The influence of sGnRH-A and antidopaminergic drug – pimozide – on prolactin mRNA synthesis in female Prussian carp (*Carassius gibelio* Bloch) *in vivo*

J. Chyb, M. Socha, P. Szczerbik, M. Sokolowska-Mikolajczyk, T. Mikolajczyk, P. Epler

Department of Ichthyobiology and Fisheries, University of Agriculture in Krakow, Krakow, Poland

ABSTRACT: Effects of salmon gonadotropin releasing hormone analogue (sGnRH-A) and antidopaminergic drug, pimozide, on the synthesis of prolactin mRNA *in vivo* in female Prussian carp (*Carassius gibelio* Bloch) during two different stages of the reproductive cycle were evaluated. The results showed that the lowest dose of sGnRH-A (5 μg/kg body weight) significantly stimulated the mRNA synthesis in fish during the recrudescence as well as during the preovulatory period, higher doses of this compound having no significant effect on prolactin mRNA synthesis. The blocker of dopamine receptors, pimozide, also potentiated prolactin mRNA synthesis – in recrudescent females it increased mRNA levels at the dose of 1 mg/kg, while in the preovulatory period all of the used pimozide doses (1, 5, and 10 mg/kg) were responsible for the increase of prolactin mRNA levels. Taken together, the above results suggest that gonadotropin releasing hormone (GnRH) is the factor responsible for the stimulation of prolactin synthesis, while dopamine has an inhibitory influence on the prolactin production.

Keywords: PRL; gonadotropin releasing hormone; dopamine; fish; mRNA synthesis; pituitary

Prolactin (PRL) is a peptide hormone produced in the pituitary gland in vertebrates. It participates in many physiological processes, among others lactation, reproduction, osmoregulation, growth, morphogenesis, metabolism, and behaviour (Nicoll and Bern, 1972). The role of PRL in the mentioned processes has been most extensively studied in mammals. In these vertebrates, PRL is primarily responsible for the regulation of mammary gland function, among others, by stimulating the expression of genes encoding proteins of milk. Mammalian PRL also affects several reproductive processes, in particular, it stimulates steroidogenesis in the *corpus luteum*, affects the increase of the number of LH receptors in the testes, accelerates the growth of ovarian follicles. It is also considered that PRL is one of the most important hormones/factors controlling the care of the offspring.

The literature on the role of PRL in fish in most cases points to the important function of this factor in controlling osmoregulation (Ball, 1961; Yada et al., 1990), as well as melanogenesis (Pickford and Kosto, 1957) or the functioning of the epidermal cell membranes (Schreibman and Kallman, 1965; Ogawa and Johansen, 1967). However the interaction of PRL with the reproductive system is much less explored. Although reports on the involvement of PRL in the reproduction in fish are relatively scarce and highly fragmented, there is evidence that this hormone may participate in a number of phenomena related to puberty and reproduction. PRL plays an important role in reproductive behaviour (e.g. parental care) in fish (Fiedler et al., 1979), also in an indirect way – affecting the biosynthesis of steroid hormones that control such behaviour (Machamer, 1971).

Both the synthesis and release of PRL from the pituitary of fish is under the control of hypothalamic factors (Meites and Nicoll, 1966; Onuma et al., 2005). One of the most important factors affecting PRL

Supported by the Polish Ministry of Science and Higher Education (Grant No. N N303 2750 33).

synthesis and secretion is gonadotropin releasing hormone (GnRH). Especially salmon GnRH (sGnRH) is one of the most important neuroendocrine factors regulating secretion of pituitary hormones. Apart from its role in reproduction, where sGnRH is, among other, responsible for the synthesis and release of gonadotropins, sGnRH is known to affect the function of growth hormone, somatolactin, and PRL. There are data demonstrating stimulatory influence of sGnRH on PRL secretion (Weber et al., 1997). Also, there is evidence of direct action of sGnRH on PRL since in fish GnRH agonist binding sites are observed in lactotrophs (Stefano et al., 1999). Other factors influencing PRL synthesis and secretion include thyrotropin-releasing hormone (TRH), dopamine (DA), somatostatin, and serotonin (James and Wigham, 1984; Williams and Wigham, 1994a). More recent studies indicate the existence of one more PRL releasing factor localized in the hypothalamus – PrRP (Fujimoto et al., 2006). Steroids produced in the gonads, such as 17β-estradiol, are also able to control the synthesis and secretion of PRL (Williams and Wigham, 1994a; Onuma et al., 2005).

