



Short-Term Effects of Human Kisspeptin on LH Secretion in Prussian Carp (*Carassius gibelio* Bloch, 1782) Females at Two Gonad Maturity Stages

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Abstract

Kisspeptin plays a critical role in the control of reproduction and puberty onset in vertebrates, fish including. To demonstrate the pattern of LH secretory responses [spontaneous and stimulated by GnRH analogue (Des Gly¹⁰, D-Ala⁶ ethylamide LHRH)] to single, intraperitoneal injection of human kisspeptin [metastatin (45-54) amide], blood samples from mature Prussian carp females (at the time of gonad recrudescence and natural spawning) were collected at 5, 10, 15 and 30 minutes after injection. To test the influence of dopamine on kisspeptin action on LH release, the dopamine antagonist - pimozide - was used together with kisspeptin and GnRH analogue. At the time of gonad recrudescence kisspeptin alone had no effect on LH secretion. At the time of natural spawning the transient increase of LH levels at 10th minute post-injection was observed. Also the combination of kisspeptin and GnRH increased LH levels in comparison to control fish at 10, 15 and 30 minutes after injections and at 10th minute kisspeptin potentiated stimulatory effects of GnRH. No influence of kisspeptin on the spontaneous, as well as GnRH-stimulated LH release was observed in pimozide-treated fish. Presented data suggest that the short-term response to kisspeptin is dependent on the stage of gonadal maturity.

Keywords: Prussian carp, human kisspeptin, LH levels.

Introduction

Kisspeptins are considered as the major regulator of the gonadotropin axis acting upstream of the GnRH cells (Tena-Sempere, 2006). Actually, two different kisspeptin peptides (kisspeptin 1 and kisspeptin 2) are found in lower vertebrates, while in mammals only the kisspeptin 1 was identified (Felip *et al.*, 2009). These peptides are involved in all aspects of sexual maturation and function of the reproductive axis: sexual differentiation of the brain, timing of puberty, regulation of gonadotropin secretion by gonadal hormones, metabolic and environmental cues (Roa, Navarro, & Tena-Sempere, 2011; Caraty, Decourt, Briant & Beltramo, 2012). In mammals kisspeptin provokes LH secretory bursts, after its administration by different routes: intracerebral, intravenous, intraperitoneal or subcutaneous (Matsui, Takatsu, Kumano, Matsumoto, & Ohtaki, 2004; Thompson *et al.*, 2004; Navarro *et al.*, 2005a,b; Shahab *et al.*, 2005). These facts are well proven, however the profiles of gonadotropin responses to systemic kisspeptin administration have not been frequently investigated. Even for mammals dose-response and time course analyses of the LH-

releasing effects of kisspeptin have been presented mainly after central intracerebroventricular administration of the peptide (Gottsch *et al.*, 2004; Navarro *et al.*, 2005a, b), whereas the response to peripheral injections or infusions remained much less defined as it usually has been monitored at single time-point (Matsui *et al.*, 2004; Thompson *et al.*, 2004). However, Tovar *et al.* (2006), Caraty *et al.* (2007) and Wang, Sun, Zhang, Zhou, and Lou (2012) demonstrated the pattern of dynamic LH secretory responses to intravenous administration of kisspeptin-1 (single and repeated) followed by serial blood sampling in mice, in cyclical or prepubertal ewes, respectively.

Recent data from experiments on mammals showed that kisspeptin is a component of the pulse generator for GnRH (and in consequence LH) secretion (Ezzat, Pereira, & Clarke, 2015; Han, McLennan, Czieselsky, & Herbison, 2015, Javed, Qamar, & Sathyapalan, 2015; Kunimura, Iwata, Ishigami, & Ozawa, 2017). In humans (women) endogenous pulsatile release of kisspeptin was demonstrated (Meczekalski, Katulski, Podfigurna-Stopa, Czyzyk, & Genazzani, 2016) and the pulses of kisspeptin were temporally coupled with LH pulses.

