**The effects of ammonia and water hardness on the hormonal,
osmoregulatory and metabolic responses of the freshwater silver catfish
*Rhamdia quelen***

Bernardo Baldisserotto^{a*†}, Juan Antonio Martos-Sitcha^{bc†}, Charlene C. Menezes^e, Cândida Toni^a, Ricardo L. Prati^d, Luciano de O. Garcia^d, Joseânia Salbego^a, Juan Miguel Mancera^c, Gonzalo Martínez-Rodríguez^b

^aDepartamento de Fisiologia e Farmacologia, Universidade Federal de Santa Maria, 97105-900 Santa Maria – RS – Brazil.

^bDepartamento de Biología Marina y Acuicultura, Instituto de Ciencias Marinas de Andalucía, 11510 Puerto Real (Cádiz), Spain.

^cDepartamento de Biología, Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz, 11510 Puerto Real (Cádiz), Spain.

^dInstituto de Oceanografia, Estação Marinha de Aquicultura, Universidade Federal do Rio Grande, Rio Grande – RS – Brazil.

^eDepartamento de Química, Universidade Federal de Santa Maria, Santa Maria, 97105-900 Santa Maria – RS – Brazil.

† Both authors contributed equally to this work

* Corresponding author

Departamento de Fisiologia e Farmacologia

Universidade Federal de Santa Maria

97105.900 – Santa Maria, RS, Brazil

Phone: +55-55-3220-9382, fax: 55-55-3220-8241

E-mail: bbaldisserotto@hotmail.com

Abstract

The aim of the present study was to assess the effects of ammonia and water hardness on endocrine, osmoregulatory and metabolic parameters in silver catfish (*Rhamdia quelen*). The specimens (60–120 g) were subjected to six treatments in triplicate, combining three levels of un-ionized ammonia (NH₃) (0.020 ± 0.008 mg/L [1.17 ± 0.47 µM], 0.180 ± 0.020 mg/L [10.57 ± 1.17 µM] and 0.500 ± 0.007 mg/L [29,36 ± 0.41 µM]) and two levels of water hardness (normal: 25 mg CaCO₃/L and high: 120 mg CaCO₃/L), and sampled after two exposure times (1 and 5 days post-transfer). Plasma cortisol, metabolites, osmolality and ionic values were determined concomitantly with the mRNA expression levels of different adenohypophyseal hormones (growth hormone, GH; prolactin, PRL; and somatolactin, SL). Previously, full-length PRL and SL as well as β-actin cDNAs from *R. quelen* were cloned. Exposure to high NH₃ levels enhanced plasma cortisol levels in fish held under normal water hardness conditions but not in those kept at the high hardness value. The increase in water hardness did not alter plasma metabolites, whereas it modulated the osmolality and ion changes induced by high NH₃ levels. However, this hardness increase did not lead to the decreased GH expression that was observed 5 days after exposure to 0.18 mg/L NH₃ in fish held at the normal water hardness level, whereas PRL expression was enhanced after one day of exposure under the increased hardness conditions. Additionally, SL expression decreased in specimens exposed for 5 days to 0.18 mg/L NH₃ and maintained at the high water hardness level. The results showed that increasing water hardness attenuated the hormonal parameters evaluated in *R. quelen* specimens exposed to high NH₃ levels, although plasma metabolism do not appear to suffer major changes.

Keywords: cortisol; growth hormone; ions; metabolites; prolactin; somatolactin.

1. Introduction

Ammonia is the principal nitrogen compound excreted by fish, primarily through the gills. Its production depends primarily on protein intake and metabolic efficiency, parameters that are species specific and can be affected by several factors such as salinity, temperature, pH and waterborne ammonia levels (Randall and Wright, 1987; Randall and Tsui, 2002; Eddy, 2005; Wright and Wood, 2012). In intensive fish culture systems, a high stocking density and food supply as well as a low frequency of water renewal may increase ammonia levels, and this increase in ammonia could compromise the growth of cultured specimens (*Sparus aurata*: Wajsbrodt et al., 1993; *Scophthalmus maximus*: Le Ruyet et al., 1997; Foos et al., 2009; *Dicentrarchus labrax*: Dosdat et al., 2003; *Rhamdia quelen*: Miron et al., 2011; Ferreira et al., 2013; *Carassius auratus*: Sinha et al., 2012a).

Among the nitrogen compounds occurring in water are ionized (NH_4^+) and un-ionized (NH_3) ammonia. High NH_3 levels may cause changes in osmolality and/or electrolyte balance (McDonald and Milligan, 1997) and endocrine alterations (El-Shebly and Gad, 2011; Sinha et al., 2012b,c) as well as decreases in food intake, metabolism and growth (Paust et al., 2011; Wajsbrodt et al., 1993; Le Ruyet et al., 1997; Lemarié et al., 2004; Pinto et al., 2007; Foss et al., 2009; Sinha et al., 2012a).

NH_3 excretion by the gills is affected by divalent cations. Ca^{2+} is more effective than Mg^{2+} in enhancing NH_3 elimination in Lahontan cutthroat trout (*Onchorhynchus clarki henshawi*) (Iwama et al., 1997; Eddy, 2005). Accordingly, it has been observed that elevated waterborne Ca^{2+} levels reduced acute NH_3 toxicity in channel catfish, *Ictalurus punctatus* (Tomasso et al., 1980) and sunshine bass (female *Morone chrysops* × male *Morone saxatilis*) (Weirich et al., 1993). However, no previous studies have explained how enhanced water hardness reduces NH_3 toxicity in fish.

Pituitary is considered as a “master gland” due to the synthesis and release of different hormones, peptides and factors that control a large number of physiological functions. Among others, prolactin (PRL), growth hormone (GH) and somatolactin (SL) are hormones belonging to the same hormone family due to their structural similarities. Although PRL has been related as an essential

hormone for playing a role in the control of the osmoregulatory process in hyposmotic environments (Manzon, 2002; McCormick, 2001), this hormone is also related to other processes as reproduction, stress and metabolism (Mancera and McCormick, 2007; Laiz-Carrión et al., 2009). The same occurs with GH, which regulates growth, intermediary metabolism and in some species present osmoregulatory effects (Sakamoto and McCormick, 2006; Mancera and McCormick, 2007). In addition, SL is related to different physiological processes, including stress response, reproduction, acid-base regulation, growth and reproduction (Vega-Rubín de Celis et al., 2004; Fukamachi and Meyer, 2007).

