Received: 26 April 2017 | Accepted: 4 December 2017

DOI: 10.1111/anu.12661

ORIGINAL ARTICLE

WILEY Aquaculture Nutrition

Cichlasoma dimerus responds to refeeding with a partial compensatory growth associated with an increment of the feed conversion efficiency and a rapid recovery of GH/IGFs axis

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Funding information
Consejo Nacional de Investigaciones Científicas y Técnicas, Grant/Award Number: 11220130100501CO; Universidad de Buenos Aires, Grant/Award Number: 20020120100280; Agencia Nacional de Promoción Científica y Tecnológica, Grant/Award Number: PID 020-2013

Abstract

Many fish species display compensatory growth (CG), a phenomenon by which fasted fish grow faster during refeeding. However, most studies use a group-housed fish approach that could be problematic in social fish when interaction between individuals is not considered or eliminated. Additionally, the growth hormone (GH)/insulin-like growth factors' (IGF-1 and IGF-2) axis is implicated in postnatal growth in vertebrates, but its relevance in CG is not fully understood. Thus, the aim of this work was to determine whether CG occurs in a social fish, *Cichlasoma dimerus*, using an individually held fish approach and secondly, to evaluate the GH/IGFs expression profile during refeeding by 3 days and 3 weeks. *C. dimerus* showed partial CG. The feed conversion efficiency (FCE) was higher in three-day-refed fish, which presented higher GH plasma and mRNA levels than controls but shown no differences in liver and muscle GH receptors (GHR1 and GHR2) and IGFs mRNA levels. Surprisingly, three-week-refed fish exhibited GHR1 and IGF-2 increments, but a reduction in GHR2 expression in muscle. These results show a strong association between GH levels, growth rate and FCE during refeeding, and a long-lasting effect of refeeding on muscular expression of GHRs and IGF-2.

KEYWORDS
fish, GHRs, growth, growth hormone, IGFs, refeeding

1 | INTRODUCTION

Maximizing growth efficiency in fish may be of great interest in fish farming, which could be accomplished at least theoretically by a phenomenon resulting from feeding manipulation: compensatory growth. In this regard, fasting and refeeding protocols have gained attention in fish research and aquaculture owing to the effects on somatic growth they may induce. CG is a phase of accelerated growth that is usually observed in many fish species during refeeding after a variable period of feed restriction (Ali, Nicieza, & Wootton, 2003; Wilson & Osbourn, 1960) as a result of hyperphagia (Jobling & Koskela, 1996; Mlglavs & Jobling, 1989; Tian & Qin, 2003; Wang, Cui, Yang, & Cai, 2000) and/or an increment in the feed conversion efficiency (FCE) (Boujard, Burel, Médale, Haylor, & Moisan, 2000), defined as the ratio between body mass gain and ingested feed (Won & Borski, 2013). CG is complete when fish can reach a final body size that matches those of control animals that were growing at optimal condition in the same period of time (Dobson & Holmes, 1984; Koppe, Pockrandt, Meyer-Burgdorff, & Gunther, 1993; Qian, Cui, Xiong, & Yang, 2000; Zhu, Cui, Ali, & Wootton, 2001), or partial, when a phase of accelerating growth takes place but animals cannot match the body size of control ones (Buckel, Letcher, & Conover,

1998; Jobling, Jørgensen, & Siikavuopio, 1993; Thorpe, Talbot, Miles, & Keay, 1990). Overcompensation may also occur (Hayward, Noltie, & Wang, 1997) when fish displaying CG growth more than controls.

Somatic growth in vertebrates is under GH/ IGFs system regulation (Duan, 1997), which comprises GH, IGF-1, IGF-2, its receptors and its binding proteins. GH is a 22- to 25-kDa pleiotropic hormone synthesized in the pituitary gland that is under multifactorial regulation and is involved in many physiological processes in the organism which includes, but is not limited to, postnatal growth, energy mobilization, reproduction, appetite and immune system (Canosa, Chang, & Peter, 2007; Chang & Wong, 2009; Gahete et al., 2009; Hattori, 2009; Johnston, Bower, & Macqueen, 2011). The biological effects of GH are mediated by a member of the class 1 cytokine receptor family that is associated with the JAK/STAT intracellular signalling pathway (Brooks, Wooh, Tunny, & Waters, 2008; Brooks et al., 2014). Interestingly, teleost fish present two types of GH receptor, named GH receptor type 1 (GHR1) and GH receptor type 2 (GHR2) (Jiao et al., 2006; Saera-Vila, Calduch-Giner, & Pérez-Sánchez, 2005), although their specificities for GH and somatolactin (SL), a hormone related to GH and prolactin (PRL), are not completely resolved (Chen, Huang, Yuen, & Cheng, 2011; Ellens, Kittilson, Hall, Sower, & Sheridan, 2013).

