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

Amrita Bakshi and Umesh Rai*

Department of Zoology, University of Delhi, Delhi 110 007, India

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

Keywords: *Channa punctatus*, kisspeptin receptors, sexual dimorphism, teleost.

KISSPEPTIN (*KISS1*) discovered as a candidate gene for the suppression of melanoma metastasis[1], encodes *kisspeptin* (*KISS1*) that belongs to RF-amide family of neuropeptides. *KISS1* has been reported to act through serpentine transmembrane receptor, GPR54/KISS1R[2]. The existence of *kisspeptin* receptor in fishes was first evidenced in *Oreochromis niloticus* from which cDNA encoding GPR54 was isolated[3]. Thereafter, multiple forms of *kisspeptin* (*kiss1*, *kiss2*) and its receptors (*kiss1r*, *kiss2r*) have been demonstrated in a number of teleosts[4–8]. *Kisspeptin 1* and 2 have been shown to activate both the receptors, though with different potencies[9]. For several years, kisspeptin has been known as the prime neuroendocrine regulator of reproduction in mammals[10] and fishes[8] as well. Moreover, only a few reports are available that describe sexual dimorphism in the expression of *kisspeptin* and its receptors in various tissues of fishes[4,5,7,11–13]. However, no report is available on sexual dimorphism in the expression of *kiss* and *kissr* in the thymus of fishes, even when *KISS1* has been described as a metastasis suppressor gene since its discovery[1]. Hence, in the present study, sex-related differential expression of *kisspeptin 1* and its receptors (*kiss1r*, *kiss2r*) was examined in primary as well as secondary lymphoid organs of *Channa punctatus*. In addition to lymphoid tissues, sexual dimorphism in the expression of *kisspeptin* and its receptors was studied in different parts of the brain, gonad and liver.

*For correspondence. (e-mail: rai_u@rediffmail.com)

Materials and methods

Animals and tissue collection

Eight male and female *C. punctatus* were procured from wild population (freshwater bodies of National Capital Region of Delhi, India) in July, when they are reported to be reproductively active[14]. After a week of acclimation, they were sacrificed using an excessive dose of 2-phenoxyethanol in water (5 ml l^{-1}). Their brain and peripheral tissues, namely spleen, thymus, head kidney, liver and one side gonad were dissected out. In order to demonstrate region-specific expression of *kisspeptin* and its receptors in the brain, it was divided into anterior, middle and posterior parts (Figure 1). The anterior part of the brain contains telencephalon, the midpart includes diencephalon, optic tectum, hypothalamus and mesencephalon, while the posterior part consists of cerebellum and medulla oblongata[15–17]. All the tissues were stored at –80°C until RNA extraction. The opposite side gonads were processed for routine histology to verify the reproductive state of fish. The Institutional Animal Ethics

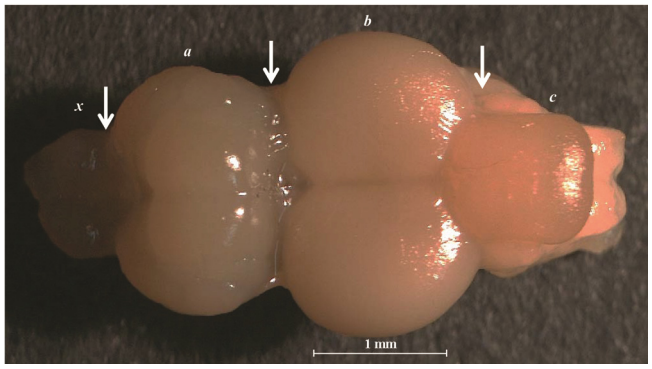

Figure 1. Dorsal view of the brain of *Channa punctatus*. Arrows indicate the precise site of cut to excise the olfactory bulbs (*x*) and divide the brain into (***a***) anterior, (***b***) middle and (***c***) posterior parts. Image was captured with Nikon SMZ-1000 stereomicroscope using NIS-BR 3.1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

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

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

FP, Forward primer; RP, Reverse primer.