In spite of the growing amount of data on factors affecting PRL release in fish, the exact mechanism of its synthesis needs further investigations, especially in the light of its still obscured role in reproduction. Therefore the aim of this study was to investigate the influence of two important hypothalamic factors controlling reproduction in fish, sGnRH and dopaminergic system, on the synthesis of mRNA for PRL in female Prussian carp during the reproductive cycle.

MATERIAL AND METHODS

Experimental animals

For all experiments 128 sexually mature female Prussian carp (*Carassius gibelio* Bloch) bred in the Fisheries Experimental Station of the Department of Ichthyobiology and Fisheries, University of Agriculture in Krakow, were used.

Drugs used in experiments

In the experiments, the following substances were used: sGnRH-A – (D-Arg^6, Trp^7, Leu^8, Pro^{9}Net)-LHRH – an analogue of salmon GnRH (Bachem AG, Bubendorf, Switzerland) and pimozide – blocker of D2-type of dopamine receptors (Sigma-Aldrich, St. Louis, USA).

In vivo experiments

The experiments were conducted in two different periods of the reproductive cycle – during gonadal recrudescence and during preovulatory period.

Before each of the *in vivo* experiments fish were harvested from the pond and transferred to 360 l aquaria. Fish underwent a 2-day adaptation to experimental conditions – to the water temperature of 20°C and to the simulated photoperiod (in the case of experiments performed in winter the ratio of light (L) to dark (D) was 8 : 16, while in the experiments conducted in spring L : D was equal to 16 : 8). Prior to any manipulations fish were anaesthetized by immersion in Propiscin (IRS, Zabieniec, Poland) solution (0.3 ml/l of water). After a two-day adaptation to these conditions the fish were weighed and subjected to intraperitoneal injections of the tested substances, depending on the experiment.

In Experiment 1, performed in January, 32 Prussian carp females (average body weight (BW) 199 ± 21 g, gonadosomatic index (GSI) 4.8) undergoing gonadal recrudescence were used. Fish were divided into 4 groups (8 fish per group) and submitted to intraperitoneal injections of the following drugs: group 1 (control) – 0.6% NaCl; group 2 (sGnRH-A 5) – sGnRH-A 5 μg/kg BW; group 3 (sGnRH-A 10) – sGnRH-A 10 μg/kg BW; and group 4 (sGnRH-A 20) – sGnRH-A 20 μg/kg BW.

In Experiment 2, conducted in May, 32 Prussian carp females at the preovulatory period (average BW 98 ± 17 g, GSI = 18.3) were intraperitoneally injected with different concentrations of sGnRH-A, according to the same experimental design as in Experiment 1.

Experiment 3 was conducted in January on 32 recrudescent Prussian carp females (average BW 201 ± 25 g, GSI = 4.8) divided into 4 groups (8 specimens per group) and submitted to intraperitoneal injections of the following drugs: group 1 (control) – saline; group 2 (PIM 1) – pimozide 1 mg/kg BW; group 3 (PIM 5) – pimozide 5 mg/kg BW; and group 4 (PIM 10) – pimozide 10 mg/kg BW.

In Experiment 4 performed in May on 32 Prussian carp females at the preovulatory period (average BW 92 ± 16 g, GSI = 18.3) the experimental protocol was analogous as in Experiment 3.

At the end of each experiment, 24 h after intraperitoneal injections, the fish were subjected to deep anaesthesia using Propiscin solution for 15 min, then killed by decapitation. The pituitary glands were taken from all the fish and individually transferred to sterile 1.5 ml Eppendorf tubes containing 0.5 ml Trizol reagent (Life Technologies, Carlsbad, USA) in order to determine the levels of mRNA for PRL.

