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# GnRH mRNA levels in male three-spined sticklebacks, *Gasterosteus aculeatus*, under different reproductive conditions☆

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ABSTRACT

In vertebrates, reproduction is regulated by the brain–pituitary–gonad (BPG) axis, where the gonadotropin-releasing hormone (GnRH) is one of the key components. However, very little is known about the possible role of GnRH in the environmental and feedback control of fish reproduction. To investigate this, full-length *gnrh2* (chicken GnRH II) and *gnrh3* (salmon GnRH) sequences of male three-spined sticklebacks (*Gasterosteus aculeatus*), which are clustered with the taxa of the same GnRH type as other Euteleostei, were cloned and annotated. *gnrh1* is absent in this species. The mRNA levels of *gnrh2* and *gnrh3* in the sticklebacks' brain were measured under breeding and post-breeding conditions as well as in castrated and sham-operated breeding fish and castrated/sham-operated fish kept under long-day (LD 16:8) and short-day (LD 8:16) conditions. Fully breeding males had considerably higher mRNA levels of *gnrh2* and *gnrh3* in the thalamus (*Th*) and in the telencephalon and preoptic area (*T + POA*), respectively, than post-breeding males. Sham-operated breeding males have higher gnrh3 mRNA levels than the corresponding castrated males. Moreover, higher *gnrh2* mRNA levels in the *Th* and higher *gnrh3* mRNA levels in the *T + POA* and hypothalamus (*HypTh*) were also found in long-day sham-operated males than in sham-operated fish kept under an inhibitory short day photoperiod. Nevertheless, *gnrh2* and *gnrh3* mRNA levels were not up-regulated in castrated males kept under long-day photoperiod, which suggests that positive feedbacks on the brain-pituitary-gonad axis are necessary for this response.


## 1. Introduction

In vertebrates, reproductive rhythms are regulated by the brain–pituitary–gonad (BPG) axis. The secretion of gonadotropins (GtH) from the pituitary is stimulated by gonadotropin-releasing hormone (GnRH) from the brain (Goos, 1991; Peter et al., 1991), although other factors are also involved. To date, 14 forms of GnRH have been identified in vertebrates, belonging to three groups (GnRH 1, 2 and 3) (reviewed in Okubo and Nagahama, 2008). To date, all studied fish species have at least two GnRH forms (GnRH2 and GnRH3) in the central nervous system, and many bony fishes have all three forms (reviewed in Okubo and Nagahama, 2008; Somoza et al., 2002). Based on available genome database (the Ensembl Genome Browser system; Ensembl release 75-February 2014), two types of GnRH, GnRH2 (chicken GnRH II) and GnRH3 (salmon GnRH), have been assembled in the stickleback genome. However, GnRH1 is absent. Even though it's neighbor genes were found on chromosome group XII, no possible GnRH homolog was present in the appropriate locus as has also recently been found in the zebrafish (*Danio rerio*) (Tostivint, 2011).

Numerous studies have shown that GnRH treatments can induce elevated plasma GtH levels and sexual maturation in many fishes, e.g., goldfish (*Carassius auratus*), African catfish (*Clarias gariepinus*), gilthead sea breams (*Sparus aurata*) and salmonids (reviewed in Peter et al., 1991). In several studies, brain GnRH peptide contents have been measured in relation to gonadal maturation in goldfish, eels (*Anguilla anguilla*), platyfish (*Xiphophorus maculatus*) and salmonids, but the results vary among species (reviewed in Amano et al., 1997). Nevertheless, recent studies showed that mRNA levels of hypophysiotropic *gnrh* increase at sexual maturation in red sea bream (*Pagrus major*) and black sea bream (*Acanthopagrus schlegeli*) (Senthilkumaran et al., 1999), salmonids (Onuma et al., 2010) and Atlantic cod (*Gadus morhua*) (Hildahl et al., 2011). Very little is known about the possible role of GnRH in the environmental and BPG-axis feedback control of seasonal reproduction.

The three-spined stickleback, *Gasterosteus aculeatus*, is one of the most studied fishes with regard to the control of seasonal reproductive cycles (Borg, 2010). Its natural breeding season is late spring to early

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summer, when photoperiods are long and temperatures are high. Breeding male sticklebacks can be easily distinguished by their bright breeding colors, with blue eyes and a red belly, and by their nesting behavior. The nest is made of algae glued together with threads of the protein spiggin produced by the kidney, which hypertrophies under androgen stimulation (Jakobsson et al., 1999; Borg, 2007). After the breeding season, these characters disappear. In the yearly cycle, the highest kidney development is found in the breeding season and the lowest in the post-breeding period in late summer (Borg, 1982). A similar pattern is also found in plasma levels of the androgen 11-ketotestosterone (Mayer et al., 1990) and in pituitary *lhβ* and *fshβ* mRNA levels (Hellqvist et al., 2006). The stickleback displays a clear-cut photoperiodic response, which makes it an ideal model to investigate how endocrine systems regulate seasonal reproduction. Long-day photoperiod induces positive androgen feedback on *lhβ* and *fshβ* in male sticklebacks (Shao et al., 2013), which. is likely to stimulate the onset of breeding. However, it is still unclear what role, if any, GnRH plays in this photoperiod-dependent maturation.

The aim of this study is to determine the role of GnRH in the regulation of seasonal reproduction in sticklebacks. To that end, brain *gnrh* mRNA levels were measured in stickleback males in different reproductive states.

## 2. Materials and methods

### 2.1. Animals

The fish were caught at the coasts of Skåne, southern Sweden, and transported to Stockholm University. They were kept in aquaria containing artificial brackish water (0.5% salinity), which was aerated and filtered. Ceramic pots and tubes provided hiding places on the sand-covered bottom. The fish were fed daily with frozen bloodworms, Artemia or mysids.

### 2.2. Experiment 1: breeding and post-breeding male, whole brain

The fish used for experiments 1 and 2 were caught in the early winter 2009 and kept in 700–1200 L aquaria under winter condition (4–8 °C and LD 8:16). In 16 October 2009, the breeding and post-breeding fish were moved to summer conditions (20 °C and LD 16:8) in 1200 L aquaria. After 2 weeks, males that showed breeding colors were placed individually into 50 L aquaria with algae as nesting material. The small aquaria were also provided with sand and filters. Eighteen males with breeding colors and nests were dissected after euthanasia with 0.1% 2-phenoxyethanol on November 16 and 25, 2009. Other nesting males were kept similarly until the breeding colors had disappeared in most fish. Seventeen post-breeding males were dissected on January 12, 2010.

### 2.3. Experiment 2: photoperiod experiment, whole brain

For the photoperiod experiment, another set of fish caught in the early winter 2009 were removed from winter condition and divided into two 1200 L aquaria, each first containing 57 males and 58 females (also for other studies, 16 of each removed at halftime). One aquarium was kept under long day photoperiod (LD 16:8) and the other one under short day (LD 8:16). The temperature was kept at 20 °C. After 5 weeks, 9 males from long day and 7 males from short day were dissected.

### 2.4. Experiment 3: castration and sham-operation experiment, whole brain

The fish used for castration experiment were collected in the winter of 2010 and kept in individual 50 L aquaria under L16:D8 and 20 °C provided with algae where their reached the breeding state. The operations were carried out on 20 nesting males (10 castrated and 10 sham-operated). Before operating, fish were anaesthetized with buffered MS-222. 1.5 mm long incisions were made into the abdominal cavity on each side, and the testes were excised with fine forceps. Sham-operated fish were treated similarly, but the testes were not removed. The incisions were closed with BV-2 (0.4 Ph. Eur.) sutures. The fish were sampled 2 weeks after operation.

### 2.5. Experiment 4: breeding and post-breeding male, brain areas

The fish were caught in March 2013 (group 1) and in October 2013 (group 2). They were kept under LD 16:8 and high temperature (15–20 °C) in 700 L aquaria since January 2014 and November 2013, respectively. Mature males in breeding condition (from groups 1 and 2) displaying nuptial colors and a hypertrophied kidney and post-breeding males (group 2, fish from group 1 were all still in breeding condition) lacking these traits were dissected on the March 4th 2014 after euthanasia with 0.025% buffered MS-222 (ethyl 3-aminobenzoate, methanesulfonic acid salt).

