Biologically active recombinant carp LH as a spawning-inducing agent for carp

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Abstract

Currently, spawning is induced in carp species by carp pituitary extract (CPE) and a combination of synthetic agonist of GnRH combined with a dopamine antagonist. The main goal of this study was the production of recombinant gonadotropins (GtHs) on a large scale to serve as an alternative to currently used agents. We produced carp (c) recombinant (r) Lh as a single chain in the methylotrophic yeast *Pichia pastoris*. Lha subunit was joined with Lhb subunit with a flexible linker of three glycine-serine repeats and six Histidines to form a mature protein, the β -subunit formed the N-terminal part and the α -subunit formed the C-terminal part. The ability of the rLh to elicit biological response was tested by *in vivo* stimulation of estradiol (E2) and $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one (DHP) and by its *in vivo* potency to induce ovulation and spawning induction. rLh tested in this work significantly enhanced both E2 and DHP secretion in a dose-dependent manner similar to the results obtained with CPE. E2 levels showed a moderate rise following the priming injection and a subsequent decrease during the rest of the trial. DHP levels were only increased after the resolving injection, approximately 5 h before spawning. At the highest dose of rLh (350 μ g/kg BW), the recombinant protein was more efficient than CPE in terms of both spawning success and fertilization rate. It is shown here that rLh can elicit the secretion of DHP *in vivo* and actually trigger spawning. These novel findings introduce the potential of utilizing recombinant gonadotropins in aquaculture.

Key Words

- carp
- LH
- FSH
- E2
- DHP

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Introduction

Under the control of the hypothalamus, especially by the action of specific neuropeptides and gonadotropin-releasing hormone (GnRH), the fish pituitary synthesizes and releases gonadotropins (GtHs). Follicle-stimulating hormone (Fsh) stimulates the recruitment and growth of early follicles and activates the synthesis and uptake of vitellogenin by E2 secretion, whereas luteinizing hormone (Lh) promotes final follicular maturation via

the secretion of DHP (reviewed by Levavi-Sivan *et al.* 2010). The action of these hormones requires binding to specific receptors anchored in the plasma membrane of target cells of the gonads (granulosa and theca cells), and through the activation of certain pathways, the secretion of sex steroids is initiated (Levavi-Sivan *et al.* 2010). The common carp (c) *Cyprinus carpio* is probably the oldest cultured and most domesticated fish in the world.

Carp (and related genera) represent 52% of the total aquaculture production from the world's inland waters with total harvest figures of over 28 million tons in 2014. In fish, as in mammals, within the same species, the α -subunit is encoded by a single gene and is common to all glycoprotein hormones, whereas different genes encode β -subunits that confer specificity to the glycoprotein hormone heterodimers (Levavi-Sivan *et al.* 2010). One of the major goals of aquaculture practices is to artificially stimulate spawning via the manipulation of the hormonal axis (Yaron 1995). Manipulating various environmental parameters, such as temperature, photoperiod, salinity, tank volume and depth, substrate vegetation and so forth showed improvements in spawning for particular species (Mylonas & Zohar 2001, Zohar & Mylonas 2001). However, in some species, hormonal treatments are the only means of consistently controlling reproduction. A heterologous GtH from human origin (human chorionic GtH; hCG) proved to be a powerful spawning-inducing agent in finfish reproduction (Zohar & Mylonas 2001). However, the response of cyprinid fish to hCG is notoriously poor (Kucharczyk *et al.* 1997, Yaron *et al.* 2009). Over the years, a variety of endocrine approaches have been considered to induce spawning in carps (reviewed by Yaron 1995, Yaron *et al.* 2002).

Gonadotropins, from various sources, have traditionally been used to stimulate folliculogenesis and ovulation in the treatment of infertility and in assisted reproductive technology (ART) in humans (Wallach *et al.* 1996). Similar to ART procedures, protocols currently used in aquaculture for spawning induction in carp is based on the use of native gonadotropins, such as calibrated carp pituitary extract (cCPE) (Levavi-Zermonsky & Yaron 1986). Females are injected twice at an 11-h interval, and 8–11 h after the second injection, they usually spawn. Males are given a single dose of cCPE, containing a calibrated dose of Lh, via injection to induce spawning (Yaron *et al.* 1984). An alternative agent, based on hypothalamic hormones (DAGIN, Ovaprim or LinPe), involves a combination of synthetic, highly potent GnRH agonist with a dopamine antagonist (Peter *et al.* 1988b, Drori *et al.* 1994). However, the use of ground pituitaries is associated with various drawbacks; the most hazardous one is the potential for disease transmission from donor fish to recipient. Furthermore, the harvest procedure for carp pituitaries is labor-intensive. The usage of the hypothalamic approach also includes some disadvantages. As acute GnRH α action is dependent on the releasable pool of Lh in the pituitary of the recipient fish, DAGIN potency is limited to spawning induction during spring and early summer and