Determination of mRNA levels for PRL

We used the method of determining the relative level of mRNA coding for PRL by means of semi-quantitative RT-PCR. In brief, pituitary glands collected after *in vivo* experiments were subjected to RNA isolation according to standard given in Trizol-chloroform protocol. Absorbance measurements showed that in samples from the experiments RNA concentration ranged from 4 to about 22 µg/µl. The isolated RNA was subjected to reverse transcription using M-MLV reverse transcriptase (Promega, Madison, USA). The resulting DNA was amplified using the polymerase chain reaction (PCR) in order to analyze the levels of transcription of mRNA for PRL, as well as in order to investigate the levels of transcription of *β-actin* gene, which is expressed equally in all tissues and developmental stages. The following pairs of primers were used in PCR reactions:

PRL: 5' -ACACATCTCAGGTCTCTTCTTG-3'
5' -GTCGGTCTGAATGAATTACTG-3'
β-actin: 5'-GGCGTAACCCTCGTAGAT-3'
5'-GTTGGTATGGGACAGAAG-3'

These primers designed based on nucleotide sequences are available on the Internet (National Center for Biotechnology Information, USA).

After optimization of the PCR conditions, the prepared samples were placed in a thermocycler (Mastercycler Personal, Eppendorf, Germany) and PCR was performed under the following conditions: denaturation (5 min at 95°C), 32 cycles of amplification consisting of denaturation for 30 s at 95°C, annealing for 60 s at 50°C and extension for 90 s at 72°C, followed by final extension for 10 min at 72°C.

After simultaneous PCR amplification of both PRL and *β-actin*, the resulting products (10 µl per sample) were run on 1.5% agarose gel stained by ethidium bromide. The captured images were analyzed using Scion Image software (Version 4.02, 2000). On each gel, 5 µl of Smartladder marker (Eurogentec, Seraing, Belgium), showing bands of known quantity and known number of base pairs, was applied in order to evaluate the exact amount of DNA in each band. The individual results corresponding to each fish were expressed as the ratio of the density of band corresponding to PRL and that of *β-actin*.

Statistical analysis

The statistical analysis was performed with the use of GraphPad Prism 5 software (Version 5.01, 2007) for MS Windows. Results of the measurements obtained in the experiments were subjected to one-way analysis of variance (ANOVA). To determine the significance between experimental groups, Tukey's post-test was used. The results were considered as significant for $P \leq 0.05$. Results on graphs were presented as mean + standard error of mean (*SEM*).

RESULTS

Effects of sGnRH-A on PRL mRNA synthesis

In recrudescent females, subjected to intraperitoneal injections of sGnRH-A, the average relative level of mRNA for PRL in the control group was 2.61 ± 0.06. The injections of sGnRH-A at the concentration of 5 µg/kg led to statistically significant ($P \leq 0.05$) increase in the relative PRL mRNA levels to 3.57 ± 0.16. Higher sGnRH-A doses of 10 and 20 µg/kg increased mRNA for PRL to 3.22 ± 0.17 and 3.17 ± 0.20, respectively, however they did not significantly differ from the values observed in the control (Figure 1A).

In case of fish in the preovulatory stage of maturity the relative mRNA levels in the control group reached 2.61 ± 0.08. The injections of sGnRH-A at a dose of 5 µg/kg evoked an increase of mRNA levels to 3.47 ± 0.22, which have been statistically higher ($P \leq 0.05$) than in the control. Five times higher sGnRH-A dose (10 µg/kg) resulted in mRNA levels of 3.03 ± 0.17, whereas in fish receiving the highest dose of sGnRH-A (20 µg/kg) the average relative mRNA levels were 2.80 ± 0.31. The latter two concentrations of sGnRH-A did not have any statistically significant influence