### 2.6. Experiment 5: photoperiod and castration experiment, brain areas

The fish were caught December 13–14, 2012. After arriving at Stockholm University, the fish were kept under a short-day photoperiod (20 °C and LD 8:16) until shipped to Taiwan by air in the summer of 2012. After a 1-week adaption period under short-day conditions, the fish were either castrated or sham-operated following the protocol shown above. Three intact fish were taken as the initial control. Five castrated and five sham-operated males were transferred to a long-day photoperiod (LD 16:8). Another five sham-operated males were kept under a short-day photoperiod. These fish were dissected 3 weeks after the operation. Three days before dissection, the operated fish were immobilized by injection of gallamine triethiodide (Flaxedil; Sigma, St. Louis, Mo. USA.) into the dorsal muscle (0.4–0.6 μg per gram of body weight) after being anaesthetized with MS-222 for a brief electroretinogram (ERG) test (for another purpose). After ERG test, the fish returned to its own aquarium for recovering. All fish used in the present study were fully recovered from the additional test.

### 2.7. Sampling

Breeding colors were noted and the fish weighed. The kidney maturity status was assessed under a stereo microscope, and the kidney was weighed. The kidney-somatic index (KSI, kidney weight/body weight × 100) was then calculated. The brain (without the pituitary, which was collected for another purpose) was dissected out after decapitation and removal of the skullcap. Tissues were immersed in 300 μl of RNAlater (Ambion) in room temperature for 2 hours, and then stored at −70 °C until analysis.

The experiments were carried out with permission from the Stockholm Northern Animal Experiment Ethical Committee and from the Institutional Animal Care and Use Committee (IACUC) of Academia Sinica.

### 2.8. Cloning and bioinformatic analysis

Peptide sequences from other species (teleosts were given the highest priority) were used to BLAST the genome databases (of the Ensembl Genome Browser System) for stickleback GnRH homologs. *In silico* predicted full-length stickleback GnRH homologs obtained from the genome were carefully confirmed with the NCBI database. Specific primers (listed in Table 1) were designed for the reverse transcription polymerase chain reaction (RT-PCR) analysis. PCR products thus obtained were subcloned into a pGEM-T Easy vector (Promega), and the nucleotide sequences were determined with an ABI 377 sequencer (Applied Biosystems). Sequence analysis was conducted with a BLASTx program (NCBI). To verify the membership of identified candidates in

**Table 1**
The primer sequences used in RT-PCR and qRT-PCR. The reference genes used in qRT-PCR were *q-rpl8* and *q-actb*.

| Gene | Primer sequence | Product size (bp) |
|---|---|---|
| *A. RT-PCR primer for cloning.* | | |
| *gnrh2* | F 5′-ATGTGTGTATCTCGGCTGGTTTTG-3′ | 258 |
| | R 5′-TCACTTTCTTTTCTGAAGCTCTCT-3′ | |
| *gnrh3* | F 5′-ATGGAAGCGAGCAGCAGAGCGATG-3′ | 273 |
| | R 5′-AAGAGAAAGTTCCAAAATAATTGA-3′ | |
| *B. qRT-PCR primer for Q-PCR.* | | |
| *q-gnrh2* | F 5′-CCGTCGGAGATTTCAGAGGAGATT-3′ | 121 |
| *Ei* = 91% | R 5′-TCTGAAGCTCTCTGGCTAAGGCAT-3′ | |
| *q-gnrh3* | F 5′-AGCGATGGTGCAGGTGTTGATGTT-3′ | 189 |
| *Ei* = 107% | R 5′-TTAAGTCTCTCTTGGGTCTGGGCA-3′ | |
| *q-rpl8* | F 5′-CGACCCGTACCGCTTCAAGAA-3′ | 143 |
| *Ei* = 101% | R 5′-GGACATTGCCAATGTTCAGCTGA-3′ | |
| *q-actb* | F 5′-ATGGGCCAGAAGGACAGCTA-3′ | 91 |
| *Ei* = 96% | R 5′-TCACAATACCGTGCTCAATGG-3′ | |

(*Ei*) indicates the efficiency for each q-PCR primer.

the core GnRH family, the deduced amino acid sequences of cloned stickleback GnRHs were aligned with ClustalW together with other GnRH orthologs amino acid sequences available in the ENSEMBL database (listed in the Supplemental table) and then subjected to phylogenetic inferences using the neighbor-joining (NJ) method (*p*-distance). Ten thousand bootstrap replicate analyses were carried out with Mega5.0. Physical gene maps of verified GnRH loci were scaled based on assemblies of the Ensembl Genome Browser. Genes located up- and downstream in the conserved synteny of GnRH were blasted against mammalian genomes to determine the highest score.

### 2.9. Real-time quantitative PCR (Q-PCR)

Andersson et al. (1995) found three major groups of GnRH-ir perikarya in the stickleback brain: in the olfactory bulb (*OB*) at the junction between the *OB* and the telencephalon, in the ventral telencephalon and preoptic area (*T + POA*) and in the ventro-lateral hypothalamus (*HypTh*). Moreover, GnRH-immunoreactive cells have also been identified in periventricular areas, including the thalamus, in the stickleback (Borg et al., 1982). Under a stereo microscope, the stickleback brain at near-freezing condition was divided by razor blade into five areas, i.e., the *OB*, the *T + POA* (not including the junction between the *OB* and the telencephalon), the *HypTh*, the cerebellum including dorsal mesencephalon (*Cer*) and the thalamus (*Th*) (Fig. 1). Total RNA was extracted and reverse transcribed from the brain parts or the whole brains according to the protocol of a previous study (Tseng et al., 2011). The RNA samples were treated with DNase (2U) using the TURBO DNA-free kit according to the manufacturer's instruction (Ambion) to remove possible genomic DNA contamination and re-concentrated by the RNeasy MinElute Cleanup Kit (Qiagen). Two micrograms of total RNA were reverse transcribed to cDNA with Super-Script III First-Strand Synthesis SuperMix (Invitrogen). Primers for all genes

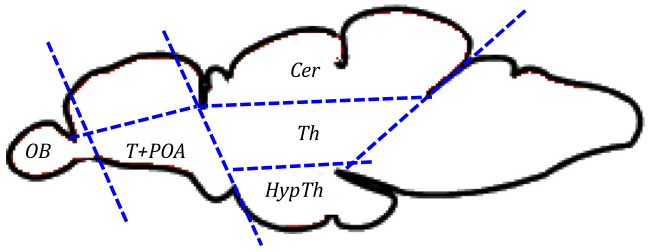


**Fig. 1.** Schematic diagram demarcing dissected areas used for expression studies. *OB*, olfactory bulb; *T + POA*, telencephalon and preoptic area; *Cer*, cerebellum and optic tectum; *Th*, thalamus; *HypTh*, hypothalamus. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

(Table 1) were designed using Primer Express Software (v2.0, Applied Biosystems). The mRNA expressions of target genes were measured by Q-PCR with the Applied Biosystems StepOnePlus System. PCR reactions contained 40 ng of cDNA, 100 nM of each primer and the Fast SYBR Green Master Mix (Applied Biosystems) for a final volume of 20 μl. All Q-PCR reactions were performed as follows: 1 cycle at 50 °C for 2 min and at 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s and at 60 °C for 1 min, using 0.05 μM of specific primers. PCR products were subjected to a melting curve analysis, and representative samples were electrophoresed to verify that only a single product was present. Control reactions were conducted with RNA-free water to determine background levels. In the whole brain breeding/post-breeding and photoperiod experiments, the *gnrh2* and *gnrh3* expression data were normalized against the ribosomal protein (*rpl8*) (ENSGACG00000002035). In other experiments, the geometric means of the expressions of two reference genes: β-actin (*actb*) (DQ018719.1) and ribosomal protein (*rpl8*) were used to normalized the target genes expression levels.

### 2.10. Statistics

The data were analysed using SPSS v.14 with a two-tailed Student's *t*-test after a normality test ($p > 0.05$) for comparisons between two groups. One-way ANOVA with Tukey's post-hoc test was used for comparisons among multiple groups.