is less potent at the beginning and end of the breeding season, with a low releasable pool (Kulikovskiy *et al.* 1996). The interval between hormone administration and initial egg release (latency) is long (more than 14 h) and highly variable (Drori *et al.* 1994). Due to these disadvantages, a reliable, low-cost product for artificial spawning induction with all year round availability in large quantities is needed. Recombinant Lh has all the advantages of the hypophyseal approach but does not carry the risk of distributing diseases and contamination. The main goal of this study was to establish a reliable spawning protocol that will use a single-chain recombinant carp Lh. We then wanted to determine if positively charged amino acid cluster, at the N-terminal portion of the carp α -subunit, increases biological activity as it does in mammals, thereby allowing us to determine if structural constraints on hormone–receptor interactions are conserved between teleosts and mammals.

Materials and methods

Fish

Common carp, *Cyprinus carpio*, were collected from the ponds of a local farm (Kibbutz Gan-Shmuel, Israel) and were reared under standard aquaculture conditions (Levavi-Zermonsky & Yaron 1986). All experimental procedures comply with the Animal Care and Use Guidelines of the Hebrew University and are approved by the local administrative panel on laboratory animal care.

Recombinant gonadotropin production and purification

The synthetic construct was based on the cDNA encoding the mature secreted form of cLh β (GenBank Accession No. M37380) and cLh α (GenBank Accession No. X56497) without their cognate signal peptide. A linker (Kasuto & Levavi-Sivan 2005) was introduced, in addition to a His-tag, to operate as a bridge between the β - and α -subunits.

Selection and expression

The procedure was generally performed according to Aizen *et al.* (2007a) and Kasuto & Levavi-Sivan (2005). Recombinant proteins were purified using nickel–nitrilotriacetic acid–agarose (Ni-NTA; Qiagen). As a negative control, *P. pastoris* was transformed with an expression vector that did not contain the cLH cDNA, and fractions were prepared in the same manner.

Prediction of proper folding of the recombinant protein

Proper folding was determined by a unified platform for automated protein structure and function prediction. Proteins models were prepared by the iterative threading assembly refinement server I-TASSER server (Roy *et al.* 2010). Visualization and superposition of the models were performed using Accelrys Discovery Studio package (version 2.5). All models superposed by their backbone atoms based on the multiple sequence alignment.

Transfection and luciferase reporter gene assay

Transactivation of the cLh receptor, transient transfection, cell procedures and stimulation protocols were generally conducted according to Aizen *et al.* (2012b).

Spawning induction in carp

Currently, protocols used in Israeli aquaculture for spawning induction in carp are based on that described by Levavi-Zermansky & Yaron (1986). Prior to spawning induction experiments, oocytes from females that were ready for induced spawning were sampled and cleared in Serra's fluid (ethanol: 40% formalin: acetic acid (6:3:1)). Then the position of the germinal vesicle (GV) was determined under a dissecting microscope according to Levavi-Zermansky & Yaron (1986) and Yaron & Levavi-Zermansky (1986). Oocyte maturation was categorized into the following stages: I, central GV; II, migrating GV; III, peripheral GV; IV, GV breakdown (GVBD); and V, ovulated eggs in the ovarian lumen. Only fish possessing over 60% oocytes at stage II were selected for the spawning experiments. Briefly, female common carp were injected with calibrated carp pituitary extract (cCPE) containing 0.07 mg cGtH/kg BW as a priming dose, and 11 h later, with cCPE containing 0.23–0.35 mg cGtH/kg BW as a resolving dose. Eight to 11 h after the second injection, the females spawned. In the current work, female carp were injected following the same protocol but with graded doses (5, 50, 100, 200 and 350 µg/kg BW) of rLh to precisely determine the dose of pure rLH that would lead to successful spawning. The priming dose was always 1/10 of the resolving dose. Each group consisted of at least six mature females, selected as ready for induced spawning (i.e. more than 60% of their oocytes contained eccentric (migrating) GV). Spawning success was determined by the number and volume of eggs spawned, percent of fertilization and embryo survival.

ELISA for E2 and DHP

E2 and DHP levels were determined by specific ELISAs according to established protocols (Hurvitz *et al.* 2005). The lowest limit of detection was 1.56 pg/mL for either E2 or DHP. The intra- and inter-assay coefficients of variation were less than 7% and 11%, respectively.

