

Preliminary Results Concerning the Influence of Human Kisspeptin on LH Secretion in Prussian Carp (*Carassius gibelio*) Females at the Stage of Ovarian Recrudescence and Spawning Season*

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The aim of the experiments was to evaluate the influence of human kisspeptin on LH secretion of Prussian carp (*Carassius gibelio*) females during ovarian recrudescence and spawning season. For the experiments, human kisspeptin KISS1 (0.1 mg kg⁻¹ of body weight – b.w.), GnRH analogue (Des Gly¹⁰, D-Ala⁶) GnRH-A (20 μg kg⁻¹ b.w.) and dopamine antagonist (pimozide) (5 mg kg⁻¹ b.w.) were used alone or in combinations. At 3, 6, 12, 24 hours after injection(s) blood samples were collected from all fish. LH levels were measured in plasma with the use of the ELISA method. KISS1 did not show any significant effects on spontaneous LH secretion in both tested seasons. At 12 hours sampling time (both stages of gonad maturity) a combination of tested compounds (GnRH-A+KISS1) significantly increased LH release in comparison with the control. In the stage of gonad recrudescence KISS1 significantly increased LH secretion evoked by pimozide at 24 hours. A combination of three components: KISS1, GnRH-A, and pimozide significantly decreased LH secretion in comparison to LH secretion evoked by GnRH-A and pimozide during stage of gonad recrudescence. These results suggest that kisspeptin is involved in seasonal control of reproduction in Prussian carp. The possible interaction of kisspeptin and the dopaminergic system is also discussed.

Key words: Kisspeptin, LH, fish, *Carassius gibelio*, season, reproduction.

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Puberty and reproduction, crucial events in the life history of all animals, are controlled by environmental factors (temperature, photoperiod) as well as endogenous factors such as hormones, neurohormones, neurotransmitters or steroids (ZOHAR *et al.* 2010).

In fish, gonad maturation (ended by spermiation or ovulation) is regulated by the hypothalamo-pituitary-gonadal (HPG) axis. A major role in this process is played by gonadoliberin (GnRH) produced in the hypothalamus. GnRH controls the release of gonadotropins (LH and FSH) from the pituitary gland (PETER & YU 1997; YU *et al.* 1997). Gonadotropins act on gonads to stimulate the synthesis of sex steroid hormones and gonadal development. Sex steroids affect GnRH and gonadotropin release by positive or negative feedback effects depending on the stage of gonad maturity

(CRIM & EVANS 1983; TRUDEAU 1997; AROUA *et al.* 2007). In many fish species, the dopaminergic system is responsible for inhibition of GnRH and LH secretion (KEBABIAN & CALNE 1979; CARDINAUD *et al.* 1998; NIEUWENHUYNS *et al.* 1998). It was shown that the strength of dopamine (DA) inhibition on LH secretion differs between species and could depend on the stage of gonad maturity and season (SOKOŁOWSKA *et al.* 1985; PETER *et al.* 1991). Strong dopamine inhibition was observed in juvenile eel in which DA is a puberty gatekeeper. In cyprinids a weak inhibitory effect of DA was observed during the initial stage of gametogenesis and strong inhibition was found during the pre-ovulatory LH surge and ovulation (SOKOŁOWSKA *et al.* 1985; VIDAL *et al.* 2004; PARK *et al.* 2007).

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The discovery of kisspeptin in 2001 (KOTANI *et al.* 2001; MUIR *et al.* 2001; OHTAKI *et al.* 2001) increased our knowledge of endocrine control of puberty and maturation. Kisspeptin is a neurohormone controlling the HPG axis *via* the GnRH system in all vertebrates, including fish (MESSAGER *et al.* 2005; VAN AERLE *et al.* 2008; ZOHAR *et al.* 2010; TENA-SEMPERE 2012; MECHALY *et al.* 2013; OGAWA & PARHAR 2013). Injections of kisspeptin increase LH secretion in goldfish, *Carassius auratus* (LI *et al.* 2009), European sea bass, *Dicentrarchus labrax* (FELIP *et al.* 2009), Yellowtail Kingfish, *Seriola lalandi* (NOCILLADO *et al.* 2012) and Striped bass, *Morone saxatilis* (BECK *et al.* 2012; ZMORA *et al.* 2012). Also, *in vitro* studies demonstrate the ability of kisspeptins to induce LH release from cultured pituitary cells (YANG *et al.* 2010; CHANG *et al.* 2012). Moreover, it was shown that kisspeptin is able to modulate activity of the HPG axis; therefore kisspeptin may play a role as a puberty gatekeeper in juvenile eel (PASQUIER *et al.* 2011).

