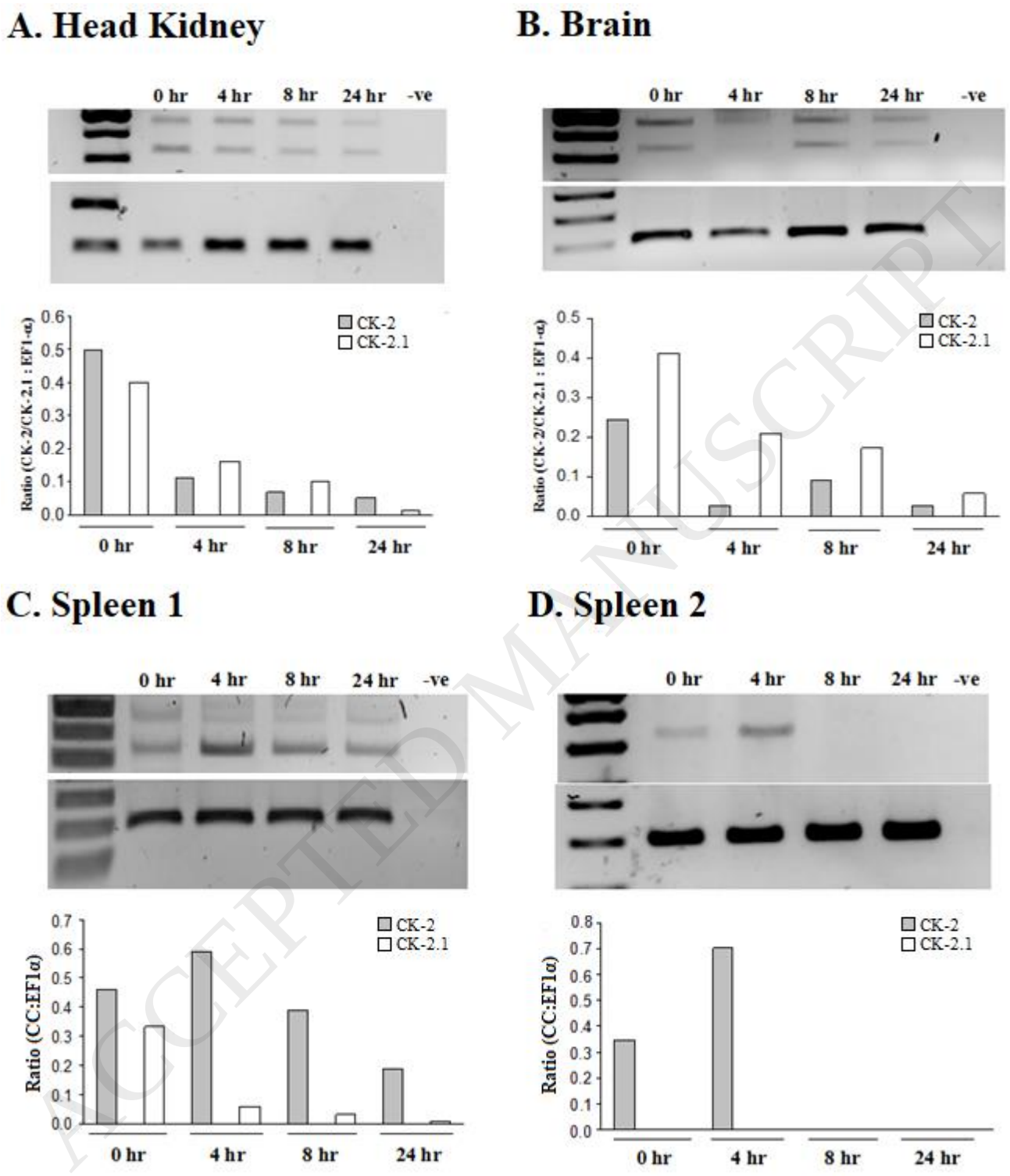

Figure 4

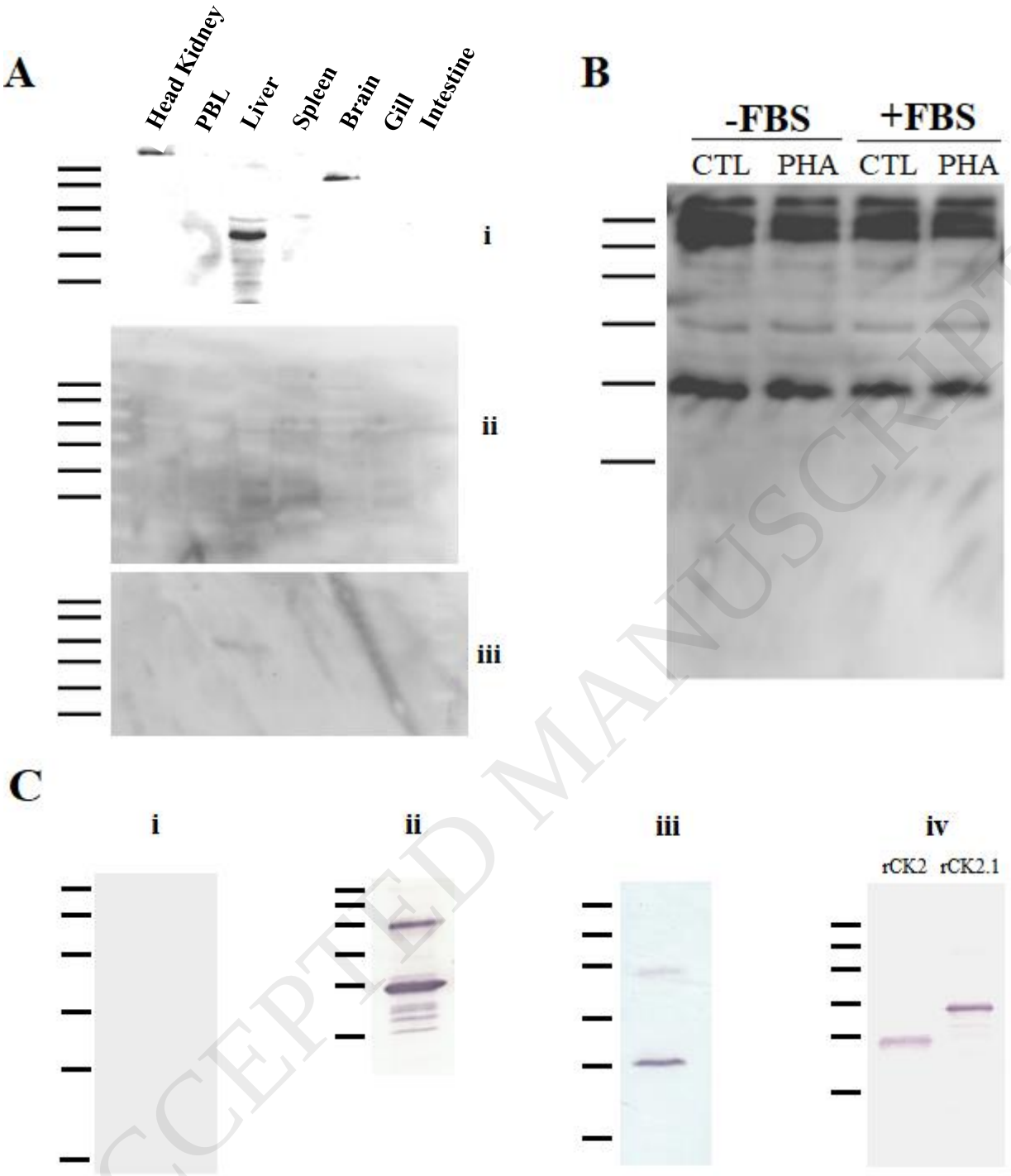

Figure 5

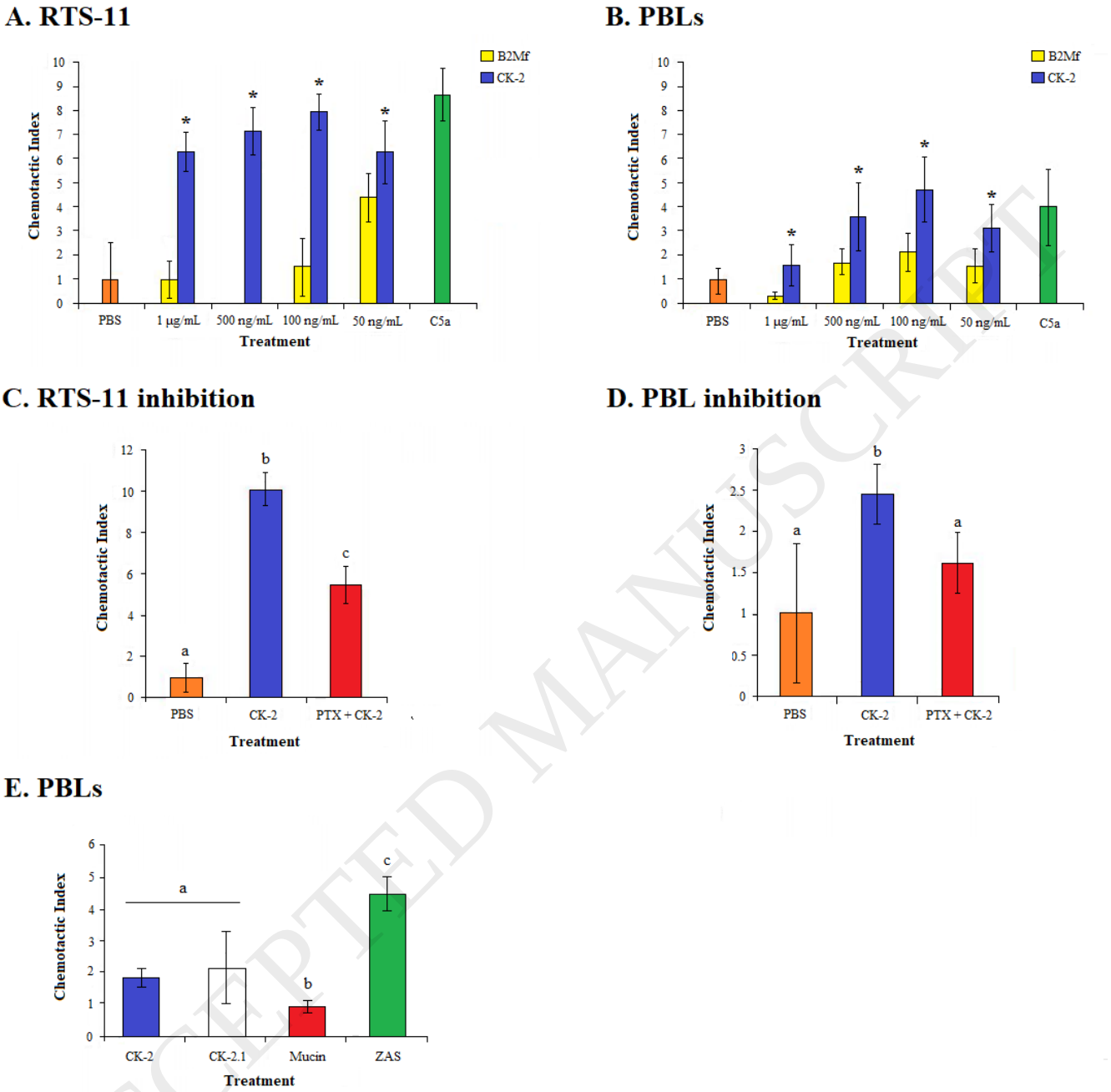

Figure 6

Tables

Table 1: Primer sequences used in this study.

Primer Name	**Sequence (5'-3')**	**Annealing Temp.**	**Application**
CK-2 sense	GCAGAAAAGCTGGTGTCGTG	53ºC	RT-PCR, genotyping
CK-2 antisense	GGAAGGTACGGATGGAGAAG		RT-PCR, genotyping
CK-2 stalk sense	GTCTGAGCTCAACATTTCTC	48ºC	Genotyping
CK-2 stalk antisense	AGGAGCTTCAGCCATTAGCA		Genotyping
CK-2 stalk SP2 antisense	GTTCTACAAGCCCCCATAAG		Genotyping
CK-2 stalk SP4 antisense	ATGGGCACATACAATACTGG		Genotyping
EF1-α sense	GAGTGAGCGCACAGTAACAC	54ºC	RT-PCR control
EF1-α antisense	AAAGAGCCCTTGCCCATCTC		RT-PCR control