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Recombinant luteinizing hormone as a tool towards understanding reproductive dysfunction in captive Macquarie perch *Macquaria australasica*

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ABSTRACT: The Macquarie perch *Macquaria australasica* is an endangered freshwater fish endemic to the Murray-Darling Basin in Australia. Captive breeding is considered an essential measure to save the species from extinction, yet its reproductive biology is not fully understood. We produced a recombinant single-chain Macquarie perch luteinizing hormone (rmpLh) in the methylotrophic yeast *Pichia pastoris*, confirmed by Western blot analysis and mass spectrometry. We developed and validated a heterologous competitive enzyme-linked immunosorbent assay (ELISA) for *M. australasica* Lh using antibodies generated against the giant grouper *Epinephelus lanceolatus* Lhb, which is 91% identical to the *M. australasica* sequence at the amino acid level. Measurement of Lh in plasma samples collected at 2 timepoints (June vs. August 2020) from captive F1 broodstock did not show a significant difference. Plasma Lh in samples from wild fish collected in September 2020, a month prior to the spawning season, were almost 10-fold higher than those of the F1 broodstock. The tools generated here will help us to understand the reproductive biology of *M. australasica* and develop reliable assisted reproduction techniques for the species.

KEY WORDS: Endangered Percichthyidae · Gonadotropins · Murray-Darling Basin

1. INTRODUCTION

The Macquarie perch *Macquaria australasica* of the family Percichthyidae is an endemic Australian freshwater fish species which was once abundant in the inland Murray-Darling Basin and across 3 coastal drainage basins, Hawkesbury-Nepean, Shoalhaven and Georges River (reviewed by Cadwallader 1978, Ingram et al. 1990). The species was declared nationally endangered in 1998, when a resident population in the Shoalhaven Basin became extinct (Ingram et al. 2000). Existing fragmented populations have low genetic diversity (Pavlova et al. 2017).

In New South Wales, efforts over the years to reliably breed *M. australasica* using various hormonal treatments yielded variable results, with fertilisation rates ranging from 0 to 60% (D. Gilligan unpubl. data). Nevertheless, an F1 generation was reared in captivity to maturity, but they have never spontaneously spawned. Data from breeding programmes in New South Wales and Victoria showed reproductive dysfunction typical of broodstock held in captivity where

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the key reproductive cues are absent (Zohar & Mylonas 2001, Mylonas et al. 2010). Oocytes from captive-held *M. australasica* broodstock did not complete vitellogenesis and final oocyte maturation (Ingram et al. 1994).

In fish held in captivity, one of the reasons preventing final gamete maturation and spawning is the failure of gonadotropin secretion from the pituitary gland (Zohar & Mylonas 2001). Recombinant gonadotropins are valuable in overcoming reproductive failure in captive broodstock and for characterising the hormonal profile of a cultured species, leading to a better understanding of its reproductive biology (Aizen et al. 2017, Molés et al. 2020, Ramos-Júdez et al. 2021).

Towards understanding the reproductive biology of *M. australasica*, we produced a recombinant Macquarie perch luteinizing hormone (rmpLH) and developed a competitive enzyme-linked immunosorbent assay (ELISA) using antibodies against the giant grouper *Epinephelus lanceolatus* Lh beta subunit (Lhb), which is 91% identical to that of *M. australasica*. Results here will enhance knowledge of the reproductive biology of *M. australasica* and development of reliable hormonal therapies for spawning captive broodstock.

2. MATERIALS AND METHODS

2.1. Fish sources and collection of plasma

All activities involving live fish were performed according to animal ethics permits DPI NSW ACEC REF 05/06 from the Department of Primary Industries, New South Wales (NSW), and ANA/20/170 from the University of the Sunshine Coast.

First generation, hatchery-reared fish (F1) were obtained after wild-caught *Macquaria australasica* were induced to spawn in 2014 using techniques similar to those described by Trueman (2007). Juvenile fish were PIT-tagged and then held in an earthen pond at the Narrandera Fisheries Centre, NSW, where they were on-grown for 6 yr under ambient environmental conditions with access to live feeds including crustaceans, insects and fish larvae that developed naturally within the pond. After 6 yr, the fish were expected to be mature but spawning within the pond was never observed.