Kisspeptin receptor expression was localized close to GnRH neurons in cichlid fish (PARHAR *et al.* 2004) and was greater in fish which were at the beginning of puberty than at pre- and post-puberty stages. These results demonstrated that GnRH cells are direct targets for kisspeptin in fish and that kisspeptin induces GnRH release, as already shown in mammals (DHILLO *et al.* 2005, 2007; MESSAGER *et al.* 2005; PLANT *et al.* 2006; CARATY & FRANCESCHINI 2008). Also, dopamine affects LH release in juvenile fish (VIDAL *et al.* 2004; PARK *et al.* 2007) which is very well documented at the time of final maturation (ovulation and spermiation) (OMELJANIUK *et al.* 1989; DULKA *et al.* 1992; TRUDEAU *et al.* 1993; TRUDEAU 1997).

Since the relation between GnRH and dopamine as well as between GnRH and kisspeptin systems has been demonstrated in fish, the crucial problem is the existence of a possible relationship between kisspeptin and the dopaminergic system. According to our knowledge there is no data showing a direct relationship between these two systems in fish: however in experiments conducted on ewes it was shown that interaction between kisspeptin and dopamine is involved in seasonal changes in reproductive function, measured by the variation in GnRH and LH secretion (GOODMAN *et al.* 2012). Also, in mice it was demonstrated that one of the sub-populations of kisspeptin neurons is responsible for synthesizing DA and projecting it to GnRH neurons (CLARKSON & HERBISON 2011).

Therefore the aim of our experiments was to examine the effects of kisspeptin on spontaneous and GnRH-stimulated LH release in Prussian carp (*Carassius gibelio* Bloch, 1782) with active and

blocked dopaminergic systems at different stages of gonad development.

Material and Methods

All 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. Two year old females of *Carassius gibelio* of average body weight (b.w.) 83 ± 17 g and gonadosomatic index (GSI) – (4.30 ± 2.06 % – stage of gonad recrudescence; 15.96 ± 3.47 % – mature fish) were transferred to glass tanks with aerated water. After 2 days of acclimatisation to experimental conditions fish were divided into 8 groups of 14 animals each. During the winter experiment (January) water temperature was $12 \pm 1^\circ\text{C}$ and winter photoperiod (L:D = 8:16) was maintained, whereas during summer experiments (May) water temperature was $20 \pm 1^\circ\text{C}$ and summer photoperiod (L:D = 16:8) was established. In both seasons, fish were given intraperitoneal (ip) injections of metastatin (45-54) amide, human (Sigma-Aldrich, USA) – kisspeptin (KISS1) at a dose of 0.1 mg kg^{-1} of b.w.; pimozide (Pim) (Sigma-Aldrich, USA) dopamine antagonist at a dose of 5 mg kg^{-1} of b.w. and (Des Gly¹⁰, D-Ala⁶ ethylamide) LH-RH (Sigma-Aldrich, USA) – GnRH analogue (GnRH-A) at a dose of $20 \mu\text{g kg}^{-1}$ of b.w. alone or in combinations. Drugs were dissolved in 0.6 % saline solution (KISS1 and GnRH-A) or solubilized in acidified ethylene glycol (pimozide). Samples of blood (200 μl) were collected from caudal veins of each fish with the use of heparinized syringes before injection (time 0) and 3, 6, 12 or 24 hours after injection. For plasma separation samples were centrifuged for 3 minutes at 14000 g. LH levels were measured in plasma with the use of the enzyme-linked immunosorbent assay method – ELISA (KAH *et al.* 1989).