According to our knowledge, there is an increasing number of publications on the role of kisspeptins in hormonal control of reproduction in lower vertebrates. For years experiments on fish were focused on kisspeptin structure, molecular identification and functional characterization of the kisspeptin/kisspeptin receptor system. Subsequent data described the response of fish to exogenous kisspeptin, showing its effects on gonadotropin release, gonad maturation, ovulation or spermiation (Li *et al.*, 2009; Felip *et al.*, 2009; Beck, *et al.*, 2012; Nocillado, Levavi-Sivan, Carrick, & Elizur, 2013; Gosiewski, Sokolowska-Mikołajczyk, Chyb, & Socha, 2015) however data on the mechanism or dynamics of kisspeptin action on hypothalamo-pituitary-gonadal axis (HPG axis) in fish are still scarce. It is possible that diurnal gonadotropin fluctuations (demonstrated in goldfish by Hontela and Peter 1983) follow the GnRH secretion changes induced by kisspeptin waves, occurring during the day. Thus, the theoretical "pulses" of kisspeptin may have a physiological meaning for gonad development. From the practical point of view it would be interesting to find out the short-term response (in minutes) measured by LH secretion changes after kisspeptin administration. Such results could have a practical meaning, as the effects of kisspeptins on fish gonad maturation have already been demonstrated (Beck *et al.*, 2012; Nocillado *et al.*, 2013; Selvaraj *et al.*, 2013). There is a potential for the application of this molecule in the control of ovulation and spermiation and that kisspeptin could be applied to improve methods of hormonally stimulated fish reproduction, currently used in aquaculture: gonadoliberin agonists and dopamine antagonists as a spawning inducing agents, as it is well established that in fish dopamine is a factor having inhibitory effects on LH secretion (Peter *et al.* 1986; Peter & Yu, 1997). The aim of the present paper was to demonstrate the spontaneous and GnRH analogue stimulated LH release in mature female of Prussian carp, following the single injection of human kisspeptin 1. The changes in plasma LH were investigated in short-term (5, 10, 15 and 30 minutes) after injection in two periods of the season: late gonad recrudescence or in the period of natural spawning; the potential interaction of kisspeptin with dopamine inhibitor - pimozide - was also investigated.

Materials and Methods

The experiments were approved by the First Local Ethical Committee on Animal Testing in Kraków 41/2011. Fish were collected from the ponds of the Fisheries Research Station of the University of Agriculture in Kraków. For the experiment 112 two year old mature females of Prussian carp (*Carassius gibelio* Bloch, 1782) of average body weight (b.wt) 105.88 ± 0.78 g and gonadosomatic index (GSI) 4.30 ± 2.06 % (February, before spawning period, at the

time of gonad recrudescence) or 15.96 ± 3.47 % (June, at the time of natural spawning period) were used (56 females for each experimental period). Two days before the beginning of the experiment fish were transferred to glass tanks (volume 300 dm^3) with aerated water. After acclimatization to the experimental conditions in each of investigated periods fish were divided into 8 groups (7 fish per group). Water temperature in the basins was kept at $12 \pm 1^\circ\text{C}$ (February) or $20 \pm 1^\circ\text{C}$ (June) and fish were exposed to a simulated natural photoperiod (in February L:D=9:15, in June L:D=16:8). All fish were individually weighed after a light anesthesia with Propiscin solution (0.3 ml l^{-1} – Żabieniec, IRS Olsztyn).

In both seasons of the year anaesthetized fish were given intraperitoneal injections (under the ventral fin) of:

-Group 1 (control group): 0.6 % saline, or

-Group 2: Des Gly¹⁰, D-Ala⁶ ethylamide LHRH, GnRH analogue (GnRHa) (Sigma-Aldrich, USA) at a dose of $20\text{ }\mu\text{g kg}^{-1}$ b.wt., or

-Group 3: metastin (45-54) amide, human kisspeptin (KISS) (Sigma-Aldrich, USA) at a dose of 0.1 mg kg^{-1} b.wt., or

-Group 4: pimozide, a dopamine antagonist (PIM) (Sigma-Aldrich, USA) at the dose of 5 mg kg^{-1} b.wt., or

-Group 5: the combination of GnRHa + PIM, or

-Group 6: the combination of GnRHa + KISS, or

-Group 7: the combination of PIM + KISS, or

-Group 8: the combination of GnRHa + PIM + KISS.