ELISA for carp Lh

Specific and homologous ELISA for the determination of cLH was performed according to Aizen *et al.* (2007b) with certain modifications. The ELISA was carried out using primary antibodies against cLHβ according to Aizen *et al.* (2012a). Recombinant cLHβα was used to create the standard curves. The wells were coated with cLHβ (produced according to Kasuto & Levavi-Sivan (2005); 0.2 ng/well), the antibodies were diluted 1:7000. The intra-assay and inter-assay coefficients of variation were 7.6% and 11.3%, respectively; sensitivity of the assay was 32 pg/mL.

Statistical analysis

All data are presented as mean ± S.E.M., and the significance of difference between group means were determined by one-way ANOVA followed by Newman–Keuls test with $P < 0.05$ using the GraphPad Prism 6.05 software (GraphPad).

Results

Optimizing linkers for recombinant carp Lh

Recombinant cLh production was performed as described previously (Aizen *et al.* 2012a), and to enhance the efficiency of GtH production, fusion between the two subunits by a flexible linker was required. The Gly–Ser linker sequence is minimally hydrophobic or charged and can therefore promote the chimerization of the subunits to fold into their native conformational structures and interact with their receptors. We tested four different linkers based on the Gly–Ser string: 3x(Gly–Ser), 5x(Gly–Ser), 3x(Gly₄Ser) and 2x(Gly₄Ser). The α- and β-subunits of cLH, with each of these linkers, were constructed and expressed in the yeast. The restriction enzyme *BtgI* was used to cut the His-tag linker. Two parts emerged (Fig. 1): one contained the β-subunit and the last His amino acid of the six His-tag; the second contained the cut His-tag and the linker

Figure 1

Insertion of different linkers: 3x(Gly-Ser); 5x(Gly-Ser); 3x(Gly₄Ser); 2x(Gly₄Ser) and mutation in the α -subunit 11–20 region, Glu(E) and Asp(N), at positions 14 and 16, respectively, to Lys(K) residues.

attached to the α -subunit. All tested linkers were added to the second part with specific primers (Table 1), ligation was performed and the new construct with the novel linker was subcloned into pPIC9K expression vector.

To better predict correct folding of the rLh bearing the various linkers, the constructs were further analyzed by I-TASSER server. According to the I-TASSER server results, the proteins with the 3x(Gly-Ser), 3x(Gly₄Ser) and 2x(Gly₄Ser) were properly folded and exhibited the same binding sites (Fig. 2A, C and D, respectively). The protein with the 5x (Gly-Ser; Fig. 2B) linker lacked an active binding site due to the distortion effect on the protein structure. The protein with the 2x(Gly₄Ser) linker distorted the protein as well, but the binding site was not affected. The four proteins were produced after their constructs were inserted into the *Pichia* vector (3x(Gly-Ser); 5x(Gly-Ser); 3x(Gly₄Ser); 2x(Gly₄Ser)) and tested for activation by the GtH receptor assay (Fig. 3).

When 3x(Gly-Ser) linker was used, and the construct was significantly more potent at stimulating cAMP production, with $EC_{50}=44.24\pm0.05$. However, when the linker was 3x(Gly₄Ser), the activity was reduced ($EC_{50}=20.81\pm0.30$; Fig. 3). The other two linkers, 5x(Gly-Ser) and 2x(Gly₄Ser) were not effective at all (Fig. 3). These results were aligned with the predictions of the

I-TASSER server, and based on these results, we continued the *in vivo* experiments with the construct containing 3x(Gly-Ser) as a linker.

Formation of carp Lh analog

After determining the most efficient linker (3xGly-Ser), the next step was to produce a cLh analog that would enhance the biological activity. The amino acid 11–20 in the α -subunit of vertebrate LH contains a cluster of lysine residues forming a domain that is critical for receptor binding and signal transduction. This domain is shown to play an important role in the evolution of glycoprotein hormone activities (Szkudlinski *et al.* 1996). Two amino acids (glutamic acid (Glu; E) and asparagine (Asn; N), at positions 14 and 16, respectively, in this region) were replaced by lysine (Lys; K) residues giving rise to a mutated cLH, E14K+N16K. The altered sequence was subcloned into pPIC9K and was expressed.

In vivo experiments

We tested the rLh in *in vivo* trials. A large amount of protein was produced from 2L media, yielding $313.66\pm2.29\mu\text{g/L}$ ($n=8$). The average time for protein

Table 1 Primers used for amplification and insertions of linkers.

