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Molecular isolation and characterization of the kisspeptin system: KISS and GPR54 in roach Rutilus rutilus: a new relevant biomarker of endocrine disruption in fish.

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Corresponding Author:	Perrine Geraudie Akvaplan Niva Tromsø, NORWAY
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Akvaplan Niva
Corresponding Author's Secondary Institution:	
First Author:	Perrine Geraudie
First Author Secondary Information:	
Order of Authors:	Perrine Geraudie
	Marie Gerbron, PhD
	Anne Lockyer, PhD
	Susan Jobling, PhD
	Christophe Minier, PhD
Order of Authors Secondary Information:	
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Suggested Reviewers:	Even Jørgensen, Professor Researcher, University of Tromsø even.jorgensen@uit.no Pr. E. Jorgensen research focus on fundamental physiological of neuroendocrine regulation of reproduction in fish.

	Augustine Arukwe, Professor Researcher, NTNU Fakultet for ingeniorvitenskap og teknologi Trondheim augustine.arukwe@ntnu.no Pr A. Arukwe has large experience in biomarker responses related to endocrine disruptions in fish including molecular Toxicology.
	Jeanette Rotchell, Pr Researcher, University of Hull j.rotchell@hull.ac.uk Pr J. Rotchell research topics are particularly relevant to review this article. She has been studying endocrine disruptions in fish using molecular tools.
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Molecular isolation and characterization of the kisspeptin system: KISS and GPR54 in roach *Rutilus rutilus*: a new relevant biomarker of endocrine disruption in fish.

Perrine Geraudie[a], Marie Gerbron[b], Anne E. Lockyer[c], Susan Jobling[c], Christophe Minier[b].

[a]: Akvaplan-Niva, High North Centre for Research on climate change and environment, Framsenteret, Tromsø, Norway.
[b]: Laboratory of Ecotoxicology, UMR-I 02 SEBIO – Normandie University, Le Havre, France.
c: Institute of Environment, Health and Societies, Brunel University London, Uxbridge. UB8 3PH United Kingdom.

E-mail address: pge@akvaplan.niva.no
*Corresponding author: Tel: +47 77 75 03 48

Abstract

The reproduction of vertebrates is regulated by endocrine and neuro-endocrine signaling molecules acting along the brain-pituitary-gonad (BPG) axis. The understanding of the neuroendocrine role played in reproductive function has been recently revolutionized since the KiSS1/GPR54 (KiSS1r) system was discovered in 2003 in human and mice. Kisspeptins, neuropeptides that are encoded by the *KiSS1* gene, have been recognized as essential in the regulation of the gonadotropic axis. They have been shown to play key roles in puberty onset and reproduction by regulating the gonadotropin secretion in mammals while physiological roles in vertebrates are still poorly known. In order to provide new knowledge to investigate basic reproductive physiology in fish as well as to assess impacts of endocrine disrupting compounds (EDCs), the KISS1/GPR54 system might constitute an appropriate biomarker. This study was designed to isolate and characterize the *KiSS1* and *GPR54* transcripts in roach *Rutilus rutilus* to investigate the role of this neurotransmitter system, i.e., gene/receptor, in fish reproduction. This work provides new knowledge on the neuroendocrine regulation in roach as well as new molecular tools to be used as biomarkers of endocrine disruption, and complete the set of biomarkers already validated in this species.

Keywords: *Roach, endocrine disruptions, neuropeptides, brain pituitary gonad axis.*