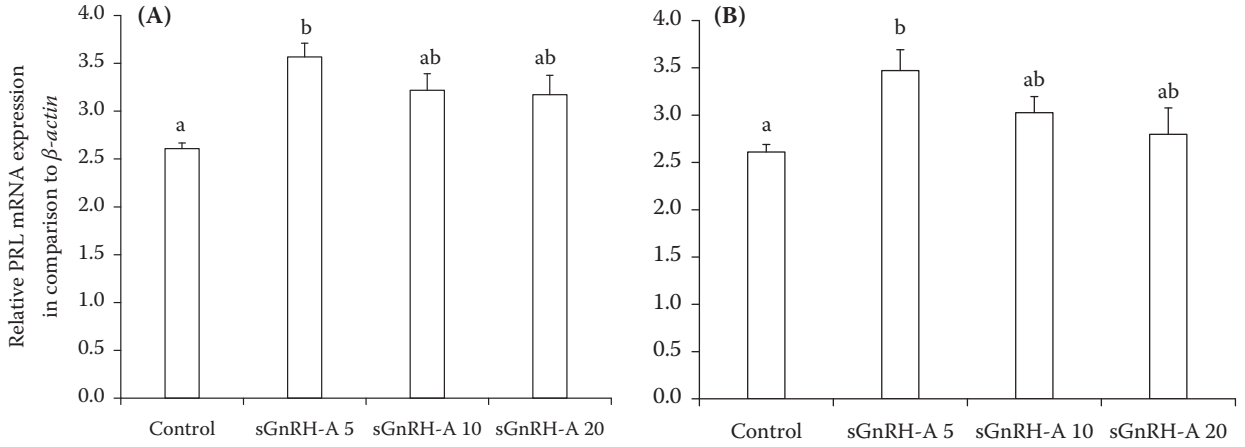

Figure 1. Relative levels of expression of mRNA encoding PRL under the influence of intraperitoneal injections of various doses of sGnRH-A in female Prussian carp pituitaries during the (**A**) recrudescence and (**B**) preovulatory period

Differences between groups were considered significant for $P \leq 0.05$. Mean values in columns marked with the same letter are not significantly different. Each column represents the average of 6–7 mRNA level measurements derived from individual fish. PRL = prolactin, sGnRH-A = salmon GnRH analogue, BW = body weight, sGnRH-A 5 = sGnRH-A 5 µg/kg BW, sGnRH-A 10 = sGnRH-A 10 µg/kg BW, sGnRH-A 20 = sGnRH-A 20 µg/kg BW

on PRL mRNA synthesis in comparison with the control (Figure 1B).

Effects of pimozide, an antidopaminergic drug, on PRL mRNA synthesis

The average relative mRNA levels in control fish at the stage of gonad recrudescence were 2.56 ± 0.06. In the group where fish received intraperitoneal injection of pimozide at a dose of 1 mg/kg (PIM 1), the relative PRL mRNA levels significantly ($P \leq 0.05$) increased to 3.02 ± 0.09. The intraperitoneal administration of PIM at the dose of 5 mg/kg evoked the relative PRL mRNA expression at the level of 2.73 ± 0.10. The highest dose of this compound (PIM 10 mg/kg) was responsible for the relative expression of mRNA at the level of 2.69 ±