Blood samples were obtained from the F1 broodstock in June 2020. As fish were expected to spawn in October, blood samples were again collected from randomly caught fish in August 2020. Fish were captured from the pond using Fyke nets and transferred to a 2000 l hatchery tank filled with ambient fresh bore water. Individual fish were anaesthetised in a 40 l tub with 25 ppm AQUI-S®, then weighed and measured for length (Table 1). Blood was collected from the caudal vein using heparinised syringes and centrifuged at 3220 × *g* for 15 min. Plasma was transferred to fresh tubes and then stored at −80°C until transport to the University of the Sunshine Coast on dry ice where it was stored at −80°C until analysis.

Wild *M. australasica* were collected from Upper Murrumbidgee and Abercrombie Rivers in central New South Wales in September 2020. Fish were transported to Narrandera Fisheries Centre and stocked in a pond similar to that for the F1 fish. Prior to the transport, blood samples were collected, and plasma was processed as described for the F1 fish. Fish were anaesthetised with 25 ppm AQUI-S® prior to blood collection. Sexes were not determined (not biopsied). The fish average (±SD) body weight was 851 ± 93 g.

2.2. Production of *M. australasica* rmpLh and analysis by Western blot and mass spectrometry

The gene sequences encoding for the mature *M. australasica Lhb* and common gonadotropin a (*cga*) subunits were retrieved from the *M. australasica* genome (NCBI GenBank BioProject PRJNA516983) by BLAST tblastn, using as a query sequence the *Epinephelus lanceolatus Lhb* (from *E. lanceolatus* transcriptome NCBI BioProject number PRJNA413272) and *cga* (GenBank accession number MH262555). The *M. australasica* Lhb amino acid sequence was deduced using the Expasy Translate tool (web.expasy.org/translate). The deduced amino acid sequence was aligned with sequences from other species of fish in the Order Perciformes by CLUSTAL W

Table 1. Average body weight and length of sampled F1 *Macquaria australasica* and their corresponding average plasma luteinizing hormone (Lh) levels (mean ± SD)

Sampling schedule		Number sampled	Body weight (g)	Length (mm)	Plasma Lh (ng ml^{-1})
Jun 2020	Male	17	534 ± 102	321 ± 14	11.2 ± 4.5
	Female	22	577 ± 120	327 ± 21	11.1 ± 4.8
Aug 2020	Male	6	556 ± 98	323 ± 14	11.8 ± 5.0
	Female	7	658 ± 75	338 ± 13	10.0 ± 1.9

(Thompson et al. 1994). The molecular weight was calculated using the Protein Molecular Weight Calculator software (https://www.bioinformatics.org/sms/prot_mw.html).

The deduced *M. australasica* Lhb and alpha (cga) mature peptide sequences were compared phylogenetically with mature Lhb, follicle stimulating beta (Fshb) and cga sequences in other fish species as well as with the corresponding golden perch *M. ambigua* sequences, which were obtained from *M. ambigua* (NCBI GenBank BioProject PRJNA556086). The phylogenetic analysis was performed in MEGA X (Kumar et al. 2018) following the maximum likelihood method and a JTT matrix-based model (Jones et al. 1992).

To produce the single-chain rmpLh, an expression construct was designed following that of Sanchís-Benlloch et al. (2017) and Dennis et al. (2020) and produced by a commercial provider (Genscript, Hong Kong). Preparation of the expression plasmid, electroporation of the expression construct into *Pichia pastoris* and identification of high-expressing transformants was as previously described (Aizen et al. 2007, Sanchís-Benlloch et al. 2017, Palma et al. 2019). Briefly, high-expressing yeast clones were grown in 1 l buffered minimal methanol medium for 72 h with shaking, and the temperature was gradually adjusted from 30 to 24°C. Methanol was added daily at 1% of the culture medium. The recombinant hormone was collected from the medium using Ni-NTA Superflow beads (Qiagen) according to the manufacturer's protocol, and then further purified using Amicon centrifugal filters (10 kDa cut-off; Millipore). Protein concentration was determined using a NanoDrop 2000 (Thermo Scientific).

Intact and deglycosylated recombinant peptides were assessed by Western blot analysis (Sanchís-Benlloch et al. 2017) with minor modifications. Briefly, peptide (~5–20 µg) was electrophoresed for 1.5 h at 160 V in gradient (8–16%) precast polyacrylamide gels (BioRad), then transferred into nitrocellulose membrane (Transblot Turbo, BioRad), blocked with BSA, and then incubated overnight with antiserum against the *E. lanceolatus* Lhb (diluted 1:4000 in the blocking buffer) (Dennis et al. 2020). The secondary antibody was goat anti-rabbit IRDye 680 LT (LI-COR Bioscience). Bands were visualised at the 700 CW channel of the LI-COR Odyssey Infrared Imaging system (LI-COR Bioscience). Positive control was deglycosylated single-chain recombinant *E. lanceolatus* Lh (rggLh).