1 Introduction

In most teleost species, dopamine negatively regulates the gonadotropin secretion via D2-type receptors (Dufour et al., 2010; Vacher et al, 2000) counteracting the stimulatory effect of GnRH on gonadotropin secretion. The agonist signal pathways via norepinephrine and seretonin secretion could stimulate luteinizing hormone (LH) release directly or via the GnRH axis (Dufour et al, 2005, 2010). Several neurotransmitters have been reported to regulate GnRH synthesis, including neuropeptide Y (NPY), gamma-aminobutyric acid (GABA) and kisspeptins (Zohar et al, 2010; Popesku et al, 2008). The KISS/GPR54 (G-coupled protein receptor 54) system encoding for kisspeptins and their receptors (GPR54 or KISS-R) has recently been discovered and has revolutionized the understanding of regulation of reproduction and puberty onset in vertebrates (De Roux et al, 2003; Seminara et al, 2003). The first studies on this system demonstrated that the inactivation of GPR54 was shown to be responsible for idiopathic hypogonadotrophic hypogonadism associated with reduced circulating LH levels (De Roux et al, 2003). Since then, the identification of this new neuroendocrine regulatory system has triggered an important research effort to understand the role and mechanism of action (MOA) of kisspeptins and GPR54. In mammals, this system is considered to be the gatekeeper of puberty and reproduction (Tena-Sempere, 2006). Characterization and role in lower trophic levels are not fully understood yet and little is known regarding kisspeptin gene regulation in fish. Two distinct kiss genes (kiss1 and kiss2) have been identified in different fish species encoding for two relatively well conserved kisspeptins (Kitahashi et al, 2009; Felip et al, 2009; Li et al, 2009). Especially, it has been suggested that KISS/GPR54 system could be the mediators between environmental cues and metabolic signals to the reproductive axis and to modulate gonadotropin secretion (Akazome et al, 2010). Two gonadotropin hormones, the follicle stimulating hormone (FSH) and luteinizing hormone (LH) are synthetised in the brain of vertebrates and are responsible in the gonadal development and maturation. FSH and LH regulate the sex steroid production in the gonads, which can in return regulate the upper part of the hypothalamo-pituitary axis, via negative or positive feedbacks (Schulz and Goos, 1999). The main sex steroids, 17-β-Estradiol and Testosterone have been reported to regulate the kisspeptin genes in the brain of fish (Kanda et al., 2008, 2012).

Among the 400 million tons of chemicals produced annually, some of them are called endocrine disrupting chemicals (EDCs) and are known to interact with the endocrine systems of wildlife and humans, causing deleterious effects on development, reproduction, physiological homeostasis and health of vertebrates (Colborn et al., 1993). Several model species have been used to investigate the impact of EDCs in wildlife, with fish having beeing intensively used as vertebrate aquatic model. The roach, *Rutilus rutilus,* has been selected as a sensitive model organism to assess the impact of xenobiotics in freshwater (Jobling et al., 2002; Tyler, 2007; Geraudie et al., 2010, 2011, 2017).

Alterations involving different hormones and signal molecules acting along the brain-pituitary-gonad axis of roach have been reported. Previous studies on roach sampled in contaminated areas showed evidence of alteration of sex steroid levels and brain aromatase activity, induction of vitellogenin, as well as an global feminization of the population (Geraudie et al., 2010, 2011, 2017; Gerbron et al., 2015). By modulating the gonadotropin secretion, the KISS1/GPR54 system seems to play a key role in the regulation of reproduction (Tena-Sempere, 2006) and could be an essential player in sex differentiation. Transcripts encoding for KiSS1 and its receptor, GPR54, have been isolated from a number of fish species including zebrafish (*Danio rerio*; Van Aerle et al, 2008), medaka (*Oryzias latipes,* Kanda et al, 2008) and goldfish (Kanda et al, 2012). It has been shown that, in fish, two distinct genes, *kiss1* and *kiss2*, encode for different kisspeptins unlike in placental mammals where only the *kiss2* has been reported (Kitahashi et al., 2009).

In regard to the potential alterations of the endocrine system by EDCs, the KISS1/GPR54 system could constitute a new relevant target and thus an excellent biomarker of endocrine disruption in fish. In order to facilitate further investigation of this neurotransmitter in roach endocrinology and reproduction, the *kiss2* and *gpr54* transcripts have been isolated and identified in roach *Rutilus rutilus*.

2 Material and methods

2.1: Fish collection:

Adult wild roach were collected by fishnets in September 2006 from a reference site located in the northern part of France, Venables (49.199371, 1.29548). The sampling site is an old sand quarry with low levels of contamination previously reported as mutagenicity of sediment extracts performed using the SOS chromotest (Cachot et al., 2006) were below the detection limit (Geraudie et al., 2010a,b). Venables has been used and validated as reference site in previous studies where no sign of endocrine disruption in roach has been found (absence of intersex fish, mean male plasma VTG concentration lower than 20 ng/ml in over (N>500 fish; Geraudie et al., 2010a; Gerbron et al., 2015).