On the other hand, insulin-like growth factor involvement in somatic growth has been already demonstrated in KO mammalian models (Baker, Liu, Robertson, & Efstratiadis, 1993; DeChiara, Efstratiadis, & Robertson, 1990; Liu, Baker, Perkins, Robertson, & Efstratiadis, 1993; Nakae, Kido, & Accili, 2001). IGFs are polypeptide hormones highly conserved in vertebrates and structurally related to insulin, sharing with it some biological actions such as glucose uptake by the cell (Duan, 1997; Wood, Duan, & Bern, 2005). Although IGF-1 is produced mainly in the liver, some studies suggest that extrahepatic IGF-1 expression may play an important role during postnatal growth (Sjögren et al., 1999; Yakar et al., 1999). On the other hand, IGF-2 involvement in fish growth is not fully understood, but several studies have demonstrated an association of IGF-2 with growth and CG in fasting and refeeding experiments (Gabillard, Kamangar, & Montserrat, 2006; Picha, Turano, Tipsmark, & Borski, 2008; Terova et al., 2007).

Cichlid fish are one of the most diverse groups of teleost fish (Nelson, Grande, & Wilson, 2016). Many of them are important in aquaculture, and many others are popular as fish for fishkeeping given its ease of breeding and rearing. However, most cichlids are known to be social fish and display aggressive behaviour with each other, a fact that may constitute a negative factor in its farming and a feature to be considered in experimental designs. In spite of these inconveniences, most studies on CG have been conducted in a group-housed fish approach, which may be misleading in terms of heterogeneity of fish reared in a tank related to differences in sex, size and hierarchy status (Ali et al., 2003). In this context, it would be desirable to study CG in cichlids but in an individually housed fish approach. In this respect, an overcompensatory growth was observed in the hybrid sunfish, *Lepomis cyanellu* x *L. macrochirus*, when fish were individually held but not when they were grouped (Hayward, Wang, & Noltie, 2000; Hayward et al., 1997).

Cichlasoma dimerus is a South American cichlid fish that shows a great adaptability to laboratory conditions, feature that makes this fish a promising biological model for studies in behaviour (Ramallo et al., 2014), toxicology (Genovese, Da Cuna, Towle, Maggese, & Nostro, 2011, Genovese et al., 2012), neuroendocrinology (Cánepa, Zhu, Fossati, Stiller, & Vissio, 2012; Di Yorio, Delgadin, Pérez Sirkin, & Vissio, 2015; Pérez Sirkin et al. 2012) and growth (Delgadin, et al., 2015). In spite of these studies, there are many relevant aspects of its biology that are not known to date. Particularly, it would be of interest to know whether *C. dimerus* is able to recover from growth retardation caused by starvation (Delgadin et al., 2015) with a CG response during refeeding.

It has been demonstrated that fasting affects several components of the GH/IGF-1 axis in many fish species (Fox et al., 2010; Fuentes et al., 2011; Picha et al., 2008). Fasting generally induces an increment in plasmatic concentration of GH and a reduction in circulating IGF-1 and liver mRNA expression of GH receptors, profile called "GH resistance." However, *C. dimerus* does not follow this profile (Delgadin et al., 2015) and therefore, it would be interesting to study how the GH/IGF-1 axis behaves in refeeding.

As most studies on CG in fish have been conducted in a group-housed manner, the aim of this work was to analyse whether CG occurs in a cichlid fish, *C. dimerus*, in a context of individually housed fish approach and the endocrine response to refeeding at the GH/IGFs axis level.

2 | MATERIALS AND METHODS

2.1 | Animals

Adult male and female *C. dimerus* (Heckel, 1840) fish were caught from the wild in "Los Esteros del Riachuelo," Corrientes, Argentina (27°12′50″S, 58°11′50″W) and transferred to 150-l aquaria, where they were kept under controlled conditions of temperature (25 ± 1°C), photoperiod (14 L:10 D) and fed daily with commercial pellets (Tetra Pond Variety Blend, Tetra®, Germany). All experimental procedures conducted in this work comply with the approval of Comisión Institucional para el Cuidado y Uso de Animales de Laboratorio, Facultad de Ciencias Exactas y Naturales, Buenos Aires, Argentina (protocol number 26).

2.2 | Experimental design

This work comprises two main experiments, named A and B, respectively. Experiment A was intended to explore the general growth profile during a long-lasting refeeding period. Based on this growth curve, a short-term refeeding experiment was designed to analyse the GH/IGF-1 axis during the CG phase (Experiment B).

