Univerzita Karlova v Praze
1. lékařská fakulta

Autoreferát disertační práce

The study of expression and function of selected nuclear receptors in *Caenorhabditis elegans*

Ing. Jaroslav Vohánka

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Předseda oborové rady:	**Prof. MUDr. Milan Elleder, DrSc.,**
Školicí pracoviště:	**Ústav dědičných metabolických poruch** **Laboratoř molekulární biologie a genetiy**
Školitel:	**MUDr. Marta Kostrouchová, CSc.**

Disertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištěné podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

Content

Abstrakt:

V genomu *Caenorhabditis elegans* je více než 280 jaderných hormonálních receptorů (NHRs) v porovnání se 48 jadernými receptory u člověka a 18 jadernými receptory u octomilky (*Drosophila*). Většina jaderných hormonálních receptorů u *C. elegans* patří do skupiny suplementárních (podpůrných) jaderných receptorů (supnrs), které vznikly následnou duplikací jednoho původního genu. Evoluční tlak, který vedl ke zmnožení jaderných hormonálních receptorů u nematod, stejně tak i funkce většiny supnrs nejsou známé. V této práci byla studována exprese sedmi genů organisovaných v klastru na chromosomu V: *nhr-206, nhr-208, nhr-207, nhr-209, nhr-154, nhr-153* a *nhr-136*.

Metodou reverzní transkripce-kvantitativní PCR a použitím transgenních linií nesoucích fůzní geny (obsahující pravděpodobné promotory) s GFP bylo zjištěno, že všech sedm genů tohoto klastru je exprimováno a pět z těchto genů má částečně se překrývající expresní profil. Exprese byla lokalizována v jícnu, ve střevě, v určitých neuronech, v análním svěrači a v samčích specifických buňkách.

Čtyři geny v tomto klastru jsou zachovány u *C. elegans* a u *C. briggsae*, zatímco tři geny jsou přítomné pouze u *C. elegans*, což ukazuje na poměrně nedávnou genovou expanzi.

V naší práci jsme zjistili, že jak část zachovaných tak všechny nezachované geny v tomto klastru odpovídají transkripčně na hladovění a tato odpověď je tkáňově specifická. Naše výsledky ukazují, že rozrůznění časové, prostorové a metabolické genové exprese je ve skupině suplementárních jaderných receptorů spojeno s evolučním tlakem.

Abstract:

The genome of *Caenorhabditis elegans* encodes more than 280 nuclear hormone receptors (NHRs) in contrast to the 48 NHRs in humans and 18 NHRs in *Drosophila*. The majority of the *C. elegans* NHRs are categorized as supplementary nuclear receptors (supnrs) that evolved by successive duplications of a single ancestral gene. The evolutionary pressures that lead to the expansion of NHRs in nematodes, as well as the function of the majority of supnrs, are not known. Here, we have studied the expression of seven genes organized in a cluster on chromosome V: *nhr-206, nhr-208, nhr-207, nhr-209, nhr-154, nhr-153 and nhr-136*.

Reverse transcription-quantitative PCR and analyses using transgenic lines carrying GFP fusion genes with their putative promoters revealed that all seven genes of this cluster are expressed and five have partially overlapping expression patterns including in the pharynx, intestine, certain neurons, the anal sphincter muscle, and male specific cells.

Four genes in this cluster are conserved between *C. elegans* and *C. briggsae* whereas three genes are present only in *C. elegans*, the apparent result of a relatively recent expansion. Interestingly, we find that a subset of the conserved and non-conserved genes in this cluster respond transcriptionally to fasting in tissue-specific patterns. Our results reveal the diversification of the temporal, spatial, and metabolic gene expression patterns coupled with evolutionary drift within supnr family members.

1. Introduction

Using a model organism helps us to understand biological functions and phenomena because of the conservation of several metabolic and developmental pathways over the course of evolution. Studying model organisms can be informative, but care must be taken when generalizing from one organism to another. Nowadays model organisms are widely used to explore gene regulatory cascades and causes and treatments for human diseases.

There are many model organisms. One of the first model systems for molecular biology was the bacterium *Escherichia coli*, a common constituent of the human digestive system. Several of the bacterial viruses (bacteriophages) that infect *E. coli* also have been very useful for the study of gene structure and gene regulation (e.g. phages Lambda and T4). From eukaryotes, several yeasts, particularly *Saccharomyces cerevisiae* (baker's yeast), have been widely used in genetics and cell biology, largely because of the simplicity with which yeast can be grown. The cell cycle in a simple yeast is very similar to the cell cycle in human cells and is regulated by homologous proteins. The fruit fly *Drosophila melanogaster* is studied, because it is easy to grow, has various visible congenital traits and has a polytene (giant) chromosome in its salivary glands that can be examined under a light microscope. The nematode *Caenorhabditis elegans* is studied because it is a simple organism, it has short generation time, it has very defined patterns of development involving fixed numbers of cells, it has a transparent body, which could be rapidly assayed for abnormalities.

Nuclear hormone receptors (NHRs) are transcription factors with many specific functions. They are important for development, metabolism, cellular differentiation and defense against xenobiotics. NHRs consist of several domains described below.

Nuclear hormone receptors form a large superfamily of transcription factors which either activate or repress expression of specific genes (Beato et al., 1995; Robyr et al., 2000; Horwitz et al., 1996).

The number of NHRs found in different genomes varies from 18 in Drosophila melanogaster, 48 in the human genome, 268 in C.briggsae and more than 284 in Caenorhabditis elegans genome (Stein et al., 2003; Enmark and Gustafsson, 2001; Maglich et al., 2001; Van Gilst et al., 2002; Robinson-Rechavi et al., 2003; Gissendanner et al., 2004; King-Jones and Thummel, 2005; Antebi, 2006).

From 284 NHRs in C.elegans only 15 receptors are homologues to vertebrate and Drosophila. These genes are placed into five major subfamilies of NHRs (Sluder, 2001; Gissendanner, 2004).

2. The aim of the study

The aim of the study was to analyze a cluster of seven genes localized on chromosome V in Caenorhabditis elegans, which were predicted as nuclear hormone receptors. All seven receptors belong to the Class I of C.elegans nuclear hormone receptors based on their P-box sequences (Gilst 2002). NHR-206, NHR-208, NHR-207 have P box sequence CNGCKA and form a small subgroup 12, and four receptors NHR-209, NHR-154, NHR-153 and NHR-136, which have P box sequence CNGCKT form the subgroup 8, (Van Gilst, 2002).

The goal of this study was

a) to determine whether all these receptors are functional NHRs

b) to characterize and localize their expression

c) to find their function

3. Materials and methods

Following *C.elegans* strains were used: **N2** – wild type animals (var. Bristol) and wild type *C.briggsae* (related nematode species) worms were kindly afforded by the CGC

Following vectors were used: **pRF4** – vector containing the *rol-6*(su1006) mutant collagen gene. Its expression causes exhibition of a helically twisted body (Mello et al., 1991), **L4440** (pPD129.36) – vector containing two convergent T7 RNA polymerase promoters with opposite orientation separated by a multicloning site, **pCR®4-TOPO®** Cloning Vector (Invitrogen, Carlsbad, CA) – vector for the direct insertion of Taq polymerase-amplified PCR (polymerase chain reaction) products (using covalently bound topoisomerase I) containing T3 and T7 polymerase promoters, **pCR®II** (Invitrogen, Carlsbad, CA) TA Cloning Kits are designed for cloning Taq-amplified PCR products directly from a PCR reaction using an overnight ligation step. The kits use a pCR vector and yield greater than or equal to 80% recombinants with greater than or equal to 90% of the recombination, **pPD95.67** – promoterless gfp vector with a nucleolar localization signal

Molecular biology methods

All molecular biology methods as gDNA and total RNA isolation, PCR, single worm PCR, reverse transcription PCR (RT-PCR), quantitative real-time PCR (qPCR), cloning, transformation, in vitro transcription as well as RNAi, RACE and densitometry analyses were performed according to standard protocols.

4. Results

4.1. Characterization of the cosmids R07B7.13 and C13C4.3

The genomic region, which contains this locus on chromosome V, is included in two cosmids, R07B7.13 and C13C4.3 and spans 17 kb (V: 12 092 022 – 12 109 114, WS207). The genes localized at this region are in close proximity to each other and include the following nhr genes starting from 5'end: *nhr-206, nhr-208, nhr-207, nhr-209, nhr-154, nhr-153* and *nhr-136*.