The silver catfish *Rhamdia quelen* is an appropriate biological model for analyzing the effects of NH_3 and water hardness on physiological processes because the effects of these factors on survival and growth have been previously assessed in larvae and juveniles of this species (Townsend and Baldisserotto, 2001; Silva et al., 2003, 2005; Townsend et al., 2003; Becker et al., 2009; Miron et al., 2008, 2011; Copatti et al., 2011a, b). An increase in water hardness has been found to decrease the harmful effects of NH_3 on the growth of silver catfish in soft water (Ferreira et al., 2013). Therefore, the aim of the present study was to assess a possible protective role of water hardness relative to the toxic effects of NH_3 , focusing on endocrine, osmoregulatory (osmolality and plasma ions concentrations [Cl^- , Ca^{2+} , Na^+]) and metabolic (plasma glucose, triglycerides, lactate and proteins) parameters. To study changes in the endocrine system related to growth and stress processes, in addition to plasma cortisol measurements, we cloned the cDNAs of two adenohypophyseal hormones (prolactin – PRL and somatolactin – SL) and of β -actin (used as a reference gene). Moreover, the effect of ammonia and water hardness on the expression of the examined hormones (PRL and SL) as well as on growth hormone (GH) was evaluated.

2. Materials and methods

2.1. Fish and experimental conditions

Juvenile silver catfish (60–120 g) were obtained from fish farming

sources in Santa Maria, southern Brazil. These juveniles were taken to the Fish Physiology Laboratory at the Universidade Federal de Santa Maria (29°43'50" S, 53°43'42" W) and maintained in 37 continuously aerated 250-L tanks ($n = 10$ fish per box; stocking density 5 kg/m³) at a natural temperature (22.2 ± 0.1 °C) and photoperiod for our latitude (November-December 2011) as well as under constant salinity (0 ppt) for one month prior to the experiment. This stocking density showed not to be stressful for this species (Barcellos et al., 2001). The juveniles were fed once daily (5% of total fish weight) with commercial pellets (Supra juvenil, 32% crude protein, Alisul Alimentos S.A., Carazinho, Brazil).

Experiments were conducted in 36 different tanks combining three different NH₃ levels (0.020 ± 0.008 mg/L [1.17 ± 0.47 µM] – control group, 0.180 ± 0.020 mg/L [10.57 ± 1.17 µM] and 0.500 ± 0.007 mg/L [29.36 ± 0.41 µM]) and two water hardness levels (normal: 25 mg/L CaCO₃ and high: 120 mg/L CaCO₃) (three replicates per treatment). These concentrations were chosen because Ferreira et al. (2013) verified that silver catfish exposed to 0.62 mg/L NH₃ presented lower growth than those exposed to 0.02 mg/L NH₃ and that the increase of hardness reduced the deleterious effect of high NH₃ on growth of this species. On day 0, specimens maintained in an extra tank containing the lowest ammonia concentration (0.02 mg/L) and the normal water hardness (25 mg CaCO₃/L) were used as “control time 0 before exposure”. The waterborne NH₃ levels were increased adding concentrated NH₄Cl (ammonium chloride) solution. The normal tap water hardness in Santa Maria city (southern Brazil) was 25 mg/L CaCO₃, and the higher hardness level (120 mg/L CaCO₃) was produced adding CaCl₂.2H₂O to dechlorinated tap water.

Uneaten food and feces were daily removed, and at least 20% of the water was replaced with water from a reservoir previously adjusted to the levels of ammonia and suitable hardness. The pH of the water (7.57 ± 0.02) was monitored three times daily with a Quimis pH meter (model 400 A, Diadema, SP, Brazil). Water hardness was measured daily with the EDTA titrimetric method (Eaton et al., 2005). Total ammonia levels were determined according to Verdouw et al. (1978), and un-ionized ammonia levels were calculated according to Colt (2002). The levels of dissolved oxygen (6.59 ± 0.06 mg/L) and temperature were measured daily with a YSI oxymeter (model Y5512 Yellow Springs, USA). Total alkalinity levels and nitrite were determined at the

beginning and end of the experiment using the method described by Boyd (1998).

Specimens were anesthetized with 50 mg/L eugenol for 3 min, weighed and measured, and sampled on days 0, 1 and 5 ($n = 10$ animals per sampling point and experimental condition, each group collected from a different tank). Blood was obtained by caudal puncture, centrifuged at 600 g for 5 min to separate the plasma, and then stored at $-20\text{ }^{\circ}\text{C}$ for subsequent analysis. Pituitaries were collected in an appropriate volume of RNA $later^{\circledR}$ (Life Technologies, USA) and stored at $-20\text{ }^{\circ}\text{C}$ prior to total RNA extraction. The methodology of this experiment was approved by the Ethics Committee on Animal Experimentation at UFSM under registration number 24/2007 for the use of laboratory animals.

2.2. Analytical techniques in plasma

Plasma osmolality was measured with a vapor pressure osmometer (Fiske One-Ten Osmometer, Fiske-VT, Norwood, USA) and expressed as mOsm/kg. Glucose, triglycerides and lactate concentrations were measured in plasma using commercial kits from Spinreact (Barcelona, Spain) (Glucose-HK Ref. 1001200; Triglycerides Ref. 1001311; Lactate Ref. 1001330) adapted to 96-well microplates. Plasma protein was analyzed by diluting the plasma 50 times and measuring protein concentration using the bicinchonic acid method with a BCA protein kit (Pierce P.O., Rockford, USA) with bovine serum albumin serving as the standard. All of these assays were run on an Automated Microplate Reader (PowerWave 340, BioTek Instrument Inc.) controlled by the KCjuniorTM program. The standards and all samples were run in duplicate.

Plasma cortisol levels (expressed in ng/mL) were measured by indirect enzyme immunoassay (EIA) in 96-well microplates as described previously by Rodríguez et al. (2000) for testosterone. Steroids were extracted from 5 μL of plasma in 100 μL RB (10% v/v PPB (Potassium Phosphate Buffer) 1 M, 0.01% w/v NaN_3 , 2.34% w/v NaCl, 0.037% w/v EDTA, 0.1% w/v BSA (Bovine Serum Albumin)) and 1.2 mL methanol (Panreac) and evaporated for 48–72 h at $37\text{ }^{\circ}\text{C}$. Cortisol EIA standard (Cat. #10005273), goat anti-mouse IgG monoclonal antibody (Cat. #400002), specific cortisol express EIA monoclonal antibody

(Cat. #400372) and specific cortisol express AChE tracer (Cat. #400370) were obtained from Cayman Chemical Company (Michigan, USA). The standards and extracted plasma samples were run in duplicate. The standard curve was run from 2.50 ng/mL to 9.77 pg/mL ($R^2 = 0.994$). The lower limit of detection (90.56% of binding, ED 90.56) was 13.02 pg/mL. The percentage of recovery was 95%. The inter- and intra-assay coefficients of variation (calculated from the duplicate samples) were $2.85 \pm 1.52\%$ and $3.99 \pm 0.65\%$, respectively. Cross-reactivity information for specific antibodies with intermediate products involved in steroid synthesis was furnished by the supplier (cortexolone (1.6%), 11-deoxycorticosterone (0.23%), 17-hydroxyprogesterone (0.23%), cortisol glucurinoide (0.15%), corticosterone (0.14%), cortisone (0.13%), androstenedione (<0.01%), 17-hydroxypregnenolone (<0.01%), testosterone (<0.01%)).

