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1 **Diurnal changes in salmon GnRH secretion in the brain of masu salmon**

2 *(Oncorhynchus masou)*

3  
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19 **Abstract**

20 The day-night changes of salmon GnRH (sGnRH), which is secreted from various  
21 brain regions, were analyzed in maturing and matured masu salmon (*Oncorhynchus*  
22 *masou*). In maturing males, the levels of sGnRH secreted from the olfactory bulb (OB),  
23 terminal nerve (TN), and ventral telencephalon and preoptic area (VT+POA) were all  
24 significantly higher during midnight than daytime. However, the contents of sGnRH in  
25 the pituitary gland during midnight were not higher than those during daytime. In  
26 maturing females, the levels of sGnRH secreted from the VT+POA were higher during  
27 midnight than daytime, and the contents of sGnRH in the pituitary gland were also  
28 higher during midnight. In matured fish, the levels of sGnRH secreted from the OB, TN  
29 and VT+POA during midnight were significantly higher than those during daytime.  
30 There were also no significant differences in the contents of sGnRH in the pituitary  
31 gland. These results suggest that a short photoperiod may be involved in diurnal  
32 secretion rhythms of sGnRH in various brain regions and the pituitary gland.

33

34 **Key words:** diurnal secretion, sGnRH, brain, pituitary gland, masu salmon

35

36 **1. Introduction**

37 Gonadotropin-releasing hormone (GnRH) is considered as one of the most  
38 important hormones in gonadal maturation and homing migration in salmonid species  
39 [21]. GnRH controls gonadal maturation through the synthesis and release of  
40 gonadotropin (GTH) in the pituitary gland [19]. In salmonid species, gonadal  
41 maturation is promoted under a short photoperiod. Moreover, it has been reported that a  
42 short photoperiod influences GnRH gene expression in masu salmon (*Oncorhynchus*  
43 *masou*) [4]. Although GnRH synthesis and secretion are thought to be corresponding to  
44 day length [3], the actual diurnal rhythms related to GnRH secretions in salmonid  
45 species have yet to be clarified.

46 In salmonid species, two molecular forms of GnRH, salmon GnRH (sGnRH) and  
47 chicken-II GnRH (cGnRH-II), are secreted from various brain regions [10]. sGnRH  
48 neurons are distributed widely from the olfactory nerve to the preoptic area (POA) [1, 2].  
49 It has been well established that sGnRH systems in the ventral telencephalon (VT) and  
50 POA are involved in GTH regulations in the pituitary gland [4]. Therefore, it has been  
51 accepted that the levels of sGnRH secreted from the VT+POA are directly associated  
52 with the relative quantity of sGnRH in the pituitary gland. In contrast, it is believed that  
53 the wide distributions of sGnRH in various brain regions act as a neuromodulator that is

54 involved in reproductive behavior [13, 14]. However, in salmonid, there have been not  
55 much works focused on sGnRH, which are not involved in GTH regulations. Therefore,  
56 further studies of sGnRH not only in VT+POA but also in the other brain regions should  
57 be done.

58 In this study, the diurnal rhythm of sGnRH secretion was assessed in various brain  
59 regions in masu salmon using an *in vitro* brain culture and time-resolved  
60 fluoroimmunoassay (TR-FIA). The difficulty in collecting secreted sGnRH from the  
61 various brain regions in living fish made us to perform the *in vitro* brain culture method.  
62 And, TR-FIA is one of the useful method to measure the contents of GnRH forms in  
63 another species [16, 18]. These experiments were performed using maturing and  
64 matured masu salmon brains to investigate diurnal rhythms of sGnRH during  
65 maturation. Moreover, the contents of sGnRH in the pituitary gland were also measured  
66 by TR-FIA.