In order to characterize the cDNAs transcribed from this cluster, we prepared cDNAs from mixed cultures of *C.elegans* N2 worms and performed PCR reactions with primers derived from genes identified by the GeneFinder program including the SL1 and SL2 splice leader primers. PCR products were cloned into pCR4 and or PCRII vectors and sequenced. This strategy confirmed the expression of mRNAs of all seven genes in the cluster as predicted by the WormBase (WS 207 data release).

The alignment of amino acid sequences of all receptors from this region was analyzed by the Clustal program [Larkin, 2007] (Fig.1). The analysis shows the strongest homology between receptors in the DNA binding domain and at the C terminal parts of the receptors. In the remaining regions, the receptors show striking sequence diversity despite that the Blast search identifies genes of this cluster as the closest homologues in the *C.elegans* genome that is in agreement with recent origin of this cluster by successive duplications, however with a significant sequence diversification. The phylogenic and cladistic analyses indicate that the genes in the cluster can be divided to two subgroups, first containing the genes that show phylogenic relation with *nhr-207* and the second clustering with a common ancestor of *nhr-153* and *nhr-136* (Fig. 13A). The analysis also indicates that the later cluster is more ancient and the genes *nhr-206*, *nhr-208* and *nhr-207* were formed by more recent duplications.

All seven receptors belong to the Class I of *C.elegans* nuclear hormone receptors based on their P-box sequences [Van Gilst, 2002]. Their classification according to the P-box sequence keeps with the division based on the overall homology estimated by the Blast program: the first three receptors, NHR-206, NHR-208 and NHR-207, have P box sequence CNGCKA and form a small subgroup of receptors in the subgroup 12 and four receptors NHR-209, NHR-154, NHR-153 and NHR-136, which have the P box sequence CNGCKT form the subgroup 8 [Van Gilst, 2002].

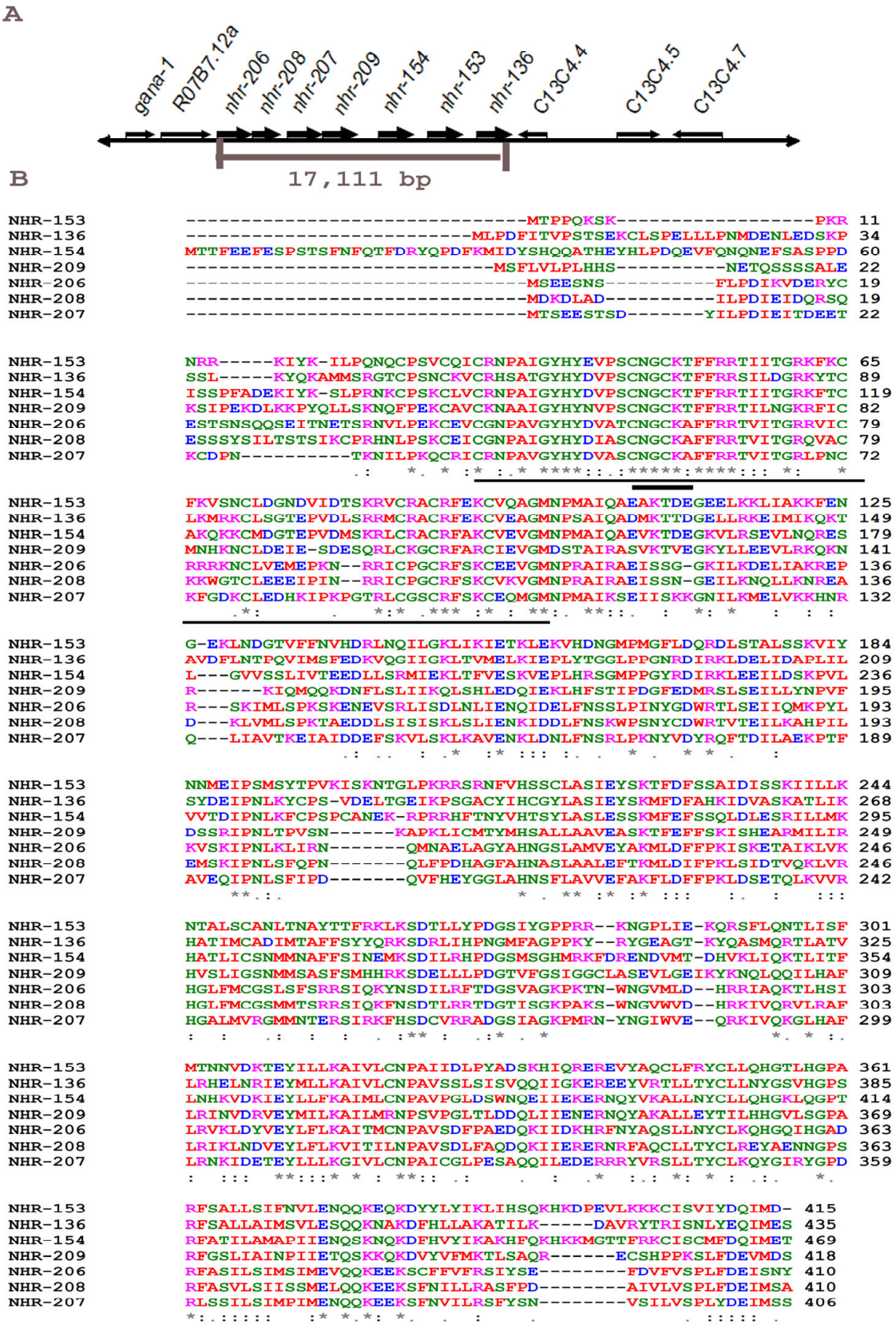

Figure 1. Genomic organization and sequence similarity of seven clustered NHRs.

A) A schematic diagram of genes localized in a region of Chromosome V. A cluster of seven *nhr* genes (thick back arrows) spanning 17 kb are bracketed by the unrelated genes *R07B7.12a* at the 5' end *C13C4.4* at the 3' end.

B) The Clustal analysis of the amino acid sequences corresponding to the seven clustered nuclear hormone receptors: NHR-206, NHR-208, NHR-207, NHR-209, NHR-154, NHR-153 and NHR-136. The alignment shows regions of high sequence conservation and regions that have substantially diversified. The highly conserved DNA binding domain (DBD, thin underline) and the sub domain involved in the contact of nuclear hormone receptors with DNA response elements (P box, thick underline). The ligand binding domains constituting the majority of approximate C- terminal halves of receptors show substantial diversification (visualized using standard color parameters, representing small and hydrophobic amino acids in red, acidic amino acids in blue, basic in magenta and hydroxyl- or amine- group containing amino acids in green color and the remaining amino acids in grey color).