All experiments presented in this work were conducted assigning one fish per tank on a closed recirculating system containing twelve 16-l tanks. This approach was elected over the classical multiple fish per tank as a mean to minimize unpredictable effects of intratank interaction between individuals on growth, feed ingestion and related

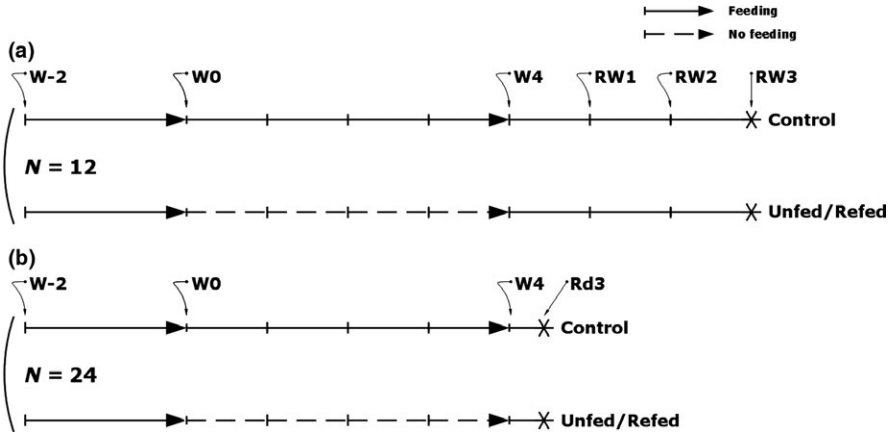

FIGURE 1 Protocols used in this work for refeeding experiments. Control groups (n = 6) were fed daily at 15g/kg WB (solid line) while treated groups (n = 6) were fasted for 4 weeks (dash lines) and then refed for 3 weeks (Experiment a) or 3 days (Experiment b). W-2 to W0 is the acclimatization period, W0 to W4 the fasting phase, Rd3 is refeeding for 3 days and RW1, RW2 and RW3 are refeeding for 1, 2 and 3 weeks. Experiment b was carried out twice (N = 24)

variables that are under study in this work, a fact that gain critical relevance as *C. dimerus* is a social cichlid fish with a marked agonistic behaviour between dominant and subordinate fish (Alonso, Cánepa, Moreira, & Pandolfi, 2011; Delgadin et al., 2015; Ramallo et al., 2014). Additionally, a "one fish per tank" arrangement allows tracking growth and other variables for each fish without tagging them.

Fish to be used in the experiments were transferred from the stock tank to the experimental device and allowed to acclimatize for 2 weeks. For Experiment A, six fish were randomly assigned to the control group fed at 15g/kg BW and six fish to the treated one, which was starved for 4 weeks and then refed for 3 weeks (N = 12). Similarly, Experiment B consisted on a control group of six animals fed at 15g/kg BW and a treated group fasted for 4 weeks and refed for 3 days (Figure 1). To increase statistical power for qPCR and ELISA data, Experiment B was run twice (N = 24). Body weight and standard length were registered at the beginning of acclimatization and fasting period, and weekly during refeeding. The amount of pellets to be administered along the experiments was set at 15g/kg of body weight per day registered at the end of acclimatization period.

As GH plasma levels and IGF-II mRNA levels were the only parameters not measured in a previous study performed in our model species in fasting conditions (Delgadin et al., 2015), a four-week fasting experiment was carried out to complete those results. This experiment was conducted following the same procedures described for experiments A and B, except that it finished at the end of fasting period.

2.3 | Sampling

At the end of the experiments, animals were anaesthetized with benzocaine 0.1% and then euthanized. Liver, pituitary gland and muscle were immediately extracted and homogenized in 500 µl TRI Reagent® (Molecular Research Center, Inc., Cincinnati, USA) for RT-qPCR assays. Part of the liver was submerged in 15 ml of Bouin's fluid for histological studies. The liver was weighted to determine the hepatosomatic index (HSI), which was calculated as liver weight/body weight × 100. Muscle samples were extracted from the posterior dorsolateral region of the fish, and scales and skin were removed.

2.4 | Growth parameters

Somatic growth was evaluated by measuring both body weight (BW) and standard length (SL). Additionally, specific growth rates (SGR) were calculated as $[\mathrm{Ln}(X_f/X_i)/t]$*1,000, where t is time measured in days and X_f and X_i denotes final and initial BW or SL, respectively.

2.5 | Feed intake and feed conversion efficiency

During the refeeding period, seven pellets were administered to each tank every day at 1,400 hr, and the number of uneaten pellets was registered at 10 min, 30 min, 60 min, 180 min and 24 hr afterwards. Thus, feed ingestion was quantified as follows: ingested pellets = 7 − X, where X is the number of pellets remaining on the surface of the tank after food administration.

Feed conversion efficiency was calculated as ($BW_f - BW_i$/food weight)*100, where BW_f and BW_i are BW at the end and the beginning of a time interval, respectively, while food weight is the amount of food ingested during the specified period.

2.6 | Liver histology

Hepatocyte size was evaluated by measuring the hepatocyte area on histological sections of liver. Thus, liver samples were immediately fixed in Bouin's fluid 1 hr at room temperature and at 4°C overnight. Once fixed, samples were dehydrated by a graded alcohol passage and embedded in paraffin for 6 hr. Liver sections of 7 µm were stained with haematoxylin–eosin (H&E) and analysed in ImagePro Plus software (Media Cybernetics, Inc., Washington, USA) to measure hepatocyte areas from twenty hepatocytes selected at random per fish.