Fish were dissected *in situ* in order to reduce the stress impact due to transportation to the laboratory. Then brain were collected and preserved in RNA laterTM (Ambion). Total RNA was extracted from the brain using SV total RNA isolation kit (Promega) according to the manufacturer's instructions. This kit includes a DNAse treatment step to remove contaminating genomic DNA. Ribonucleic acid (RNA) concentrations and purity were measured at 260 and 280 nm using a NanoDrop®. cDNA was synthesized from 1 µg total RNA using cDNA Synthesis with SuperScript® III First-Strand Synthesis System for RT-PCR kit (Invitrogen). Briefly, 1 µg total RNA was incubated for 5 min at 65°C with 1 µL of random hexamers (50 ng/µL), 1 µL of dNTP mix (10 mM) and RNAse-free water in a volume of 10 µL. Then, cDNA was synthesized in a 20 µL volume: 10 µL of the previous RNA solution with 10 µL of reaction mixture containing 1 µL of SuperScript® III RT (200 U/mØ), 2 µL of 10 X RT reaction buffer, 4

µL $MgCl_2$ (25mM), 2 µL of 0.1 M DTT and 1 µL of RNase OUT (40 U/µl). The reverse transcription was carried out for 10 min at 25°C, 50 min at 50°C and then terminated 5min at 85°C. Finally, 1 µL of RNase H (2 U/µL) was added to cDNAs and incubated 20 min at 37°C before being stored at -20°C.

The degenerate primer sets used for PCR (Table 1) were designed from *kiss1*, *kiss2* and *GPR54* cDNA sequences available on the Genbank database using the iCODEHOP software: (http://dbmi-icode-01.dbmi.pitt.edu/i-codehop-context/iCODEHOP/view/PrimerAnalysis).

PCR was performed using AmpliTaq gold® DNA polymerase (Invitrogen) under the following conditions: 95°C/10 min, 17 cycles of 95°C/30 s, 58°C/30 s diminishing of 0.5°C every cycle, 72°C/30 s, followed by 30 cycles of 95°C/30 s, 50°C/30 s, 72°C/30 s finished by 72°C/2 min. The amplified PCR products were cloned into pGEM®-T Easy Vector System (Promega) and transformed into competent JM109 cells (Promega). Amplicons displaying expected size were sequenced by the MilleGen® company (Labège, France) with ABI3130XL sequencer (Applied Biosystems) by using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Courtaboeuf, France). The identity of the PCR products was verified by Blast analysis.

3. Results

Partial sequences were obtained for gpr54 and kiss2 genes but not for kiss1. Degenerate primer pair GPR54F-GPR54R allowed amplification of a fragment of 498 bp, which, after Blast analysis appeared to correspond to a part of the gpr54a transcript also called kiss1 receptor (kiss1r). The alignment from other available gpr54a sequences of other species underlined a high conservation degree within cyprinidae fish, with identities of 96 % with fathead minnow *Pimephales promelas* (GenBANK accession number EF672266.1), 95 % for goldfish *Carassius auratus* (GenBANK accession number FJ465139.1) and 92 % for zebrafish *Danio rerio* (GenBANK accession number NM_001105679.1). The sequence homologies between these species are presented in Figure 1.