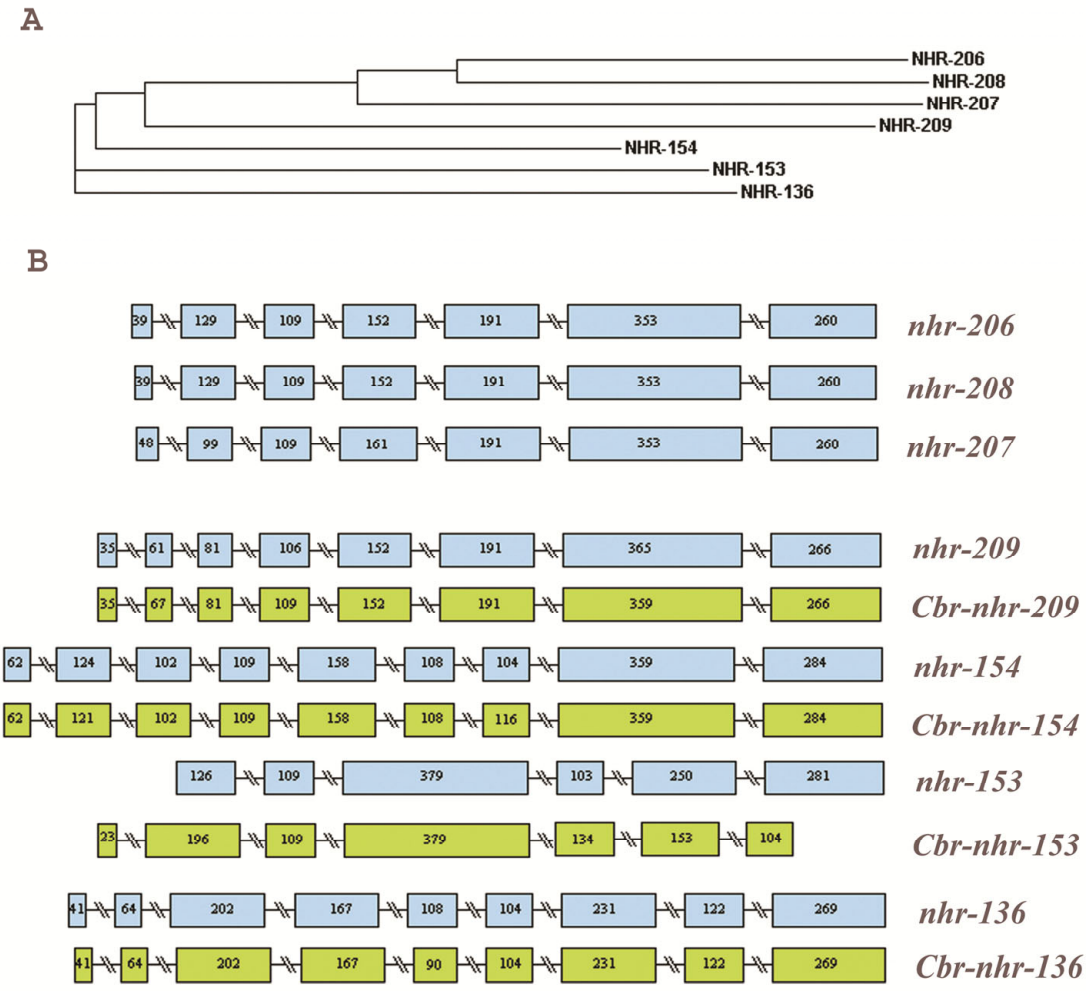

Figure 2. Evolutionary relatedness and intron-exon similarities among the seven clustered *nhrs*.

A) The phylogram of the seven clustered NHRs, as calculated from protein sequences using the Clustal program, indicated that NHR-209, NHR-54, NHR-153 and NHR-136 group together while NHR-206, NHR-208 and NHR-207 represent more recent duplications and are most closely related to NHR-209.
B) The coding region organization of the seven clustered *C.elegans nhr* genes and their closest homologs in *C.briggsae.* The diagram shows differences between recently duplicated genes present only in *C.elegans* and genes conserved between *C.elegans* and *C.briggsae*. While the recently duplicated genes have almost identical exon sizes, the conserved genes show wider diversity of exon sizes between genes in the particular species but are conserved between these closest corresponding *C.elegans* and *C.briggsae* homologues.

4.2. *nhr-207* and *nhr- 209* are organized in an operon

The intergenic regions between genes of the studied cluster vary from 270 to 1277 bp. A 270 bp long region of a putative promoter or an intergenic region is upstream of *nhr-206*. Preceeding *nhr-206*, Wormbase identifies a pseudogene R07B7.12. The other regions have sizes of 651, 338, 542, 1277, 1141, 1156 bp. This suggests that some genes of the cluster may be organized in operons.

In order to distinguish between individually organized genes and genes expressed from operons, we prepared cDNA from mixed stages of N2 animals and performed multiple PCRs with primers specific for splice leader 1 (SL1) and splice leader 2 (SL2) together with gene specific reverse primers. This strategy identified trans-splicing with SL1 primer in the case of *nhr-154* and its 13 bp long 5'UTR (tatagtggcagcc). The trans-splicing with SL2 splice leader was detected in the case of *nhr-209* along with the 257bp long 5'UTR. Surprisingly, trans-splicing was not detected in the remaining genes of the studied cluster, despite that the expression of predicted mRNAs was readily amplified using gene specific primers derived from the predicted sequences. Keeping with our results, WormBase lists SL1 splice leader for *nhr-154*.

The same region and splice leader SL2 for *nhr-209* was also found by the RACE method.

4.3 Expression of studied genes

We have found that all genes are expressed during all developmental stages at a similar and relatively stable expression level accept *nhr-154.* We started with semi-quantitative PCR. The expression level was confirmed by real-time PCR.

We confirmed that *ama-1* could be used as a reference gene in our study. There were treated worms (stressed by limitation of food) and control N2 worms. We have found that the confidential interval and standard deviation is close to one (no expression changes). After this confirmation we were sure that ama-1 can be used as a reference (housekeeping) gene in this study.

PCR amplification for each amplicon was measured and calculated using the formula $N=10^{-1/slope}$. For the actual calculations, the base of the exponential amplification function was used (e.g. 1.96 means 96% amplification efficiency). The formula of regression was calculated automatically by the LighCycler® software. Each specific amplicon was diluted ten times. This diluent was used as a template for real-time PCR. To visualize Cp values absolute quantification using 2^{nd} derivation maximum was used. Cp values were used to create curve, formula of regresion and efficiency value.

Quantitative PCR has confirmed that the expression level of all genes is relatively stable contrary to *nhr-154* which was expressed at a high level in embryos and L1 larvae and than the expression decreased. High levels of expression of *nhr-154* were observed again in young adults (**Fig. 3**). Using qPCR we have found that expression level of *nhr-206*, *nhr-208* and *nhr-207* is expressed in hundreds of copies in contrary to *nhr-209*, *nhr-154*, *nhr-153* and *nhr-136* were the expression is in thousands of copies (expressed in absolute values). The values detected for *C. brigssae* orthologues *Cbr-nhr-209*, *Cbr-nhr-154*, *Cbr-nhr-153* and *Cbr-nhr-136* were similarly large **(Fig. 4)** as in the case of *C.elegans* orthologues suggesting that the expression of conserved genes at standard laboratory conditions is bigger in comparison with the expression of genes that arouse as new members of the family the in *C.elegans* genome.

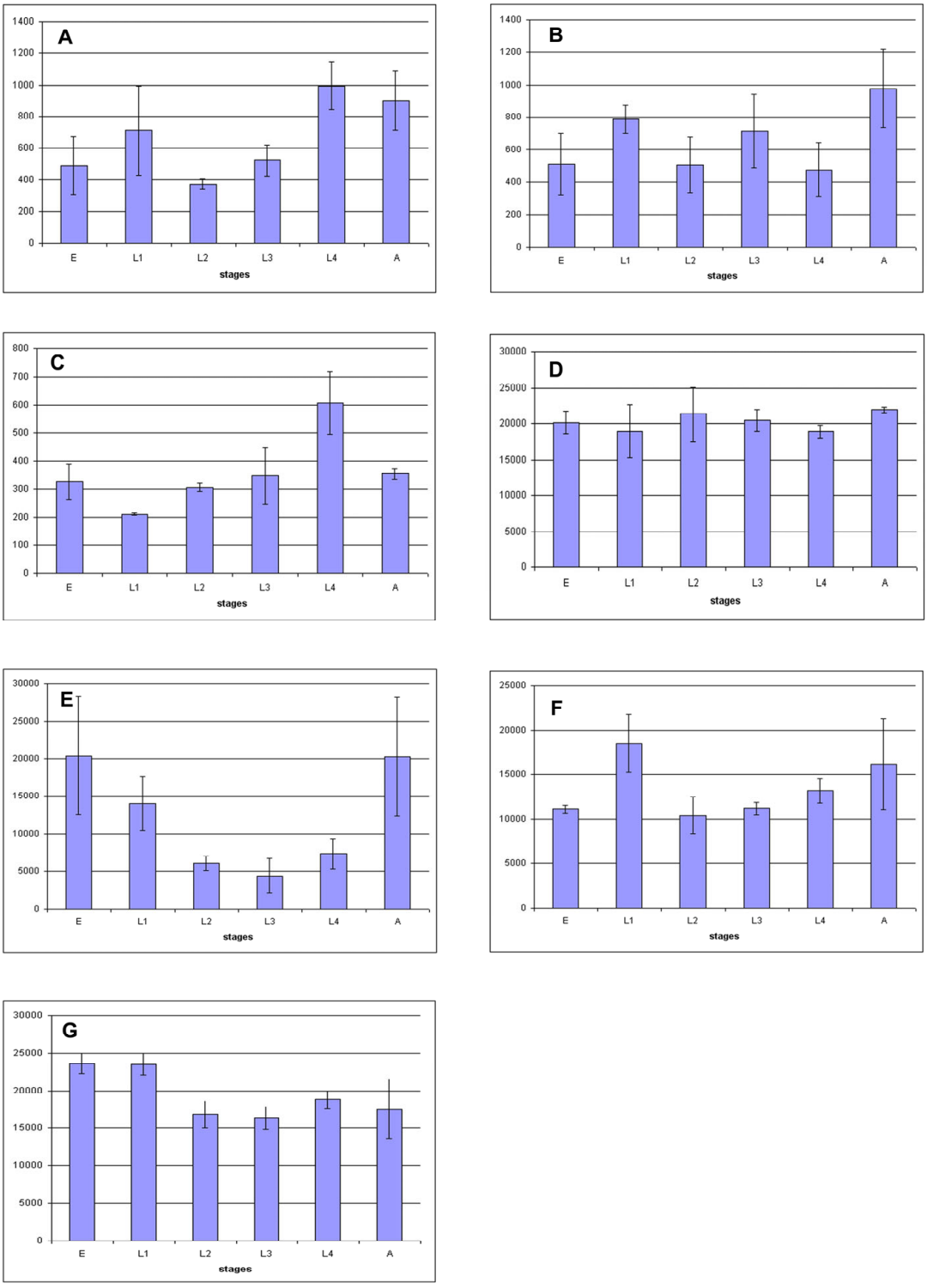

Fig.3 The expression level of *nhr-206, nhr-208, nhr-207, nhr-209, nhr-154, nhr-153, nhr-136*