To further confirm the production of rmpLh, purified peptide was subjected to in-solution digestion and then analysed by uHPLC tandem mass spectrometry (Sciex X500R QTof) following Ni et al. (2018).

2.3. Development of *Macquaria australasica* Lh ELISA and measurement of plasma Lh

Using the rmpLh, a competitive ELISA was developed according to that of *E. lanceolatus* Lh (Dennis et al. 2020). The serially diluted rmpLh, *M. australasica* plasma, maximum binding (Bo) and non-specific binding (NSB) reactions were prepared in phosphate-buffered saline–0.1% Tween 20 (PBST) with 0.1% BSA. The primary antibody was *E. lanceolatus* Lhb antiserum diluted 1:10 000. ELISA plate (Nunc Maxisorp) wells were coated with 100 µl of rggLhb at 100 ng ml^{-1} in 50 mM sodium carbonate (pH 9.6). The rmpLh and rggLh were serially diluted 2-fold from 100 ng ml^{-1} down to 50 pg ml^{-1}. To obtain *M. australasica* plasma that can be serially diluted to test for parallelism with the standard curve, plasma samples were pooled to a volume of 1 to 1.2 ml and then lyophilised and finally resuspended to 350 µl in PBST–0.1% BSA. Intra-assay coefficient of variation was determined from 4 replicates of a standard of the same concentration on a plate while inter-assay coefficient of variation was determined from a standard at 25 ng ml^{-1} from duplicate wells in 3 separate assays. The detection limit was defined as the lowest concentration of standard whose optical density (OD) B/Bo ratio was equivalent to 95%, where B is the OD of the standard and Bo is the maximum binding OD. We assessed the specificity of the Lh ELISA by running a serially diluted rmpLh and rggLh preincubated with yellowtail kingfish *Seriola lalandi* Fshb antiserum and assayed on wells coated with recombinant *S. lalandi* Fshb.

Following validation, levels of plasma Lh were determined from F1 *M. australasica* samples collected in June 2020 and August 2020. Plasma samples from 11 wild *M. australasica* collected from Upper Murrumbidgee and Abercrombie Rivers in central New South Wales in September 2020 were also analysed for Lh levels.

2.4. Statistical analysis

Data were logit-transformed prior to analysis. Parallelism analysis was performed using the chi-squared statistic on IBM SPSS Statistics 27. An analysis of covariance was also performed using the Excel covariance statistical tool to determine whether there were

FQLPPCQLINQTVSLEKEGCSKCHPVETTICSGHCITKDPVIKIPFSNVYQHVCTYRDFYYKTFELPGCPPGVDPT
VTYPVALSCHCGRCAMDTSDCTSESLHPNFCLNDIPFYYGSGSHHHHHHGSGSYPNIDLSLVGCEECTLRKNNVFS
RDRPIYQCMGCCFSRAYPTPLKAMRTMNIPKNITSEATCCVAKHSYEVQSPHLFLFQTEVAGTGIRVRNHTDCHCS
TCYYFHK*

Fig. 1. Recombinant *Macquaria australasica* luteinizing hormone (rmpLh) designed for expression in the yeast *Pichia pastoris*. Lhb subunit (green) is linked with the cga subunit (purple) via a histidine tag (red) enclosed by flexible hinges (blue). The asterisk represents the stop codon. The predicted molecular weight of rmpLh is 26.4 kDa

significant differences in the regression lines generated. Plasma Lh levels are expressed as mean ± SD. Significant difference was accepted if p was ≤0.05.

3. RESULTS

3.1. *Macquaria australasica* cga and Lhb subunit sequences

Using the *cga* of *Epinephelus lanceolatus* as the query sequence, a deduced peptide corresponding to 101 residues was obtained; however, it lacks 5 residues at the N-terminus immediately after the conserved signal sequence cleavage site in other teleosts (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/n051p285_supp.pdf). As it is possible that the sequence represents a gap in the current available genome sequence, the most conserved residues (YPNID) from other species in the Order Perciformes were added in the expression construct (Fig. 1).