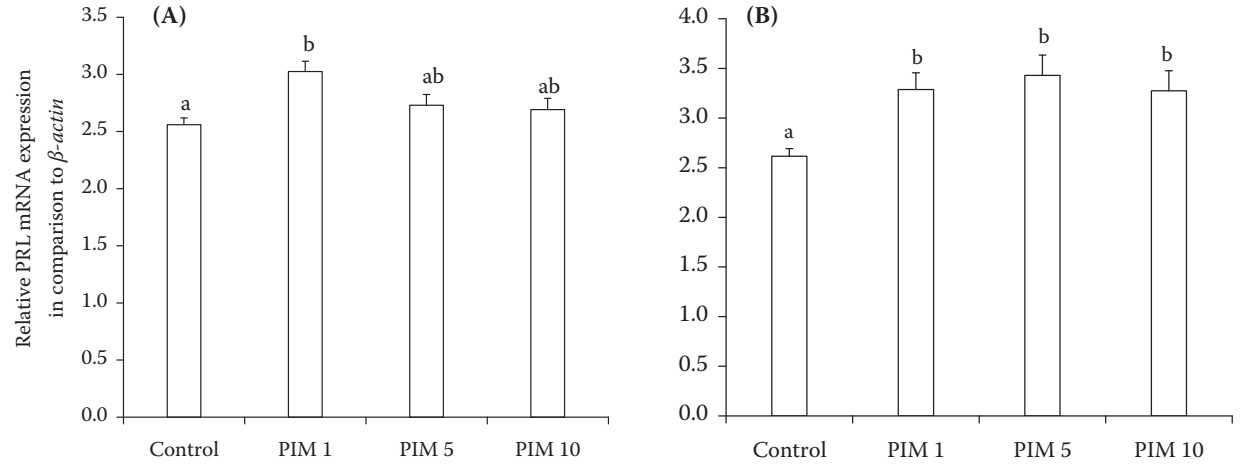

Figure 2. Relative levels of expression of mRNA encoding PRL after intraperitoneal injections of various doses of PIM in female Prussian carp pituitaries during the (**A**) recrudescence and (**B**) preovulatory period

Differences between groups were considered significant for $P \leq 0.05$. Mean values in columns marked with the same letter are not significantly different. Each column represents the average of (**A**) 6 mRNA and (**B**) 6–7 mRNA level measurements derived from individual fish

PRL = prolactin, PIM = pimozide, BW = body weight, PIM 1 = pimozide 1 mg/kg BW, PIM 5 = pimozide 5 mg/kg BW, PIM 10 = pimozide 10 mg/kg BW

0.10. There were no significant differences between PIM 5- and PIM 10-treated groups and the control (Figure 2A).

As regards preovulatory stage, the relative mRNA levels in the control group reached 2.62 ± 0.08. The treatment of fish with pimozide at the dose of 1 mg/kg evoked statistically significant ($P \leq 0.05$) rise of average mRNA levels to 3.29 ± 0.17. Five times higher dose of pimozide (PIM 5 mg/kg) was even more potent, significantly ($P \leq 0.05$) increasing PRL mRNA levels to 3.43 ± 0.21. The highest dose of PRL – 10 mg/kg – was also responsible for a significant ($P \leq 0.05$) elevation of mRNA synthesis – in this case the levels of mRNA for PRL reached 3.27 ± 0.20 (Figure 2B).

DISCUSSION

In our *in vivo* study the analogue of sGnRH-A evoked a significant increase in the relative levels of mRNA for PRL in both stages of gonad maturation. The stimulatory effects of sGnRH on PRL synthesis were more recently documented using *in vitro* techniques by Zhao et al. (2010) in goldfish (*Carassius auratus*) – a species closely related to Prussian carp. In this species sGnRH at a concentration of 100nM significantly increased PRL mRNA synthesis in dispersed pituitary cells. The above observations are to a certain extent consistent with the results obtained by Bhandari et al. (2003), who showed that sGnRH analogue stimulated the PRL mRNA synthesis in masu salmon (*Oncorhynchus masou*). Also Onuma et al. (2005) observed the stimulatory action of sGnRH on PRL mRNA synthesis in primary pituitary cells of masu salmon. The above findings show that sGnRH is not only a stimulator of PRL release, as demonstrated by Weber et al. (1997), but also plays a positive role in the synthesis of PRL.

Interesting is that in our study the stimulation of PRL synthesis was visible only after the administration of the smallest dose of sGnRH-A (5 μg/kg), whereas higher sGnRH-A doses did not significantly affect PRL synthesis. This would suggest that the concentration of GnRH that couples to their binding sites, could be an important factor influencing the response of GnRH to PRL synthesis. Indeed, there are data suggesting that high concentration of GnRH can lead to the desensitization of pituitary cells to GnRH by down-regulation of GnRH receptors. The latter can be supported by the data obtained by Onuma et al. (2005), who observed that sGnRH at the concentration of 1 ng/ml stimulated PRL synthesis before ovulation, but 100 times higher concentration of this compound did not evoke any significant effect at any of the stages of the reproductive cycle of masu salmon.