The dose of GnRHa or KISS for 1 kg of b.wt was dissolved in 1 ml of acidified saline, whereas PIM was prepared as a solution in acidified ethylene glycol (Gosiewski *et al.*, 2015). The selection of doses was justified by the earlier *in vivo* experiments on Prussian carp by Gosiewski *et al.* (2015).

Before injection and 5, 10, 15 and 30 minutes after injection the blood samples ($150\text{ }\mu\text{l}$) were taken from the caudal vasculature with a 23-gauge needle attached to a 1-ml heparinized syringe. For plasma separation, samples were centrifuged for 3 minutes at 14000 g . Plasma was frozen at -20°C until LH levels were determined by the ELISA method (Kah, Pontet, Nunez Rodriguez, Calas, & Breton, 1989). Sensitivity of the assay was in the range of $0.6\text{--}100\text{ ng ml}^{-1}$ with intra- and inter-assay coefficients of variance (CV) of 5 and 9%, respectively.

LH concentrations were analyzed using GraphPad Prism statistical software (version 5, USA). All data were expressed as the percent of pre-treatment values (plasma LH levels before injection) and presented on the graphs as mean \pm SEM. A nonparametric two-tailed Mann-Whitney test (U-test) was performed and the differences between groups were considered significant at $P<0.05$.

Results

The Effects of KISS on the Spontaneous LH Secretion

At the time of gonad recrudescence kisspeptin had no effect on LH secretion (Figure 1A), but at the time of natural spawning the transient, significant increase at 10 minutes post-injection was observed (Figure 1B). After next 5 minutes the levels of LH in KISS-injected fish and the controls were not different.

The Effects of KISS on GnRHa Stimulated LH Secretion

GnRHa or kisspeptin, given alone, did not affect LH secretion at the gonadal recrudescence period (Figure 1A). At 10 minutes after injection the

combination of KISS and GnRHa significantly increased LH concentration only in comparison to KISS alone injected fish.

At the spawning period kisspeptin or GnRHa alone significantly increased LH concentration after 10 and 30 minutes post-injection, respectively (Figure 1B). Statistically significant increase of LH levels in comparison to control fish was observed by the action of the combination of both hormones at 10, 15 and 30 minutes after administration. At 10 minutes post-injection in fish receiving GnRHa + KISS gonadotropin levels were significantly higher than in GnRHa alone treated fish.

The Effects of KISS on the Spontaneous LH Secretion in the Presence of Dopamine Receptor Blocker - Pimozide

Either during gonadal recrudescence (Figure 2A)