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R.rutilus   AGCAGGTGACCGTGCAGGCGACGTGCATCACTCTTGCGGCGATGAGTGGAGACCGTTGCT
P.promelas  AACAGGTGACTGTACAGGCGACGTGCATCACTCTTACGGCGATGAGTGGAGACCGTTGCT
C.auratus   AACAGGTGACCGTACAGGCGACGTGCATCACTCTTACGGCAATGAGTGGAGACCGTTGCT
D.rerio     AACAGGTGACGGTACAGGCGACGTGCATCACTCTCACGGCCATGAGTGGAGACCGATGTT
            * ******** ** ********************  **** ************** ** *
R.rutilus   ATGTGACTGTGTATCCTCTGAAATCCCTGCACCATCGGACCCCTCGTGTTGCAATGATTG
P.promelas  ATGTGACTGTGTATCCTCTGAAATCCCTCCACCATCGGACCCCTCGTGTCGCAATGATTG
C.auratus   ATGTGACTGTGTATCCTCTGAAATCCCTGCACCACCGAACCCCTCGTGTCGCAATGATTG
D.rerio     ATGTGACTGTGTATCCTCTCAAATCCCTGCACCATCGCACCCCTCGTGTTGCTATGATTG
            ****************** ******** ***** ** ********** ** *******
R.rutilus   TTAGCATCTGTATCTGGATCGGTTCCTTCATTCTTTCCATACCAATCTTCCTGTACCAGA
P.promelas  TTAGCATCTGTATCTGGATAGGTTCCTTCATTCTTTCCATACCAATCTTCCTGTACCAGA
C.auratus   TTAGCATCTGTATCTGGATCGGTTCCTTCATCCTTTCCATACCAATCTTCCTGTACCAGA
D.rerio     TTAGCATATGCATATGGATTGGTTCCTTCATTCTTTCCATACCGATCTTCCTGTACCAGA
            ******* ** ** ***** ********** ********** ***************
R.rutilus   GGCTTGAGGACGGCTATTGGTATGGACCAAGAAAATACTGCATGGAGAGGTTTCCATCAA
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P.promelas GGCTTGAGGACGGCTATTGGTACGGACCAAGAAAGTACTGCATGGAGAGGTTTCCATCAA
C.auratus  GGCTTGAGGATGGCTTTTGGTATGGACCAAGAAAATACTGCATGGAGAGGTTTCCATCAA
D.rerio    GGCTGGAAGACGGCTATTGGTATGGACCAAGAAAATACTGCATGGAAAGGTTTCCATCAA
           **** ** ** **** ****** ********** ********** *************
R.rutilus  AGGCCACTGAGAAGGCTTTCATCCTCTATCAGTTCATAGCTGTGTATCTATTGCCTGTCA
P.promelas AGGCCACTGAAAAGGCTTTCATCCTTTATCAGTTCATAGCTGTTTATCTACTGCCTGTCA
C.auratus  AGACCCACGAGAAAGCTTTCATCCTCTATCAGTTCATAGCCGTATATCTACTGCCTGTCA
D.rerio    AGACCCATGAGAAAGCTTTCATCCTCTATCAGTTCATAGCTGTGTATCTACTGCCTGTCA
           ** **   ** ** ********** ************** ** ****** *********
R.rutilus  TTACCATCTCCTTCTGTTATTCCTTCATGTTGAAGAGAGTGGGACAGGCCTCTGTGGAAC
P.promelas TTACCATCTCCTTCTGTTATTCCTTCATGTTGAAGAGAGTGGGACAGGCCTCTGTAGAAC
C.auratus  TTACCATCTCCTTCTGTTATTCCTTCATGCTGAAGAGAGTGGGACAAGCCTCTGTGGAAC
D.rerio    TTACCATCTCTTTCTGTTATTCCTTCATGTTGAAAAGAGTGGGACAGGCTTCGGTGGAAC
           ********** ****************** **** ********** ** ** ** ****
R.rutilus  CAGTGGATAACAGCCATCAGGTCCACCTGCTCTCAGAGAGAACTATCTCCATTAGGAGTA
P.promelas CAGTGGATAACAGCCATCAGGTCCACCTGCTCTCAGAGAGAACTATCTTCATTAGGAGTA
C.auratus  CAGTGGATAACAACCATCAGGTCCACCTGCTCTCAGAGAGAACTATCTCCATTAGGAGTA
D.rerio    CAGTGGATAACAACCATCAGGTCCATCTTCTCTCAGAGAGAACCATCTCCATCCGGAGCA
           ************ ************ ** ************** **** ***  **** * 
R.rutilus  AGATTTCCAAAATGGTAGTGGTCATTGTTGTCCTCTTCACCATTTGCTGGGGGCCCATAC
P.promelas AGATTTCCAAAATGGTAGTGGTCATTGTTGTCCTCTTCACCATTTGCTGGGGTCCCATTC
C.auratus  AGATTTCCAAAATGGTAGTGGTCATTGTTGTTCTCTTCACCATCTGCTGGGGTCCCATTC
D.rerio    AGATTTCCAAAATGGTAGTGGTCATAGTTGTCCTCTTCACCATCTGCTGGGGCCCCATTC
           ************************ ***** ********** ******** ***** * 
R.rutilus   AGATCTTCGTGCTGTTCC
P.promelas  AGATCTTTGTCCTGTTCC
C.auratus   AGATCTTTGTCCTGTTCC
D.rerio     AGATCTTTGTTCTGTTCC
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Figure 1: Multiple alignment of gpr54a (kiss1r) sequences, including the partial sequence of R. rutilus characterized in this study. Stars represent base homology for all species.

For the *kiss2* gene, a transcript of 297 bp was isolated. The conservation degree with other cyprinid species was not as high as for the *grp54a* gene (Figure 2). The percentage of homology was the highest with zebrafish (84 %; GenBANK access number EU853684.1) and carp *Cyprinus carpio* (84 %; GenBANK access number JQ715608.1) while it was of 79 % with goldfish (GenBANK access number GQ141877.1).