The picture shows the expression level in individual larval stages and studied genes. The expression of the seven clustered *C.elegans* nhr genes during development was analyzed by RT-qPCR revealing that all genes are expressed under standard laboratory growth conditions. The recently duplicated genes within the cluster (*nhr-206*, *nhr-208* and *nhr-207*) are

expressed at substantially lower levels than the genes that are conserved between *C.elegans* and *C.briggsae*. All genes, with the exception of *nhr-154*, are expressed at relatively constant levels throughout development in *C.elegans* . The expression of *nhr-154* decreases in mid-larval stages with the lowest levels detected at the L3 stage.

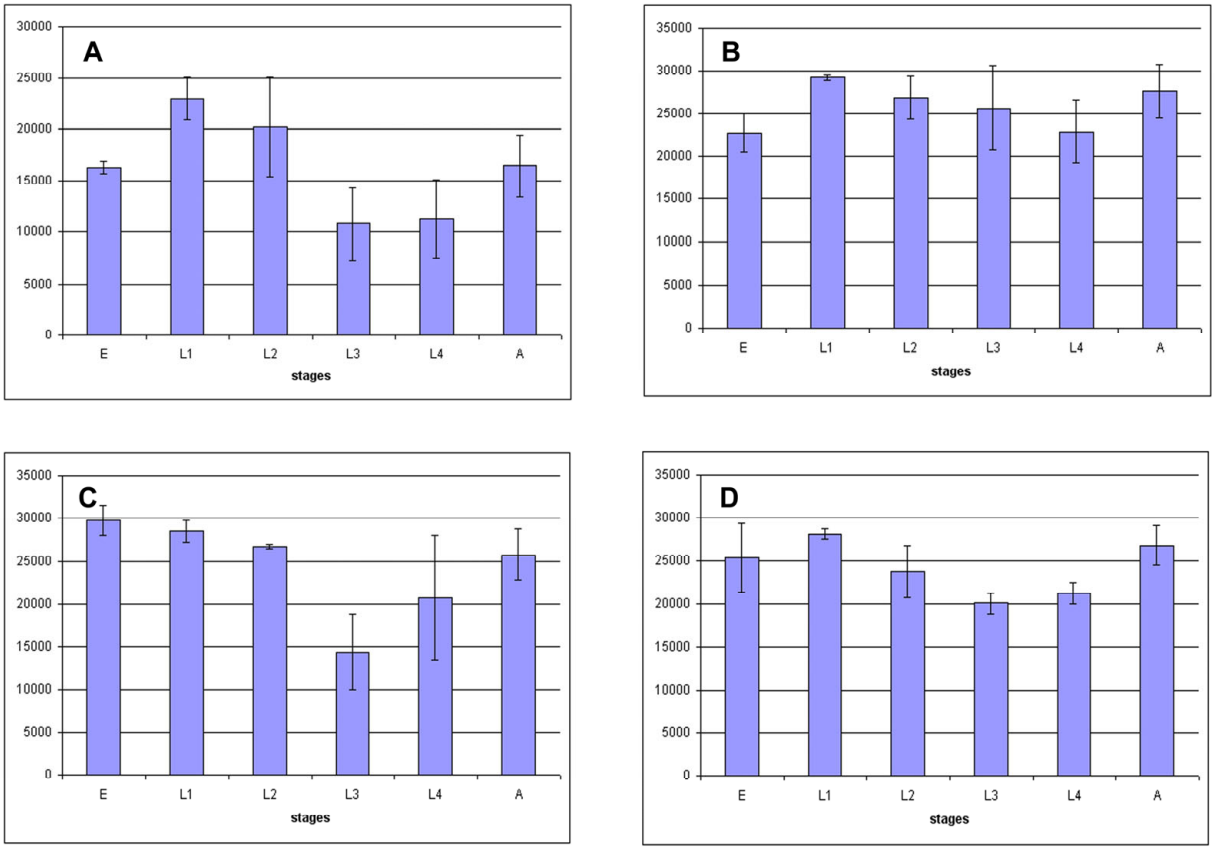

Fig.4 Gene expression of *Cbr-nhr-209, Cbr-nhr-154, Cbr-nhr-153* and *Cbr-nhr-136*
Analysis of the expression of *C.briggsae* nhr cluster orthologs by RT-qPCR. The expression of Cbr-*nhr-209*, Cbr-*nhr-154*, Cbr-*nhr-153*, Cbr-*nhr-136* is relatively stable during development and shows levels comparable to their *C.elegans* orthologs.

4.4 GFP expression

In order to determine expression patterns for nhr studied genes, we prepared various transgenic lines expressing GFP as transcriptional fusion constructs. Our strategy was to have two different promoter lenthts. The shorter promoter region was located upstream of the gene but only in the intergenic region. The longer promoter region was approximately 1-2 kb and usually took a 3´end of the previous gene. Fig.5 shows the organization of the studied genes on chromosome V, their orientation and according to our strategy promoter regions.

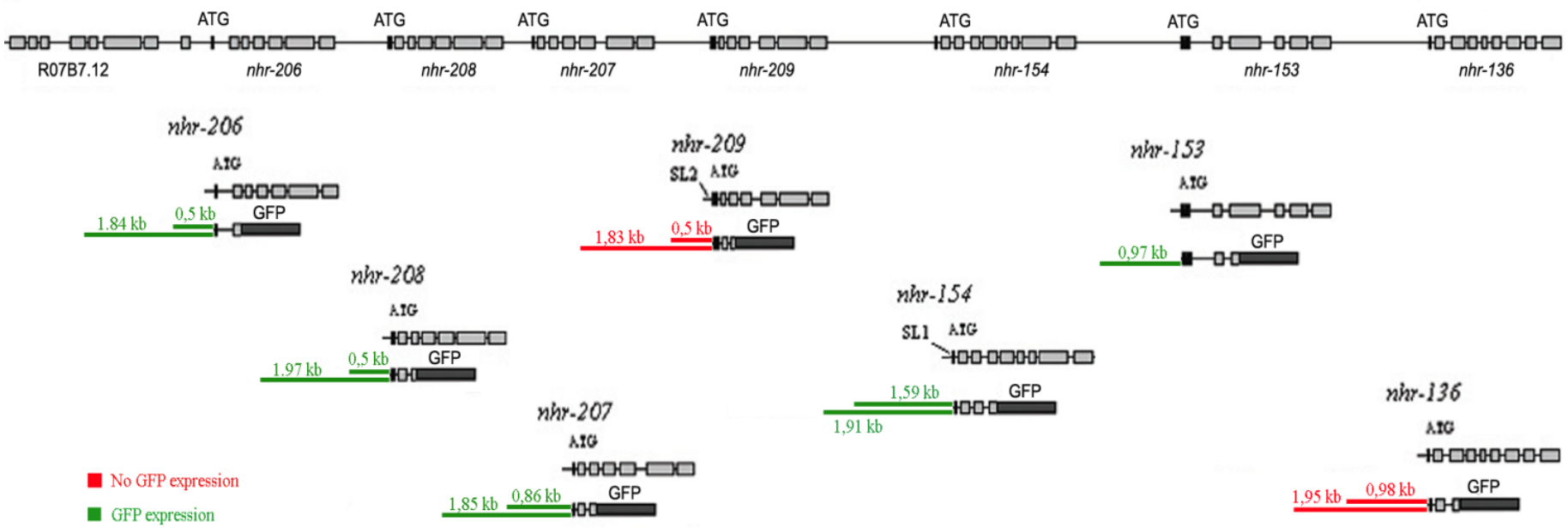

Fig.5 Organization on chromosome V and GFP promoters

The picture shows organization, gene orientation, gene and intergenic sizes. There are promoter regions which were tested using GFP constructs. Green and red colors show if GFP expression was visible.