We have not observed important seasonal differences in the potency of sGnRH-A to stimulate PRL synthesis. That would suggest that action of sGnRH on PRL synthesis does not depend on the stage of reproductive cycle, contrary to the well-known seasonal changes in the responsiveness of gonadotropins secretion (especially LH) to GnRH, as shown by Sokolowska et al. (1985).

Our studies also showed the stimulatory influence of pimozide – the selective blocker of D2-type of dopamine receptors – on PRL mRNA synthesis. This compound stimulated PRL synthesis in both stages of gonad maturity. Since pimozide evokes the blockage of D2 receptors, and in consequence blocks the action of dopamine at the level of pituitary (Peter et al., 1986), the above results would suggest that dopamine exerts an inhibitory action on the synthesis of PRL in Prussian carp. The support for this hypothesis comes from the work of Tse et al. (2008) who demonstrated that dopamine inhibits PRL mRNA synthesis in goldfish (*Carassius auratus*).

We have also demonstrated that this stimulatory influence of pimozide on PRL mRNA synthesis depends on the stage of the reproductive cycle, since in recrudescent females only the smallest dose of PIM was able to significantly elevate PRL mRNA synthesis whereas in fish during the preovulatory period all doses of pimozide evoked a significant increase in the amount of mRNA for PRL. The seasonal changes of pimozide effectiveness in goldfish was also observed by Sokolowska et al. (1985), who investigated the role of the dopaminergic system in the gonadotropin release.

Taken together, the above results suggest that in Prussian carp sGnRH as well as dopamine play an important role as the factors regulating PRL production. GnRH has a stimulatory influence on PRL synthesis, and dopamine is an inhibitory factor in this process. Moreover, these data suggest that the impact of dopamine on PRL synthesis depends on the stage of the reproductive cycle, being more prominent before ovulation than during the recrudescence. This in consequence indicates the existence of the relationship between reproduction and PRL synthesis in fish.

REFERENCES

Ball N.J. (1961): Prolactin and osmoregulation in teleost fishes: A review. General and Comparative Endocrinology, Supplement 2, 10–25.

Bhandari R.K., Taniyama S., Kitahashi T., Ando H., Yamauchi K., Zohar Y., Ueda H., Urano A. (2003): Seasonal changes of responses to gonadotropin-releasing hormone analog in expression of growth hormone/prolactin/somatolactin genes in the pituitary of masu salmon. General and Comparative Endocrinology, 130, 55–63.

Fiedler K., Brus R., Christ H., Lotz-Zoller R. (1979): Behavioural effects of peptide hormones in fishes. In: Wuttke A., Weindl K., Vogt K.H., Dries R.R. (eds): Brain and Pituitary Peptides. S. Karger AG, Basel, Switzerland, 65–70.

Fujimoto M., Sakamoto T., Kanetoh T., Osaka M., Moriyama S. (2006): Prolactin-releasing peptide is essential to maintain the prolactin level and osmotic balance in freshwater teleost fish. Peptides, 27, 1104–1109.

James A.V., Wigham T. (1984): Evidence for dopaminergic and serotonergic regulation of prolactin cell activity in the trout (*Salmo gairdneri*). General and Comparative Endocrinology, 56, 231–239.

Machemer L. (1971): Synergistische Wirkung von Sauger-Prolaktin und Androgen beim Schaumnestbau des Paradiesfisches, *Macropodus opercularis* L. (Anabantidae). Zeitschrift für Tierpsychologie, 28, 33–53.

Meites J., Nicoll C.S. (1966): Adenohypophysis: prolactin. Annual Review of Physiology, 28, 57–88.

Nicoll C.S., Bern H.A. (1972): On the actions of prolactin among the vertebrates: Is there a common denominator? In: Wolstenholme G.W.E., Knight J. (eds): Lactogenic Hormones. Churchill Livingstone, London, UK, 299–317.