The obtained LH concentrations were analysed using GraphPad Prism statistical software (version 5 GraphPad Software, USA). All data were expressed as the percent of pre-treatment value (measured at time 0) and presented as mean \pm SEM. A non-parametric Mann-Whitney test (U-test) with Bonferroni correction was performed. Differences between groups were considered significant at $P < 0.05$.

Results

The effects of KISS1 on the spontaneous or GnRH-A stimulated LH secretion in fish at the stage of gonad recrudescence or in mature fish (Fig. 1).

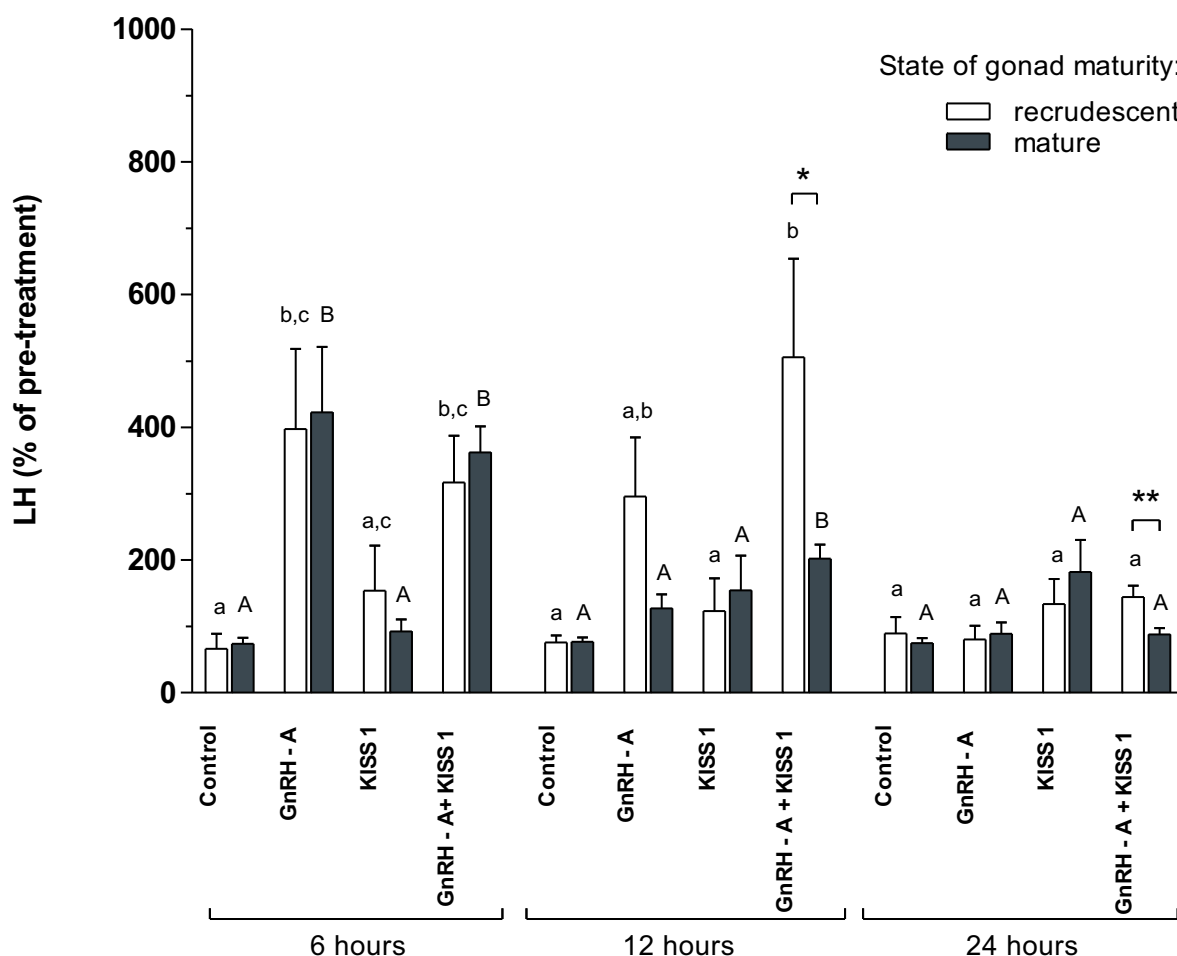


Fig. 1. Plasma LH levels expressed as the percentage of pretreatment (mean \pm S.E.M) levels in Prussian carp injected with KISS1 (0.1 mg kg^{-1} b.w.) or GnRH-A ($20 \mu\text{g kg}^{-1}$ b.w.) or their combination at the time of gonad recrudescence or in mature fish. Different letters above the bars represent significant differences ($P < 0.05$) between groups within sampling time (lower case letters concern recrudescent fish and capital letters concern mature fish). Asterisks above square brackets represent significant differences between recrudescent and mature fish. Number of fish per group: $n = 14$.

At 3 hours after injection there were no significant changes in LH levels between all tested drugs (data not shown).