Chloride (Cl^-) was analyzed by diluting the plasma 2-fold in Milli-Q water and measured using a commercial kit available from Spinreact (chloride, thiocyanate-Hg colorimetric, Ref. 1001360, Barcelona, Spain), whereas values of calcium (Ca^{2+}) and sodium (Na^+) electrolytes were obtained by diluting the plasma samples 100-fold in Milli-Q water and were measured using an Iris Intrepid plasma spectrometer (Thermo Elemental) in the AAS-ICP of the Central Service for Science and Technology (University of Cádiz, Spain). All of these dilutions were performed in tubes pre-treated with nitric acid and washed with Milli-Q water to avoid any disturbance.

2.3. Molecular cloning of full cDNA sequences for PRL, SL and β -actin

First, a set of degenerate primers was designed according to the sequences of cDNA most highly conserved between different species for prolactin (*Silurus meridionalis*, GenBank acc. no. EU177781; *Ictalurus punctatus*, GenBank acc. no. AF267990; *Heteropneustes fossilis*, GenBank acc. no. AF372653), somatolactin (*Ictalurus punctatus*, GenBank acc. no. AF267991; *Silurus meridionalis*, GenBank acc. no. EU131896 and EU131896; *Danio rerio*, GenBank acc. no. NM_001037706; *Salmo salar*, GenBank acc. no. NM_001141618) and β -actin (*Pelteobagrus fulvidraco*, GenBank acc. no. EU161066; *Leiocassis longirostris*, GenBank acc. no. JN833582; *Clarias*

batrachus, GenBank acc. no. EU527190; *Clarias gariepinus*, GenBank acc. no. HM768299; *Heteropneustes fossilis*, GenBank acc. no. FJ409641). Subsequently, degenerate primers were synthesized by IDT® (Integrated DNA Technologies) and supplied by BIOMOL. The nucleotide sequences are shown in Table 1.

Total RNA was isolated from complete brains using a NucleoSpin® RNA II kit (Macherey-Nagel) with an appropriate volume of RA1 and on-column RNase-free DNase digestion according to the manufacturer's protocol. The amount of RNA was spectrophotometrically measured at 260 nm with the BioPhotometer Plus (Eppendorf), and its quality was determined in an Agilent 2100 Bioanalyzer using the RNA 6000 Nano Kit (Agilent Technologies). After reverse transcription to obtain the first-strand cDNA (Super Script III, Life Technologies™), PCR amplification was performed with the proofreading VELOCITY DNA Polymerase (BIOLINE) and samples were cycled (98 °C, 5 min; [98 °C, 30 s; 65 to 55 °C in touchdown, 30 s; 72 °C, 1 min] X 35 cycles; 72 °C, 10 min). Subsequently, 1 U of Platinum Taq DNA Polymerase (Invitrogen, Life Technologies) was applied to add a single adenine (A) as an overhang to each extreme of the PCR product for the next step in a unique step of 10 min at 72 °C. PCR products were identified by agarose gel electrophoresis and ligated with the TOPO TA Cloning® Kit for Sequencing (Invitrogen™, Life Technologies™) into the pCR®4 TOPO® vector. A single clone for each type of cDNA was sequenced in both strands, using M13 Forward (-20) and M13 Reverse primers, by the dideoxy method at the Bioarray S.L. sequencing facilities (Alicante, Spain). The sequence homology for all the PCR products was confirmed by *blastn* using the NCBI website (<http://www.ncbi.nlm.nih.gov/>).

Using total RNA as the template, the 5' and 3' ends of prolactin, somatolactin and β -actin mRNAs were amplified using 5' and 3' Rapid Amplification of cDNA Ends (FirstChoice® RLM-RACE kit, Life Technologies™). Specific forward primers were designed in the three fragments previously cloned (see above) at two different positions (Table 1) and used in combination with the 3'RACE Outer or Inner primers supplied in the kit to amplify the 3' ends. For 5' RACE amplifications, specific reverse primers for each of the three fragments were designed (Table 1) and used in combination with the 5'RACE Outer or Inner primers supplied in the kit. The primers were designed to achieve

an overlap of at least 150 bp between the RACE clones and the previously obtained partial cDNAs. The cloning and sequencing of PCR products were performed as described above. eBiox (v1.5.1) software was used for fragment assembly. Translation of the sequences to the open reading frame (ORF) was performed with eBiox (v1.5.1) software. A homology analysis of putative protein sequences was run with blastp at the NCBI website.

Amino acid sequences were retrieved from the NCBI protein database (www.ncbi.nlm.nih.gov/pubmed, accessed in January 2013). A phylogenetic analysis of all translated sequences was performed using MEGA5 software (Tamura et al., 2011) with the Neighbor-Joining algorithm based on amino acid differences (p-distances) and pairwise deletions. The reliability of the tree was assessed with the bootstrap method (1,000 replicates).

2.4. Quantification of mRNA expression levels

Total RNA isolation, quantification and the assessment of quality were performed as previously described. First, various amounts of cDNA were applied in triplicate (6 serial 1/10 dilutions from 10 ng to 100 fg per reaction) to check the assay linearity and the amplification efficiency for each one of the designed specific pair of primers. Primers for *R. quelen* GH were designed from the complete sequence available in GenBank (Accession number EF101341). Although the assay was linear along the 6 serial dilutions (PRL: $r^2 = 0.998$, efficiency (E) = 0.95; SL: $r^2 = 0.994$, E = 1.02; GH: $r^2 = 0.998$, E = 0.98; β -actin: $r^2 = 0.999$, E = 1.04), 1 ng of cDNA per reaction was used in qPCR reactions after synthesizing the first-strand cDNA using a qSCRIPT™ cDNA Synthesis Kit (Quanta Biosciences). qPCR was performed with Fluorescent Quantitative Detection System (Eppendorf Mastercycler® ep *realplex*² S). Each reaction mixture (10 μ L) contained 0.5 μ L at 200 nM of each specific forward and reverse primer and 5 μ L of PerfeCTa SYBR® Green FastMix™ (Quanta Biosciences). The nucleotide sequences of the specific primers used for qPCR are shown in Table 1. The PCR thermal profile was as follows: 95 °C, 10 min; [95 °C, 30 s; 60 °C, 45 s] X 40 cycles; *melting curve* [60 °C to 95 °C, 20 min], 95 °C, 15 s). β -actin was used as the reference gene due to its low variability (less than 0.35 C_T, whit any differences detected between experimental groups)

under our experimental conditions. A relative gene quantification was performed using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001).