2.7 | Plasma GH levels

A competitive indirect enzyme-linked immunosorbent assay (ELISA) developed for pejerrey *Odontesthes bonariensis* (Atheriniformes, Atherinopsidae) growth hormone (Simó, unpublished results) was performed to determine the *C. dimerus* GH levels on plasma samples. This ELISA was later validated in *C. dimerus* by Western blot analysis and immunohistochemistry. Western blot analysis showed a single

TABLE 1 5′ to 3′ primer sequences for qPCR assays

Primer	5′-3′ sequence
IGF-1 forward	CTCCCAAGATTTCTCGCTCTG
IGF-1 reverse	CCCTTCTCCGCTTTACTAACC
IGF-2 forward	GTAGAGGAGTGTTGTTTCCGT
IGF-2 reverse	CCTGTAGAGAGGTGGCTGAC
GH forward	GTCAGTCGTGTGTGTTTGGGTGTC
GH reverse	CGAGCAGGTGGAGGTGTTGG
GHR1 forward	CACCCAAAATCAAAGGCATCG
GHR1 reverse	GGCTCATCTTGGTAGAAATCTGG
GHR2 forward	ACTGCTCTCCACTCTCAATTG
GHR2 reverse	AAAGGTGATGGTTCTGGGTC
ARP forward	ACTGTGGGAGCAGACAATG
ARP reverse	TCCAGTGCAGGATTGTTCTC
18S forward	GGAGTATGGTTGCAAAGCTG
18S reverse	TCTGTCAATCCTTTCCGTGTC

band at the expected molecular weight that is not observed when pjGH antibody is omitted. Immunohistochemistry revealed GH-ir cells surrounding the neurohypophysis at the proximal *pars distalis* level. This arrangement in the pituitary gland corresponds to somatotropic cells that were previously described for this species (Pandolfi, Paz, Maggese, Meijide, & Vissio, 2001).

Recombinant pejerrey growth hormone (pjGHr) solution (2,000 ng/ml) was serially diluted 1:2 in PBS Tween (PBST) 0.05% to a final concentration of 3.9 ng/ml, and samples were used to obtain the standard curve. In a similar way, 80 µl of plasma samples was diluted 1:2 in PBST 0.05% for triplicate analyses. The recombinant hormone and plasma dilutions were then mixed 1:1 with primary antibody (Rabbit anti-pjGH serum) (Sciara, Rubiolo, Somoza, & Arranz, 2006) in PBST 0.05% at a dilution of 1:30,000 in 1.5-ml centrifuge tubes. To determine the maximum binding capacity, a blank-zero tube (Bo) was prepared using a 1:1 mixture of PBST 0.05% and primary antibody (1:30,000 dilution). Additionally, a non-specific binding (NSB) sample was carried out using a 1:1 mixture of PBST 0.05% without anti-pjGH. All the samples were incubated at RT for 3.45 hr with continuous gentle shaking.

One hour later, immunoplates (Microlon 96 wells, flat-bottom, high binding) were coated with 100 µl of 2,000 ng/ml of pjGHr in 20 mM TRIS-0.05 M EDTA pH 9 (TE), with additional wells for non-specific binding (NSB) coated with 100 µl of 2,000 ng/ml of BSA (Bovine Serum Albumin, Premium Quality, Sigma-Aldrich, USA) in TE buffer and incubated for 2 hr at 37°C. Plates were washed three times with 300 µl of washing solution (0.01 M NaPO4, 0.15 M NaCl, and 0.1% Tween 20, pH 7.6), 2 min per wash. Then, all wells were blocked with 200 µl of 4% W/V non-fat dry milk in PBST (0.01 M NaPO4, 0.15 M NaCl and 0.05% Tween 20, pH 7.4) 30 min at 37°C without shaking, and finally, wells were washed three times with washing solution. After 3.45 hr of anti-pjGH incubation with the hormone, 100 µl of each sample was pipetted into individual coated wells and incubated overnight at 4°C. Following this step, each well was washed three times with washing solution. Then, each well was incubated with 100 µl of peroxidase conjugated affinipure goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc., USA) secondary antibody diluted 1:2,500 in PBST 0.05% and incubated at 37°C for 1 hr. After that, the plate was washed three times and incubated with 100 µl of TMB reveller agent (3,3′,5,5′-tetrametilbenzidinea and hydrogen peroxide; Winner Lab, Rosario, Argentina) at RT for 30 min. Finally, the reaction was stopped with 50 µl of HCl and absorbance of each sample was determined at 450 nm using BioHit plate reader.