At all sampling times (3, 6, 12 and 24 hours after injection) there were no significant changes in spontaneous LH secretion in KISS1 treated fish, neither at the time of gonad recrudescence nor in mature fish. GnRH-A alone stimulated LH secretion in comparison with the control group only at 6 hours after injection (in both stages of gonad maturity). At 12 hours sampling time (both stages of gonad maturity) the combination of tested agents (GnRH-A+KISS1) significantly increased LH release in comparison with control, despite the fact that each of them alone did not significantly change LH secretion.

At 12 hours sampling time the potentiation of GnRH-A action by KISS1 was observed in mature fish: secretion of LH was higher in combined treatment than in fish receiving only GnRH-A.

Comparison of LH secretion in mature fish and those having gonads at the stage of recrudescence showed that only combined treatment (GnRH-A+KISS1) at 12 and 24 hours post treatment caused higher stimulation of LH secretion in fish at the time of gonad recrudescence ($P < 0.0158$; $P < 0.0046$, respectively).

The effects of KISS1 on LH secretion in fish treated with pimozone at the stage of gonad recrudescence or in mature fish (Fig. 2).

At all sampling times in mature fish pimozone alone significantly increased LH release in comparison to the control group. The combination of KISS1 with pimozone was very effective in stimulation of LH secretion in comparison to saline (control group): the differences were highly significant. This combined treatment after 24 hours evoked significantly higher LH release in recrudescent fish than pimozone alone, demonstrating the potentia-