2.5. Statistical analysis

The homogeneity of the variances between treatments was tested with a Levene test. Due to the presence of homogeneous variances in the metabolic data, statistical differences were analyzed by two-way ANOVA with each i) combination of ammonia levels and water hardness (6 groups) and ii) time as main factors, followed by post-hoc comparison made with the Tukey's test. Moreover, Student t-test was used to compare both water hardness used at the same level of NH_3 . Statistica software (version 7) was used for these analyses. The data on gene expression were not homoscedastic and were analyzed using the Scheirer-Ray-Hare extension of the Kruskal-Wallis test followed by a Nemenyi test. The minimum level of significance was 95% ($p < 0.05$).

3. Results

3.1. Mortality

Exposure to different levels of water hardness did not change the mortality of silver catfish caused by exposure to NH_3 . Fish subjected to 0.18 mg/L NH_3 showed a mortality of $63.9 \pm 9.4\%$ at normal water hardness (25 mg/L CaCO_3) and of $57.6 \pm 9.5\%$ at high water hardness (120 mg/L CaCO_3). In addition, one-half of the specimens that survived the exposure to 0.18 mg/L NH_3 at normal water hardness for 5 days showed total RNA of low quantity and quality in both brain and pituitary samples. Exposure to 0.50 mg/L NH_3 at both CaCO_3 concentrations induced 100% mortality within 5 days (Figure 1).

3.2. Cortisol and metabolites

At normal water hardness, the exposure of *R. quelen* specimens to both 0.18 and 0.5 mg/L NH_3 levels for one day significantly increased plasma cortisol levels compared to the lowest NH_3 concentration. However, these values

increased significantly in specimens subjected to high hardness only in those animals exposed to the highest NH_3 level. After 5 days, the fish subjected to 0.18 mg/L NH_3 and normal hardness showed significantly higher plasma cortisol levels than both control groups and than those subjected to high hardness (Figure 2).

The plasma glucose levels in specimens exposed to 0.50 and 0.18 mg/L NH_3 at normal and high water hardness, respectively, increased significantly on the first day of the experiment. After 5 days of exposure to 0.18 mg/L NH_3 , however, these values were significantly lower than their respective controls at the same water hardness, whereas the controls at high hardness showed increased plasma glucose values relative to those specimens kept at normal water hardness (Figure 3A). Plasma lactate (Figure 3B) and triglycerides (Figure 3C) values did not show any significant change at day 1 of exposure and showed a similar pattern of glucose changes at day 5. However, the plasma protein levels were not affected significantly by water hardness or NH_3 exposure (Figure 3D).

3.3. Osmoregulatory parameters

Plasma osmolality increased linearly relative to NH_3 levels on the first day of the experiment at normal water hardness (25 mg CaCO_3/L). However, the specimens subjected to the highest water hardness (120 mg CaCO_3/L) did not show such an increase. On the fifth day, *R. quelen* specimens exposed to 0.18 mg/L NH_3 at normal hardness and both 0.18 and 0.5 mg/L NH_3 groups at the higher water hardness showed plasma osmolality levels that were significantly lower than those at the previous sampling point (Figure 4A).

Exposure to both 0.18 and 0.5 mg/L NH_3 concentrations increased, but not significantly, the plasma Na^+ and Cl^- levels (Figures 4B and 4C). However, on the first day of exposure, the plasma Ca^{2+} values showed a direct linear relationship with NH_3 levels when the normal water hardness was used, as well as and a U-shaped relationship at the highest hardness (Figure 4D). The control fish (0.02 mg/L NH_3 and 25 mg CaCO_3/L) showed significantly lower plasma Na^+ and Cl^- levels on the fifth day of exposure to high water hardness and

higher plasma Ca^{2+} levels from the first day of exposure to high water hardness (Figures 4B, 4C and 4D).

3.4. Molecular cloning of full cDNA sequences for PRL, SL and β -actin

The full-length silver catfish PRL, SL and β -actin cDNAs consisted of 912, 889 and 1814 bp, respectively (Figures 5, 6 and 7). The complete open reading frame (ORF) inferred from the full-length cDNA of PRL and SL in *R. quelen* clustered as an independent branch for each one, as shown by a phylogenetic analysis using the translated sequences in other fish species (Figure 8).

The nucleotide sequence of silver catfish *R. quelen* PRL showed 91% identity with the PRL gene of channel catfish *I. punctatus*, 84–86% identity with the PRL of two other Siluriformes, *H. fossilis* and *S. meridionalis*, and over 70% identity with the PRL of several other teleosts. The *R. quelen* SL sequence showed approximately 80% identity with *I. punctatus* SL and more than 68% with the SLs of several other teleosts. Although 2 different types of SL, namely, $\text{SL}\alpha$ and $\text{SL}\beta$, have been identified in other teleosts (Zhu et al., 2004), *R. quelen* only showed a single isoform of this sequence, with a high identity with both types of *S. meridionalis* (78–81%) and *D. rerio* SL (69–72%) but clustering with the $\text{SL}\alpha$ type (Figure 8). The β -actin sequence of *R. quelen* is very similar to that of other Siluriformes, such as *Tachysurus fulvidraco* (97% identity), and showed more than 90% identity with the β -actin sequences of several other teleosts. Moreover, the phylogenetic analysis of the silver catfish indicates that both PRL and SL sequences cloned belongs to the GH/PRL adenohypophyseal hormone family (Mancera and Fuentes, 2006), corresponding to two different clusters (Figure 8).

3.5. Hormonal expression

The values of GH expression were not significantly affected by water hardness or NH_3 exposure at day 1. However, GH expression decreased significantly compared with the control group (0.02 mg/L NH_3 and 25 mg CaCO_3/L) after 5 days of exposure to 0.18 mg/L NH_3 in specimens held under

normal hardness (Figure 9A). The PRL expression increased significantly in specimens held under normal water hardness and exposed to 0.18 mg/L NH_3 for one day. However, such an increase was not observed in specimens under a similar NH_3 concentration and maintained at high hardness. After 5 days, PRL expression in the fish exposed to 0.18 mg/L NH_3 did not differ significantly from that of the controls (Figure 9B). SL expression only decreased on the fifth day of exposure to 0.18 mg/L NH_3 and high hardness relative to SL expression under the same treatment at the previous sampling point (Figure 9C).

4. Discussion

The lethal NH_3 concentration (96 h) for *R. quelen* kept at pH 7.5 is within the 1.2–1.45 mg/L range (Miron et al., 2008; Ferreira et al., 2013). In the present study, however, mortality was total in specimens exposed for 5 days to 0.5 mg/L NH_3 at both water hardness levels, whereas no significant effect of water hardness on mortality was observed in those specimens subjected to 0.18 mg/L NH_3 . These results are consistent with the lack of an effect of water hardness on lethal NH_3 concentration observed in a previous study with the same species (Ferreira et al., 2013). In addition, specimens weighing 60–120 g (present study) appear to be more sensitive to NH_3 than those weighing 1.85–11.0 g (Miron et al., 2008; Ferreira et al., 2013), suggesting that in this species, resistance to NH_3 could be associated with age and/or size. Further studies are necessary to evaluate these hypotheses as well as the possible application of these results to *R. quelen* aquaculture.

