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2 **Tissue-specific sexual dimorphism in expression of kisspeptin**
3 **and its receptors in spotted snakehead *Channa punctatus***

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19 **Abstract**

20 **The present study paves the way for novel aspects of kisspeptin in regulation of fish physiology,**
21 **importantly, immunity and metabolic activities. The level of kisspeptin (*kiss1*) and its receptors**
22 **(*kiss1r*, *kiss2r*) expression was observed in different regions of brain, primary and secondary**
23 **lymphoid organs, liver and gonads of male and female *Channa punctatus*, suggesting a wider**
24 **role of kisspeptin in regulation of fish physiology. Further, expression profile of *kiss1*, *kiss1r* and**
25 ***kiss2r* revealed sexual dimorphism depending on tissues. Surprisingly, insignificant correlation**
26 **was observed between expression of *kiss1* and its receptors.**

27 **Keywords:** Kisspeptin, kisspeptin receptors, teleost, sexually dimorphic expression.

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40 **Introduction**

41 KISSPEPTIN (*KISS1*) discovered as a candidate gene for suppression of melanoma metastasis¹ encodes
42 kisspeptin (KISS1) that belongs to RF-amide family of neuropeptides. KISS1 has been reported to act
43 through serpentine transmembrane receptor, GPR54/KISS1R². The existence of kisspeptin receptor in
44 fishes was first evidenced in *Oreochromis niloticus* from which cDNA encoding GPR54 was isolated³.
45 Thereafter, multiple forms of kisspeptin (*kiss1*, *kiss2*) and its receptor (*kiss1r*, *kiss2r*) are demonstrated in
46 a number of teleosts⁴⁻⁸. Kisspeptin 1 and 2 have been shown to activate both the receptors, though with
47 different potencies⁹. For several years, kisspeptin is known as prime neuroendocrine regulator of
48 reproduction in mammals¹⁰ and fishes⁸ as well. Moreover, only a few reports are available that describe
49 sexual dimorphism in expression of kisspeptin and its receptors in various tissues of fishes^{4,5,7,11-13}.
50 Nonetheless, no report is available on sexual dimorphism in expression of *kiss* and *kissr* in thymus of
51 fishes even when *KISS1* is described as metastasis suppressor gene since discovery¹. Hence, in the current
52 study, sex-related differential expression of kisspeptin 1 and its receptors (*kiss1r*, *kiss2r*) was examined in
53 primary as well as secondary lymphoid organs of *Channa punctatus*. In addition to lymphoid tissues,
54 sexual dimorphism in expression of kisspeptin and its receptors was investigated in different parts of
55 brain, gonad and liver.

56 **Materials and methods**

57 *Animals and tissue collection*

58 Eight male and female *C. punctatus* were procured from wild population (freshwater bodies of National
59 Capital Region of Delhi, India) in the month of July when they are reported to be reproductively active¹⁴.
60 After a week of acclimation, they were sacrificed using an excessive dose of 2-phenoxyethanol in water (5
61 ml L⁻¹). Their brain and peripheral tissues namely spleen, thymus, head kidney, liver and one side gonad
62 were dissected out. In order to demonstrate region-specific expression of kisspeptin and its receptors in
63 brain, it was divided into anterior, middle and posterior parts as shown in Figure 1. The anterior part of

64 brain contains telencephalon, midpart includes diencephalon, optic tectum, hypothalamus and
65 mesencephalon, and posterior part consists of cerebellum and medulla oblongata^{15,16,17}. All the tissues
66 were stored at -80°C until RNA extraction. The opposite side gonads were processed for routine histology
67 to verify the reproductive state of fish. The Institutional Animal Ethics Committee of the Department of
68 Zoology, University of Delhi has approved the experimental protocol followed for the current study.

69 *RNA extraction, cDNA preparation and variation in gene expression*

70 Total RNA isolated from lymphoid organs (spleen, head kidney), liver, gonads and different parts of brain
71 using TRIzol (SIGMA, USA) was quantified and subjected to integrity validation. RNA samples with
72 optimal ratio of optical density ($A_{260/280}$) ranging between 1.8 and 2.0 were selected for cDNA
73 preparation. In brief, one microgram RNA, after DNase (Thermo Scientific, USA) treatment, was
74 processed for cDNA synthesis following manufacturer's protocol (Cat# K1622, Thermo Scientific, USA).
75 In order to identify the transcripts encoding kisspeptin and its receptors, polymerase chain reaction (PCR)
76 was conducted using gene-specific primers for *kiss1*, *kiss1r* and *kiss2r* (Table 1). The primers for different
77 genes were designed (GeneRunner Version 3.05, Hastings Software Inc.) from their conserved region
78 following multiple sequence alignment (CLUSTAL omega, Supplementary Figure 1). The PCR products
79 were sent for commercial sequencing. The obtained sequences were verified using BLASTn and
80 submitted to the NCBI (GenBank Accession Number: *kiss1* - MG637276, *kiss1r* - MG637277 and *kiss2r* -
81 MG637278). In order to examine tissue-wise variation in same sex or sex-related variation in same tissue
82 in level of *kiss1*, *kiss1r* and *kiss2r* expression following real-time quantitative PCR, the obtained
83 sequences from PCR products were used to design qPCR primers (Table 2) for respective genes. The melt
84 curve analysis was performed to validate the specificity of primers. A single peak was obtained, indicating
85 the existence of a single product. To reaffirm, the amplified product of each gene was resolved in 1%
86 agarose gel and a single band (Supplementary Figure 2) was visualized by staining with ethidium
87 bromide. For evaluating efficiency of qPCR primers, a standard curve was made using 2-fold serial
88 dilutions of ovarian cDNA. The percentage efficiency of qPCR primers is listed in Table 2. Also,

89 expression of *18S rRNA* was estimated in each sample as reference gene using specific primers (Table 2)
90 designed from its nucleotide sequence of *C. punctatus* available with NCBI (GenBank accession number
91 KX710184.1). The reaction was carried out using power SYBR Green (Cat# 4367659, Applied
92 Biosystems, USA) following manufacturer's protocol.

93 *Statistical analysis*

94 The relative expression of *kiss1*, *kiss1r* and *kiss2r* was calculated upon normalization with *18S rRNA*. Fold
95 change in expression of each gene was calculated using $2^{-\Delta\Delta C_T}$ method¹⁸ considering female as reference
96 to assess sex-related variation in a specific tissue. To demonstrate tissue-wise variation in expression of a
97 gene in the same sex, fold change was calculated where a tissue showing least expression was considered
98 as reference. Further, fold change of tissue-wise gene expression was log-transformed. Student's t-test was
99 employed to analyse sex-related marked ($p < 0.05$) difference in expression of a gene in a specific tissue
100 (male vs female). Data are shown as mean \pm standard error of mean. The correlation between *kiss1-kiss1r*
101 and *kiss1-kiss2r* was analyzed by Pearson's correlation test using $\Delta C_t (= C_t \text{ target gene} - C_t \text{ 18S rRNA})$ values.
102 Statistical analysis was carried out using GraphPad Prism 5 software (La Jolla, CA).

103 **Results and discussion**

104 In this study, partial sequences of *kiss1*, *kiss1r* and *kiss2r* comprising of 130, 715 and 538 base pairs
105 encoded predicted proteins of 32, 231 and 156 amino acids, respectively. The expression of *kiss1* and its
106 receptors *kiss1r/kiss2r* was observed in a wide variety of tissues, immensely in midbrain and gonads while
107 moderately in liver and immune organs (i.e., spleen and head kidney) of both male and female *C.*
108 *punctatus* (Figure 2 a, b). Tissue distribution of kisspeptin system in this study suggests its direct
109 involvement in control of gonadal functions, immunity and metabolism. Further, relative mRNA
110 expression of *kiss1*, *kiss1r* and *kiss2r* in different tissues of male when compared with that in respective
111 tissues of female *C. punctatus* revealed tissue-specific sex-related differential expression of kisspeptin
112 system (Figure 3 a-d).