Primer	Sequence
2x(Gly ₄ Ser)-linker	CCACGGAGGTGGTGGATCTGGAGGTGGTGGATCTTACCCAAGAA
3x(Gly ₄ Ser)-linker	CCACGGAGGTGGTGGATCTGGAGGTGGTGGATCTGGAGGTGGTGGATCTTACCCAAGAA
5xGS-linker	CCACGGTTCTGGTTCTGGTTCTGGTTCTGGATCTTACCCAAGAA
carpLHba-1F	GAATTCTTACTTGCCACCATGTG
carpLHba-689R	GCGGCCGCTTAGGACTTGTGGTAGTAA
pic-seq-F	TACTATTGCCAGCATTGCTGC
pic-seq-R	GGCAAATGGCATTCTGACATCCTC

Figure 2

Models of rcLh $\beta\alpha$ with different linkers using the I-TASSER server. The resulting models for (A) 3x(Gly-Ser); (B) 5x(Gly-Ser); (C) 2x(Gly₄Ser); (D) 3x(Gly₄Ser); the common α -subunit is marked in purple, the β -subunit in light blue, the His-tag in red and the linker in orange. Binding sites in the α -subunit (residues 192, 199, 218) are marked in dark purple and in the β -subunit (14, 26, 60–64, 75–79) in dark blue.

production was 3 days of growth and 3 days of induction followed by 4 days of purification and validation. CPE served as a positive control, whereas saline as a negative one. Mature female carp were examined and found ready to spawn (i.e. more than 60% of their oocytes contained eccentric (migrating) GV's (Levavi-Zermonsky & Yaron 1986, Yaron & Levavi-Zermonsky 1986, Yaron *et al.* 2009)). rcLh $\beta\alpha$ was tested at doses of: 5, 50 and 100 μ g/kg BW, all doses administered were followed by increased level of both E2 and DHP, the response being dependent on the dose (Fig. 4A). After injection by either CPE or

100 μ g/kg BW rcLh $\beta\alpha$, E2 levels followed a similar pattern: a rise after the first injection and a subsequent decrease throughout the rest of the trial. However, E2 levels in fish injected with 5 or 50 μ g/kg BW did not reach the levels as high as in those of fish treated with CPE (Fig. 4A). As shown in Figure 4B, rcLh $\beta\alpha$ at 50 and 100 μ g/kg BW was successful at elevating DHP levels to 4.17 ± 1.43 ng/mL after the priming injection similar to the effect observed after CPE injection. No change was observed in DHP levels in the control fish or the lowest dose (5 μ g/kg BW) as the priming injection (Fig. 4B). After the resolving injection,

Figure 3

rcLHs induced CRE-derived transcriptional activity. COS-7 cells were transiently co-transfected with cLHR and with the reporter plasmid pCRE-LUC. Cells were stimulated for 6 h with cLH β with different linkers (3xGly-Ser, 5xGly-Ser, 3xGly₄Ser and 2xGly₄Ser) at different concentrations. Luciferase activity was determined, and results are presented as % of maximum response. Each assay was repeated at least three times. Data are presented as mean $\pm$ S.E.M. of a representative experiment, performed in triplicate. The EC₅₀ values are calculated from the nonlinear regression curve.

hormone levels in fish injected with CPE continued to rise, resulting in spawning, whereas rcLh β at doses of 5, 50 and 100 μ g/kg maintained the same level of DHP, and no spawning was observed. All females injected with CPE spawned, while none of the rcLh β -injected or control fish spawned (Table 2). CPE priming injection was followed by an increase in cLH levels up to 607.23 ± 10.56 ng/mL (Fig. 4C). As expected, injection of the resolving dose was followed by a further increase in LH, reaching a level of 954.47 ± 26.50 ng/mL. The rcLh β at the 100 μ g/kg dose induced similar levels as the CPE treatment after the priming injection, but LH levels reached only 547.93 ± 88.15 ng/mL following the resolving dose.