67

## 68 **2. Materials and methods**

### 69 **2.1. Fish**

70 Masu salmon reared at the Toya Lake Station, Field Science Center for Northern  
71 Biosphere, Hokkaido University, were used in the present study. Fish were kept in a  
72 2-ton circular tank under a natural photoperiod with a continuous flow of spring water

73 between 9.6 and 10.9°C. Under natural conditions, masu salmon generally mature in  
74 September. Therefore maturing fish were sampled from late June to July (15 light (L): 9  
75 dark (D)) and matured fish were sampled in mid-September (12L: 12D). The fork length,  
76 body weight, gonadosomatic index (GSI) and number of fish are shown in Table 1.

77

## 78 ***2.2. Sample collection and culture in vitro***

79 Fish were collected during daytime (13:00-14:00h) and during midnight  
80 (01:00-02:00h) and anesthetized by 0.05% FA-100 (4-allyl-2-methoxyphenol; DS  
81 Pharma Animal Health, Osaka, Japan). After decapitation, pituitary glands and brains  
82 were collected. Brains were divided into 3 regions (olfactory bulb: OB; terminal nerve:  
83 TN; VT+POA) as shown in Fig. 1 referring to the distribution of sGnRH neurons in  
84 masu salmon brains [1]. During midnight, fish were temporarily handled, and brains and  
85 pituitary glands were collected under weak yellow light. The 3 brain regions were then  
86 incubated in 1 ml salmon ACSF (140 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>,  
87 10 mM glucose and 5 mM HEPES, pH 7.5; Wako Pure Chemical, Osaka, Japan) [11] at  
88 15°C for 2 hours under either light or dark environments. The incubation time was  
89 determined using sockeye salmon brain (*O. nerka*) (Fig. 2). After incubation, 900 µl of  
90 each medium was placed into sample tubes and mixed with 100 µl of 1N HCl. Each

91 medium solution was lyophilized under a vacuum condition and the pellets were  
92 reconstituted in an assay buffer (20 mM sodium phosphate buffer, 0.9% NaCl, 0.1%  
93 BSA, 20  $\mu$ M diethylenetriamine-N,N,N',N'',N'''-pentaacetic acid, 0.01% Tween 40, pH  
94 7.2; all chemicals were purchased from Wako except for BSA was purchased from  
95 Sigma-Aldrich, St. Louis, MO, USA). For sGnRH extraction in the pituitary gland,  
96 samples were mixed with 1 ml of 0.1N HCl and homogenized by sonication. After  
97 centrifugation at 10,000 g for 30 min at 4°C, the supernatant was lyophilized under a  
98 vacuum condition and reconstituted in an assay buffer.

99

### 100 **2.3. Time-Resolved fluoroimmunoassay**

101 To measure the contents of sGnRH in each sample, TR-FIA was performed  
102 following the method of Yamada *et al.* [20]. Fifty  $\mu$ l of each extracted sample and  
103 biotinylated sGnRH (1 ng/ml) that was prepared following the method of Yamada *et al.*  
104 [20] were applied to 96-well microtiter plates (PerkinElmer Finland Oy, Turku, Finland),  
105 in which 100  $\mu$ l of sGnRH antibody that was prepared following the method of Pham *et*  
106 *al.* [16] was immobilized by physical adsorption. Following incubation for 18 hours at  
107 4°C, Eu-labeled streptavidin (PerkinElmer Finland Oy) was placed into each well and  
108 incubated for 18 hours at 4°C, and then thoroughly washed to remove any unbound

109 Eu-labeled streptavidin. Fluorescence intensity from dissociated Eu was then measured  
110 by a microplate reader (Infinite F500, Tecan, Männedorf, Switzerland). The intra- and  
111 inter- assay variation of sGnRH were 9.9% and 14.7%. Displacement curve of extracted  
112 samples paralleled with the sGnRH standard curves.

113

#### 114 ***2.4 Statistical analysis***

115 Values are presented as means  $\pm$  SEM. Statistical significance was determined  
116 using Student's *t* test and Welch's *t* test. The differences were considered significant at  
117  $p < 0.05$ .