113 *Tissue-specific sexual dimorphism in expression of kisspeptin and its receptors*

114 *Brain*

115 The profile of *kiss1*, *kiss1r* and *kiss2r* in different regions of brain of *C. punctatus* (Figure 2) is in
116 agreement with observations in other teleosts^{15,19-26} in which highest expression of kisspeptin and its
117 receptors has been shown in the midbrain. This is consistent with the facts that (i) major neuronal
118 population expressing kisspeptin and its receptors reside in the hypothalamus²⁷ that lies in the mid region
119 of fish brain²⁸ and (ii) kisspeptin stimulates GnRH-secreting hypothalamic neurons and consequently,
120 hypophyseal-gonadal axis in fishes¹¹, as in mammals²⁹. The expression of *kiss1*, *kiss1r* and *kiss2r* was also
121 observed in the anterior and posterior brain of *C. punctatus*. The results of current study are in
122 concordance with a report in *Carassius auratus*²⁰ where brain kisspeptin system has been proposed to act
123 as a neurotransmitter. Regarding sexually dimorphic expression of kisspeptin 1 and its receptors in
124 various regions of brain of *C. punctatus* (Figure 3 a), anterior and posterior regions exhibited sexual
125 dimorphism only for *kiss1* being considerably ($p < 0.05$) high in female and not for its receptors, *kiss1r*
126 and *kiss2r*. A high *kiss1* expression in anterior and posterior brain of female *C. punctatus* could be seen in
127 light of the reports that kisspeptin influences the secretion of brain neurotransmitters^{30,31} exhibiting sexual
128 dimorphism^{30,32,33}. The midbrain, however, exhibited sex-related differential expression for all the three
129 genes *kiss1*, *kiss1r* and *kiss2r*, with considerably ($p < 0.05$) higher level in male as compared to female.
130 Our results in midbrain are in consonance to a study in *Oryzias latipes* where greater number of kiss1-
131 expressing neurons is shown in hypothalamic nuclei of the male than female³⁴. On the contrary, no sex-
132 related difference in expression of *kiss* and *kissr* is reported in any region of brain of *Odontesthes*
133 *bonariensis*³⁵. Several other studies in fishes in which total brain has been used for sex-related differential
134 expression of kisspeptin system report inconsistent results, from no sex-related difference^{11,13,35-39} to high
135 expression in female^{4,7,11,12,20,39} or male¹³. The cause and physiological significance of sex-related
136 differential expression of kisspeptin 1 and its receptors in different regions of brain need to be explored to
137 address this dichotomy.

138 *Gonads*

139 In the current study, tissue-wise distribution showed a substantial expression of *kiss1*, *kiss1r* and *kiss2r* in
140 both, testis and ovary, of *C. punctatus* (Figure 2). Similar observations on gonadal expression of
141 kisspeptin and its receptors have been made in other teleosts^{4-6,15,40-42} and mammals⁴³⁻⁴⁷. Based on our
142 observation and other reports, it is obvious that kisspeptin, in addition to hypothalamo-hypophyseal
143 axis^{11,29}, directly regulates the gonadal functions. When sex-related differential expression was examined
144 in gonads, expression of *kiss1* was found to be significantly ($p < 0.05$) higher in testis than ovary of *C.*
145 *punctatus* (Figure 3 b). Our observation is in concordance with reports in *Danio rerio*¹⁵, *O. latipes*^{11,34} and
146 *C. auratus*^{20,41}, however, no sex-related difference in gonadal *kiss1* expression is seen in *Dicentrarchus*
147 *labrax*¹¹, *Scomber japonicas*⁴ and *Sebastes schlegeli*¹². Regarding kisspeptin receptors, expression of
148 *kiss1r* in gonads of *C. punctatus* did not show any sex-related difference though *kiss2r* was significantly
149 ($p < 0.05$) high in ovary than testis (Figure 3 b) which is contrary to its ligand *kiss1* expression. Unlike the
150 present study, mRNA levels of *kiss1r* in *Pimephales promelas*³⁸ and *D. rerio*¹⁵, *kiss2r* in *C. auratus*⁴¹ and
151 *Gobiocypris rarus*¹³, and both *kiss1r* and *kiss2r* in *S. japonicus*⁵ showed a higher expression in testis than
152 ovary. However, sexual dimorphism in expression of kisspeptin receptor has been reported to be absent in
153 gonads of *O. niloticus*³⁶, *D. labrax*¹¹, *Seriola lalandi*³⁷, *Anguilla anguilla*²¹ and *Cynoglossus semilaevis*⁷.
154 Surprisingly, reports on sexually dimorphic expression of kisspeptin and its receptors are lacking in
155 gonads of mammals.

156 *Liver*

157 The metabolic relevance of kisspeptin has emerged since its mRNA detection in liver of mice⁴³ and rat⁴⁸.
158 Kisspeptin has been proposed to be involved in glucose homeostasis⁴⁹ and protecting liver from oxidative
159 stress⁵⁰. The presence of kisspeptin system has been shown in liver of a number of teleosts though its
160 physiological significance has not been explored so far. In the current study, kisspeptin and its receptors
161 expression in liver of male and female *C. punctatus* revealed sex-related marked difference in mRNA
162 level of *kiss1* and *kiss2r*, but not *kiss1r* (Figure 3 c). The hepatic *kiss1* expression was markedly ($p < 0.05$)
163 high in female when compared to male. In contrast, hepatic *kiss2r* expression in female was considerably

164 ($p < 0.05$) lower than male *C. punctatus*. Nevertheless, studies in teleosts have shown no sexually
165 dimorphic expression of hepatic kisspeptin and its receptors^{7,11}, except *S. schlegeli*¹² in which male-
166 dominant kisspeptin receptor expression is reported in liver. To our knowledge, sex-related differential
167 expression of hepatic kisspeptin system has not been studied in mammals so far. Nevertheless, taken
168 together, we speculate a prime role of liver-derived kisspeptin in managing oxidative stress and energy
169 balance in fishes.

170 *Immune organs*

171 Regardless of the fact that *kiss* is a metastasis suppressor gene and mRNA for kisspeptin and its receptor
172 has been shown in spleen^{43,48} and thymus^{43,44,48} of mammals, its role in immunity has not been explored so
173 far. In fishes, several reports are available on expression of kisspeptin system in secondary lymphoid
174 organ, i.e. spleen^{5-7,11,12,15,20,21,35}. In case of primary lymphoid organs, a single study is available in head
175 kidney¹² while no such effort has been made for thymus of fishes. In the current study, tissue-wise
176 distribution of *kiss1*, *kiss1r* and *kiss2r* showed a comparatively low expression of these genes in primary
177 as well as secondary immune organs (Figure 2). Despite low level of expression, a marked sex-related
178 variation was noticed in transcript levels of *kiss1*, *kiss1r* and *kiss2r* in all the immune organs, spleen, head
179 kidney and thymus, of *C. punctatus* (Figure 3 d). The dimorphic expression pattern of *kiss1* was found to
180 be same in primary and secondary lymphoid organs, with considerably ($p < 0.05$) high mRNA levels in
181 female as compared to that of male *C. punctatus*. A relatively similar expression pattern was observed for
182 *kiss1r* and *kiss2r* in spleen and thymus of *C. punctatus*. Nonetheless, no sex-related marked difference in
183 expression of *kiss1r* and *kiss2r* was observed in its head kidney. Studies on sex-dependent expression of
184 kisspeptin system in immune organs are meagre in fishes and largely confined to spleen^{5,11,12}. The splenic
185 *kiss1* expression in *D. rerio*¹¹ and *kiss1r* in *S. japonicas*⁵ has been reported to be higher in males than
186 females, while no sex-related difference has been observed for kisspeptin and its receptor in *S. schlegeli*¹²
187 and *C. semilaevis*⁷. With regard to primary lymphoid organ, a single study is available in teleosts wherein
188 noticeable sexual dimorphism has been demonstrated only for *kiss1*, being higher in head kidney of male

189 *S. schlegelii*¹² than female and not for kisspeptin receptor. However, importance of sex differences in
190 expression of kisspeptin system in immune organs has not been explored till date.

191 *Correlation analysis*

192 The correlation analysis did not exhibit significant relationship between expression of *kiss1* and its
193 receptors *kiss1r/kiss2r* at 95% confidence interval in any tissue of *C. punctatus* (Figure 4). This is in
194 concordance with a recent report in another Perciformes *D. labrax*⁵¹ in which no significant correlation is
195 seen between hypothalamic expression of kisspeptin (*kiss1/kiss2*) and its receptors (*kiss1r/kiss2r*) during
196 advanced stages of oogenesis when their maximal expression level has been recorded. In contrast, direct
197 correlation between *kiss2* and *kiss1r* expression has been reported in pituitary and brain of *Takifugu*
198 *niphobles*⁵² during reproductively active spawning phase. Nonetheless, in general, even when significant
199 correlation between ligand and its receptor is not obvious, it is evidenced that maximal functional
200 responses of cells get altered with marked alteration in expression of either ligand or its receptor or both⁵³.