A complete mature Lhb deduced sequence was obtained from *M. australasica* genome following the query with the mature *E. lanceolatus* Lhb subunit (Fig. 1; Fig. S2A) with the 2 sequences different only in 5 residues (Fig. S2B). Phylogenetic analysis revealed the evolutionary proximity between *M. australasica* and *M. ambigua* Lhb subunit sequences and their similarity with those from the Order Perciformes (Fig. 2).

3.2. Confirmation of rmpLh by Western blot analysis and mass spectrometry

Using the *E. lanceolatus* Lhb antibodies, a band of the predicted size (24.6 kDa) corresponding to rmpLh was observed from deglycosylated recombinants (Lane 2 in Fig. 3), indicating the expression of rmpLh in *P. pastoris*. The expected 24 kDa size of the positive control (rggLh) was also observed (Lane 1 in Fig. 3).

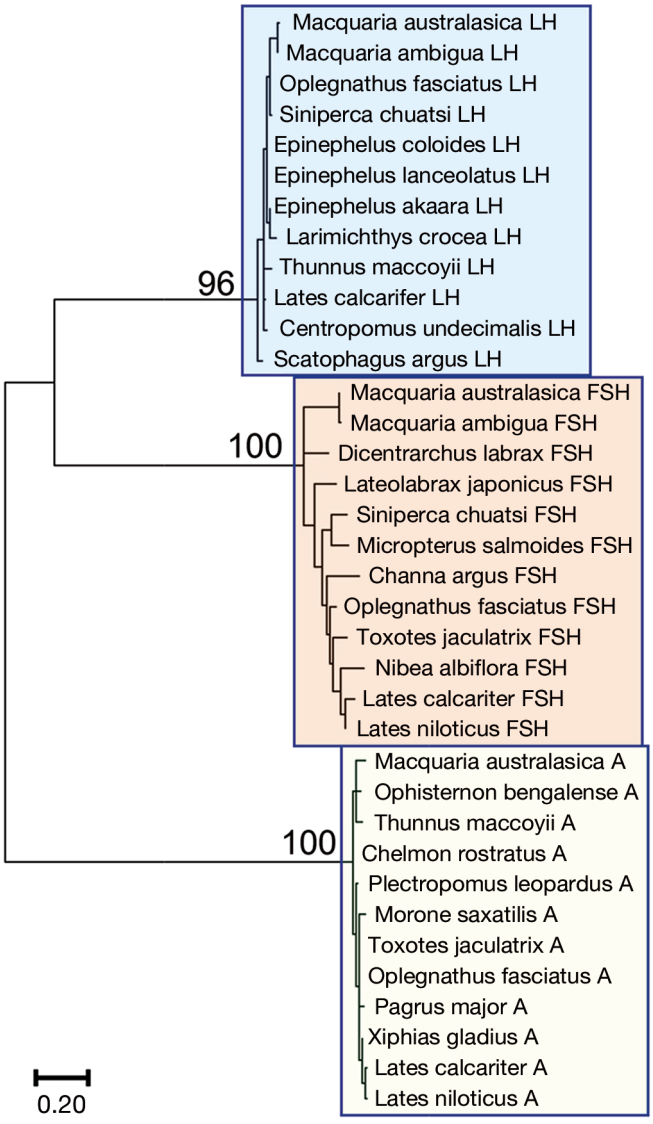

Fig. 2. Evolutionary analysis of *M. australasica* luteinizing hormone (Lh) subunits using the maximum likelihood method. The *M. australasica* and *M. ambigua* Fshb sequences were obtained by a BLAST search of publicly available genome sequences (NCBI GenBank BioProjects PRJNA 516983 and PRJNA556086) using as a query the *Epinephelus lanceolatus* Fshb sequence. The accession numbers of all sequences included for analysis are listed in Table S1 in the Supplement

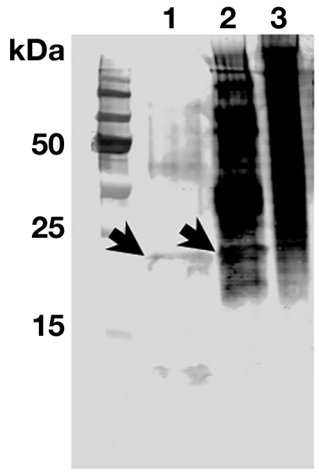

Fig. 3. Western blot analysis of rmpLh using rggLhb antibodies. Lane 1: deglycosylated rggLh (positive control); Lane 2: deglycosylated rmpLh; Lane 3: intact rmpLh. Arrowheads indicate the expected band size of rmpLh and rggLh