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

Gene	Primer	5′→3′ primer sequence	Amplicon size (bp)	Efficiency (%)
18S rRNA	RT18FP	CTGAACTGGGGCCATGATT	100	100
	RT18RP	CTTTCGCTTTCGTCCGTCT		
kiss1	RTK1FP	GAGATTTAAGTCATGCACC	101	108.8
	RTK1RP	ACATTTACCAGGAGACGA		
kiss1r	RTKr1FP	TTCACCGCCACACTTTAC	98	98.3
	RTKr1RP	GACAGCTCAGGCAACATG		
kiss2r	RTKr2FP	TCGGCTCTTTTATCCTG	132	97.8
	RTKr2RP	GGCTTTCATCCTCTACC		

FP, Forward primer; RP, Reverse primer.

Committee of the Department of Zoology, University of Delhi has approved the experimental protocol followed in the present study.

RNA extraction, cDNA preparation and variation in gene expression

Total RNA isolated from lymphoid organs (spleen, head kidney, thymus), liver, gonads and different parts of the brain using TRIzol (Sigma, USA) was quantified and subjected to integrity validation. RNA samples with optimal ratio of optical density ($A_{260/280}$) ranging between 1.8 and 2.0 were selected for cDNA preparation. In brief, 1 µg RNA, after DNAase (Thermo Scientific, USA) treatment, was processed for cDNA synthesis following the manufacturer's protocol (Cat# K1622, Thermo Scientific, USA). In order to identify the transcripts encoding *kiss-peptin* and its receptors, polymerase chain reaction (PCR) was performed using gene-specific primers for *kiss1*, *kiss1r* and *kiss2r* (Table 1). The primers for different genes were designed (GeneRunner Version 3.05, Hastings Software Inc., Hastings, New York, USA) from their conserved regions following multiple sequence alignment (Clustal Omega, Figure S1). The PCR products were sent for commercial sequencing. The obtained sequences were verified using BLASTn and submitted to NCBI (GenBank accession number: *kiss1* – MG637276, *kiss1r* – MG637277 and *kiss2r* – MG637278). In order to examine tissue-wise variation in the same sex or sex-related variation in the same tissue in the expression level of *kiss1*, *kiss1r* and *kiss2r* following real-time quantitative PCR (qPCR), the obtained sequences from PCR products were used to design qPCR primers (Table 2) for the respective genes. The melt curve analysis was performed to validate the specificity of primers. A single peak was obtained, indicating the existence of a single product. To reaffirm, the amplified product of each gene was resolved in 1% agarose gel and a single band (Figure S2) was visualized by staining with ethidium bromide. For evaluating the efficiency of qPCR primers, a standard curve was made using two-fold serial dilutions of ovarian cDNA. Table 2 enlists the percentage efficiency of qPCR primers. Also, expression of *18S rRNA* was estimated in each sample as reference gene using specific primers (Table 2) designed from the nucleotide sequence of *C. punctatus* available with NCBI (GenBank accession number KX710184.1). The reaction was carried out using power SYBR Green (Cat# 4367659, Applied Biosystems, USA) following the manufacturer's protocol.

Statistical analysis

The relative expression of *kiss1*, *kiss1r* and *kiss2r* was calculated upon normalization with *18S rRNA*. Fold change in expression of each gene was calculated using $2^{-\Delta\Delta C}{}_{T}$ method[18], considering female as reference to