201 **Conclusion**

202 In addition to different parts of brain and gonads, sex-dependent expression of *kiss1*, *kiss1r* and *kiss2r* in
203 liver and immune organs paves the way for several novel aspects of possible involvement of kisspeptin in
204 regulation of peripheral functions in fish including metabolic activity and immunity. However, cause and
205 physiological significance of tissue-specific sex-dependent variations in expression of *kiss1*, *kiss1r* and
206 *kiss2r* needs to be explored in order to reach to a logical conclusion.

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396 **Table 1.** RT-PCR primers for *kiss1*, *kiss1r* and *kiss2r* in *Channa punctatus*

Gene	5'→3' primer sequence	Amplicon size (bp)	Primer name
<i>kiss1</i>	TGTCAACAGAGGTCTAC	130	K1FP
	GAGTTGAAGTTGTATGAGG		K1RP
<i>kiss1r</i>	GTCATCCATGTGGTCAC	715	K1rFP
	CCAGATGAAAGAAAGTG		K1rRP
<i>kiss2r</i>	TCCAAACACAGGCAGATGAG	538	K2rFP
	AGATCTGGATGGGACCCC		K2rRP

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398

Forward primer (FP), Reverse primer (RP)

399 **Table 2.** Real-time quantitative PCR primer sequences of *18S rRNA*, *kiss1*, *kiss1r* and *kiss2r* in

400

Channa punctatus

Gene	Primer name	5'→3' primer sequence	Amplicon size (bp)	Efficiency
<i>18S rRNA</i>	RT18FP	CTGAACTGGGGCCATGATT	100	100%
	RT18RP	CTTTCGCTTTCGTCCGTCT		
<i>kiss1</i>	RTK1FP	GAGATTTAAGTCATGCACC	101	108.8%
	RTK1RP	ACATTACCAGGAGACGA		
<i>kiss1r</i>	RTKr1FP	TTCACCGCCCACTTTAC	98	98.3%
	RTKr1RP	GACAGCTCAGGCAACATG		
<i>kiss2r</i>	RTKr2FP	TCGGCTCTTTTATCCTG	132	97.8%
	RTKr2RP	GGCTTTCATCCTCTACC		

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FP: forward primer, RP: reverse primer

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409 **Figure legends**

410 **Figure 1.** Dorsal view of the brain of *Channa punctatus*. Arrows indicate the precise site where cut
411 was given to excise the olfactory bulbs (**x**) and divide the brain into **a**, anterior, **b**, middle and **c**,
412 posterior parts. Image was captured with Nikon SMZ-1000 stereomicroscope using NIS-BR 3.1
413 software (magnification $\times 0.8$).

414 **Figure 2.** Tissue-wise expression of kisspeptin 1 (*kiss1*) and its receptors (*kiss1r* and *kiss2r*) in **a**,
415 male and **b**, female *C. punctatus*. The expression of each gene was quantified following real-time
416 quantitative PCR (qPCR). For each tissue, two technical replicates were used. The data is shown as
417 fold change in gene expression (Mean \pm SEM; N = 8 for each sex).

418 **Figure 3.** Sex-related variation in *kiss1*, *kiss1r* and *kiss2r* expression in **a**, various parts of brain, **b**,
419 gonads, **c**, liver and **d**, immune organs of adult *C. punctatus*. Asterisks (*) on bars denote significant
420 ($p < 0.05$) difference between individual gene expression in male and female.

421 **Figure 4.** Correlation curves representing relation between gene expression of ligand *kiss1* and its
422 receptors *kiss1r* and *kiss2r* in tissues of **a**, male and **b**, female *C. punctatus*. Values above the curve
423 shows Pearson's coefficient calculated using $\Delta C_t (= C_{t \text{ target gene}} - C_{t \text{ 18S rRNA}})$ values.