2.8 | GH, GHR1, GHR2, IGF-1 and IGF-2 mRNA expression

Gene expression was analysed by RT-qPCR on pituitary gland, liver and muscle. After fish euthanization, whole pituitary gland and 50 mg of liver and muscle were extracted and immediately homogenized in 500 µl of TRI Reagent (Molecular Research Center, Inc., Cincinnati, USA) for total RNA extraction following manufacturer's instructions. RNA quantification and purity were determined using NanoDrop 2000 UV–vis spectrophotometer (Thermo Scientific®, USA). To eliminate possible genomic DNA contamination, all samples were DNase I-treated (Sigma-Aldrich, USA) starting from 2 µg of total RNA according to manufacturer's instructions. Then, first-strand cDNA synthesis was performed with M-MLV enzyme (Promega, Madison WI, USA) for 50 min at 37°C followed by 10 min at 70°C using random oligomers as reaction primers in a 10-µl final volume. RT-qPCRs were conducted in a 10-µl final volume with 5 µl of 2X FastStar Universal SyBR green Master (Roche, Switzerland), 1.5 µl of forward/reverse primer mix (Table 1; GH: 100 nM; GHR1: 250 nM; GHR2: 250 nM; IGF-1: 650 nM and IGF-2: 650 nM), 2.5 µl of cDNA template and 1 µl of water. qPCR protocols were as follows: 10 min of initial denaturation at 95°C, then 40 cycles of denaturation at 95°C for 30 s and annealing/elongation at 60°C for 30 s, followed by a melting curve from 65°C to 95°C to detect possible non-specific PCR products. All primers were designed based on the respective *C. dimerus* cDNA sequences to give an amplicon size between 100 and 130 base pairs. Samples were run in duplicate, and no template controls were performed in every run for each set of primers. Raw fluorescence data from qPCR were exported to LinRegPCR software and analysed to obtain the initial amount of fluorescence per reaction (N_0), which is directly related to the starting amount of cDNA (Ramakers, Ruijter, Deprez, & Moorman, 2003). N_0 determination by this methodology is efficiency based and is carried out by LinRegPCR software (Ramakers et al., 2003; Ruijter et al., 2009). Briefly, individual efficiencies are determined for each reaction based on a linear regression between fluorescence and cycles over the linear phase of the PCR after a LN transformation. Then, a general amplicon efficiency is determined by averaging individual efficiencies for a given qPCR run and tissue, which is used to obtain individual N_0. The response variable for qPCR data was $E_{GOI} = N_0^{GOI}/N_0^{RG}$, where E_{GOI} is the expression of a gene of interest (GOI) normalized against a reference gene (RG), N_0^{GOI} is the initial fluorescence of a GOI and N_0^{RG} the initial fluorescence of RG. Reference genes used in this work were acidic ribosomal protein (ARP) for liver and muscle and 18S for pituitary gland.

TABLE 2 Growth parameters over time in refeeding experiments. Controls (n = 6) were continuously fed at 15g/kg BW while treated (n = 6) were starved for 4 weeks and refed for 3 weeks (A) or 3 days (B)

		BW (g)		SL (cm)	
Experiment	Time	Control	Treated	Control	Treated
A	W-2	9.12 ± 0.74	8.55 ± 0.42	5.69 ± 0.14	5.61 ± 0.12
	W0	10.44 ± 0.95	10.32 ± 0.50	5.97 ± 0.18	5.89 ± 0.13
	W4	12.84 ± 1.04	9.31 ± 0.43	6.37 ± 0.18	5.85 ± 0.11
	RW1	13.21 ± 1.04	10.05 ± 0.44**	6.44 ± 0.17	6.00 ± 0.13**
	RW2	13.65 ± 1.04	10.48 ± 0.43**	6.47 ± 0.19	6.04 ± 0.12**
	RW3	14.30 ± 1.09	11.09 ± 0.46**	6.57 ± 0.18	6.16 ± 0.11**
B	W-2	8.17 ± 0.34	8.29 ± 0.48	5.56 ± 0.07	5.59 ± 0.08
	W0	9.24 ± 0.48	9.04 ± 0.61	5.76 ± 0.08	5.81 ± 0.10
	W4	10.98 ± 0.77	8.21 ± 0.57**	6.09 ± 0.10	5.72 ± 0.08**
	RD3	11.24 ± 0.87	8.96 ± 0.68**	6.13 ± 0.11	5.79 ± 0.09**

W-2, beginning of acclimatization; W0, beginning of fasting; W4, end of fasting; RW1, RW2 and RW3, refeeding for 1, 2 and 3 weeks, respectively; RD3, 3 days of refeeding. BW, body weight; SL, standard length. Table shows mean ± *SEM*. **p < .01 between control and treated groups in the same row.

TABLE 3 Specific growth rates (SGR) for body weight (BW)/standard length (SL) and feed conversion efficiency (FCE) measured weekly during 3 weeks of refeeding in experiment A and after 3 days of refeeding (RD3) in experiment B

	SGR BW (‰)		SGR SL (‰)		FCE (%)	
Time	Control	Treated	Control	Treated	Control	Treated
RW1	4.21 ± 0.40	10.98 ± 1.22***	1.59 ± 0.44	3.55 ± 0.38**	1.88 ± 0.32	3.71 ± 0.29***
RW2	4.85 ± 1.15	6.09 ± 1.39	0.42 ± 0.83	1.21 ± 0.61	2.22 ± 0.50	2.17 ± 0.46
RW3	5.19 ± 0.47	6.17 ± 0.99	1.73 ± 0.59	2.14 ± 0.85	3.26 ± 0.46	3.01 ± 0.42
RD3	7.00 ± 3.47	28.00 ± 5.87*	2.15 ± 1.31	3.83 ± 2.24	3.61 ± 2.16	17.09 ± 3.93*

Table shows mean ± *SEM*. *p < .05; **p < .01; ***p < .001 between control (n = 6) and treated (n = 6) groups in the same row.