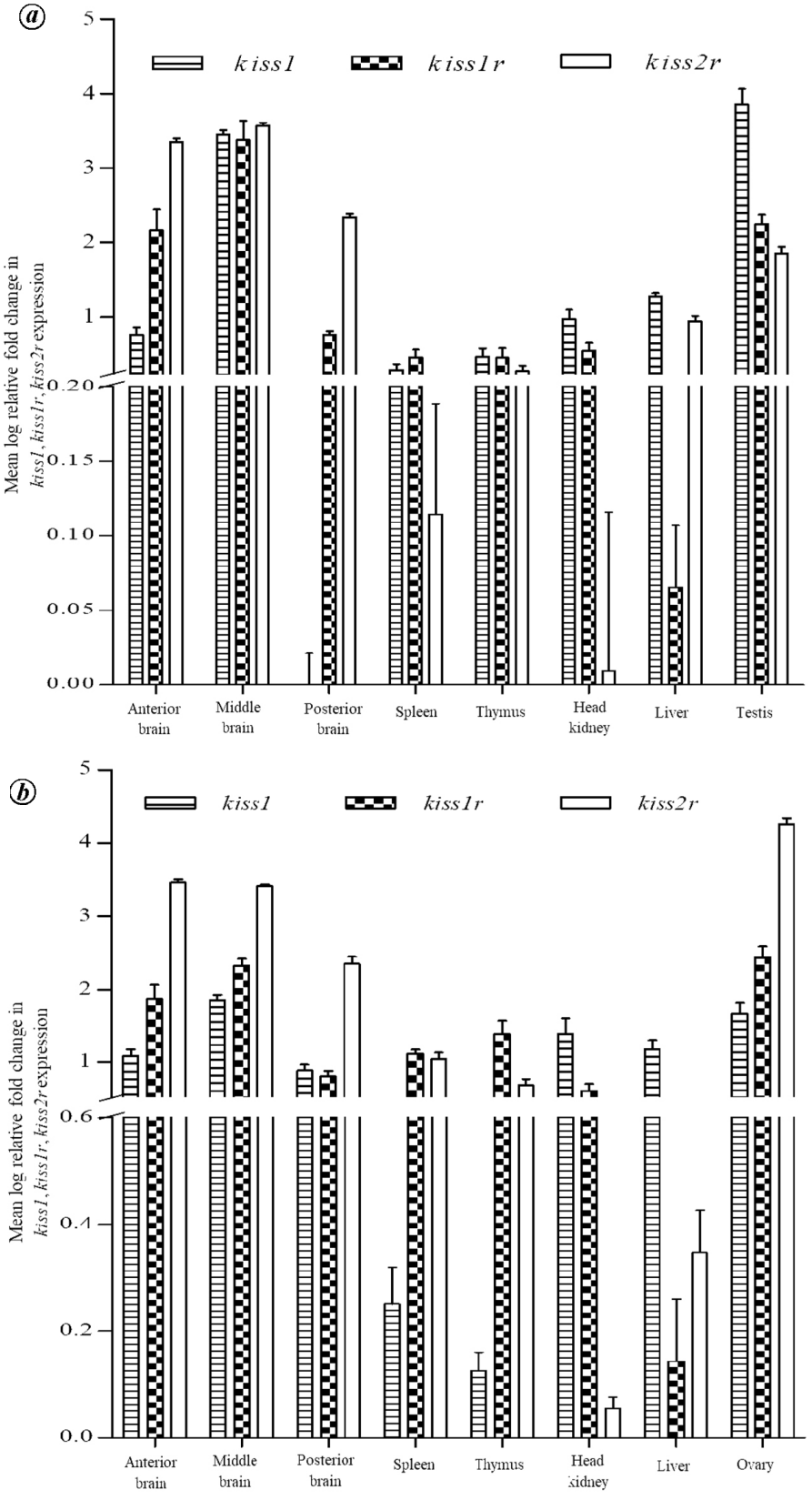

Figure 2. Tissue-wise expression of *kisspeptin 1* (*kiss1*) and its receptors (*kiss1r* and *kiss2r*) in (***a***) male and (***b***) female *C. punctatus*. The expression of each gene was quantified following real-time quantitative PCR (qPCR). For each tissue, two technical replicates were used. Data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