The increased plasma cortisol levels due to NH_3 exposure indicated a clear activation of the stress system in the specimens subjected to this toxicant. Similar results have been observed in the goldfish *Carassius auratus* and the common carp *Cyprinus carpio* exposed to 1.0 mg/L NH_3 over 4 days (Sinha et al., 2012b, c). Moreover, acute exposure (12 h) to 0.4 mg/L NH_3 also increased plasma cortisol levels in both species (Liew et al., 2013). In addition, freshwater- and seawater-adapted rainbow trout *Oncorhynchus mykiss* exposed to 0.29 mg/L NH_3 presented higher plasma cortisol levels after 4 h, and the levels returned to control values after 24 h in freshwater-adapted specimens but not in seawater-adapted ones (Wood and Nawata, 2011). This evidence suggests that

in terms of cortisol values, the stress response induced by NH_3 toxicity is species specific.

The increase in water hardness from 25 to 120 mg/L CaCO_3 did not affect mortality in the groups subjected to various NH_3 levels. However, increased plasma cortisol was not observed in the group maintained at high water hardness values in those specimens of *R. quelen* exposed to 0.18 mg/L NH_3 for a period of 5 days. Therefore, a protective effect of CaCO_3 on mortality could be expected under long-term NH_3 exposure. Furthermore, the RNA degradation in the whole brains and pituitaries of fish exposed to 0.18 mg/L NH_3 and normal water hardness was not observed in the specimens maintained under high water hardness. This result also suggests a protective effect of CaCO_3 on the neurotoxic effects of NH_3 .

The chronic activation of the stress system due to high plasma cortisol levels induced a tertiary stress response accompanied by a decreased growth rate (Wendelaar Bonga, 1997). Our results are consistent with the finding that high water hardness improves the growth of *R. quelen* specimens exposed to sublethal NH_3 levels (Ferreira et al., 2013). A possible explanation of these results is that Ca^{2+} plays a role in the avoidance of the plasma cortisol increase induced by exposure to this toxin in this species, as demonstrated by Carneiro et al. (2009).

Exposure to high NH_3 levels increased the plasma glucose level in *R. quelen* specimens on the first day but decreased it after 5 days. Stress system activation due to NH_3 exposure is known to increase plasma cortisol levels in several teleosts (*C. auratus* and *C. carpio*: Sinha et al., 2012b,c; *R. quelen*: present results). This hormone, acting as a glucocorticoid, stimulates carbohydrate metabolism, enhancing glucose release, glycogenolysis and gluconeogenesis at the hepatic level (Wendelaar Bonga, 1997). This cortisol enhancement could explain the pattern of changes in glucose observed with an increase of this fuel-metabolite at the first stage of the induced stress and the subsequent decrease in glucose values during the duration of the experiment in the NH_3 -treated group. A previous study of *R. quelen* has demonstrated decreases in hepatic glycogen, muscle glucose and glycogen in specimens subjected to high NH_3 levels over a period of 4 days (Miron et al., 2008). This finding is consistent with the plasma glucose depletion observed in our study.

Similarly, exposure to high NH_3 levels reduced plasma triglyceride levels in *R. quelen* specimens after 5 days. This effect becomes important after the plasma glucose is consumed. However, the levels of plasma lactate and protein were not affected by this exposure. Freshwater-adapted *O. mykiss* also showed lower plasma glucose levels, with no effect on plasma lactate levels, after 24 h of exposure to 0.29 mg/L NH_3 (Wood and Nawata, 2011). These results suggest that *R. quelen* used additional carbohydrate and lipids rather than proteins, as shown by the absence of change in this parameter. These metabolites appear to supply the energy needed to increase ammonia excretion at high NH_3 levels. High water hardness did not alter the metabolic changes produced by high NH_3 levels.

In our experiment, *R. quelen* specimens exposed to the high water hardness level showed higher plasma glucose, lactate and triglyceride levels after 5 days, suggesting that the specimens were subjected to metabolic reorganization. However, plasma cortisol values did not change, suggesting not only that no stress occurs in this situation but also that water hardness serves as a protective agent. Our Research Group has previously shown that the exposure of *R. quelen* specimens to as much as 180 mg/L CaCO_3 at pH 7.0 for 30 days did not change growth relative to the control group (Copatti et al., 2011a), suggesting the absence of stress in specimens subjected to high water hardness levels or even a need for additional time (> 5 days) to reach normal values for these parameters. Exposure for one day to higher NH_3 levels increased plasma osmolality and Ca^{2+} values but did not significantly increase plasma Na^+ and Cl^- levels. However, increased water hardness prevented NH_3 effects on these parameters. Previous experiments with *R. quelen* exposed to 0.10 mg/L NH_3 at 20 mg/L CaCO_3 for one day found that these specimens showed significant increases in plasma Na^+ , Cl^- and K^+ (Becker et al., 2009). Moreover, acute ammonia exposure (2–3 h) increased the ventilation rate in *O. mykiss* (Zhang et al., 2011), potentially causing higher ion losses by diffusion. In fact, an increase in net Na^+ loss (which would lead to lower Na^+ plasma levels) is known to occur after a 27-h exposure of *O. mykiss* to 0.11 mg/L NH_3 (Twitchen and Eddy, 1994) and after a 12-h exposure of *O. mykiss*, *C. carpio* and *C. auratus* to 0.4 mg/L NH_3 (Liew et al., 2013). However, the hyperventilatory response produced by high NH_3 levels is abolished in

chronically exposed *O. mykiss* (Zhang et al., 2011); moreover, by 12 h and continuing over a period of 7 days, Na^+ uptake was found to increase in all three species. It is possible that this increase is due to the activation of the branchial apical " $\text{Na}^+/\text{NH}_4^+$ exchange metabolon" which involves several membrane transporters and Rh glycoproteins (Wright and Wood, 2012). It is possible that the higher ion levels observed in *R. quelen* after NH_3 exposure are produced by the same mechanism. Additionally, other alternative and/or complementary hypotheses can be proposed. Cortisol has been found to increase tight junction protein (occludin) expression and reduce paracellular permeability in cultured *O. mykiss* gill cells (Kelly and Chasiotis, 2011). In addition, this hormone has been found to induce Na^+ uptake in *D. rerio* (Kumai et al., 2012). As a result, the higher cortisol levels observed in *R. quelen* exposed to NH_3 might serve to reduce net ion loss and, consequently, increase the values of plasma ions. In addition, a 4-day sustained elevation of cortisol has been found to decrease serum Cl^- levels in *C. auratus*, suggesting a detrimental effect on ionoregulatory tissues other than gills (Chasiotis and Kelly, 2012). This finding is consistent with the lower plasma Na^+ , Ca^{2+} and osmolality observed in *R. quelen* specimens exposed to high NH_3 at day 5 compared with the initial values. However, the relationship between plasma ions and cortisol levels was found only in *R. quelen* maintained at the lowest water hardness.