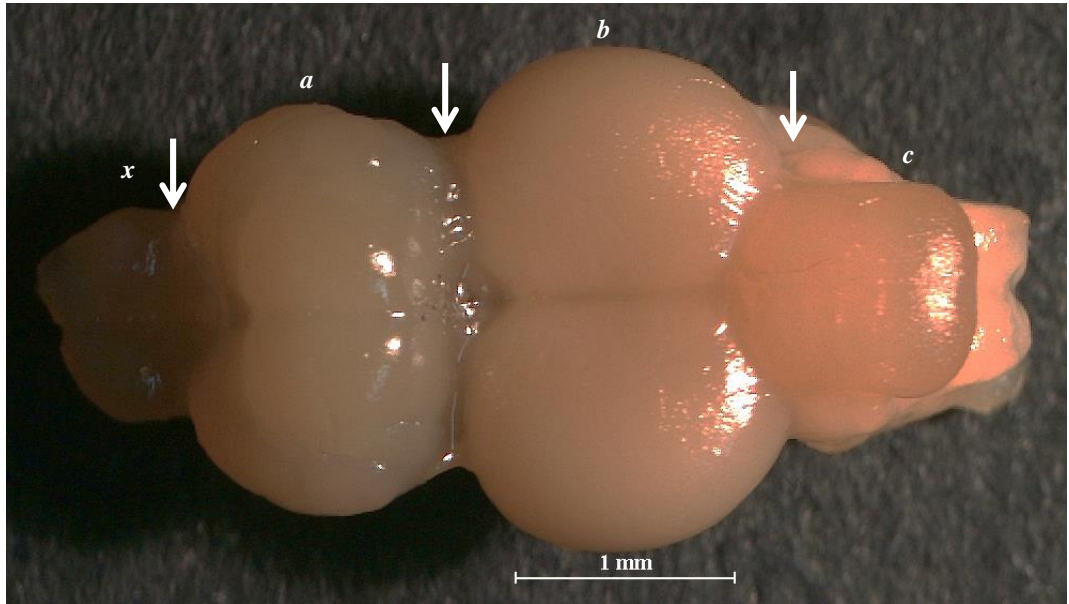


Figure 1

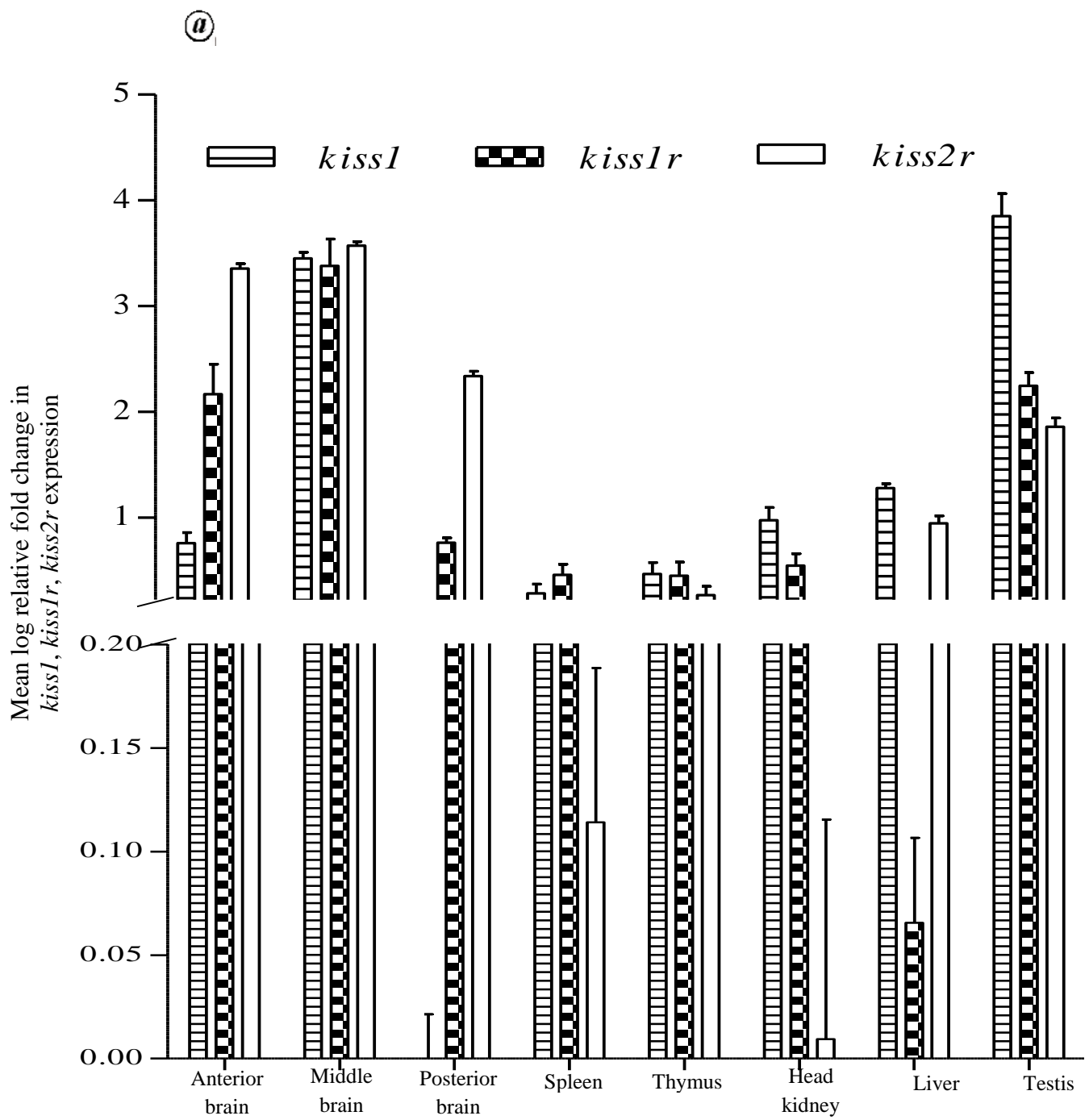


Figure 2 a

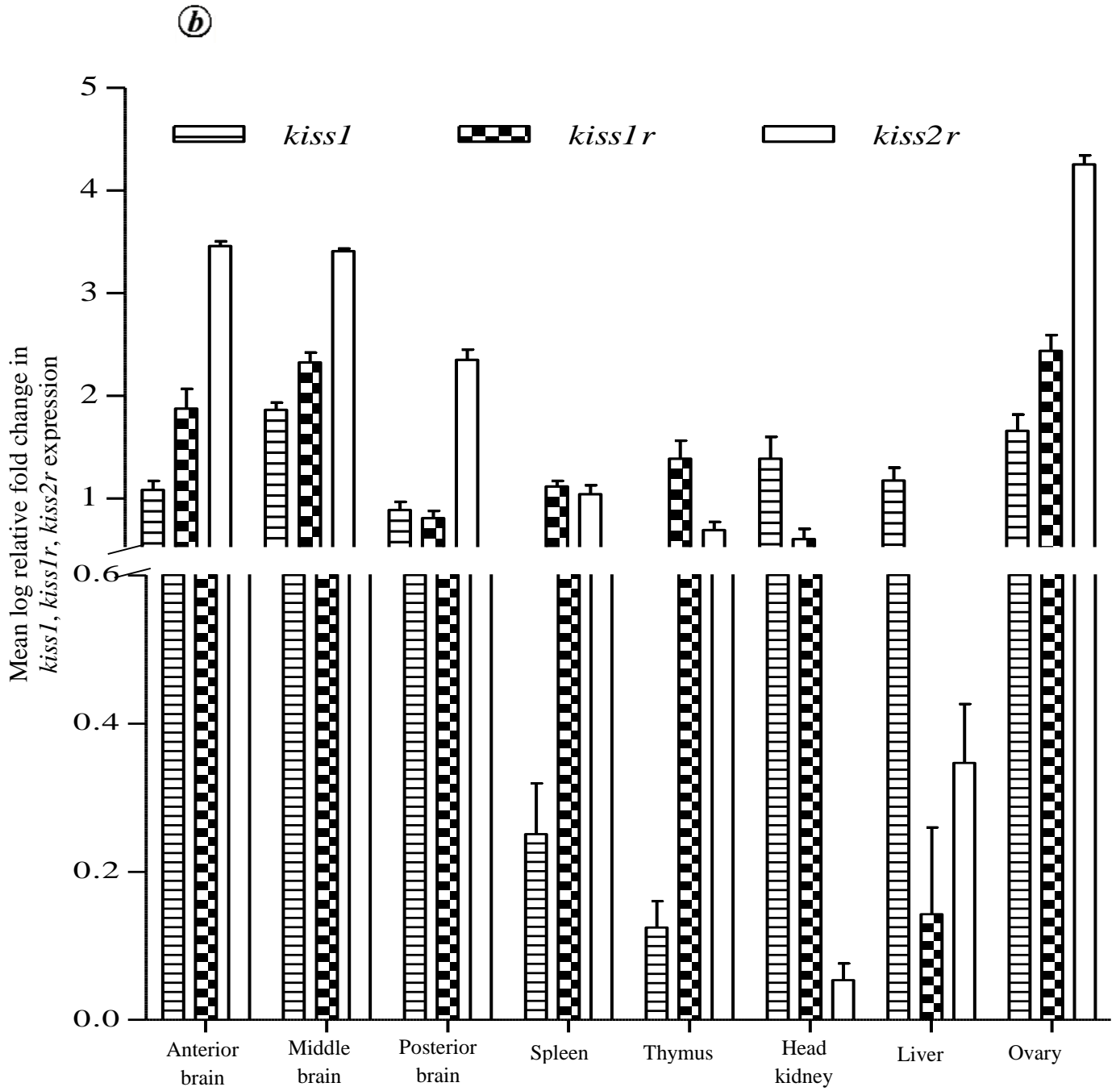


Figure 2 b