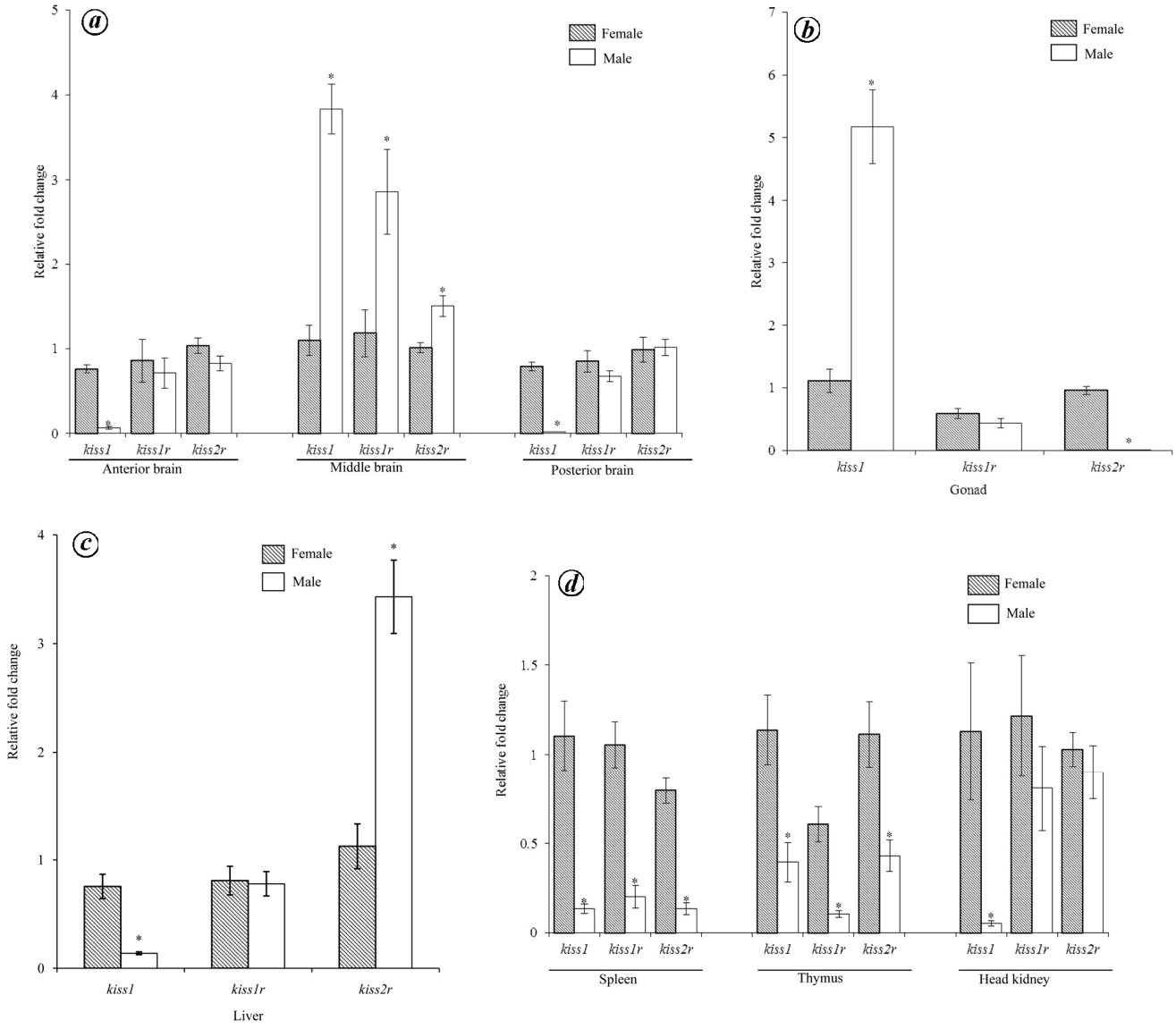

Figure 3. Sex-related variation in *kiss1*, *kiss1r* and *kiss2r* expression in (***a***) various parts of the brain, (***b***) gonads, (***c***) liver and (***d***) immune organs of adult *C. punctatus*. Asterisks (*) on bars denote significant ($P < 0.05$) difference between individual gene expressions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

assess sex-related variation in a specific tissue. To demonstrate tissue-wise variation in the expression of a gene in the same sex, fold change was calculated where a tissue showing least expression was considered as reference. Further, fold change of tissue-wise gene expression was log-transformed. Student's *t*-test was employed to analyse sex-related marked ($P < 0.05$) difference in the expression of a gene in a specific tissue (male versus female). Data are shown as mean ± standard error of mean (SEM). The correlation between *kiss1* and *kiss1r* as well as *kiss1* and *kiss2r* was analysed by Pearson's correlation test using ΔC_t ($=C_{t\ \text{target gene}} - C_{t\ 18S\ rRNA}$) values. Statistical analysis was carried out using GraphPad Prism 5 software (La Jolla, CA).

Results and discussion

In this study, partial sequences of *kiss1*, *kiss1r* and *kiss2r* comprising of 130, 715 and 538 base pairs encoded predicted proteins of 32, 231 and 156 amino acids respectively. The expression level of *kiss1* and its receptors *kiss1r*/*kiss2r* was observed in a wide variety of tissues; it was high in the midbrain and gonads while moderate in the liver and immune organs (i.e. spleen and head kidney) of both male and female *C. punctatus* (Figure 2 *a* and *b*). Tissue distribution of *kisspeptin* system in this study suggests its direct involvement in the control of gonadal functions, immunity and metabolism. Further, relative mRNA expression of *kiss1*, *kiss1r* and *kiss2r* in different

tissues of male when compared to female *C. punctatus* revealed tissue-specific sex-related differential expression of *kisspeptin* system (Figure 3 *a–d*).