## 3. Results

### 3.1. Genomic prediction, identification and gene structures of stickleback GnRH

Based on *in silico* sequence cloning from the Ensembl genome database, two stickleback GnRH homologs, GnRH2 (also called chicken GnRH II, cGnRHII) and GnRH3 (also called salmon GnRH, sGnRH), were carefully predicted, cloned and annotated (Fig. 2). The complete cDNA sequences of the stickleback GnRH coding regions were about 255–270 bp, which were submitted to the NCBI database (BankIt1514965 for *gnrh2* and BankIt1514974 for *gnrh3*). To further identify these two novel stickleback GnRH-like genes, comprehensive searches were performed to confirm these orthologs and compare their genomic loci based on their genome sequence databases (Figs. 3 and 4). In the phylogenetic analysis using the neighbor-joining method, each type of *gnrh* formed a monophyletic group. The two newly cloned *gnrh2* (*G. aculeatus* GnRH2) and *gnrh3* (*G. aculeatus* GnRH3) were grouped in the GnRH2 and GnRH3 clusters with high statistical support, respectively. Moreover, the *G. aculeatus* GnRH2 was nested within the clade formed by GnRH2 taxa from other Teleostei with high statistical support, as was the *G. aculeatus* GnRH3 (Fig. 5). In the genome map of sticklebacks, the sequences of *gnrh2* and *gnrh3* are located at different chromosomes, indicating that these two GnRH variants in sticklebacks have their own respective syntenies. In addition, both of them are composed of three exons, which represents the typical GnRH exon organization of genomes. Neighboring genes located in the conserved syntenies of the mammalian GnRH1 were also examined in stickleback genome databases, but no stickleback orthologs could be found (Fig. 6).

### 3.2. Experiment 1: breeding and post-breeding, whole brains

Hypertrophied kidneys and red breeding colors were observed in all 18 fully breeding males, but only in one out of 16 post-breeding males in breeding/post-breeding experiment. There was no significant difference found in the body weight between breeding and post-breeding males. KSI was higher in breeding (3.73% ± 0.98%) than that in post-breeding fish 0.82% ± 0.14% ($p < 0.001$).

In the whole brains, mRNA levels of both *gnrh2 and gnrh3* were significant higher in fully breeding males than those in post-breeding males ($p < 0.001$ in GnRH3; $p < 0.05$ in GnRH2) (Fig. 7A).

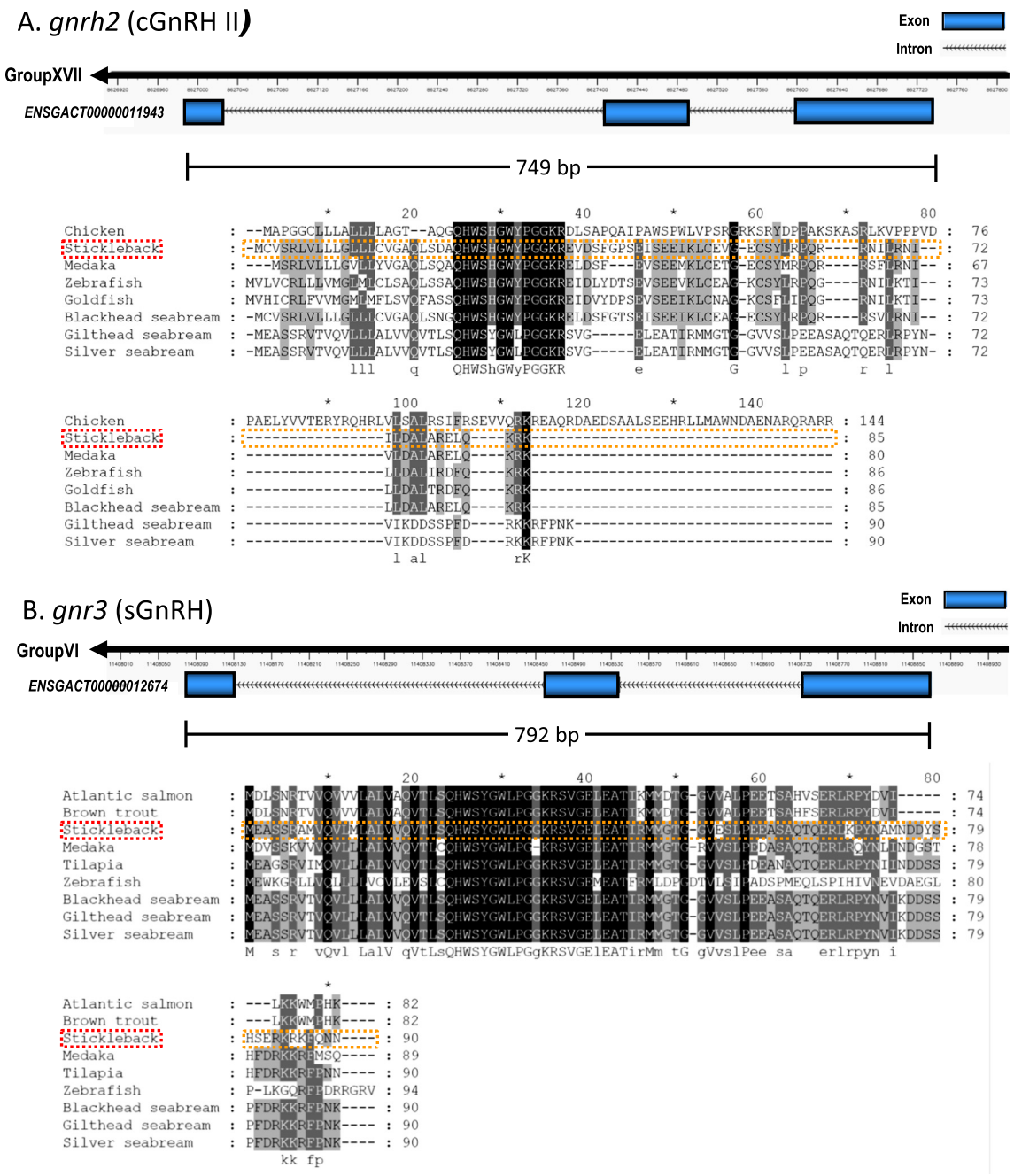


**Fig. 2.** Multiple alignment of the deduced amino acid sequences and gene structures encompassing *gnrh2* (A) and *gnrh3* (B). The GnRH sequences that the stickleback GnRHs were compared with have been sourced from the NCBI database. Residues in the consensus sequence with a black background represent completely conserved amino acids. Residues that are 80% or more conserved, and 62% or more conserved, are marked with dark gray and light gray, respectively. Exons are shown in blue squares separated by introns drawn as lines. The exons and introns are shown as original full-sized in respective regions of chromosome. Chr., the chromosome. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

### 3.3. Experiment 2: photoperiod experiment, whole brain

In the photoperiod experiment, both hypertrophied kidneys and breeding colors were shown in some fish in the LD 16:8 group (4/9), but not in the LD 8:16 group. KSI in the long photoperiod group (KSI = 1.94% ± 0.15% mean ± SE) was higher than that in the short photoperiod group (KSI = 0.69% ± 0.10%, $p < 0.001$). The GnRH2 mRNA levels in the long photoperiod group were significantly higher than those in the short photoperiod group ($p < 0.05$) (Fig. 7B), but there was no significant difference in GnRH3 mRNA levels between the two photoperiods ($p = 0.247$) (Fig. 7B).

### 3.4. Experiment 3: castration and sham-operation experiment, whole brain

The body weight of sham-operated breeding males (1.99 ± 0.03 g, $p < 0.05$) was higher than that of castrated breeding males (1.87 ± 0.04 g), and the KSI in sham-operated fish (KSI = 1.87% ± 0.13%, $p < 0.001$) was higher than in castrated males (KSI = 0.76% ± 0.11%). Breeding color was displayed by all sham-operated males and by some castrated males (3/10), but the breeding color of the latter was less pronounced. The mRNA levels of *gnrh3* were higher in sham-operated than in castrated males ($p < 0.05$), but differences in *gnrh2* mRNA levels were not significant ($p = 0.055$) (Fig. 7C).