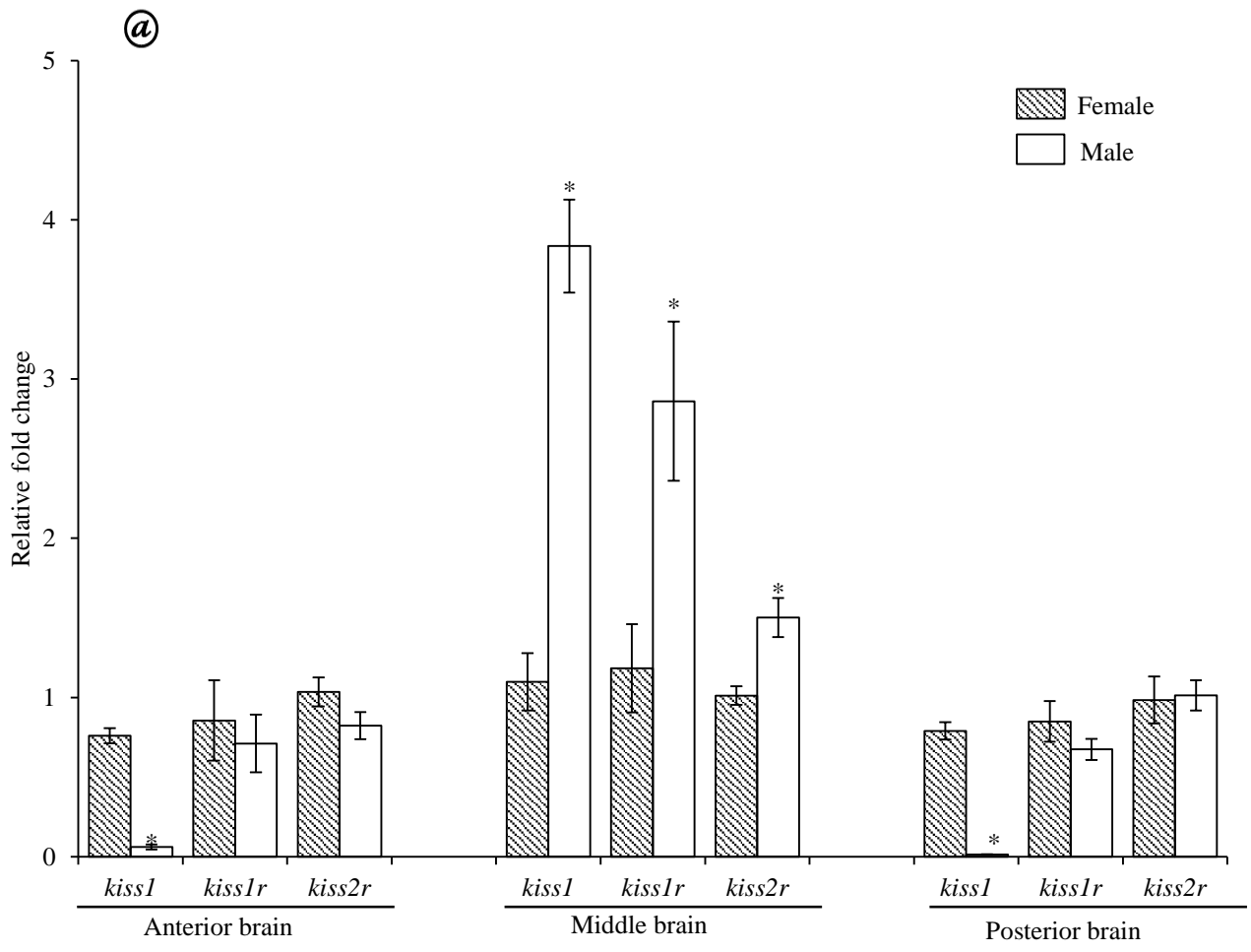


Figure 3 a

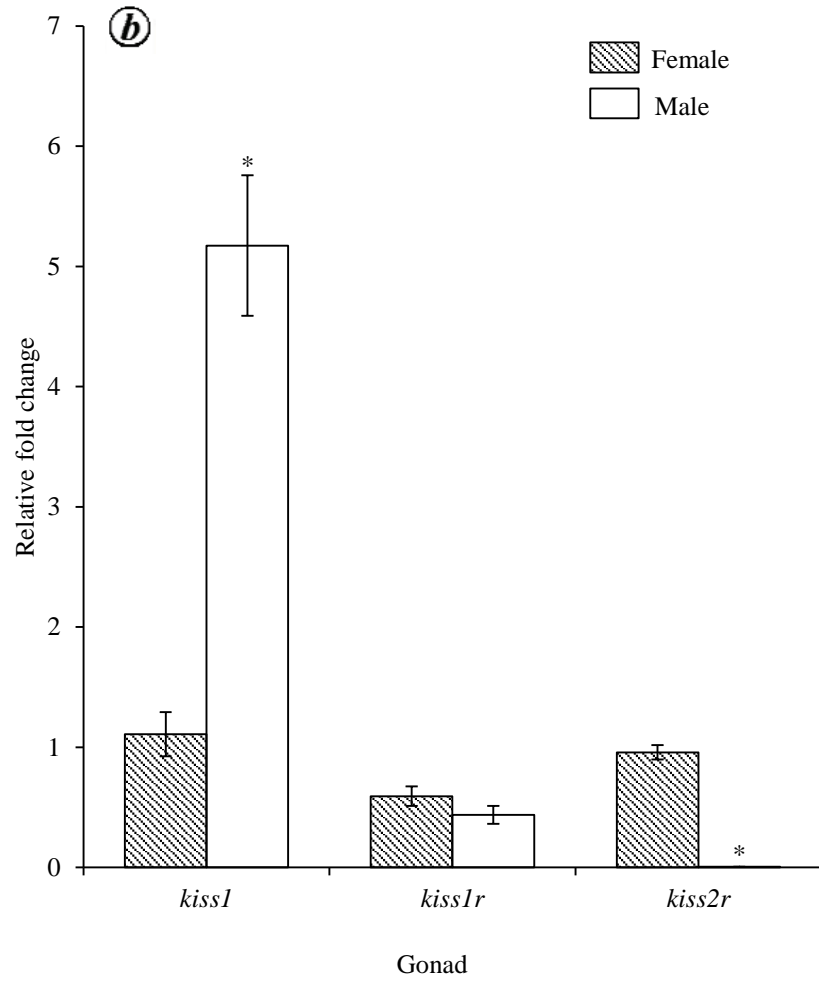


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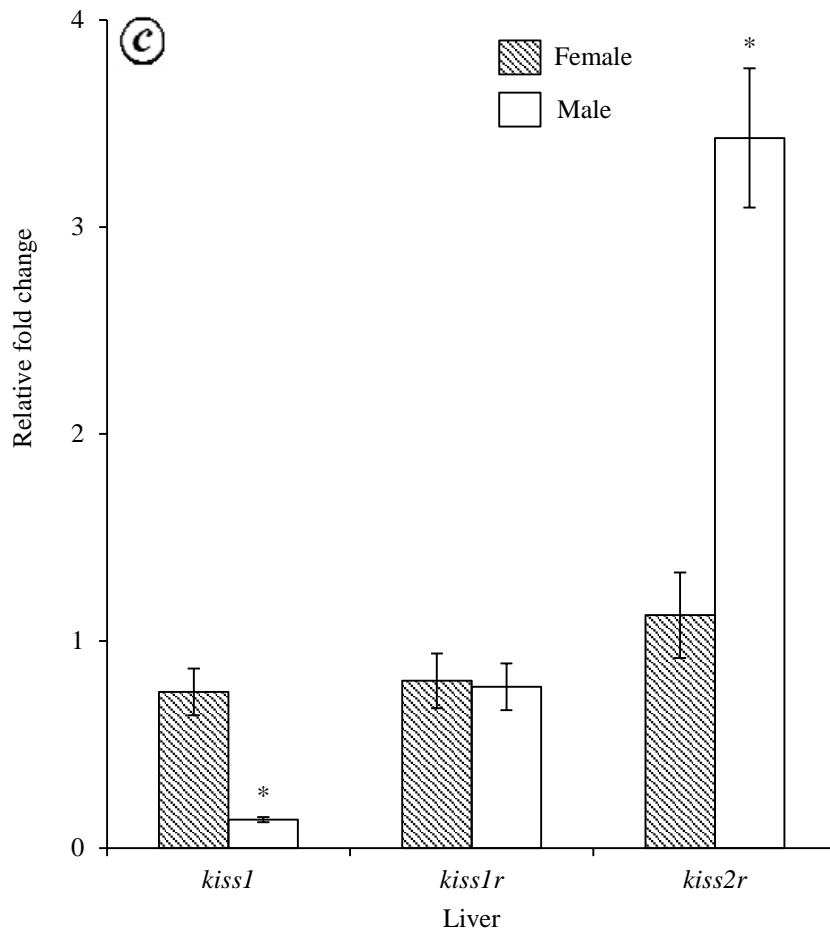