We next tested higher doses of rcLh β (200 μ g/kg) and rcLh β that was mutated in the α -subunit (rcLh β -mut; E14K+N16K) at two doses (100 μ g/kg and 200 μ g/kg). In fish that were injected with 200 μ g/kg BW, rcLh β -mut and rcLh β , we found the same trend: elevated levels of E2 and DHP appeared after the priming injection and again after the resolving injection, DHP reached 3.23 ± 1.01 ng/mL; the CPE group reached a level of 21.62 ± 5.84 ng/mL (Fig. 5A and B). Injection of the CPE priming dose was followed by an increase in cLH levels up to 256.46 ± 13.39 ng/mL (Fig. 5C). Injection of the resolving dose increased LH levels, reaching 697.88 ± 4.45 ng/mL. Consistent with the DHP results, the LH levels showed a similar pattern. In the 200 μ g/kg BW rcLh β -mut and 200 μ g/kg BW rcLh β groups, three out of six females actually spawned (25% of fertilization), while fish injected with 100 μ g/kg, no spawning was observed. In the CPE-injected group, all females spawned (65% of

Figure 4

Concentration of E2 (A), DHP (B) and cLH (C) in the plasma of female carp, after injection of carp pituitary extract (CPE) or different doses of cLH β (5, 50 and 100 μ g/kg). Black arrows indicate priming (at 12:00h) and resolving (at 22:00h) injections, respectively. Data are presented as mean $\pm$ S.E.M.; Statistically significant differences from controls are marked by asterisks (* P < 0.05, ** P < 0.01 and *** P < 0.001).

fertilization) (Table 2). A higher dose of rcLh β (350 μ g/kg) was tested, and the priming injection caused elevation of E2, whereas the resolving injection triggered an increase in DHP (Fig. 6A and B). E2 levels of the rcLh β -injected females did not reach as high levels as the CPE-treated group. DHP levels, both in rcLh β and CPE-injected fish, reached 21.64 ± 13.86 ng/mL and 19.21 ± 5.69 ng/mL, respectively after the resolving injection. As expected, injection of the CPE and rcLh β was followed by an increase in cLH circulatory levels. In fish injected with rcLh β (350 μ g/kg BW), eight out of ten females spawned (22% of fertilization), fish injected with saline did not spawn at all, whereas in the CPE-injected group, all females spawned (31.5% of fertilization) (Table 2).

Table 2 Female body weight, oocyte diameter and latency period are expressed as mean $\pm$ S.E.M.

Treatment	No. of injected females	No. of spawned females	Female body weight (g) ¹	Oocyte diameter (μ m) ²	Latency period (h) ³	Spawning success (%) ⁴	Fertilization rate (%) ⁵
Exp.1							
Control (Saline)	6	0			–	–	–
rcLH β α (5 μ g/kg)	6	0			–	–	–
rcLH β α (50 μ g/kg)	6	0	153.90 $\pm$ 6.86	1290 $\pm$ 20.20	–	–	–
rcLH β α (100 μ g/kg)	6	0			–	–	–
CPE	6	6			10.5	100	93
Exp.2							
Control (Saline)	6	0			–	–	–
rcLH β α (200 μ g/kg)	6	3	186.9 $\pm$ 10.78	1310 $\pm$ 34.76	12	50	25
rcLH β α (100 μ g/kg) mut	6	0			–	–	–
rcLH β α (200 μ g/kg) mut	6	3			12	50	23
CPE	6	6			10.5	100	65
Exp.3							
Control (Saline)	10	0			–	–	–
rcLH β α 350 μ g/kg	10	8	569.71 $\pm$ 62.76	1370 $\pm$ 10.34	10.5	80	31.5
CPE	10	10			10.5	100	22

¹Mean $\pm$ S.E.M. of female BW (g) in experiment group. ²Mean $\pm$ S.E.M. of oocyte diameter (μ m) in experiment group. ³Latency periods: time (h) between resolving injection and spawning. ⁴Spawning success (%): the number of females that ovulated after injection, divided by the total number of injected females. ⁵Fertilization (%): the number of eggs that were fertilized divided by the total number of eggs sampled.

Figure 5 Concentration of E2 (A), DHP (B) and cLH (C) in the plasma of female carp, after injection of carp pituitary extract (CPE), cLH β α (200 μ g/kg) and cLH β α (mut) at different doses (100 and 200 μ g/kg). Black arrows indicate priming (at 12:00h) and resolving (at 22:00h) injections, respectively. Data are presented as mean $\pm$ S.E.M. Statistically significant differences from controls are marked by asterisks (* P < 0.05, ** P < 0.01 and *** P < 0.001).