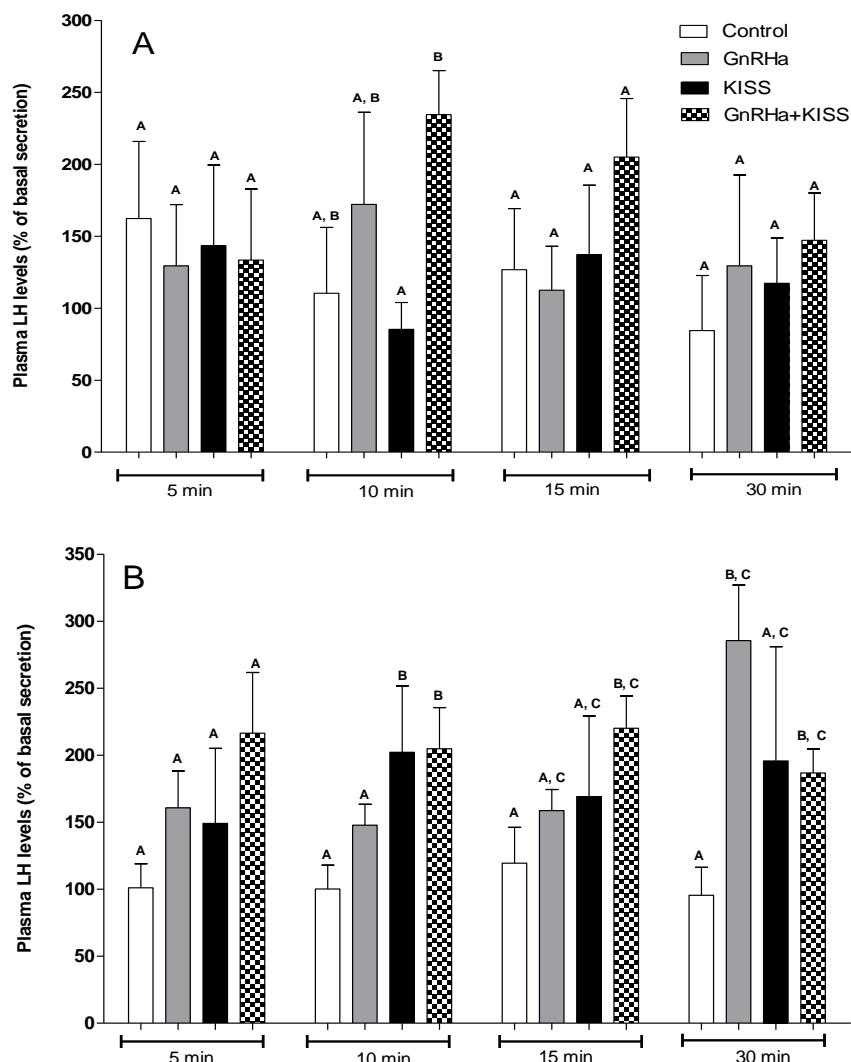


Figure 1. GnRHa-stimulated LH release expressed as the percentage of the pre-treatment levels (mean \pm SEM) before spawning period at the time of gonadal recrudescence, T=12°C (A) and at the time of natural spawning period, T=20°C (B) in Prussian carp females in response to kisspeptin administration. Blood sampling time: before injection and 5, 10, 15 and 30 minutes post-injection. Different letters above the bars represent significant differences ($P<0.05$) between groups within the sampling time (min). Number of fish per group: n = 7.

or spawning period (Figure 2B) no influence of kisspeptin on LH secretion was found.

The Effects of KISS on GnRHa Stimulated LH Secretion in the Presence of Dopamine Receptor Blocker - Pimozide

In both investigated seasons (Figure 3A and 3B) pimozide did not change GnRHa-stimulated LH secretion under the influence of kisspeptin.

Discussion

Experiments of the presented work demonstrated the *in vivo* response of Prussian carp to human kisspeptin, measured by plasma LH levels monitored in minutes (5, 10, 15 or 30) after the administration of

the peptide. Data of this type are scarce as, according to our knowledge, there is just one publication presenting the short-term (within one hour) effects of kisspeptin administration on *in vivo* LH secretion in European sea bass *Dicentrarchus labrax* (Felip *et al.*, 2009). The use of mammalian kisspeptin for fish was already described by Filby, van Aerle, Duitman, and Tyler (2008), who demonstrated that intraperitoneal injection of this peptide (2.6 mg kg⁻¹ b.wt.) resulted in statistically significant increase of kisspeptin receptor as well as GnRH3 gene expression in immature and mature fathead minnow (*Pimephales promelas*) at 10 hours after hormone administration. These results indicate that nonspecific kisspeptin can affect GnRH system of another species, even so distant in systematic classification as fish and mammals (Li *et al.*, 2009). The changes in the expression of kisspeptin