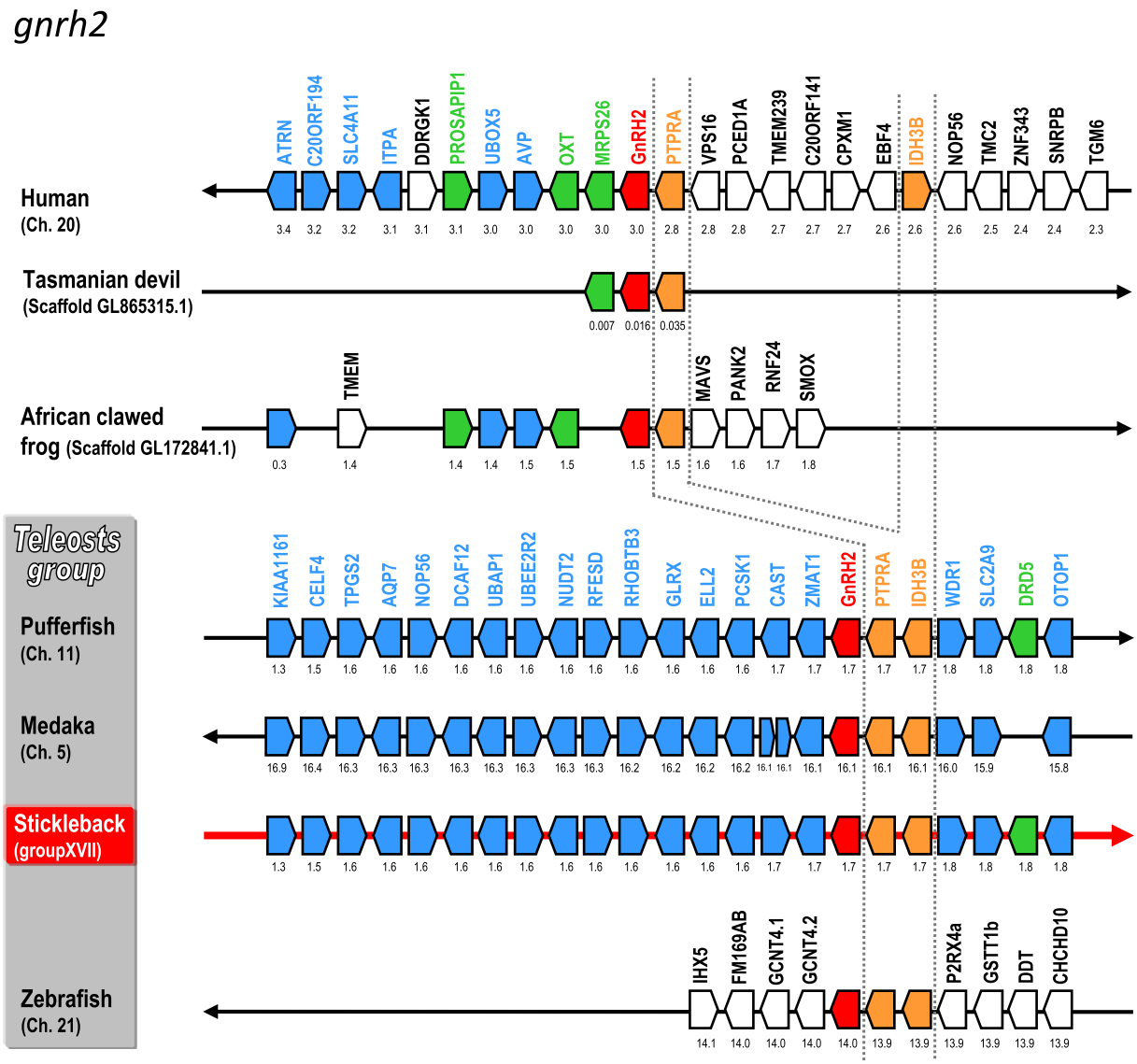


**Fig. 3.** Synteny map comparing orthologs of the *gnrh2* locus and the genes flanking it in three-spined sticklebacks, humans (*Homo sapiens*), Tasmanian devils (*Sarcophilus harrisii*), African clawed frogs (*Xenopus laevis*), pufferfish (*Tetraodon nigroviridis*), medaka (*O. latipes*) and zebrafish (*D. rerio*). The GnRH neighboring transcripts were identified using the Ensembl genome browser system. Chr., chromosome. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

### 3.5. Experiment 4: breeding and post-breeding, brain areas

Breeding features were observed in all 6 mature males from groups 1 and 2 but not in any post-breeding male from group 2. The mature fish from groups 1 and 2 did not differ in any respect and were treated together. The KSI was higher in mature (2.91% ± 0.31%) than that in post-breeding fish (1.40% ± 0.08%) ($p < 0.001$). No significant difference was found in the body weight between the two groups. *gnrh3* mRNA were found in all investigated brain areas, whereas *gnrh2* mRNA was not detectable in the *OB* and *T + POA* areas and extremely low in *HypTH*. In *Cer* and *Th*, both *gnrh2* and *gnrh3* mRNA levels were present, but *gnrh3* mRNA levels were barely detected. The *gnrh2* mRNA levels in the *Th* and *gnrh3* mRNA levels in the *T + POA* were significantly higher in mature males than those in post-breeding males (Fig. 8). Breeding males had slightly higher *gnrh3* mRNA levels in the *HypTH* than post-breeding males, but the difference was not significant ($p = 0.053$) (Fig. 8).

### 3.6. Experiment 5: photoperiod and castration experiment, brain areas

Both hypertrophied kidneys and breeding colors were observed in most (3/5) sham-operated fish kept under a long-day photoperiod, but not in the initial controls, long-day castrated fish or short-day sham-operated fish. The KSI in the long-day sham-operated males (KSI = 2.91% ± 0.33%) was higher than that in the initial controls (KSI = 1.31% ± 0.23%) ($p < 0.05$), long-day castrated males (KSI = 1.18% ± 0.29%) ($p < 0.001$) or short-day sham-operated males (KSI = 1.26% ± 0.14%) ($p < 0.05$), whereas there was no difference in body weight. Long-day sham-operated males had significantly higher *gnrh3* mRNA levels in the *T + POA* and *HypTh* than the initial controls, long-day castrated males or short-day sham-operated males (Fig. 8). Moreover, *gnrh2* gene mRNA levels in the *Th* were higher in long-day sham-operated males than in the other groups, whereas such a difference was not found in the *Cer* or *HypTh*. No differences in both *gnrh2* and *gnrh3* mRNA levels were found in any brain area among long-day