Discussion

The present study demonstrates the use of recombinant cLh for spawning induction in carp species. Biological availability was proven through *in vivo* stimulation of E2 and DHP secretion and by its potential to induce ovulation and spawning. For the last three decades, two main spawning induction agents have been used for spawning induction in carp species: CPE and an agent that is based on hypothalamic hormones like DAGIN, LinPe or Ovaprim (Peter *et al.* 1988a, Drori *et al.* 1994, Brzuska 2005). The CPE is mainly used to support spawning at the beginning and at the end of the seasonal reproduction period, whereas the rest are commonly used in mid-season. The use of native pituitaries, however, has some drawbacks, the most important is the transmission of hazardous material from donor fish to recipient broodstocks. Moreover, collection of pituitaries has to be carried out by trained stuff and is labor-intensive, which makes it expensive. In recent years, there has been a shortage of carp glands in the international market due to herpes virus disease among carp and koi (Davidovich *et al.* 2007), which lead to a legal restriction for importing fresh and processed pituitaries in several countries. The DAGIN/LinPe/Ovaprim method is widely used, but has some shortcoming. As acute GnRH α action is dependent on the endogenous releasable pool of Lh in the pituitary of the recipient fish, their potency is limited to spawning induction during spring and early summer and is less potent at the beginning and end of

Figure 6

Concentration of E2 (A), DHP (B) and cLH (C) in the plasma of female carp, after injection of carp pituitary extract (CPE) or cLHβα (350 μg/kg). Black arrows indicate priming (at 12:00 h) and resolving (at 22:00 h) injections, respectively. Data are presented as mean ± S.E.M. Statistically significant differences from controls are marked by asterisks (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).

the breeding season when this pool of the hormone is generally low (Kulikovsky *et al.* 1996, Yaron *et al.* 2009). In addition, when using DAGIN, the latency is relatively long and variable (Drori *et al.* 1994). Due to these disadvantages, a need for a new approach emerged and the use of recombinant GtHs was suggested. It was anticipated that the next generation of spawning induction agents would involve the use of molecular tools to produce recombinant GtHs at an affordable cost (Yaron *et al.* 2009). Earlier work conducted in our laboratory showed that the *P. pastoris* expression system can be used to achieve single-chain tethered LH and FSH of tilapia (Kasuto & Levavi-Sivan 2005, Aizen *et al.* 2007a). We used a *P. pastoris* expression system to produce rLH in large scale. Most of the GtHs expressed to date in *P. pastoris* in tethered form have been hCGs (Sen Gupta & Dighe 1999, 2000).

In hCG, last 30 amino acids of the β-subunit contain a C-terminal peptide (CTP) that plays a pivotal role as a linker (Sugahara *et al.* 1995). Only when the CTP is placed on either the C-terminal end of the FSH or LH β-subunits or in the N-terminal region of the α-subunits, assembly, secretion and signal transduction of the dimers are comparable to the wild-type hormones (Fares *et al.* 1992, Furuhashi *et al.* 1995, Sugahara *et al.* 1995). As cLh lacks any CTP-like structure at its C terminus, the fusion between the two subunits needed to be bridged by a flexible linker. A bridge consisting of Gly-Ser was chosen due to the fact that the linker sequence had to be minimally hydrophobic or charged, to maximize the opportunity of chimerization of the subunits to fold into their native conformational structures. This is crucial for their receptor-binding ability. Previously (Kasuto & Levavi-Sivan 2005, Aizen *et al.* 2007a), we used a linker that consisted of three pairs of Gly-Ser that provided a certain distance between the α- and β-subunits. In recent years, progress in the production of recombinant GtHs has made the generation of single-chain analogs of originally dimeric proteins more feasible (Adams & Boime 2008). The single-chain technology involves transfection of cells with DNA encoding both subunits combined in a single gene construct. Over the last decade, the use of computational (*in-silico*) methods has been developed and applied as a tool for pharmacological hypothesis, development of active component and studying their biological activities. We used an *in-silico* method that was further verified by *in vivo* experiments. Using modeling approach, we analyzed the sequences and the predicted structures of the proteins we intended to produce with regards to their native conformational structures and folding. Using the I-TASSER platform (Roy *et al.* 2010), four linkers were tested based on combinations of Gly-Ser repeats: 1. 3x(Gly-Ser), 2. 5x(Gly-Ser), 3. 3x(Gly4Ser), 4. 2x(Gly4Ser). The model construct bearing the 5x(Gly-Ser) linker showed no deformity but lacked an active binding site as well, probably due to the length of the linker that extended the distance between the subunits, which was too long for forming a binding pocket. All other constructs (1, 3 and 4) showed structural similarity and presented the same predicted binding pocket. Using these data, we were able to produce recombinant GtHs bearing linkers that showed proper folding; furthermore, the *in-silico* method was found to be reliable in predicting the constructs' potency for GtHR activation. The rLhβα was examined for its ability to stimulate cAMP production in COS-7 cells expressing cLhR. This enabled us to compare the potency of different rLHs with the activity of native