118

#### 119 ***2.5. Ethics statement***

120 This study (No. 23-2) has been carried out under the control of the committee  
121 following the "Guide for the Care and Use of Laboratory Animals in Field Science  
122 Center for Northern Biosphere, Hokkaido University" and Japanese Governmental Law  
123 (No.105) and Notification (No.6).

124

### 125 **3. Results**

#### 126 ***3.1. Diurnal changes in secretion of sGnRH***

127 In maturing males, the levels of sGnRH secreted from the OB, TN and VT+POA  
128 showed significant diurnal changes (Fig. 3A-C). The secreted sGnRH levels during  
129 midnight were 3-10 times higher than those during daytime in the OB, TN and  
130 VT+POA (Fig. 3A-C). However, in maturing females, significant diurnal changes in the  
131 secreted sGnRH levels were only observed in the VT+POA (Fig. 3F). In the OB and TN,  
132 the levels of secreted sGnRH tended to be higher during midnight, nevertheless no  
133 significant diurnal changes were shown (Fig. 3D, E).

134 In matured males and females, the levels of sGnRH secreted from the OB, TN and  
135 VT+POA showed significant diurnal changes (Fig. 3A-F). During midnight, the  
136 secreted sGnRH levels were 2-5 times higher than those during daytime. Out of all  
137 sGnRH secretion levels examined, the only secretion that showed significant diurnal  
138 changes among both maturing and matured fish of both sexes was sGnRH in the  
139 VT+POA (Fig. 3C, F). In general, the secreted sGnRH levels during midnight in  
140 maturing fish were higher than those in matured fish.

141

### 142 ***3.2. Diurnal changes in the content of sGnRH in the pituitary gland***

143 The contents of sGnRH in the pituitary gland showed significant diurnal changes in  
144 maturing females (Fig. 4B). The contents of sGnRH in the pituitary gland were higher

145 than those during daytime. In maturing males and matured fish, the contents of sGnRH  
146 in the pituitary gland showed no diurnal changes (Fig. 4A, B).

147

#### 148 **4. Discussion**

149 In the present study, the diurnal changes of sGnRH secretions in various brain  
150 regions were examined. The levels of sGnRH showed nocturnal elevations with the  
151 exception of those secreted from the TN in maturing female. In all cases, the levels of  
152 sGnRH secreted from the VT+POA showed significant diurnal changes. It is possible  
153 that the activity of sGnRH systems is accelerated under dark environments suggesting  
154 that salmonids are short-day breeders.

155 In medaka (*Oryzias latipes*), medaka GnRH (mdGnRH) neuron firing rates were  
156 elevated during the evening [9]. mdGnRH neurons are responsible for synthesizing  
157 mdGnRH that stimulate the pituitary gland. The luteinizing hormone (LH) surge also  
158 occurs during the night in medaka. In salmonids, no clear LH surges were observed [12].  
159 On the other hand, it is reported that plasma melatonin show diurnal rhythms in masu  
160 salmon reared under a long day photoperiod and pre-spawning chum salmon (*O. keta*)  
161 [6, 17]. Although it is well established that melatonin controls the diurnal regulation of  
162 reproduction in many vertebrates, the role of melatonin in fish reproduction has not

163 been clarified [8]. Moreover, the diurnal pattern of plasma melatonin is similar to  
164 sGnRH secretion patterns from the VT+POA observed in the present study. Since  
165 plasma melatonin undergoes nocturnal elevation, there may be an indirect relationship  
166 between melatonin and sGnRH.

167       During the course of the present study, the characteristics of diurnal secretion  
168 patterns of sGnRH in the OB, TN and VT+POA were similar in that sGnRH levels were  
169 higher during midnight than daytime. In sockeye salmon, the ontogenic origins of  
170 sGnRH neuronal somata were the olfactory placode, and these somata migrated to wide  
171 brain regions between the olfactory nerve and preoptic area [15]. Therefore, it is  
172 considered that control mechanisms of sGnRH neuronal systems may be regulated by  
173 same factors.