As Ca^{2+} has been found to decrease gill membrane permeability (Wendelaar Bonga et al., 1983), it could decrease the initial ion loss caused by the hyperventilation produced by ammonia. However, because it is probable that this hyperventilation does not persist, this hypothesis does not explain the protective effect of Ca^{2+} relative to chronic NH_3 exposure.

The pituitary gland plays an important role in the endocrine control of several physiological processes mediated by various hormones, such as growth hormone (GH), prolactin (PRL) or somatolactin (SL). In the Nile tilapia (*Oreochromis niloticus*), GH plasma levels have been found to decrease proportionally to waterborne NH_3 levels (in the 0.01–0.6 mg/L range) (El-Shebly and Gad, 2011). In addition, GH values and the expression of insulin growth factor I (IGF-I) receptors have also been found to decrease in *C. auratus* and *C. carpio* exposed to 1.0 mg/L NH_3 at 10 and 21 days (Sinha et al., 2012b, c). The decrease in GH expression in *R. quelen* after 5 days of exposure to 0.18

mg/L NH_3 at normal water hardness, together with the increased levels of plasma cortisol, are consistent with previous results for teleosts (see above) and confirm the inhibitory effect of cortisol, at least via the inhibition of GH expression, on the growth system in *R. quelen*. In addition, in those specimens maintained at the highest water hardness, the increase in plasma cortisol levels and the GH expression was reduced. This finding agrees with our previous observation that high water hardness improves the growth of *R. quelen* specimens exposed to sublethal levels of NH_3 (Ferreira et al., 2013).

PRL is a pleiotropic hormone involved in various physiological processes such as osmoregulation, stress and metabolism (Manzon, 2002; Sangiao-Alvarellos et al. 2006; Laiz-Carrión et al., 2009). Accordingly, confinement stress is known to increase plasma PRL levels (Avella et al., 1991; Auperin et al., 1995; Weber and Grau, 1999) as well as PRL expression (Laiz-Carrión et al., 2009). In addition, it has been shown that PRL treatment increases plasma cortisol levels in certain teleost species (Wendelaar Bonga, 1997; Sangiao-Alvarellos et al., 2006), reinforcing the idea that PRL is involved in the stress pathways of teleosts. The exposure of *R. quelen* to NH_3 induced stressful conditions that were reflected in high plasma cortisol levels (see above). Accordingly, the increased PRL expression observed under these conditions after one day of exposure could also be related to a possible role of this hormone in stress response in this species. However, *R. quelen* subjected to NH_3 but under high water hardness did not show this increase in PRL expression. This finding, similar to that observed for plasma cortisol levels, indicates a protective role of Ca^{2+} in stress system activation. As an increase in waterborne Ca^{2+} levels has been found to decrease PRL secretion in *O. mossambicus* (Wendelaar Bonga et al., 1985), another possibility is that the increase in water hardness itself inhibited PRL expression in *R. quelen*.

It has been suggested that SL is involved with the regulation of sexual maturation (Benedet et al., 2008), of metabolism (Vargas-Chacoff et al., 2009), of mitochondria-rich gill cells in fish exposed to acidic water (Furukawa et al., 2010) and of background color adaptation (Cánepa et al., 2012) as well as responses to crowding (Laiz-Carrión et al., 2009) and handling stress (Khalil et al., 2012). SL may even favor hypercalcemic action (Kaneko and Hirano, 1993; Kakizawa et al., 1993). However, no clear role of SL in NH_3 exposure or Ca^{2+}

regulation in *R. quelen* has been demonstrated in the present study. SL expression only decreased in *R. quelen* specimens exposed for 5 days to 0.18 mg/L NH₃ at 120 mg/L CaCO₃, suggesting that exposure to high water hardness (as well as high Ca²⁺ environments) decreased SL cell activity. Thus, the level of this hormone could reflect its participation in hypercalcemic action, as previously demonstrated in *O. mykiss* (Kakizawa et al., 1993). Additionally, this phenomenon could be species specific, as suggested by the finding in *C. auratus* that the levels of expression of this hormone did not change significantly after exposure to 1.0 mg/L NH₃ (Sinha et al., 2012c).

5. Conclusions

Irrespective of water hardness levels, short-term exposure to 0.50 mg/L NH₃ is lethal to *R. quelen* specimens in the size range (60–120 g) studied. This NH₃ level also activates the stress system, as indicated by high plasma cortisol levels and PRL expression, and decreases the expression of GH. Moreover, SL action can be related to an interaction between stress processes and Ca²⁺ regulation. An increase in water hardness offers a degree of protection to *R. quelen* specimens exposed to 0.18 mg/L NH₃. This conclusion is based not only on the less marked hormonal changes observed for this treatment but also on the fact it did not alter the modifications to plasma metabolism and plasma ions induced by the NH₃.

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Table 1. Degenerate primers designed for molecular identification of partial cDNA PRL, SL and β -actin sequences, as well as specific primers used for 5' and 3' Rapid Amplification cDNA Ends (RACE) and semi-quantitative expression by RT-PCR.

Degenerate	Nucleotide sequence	Size
<i>RqPRL_Fw1</i>	5'-GAGCKTCHCAACTHTCHGACAA-3'	480 bp
<i>RqPRL_Rv1</i>	5'-ATYTTGTGSGAGTYCTGCGGAA-3'	
<i>RqSL_Fw1</i>	5'-CTRGAYCGAGYSATCCARCAT-3'	515 bp
<i>RqSL_Rv1</i>	5'-AGCAGYTTKAGRAAGGTCTCCA-3'	
<i>Rqβ-actin_Fw1</i>	5'-ACCACAGCYGARMGKGAAAT-3'	336 bp
<i>Rqβ-actin_Rv1</i>	5'-TCCKGTCWGCRAATGCCAGGGT-3'	