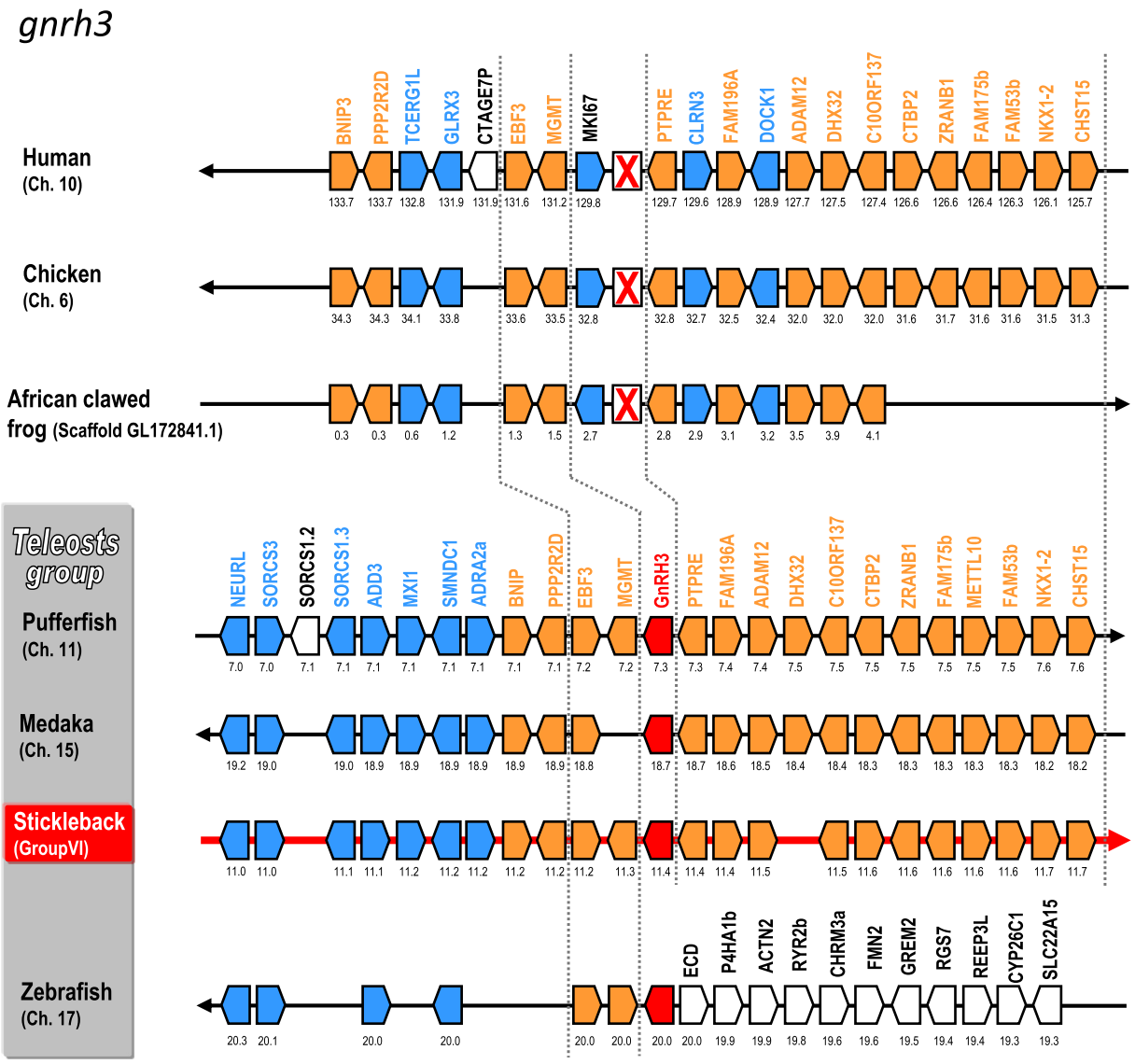


**Fig. 4.** Synteny map comparing orthologs of the *gnrh3* locus and the genes flanking it in three-spined sticklebacks, humans (*Homo sapiens*), Tasmanian devils (*Sarcophilus harrisii*), African clawed frogs (*Xenopus laevis*), pufferfish (*Tetraodon nigroviridis*), medaka (*Oryzias latipes*) and zebrafish (*D. rerio*). The GnRH neighboring transcripts were identified using the Ensembl genome browser system. Chr., the chromosome. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

castrated fish, short-day sham-operated fish or the initial controls (Fig. 9).

## 4. Discussion

Genes for GnRH2 and GnRH3, but not for GnRH1, were present in the stickleback genome. GnRH3 (salmon GnRH) peptide has also been tentatively identified in stickleback brains using HPLC (Andersson et al., 1995). The syntenies of GnRH1 are variant-specific and conserved among the vertebrates, which have this variant, e.g., humans (*Homo sapiens*), chickens (*Gallus gallus*), western clawed frogs (*Xenopus tropicalis*), medaka (*Oryzias latipes*) and spotted green pufferfish (*Tetraodon nigroviridis*). However, according to the above phylogenetic analysis, as well as comprehensive genome location searches for conserved syntenies of GnRH1 in other species, GnRH1 has been lost in some teleost species including sticklebacks and zebrafish (Fig. 6) as previously inferred (Tostivint, 2011). Our phylogenetic results were in agreement with earlier studies (Kah et al., 2007; Guilgur et al., 2007), where GnRH3 and GnRH1 clades were regarded as sister-clades to each other, both belonging to the forebrain lineage and distinct from the GnRH2 clade (midbrain lineage) (Kah et al., 2007; Guilgur et al., 2007). It is generally believed that GnRHs of the forebrain lineage, especially GnRH1, are involved in the regulation of reproduction (reviewed in Okubo and Nagahama, 2008). Nevertheless, in the forebrain, GnRH1 and GnRH3 may take over the function of each other when one of them is lost (reviewed in Kah et al., 2007; Okubo and Nagahama, 2008). In species that do not have GnRH1, e.g., salmon, zebrafish and goldfish, GnRH3 neurons are present in the preoptic area and terminal nerve ganglion and project their axons into the pituitary. In these cases, GnRH3 would act as the main stimulator of GtH release (reviewed in Okubo and Nagahama, 2008; Zohar et al., 2010).

Using immunocytochemistry, distinct groups of GnRH-ir perikarya have been found in the stickleback brain: in the nucleus olfactoretinalis (in the *OB* region of the present study), in the nucleus anterior periventricularis (*T + POA*) and in the nucleus lateralis tuberis (*HypTh*) (Andersson et al., 1995) as well as the GnRH-ir cells discovered in periventricular areas located in the *Cer* and *Th* regions of this study (Borg et al., 1982). Moreover, the proximal pars distalis of the pituitary, housing the gonadotropic cells, is innervated by GnRH-ir positive fibers, whereas GnRH-ir perikarya are absent there (Andersson et al., 1995).

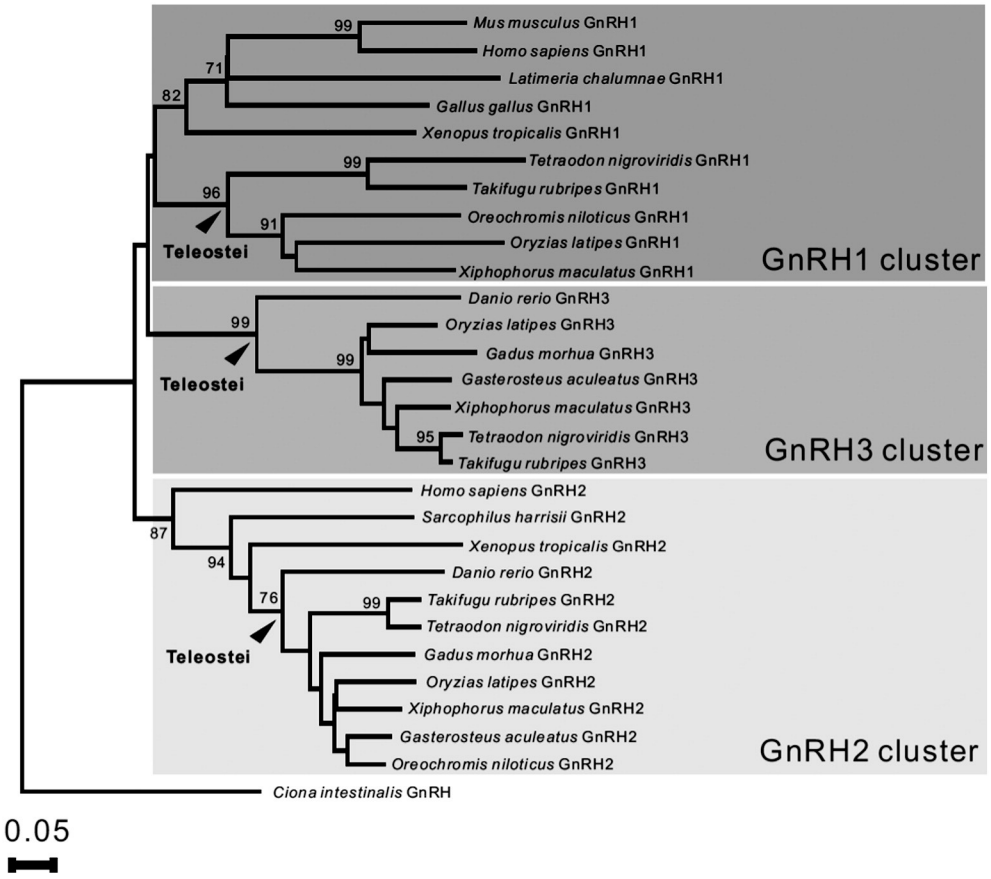


**Fig. 5.** Phylogenetic tree of prepro-GnRH sequences obtained by the neighbor-joining (NJ) method with $10^5$ bootstrap replicates using Mega 5.0. Numbers on branches are NJ bootstrap values (those below 70% are not shown).