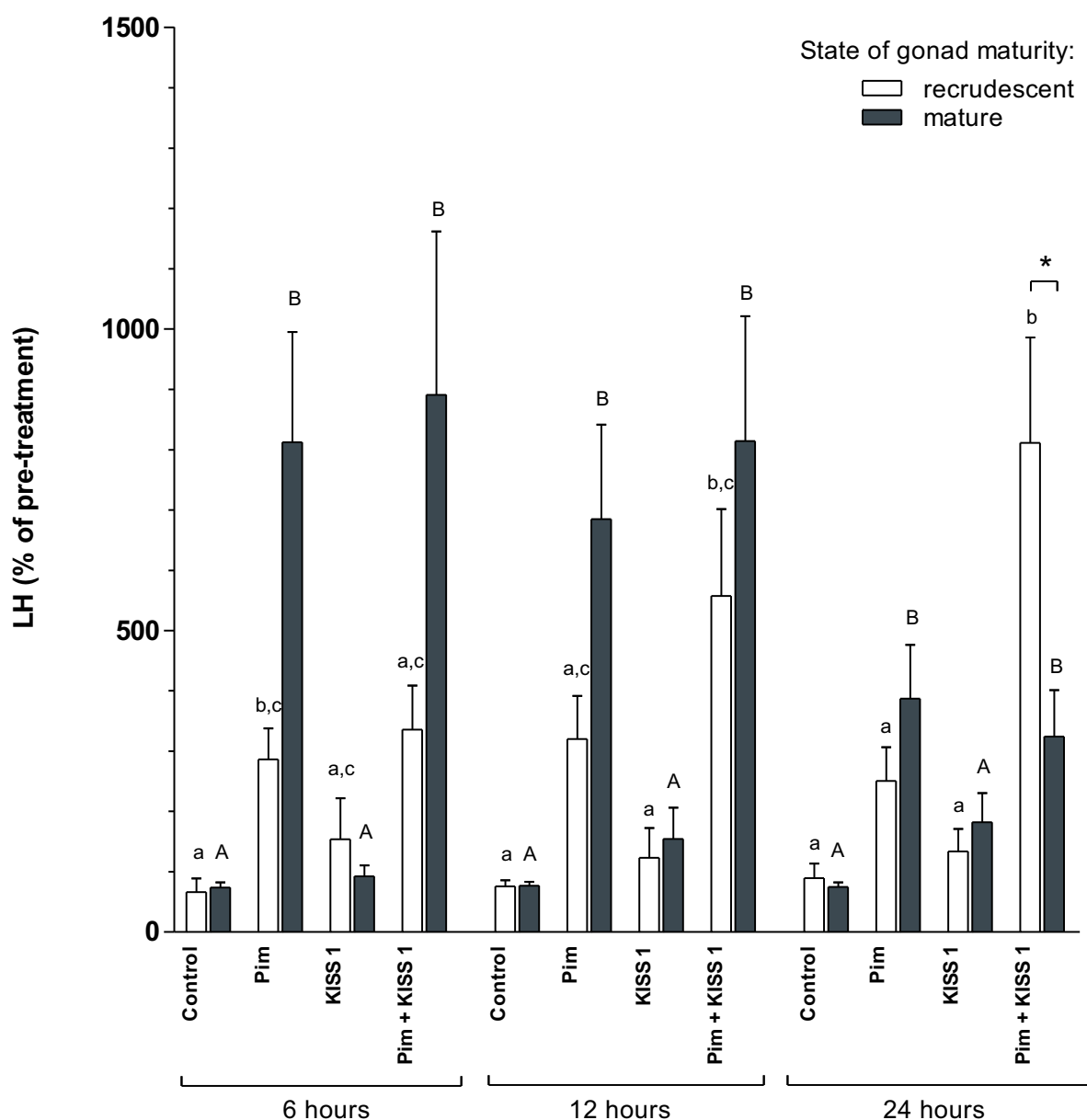


Fig. 2. Plasma LH levels expressed as the percentage of pretreatment (mean \pm S.E.M) levels in recrudescent and mature Prussian carp injected with KISS1 ($0.1 \text{ mg kg}^{-1} \text{ b.w.}$) or pimozone (Pim, $5 \text{ mg kg}^{-1} \text{ b.w.}$) or their combination. Different letters above the bars represent significant differences ($P < 0.05$) between groups within sampling time (lower case letters concern recrudescent fish and capital letters concern mature fish). Asterisk above square brackets represent significant differences between recrudescent and mature fish. Number of fish per group: $n = 14$.

tion effect of KISS1 on pimozone action. In mature fish this potentiation was not observed.

The only significant difference between fish in different stages of maturity was found at 24 hours after treatment with pimozone+KISS1: in mature fish LH secretion was lower than in fish with gonads during recrudescence.

The effects of KISS1 on LH secretion in fish treated with a combination of GnRH-A and pimozone in fish at the stage of gonad recrudescence or in mature fish (Fig. 3).

LH secretion in fish treated with GnRH-A+pimozone or GnRH-A+pimozone+KISS1 was extremely high (several thousand percent in relation to pre-treatment) and was significantly higher than in control fish at both investigated stages of gonad maturity.

At 6 hours sampling time KISS1 significantly reduced the response to the combination of GnRH-A+pimozone in fish with recrudescing gonads, but not in mature fish.