cLh purified from carp pituitaries. We found that the only rLh carrying the 3x(Gly-Ser) linker could activate cLHR opposite to the 5x(Gly-Ser) construct, which showed no binding site in the modeling analysis, was also found to lack any activation of the receptor, which implies that the *in-silico* method is useful in predicting which linkers have the potential to produce a successful single-chain molecule. The bioactivity of the single-chain analogs demonstrated that the folding of the α and β components into the configuration required to associate with the GtHR is not impaired by the single-chain organization of the α and β domains. The correct folding is likely facilitated by the intervening linker sequence. Importantly, the high proportion of Ser and Gly residues in the linker sequence makes this segment highly flexible and allows the α and β domains of the single chain to fold into the active configuration. An additional benefit of incorporating the linker sequence into the single chain is that, much like the dimeric CTP-modified GtHs, the CTP linker or CTP portion of the single chain reduces the rate of clearance (Fares *et al.* 1992). In this regard, recombinant human FSH produced in the CHO system bearing a Gly-Ser-based linker with the addition of N-linked glycosylation sites showed that the FSH analogs had higher biological capability. The addition of multiple carbohydrate side chains does not appear to interfere with protein folding and in fact, amplified glycosylation further enhances ovarian follicle growth in rats (Weenen *et al.* 2004). Furthermore, N-linked analogs are easier to design because signal sequences are known for N-linked glycosylation.

In the current work, after optimizing the protein with a suitable linker, the ability of rLh to elicit a biological response was examined by *in vivo* stimulation of E2 and DHP secretion and by its potency to induce ovulation and spawning in mature carp females. The use of rLh administered in two injections, as described in Levavi-Zermonsky & Yaron (1986), resulted in elevated E2, DHP and cLh levels, culminating actual spawning at the high-dose treatment (350 µg/kg). All doses tested in this work significantly enhanced both E2 and DHP secretion in a dose-dependent manner, similar to injection of the native GtHs present in the CPE. E2 levels after injection with either CPE or graded doses of rLH followed a similar pattern: a rise after the priming injection and a subsequent decrease throughout the remaining duration of the trial. DHP levels were elevated after the resolving injection, similar to the effect observed with CPE. These results are in line with previous findings in carp (Levavi-Zermonsky & Yaron 1986, Kime & Bieniarz 1987)

as well as in other fish species (Levavi-Sivan *et al.* 2010). Proceeding resolving injection, DHP levels in the fish injected with CPE continued to rise, with a peak at 0300h, 5h before spawning. Only the high-dose treatment of 350 µg/kg rLh mimicked the CPE treatment with regard to DHP levels. The 200 µg/kg rLh elicited DHP increase but with lower levels, and hence, a lower rate of spawning. It is assumed that the moderate increase in cLh after the priming injection stimulated the formation of the maturation-inducing hormone (MIS) DHP receptors on the plasma membrane of the oocytes rendering them sensitive to the MIS surge. Indeed, in sea trout, 6h after exposure to GtH, a two- to four-fold increase in oocyte and ovarian MIS receptors and the development of oocyte maturational competence (OMC: the ability to complete oocyte maturation *in vitro* in response to exogenous MIS (Thomas *et al.* 2001)) were observed. The decrease in E2 levels that was concomitant with the peak in DHP demonstrates a shift in the steroidogenic pathway from the formation of estrogens towards the formation of progestogens; this may involve a decrease in 17–20 lyase activity and a rise in 20 β hydroxysteroid dehydrogenase activity. In a number of teleost species, two genes encoding 17 α hydroxylase (P450c17) have been detected. One is P450c17-I, which is similar to that in tetrapods and also displays the lyase activity that produces C19 steroids (androstenedione or dehydroepiandrosterone) which may serve as a precursor for estrogens. This type of P450c17 is expressed in the ovarian granulosa cells of vitellogenic follicles. P450c17-II encodes a 17 α hydroxylase that is devoid of lyase activity and fully expressed in oocytes only during final oocyte maturation (Zhou *et al.* 2007, Nagahama & Yamashita 2008). Irrespective of the mechanism leading to the steroidogenic shift, E2 and its 7TMD receptors (GPR30=GPER) in the oocytes maintain meiotic arrest. Therefore, the decrease in E2 is essential for the resumption of meiosis during oocyte maturation (Pang & Thomas 2009, Pang & Thomas 2010). Recent work by Majumder *et al.* (2015) demonstrated the presence of such a mechanism in the common carp. The rLh managed to elicit spawning as the CPE treatment. In the 200 µg/kg treatment, only 50% of the females spawned and a low percentage of fertility was achieved (around 25%). Moreover, the latency period in females treated with rLh was about 1.5 to 3 h longer than that in CPE-treated fish; this could be related to the fact that the DHP peak occurred later, and therefore, the spawning was delayed.