4.4.1. The expression of nhr-206::GFP

Since we did not find trans-splicing with the SL2 splice leader in the case of *nhr-206*, that would show possible expression from an operon with the preceding gene, we used as a putative promoter the 270 bp long sequence that is in front of *nhr-206* together with two differently long regions included in R07B7.12. Both strategies (putative promoters with the length of 511 bp and 1839 bp) lead to functional promoters with an identical expression pattern.

The expression of *nhr-206* GFP reporter transgenes starts during the comma stage of embryogenesis, and is initially seen in four unidentified cells localized in the head region. By the 2-fold stage, embryonic expression is observed in the pharynx with weaker expression in the intestine. This expression pattern continues throughout all larval stages and in adults with pronounced anterior pharyngeal expression. The reporter genes were also strongly expressed in rectal gland cells, the anal sphincter, and in epidermal cells in the tail. Weaker expression was also observed in the vulva and spermatheca. In males, expression was visible in male specific neurons of the tail and rays. The pattern of expression is very similar to that described by Reece-Hoyes and coworkers in their high throughput screen (Reece-Hoyes et al., 2007).

4.4.2. The expression of nhr-208::GFP

The expression of *nhr-208* GFP reporters started in embryos at the 1.5-fold stage within the pharynx, intestinal sphincter, and epidermal cells in the tail. By the 3-fold stage of embryogenesis, additional expression was observed in rectal gland and surrounding cells. During all larval stages, strong expression of the transgenes was visible in the pharyngeal and unidentified head neurons, the pharyngeal-intestinal valve cell, the posterior part of the intestine, the intestinal sphincter, two rectal gland cells, the intestinal-rectal valve cell, and the epidermal hyp10 cell. In males, the expression was seen in several rays (6-8) and other male specific neurons. This pattern was similar to that described by Reece-Hoyes and coworkers (using a 653 bp long promoter), although our reporters did not result in expression in the excretory cell and vulva.

4.4.3. The expression of nhr-207::GFP

The expression of *nhr-207* GFP reporters began in 1.5-fold embryos in pharyngeal and epidermal cells. In 3-fold stage embryos, expression was observed in pharyngeal neurons, intestinal cells, the intestinal-rectal valve, and the sphincter. This pattern of expression was present during all larval stages and in adults. In larvae and in adults, additional expression was observed in the pharyngeal-intestinal valve and spermathecae. In males, the expression was seen in male specific

neurons, including rays. Our results overlap those reported by Reece-Hoyes and coworkers (using a 340 bp long promoter).

4.4.4. The expression of nhr-209::GFP

In search of a putative promoter of *nhr-209*, we prepared six transgenic lines with a construct made from the short upstream region (530 bp) and four lines with a construct made from a long upstream region of ***nhr-209*** (1834 bp), but we did not see any GFP expression. We also attempted to create a construct with the promoter of *nhr-207*, the complete genomic sequence of *nhr-207*, the intergenic region between *nhr-207* and *nhr-209* and fusion of *gfp* gene with the second exon of *nhr-209* (by overlapping PCR products injected with a linearized pRF4plasmid) but we did not observe GFP expression in the progeny of injected mothers.

4.4.5. The expression of nhr-154::GFP

The expression of *nhr-154* GFP reporter transgenes was first detected in the 2-fold stage embryo within the developing pharynx and in precursors of several unidentified head neurons. By the three-fold stage of embryogenesis, expression was seen in the pharynx and throughout the intestine, a pattern reminiscent of the developmental transcription factor pha-4 (Smith and Mango, 2007; Updike and Mango, 2006). In the L1 and L2 stages, the reporter gene expression was strong in the pharyngeal muscles (anterior and posterior bulbs, predominantly), in unidentified head neurons, the intestine, and in the intestinal-rectal valve or sphincter cell. Consistent with our RTqPCR analysis, GFP reporter gene expression decreased in subsequent larval stages (L3, L4) so that by the adult stage only pharyngeal expression persistence was reproducibly observed. This suggested that the peak of expression in adults detected by RT-qPCR might be due to the embryos inside gravid adults in these samples. No obvious differences in pattern were seen between the two promoter lengths tested.

4.4.6. The expression of nhr-153::GFP

The expression of *nhr-153* GFP reporter transgenes was first detected in the 2-fold stage embryo in the pharyngeal and intestinal cells. In all larval stages, the reporter gene expression was very strong in the posterior bulb of the pharynx and in all intestinal cells as well as the intestinal-rectal valve or rectal gland cells. Expression was also seen in several unidentified neurons near the posterior pharyngeal bulb and in the tail. In males, very strong reporter expression was observed in some of the ray-associated neurons.

4.4.7. The expression of nhr-136::GFP

The construct ***nhr-136::gfp*** was prepared in the same way as other constructs and we prepared 12 transgenic lines, with promoter lengths 951 and 1900 bp, but we did not observe GFP expression despite that the transgene was detected in transgenic animals by PCR and the integrity of the construct was confirmed by sequencing.

Summarized results of reporter gene expression for *C.elegans* clustered nhrs. The intensity of the expression was scored from low (+) to high (+++) in each cell or tissue type. Absence of expression is indicated (-). If specific developmental stages are not reported, expression was observed in all larval stages and in adults.

Reporter Gene	*nhr-206*	*nhr-208*	*nhr-207*	*nhr-209*	*nhr-154*	*nhr-153*	*nhr-136*
Start of emb. expression	Comma	1.5-fold	1.5-fold	-	2-fold	2-fold	-
Phar. Muscles	+++	-	+	-	+++	-	-
Head Neurons	+++	+++	++	-	++	+++	-
Phar. – Intest. Valve	-	-	+++	-	-	-	-
Intestine	+	+	+	-	+++ L1, L2, L3	+++	-
Int.- Rectal Valve	-	+	++	-	+	+	-
Rectal Glands	+++	+++	+	-	+	+	-
Int. Sphincter	+	+	+	-	+	-	-
Tail Neurons	-	+	-	-	-	+	-
Epidermal cells in head	++	+	-	-	+	-	-
Epidermal cells in tail	++	++	-	-	-	+	-
Spermatheca	+++	-	+++	-	-	-	-
Vulva	++	-	++	-	-	-	-
Rays	+ R6	+ R6, 7, 8	+ R6	-	-	-	-
Ray Neurons	+	+	+	-	-	+	-
Male Specific Neurons	++	-	++	-	-	-	-

4.5. The expression of clustered nuclear hormone receptors responds to fasting

We performed 6 hour and 54 hour fasting experiments in synchronized larvae L1 of *C.elegans* and *C.briggsae* and assayed the expression of clustered *nhr*s by reverse transcription – quantitative PCR. This strategy revealed that the expression of *nhr-206, nhr-208, nhr-207, nhr-153* and *nhr-136* was higher in starving animals. The expression of *ama-1* was not affected by six-hour or 54 hour starvation.

Analysis of the expression of *C.briggsae* orthologues, the Cbr-*nhr-209, Cbr-nhr-154, Cbr-nhr-153*, and *Cbr-nhr-136* showed that the first gene in *the C.briggsae* cluster, the *Cbr-nhr-209* was also elevated, approximately twice, in starving larvae (Fig. 7).

In order to see if the up-regulation of gene expression at fasting conditions is not caused by errors in the experimental setting, we assayed the expression of genes that are known to be affected by feeding status. We have chosen acs-2, fat-7 and nhr-49 that are known to be elevated, decreased and not affected by fasting, respectively [Van Gilst, 2005]. As expected, acs-2 was up-regulated approximately 2-fold by fasting, fat-7 decreased approximately 5 to 10 times and the expression of nhr-49 did not change (Fig. 6).