Figure 3 c

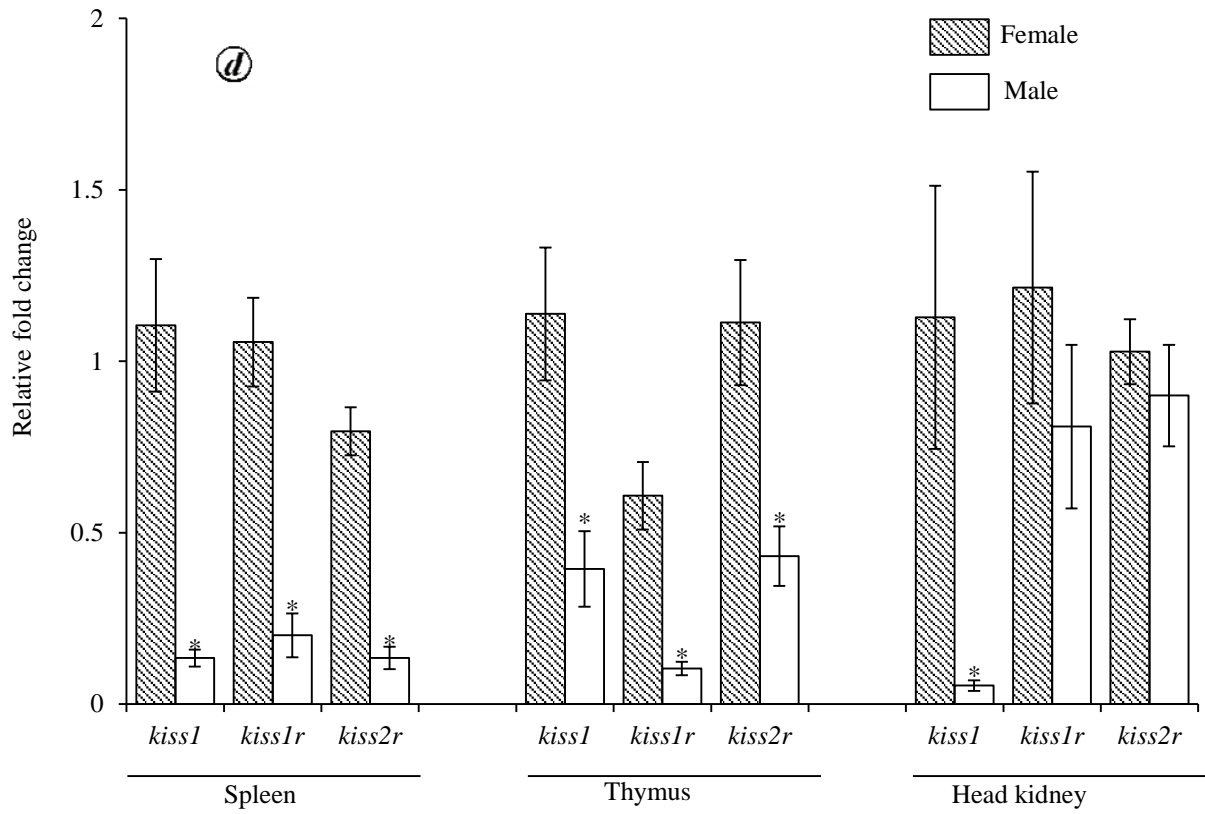


Figure 3 d

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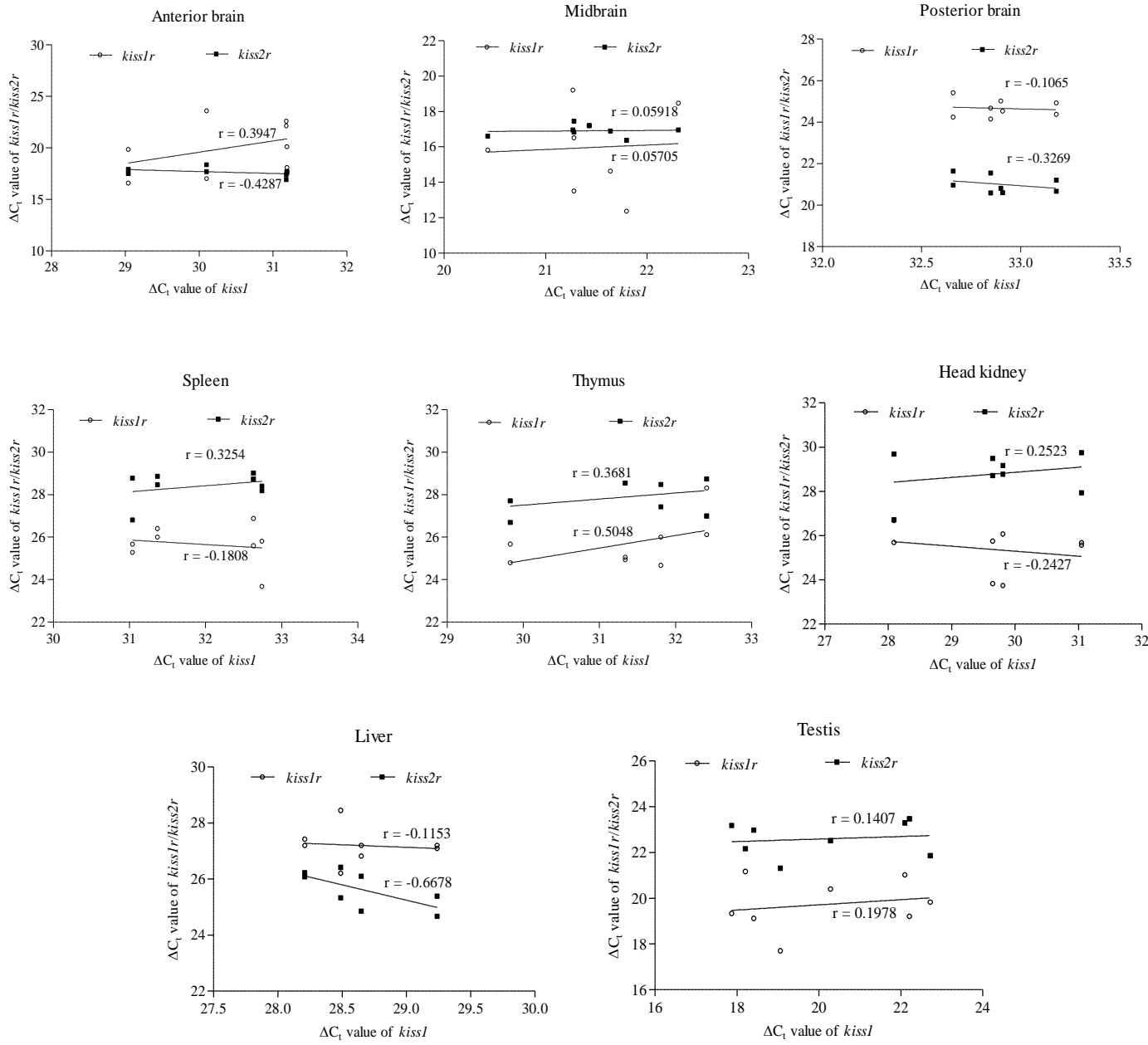


Figure 4 a

b

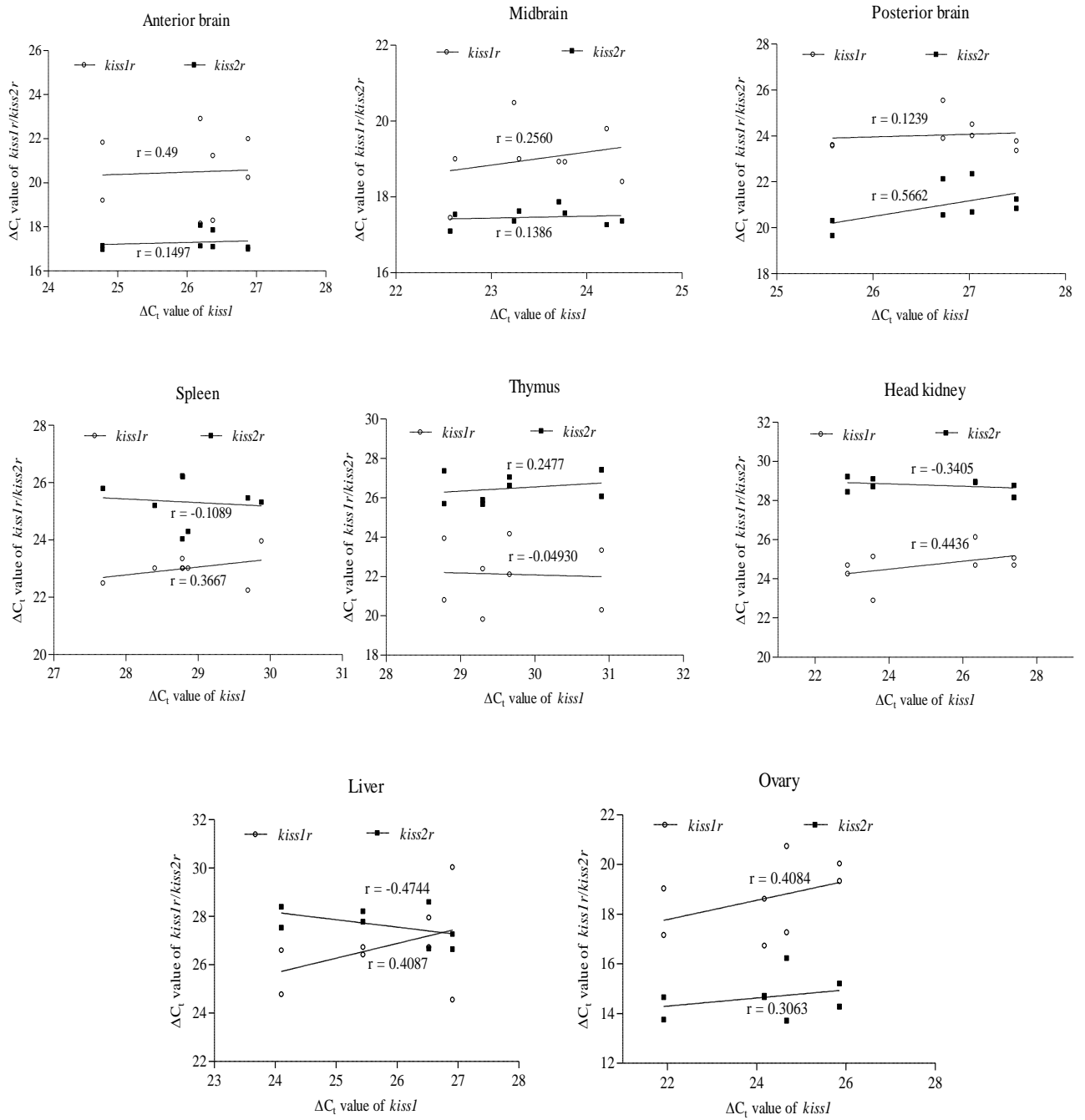


Figure 4 b

Supplementary file

Supplementary Figure 1. The nucleotide sequences of **a**, *kiss1*, **b**, *kiss1r* and **c**, *kiss2r* from different teleosts (*kiss1*: *Thunnus thynnus* JX459926, *Scomber japonicus* GU731672, *Seriola lalandi* HQ449729; *kiss1r*: *Scomber japonicus* JX982322, *Anoplopoma fimbria* KP677563, *Morone saxatilis* KJ725183, *Dicentrarchus labrax* JN202446; *kiss2r*: *Anoplopoma fimbria* KP677564, *Morone saxatilis* GU351869, *Dicentrarchus labrax* JN202447) were used for multiple sequence alignment to generate primers for RT-PCR of respective genes. The highlighted part of a gene sequence represents the primers used in the current study.