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

Brain: The profile of *kiss1*, *kiss1r* and *kiss2r* in different regions of the brain of *C. punctatus* (Figure 2) is in agreement with observations in other teleosts[15,19–26], in which the highest expression of kisspeptin and its receptors has been shown in the midbrain. This is consistent with the facts that: (i) major neuronal populations expressing *kisspeptin* and its receptors reside in the hypothalamus[27] that lies in the mid region of fish brain[28] and (ii) *kisspeptin* stimulates GnRH-secreting hypothalamic neurons and consequently, hypophyseal–gonadal axis in fishes[11], as in mammals[29]. The expression of *kiss1*, *kiss1r* and *kiss2r* was also observed in the anterior and posterior brain of *C. punctatus*. The results of the present study are in concordance with a report in *Carassius auratus*[20], where brain *kisspeptin* system has been proposed to act as a neurotransmitter. Regarding sexually dimorphic expression of *kisspeptin 1* and its receptors in various regions of the brain of *C. punctatus* (Figure 3 *a*), the anterior and posterior regions exhibited sexual dimorphism only for *kiss1* being considerably ($P < 0.05$) high in female and not for its receptors *kiss1r* and *kiss2r*. A high *kiss1* expression in the anterior and posterior brain of female *C. punctatus* could be seen in light of the reports that *kisspeptin* influences the secretion of brain neurotransmitters[30,31], exhibiting sexual dimorphism[30,32,33]. The midbrain, however, exhibited sex-related differential expression for all the three genes *kiss1*, *kiss1r* and *kiss2r*, with considerably ($P < 0.05$) higher level in male compared to female. Our results for the midbrain are in consonance with a study in *Oryzias latipes*, where greater number of *kiss1*-expressing neurons is shown in hypothalamic nuclei of male than female[34]. On the contrary, no sex-related difference in expression of *kiss* and *kissr* was reported in any region of the brain of *Odontesthes bonariensis*[35]. Several other studies in fishes in which total brain has been used for sex-related differential expression of *kisspeptin* system reported inconsistent results, from no sex-related difference[11,13,35–39] to high expression in female[4,7,11,12,20,39] or male[13]. The cause and physiological significance of sex-related differential expression of the *kisspeptin 1* and its receptors in different regions of brain need to be explored to address this dichotomy.

Gonads: In the present study, tissue-wise distribution showed substantial expression of *kiss1*, *kiss1r* and *kiss2r* in both testis and ovary of *C. punctatus* (Figure 2). Similar observations on gonadal expression of *kisspeptin* and its receptors have been made in other teleosts[4–6,15,40–42] and mammals[43–47]. Based on our observations and other reports, it is obvious that *kisspeptin*, in addition to the hypothalamo-hypophyseal axis[11,29], directly regulates gonadal functions. When sex-related differential expression was examined in the gonads, expression of *kiss1* was found to be significantly ($P < 0.05$) higher in testis than ovary of *C. punctatus* (Figure 3 *b*). Our observation is in concordance with reports in *Danio rerio*[15], *O. latipes*[11,34] and *C. auratus*[20,41]; however, no sex-related difference in gonadal *kiss1* expression was seen in *Dicentrarchus labrax*[11], *Scomber japonicas*[4] and *Sebastes schlegeli*[12]. Regarding *kisspeptin* receptors, expression of *kiss1r* in the gonads of *C. punctatus* did not show any sex-related difference though *kiss2r* was significantly ($P < 0.05$) high in ovary than testis (Figure 3 *b*), which is contrary to its ligand *kiss1* expression. Unlike the present study, mRNA levels of *kiss1r* in *Pimephales promelas*[38] and *D. rerio*[15], *kiss2r* in *C. auratus*[41] and *Gobiocypris rarus*[13], and both *kiss1r* and *kiss2r* in *S. japonicus*[5] showed higher expression in testis than ovary. However, sexual dimorphism in the expression of *kisspeptin* receptor has been reported to be absent in the gonads of *O. niloticus*[36], *D. labrax*[11], *Seriola lalandi*[37], *Anguilla anguilla*[21] and *Cynoglossus semilaevis*[7]. Surprisingly, reports on sexually dimorphic expression of *kisspeptin* and its receptors are lacking in the gonads of mammals.