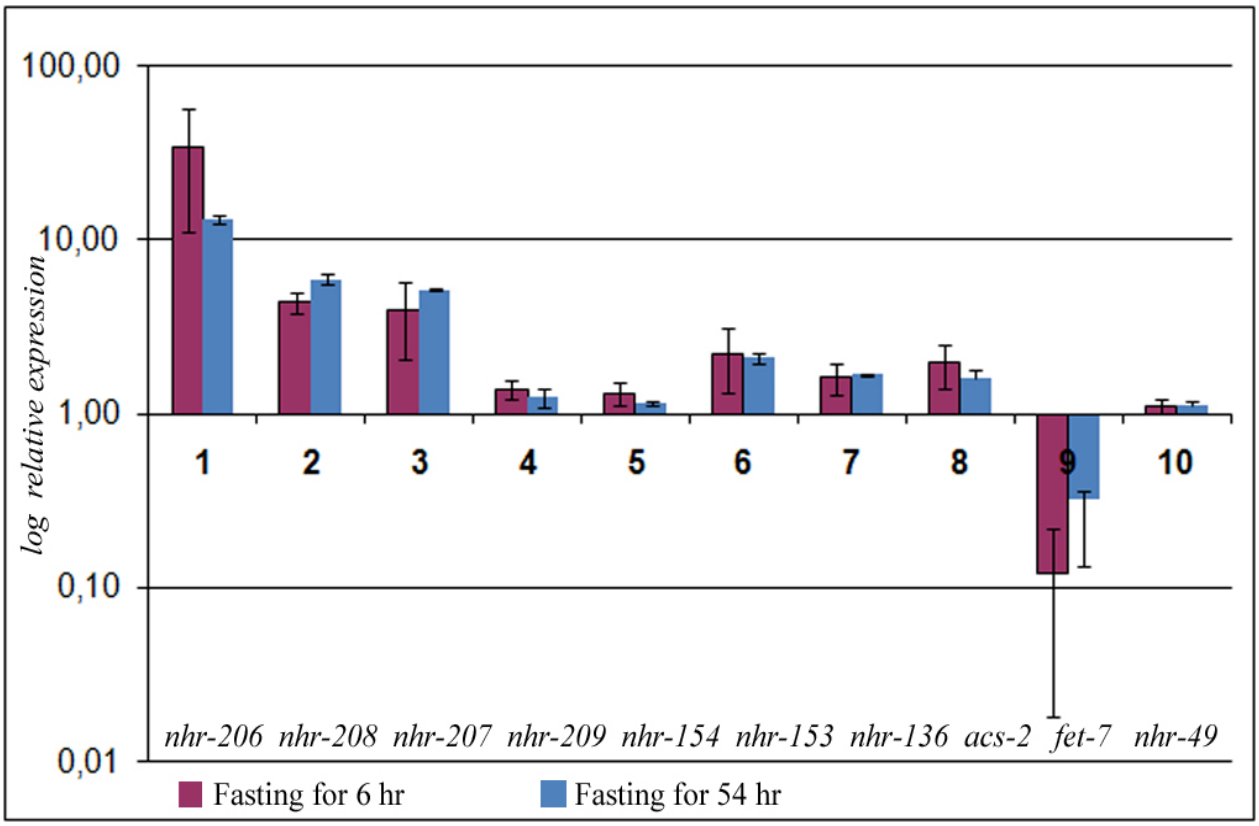

Figure 6. The fasting experiment of *C.elegans*

The picture shows the expression of seven clustered *C.elegans nhrs* during fasting and analyzed by RT-qPCR. Expression of the recently duplicated *C.elegans* genes *nhr-206, nhr-208 and nhr-207* is strongly induced in animals after either 6 hours (violet columns) or 54 hours (blue columns) of fasting. The results are shown in a logarithmic scale where the value of one is equal to unchanged expression compared to fed control animals. Values greater than one indicate up-regulation and levels less than one represent down-regulation during fasting conditions. The expression of genes reported previously to be affected by fasting (Van Gilst et al., 2005b), *acs-2* (increased), *fat-7* (decreased) and *nhr-49* (unchanged) were included as additional controls for these experiments.

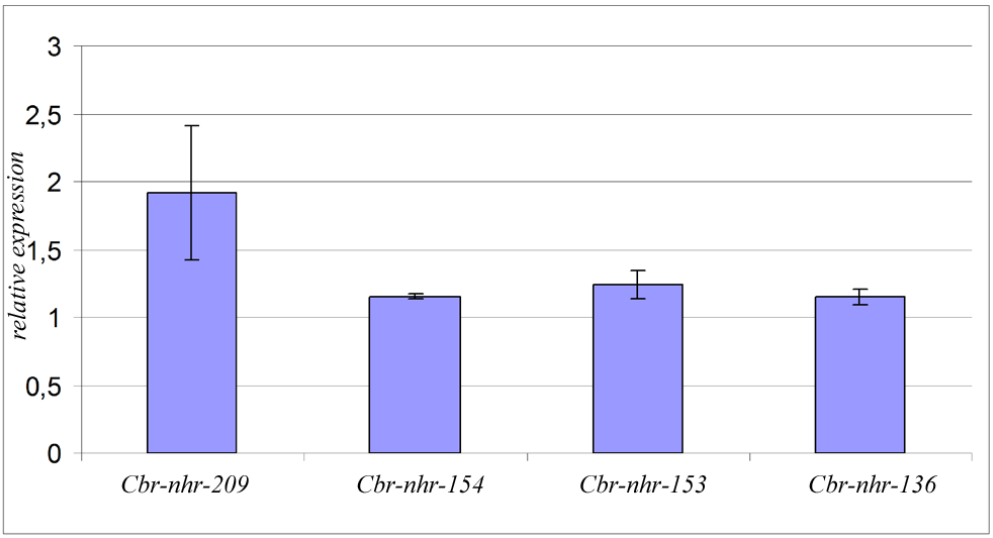

Figure 7. The fasting experiment of *C.briggsae*

The picture shows the expression of *C.briggsae* homologues *Cbr-nhr-209*, *Cbr-nhr-154*, *Cbr-nhr-153* and *Cbr-nhr-136.* The expression was analyzed by RT-qPCR in animals after 6 hours of fasting. Only *Cbr-nhr-209* showed an increase of expression (2-fold) during fasting.

Although we did not perform fully calibrated experiments that would take in account the possible differences in the efficiency of reverse transcription of individual genes and our results were normalized against *ama-1*, the results showed consistently higher reading values for the expression of genes conserved between *C.elegans* and *C.briggsae*. The values of conserved genes were 5 to 10 times higher than the values obtained in the cases of more recently duplicated genes.

4.6. Densitometric analyses

Since the up-regulation of gene expression of certain clustered *nhrs* detected by RT-qPCR was substantial, we wanted to see if the effect of starvation may be detected also by gfp fusion transgenes. The elevated expression of GFP fusion trasgenes was clearly visible in intestinal cells in case of *nhr-206*, *nhr-208* and *nhr-207*. To confirm this, we performed densitometric analyses of sets of pictures taken randomly at constant setting. The densitometric analyses confirmed the elevated expression in *gfp* fusion genes in intestinal cells of transgenic animals expressing *nhr-206*, *nhr-208* and *nhr-207* and in pharynx of animals expressing gfp fusion genes of *nhr-153* and *nhr-154*.