2.9 | Statistical analysis

Results are presented as mean ± standard error of the mean (*SEM*). One-way ANOVAs were run whenever two means were to be compared. Body weight, standard length, SGR(BW) and SGR(SL) were analysed by repeated measures ANCOVAs followed by simple effects post hoc comparisons. For qPCR and ELISA data from Experiment B, which was run twice, experiment replication was included in the ANOVA model as a random factor. As each replication of Experiment B was run independently, data cannot be pooled in a graph and therefore only date of one experiment is shown in figures. All assumptions were tested for each test and if not met, a Ln(x) transformation was carried out. Data from feed intake were analysed by Cochran–Mantel–Haenszel test, with time and treatment as categorical variables and days as strata.

3 | RESULTS

3.1 | Effect of 3 weeks of refeeding on somatic growth parameters

Control and treated fish grew equally from the acclimatization to the beginning of the experiment period as they did not show differences in BW and SL (Table 2). Four weeks of fasting, on the other hand, resulted in a reduced body size of unfed fish compared to controls, both in BW and SL (Table 2). After 3 weeks of refeeding, BW and SL were reduced in refed animals compared to continually fed ones (p < .05, Table 2). However, refeeding resulted in an increment of SGR(BW) and SGR(SL) in refed fish compared to control ones during the first week of treatment (p < .05), differences that were not observed at week 2 and week 3 of refeeding (p > .05, Table 3). In addition, FCE was higher in refed fish only during the first refeeding week (p < .05), but not at weeks 2 and 3 (p > .05, Table 3). On the other hand, both control and refed animals showed no differences in feed intake profile during the complete 3 weeks of refeeding (p = .95, Figure S1). Both HSI and hepatocyte area were not different between treatments after 3 weeks of refeeding (p > .05, Table 4).

3.2 | Effect of 3 days of refeeding on somatic growth parameters

As for the previous experiment, control and treated animals did not differ in BW and SL at the beginning of acclimatization and at the beginning of the experiment. Likewise, 4 weeks of fasting reduced BW and SL of unfed fish compared to fed ones (Table 2). Three days of refeeding resulted in elevated SGR(BW) in refed fish compared to controls (p < .05), although SGR(SL) were not different between

TABLE 4 Hepatosomatic index (HSI) and hepatocyte area after 3 weeks (RW3) or 3 days (RD3) of refeeding in control (n = 6) and treated (n = 6) fish

	HSI (%)		Hepatocyte area (μm^2)	
Time	Control	Treated	Control	Treated
RW3	2.0 ± 0.2	2.5 ± 0.2	174.1 ± 17.0	197.0 ± 16.0
RD3	2.3 ± 0.6	2.8 ± 0.4	175.7 ± 19.6	196.4 ± 7.8

Table shows mean ± *SEM*.

treatments (p > .05, Table 3). Refed animals showed higher FCE values than control ones (p < .05, Table 3), but both HSI and hepatocyte areas were not different between them (p > .05, Table 4).

3.3 | Effect of refeeding on GH/IGF-1 axis

3.3.1 | Pituitary gland

After 3 days of refeeding, GH mRNA expression was higher in refed fish compared to controls (p < .05), but returns to control levels at week 3 (p > .05, Figure 2a). GH plasma levels are not different between control and four-week-fasted fish (p > .05). Three-day-refed fish showed higher plasma levels of GH compared to control fish (p < .01). No differences were observed between treatments after 3 weeks of refeeding (p > .05, Figure 2b).

3.3.2 | Liver

Three-day and three-week-refed fish presents no differences in liver GHR1 and GHR2 mRNA expression levels (p > .05, Figure 3a,b). IGF-1 and IGF-2 mRNA expressions compared to control animals were not statistically different after 3 days and 3 weeks of refeeding (p > .05, Figure 3c,d). IGF-2 mRNA expression was not different between control and four-week-fasted fish (p > .05).

3.3.3 | Muscle

GHR1 mRNA expression was not altered by 3 days of refeeding (p > .05) but was higher in 3-week-refed fish (p = .0286, Figure 4a). On the other hand, GHR2 mRNA levels were not different between control and 3-day refed fish (p > .05). However, 3 weeks of refeeding resulted in lower levels of GHR2 mRNA in refed fish compared to controls (p = .0072, Figure 4b). No differences were observed in IGF-1 mRNA expression between controls and 3-day- and 3-week-refed fish (p > .05, Figure 4c). IGF-2 expression was higher in refed fish at week 3 of refeeding (p = .0031), but no differences were observed at day 3 (p > .05, Figure 4d) or four-week fasting (p > .05).