Supplementary Figure 2. Agarose gel electrophoresis images showing PCR products of **a**, *18S rRNA*, **b**, *kiss1*, **c**, *kiss1r* and **d**, *kiss2r* of *Channa punctatus* (Lane1: 100 bp DNA Ladder, Lane 2: Specific gene product, Lane 3: No template control).


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morone          ATCATCTACAGCTTCTGGCTGTCTACCTGCTGCCCTGCTCACCATCACCGCCTGTTAC
dicentrarchus  ATCATCTACAGCTTCTGGCCGTCTACCTGCTGCCCTGCTCACCATCACCGCCTGTTAC
*****

scomber        GCCTTCATGCTGAAGCGCATGGGCCAGACCAGCGTGAATCCCATGACAGTAGCTACCAA
anoplopoma     GCCTTCATGCTCAAGCATATGGGGCAAGCCAGCGTGAATCCTAGCGACGGCAGCTACCAA
morone         ACCTTCATGCTCAAGCGCATAGGGCAGCCAGCGTGAATCCCATCGACAGCAGCTACCAA
dicentrarchus  GCCTTCATGCTCAAGCGCATGGGGCAGCCAGCGTGAATCCCATCGACAGCAGCTACCAA
*****

scomber        CTTCAGGCTCAGGTAGAACGTGCAGCAGCAGTGCGGGCAAGAGTCTCTCGTATGGTGGTG
anoplopoma     CTCCAGGCTCAGGTGGAGCGAGCGCGCGGTGCGGGCGCGAGTCTCCCGGATGGTGGTG
morone         CTTCAGGCTCAGGCAGAGAGAGCAGCAGCAGTGCGGGCGCGGTCTCCCGGATGGTGGTG
dicentrarchus  CTTCAGGCTCAGGCAGAGAGAGCAGCAGCAGTGCGGGCGCGGTCTCCCGGATGGTGGTG
** ***** ** * ** * ** ***** * **** * *****

scomber        GTGATGGTGGCCCTGTTCTCATCTGCTGGGGCCCCATTTCAGGTCTGCATTCTGCTGCAA
anoplopoma     GTGATGGTGGCCCTGTTCTCATCTGCTGGGGCCCGATCCAGGTCTGCATCCTCCTGCAA
morone         GTGATGGTAGCTCTGTTCTCATCTGCTGGGGCCCCATCCAGGTCTGCATCCTCCTGCAG
dicentrarchus  GTGATGGTGGCTCTGTTCTCATCTGCTGGGGCCCCATCCAGGTCTGCATCCTCCTGCAG
***** ** ***** ***** ** ***** ** *****

scomber        ACGTTTGGCTTCCGCAGTTATGTTCTCTACAAGCTGAAGATTTGGGGTCACTGTATGTCT
anoplopoma     GCTTTTGGCCTCCGCAGTTACGTTCTATAACAAGCTGAGGATTTGGGGTCACTGCATGTCT
morone         GCTTTTGGCCTCCGCAGTTACGTTCTATAACAAGCTGAAGATTTGGGGTCACTGCATGTCT
dicentrarchus  GCTTTTGGCCTACGCAGTTACGTTCTATAACAAGCTGAAGATTTGGGGTCACTGCATGTCT
* ***** * ***** ***** ** ***** ***** ***** *****

scomber        TACTCCAACTCCTCTGTCAACCCGCTTGTATTATGCCTTCATGGGTAACAACCTTCAGAAAG
anoplopoma     TACTCCAACTCCTCTGTCAACCCGCTGGTTTACGCCTTCCTGGGCAACAACCTTCAGAAAG
morone         TACTCCAACTCCTCCATCAACCCGCTGGTTTACGCCTTCATGGGAAATAACTTCAGAAAG
dicentrarchus  TACTCCAACTCCTCCGTCAACCCACTGGTTTATGCCTTCATGGGAAACAACCTTCAGAAAG
***** ***** ** ***** ***** ***** ** ***** *****

scomber        GCCTTCAAGCAGCCTTCCCTGCCATGTTTCTGTGGCGTACACTGCGAAAAGTCAGGGTA
anoplopoma     GCCTTCAAGAATGCCTTCCCCCATATTTCTGTGGCGCATGAGGGGGAGAGTCAGGGTG
morone         GCCTTCAAGCAGCCTTCCCTGCCATATTTCTGTGGCGCACGAGGGGGAGAGTCAGGGTG
dicentrarchus  GCCTTCAAGCAGCCTTCCCTGCCATATTTCTGTGGCGCACGAGGAGAAGAGTCAGGGTG
***** * ***** ***** ***** * * * * * *****

scomber        GGCAACATGGACACGGAGGAAGGGGCAGAGATGGATCATCAGACACCCAAAGGAGAAGCA
anoplopoma     GGAGACATGGACGACAGGAACGGGGAGAGATGGAACGCCAGGCACCCAAAGGAGAAGCA
morone         GGAAACATGGATGCAGAGGAAGGGGGAGAGATGGATCGCCAGGCACCCAAAGGAGAAGCA
dicentrarchus  GGAAACATGGATGCAGAGGAAGGGGGAGAGATGGATCGCCAGGCACCCAAAGGAGAAGCA
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scomber        GAGATGCACTTTCTTTCATCTGGGTCCTAA
anoplopoma     GAGATGCACTTTCTTTCATCTGGGTCCTAA
morone         GAGATGCACTTTCTTTCATCTGGGTCCTAA
dicentrarchus  GAGATGCACTTTCTTTCATCTGGGTCCTAA
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Supplementary Figure 1 b