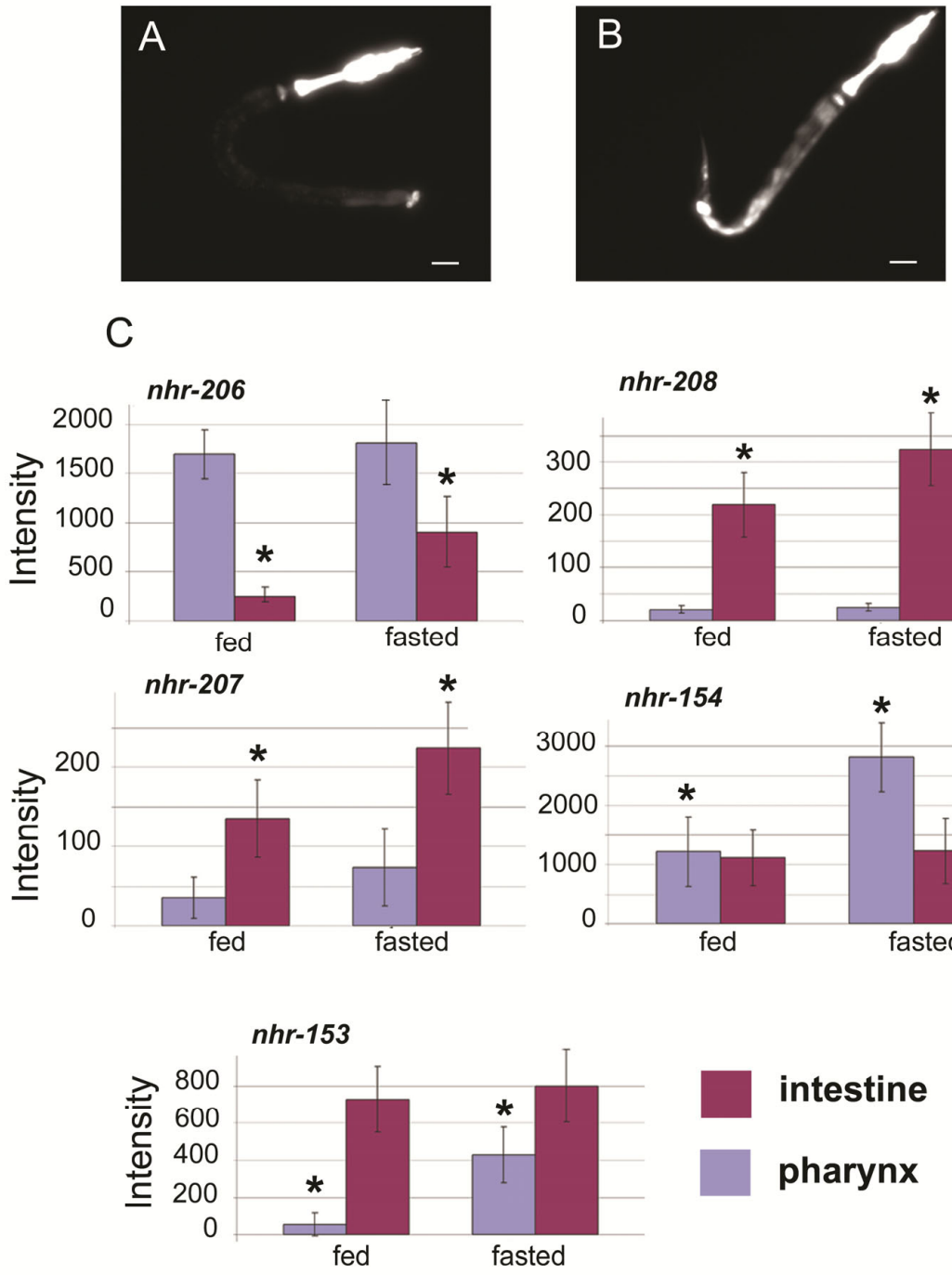

Figure 8. Responses of GFP reporter genes to fasting and feeding conditions.

The picture shows changes in reporter gene expression in response to feeding (A) or fasting (B) and could be easily visualized like in this representative image for *nhr-206::gfp*. The change in GFP expression in either the pharynx or intestine was quantified by densitometric analysis during fed or fasting conditions for the reporters *nhr-206::gfp*, *nhr-208::gfp*, *nhr-207::gfp, nhr-154::gfp, nhr-153::gfp*. Calculations were based on capturing the anterior area of animals coving the pharynx (gray columns) or the whole body and intestine, excluding the pharynx (dark columns); note that the intensity scale was adjusted for each reporter gene. The data revealed that *nhr-206*, *nhr-208,* and *nhr-207* reporters each showed significant (2- to 4-fold), intestinal-specific up-regulation of expression in

response to fasting. In contrast, *nhr-154* and *nhr-153* reporters showed significant (2- to 8-fold), pharyngeal-specific increases in expression in response to fasting. Standard deviations are indicated in each column. An asterisk (*) indicates the probability of the result in the Student T test at $P<0.05$ compared with null hypothesis. Scale: 20 μm. The picture shows densitometry changes during the starving experiment. Two areas (pharyng and gut) were compared by the densitometry method. Panel A: *nhr-206*::GFP; B: *nhr-208*::GFP; C: *nhr-207*::GFP; D: *nhr-154*::GFP; E: *nhr-153*::GFP

4.7. Study of function

The function of all studied nuclear hormon receptors in *C.elegans* was studied by several approaches. We blocked the natural function of the gene by RNA interference. We prepared gene specific dsRNA in vitro and introduced it into the animals and then processed into 21-24 bp long RNA duplexes (small interfering RNAs – siRNAs) by the RNaseIII enzyme Dicer. After unwinding, siRNA single strands were incorporated into the multi-subunit RNA-induced silencing complex (RISC) and lead the complex to cleave naturally produced RNA with a sequence identical to the siRNAs (Fire et al., 1991; Timmons and Fire, 1995, Voinnet, 2005). dsRNA was introduced into worms either by soaking, feeding or by microinjecting. To increase RNAi effect in this study cluster (R07B7 and C13C4.3) we blocked one, two, three and in the R07B7 cluster all four genes.

4.7.1. RNA mediated interference

Double stranded RNA was also used for induction of RNA interference by the soaking method (at concentration 2 mg/ml). Hermaphrodites were soaked in dsRNA and transferred every 12 hours to new plates for a total of a 4 day period. The embryonic lethality and changes of larval phenotype were recorded at least twice a day on individual plates kept at 22ºC.

To check a possible non-specific effect of prepared dsRNA a control dsRNA, which was prepared from the (non-coding) promoter region of *nhr-60* was used (Simeckova, 2007 #21).

For feeding, the same constructs were transformed into bacteria E. coli HT 115. After the induction, bacteria produced gene specific dsRNA. Worms were grown on these bacteria.

All methods (soaking, feeding, microinjecting) have had very similar results. We did not observed any morphological phenotypes as a result of RNAi but a marginal increase of embryonic lethality was found when more genes were inhibited simultaneously (Table 1).

RNAi results								
A. RNAi of individual genes made by soaking								
nhr gene	*206*	*208*	*207*	*209*	*154*	*153*	*136*	control water
Soaked Hermaphrodites	11	12	5	4	6	18	14	19
Progeny Scored	1227	1275	571	587	1467	2485	2027	2418
Embryonic Arrest	0	34 (3%)	0	0	20 (1.4%)	36 (1.4%)	10 (0.5%)	20 (0.8%)

B. RNAi of individual genes made by microinjection								
nhr gene	*206*	*208*	*207*	*209*	*154*	*153*	*136*	Control P*nhr-60*
Injected Hermaphrodites	9	21	18	14	6	9	10	18
Progeny Scored	1252	4536	2993	3175	1467	921	1370	2815
Embryonic Arrest	39 (3%)	37 (0.8%)	77 (2.6%)	119 (3.8%)	20 (1.3%)	9 (1%)	40 (2.9%)	26 (0.9%)

C. RNAi of combinations of two genes by microinjections					
Combinations of *nhr* genes	*206 + 208*	*207 + 209*	*154 + 153*	*154 + 136*	*153 + 136*
Injected Hermaphrodites	ND	ND	9	7	10
Progeny Scored	ND	ND	844	579	1481
Embryonic Arrest	ND	ND	14 (1.6%)	12 (2%)	18 (1.2%)

D. RNAi of combinations of three, four and seven genes by microinjections				
Combinations of *nhr* genes	*154+153+ 136*	*206+208+207+ 209*	*209+154+153+ 136*	*206+208+207+ 209+154+153+136*
Injected Hermaphrodites	8	25	32	31
Progeny Scored	662	4664	4910	5148
Embryonic Arrest	32 (4.8%)	75 (1.6%)	90 (1.8%)	129 (2.5%)

Table 1. RNAi results

The table shows summarized results of RNA-mediated inhibition (RNAi) of individual genes by soaking in solutions containing double stranded RNA (Part A), microinjection of dsRNA (Part B), by microinjection of combinations of dsRNA for two selected genes (Part C), or by microinjection of combinations of dsRNA for three, four and all seven genes (Part D). No developmental defects were observed in larvae and adult animals, but slight increase (approximately 5%) in embryonic lethality was observed when a combination of dsRNA for three conserved genes was used for microinjections.

5. Discussion

The presented thesis focused on functional study aimed at elucidation of biological role of seven nuclear hormone receptors that are found in *C. elegans* genome on chromosome V in the region 12,092,022-12,109,114; WS207. The genomes of Rhabditidae species that were sequenced to date contain unexpectedly large number of sequences that are recognized by sequence comparison as potential NHRs. The genome of *C. elegans* contains 284 potential NHRs and the genome of *C. briggsae* and *C. romanei* contain 232 respectively 256 potential NHRs (Anteby 2006; Haerty 2008).

This large number of NHRs in nematode species contrasts to only 18 NHRs in the genome of *Drosophila* and mammals have 48 to 50 such genes. The gene cluster that was chosen for this study has several interesting features. Although several clusters of candidate NHRs can be found on the chromosome V, the cluster V: 12,092,022-12,109,114; WS207 seems to be unusually condensed, contains genes with the same orientation and bears characteristics of a recent origin by gene duplication. In this work we hoped that the expressional and functional analysis of this cluster may yield data that may be related to biological role of recently duplicated NHRs and shed some light on the evolutionary mechanisms and selection pressure that is connected to the process of enlargement of NHR family of genes in Rhabditidae.