Hence, GnRH(s) that induces GtH release are probably synthetized in the brain and then projected to pituitary. In the present study, *gnrh3* expression was found in all investigated areas, and *gnrh2* mRNA levels were detected in the cerebellum, thalamus and hypothalamus. The distribution pattern of *gnrh3* expression among stickleback brain areas was largely similar to the distribution pattern of GnRH3 neurons in masu salmon (*Oncorhynchus masou*) and chum salmon (*Oncorhychus keta*) (Amano et al., 1991; Kudo et al., 1996). In goldfish, on the other hand (Kim et al., 1995), not only GnRH3 but also GnRH2 are synthesized in the forebrain, including the preoptic area, suggesting that both GnRH2 and GnRH3 may have a synergistic function on reproduction in that species. However, although gnrh2 mRNA expressions were detected in the hypothalamus of sticklebacks, the levels were far less than those found in the thalamus. It is unclear whether the low gnrh2 expression found in the hypothalamus is due to the GnRH-ir cells in the thalamus. In stickleback, the higher levels of *gnrh2* mRNA in the thalamus of mature than in post-breeding males and in long-day than in short-day fish suggest that also GnRH2 is related to the reproductive condition, though hardly directly stimulating GtH release. The regulation of reproductive endocrine system, especially inducing the release of GtH, is an important function of GnRHs. However, GnRHs present in the other areas of brain may also have the roles in reproduction. Both GnRH2 and GnRH3 act as neuromodulators, and they have been implicated in the control of reproductive behaviour (reviewed in Okubo and Nagahama, 2008). In mammals, treatments of GnRH2 can enhance female reproductive behaviour and reduce food intake (Temple et al., 2003; Kauffman and Rissman, 2004; Barnett et al., 2006). Administration of GnRH2 also promotes female goldfish spawning behavior. Volkoff and Peter (1999) observed that injection of 0.1 to 0.5 ng/g of native GnRH2 or 3 results more spawning acts than the fish injected with saline. Furthermore, the lesion of terminal nerve of male dwarf gouramis (*Colisa lalia*), which suppresses nest-building behavior, suggests an importance of non-hypophysiotropic GnRH neurons in reproductive function (Yamamoto et al., 1997)

In previous reports, relations vary between reproductive conditions and the GnRH system of fish. No significant difference in GnRH2 and GnRH3 peptide contents in different brain areas was found between mature and immature rainbow trout (*Oncorhynchus mykiss*) (Okuzawa et al., 1990) or between mature and immature goldfish (Yu et al., 1987). However, in gilthead sea breams (*S. aurata*), red sea breams (*Pagrus major*) and European sea bass (*Dicentrarchus labrax*), the GnRH1 (sbGnRH) peptide levels in the pituitary increase during the spawning season (Holland et al., 1998; Senthilkumaran et al., 1999; Rodríguez et al., 2000).

The mRNA levels of *gnrh*s in the brain can also change following the reproductive conditions. For example, the *gnrh3* mRNA levels in the terminal nerve of chum salmon (*Oncorhynchus keta*) are higher in mature males than in immature males (Onuma et al., 2010). *gnrh1* mRNA levels reach their apex in the natural breeding season and decline rapidly afterwards in red sea bream and Brazilian flounder (*Paralichthys orbignyanus*) (Okuzawa et al., 2003; Campos et al., 2011). On the other hand, both *gnrh1* and *gnrh3* mRNA levels in European sea bass remain unchanged between January (spermatogenesis) and March (early spawning) when both *fshb* and *lhb* mRNA level increase). In Atlantic cod (*G. morhua*), the brain *gnrh3* mRNA level increases significantly in the spawning stage, whereas no such change was found in the *gnrh2* mRNA level (Hildahl et al., 2011). (Hildahl et al., 2011), as in other fishes lacking GnRH1 (reviewed in Okubo and Nagahama, 2008; Zohar et al., 2010). In the present study, higher mRNA levels of *gnrh2* and *gnrh3* were found in the whole brain of breeding than of post-breeding sticklebacks. Those results were consistent with the observations by Andersson et al. (1992) that the capacity of high-affinity GnRH binding

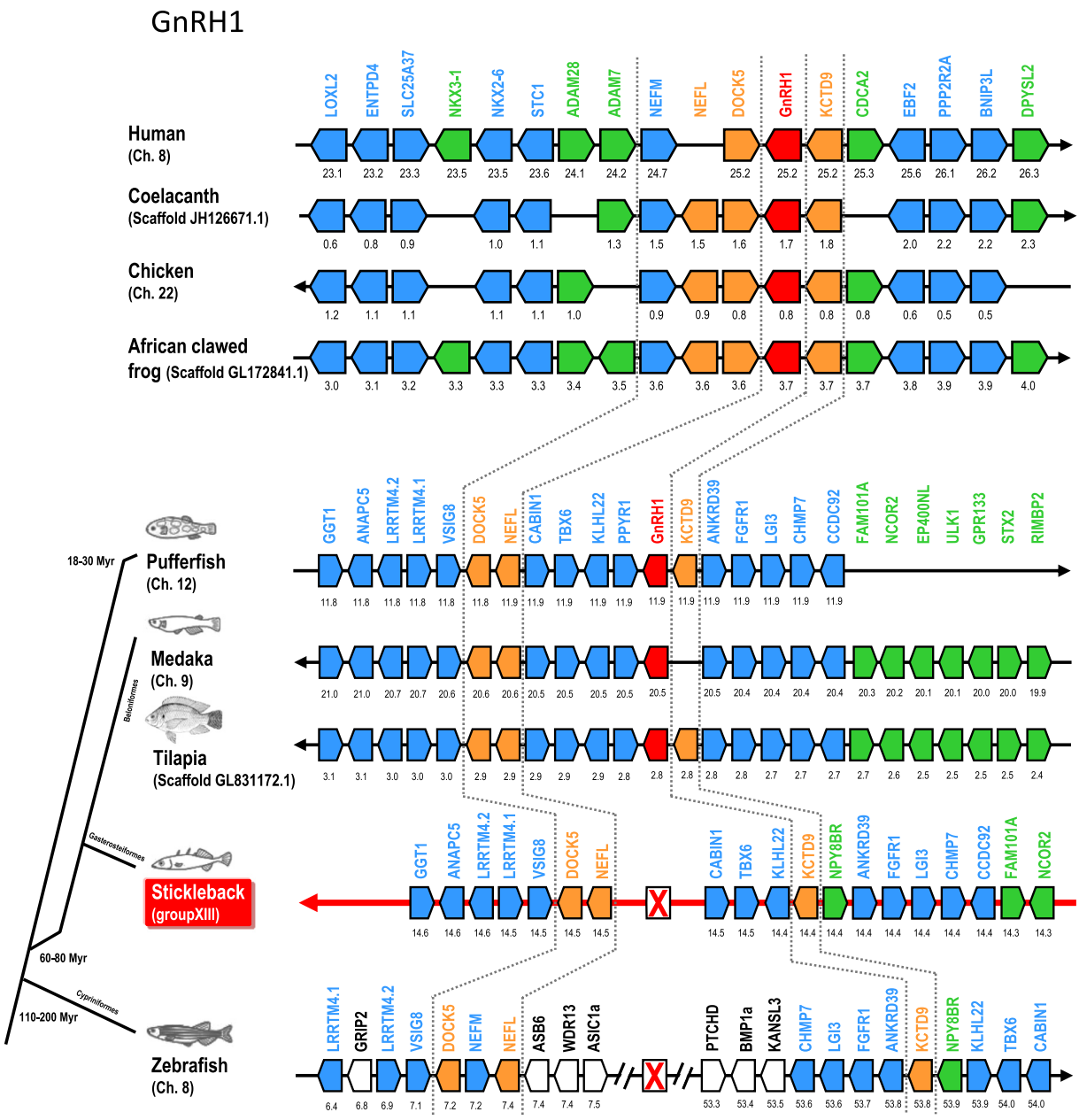


**Fig. 6.** Synteny map comparing orthologs of the *gnrh1* locus and the genes flanking it in human (*Homo sapiens*), coelacanth (*Latimeria chalumnae*), chicken (*Gallus gallus*), African clawed frog (*Xenopus laevis*), pufferfish (*Tetraodon nigroviridis*), medaka (*Oryzias latipes*), tilapia (*Oreochromis niloticus*) and zebrafish (*Danio rerio*). The GnRH neighboring transcripts were identified using the Ensembl genome browser system. Chr., the chromosome. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

sites (presumably representing receptors) was high in sticklebacks during the summer breeding season but not detectable in post-breeding fish. The higher mRNA levels of *gnrh3* in the pre-optic area and hypothalamus of mature fish and the sham-operated fish kept under long day suggest that it is the dominant GtH releasing GnRH form in sticklebacks. However, The higher levels of *gnrh2* mRNA in the thalamus in breeding than in non-breeding fish suggest that also GnRH2 is likely to be relative to the reproductive condition, although hardly stimulating GtH release directly.