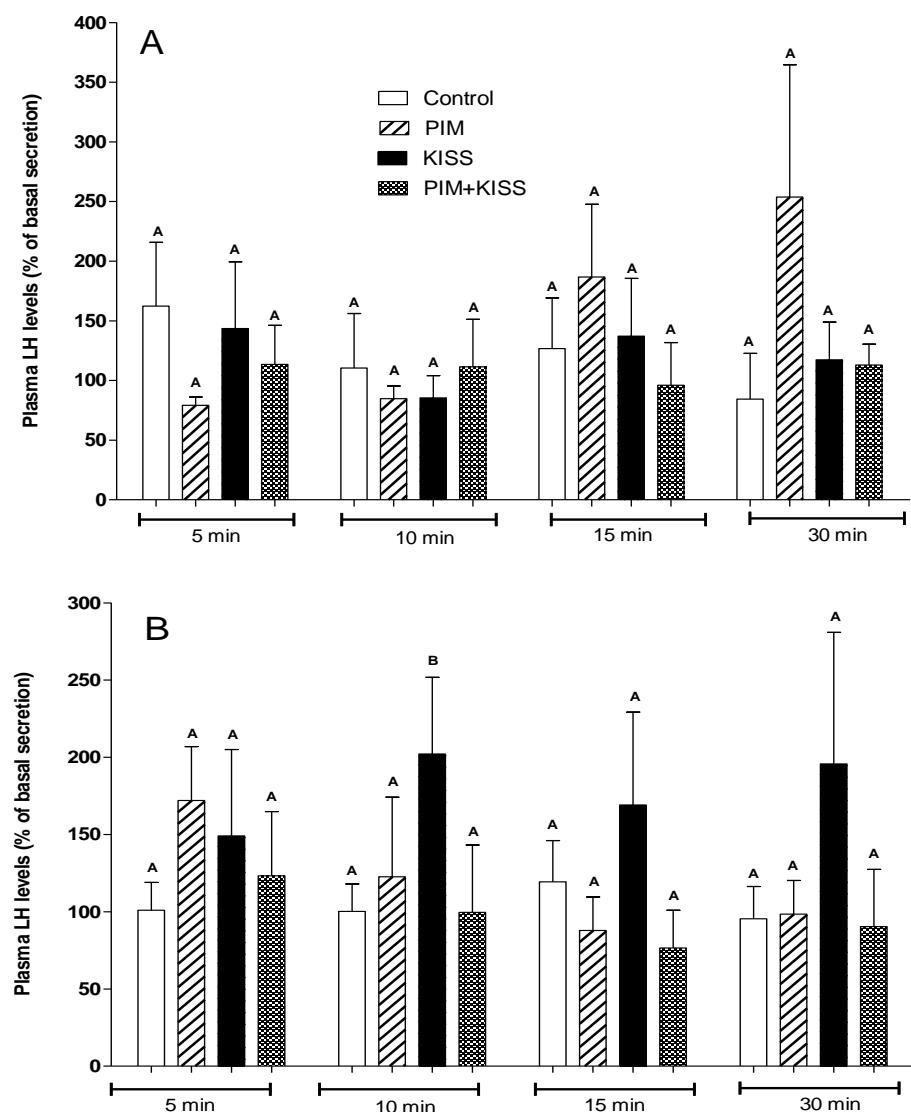


Figure 2. The spontaneous LH release in the presence of dopamine receptor blocker - pimozide, expressed as the percentage of the pre-treatment levels (mean ± SEM) before spawning period at the time of gonadal recrudescence, T=12°C (A) and at the time of natural spawning period, T=20°C (B) in Prussian carp females in response to kisspeptin administration. Blood sampling time: before injection and 5, 10, 15 and 30 minutes post-injection. Different letters above the bars represent significant differences (P<0.05) between groups within the sampling time (min). Number of fish per group: n = 7.