The data found in this study indicated, that the first three receptors in the locus are likely to be the most recently duplicated and are not conserved in the closest nematode *C. briggsae*. There is of course also a possibility, that the genes in *C. briggsae* were lost during the separate evolution. Although this seems to be unlikely, large deep-in sequencing employing the new sequencing methods that are powered to provide a more complete and detailed whole genome sequencing of a bigger number of Rhabditidae species may answer to this question.

In our work, we found that the closest homologue of *C. briggsae* genes located on the corresponding chromosomal location in *C. elegans* genome is NHR-136. The other four receptors have orthologs in *C. briggsae*. These receptors, which are present in *C. elegans* and in *C. briggsae* (NHR-209, NHR-154, NHR-153, and NHR-136), despite a pronounced diversification of their coding sequence have almost identical sizes of exons. This supports the conservation of the overall structure of these receptors and their functionality as classical NHRs.

We have found that each gene in the *C. elegans* cluster is expressed in one isoform. The same result was found in case of conserved members of the studied gene cluster. This contrasts with many *C. elegans* NHRs and with vertebrate NHRs (the data can be retrieved from www.receptors.org/NR for all NHRs and www.wormbase.org for *C.elegans* and is in agreement with the possibility that the recently duplicated receptors are compared to the later named receptors, which are functionally more restricted and specialized.

The tight organization of studied genes in the cluster suggested that several receptors could be organized in operons (Blumental 2002, 2005). Contrary to the prediction, only two studied genes were found to be organized in the operon. The intergenic region separating *nhr-207* and *nhr-209* is quite small (270 bp) to contain a functional promoter if the sequence that is already in the coding region of *nhr-207* or some more distant sequence is not included in the formation of the functional promoter for *nhr-209* . While we have found the SL1 in front of *nhr-154* (the same result is stated by WormBase), we found the SL2 in front of *nhr-209*. This proves that *nhr-207* and *nhr-209* are transcribed from same mRNA and indicates that they have, at least for the operon base expression the same expression pattern.

The data presented in this thesis characterized the stage specific as well as the cell and tissue specific expression pattern for all genes of the studied cluster. We have found that the expression level of all *C. elegans* genes (*nhr-206, nhr-208, nhr-207, nhr-209, nhr-154, nhr-153* and *nhr-136*) starts in embryogenesis and is present in all larval stages to adulthood. The exception is *nhr-154*, which expression is high in embryos and early larval stages and later decreases. Interestingly the expression in conserved genes in *C. elegans* and *C. briggsae* (*Cbr-nhr-209, Cbr-nhr-154, Cbr-nhr-153* and *Cbr-nhr-136*) is app. 10x higher at standard laboratory conditions in comparison with the expression of genes that arouse as new members of the family in the *C. elegans* genome.

The expression of *nhr-206* is similar to that described by Reece-Hoyes and coworkers in their high throughput screen (Reece-Hoyes et al., 2007). For *nhr-208* we found similar expression profile, although our reporters did not result in expression in the excretory cell and vulva and *nhr-207* our results overlap those reported by Reece-Hoyes and coworkers (Reece-Hoyes et al., 2007). From conserved genes, we prepared transgenic lines only for two genes *nhr-154* and *nhr-153*. Their expression is mainly in intestine, but varies during the development (the expression of *nhr-154*::GFP lines decreases from the middle larval stages to adulthood and we confirmed the expression found by RT Q PCR). We were not successful in preparation of transgenic lines for *nhr-209* and *nhr-136*. In the case of *nhr-209*, the localization of this receptor in an operon with upstream gene *nhr-207* suggests that the expression could be the same as it is in case of *nhr-207*::GFP. The transgenes that contained the intergenic region preceding the ATG of *nhr-209* did not yield any GFP expression. Similarly, transgenes that contained in addition to the intergenic sequence also large part of the preceding *nhr-207* did not show expression of GFP as they do not contain functional promoter. This further supports the expression of *nhr-209* from the operon with *nhr-207*.

The second gene for which we were not able to prepare the GFP expressing transgenic lines was *nhr-136*. This gene is localized in the end of studied locus and in front of ATG has region which could be sufficient to function as promoter for about 2000bp. We prepared many transgenic lines, which contained the experimental transgenes, but we did not see the GFP expression. By PCR from single

worms (rollers) we confirmed that not expressing transgenic lines carry the promoter region of *nhr-136*. This indicates that other distant factors and chromosomal arrangement are important for expression of this gene. This also suggests that chromosomal arrangement is at least partially important for functionality of multiplied *nhr* genes in Rhabditidae.

To study the function of clustered genes, we decided to inhibit them by RNA interference (Timmons,L 1998, 2003). We prepared double stranded RNA for all seven genes and attempted to inhibit the gene function of individual genes. Since we did not see any observable phenotype or changes of development, we also inhibited combinations of clustered genes simultaneously in order to see if there is a functional redundancy of clustered genes. The combinations of two, three, four and all seven genes did not show visible phenotypical changes. We found only slightly higher embryonic lethality in case of some combinations of inhibited genes (Table 1).

In our previous work on NHR-40, we found that the penetrance of its developmental phenotype was affected by feeding status and environmental conditions such as feeding and temperature (Brozova 2006, Pohludka, 2008). Since the clustered *nhrs* belong to the same subgroup of NHRs, we searched if their expression and loss of function are affected by feeding status.

As a metabolic stress, we used fasting implemented in two separate protocols. The first protocol consisted of short fasting which was done after synchronization of two paired cultures overnight and fasting was done for 6 hours while the control group of worms was on plate with food (Gilst 2005). Long fasting was done by keeping the synchronized worms 2 days (48 hours) without food and than one culture was subjected to fasting for the next 6 hours while the control group of the worms was kept on plates with food during the same period of time.

The expression of the conserved genes during fasting experiment was 5 to 10 times lower than the values obtained in case of more recently duplicated genes.

Interestingly, fasting lead to recording of values of the more recently duplicated genes that were reaching levels observed for conserved genes both during fasting and feeding. Although it can not be ruled out that some metabolites that are elevated during fasting, such as metabolites of beta-oxidation may cause activation of gene expression of fasting-up-regulated genes, it seems more likely that the more recently duplicated genes are repressed at feeding status and a de-repression is bringing the expression of fasting-responsive genes to the level seen for conserved genes.

Moreover we found that the expression of genes at fasting changed their expressional patterns. All three recently duplicated genes (*nhr-206, nhr-208, nhr-207*) which were strongly expressed in pharynx and only slightly expressed in intestine after fasting had much higher expression in intestine while the expression in pharynx was not changed. The expression of *nhr-154* and *nhr-153* genes from conserved group had slightly increased expression after fasting, their expression in intestine did not change, but the expression in pharynx was increased. The expression of *nhr-209* and *nhr-136* did not

change. This agreement between the data obtained by RT Q PCR and expression in transgenes supports the validity of the data obtained by isolated genes expressed as transgenes and indicates that the diversified promoters constitute the target of the functional evolutional pressure. The second support of this concept is the fact, that the multiplied genes show overlapping (although partially diversified) expression patterns despite the sequence dissimilarity which did not allow identifying regulatory elements by pattern recognition computer programs.

Together the data presented in this thesis support the concept that the multiplied nhrs in Rhabditidae are functional genes that have specific regulatory functions. Our data support the concept that NHRs in Rhabditidae constitute a very dynamic end evolutionally flexible gene family, where individual members may accept new functions, most likely in tuning and orchestrating the metabolic and developmental needs.

6. Conclusions

The goal of this study was to determine if the members of the superfamily of nuclear receptors that are predicted based on sequence homology in the *C. elegans* genome are functional genes and if so, to characterize their functions.

For this study, we selected a gene cluster consisting of seven genes arranged as tandem repeats.

Since the tandem arrangement of genes indicated recent and successive gene duplications, we hypothesized that the expressional and functional data may shed light at the biological mechanisms that project to the multiplication of this gene family.

The presented work:

proved that the selected recently duplicated nuclear receptors are functional genes

characterized the pattern of their tissue specific expression

established that receptors acquired species specific metabolism dependent expression regulation

The data presented in this thesis contributed to understanding of the biological role of selected members of the multiplied superfamily of nuclear receptors in Rhabditidae by narrowing their function towards involvement of this genes in metabolism.

Our data indicate that the duplicated members of NHR family acquire new species specific functions.

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