In mammals, photoperiodic effects on GnRH neurons have been shown in several studies (reviewed in Kriegsfeld and Bittman, 2010). However, the role of GnRH in photoperiodic responses in teleosts is inconclusive. Long day photoperiod has been shown to delay sexual maturation and spawning in European sea bass (*D. labrax*) (reviewed in Zanuy et al., 1995). Under natural photoperiod, pituitary GnRH1, 2 and 3 contains in European sea bass are higher in November than January/March/May. However, there was no difference in the GnRHs peptide contents in the pituitary between European sea bass kept under artificial long or short artificial photoperiods (Rodríguez et al., 2004). In male masu salmon, the number of neurons expressing *gnrh3* mRNA increases by decreasing day length, which in this species stimulates final maturation, suggesting that activated GnRH3 synthesis is involved in the maturation of this fish (Amano et al., 1994). Moreover, the number of *gnrh3*-expressing neurons in the POA of castrated male masu salmon increased under LD 8:16 after 1 month, whereas they remained few in castrated males under LD 16:8 (Amano et al., 1999). However, no significant difference was found in the *gnrh3*-expressing neurons in the POA of sham-operated fish kept under LD 8:16 or LD 16:8 after 2 months (Amano et al., 1999). It is possible that the increase of *gnrh3*-expressing neurons in short-day castrated salmon with low plasma

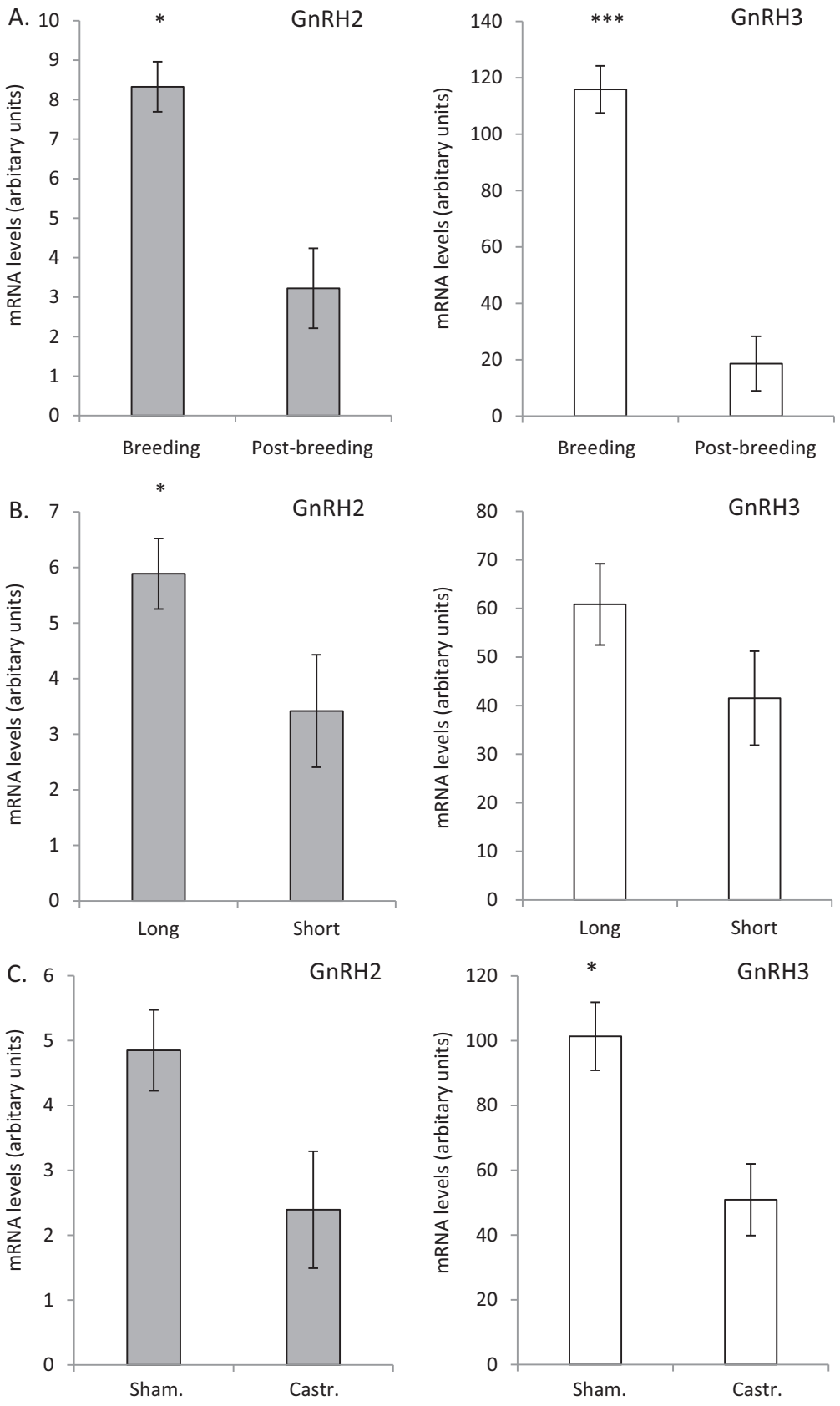


**Fig. 7.** GnRHs mRNA levels in the whole brain of (A) breeding ($n = 8$) or post breeding ($n = 16$) stickleback males, (B) the males kept under different photoperiods (long: L16:D8 ($n = 9$); short: L8:D16 ($n = 7$)) and (C) castrated ($n = 10$) or sham-operated ($n = 9$) breeding males. Mean ± SE shown (***$p < 0.001$; *$p < 0.05$).