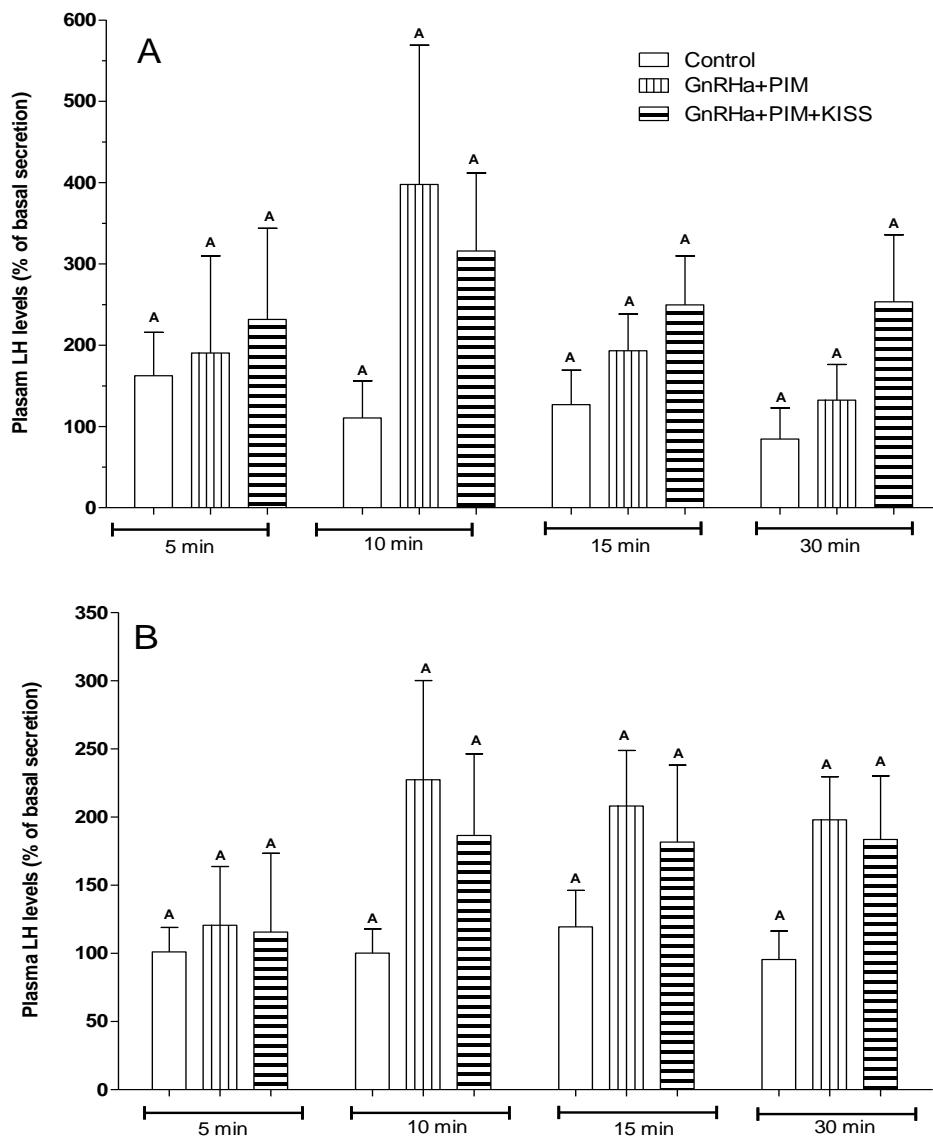


Figure 3. GnRHa-stimulated LH release in the presence of dopamine receptor blocker - pimozide, expressed as the percentage of the pre-treatment levels (mean \pm SEM) before spawning period at the time of gonadal recrudescence, T=12°C (A) and at the time of natural spawning period, T=20°C (B) in Prussian carp females in response to kisspeptin administration. Blood sampling time: before injection and 5, 10, 15 and 30 minutes post-injection. Different letters above the bars represent significant differences ($P<0.05$) between groups within the sampling time (min). Number of fish per group: n = 7.

receptor genes, induced by the exogenic kisspeptin demonstrated its autoregulatory effect on its own receptors. The results confirm the central (brain level) effect of intraperitoneally administered kisspeptin, which does not exclude its direct action on the level of pituitary. Also Selvaraj *et al.* (2013) have shown that subcutaneous injection of kisspeptin modulates the level of the brain GnRH and pituitary FSH β and LH β . Central action of peripherally administered kisspeptin was also confirmed in mammals: in rat subcutaneous injection increased FSH and LH levels (Matsui *et al.*, 2004). This effect was blocked by the earlier administration of GnRH antagonist. The authors suggest that kisspeptin directly stimulates

GnRH nerve terminals at the median eminence from the periphery or stimulates GnRH perikarya after crossing the blood-brain barrier *via* specific transporter or ventricular organs that lack a blood-brain barrier. Also, in rhesus monkey the comparable LH-releasing action of centrally and peripherally administered kisspeptin was found (Shahab *et al.*, 2005). The action of kisspeptin was probably exerted within the region of arcuate nucleus (ARC) and median eminence - an organ that possesses fenestrated capillaries highly permeable to circulating factors.

The dynamics of kisspeptin effects on the level of hypothalamus and/or pituitary gland and, as a consequence of this action, the secretion of

gonadotropins are not yet well defined in fish. These dynamics may be dependent on many external environmental factors (especially water temperature and photoperiod) and internal ones (hormonal milieu with the most important sex steroid levels connected with the stage of gonad maturity or energy balance).