The rmpLh was further subjected to in-solution trypsin digestion, followed by LC-MS/MS analysis. High-confidence MS/MS spectra corresponding to different peptide segments of the whole sequence were detected, associated with the extremely low p-values (Fig. S3). Furthermore, the MS/MS spectra showed nearly 100% coverage of the sequence, confirming the identity of rmpLh. The post-translational modifications detected are likely to have been either introduced by sample preparation, or during the recombinant protein production. The results confirmed the peptide sequence corresponding to the designed recombinant.

3.3. *Macquaria australasica* Lh ELISA

Chi-squared analysis confirmed the parallelism between *M. australasica* Lh standard and serially diluted *M. australasica* plasma ($p = 0.043$), and between *M. australasica* Lh standard and *E. lanceolatus* Lh ($p = 0.032$) (Fig. 4). Furthermore, covariance analysis between the *M. australasica* and *E. lanceolatus* Lh standard curves did not show significant differences between the 2 data sets ($p > 0.05$). In contrast, serially diluted rmpLh pre-incubated with rytkFshb antiserum and assayed on wells coated with rytkFshb did not result in parallelism with *M. australasica* and *E. lanceolatus* Lh standard curves (Fig. S4). The intra- and inter-assay coefficients of variation for Lh ELISA were 3.3 and 14.4%, respectively. The detection limit was 200 pg ml^{-1}.

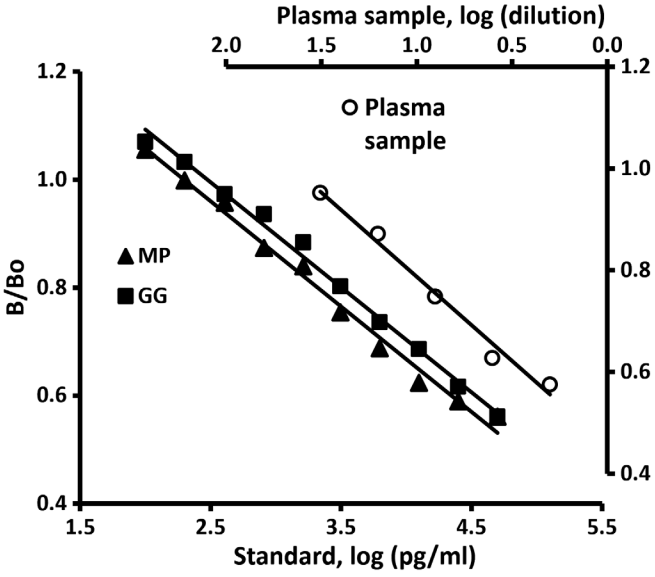

Fig. 4. Parallelism of recombinant *M. australasica* (MP) and *Epinephelus lanceolatus* (GG) luteinizing hormone (Lh) standard curves and a serially diluted *M. australasica* plasma sample. B: optical density (OD) of the standard; Bo: maximum binding OD

3.4. Lh plasma levels in captive and wild-caught *Macquaria australasica* broodstock

The mean plasma Lh in F1 male and female fish that were sampled in June 2020 and in August 2020 ranged between 8 and 17 ng ml^{-1} (Table 1). The mean levels did not vary between sexes and between sampling points. In the 11 wild-caught fish sampled close to the spawning season (September 2020), the plasma Lh levels were higher than 200 ng ml^{-1} in 6 fish while 5 fish had Lh levels between 3 and 38 ng ml^{-1} (Table 2).

Table 2. Plasma Lh levels from wild *M. australasica* collected from the Upper Murrumbidgee and Abercrombie Rivers, New South Wales, Australia, in September 2020

Fish code	Body weight (g)	Lh (ng ml^{-1})
WS2	865	>200
WS3	818	>200
WS4	778	>200
WS5	1047	5.5
WS6	915	37.2
WS9	794	>200
WS10	788	>200
WS11	756	32.9
WS12	790	>200
WS13	951	3.1
WS14	767	3.8

4. DISCUSSION

We produced a recombinant single-chain *Macquaria australasica* Lh in the yeast *Pichia pastoris* according to the *Lhb* and *cga* subunit-encoding sequences retrieved from the annotated *M. australasica* genome (Pavlova et al. 2022). Alignment and phylogenetic analysis showed high similarity between *M. australasica* and *Epinephelus lanceolatus* sequences. Western blot analysis and mass spectrometry confirmed the production of rmpLh. As the *M. australasica* and *E. lanceolatus* Lhb sequences were 91% identical, and the results of the Western blot analysis indicated cross-reactivity of rmpLh with heterologous *E. lanceolatus* Lhb antiserum, we utilised these antibodies to develop an ELISA for *M. australasica* Lh and subsequently measured circulating Lh levels in wild and captive-bred sexually mature fish.