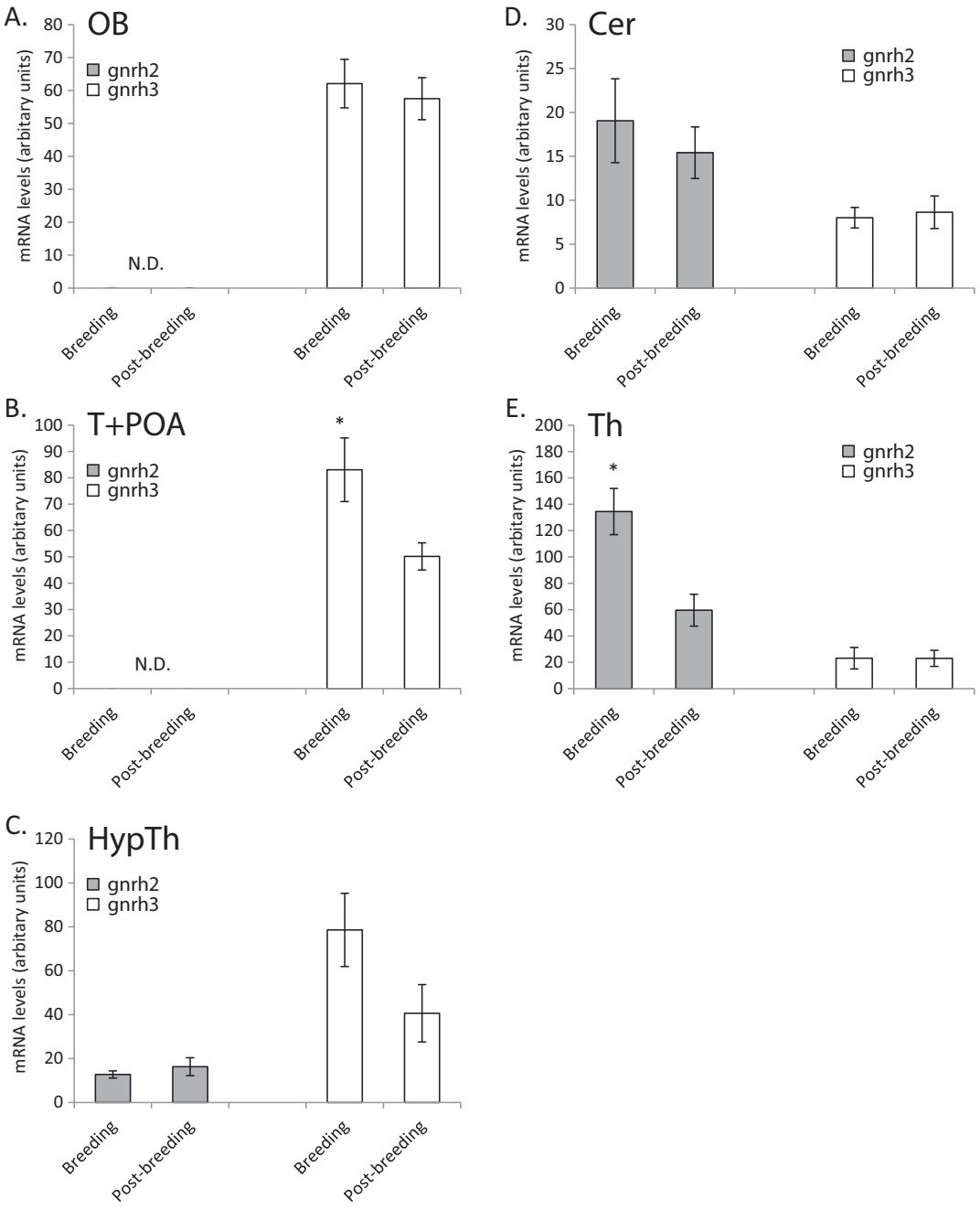


**Fig. 8.** *gnrh2* and *gnrh3* mRNA levels in different brain areas of breeding ($n = 6$) and post-breeding ($n = 6$) stickleback males: (A) *OB*, olfactory bulb; (B) *T + POA*, telencephalon with preoptic area; (C) *Cer*, cerebellum and optic tectum; (D) *Th*, thalamus; (E) *HypTh*, hypothalamus. Means ± SE are shown (*$p < 0.05$).

testosterone levels is due to negative steroid feedback effects on GnRH neurons, which do not occur under a long-day photoperiod. In another study, the mRNA level of *gnrh3* in stickleback whole brains significantly decreased, rather than increased, when the fish had been transferred to a long-day photoperiod for 5 days (O'Brien et al., 2012). The reason for this decrease under the stimulatory photoperiod is unknown, but the effect hardly supports a role of GnRH3 in the photoperiodic stimulation of reproduction. Although a long-day photoperiod up-regulated *gnrh2* and *gnrh3* mRNA levels in various brain areas of sham-operated males, no such stimulatory effects were found in castrated males. This suggests that the increase of *gnrh2* and *gnrh3* mRNA levels in long-day sham-operated fish was not controlled directly by day length. The light signal may influence the endocrine system, e.g., changing the feedback mechanism on the BPG axis (Hellqvist et al., 2008; Shao et al., 2013), or mediate other substances that can regulate GtH, e.g., kisspeptins (Felip et al., 2009; Alvarado et al., 2013) or dopamine (reviewed in Peter et al., 1991).

To summarize, *gnrh2* and *gnrh3* mRNA are present in the stickleback brain, *gnrh2* primarily in the cerebellum and in the thalamus and *gnrh3* primarily in telencephalon-preoptic area, in the olfactory bulbs and in the hypothalamus. The levels in the olfactory bulbs and in the cerebellum are similar under the different reproductive conditions, whereas the levels of *gnrh2* in the thalamus and of *gnrh3* in the telencephalon-preoptic area are higher in fish under breeding condition than in fish that are non-mature due to castration, non-stimulatory photoperiod or post-breeding condition. This is also largely consistent with the levels in whole brains, though not all results are significant. The results suggest that both GnRHs play a role in seasonal reproduction. Further,

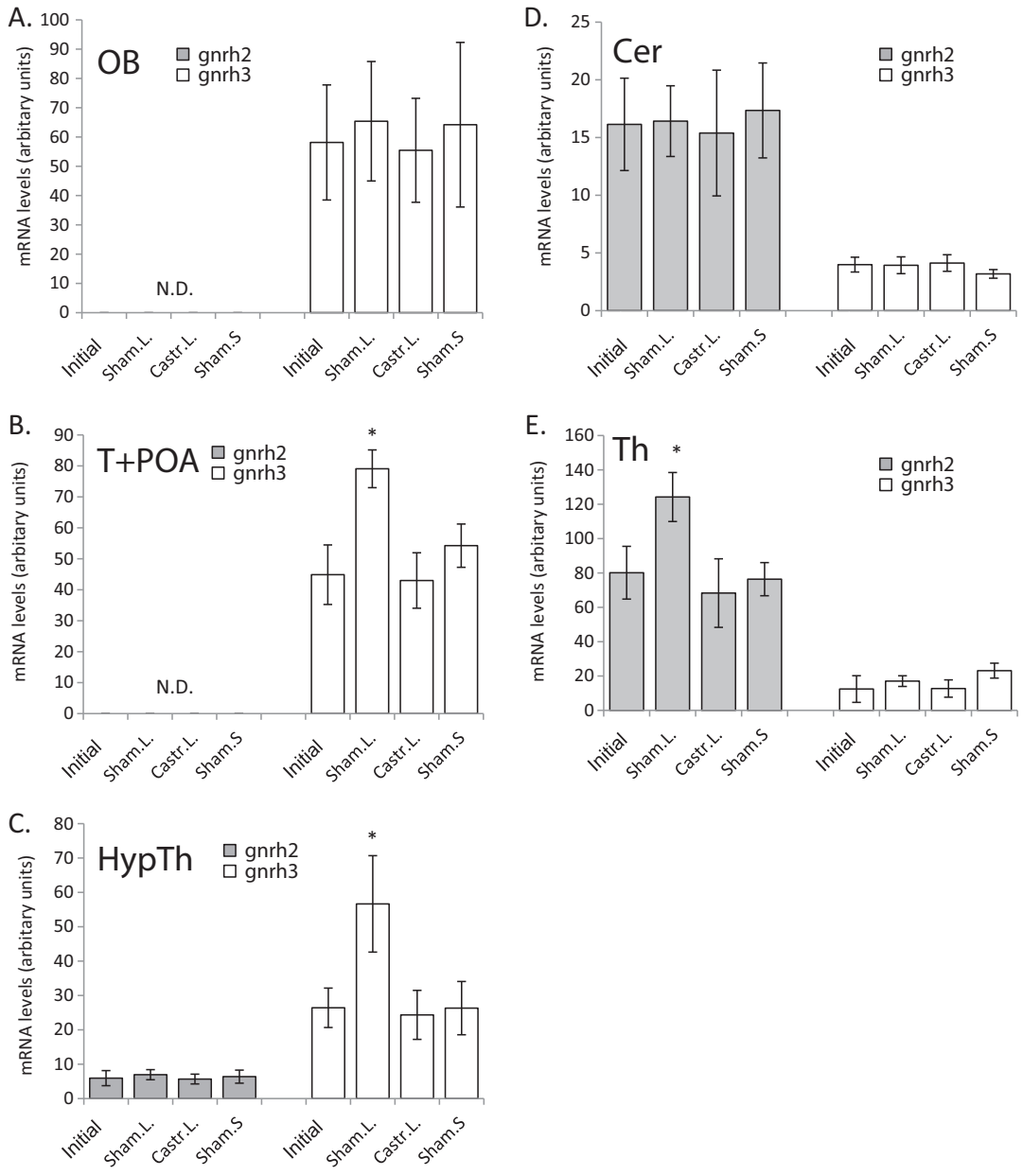


**Fig. 9.** *gnrh2* and *gnrh3* mRNA levels in different brain areas of sham-operated stickleback males under different photoperiods (long = LD 16:8, $n = 5$; short = LD 8:16, $n = 5$), castrated males under a short-day photoperiod ($n = 5$) and the initial controls ($n = 3$): (A) *OB*, olfactory bulb; (B) *T + POA*, telencephalon with preoptic area; (C) *Cer*, cerebellum plus optic tectum; (D *Th*, thalamus; (E) *HypTh*, hypothalamus. Means ± SE are shown (*$p < 0.05$ higher than each of the other groups).

as the increase in *gnrh* mRNAs under long photoperiod is dependent on the presence of the gonads, it appears that feedback effects on the BPG axis are critical to induce *gnrh*s expression.

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