Primer name	Nucleotide sequence	Position	Direction
5' RACE			
PRL 5'outer	5'-GTCCTGCAGCTCTCTGGTCTT-3'	457 - 477	Reverse
PRL 5'inner	5'-GATCTGACCAAGCCATGAGAAG-3'	370 - 391	Reverse
SL 5'outer	5'-TAGTCCCTCAGGACGTGCTCT-3'	507 - 527	Reverse
SL 5'inner	5'-ACAAGCACTCCCTGCTTAAGG-3'	615 - 635	Reverse
β -act 5'outer	5'-CGGATATCGACGTCACACTTC-3'	948 - 968	Reverse
β -act 5'inner	5'-CTCCATACCCAGGAAAGATGG-3'	889 - 909	Reverse
3' RACE			
PRL 3'outer	5'-CCTGTCTCTGGTTCGCTCTCT-3'	351 - 371	Forward
PRL 3'inner	5'-TCTCGCTATGCTGTCATCTGAA-3'	393 - 414	Forward
SL 3'outer	5'-TCCAGCACGCTGAGCTGATCT-3'	194 - 214	Forward
SL 3'inner	5'-AAGGTCATGGGGGAAACTCTT-3'	284 - 304	Forward
β -act 3'outer	5'-ACTGCTGCCTCTTCCTCCTCT-3'	784 - 804	Forward
β -act 3'inner	5'-ATTGGCAATGAGAGGTTTCAGG-3'	847 - 867	Forward

qPCR primers	Nucleotide sequence	Size amplified
<i>qPCR-PRL_Fw</i>	5'-CCTGTCTCTGGTTCGCTCTCT-3'	127 bp
<i>qPCR-PRL_Rv</i>	5'-GTCCTGCAGCTCTCTGGTCTT-3'	
<i>qPCR-SL_Fw</i>	5'-TCCAGCACGCTGAGCTGATCT-3'	111 bp
<i>qPCR-SL_Rv</i>	5'-AAGAGTTTCCCCCATGACCTT-3'	
<i>qPCR-GH_Fw</i>	5'-GGACAAACCACCCTAGACGAG-3'	116 bp
<i>qPCR-GH_Rv</i>	5'-TTCTTGAAGCAGGACAGCAGA-3'	
<i>qPCR-βact_Fw</i>	5'-GAAGTGTGACGTCGATATCCG-3'	112 bp
<i>qPCR-βact_Rv</i>	5'-CCTGAACCTCTCATTGCCAAT-3'	

Figure captions

Figure 1. Mortality ratio observed in *R. quelen* specimens exposed for five days to different NH₃ and water hardness levels. No significant difference was observed between water hardness levels in the same NH₃ level.

Figure 2. Plasma cortisol levels in *R. quelen* specimens exposed to different NH₃ and water hardness levels (n = 10). Different lowercase letters indicate significant differences between treatments on the same day of exposure, and different capital letters indicate significant differences between days of exposure under the same treatment ($p < 0.05$, two-way ANOVA followed by Tukey's test).

Figure 3. Plasma levels of (A) glucose, (B) lactate, (C) triglycerides and (D) proteins in *R. quelen* specimens exposed to different NH₃ and water hardness levels. Further details as in legend to Figure 2. *Significantly different from the same level of NH₃ and normal water hardness level ($p < 0.05$, Student t-test).

Figure 4. Plasma levels of (A) Na⁺, (B) Cl⁻, (C) Ca²⁺ and (D) osmolality in *R. quelen* specimens exposed to different NH₃ and water hardness levels. Further details as in legend to Figures 2 and 3.

Figure 5. Nucleotide and deduced amino acid sequences from *R. quelen* PRL cDNA. The start and stop codons are represented in italics, bold and underlined. The deduced amino acid sequence is displayed in bold capital letters above the underlined and italic nucleotide sequence. GenBank accession number KC195971.

Figure 6. Nucleotide and deduced amino acid sequences from *R. quelen* SL cDNA. The start and stop codons are represented in italics, bold and underlined. The deduced amino acid sequence is displayed in bold capital letters above the underlined and italic nucleotide sequence. GenBank accession number KC195972.

Figure 7. Nucleotide and deduced amino acid sequences from *R. quelen* β-actin cDNA. The start and stop codons are represented in italics, bold and

underlined. The deduced amino acid sequence is displayed in bold capital letters above the underlined and italic nucleotide sequence. GenBank accession number KC195970.

Figure 8. Phylogenetic tree of PRL and SL sequences from several teleosts using Neighbor-Joining analysis (Saitou and Nei, 1987) based on amino acid differences (p-distance). Reliability of the tree was assessed by bootstrapping (1,000 replications) (Felsenstein, 1985). The evolutionary distances were computed using the Poisson correction method (Zuckerandl and Pauling, 1965). Phylogenetic analyses were conducted in MEGA5 (Tamura et al., 2011). GenBank and NCBI Reference Sequences accession numbers are as follows: *Rhamdia quelen* PRL (amino acid sequence deduced from KC195971 nucleotide sequence), SL (amino acid sequence deduced from KC195972 nucleotide sequence); *Silurus meridionalis* PRL (ABX38813) and SL (ABY26308); *Heteropneustes fossilis* PRL (AAK53436); *Oncorhynchus mykiss* PRL (NP_001118205); *Coregonus autumnalis* PRL (AAA51434); *Danio rerio* PRL (AAN08916), SLa (NP_001032795) and SLb (NP_001032763); *Cyprinus carpio* PRL (CAA31060) and SL (ADE60529); *Schizothorax prenanti* PRL (ACX31825) and SL (ACX31826); *Tinca tinca* PRL (ABJ90338); *Ctenopharyngodon idella* PRL (ABU49656) and SL (ABN46996); *Hypophthalmichthys molitrix* PRL (CAA43386); *Ictalurus punctatus* SL (NP_001187017); *Oreochromis mossambicus* SL (BAG50585); *Cichlasoma dimerus* SL (ABM74055); *Sparus aurata* SL (AAA98734); *Rutilus rutilus* SL (AEZ02842); and *Carassius auratus* SL (ACB69758)..

Figure 9. The expression of (A) GH, (B) PRL and (C) SL in *R. quelen* specimens exposed to different NH₃ and water hardness levels (n = 10). Different lowercase letters indicate significant differences between treatments on the same day of exposure. Different capital letters indicate significant differences between days of exposure under the same treatment. *Significantly different from the same level of NH₃ at normal water hardness ($p < 0.05$, Kruskal-Wallis, Nemenyi test).