The 11–20 region in the α -subunit is a cluster of basic residues present in all vertebrates except hominoids (apes and humans) and has been recognized as an important motif in the evolution of GTH bioactivity in primates (Szkudlinski 2004). Carp, catfish and goldfish possess lysine in positions 11, 13 and 20, and bovines have lysine residues in positions 11, 13, 16 and 20, whereas humans have no lysine residues between positions 11–20 of their α -subunit (Szkudlinski 2004). As the insertion of basic amino acids at positions 11–20 increase the binding and activation of the LH receptor in humans (Szkudlinski *et al.* 1996), we assumed that this is an ancient and conserved feature of glycoprotein hormones and their receptors, having evolved before the divergence of the LH and TSH-Rs. Surprisingly, the mutated cLh (E14K+N16K) did not exhibit any difference in hormone levels compared to the native rLh and resulted in the same spawning ratio, fertility and latency period. Thus, the Lys substitution in the carp α -subunit had no effect on the binding to its receptor or activation of rLh, an effect that was prominent when applied to hCG (Szkudlinski *et al.* 1996). This can be explained by the large amount of basic residues which already exist in the carp 11–20 region in the α -subunit (3 basic residues (Lys) at positions 11, 13 and 20). Recently, Miller and coworkers (Miller *et al.* 2012) found that the four positively charged lysines that substituted for neutral or negatively charged amino acids within positions 11–20 of the glycoprotein hormone α -subunit, significantly increased the biological activity of hTSH in goldfish and in mammals. It should be noted that a recombinant human FSH analog based on mutated residues in the same region (Q13R+E14R+P16R+Q20R) increased T_4 response *in vivo* in goldfish, whereas recombinant hFSH exhibited no thyrotrophic activity (Miller *et al.* 2012). Nevertheless they noted that the protein structure of GTH β -subunit in goldfish could also have contributed to the heterothyrotropic activity of the gonadotropin dimers. The promiscuity of the GTH and TSH receptors in fish in general and carp in particular need further investigation.

To date, only a few studies have shown *in vivo* use of recombinant GtHs in fish. In eel, the biological activity of recombinant GtHs was demonstrated in various *in vitro* assays. However, these recombinant GtHs exhibited little activity in the gonads when administered *in vivo* (Kazeto *et al.* 2008). Interestingly, recombinant FSH of Japanese eel was able to induce testicular growth and spermatogenesis in immature eels (Kamei *et al.* 2006, Ohta *et al.* 2007). In the orange-spotted grouper (g), treatment with rgLh resulted in marked increases in the mRNA levels of Lh β in the pituitary, hypothalamus

and gonad of immature groupers. The mRNA levels of cyp19a1a and cyp19a1b also rose dramatically after injecting rgLh intraperitoneally (Cui *et al.* 2007). In Manchurian trout, single injection with trFsh, but not trLh, significantly increased mean GSI and follicle diameters compared with those of control fish, 3 days after injection (Ko *et al.* 2007). In goldfish, the production of single-chain Fsh and Lh was tested *in vivo* using male goldfish, female bitterling Rhodeus (*Ocellatus ocellatus*) and male Japanese eel. Injection of silkworm hemolymph containing single-chain FSH or LH induced milt production in male goldfish. Single-chain LH induced ovulation in bitterlings but single-chain FSH showed lower potency (Hayakawa *et al.* 2008). Recently, an injection of recombinant single-chain Fsh in sea bass increased the plasma levels of Fsh, which led to an increased 11-KT levels as well (Mazón *et al.* 2014).

Manipulation of reproduction processes by the administration of recombinant GtHs are frequently used in ART (Andersen & Krummen 2002), but it is still not commonly used in livestock, and its application in aquaculture is essentially non-existent (Levavi-Sivan *et al.* 2008, Levavi-Sivan *et al.* 2010). The production of active GtHs in fish species can open a new age in aquaculture, as specific homologous treatments can be designed for the different reproductive disorders found in the domestication of new species.

To conclude, to the best of our knowledge, this is the first time that the production of a recombinant glycoprotein in fish, i.e. cLH, has been shown to elicit the production and secretion of DHP in *in vivo* trials and elicit actual spawning. These novel findings raise the possibility of using the recombinant hormone as a substitute for the currently used CPE and DAGIN/LinPe in carp spawning and in other fish species.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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