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R.rutilus  AGCTATCTTCACGGATATGGATACACCTGA------AGCCAGTCCAGACTCCAAGCAGCG
D.rerio    AGCAATACTCACTGACATGGACACACCAGA------GCCTATGCCAGACCCCAAACCGCG
C.carpio   ------------------------------------------------------------
C.auratus  AGCATCATTCACGGACATGGATATATCTGATTCTGAGCCCGTTCCAGACTCCAAGCAGCA

R.rutilus  CTATCTCTCAATGGAGCGGAGGCAATTTGACGAGCCCAACGCTTCGGACGACGGAAGCCT
D.rerio    TTTTCTGTCAATGGAGCGAAGGCAGTTTGAGGAGCCCAGCGCTTCTGACGACGCAAGTCT
C.carp     ----CTGT-GGTGGAGCGGAGGCAGTTTGACGAGCCCAGCACTTCAGACGACGCAAGCCT
C.auratus  CTATCTCTCAGTGGAGCGGAGGCAGTTTGATGAGCCCAGTTCTTCAGACGATGCAAGCCT
               ** *   ******* ***** ***** *******   **** ***** * *** **
R.rutilus  TTGCGTTTTCATCCAAGAAAAAGATGAATTGAGTAAAATTTCCTGCAAACATCGATTAAC
D.rerio    TTGCTTTTTCATCCAGGAAAAAGACGAAACGAGTCAAATATCCTGCAAACATCGACTAGC
C.carpio   TTGCTTTTTCATTCAAGAAAAAGACGATTCGAGTCATATTTCCTGCAAACATCGATTAAC
C.auratus  TTGTTTTTTCTTCCAAGAAAAAGACGAATCGACACATATTTCCTGCCAACATCGATTACC
           ***  ***** * ** ******** **   **   * ** ****** ******** ** *
R.rutilus  ACGCAGTAAATTCAACTACAATCCGTTCGGGCTGCGTTTTGGGAAGCGAAATGAAGCGAC
D.rerio    ACGCAGTAAATTCAACTACAATCCGTTTGGGCTGCGATTCGGAAAGAGAAACGAAGCGAC
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C.carpio    ACGCAGTAAATTCAACTACAACCCGTTTGGGCTGCGCTTTGGGAAGCGAAATGAAGCGAC
C.auratus   ACGCAGTAAATTCAACTACAACCCGTTTGGGCTGCGCTTTGGGAAGCGAAATGAAGCGCC
            ******************** ***** ******** ** ** *** **** ****** *
R.rutilus   AACT---GACTCTGACAGACCCAAACACGAGCACCTGCTCCCTATGATGCTCTACCTGCG
D.rerio     AACCAGCGACTCTGACAGACTCAAACACAAGCACCTGCTGCCAATGATGCTTTACCTGAG
C.carpi     TACT---GACACCGACAGACCCAAACACAAGCACCTGCTGCCAATGATGCTTTTCCTGAG
C.auratus   AACT---------GACAGACCCAAACAC------CTGCTGCCAATGATGATTTACCTGAG
             **          ******* *******      ***** ** ****** * * **** *
R.rutilus       AAAGCA
D.rerio         AAAGCA
C.carpio        AAAACA
C.auratus       AAAACA
                *** **
```

Figure 2: Multi alignment of kiss2 sequences, including the partial sequence of R. rutilus characterized in this study. Stars represent base homology for all species.

4 Conclusions

This study has successfully isolated and characterized two partial transcripts (*gpr54a* and *kiss2*) implicated in the neuroendocrine regulation of kisspeptin system, KISS/GPR54 of roach. These sequences may now be developed to provide new molecular tools which can be used as relevant biomarkers for neuroendocrine disruptions in fish, as well as contributing to fundamental knowledge on upper endocrine regulation of the hypothalamus pituitary gonad axis which is still poorly understood in aquatic species.

Acknowledgments

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Tables:

Oligo name	Sequence (5’-3’)
GPR54F	AGCAGGTGACCGTGCARGCNACNTG
GPR54R	GGAACAGCACGAAGATCTGDATNGGNCC
KISS1F	GTGCTGCGAGGAACAGAYACNMGNCC
KISS1R	TCCGAAGGAGTTCAGGTTRTARTANGC
KISS2F	GCTATGCGAGCTATCTTCACNGAYATGGA
KISS2R	TGCTTTCGCAGGTAGADCATCATNGGNA

Table 1: Degenerate primers used for PCR.