4 | DISCUSSION

Three weeks of refeeding resulted in a partial CG registered during the first week and was associated with an improved FCE and high

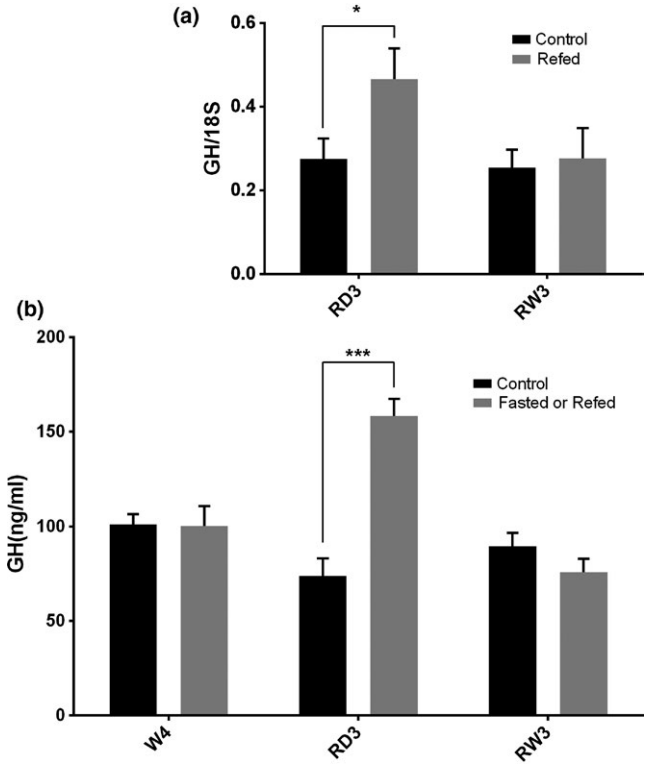

FIGURE 2 (a) GH mRNA levels in control (black bar) and refed (grey bar) fish for 3 days and 3 weeks. 18S was used as reference gene. (b) GH plasma levels in control (black bar) and 4-week fasted (4W) or refed (grey bar) fish for 3 days and 3 weeks as obtained by ELISA. *p < .05, ***p < .001. n = 6 fish per group, except for RD3, where n = 12 fish per group

plasma and transcript levels of GH. Moreover, nutritional status and GH/IGFs axis were completely restored after 3 days of refeeding. The effect of fasting on growth has been previously described for *C. dimerus* (Delgadin et al., 2015) using the same "one fish per tank" approach described in this study, in order to discard the effect of social interaction that leads to a heterogeneous somatic growth (Delgadin, Pérez Sirkin, Karp, Fossati, & Vissio, 2014). The effect of 4 weeks of fasting in *C. dimeurs* resembles those obtained in other fish species, with a characteristic reduction in body weight and a complete abolition of longitudinal growth. The present work included a period of 4 weeks of starvation before to refeeding experiment and showed a perfect agreement with those results (Table 2). In order to determine a possible CG response in this species, fish were then submitted to a three-week refeeding period. Results indicate that refed fish were not able to catch up body size of control ones, as body weight and total length were still lower in refed fish at the end of the experiment. However, a detailed inspection of the growth curve during refeeding shows a first week of accelerated growth both in BW and SL (Table 2), which is evident by analysing SGR(BW) and SGR(SL), both parameters increased in refed fish (Table 3). On the contrary, second and third week of refeeding shows no statistical differences in SGRs, accounting for a loss of the previous accelerated growth. Based on this growth profile, this work demonstrates that *C. dimerus* presents partial CG as a response to fasting. Similar results were observed in other cichlid