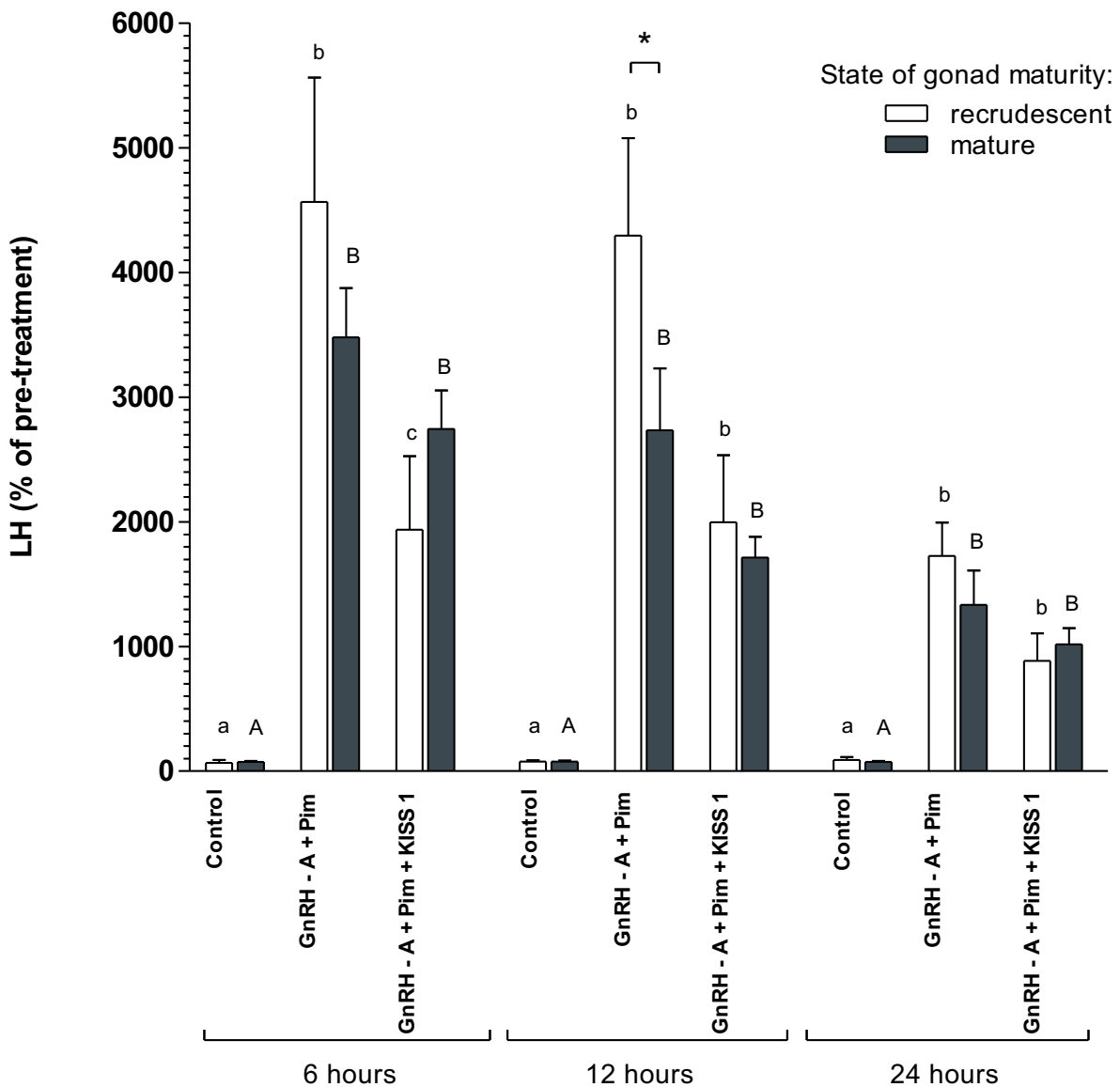


Fig. 3. Plasma LH levels expressed as the percentage of pre-treatment (mean±S.E.M) levels in recrudescent and mature Prussian carp injected with KISS1 ($0.1 \text{ mg kg}^{-1} \text{ b.w.}$), GnRH-A ($20 \mu\text{g kg}^{-1} \text{ b.w.}$) or pimoziide (Pim, $5 \text{ mg kg}^{-1} \text{ b.w.}$) or their combination. Different letters above the bars represent significant differences ($P < 0.05$) between groups within sampling time (lower case letters concern recrudescent fish and capital letters concern mature fish). Asterisk above square brackets represent significant differences between recrudescent and mature fish. Number of fish per group: $n = 14$.