Liver: The metabolic relevance of *kisspeptin* has emerged since its mRNA detection in the liver of mice[43] and rats[48]. *Kisspeptin* has been proposed to be involved in glucose homeostasis[49] and protecting the liver from oxidative stress[50]. The presence of *kisspeptin* system has been shown in liver of a number of teleosts though its physiological significance has not been explored so far. In the present study, the expression of *kisspeptin* and its receptors in the liver of male and female *C. punctatus* revealed sex-related marked difference in mRNA level of *kiss1* and *kiss2r* but not *kiss1r* (Figure 3 *c*). The hepatic *kiss1* expression was markedly ($P < 0.05$) high in female when compared to male. In contrast, hepatic *kiss2r* expression in female was considerably ($P < 0.05$) lower than male *C. punctatus*. Nevertheless, studies in teleosts have shown no sexually dimorphic expression of hepatic *kisspeptin* and its receptors[7,11], except *S. schlegeli*[12] in which male-dominant kisspeptin receptor expression was reported in the liver. To our knowledge, sex-related differential expression of hepatic *kisspeptin* system has not been studied in mammals so far. Nevertheless, taken together, we speculate a prime role of liver-derived *kisspeptin* in managing oxidative stress and energy balance in fishes.

Immune organs: Regardless of the fact that *kiss* is a metastasis suppressor gene, and mRNA for *kisspeptin* and its receptor has been shown in the spleen[43,48] and thymus[43,44,48] of mammals, its role in immunity has not been explored so far. In fishes, several reports are available

on the expression of *kisspeptin* system in secondary lymphoid organs, i.e. spleen[5–7,11,12,15,20,21,35]. In case of primary lymphoid organs, a single study is available in head kidney[12], while no such effort has been made for thymus of fishes. In the present study, tissue-wise distribution of *kiss1*, *kiss1r* and *kiss2r* showed a comparatively low expression of these genes in primary as well as secondary immune organs (Figure 2). Despite low level of expression, a marked sex-related variation was noticed in transcript levels of *kiss1*, *kiss1r* and *kiss2r* in all the immune organs, spleen, head kidney and thymus of *C. punctatus* (Figure 3 *d*). The dimorphic expression pattern of *kiss1* was found to be the same in primary and secondary lymphoid organs, with considerably ($P < 0.05$) high mRNA levels in female compared to male *C. punctatus*. A relatively similar expression pattern was observed for *kiss1r* and *kiss2r* in spleen and thymus of *C. punctatus*. No sex-related marked difference in the expression of *kiss1r* and *kiss2r* was observed in its head kidney. Studies on sex-dependent expression of *kisspeptin* system in immune organs are meagre in fishes and largely confined to the spleen[5,11,12]. The splenic *kiss1* expression in *D. rerio*[11] and *kiss1r* in *S. japonicas*[5] has been reported to be higher in male than female, while no sex-related difference has been observed for *kisspeptin* and its receptors in *S. schlegeli*[12] and *C. semilaevis*[7]. With regard to primary lymphoid organ, a single study is available in teleosts wherein noticeable sexual dimorphism has been demonstrated only for *kiss1*, being higher in head kidney of male *S. schlegeli*[12] than female and not for kisspeptin receptor. However, the importance of sex differences in the expression of *kisspeptin* system in immune organs has not been explored till date.

Correlation analysis: The correlation analysis did not exhibit significant relationship between expression of *kiss1* and its receptors *kiss1r*/*kiss2r* at 95% confidence interval in any tissue of *C. punctatus* (Figure 4). This is