Mortality (%)

100
80
60
40
20
0

- 0.18 mg/L NH_3 - 25 mg CaCO_3 /L
- 0.50 mg/L NH_3 - 25 mg CaCO_3 /L
- 0.18 mg/L NH_3 - 120 mg CaCO_3 /L
- 0.50 mg/L NH_3 - 120 mg CaCO_3 /L

Days of exposure

Days of exposure

[illegible]

	M N	2
5' aggtacggttacgaattcagactaaacttcttacatccaacaatc <u>at gaac</u>		51
A I R V F Q V C I G I L F S S V N G		20
gcaat cagagt t t t ccagggt t t gt at agggat ct t gt t cagct ct gt aaacgga		105
F T G T A L D C S D P D F M G A R C		38
t t caccggcacggct ct ggact gct cagat ccagat t t t at gggagct cgat gc		159
T I S V E K L L D R A I Q H A E L I		56
acaat t t ct gt ggaaaagct t ct ggat cgagccat ccagcacgct gagct gat c		213
Y R M S D E A R T L F E E M F I P L		74
t accgcat gt cagacgaggccaggact t t gt t t gaggagat gt t cat ccct ct a		267
S I S T H Q G H G G N S C T S N L V		92
t cgat at ccact cat caagggt cat gggggaaact ct t gcacat caaacct t gt g		321
R V P T S K L E I Q Q I S D K W L L		110
cgcggt cccaacat ccaaact cgaaat t caacaaat ct cggacaaat ggct cct t		375
H S I W I L V Q F W I D P L A D L Q		128
cact ccat ct ggat t ct ggt t cagt t ct ggat cgat ccct t ggct gacct gcag		429
D S L D S Y D N A P S S L L H K T R		146
gat t ct ct ggacagt t acgat aacgccccagct ccct cct ccacaagaccagg		483
W M S T K L M N L K Q G V L V L T S		164
t ggat gt ccact aagct gat gaacct t aagcaggaggt gct t gt cct cagaggt		537
K M L D E G T L E L E N N E S L S H		182
aagat gct ggat gaaggaact ct ggagct ggagaat aacgagagct t gt cacac		591
Q V G T P A M A E H V L R D Y A V L		200
cagggt t ggcacccccgccat ggcagagcacgt cct gagggact acgccgt gct g		645
S C F R K D A H K M E T F L K L L R		218
t cct gct t cagaaaggacgcccacaagat ggagacat t t ct gaaact gct t aga		699
C R Q T D S R S C S L F		230
t gccgt caaacagat agt cggagct gct ct ct ct t ct agccgtgaggggaagatc		753
tgcagtgggttctgtactgtggatcttaaaaaatggaattttaatattacacagt		807
taaacaaatttttaaagtctggagaaatcctaataaagatacatatttttgcaa		861
atatt-pol yA t ai l 3'		865

5'	gaaaaacttttgagctcctccacacgcagctagtgcggaatatcatctaacca	51
	aaacctttttatttcttaagccgacaaaaccccaaactttaagttcagccatggat	105
	D E I A A L V V D N G S G M C K A G	20
	gat gagat t gccgcact ggt t gt t gacaacggat ccggt at gt gcaaggct gga	159
	F A G D D A P R A V F P S I V G R P	38
	<u>t t cgct ggagat gat gct ccccggt gct gt ct t cccat ccat cgt ggggt cgccca</u>	213
	R H Q G V M V G M G Q K D S Y V G D	56
	agacat cagggt gt gat ggt t ggt at gggacagaaggacagct at gt t ggt gat	267
	E A Q S K R G I L T L K Y P I E H G	74
	gaggcccagagcaaaagagggt at cct gaccct gaagt accccat t gaggacggt	321
	I V T N W D D M E K I W H H T F Y N	92
	<u>at t gt caccaact gggat gacat ggagaagat ct ggcat cacacct t ct acaac</u>	375
	E L R V A P E E H P V L L T E A P L	110
	gagct gcgt gt t gcccccgaggagcaccgcgt cct gct t accgagggt cccct g	429
	N P K A N R E K M T Q I M F E T F N	128
	aaacccaaagccaacagggaaaagat gaccagat cat gt t cgagacct t caac	483
	T P A M Y V A I Q A V L S L Y A S G	146
	<u>acccagccat gt acgt t gccat ccagggt gt gct gt ccct gt at gcct ct ggt</u>	537
	R T T G I V M D S G D G V T H T V P	164
	cgt accact ggt at cgt gat ggact ct ggt gat ggt gt gaccacact gt gcc	591
	I Y E G Y A L P H A I L R L D L A G	182
	at ct at gaagggt t acgcccgt gcccct gccat cct ccgt ct ggact t ggct ggt	645
	R D L T D Y L M K I L T E R G Y S F	200
	<u>cgt gacct gact gact acct cat gaagat cct gaccgagagagggt acagct t c</u>	699
	T T T A E R E I V R D I K E K L C Y	218
	accaccacagccgagagagaaaat t gt ccgt gacat caaggagaagct gt gct ac	753
	V A L D F E Q E M G T A A S S S S L	236
	gt cgccct cgact t cgagcaggagat gggcact gct gcct ct t cct cct ct ct g	807
	E K S Y E L P D G Q V I T I G N E R	254
	<u>gagaagagct at gagct gcccgacggacaggt cat caccat t ggcaat gagagg</u>	861
	F R C P E A L F Q P S F L G M E S C	272
	t t caggt gt cccgagggt ct ct t ccagccat ct t t cct ggggt at ggagt cct gt	915
	G I H E T T F N S I M K C D V D I R	290
	ggaat ccacgagaccacct t caact ccat cat gaagt gt gacgt cgat at ccgt	969
	K D L Y A N T V L S G G T T M Y P G	308
	<u>aaggat ct gt at gccaacact gt act gt ct ggcggt accaccat gt accct ggc</u>	1023
	I A D R M Q K E I T S L A P S T M K	326
	at t gcagacaggat gcagaaggaaat cacat ccct ggcccct agcacaat gaaa	1077
	I K I I A P P E R K Y S V W I G G S	344
	at t aagat cat t gcccacact gagcgt aaat act ct gt ct ggat cggagggt t cc	1131
	I L A S L S T F Q Q M W I S K Q E Y	362
	<u>at cct ggcct ccct gt ccacct t ccagcagat gt ggat cagcaagcaggagt ac</u>	1185
	D E S G P S I V H R K C F	375
	gat gagt ct ggaccct ccat cgt ccaccgcaaat gct t ct aaacagactgtttac	1239
	cacttcacgtcgactcaatctgcgcagagaaaatacctgaaacgacgacaacatt	1293
	ggcatggcttttgtttttttggcgcttgactcaggatctaaaaactggaacgg	1347
	tgaagggtgacgacaatgttttttggcaataagcatccccgaagttctacaatg	1401
	catctgaggactcaaagtactttttttgttttttggtttttgtttttgttcct	1455
	tttttagtcattccaaatgtttgttaaatgcattgttcaaacttattttgocctcta	1509
	tgaaggctgcccagtaattgggagcataacttaacattgtagtattgcttgtagt	1563
	taaatttatgtaaccgaaaacaatgtctgggtttttgtactttcagccttaatac	1617
	tgggtccttttgttttctttgtgttacaaaatggaaatacaaagcttttaccttt	1671
	attcaaaatgtaaagggttcatttcccttgggcaaacataaaagctgtatggaa	1725
	catggtggtgccagacacttggtggggccaacctgtacactgactaattcaatt	1779
	ccaataaagtgcacatgt-pol yA tail	3'
		1797