Discussion

To date, most of the evidence for the role of kisspeptins in the onset of puberty and the activation of the HPA axis in fish comes from gene expression analyses. There are however, studies showing that *in vivo* exogenous kisspeptin administration accelerates gonadal development (BECK *et al.* 2012; ZMORA *et al.* 2012). The mechanism and the site of action (hypothalamus and/or pituitary gland) in this process are not well known. There are data showing that kisspeptin induces GnRH release from the hypothalamus and also acts directly at the

pituitary (NOCILLADO *et al.* 2007; YANG *et al.* 2010; ZOHAR *et al.* 2010; CHANG *et al.* 2012). According to our knowledge there is no information on the interaction of kisspeptin with other systems except for GnRH involved in the control of LH secretion, especially with the dopaminergic pathway having a crucial role in gonadotropin release inhibition in fish.

In our experiments on Prussian carp, intraperitoneal injections of human kisspeptin at a dose of $0.1 \text{ mg kg}^{-1} \text{ b.w.}$ showed no effect of KISS1 on the spontaneous secretion of LH, neither in mature nor recrudescent animals (Fig. 1) despite the fact that

earlier pilot experiments demonstrated that kisspeptin (at 0.01 and 0.1 mg kg⁻¹ b.w.) significantly stimulated spontaneous secretion of LH in fish with gonads at the stage of recrudescence (data not shown). A full explanation of this lack of response in the present study in comparison to earlier tested doses of this hormone is not possible at the moment and hopefully the next series of experiments will confirm the ability of this type of kisspeptin to affect spontaneous gonadotropin release in Prussian carp. A comprehensive assessment of the effects of different kisspeptins on LH secretion in fish is not possible because only limited data from *in vivo* experiments are available. Existing results showed that different kisspeptins affected some elements of the HPA axis: stimulation of LH release by goldfish kisspeptin 1 was observed by LI *et al.* (2009) or increased expression of *kiss1r* coding gene in fathead minnow by mammalian kisspeptin (FILBY *et al.* 2008). Human kisspeptin, used in our experiments, could have a lower ability to stimulate LH secretion in Prussian carp than the species-specific peptide, possibly causing the non-significant effect on spontaneous LH release. According to our knowledge there is no other data on the use of human kisspeptin in fish.

Admittedly, changes of the spontaneous release of LH under the influence of human kisspeptin were not demonstrated in both tested seasons but yet an additive effect of kisspeptin and GnRH-A on LH release was found: 12 hours after injection of both hormones, LH concentrations were significantly higher in comparison to the control while GnRH-A or kisspeptin given alone had no statistically significant effect on LH secretion (Fig. 1). The stimulatory effect of KISS1+GnRH-A on LH release was observed in mature fish as well as in those with recrudescing gonads and the only difference was that in recrudescing fish this additive effect lasted longer, up to 24 hours after injection. It should be noted that GnRH-A given alone stimulated significant LH release in both seasons, but this stimulation was observed only up to 6 hours post-injection (Fig. 1).

From the data obtained in mammals or in fish it is clear that kisspeptin may affect LH secretion directly, at the pituitary level (GUTIÉRREZ-PASCUEL *et al.* 2007; SUZUKI *et al.* 2008; YANG *et al.* 2010; CHANG *et al.* 2012) as well as indirectly, *via* other hypothalamic factors, such as the GnRH system (IRWIG *et al.* 2004; MESSEGER *et al.* 2005; ZOHAR *et al.* 2010; TENA-SEMPERE 2012; MECHALY *et al.* 2013; OGAWA & PARHAR 2013). In our experiments (both seasons) the potentiating effect of kisspeptin on GnRH-A action was also found: LH levels in groups receiving both drugs were significantly higher than in fish treated only with GnRH-A, at 12 hours in mature fish or at 24 hours

at the time of gonad recrudescence (Fig. 1). Based on our results we cannot explain at which level (hypothalamic or pituitary) this potentiation occurred. Interestingly, this potentiation was found regardless of the season: in both mature and recrudescing fish the secretion of gonadotropin, expressed as the percent of pretreatment, was very similar and the differences between seasons were small, which is quite unusual because the seasonality of the reproductive axis sensitivity to hormonal treatment is well recognized in fish (CHANG & PETER 1983; SOKOLOWSKA *et al.* 1985; PETER *et al.* 1986; PETER 1991; KIM *et al.* 2011).