In Prussian carp, intraperitoneal kisspeptin administration in the period of gonad recrudescence (February) had no impact on LH levels measured three times every 5 minutes and then after a further 15 minutes (Figure 1A). The same treatment performed at the time of natural spawning showed the transient, significant increase of plasma LH concentrations at 10 minutes' post injection, lasting less than 5 minutes (Figure 1B). After next 5 minutes the levels of LH in KISS-injected fish and the controls were not different. This difference in response to kisspeptin depending on the season when experiments were performed was expected as the seasonality in kisspeptin concentration or gene expression in the brain was shown in several fish species: grass puffer *Takifugu niphobles* (Shahjahan, Motohashi, Doi, & Ando, 2010), Nile tilapia *Oreochromis niloticus* (Parhar, Ogawa, & Sakuma, 2004), cobia *Rachycentron canadum* (Mohammed, Benninghoff, Holt, & Khan, 2007), grey mullet *Mugil cephalus* (Nocillado, Levavi-Sivan, Carrick, & Elizur, 2007) fathead minnow *Pimephales promelas* (Filby et al., 2008), Atlantic halibut *Hippoglossus hippoglossus* (Mechaly, Viñas, Murphy, Reith, & Piferrer, 2010), Senegalese sole *Solea senegalensis* (Mechaly, Viñas, & Piferrer, 2009) and zebrafish *Danio rerio* (Biran, Ben-Dor & Levavi-Sivan, 2008). Also in *in vivo* experiment Gosiewski et al. (2015) demonstrated a different response to kisspeptin at the time of gonad recrudescence and at the time of natural spawning (depending on the stage of gonad maturity, i.e. steroid hormone levels at the time of hormone administration). Gonadal steroid levels seem to be of special importance in case of kisspeptin action on the hypothalamo-pituitary-gonadal axis in fish, since in mammals kisspeptin is responsible for the transmission of the feedback effects of sex steroids on gonadotropin release (Tena-Sempere, 2005; Smith, 2008; Roa, Aguilar, Dieguez, Pinilla, & Tena-Sempere, 2008; d'Anglemont de Tassigny & Colledge, 2010). Also in fish this can be true, as there are data showing that in the hypothalamus of these vertebrates the cells expressing kisspeptin co-express estrogen receptors, suggesting the role of kisspeptin in the steroid feedback on gonadotropin release (Kanda et al., 2008; Escobar et al., 2010, 2013; Servili et al., 2011; Cowan, Davie, & Migaud, 2012).

The lack of response to kisspeptin at the time of gonadal recrudescence may also be connected with environmental factor characteristic to winter season (low water temperature), which is, for this species, the period of gonadal restoration. Kisspeptin can be one of the links between the seasonal changes in temperature and photoperiod and GnRH neurons

leading to onset of puberty or adult fish cyclic reproduction - kisspeptin neurons have been localized in the brain areas involved in the perception of environmental and metabolic cues (Escobar et al., 2010; Servili et al., 2011). The role of environmental temperature in kisspeptin action on LH secretion in zebrafish demonstrated Shahjahan, Kitahashi, Ogawa, and Parhar (2013).

At the time of gonad recrudescence there was no response to GnRHa or kisspeptin alone, and the combination of both caused momentary increase in LH concentration at 10 minutes post-injection in comparison with kisspeptin-alone injected fish (Figure 1A). During that period the sensitivity of HPG axis (number of receptors and/or their affinity to the ligands) is lower (Jodo et al., 2005; Shahjahan et al., 2010) and the extended time to respond to exogenous hormone (hours, but not minutes) may be needed.

At the time of natural spawning, unlike in gonadal recrudescence period, there was a significant increase in blood LH concentration caused by GnRHa alone (30 minutes after injection) as well as by the combination of kisspeptin and GnRHa at 10, 15 and 30 minutes post-injection (Figure 1B). In the tenth minute the potentiation of analogue-stimulated LH release by kisspeptin was observed: statistically significant difference between KISS+GnRHa and GnRHa group was found. Five minutes later there was no difference between these groups. The difference in the response to GnRHa and/or kisspeptin between the seasons when experiment was performed may be a consequence of the stage of gonad maturity (different sex steroid levels regulating LH secretion in such a way as to ensure reproductive success. In this regulation kisspeptin may play important role, as it was proved in mammals.