174       While sGnRH secreted from the VT+POA showed nocturnal elevation, the contents  
175 of sGnRH in the pituitary did not show any clear diurnal changes except in maturing  
176 females. In the previous study using masu salmon, short photoperiodic manipulation did  
177 not affect the pituitary LH levels [7]. It is suggested that LH does not have the diurnal  
178 rhythm corresponding to the environmental photoperiod. In contrast, we compared the  
179 contents of sGnRH in the pituitary gland between the daytime and the midnight by  
180 using immature sockeye salmon, and found that clear nocturnal elevations of sGnRH

181 levels in the pituitary gland were observed in both sexes (unpublished data). And in the  
182 previous study using chum salmon, it was reported that the diurnal endocrine rhythms  
183 may be attenuated in the pre-spawning fish [17]. From these results, it is considered that  
184 the diurnal sGnRH activity in the pituitary gland may not be involved in regulation of  
185 the diurnal LH activity and may be attenuated during sexual and gonadal maturation.

186 In general, it is believed that the ovulating time of salmonid species is not  
187 corresponding to the environmental photoperiod. Therefore, the present results suggest  
188 that diurnal changes of the VT+POA sGnRH may not be directly involved in  
189 reproductive diurnal rhythms. However, we show that sGnRH exhibit diurnal secretion  
190 rhythms in both sexual maturing and matured masu salmon. These results conclude that  
191 a short photoperiod may be deeply involved in diurnal secretion rhythms of sGnRH in  
192 various brain regions.

193

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200

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263

264 **7. Figure captions**

265 **Fig.1.** Divided 3 brain regions for the incubation, OB (A), TN (B) and VT + POA (C).

266 **Fig.2.** Secreted sGnRH from VT + POA in various time from the start of incubation,  
267 0-1hour (H), 1-2 hour (H) and 2-3 (H). We used reared sockeye salmon to determine the  
268 incubation time. Values are in means  $\pm$  SEM (n=5-6).

269 **Fig.3.** Diurnal changes in sGnRH secretion from the OB (A, D), TN (B, E) and  
270 VT+POA (C, F) in male and female fish. Values are in means  $\pm$  SEM of males (n=3-9)  
271 and females (n=4-9) during daytime (open bars) and midnight (shaded bars). \*\*  $p < 0.01$   
272 and \* $p < 0.05$  indicate statistical significances.

273 **Fig.4.** Diurnal changes in the contents of sGnRH in the pituitary gland in male (A) and  
274 female (B) masu salmon. Values are in means  $\pm$  SEM (n=3-6) during daytime (open  
275 bars) and midnight (shaded bars). \*  $p < 0.05$  indicates statistical significance.

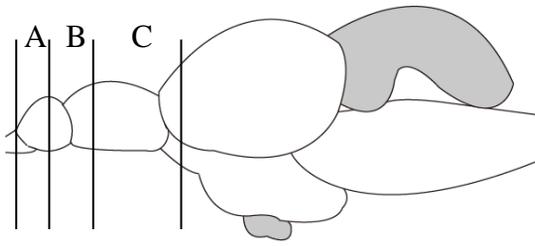


Fig. 1.

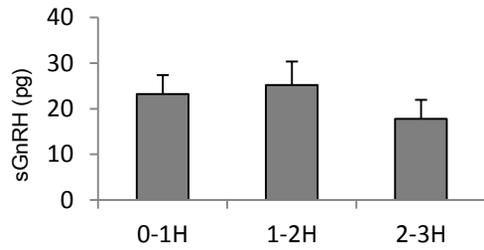


Fig. 2.