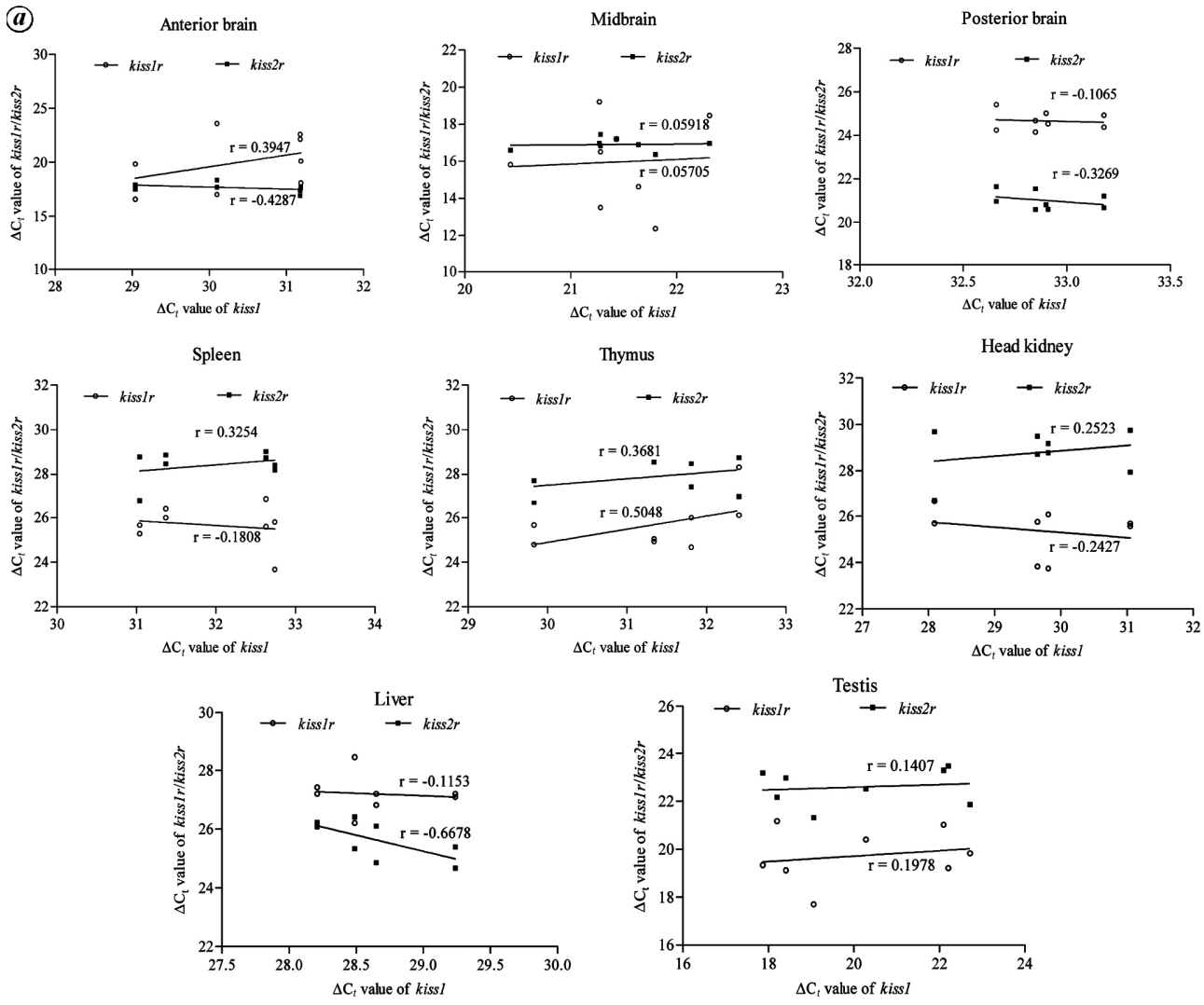

Figure 4 *a*. Correlation curves representing relation between gene expression of ligand *kiss1* and its receptors *kiss1r* and *kiss2r* in tissues of male *C. punctatus*. Values above the curve shows Pearson's coefficient calculated using ΔC_t (= $C_{t\ \text{target gene}} - C_{t\ 18S\ rRNA}$) values.