Results obtained in the present paper can be discuss with the results of Felip et al. (2009) who measured LH secretion in sexually immature and mature European sea bass after *in vivo* kisspeptin 1 or kisspeptin 2 administration, starting from 15 up to 240 minutes post-injection. They found that in immature fish the maximum LH levels were found after 120 minutes, with twice stronger effects evoked by kisspeptin 2. After another two hours the level of LH returned to the control value. In mature males only kisspeptin 2 caused significant LH level increase also at 120 minutes post-injection.

In mammals, there are data showing the pattern of dynamic LH secretory responses to systemic injection of kisspeptin (single and repeated) followed by serial blood sampling (every 15 minutes after hormone administration) in mice (Tovar et al., 2006). In case of single hormone administration kisspeptin effectively stimulated LH release within 15 min of injection, with maximum levels ranging between 4- to 8-fold increase over preinjection values and vehicle-injected controls. LH levels had returned to preinjection values at 45-105 min after injection, depending on the dose of the peptide. Repeated

administration of kisspeptin (the highest dose) caused a series of associated LH secretory bursts, which were similar to each other and analogous to those induced by the single injection of kisspeptin. It is possible that such a quick response to kisspeptin is important for the modulation of the pulsatile mode of GnRH release in mammals. According to Dungan, Clifton, and Steiner (2006) the LH-releasing effect is mediated *via* activation of GnRH at the hypothalamus and the rapid response to peripheral administration suggests that systemically delivered kisspeptin may regulate GnRH release directly at GnRH neuron nerve terminals located at the median eminence-arcuate nucleus complex, which is mostly placed outside the blood-brain barrier.

In ewes, acute intravenous or subcutaneous administration of kisspeptin induced only a short-lived stimulation of LH release (Caraty *et al.*, 2007) - after maximum response at 10 minutes post-injection, LH levels decreased in a consequence of peptide short half-life.

In fish, the stimulating effects of GnRH or other neurohormones regulating LH synthesis and secretion is masked by the inhibitory influence of dopamine and it has to be taken in the consideration in the *in vivo* experiments. Peter *et al.* (1985) demonstrated that in order to test the potency of different GnRH analogues the removal of dopamine inhibition by the dopamine receptor antagonists is necessary, otherwise the masking effects of dopamine inhibition on LH release is so strong that it interferes with the action of other neurohormones. In the presented *in vivo* experiments this aspect of LH release control was also taken into account, however pimozide (given to block dopamine inhibition) injected together with kisspeptin had no effects on its action on the spontaneous or GnRHa-stimulated LH release, measured in minutes after hormone injection, in both investigated seasons. This lack of the effects of pimozide on LH profiles is not very surprising, as another results of 24-hour lasting experiments (Gosiewski *et al.*, 2015) showed that the combination of pimozide and kisspeptin strongly stimulated LH release in Prussian carp during gonad recrudescence as well as at the time of natural spawning, but the effects were observed no sooner than at 3 hours after pimozide administration. In this short-term investigation the time between injection and blood sampling (from 5 to 30 minutes) was too short for the pimozide to block dopamine action on LH release. Other data (Sokolowska, Peter, Nahorniak, & Chang, 1985) demonstrated that pimozide blocks the inhibitory influence of dopamine at least 3 hours after its administration, and only then the stimulation by endo- or exogenous gonadoliberin can pursue. Because of this methodological deficiency in the presented data on Prussian carp, the potential effects of kisspeptin on the short-term release of LH in Prussian carp with blocked dopaminergic receptors should be verified in the future in experiment performed on fish pretreated with pimozide at several

hours before kisspepin or kisspeptin and GnRHa administration.

In summary, human kisspeptin in a very limited way affected LH secretion shortly after systemic administration (few or several minutes). Significant and long-lasting *in vivo* stimulation occurs after hours post-injection (Gosiewski *et al.*, 2015). The physiological meaning of the transient rise in blood LH concentrations, shortly after kisspeptin administration is hard to explain. In order to find an answer to this question it seems necessary to perform the experiment with frequent blood sampling, lasting at least the first 3 hours after hormone injection, also with the use of kisspeptin 2, since there are data suggesting differential action of both peptides on LH secretion during gonadal recrudescence and the period of ovulation (Zmora *et al.*, 2012). Data on the dynamics of LH release under the influence of kisspeptin are important for the use of this peptide as a potential preparation for the improvement of hormonally stimulated reproduction in aquaculture.

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