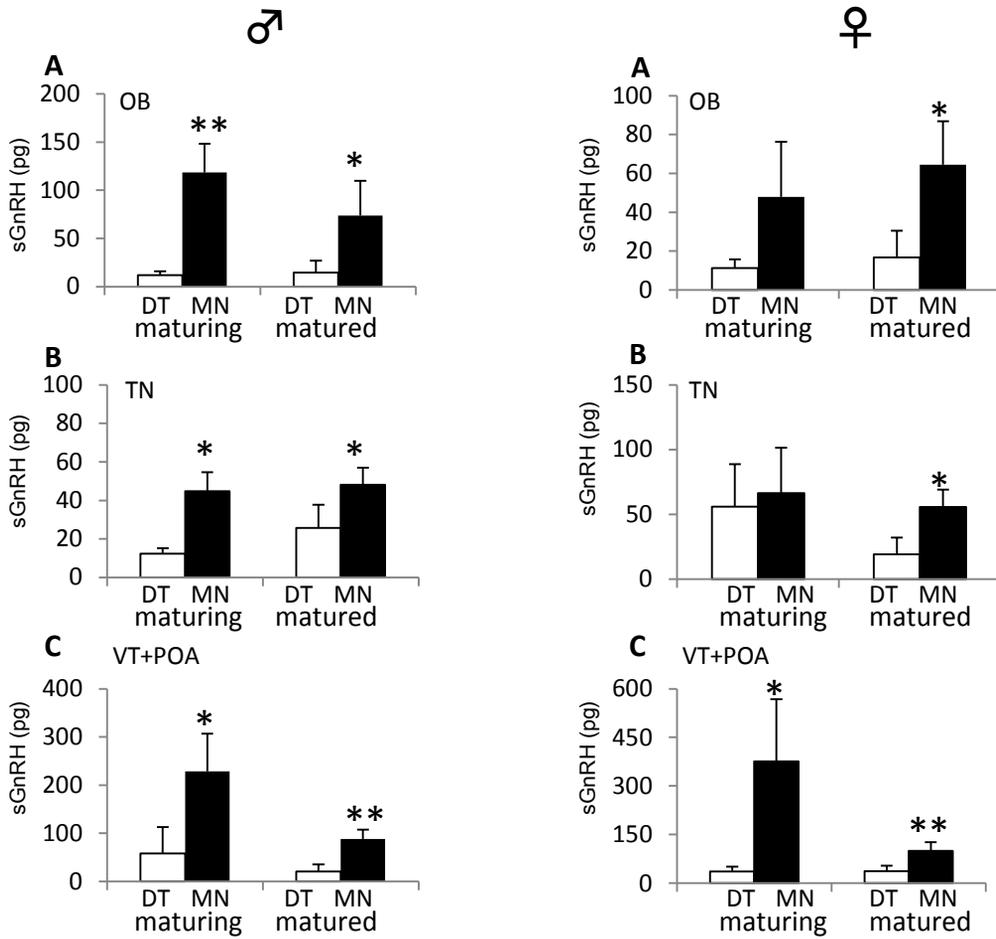


Fig. 3.

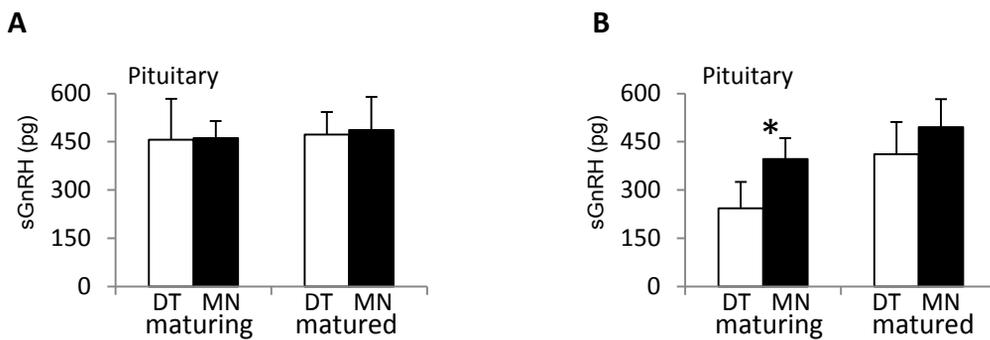


Fig. 5.