To test the possibility of indirect action of kisspeptin on LH secretion, i.e., *via* a different pathway than the GnRH system, pimoziide (dopamine receptor antagonist) was administered to Prussian carp receiving kisspeptin. Extremely strong stimulation of LH release (Fig. 2), as a consequence of blocking of DA receptors, was shown during spawning season. Pimoziide given alone evoked almost an 8-fold increase of LH secretion, lasting up to 24 hours, when compared to the control group. This response was even stronger than to GnRH-A alone (Fig. 1). The data on fish (CHANG & PETER 1983; SOKOLOWSKA *et al.* 1985; PETER *et al.* 1986; SOKOLOWSKA-MIKOLAJCZYK *et al.* 2002a; 2002b) show that differential response of LH secretion to exogenous drugs or hormones (pimoziide, GnRH analogues) exists at different stages of gonad maturity: usually weaker effects are observed at the time of gonad recrudescence. This was not the case in the present investigation. This observation is indirectly supported by findings obtained in mammals. SZAWKA *et al.* (2010) demonstrated that kisspeptin inhibited DA neurons in ovariectomized (OVX) rats treated with estradiol (E2).

Moreover, the combination of pimoziide and kisspeptin caused strong stimulation of LH release in comparison to the control group, especially evident in mature fish (Fig. 2). This was likely due to the strong action of pimoziide alone. In recrudescing fish this stimulation at 24 hours post injection was two-fold higher than in fish receiving only pimoziide, showing that kisspeptin was able to still potentiate the LH releasing ability of pimoziide despite its strong action. Because such an effect was found in recrudescing fish only, it may suggest that kisspeptin is more important for acceleration of the maturation process in the new season than for final oocyte maturation.

The fact that kisspeptin can potentiate the stimulatory effects of GnRH-A or pimoziide on LH secretion but at the same time was not effective when given alone, may suggest that the action of human kisspeptin is rather indirect, through the dopaminergic system. The elimination or diminution of do-

pamine inhibition on LH secretion by pimozide is sometimes crucial to demonstrate the potential of other hormones involved in gonadotropin secretion regulation in fish (PETER *et al.* 1986). In the conditions of our experiment this reduction enabled kisspeptin, inactive when given alone, to mark its stimulatory potential in the control of LH release (significantly important stimulation of KISS1 administered with pimozide in comparison to pimozide alone). However, in the case of the combined treatment with GnRH-A and kisspeptin in fish with blocked dopaminergic system by pimozide, the response to kisspeptin was reversed: at 6 hours post treatment in recrudescing fish a statistically significant reduction of LH secretion was observed when compared with pimozide and GnRH-A injected fish (Fig. 3). Based on our results it is not possible to explain this change in the direction of kisspeptin action on LH release, especially since kisspeptin acts in vertebrates (also in fish) as a central processor/mediator of several inputs including photoperiod, energy balance status of the organism or steroid hormone feedback and the final LH secretion is the result of the response to all these factors (DUNGAN *et al.* 2006; ZMORA *et al.* 2012).

According to our knowledge there is no evidence on dopamine and kisspeptin interaction in GnRH (and LH) release in fish, however the relationships between kisspeptin and GnRH and also between dopamine and GnRH (and LH) were already established (see Introduction) which is why dopamine involvement in these processes (dopamine-kisspeptin-GnRH) is highly likely. Besides the inhibitory action of kisspeptin on DA neurons (SZAWKA *et al.* 2010), there is information that in mammals (anestrous ewes) dopaminergic neurons act *via* kisspeptin neurons to inhibit GnRH (and LH) secretion (GOODMAN *et al.* 2010; 2012). Also, in mice, CLARKSON and HERBISON (2011) described a subpopulation of kisspeptin neurons synthesizing dopamine and projecting to GnRH neurons. It is possible then that in fish, kisspeptin neurons may integrate many hormonal and environmental signals and that dopamine inhibition on GnRH release is partly direct (at the level of GnRH producing neurons) and partly indirect through diminution of stimulatory action of kisspeptin on LH secreting cells. Our results are still preliminary and need to be verified by further *in vivo* and also *in vitro* experiments.

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