© *kiss2r* nucleotide sequence alignment

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Anoplopoma      ATGTACTCCTCCGAGGAGCTCTGGAACACCACCGAGCAGGTCTGGATCAACGGCTCCAAG
Morone          ATGTACTCCTCCGAGGAGCTCTGGAACACCACCGAGCAGGTCTGGATCAACGGCTCAGAG
Dicentrarchus  ATGTACTCCTCCGAGGAGCTCTGGAACACCACCGAGCAGGTCTGGATCAATGGCTCAGAG
*****
Anoplopoma      GCAAACCTTCTCTCTGCAAAGAC---ATGGAGATGATGAGGAGGAGGAAGAAGATCAGCAC
Morone          GCAAACCTTCTCTCTTGAAGAAGACGTGGAGACAATGAGGAGGAGGAAGGGGAACAACAC
Dicentrarchus  GCAAACCTTCTCTCTGGAAGAAGACGTGGAGACAATGAGGAGGAGGAAGGAGAACAGCAC
*****
Anoplopoma      CCCTTCCTCACAGATGCCTGGCTGGTCCCTCTCTTCTTCTCCCTCATAATGCTGGTCGGA
Morone          CCTTTCCTCACCGATGCCTGGCTGGTCCCTCTCTTCTTGGCCCTCATCATGCTGGTCGGA
Dicentrarchus  CCTTTCCTCACCGATGCCTGGCTGGTCCCTCTCTTCTTGGCCCTCATCATGCTGGTCGGA
** *****
Anoplopoma      CTGGTGGGCAACTCTCTGGTTATATATGTCATTTCCAAACACAGGCAGATGAGGACGGCA
Morone          CTGGTGGGCAACTCTCTGGTTATTTATGTAATTTCCAAACACAGGCAGATGAGAACAGCA
Dicentrarchus  CTGGTGGGCAACTCTCTGGTTATTTATGTAATTTCCAAACACAGGCAGATGAGAACAGCA
*****
Anoplopoma      ACCAACTTCTACATTGCAAACCTGGCTGCCACTGACATCATTTTTCTGGTGTGCTGCGTC
Morone          ACCAACTTCTACATAGCAAACCTGGCTGCGACTGACATCATCTTCTTGGTGTGCTGCGTC
Dicentrarchus  ACCAACTTCTACATAGCAAACCTGGCAGCGACTGACATCATCTTCTTGGTGTGCTGCGTC
*****
Anoplopoma      CCCTTCACTGCCACCCTCTATCCTCTCCCTGGATGGATCTTTGGCAACTTCATGTGCAAA
Morone          CCCTTCACTGCCACTCTCTATCCTCTCCCTGGATGGATCTTTGGCAACTTCATGTGCAAA
Dicentrarchus  CCCTTCACTGCAACTCTCTATCCTCTCCCTGGATGGATATTTGGCAACTTCATGTGCAAA
*****
Anoplopoma      TTTGTTGCCTTTCTACAGCAGGTGACAGTCCAAGCCACCTGCATCACTCTGACAGCTATG
Morone          TTTGTTGCCTTTCTACAGCAGGTGACAGTCCAAGCTACCTGTATCACTCTGACAGCTATG
Dicentrarchus  TTTGTTGCCTTTCTACAGCAGGTGACAGTCCAAGCCACCTGTATCACTCTGACAGCTATG
*****
Anoplopoma      AGCGGGGACCGATGTTACGTACAGTCTACCCCTCTGAAATCTCTCCGCCACCGTACCCCA
Morone          AGTGGTGACCGCTGTTACGTACAGTCTACCCCTCTGAAATCTCTACGCCACCGCACCCCG
Dicentrarchus  AGCGGTGACCGCTGTTACGTACAGTCTACCCCTGAAATCTCTCCGCCACCGCACTCCG
** *
Anoplopoma      AGAGTGCCATGATTGTGTCAGCATCTGCATTTGGATTGGCTCCTTCACTCTGTCTACCCCA
Morone          AAAGTAGCCATGATCGTCAGCATCTGCATTTGGATTGGCTCCTTCACTCTGTCCACCCCG
Dicentrarchus  AAAGTAGCCATGATTGTGTCAGCATCTGCATTTGGATTGGCTCCTTCACTCTGTCCACCCCG
* *
Anoplopoma      ATTTTAATGTACCAGCGTATAGAGGAGGGTTACTGGTACGGCCCGAGGCAGTACTGCATG
Morone          ATTTTAATGTACCAGCGTATAGAGGAGGGTTACTGGTACGGCCCGAGGCAGTACTGCATG
Dicentrarchus  ATTTTAATGTACCAGCGTATAGAGGAGGGTTACTGGTACGGCCCGAGGCAGTACTGCATG
*****
Anoplopoma      GAGAGATTCCCTCCTAAGACACACGAGAGGGCTTTTCATCCTCTATCAGTTTATTGCTGCC
Morone          GAGAGATTCCCTCCTAAGACACATGAGAGGGCTTTTCATCCTCTACCAGTTTATTGCTGCA
Dicentrarchus  GAGAGATTCCCTCCTAAGACACATGAGAGGGCTTTTCATCCTCTACCAGTTTATTGCTGCA
*****
Anoplopoma      TACCTGCTGCCTGTCTCACCATCTCCTTCTGCTACACTCTGATGGTGAAGAGGGTGGGC
Morone          TATCTGCTGCCTGTCTCACTATTTCTTCTGCTACACTCTGATGGTGAAGAGGGTGGGC
Dicentrarchus  TATCTGCTGCCTGTCTCACTATTTCTTCTGCTACACTCTGATGGTGAAGAGGGTGGGC
** *****
Anoplopoma      CAGCCCACTGTGGAGCCTGTAGACAACAACCTATCAGGTAAACCTCCTGTCTGAGAGAAC
Morone          CAACCCACTGTAGAGCCTGTAGACAACAACCTATCAGGTAAACCTCCTGTCTGAAAGAAC
Dicentrarchus  CAACCCACTGTAGAGCCTGTAGACAACAACCTATCAGGTAAACCTCCTGTCTGAAAGAAC
** *****

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Anoplopoma ATCAGTATCAGGAGTAAAGTCTCCAAGATGGTGGTAGTAATCGTCCTCCTCTTCGCCATA
Morone ATCAGTATCAGGAGCAAAGTCTCCAAGATGGTGGTAGTAATCGTCCTCCTCTTCGCTGTC
Dicentrarchus ATCAGTATCAGGAGCAAAGTCTCCAAGATGGTGGTAGTAATCGTTCCTCCTCTTCGCTGTC

Anoplopoma TGCTGGGGACCCATCCAGATCTTCATCCTATTCCAGTCTTTCTACCCAACTATCAGCAC
Morone TGCCGGGGTCCCATCCAGATCTTCGCTCTCTTTTCAGTCTTTCTACCCAACTACCGGCC
Dicentrarchus TGCTGGGGTCCCATCCAGATCTTCGCTCTCTTTTCAGTCTTTCTACCCAACTACCGGCC
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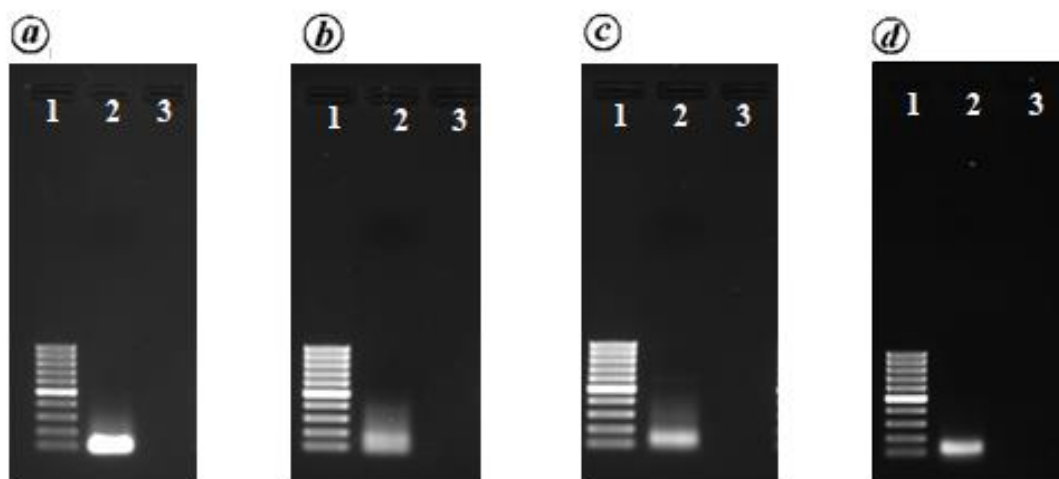
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Dicentrarchus AACTACGCCACATACAAGATCAAGACGTGGGCCAACTGCATGTCCTACGCCAACTCTTCT
** **

Anoplopoma GTCAACCCCATAGTTTACGGATTTCATGGGAGCCACCTTCCAAAAGTCCTTCAAGAAGACC
Morone GTCAACCCCATAGTTTACGGTTTTCATGGGAGCCACTTTCAAAAGTCCTTCAAGAAAACC
Dicentrarchus GTCAACCCCATAGTTTATGGTTTTCATGGGAGCCACTTTCAAAAGTCCTTCAAGAAAACC

Anoplopoma TTCCCGTTTCTGTTCAAGCACAAAGTTCAGAGATAGCAGCATGGCTTCCAGGACTGCCAAC
Morone TTCCCATTTCTGTTCAAGCACAAAGTTCAGAGATAGCAGCATGGCTTCCAGGACTGCCAAC
Dicentrarchus TTCCCATTTCTGTTCAAGCACAAAGTTCAGAGATAGCAGCATGGCTTCCAGGACTGCCAAC

Anoplopoma GCCGAGATCAAGTTTGTGCTGCGGAGGGAGGCAACAATAACAATGCAACAACCTGA
Morone GCTGAGATAAAGTTTGTGCTGCGAGGAAGGCAACAATAACAATGCAATGAATTGA
Dicentrarchus GCTGAGATAAAGTTTGTGCTGCGGAGGAAGGCAACAATAACAATGCAATGAATTGA
** **

Supplementary Figure 1 c



Supplementary Figure 2