Ogawa M., Johansen P.H. (1967): A note on the effects of hypophysectomy on the mucous cells of the goldfish, *Carassius auratus*. Canadian Journal of Zoology, 45, 885–886.

Onuma T., Ando H., Koide N., Okada H., Urano A. (2005): Effects of salmon GnRH and sex steroid hormones on expression of genes encoding growth hormone/prolactin/somatolactin family hormones and a pituitary-specific transcription factor in masu salmon pituitary cells *in vitro*. General and Comparative Endocrinology, 143, 129–141.

Peter R.E., Chang J.P., Nahorniak C.S., Omeljaniuk R.J., Sokolowska M., Shih S.H., Billard R. (1986): Interaction of catecholamines and GnRH in regulation of gonadotropin secretion in teleost fish. Recent Progress in Hormone Research, 42, 513–548.

Pickford G.E., Kosto B. (1957): Hormonal induction of melanogenesis in hypophysectomized killifish (*Fundulus heteroclitus*). Endocrinology, 61, 177–196.

Schreibman M.P., Kallman K.D. (1965): The effects of hypophysectomy on freshwater survival and scale mucous cells in two species of freshwater fish. American Zoologist, 5, 728–736.

Sokolowska M., Peter R.E., Nahorniak C.S., Chang J.P. (1985): Seasonal effects of pimozide and des Gly10 (D-Ala6) LH-RH ethylamide on gonadotrophin secretion in goldfish. General and Comparative Endocrinology, 57, 472–479.

Stefano A.V., Vissio P.G., Paz D.A., Somoza G.M., Maggese M.C., Barrantes G.E. (1999): Colocalization of GnRH binding sites with gonadotropin-, somatotropin-, somatolactin-, and prolactin-expressing pituitary cells of the pejerrey, *Odontesthes bonariensis*, *in vitro*. General and Comparative Endocrinology, 116, 133–139.

Tse M.C., Wong G.K., Xiao P., Cheng C.H., Chan K.M. (2008): Down-regulation of goldfish (*Carassius auratus*) prolactin gene expression by dopamine and thyrotropin releasing hormone. General and Comparative Endocrinology, 155, 729–741.

Weber G.M., Powell J.F., Park M., Fischer W.H., Craig A.G., Rivier J.E., Nanakorn U., Parhar I.S., Ngamvongchon S., Grau E.G., Sherwood N.M. (1997): Evidence that gonadotropin-releasing hormone (GnRH) functions as a prolactin-releasing factor in a teleost fish (*Oreochromis mossambicus*) and primary structures for three native GnRH molecules. Journal of Endocrinology, 155, 121–132.

Williams A.J., Wigham T. (1994a): The regulation of prolactin cells in the rainbow trout (*Oncorhynchus mykiss*). 1. Possible roles for thyrotropin-releasing hormone (TRH) and oestradiol. General and Comparative Endocrinology, 93, 388–397.

Williams A.J., Wigham T. (1994b): The regulation of prolactin cells in the rainbow trout (*Oncorhynchus mykiss*). 2. Somatostatin. General and Comparative Endocrinology, 93, 398–405.

Yada T., Takahashi K., Hirano T. (1990): Seasonal changes in seawater adaptability and plasma levels of prolactin and growth hormone in landlocked sockeye salmon (*Oncorhynchus nerka*) and amago salmon (*O. rhodurus*). General and Comparative Endocrinology, 82, 33–44.

Zhao E., Grey C.L., Zhang D., Mennigen J.A., Basak A., Chang J.P., Trudeau V.L. (2010): Secretoneurin is a potential paracrine factor from lactotrophs stimulating gonadotropin release in the goldfish pituitary. American Journal of Physiology, 299, 1290–1297.

Received: 2011–10–28
Accepted after corrections: 2012–08–13

Corresponding Author

Dr hab. Jaroslaw Chyb, University of Agriculture in Krakow, Department of Ichthyobiology and Fisheries, ul. Spiczakowa 6, 30-198 Krakow, Poland

Tel. +48 126 375 176, fax +48 126 385 979, e-mail: j.chyb@ur.krakow.pl