The validity of the Lh ELISA was confirmed by the parallelism of the serially diluted *M. australasica* Lh standard with the serially diluted *M. australasica* plasma, indicating the suitability of *E. lanceolatus* Lhb antiserum for the *M. australasica* Lh ELISA. The *M. australasica* and *E. lanceolatus* mature Lhb sequences differ only in 5 of the 115 amino acid residues, which is consistent with the cross-reactivity of the heterologous antibody and the similarity of the Lh standard curves of the 2 species. In contrast, assays using antiserum against the recombinant yellowtail kingfish *Seriola lalandi* Fshb (rytkFshb) on wells coated with rytkFshb did not result in a similar displacement curve, suggesting specificity of the Lh ELISA.

We retrieved the mature Lhb sequence of *M. ambigua*, considered as a sister species of *M. australasica* (Pavlova et al. 2022). The Lhb sequence of the sister species differed only in 1 amino acid residue, suggesting that the Lh ELISA developed for *M. australasica* is also applicable to *M. ambigua*.

We obtained plasma samples from captive F1 *M. australasica* broodstock in June 2020, and from 5 males and 6 females again in August 2020. These broodstock are sexually mature but have never been observed to spawn spontaneously in captivity. As the spawning season at that location is October, fewer fish were sampled for the second time, in August, to minimise handling stress and maximise potential spawning success in the remaining broodstock. The plasma Lh in F1 males and F1 females did not vary in both sexes during the 2 sampling points and were within the ranges reported for *Epinephelus* spp. (Palma et al. 2019, Dennis et al. 2020) and other species of fish (Aizen et al. 2007, Chauvigné et al. 2015, Nyuji et al. 2016). In contrast, the Lh plasma levels in 6 out of 11 fish that were collected from the wild in September 2020, a month prior to when they are expected to spawn, had much higher levels (>200 ng ml^{-1}) than from the captive broodstock that were sampled in August. We interpret these results to represent a hormonal surge towards spawning in these individuals. Similar values (>200 ng ml^{-1}) were reported in Atlantic bluefin tuna *Thunnus thynnus* (Rosenfeld et al. 2012) and greater amberjack *Seriola dumerilii* (Nyuji et al. 2019) that underwent ovarian maturation and ovulation after treatment with gonadotropin-releasing hormone analogue (GnRHa), suggesting that considerable increase in plasma Lh levels does occur, depending on maturation stage. Although exact comparison between the captive and wild *M. australasica* could not be made because of the differences in the timing of sample collection, nevertheless, the data from captive and wild fish altogether suggest that the Lh levels in captive fish were not undergoing a significant increase towards the spawning season, which may explain the lack of any spontaneous spawning. We were not able to measure the pituitary levels of Lh in captive and wild fish, as it was not possible to sacrifice these fish; however, we hypothesise that failure occurs in the release of pituitary Lh, which is a typical hormonal dysfunction in fish broodstock held in captivity (Zohar & Mylonas 2001, Mylonas et al. 2010). We have successfully induced spawning in captive F1 broodstock after GnRHa treatment (J. Nocillado et al. unpubl. data), supporting our hypothesis.

The production of recombinant gonadotropins has enabled the characterisation of the hormonal profile of many cultured species of fish, advancing the comparative understanding of reproductive biology in teleosts (reviewed by Molés et al. 2020). Evidence of the value of recombinant gonadotropins in overcoming reproductive dysfunctions in captive broodstock is also growing (Aizen et al. 2017, Chauvigné et al. 2017, 2018, Molés et al. 2020, Ramos-Júdez et al. 2021). The recombinant *M. australasica* Lh that we produced here and that the Lh ELISA validated can be used to help refine the assisted reproduction protocol of this species in captivity. Together with sound genetic management, future efforts can assist in the restoration of this once-abundant iconic Australian species.

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