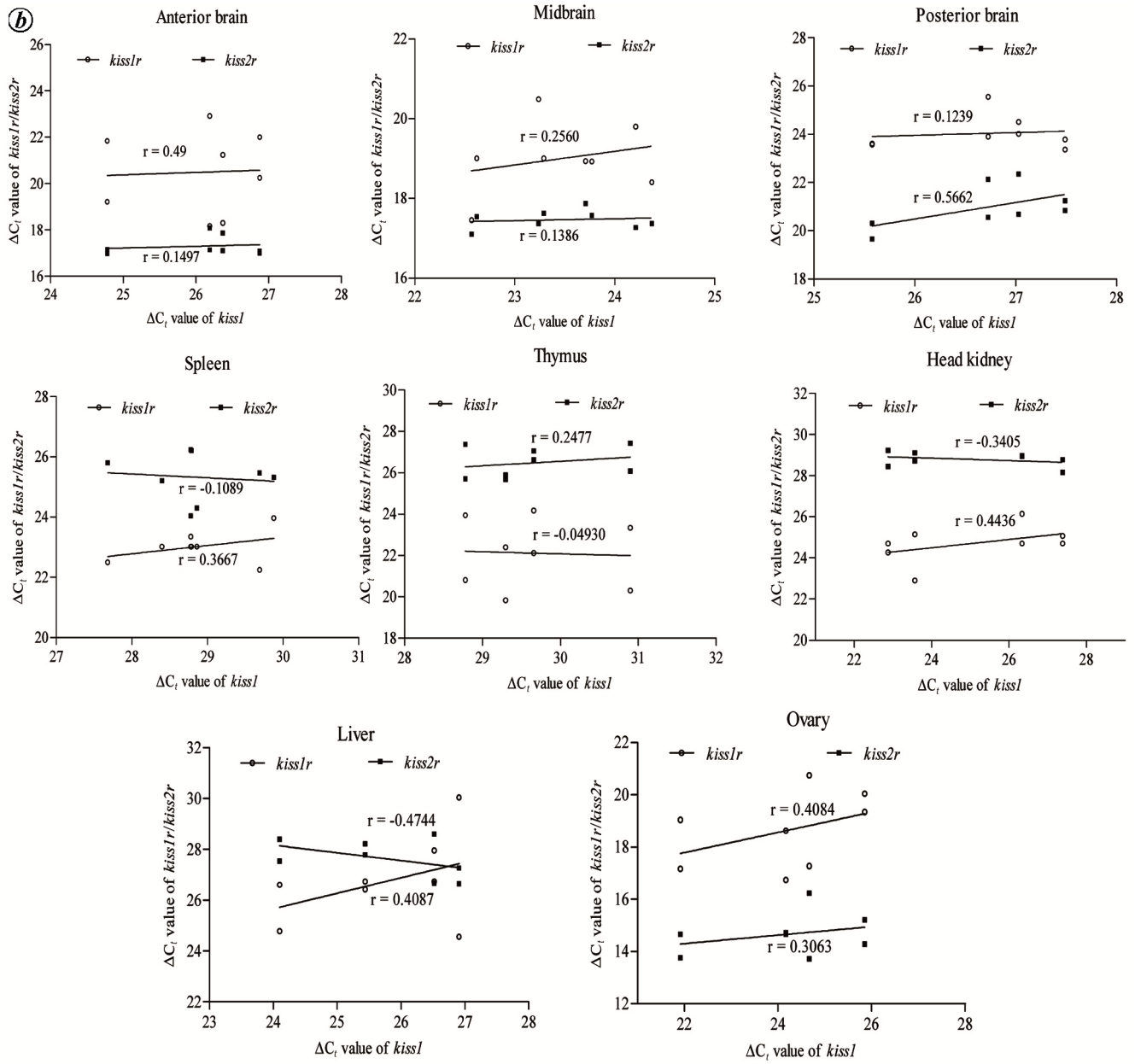

Figure 4 *b*. Correlation curves representing relation between gene expression of ligand *kiss1* and its receptors *kiss1r* and *kiss2r* in tissues of female *C. punctatus*. Values above the curve shows Pearson's coefficient calculated using ΔC_t (= $C_{t\ \text{target gene}} - C_{t\ 18S\ rRNA}$) values.

in concordance with a recent report in another Perciformes *D. labrax*[51], in which no significant correlation was seen between hypothalamic expression of kisspeptin (*kiss1*/*kiss2*) and its receptors (*kiss1r*/*kiss2r*) during advanced stages of oogenesis when their maximal expression level was recorded. In contrast, direct correlation between *kiss2* and *kiss1r* expression has been reported in pituitary and brain of *Takifugu niphobles*[52] during reproductively active spawning phase. Nonetheless, in general, even when significant correlation between ligand and its receptor is not obvious, it is evidenced that maximal functional responses of cells get altered with marked alteration in the expression of either ligand or its receptor, or both[53].

Conclusion

In addition to different parts of the brain and gonads, sex-dependent expression of *kiss1*, *kiss1r* and *kiss2r* in the liver and immune organs paves the way for several novel aspects of possible involvement of kisspeptin in the regulation of peripheral functions in fish, including metabolic activity and immunity. However, cause and physiological significance of tissue-specific sex-dependent variations in

the expression of *kiss1*, *kiss1r* and *kiss2r* need to be explored in order to reach to a logical conclusion.

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