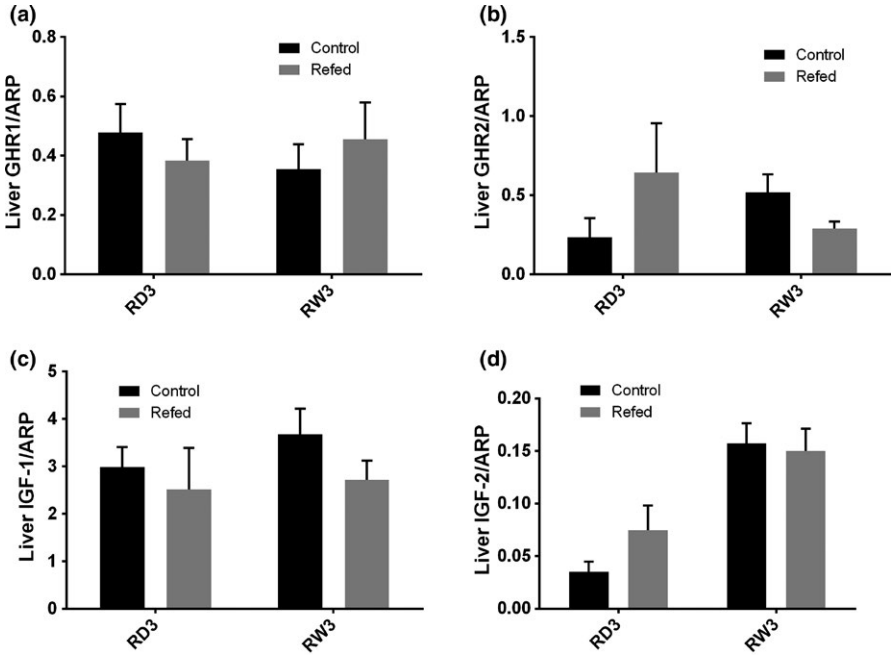

FIGURE 3 Liver mRNA levels of GHR1 (a), GHR2 (b), IGF-1 (c) and IGF-2 (d) normalized against ARP in control and refed fish for 3 days and 3 weeks. $n = 6$ fish per group, except for RD3, where $n = 12$ per group

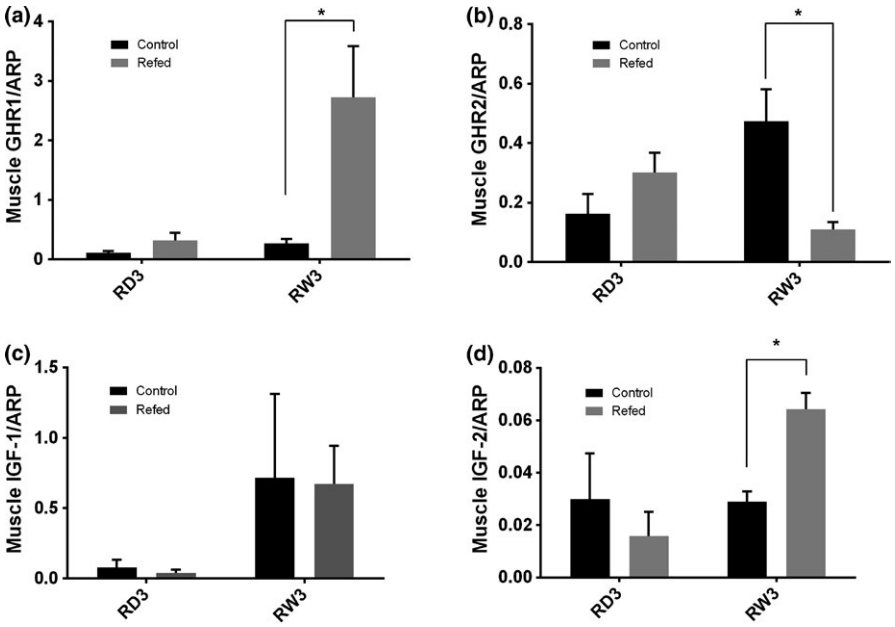

FIGURE 4 Muscle GHR1 (a), GHR2 (b), IGF-1 (c) and IGF-2 (d) expression in control and refed for 3 days and 3 weeks fish. ARP was used as reference gene. *$p < .05$. $n = 6$ fish per group, except for RD3, where $n = 12$ per group

fish, the tilapia *Oreochromis mossambicus* (Fox et al., 2010), where after 4 weeks of fasting and 8 weeks of refeeding, fish presented a CG response but with lower body weight than continuously fed controls at the end of the experiment. Other studies with similar results, although with slightly different experimental protocols, were obtained in *Seriola quinqueradiata* (Fukada, Murashita, Furutani, & Masumoto, 2012), *Oncorhynchus mykiss* (Montserrat, Gabillard, Capilla, Navarro, & Gutiérrez, 2007), *Sparus aurata* (Peres, Santos, & Oliva-Teles, 2011), *Lates calcarifer* (Tian & Qin, 2003) and in *O. mossambicus X O. niloticus* (Wang et al., 2000). Some species on these studies showed full CG when the fasting period was set to 1 week long (Monserrat et al., 2007; Tian & Qin, 2003; Wang et al., 2000). This raises the possibility that a shorter period of fasting could have caused a full CG in *C. dimerus*, which remains to be solved in further experiments.

Interestingly, the FCE was higher during the first week of refeeding, but returns to control levels at weeks 2 and 3. The FCE profile matches that of SGRs and suggests that the accelerated growth observed during week 1 of refeeding may be due, at least in part, to the increment in FCE. On the other hand, it is known that CG may be also a result of hyperphagia (Ali et al., 2003). Under the experimental design used in this study, hyperphagia during refeeding can be discarded as food administration and feed ingestion were equal for all

fish. Moreover, the feed intake profile during refeeding shows that control and refed fish did not differ in feed ingestion behaviour at any point (Figure S1). However, alternative experimental designs, for instance, by adjusting the amount of feed by body weight changes or by feeding to satiation, could have shown other results. Together, this work suggests that partial CG observed in *C. dimerus* is due to an increment in the FCE. In the hybrid striped bass, Picha et al. (2008) have shown a partial CG response to 3 weeks of fasting along with hyperphagia and an increment FCE during the accelerated period of growth. Other studies, however, assigned CG only to hyperphagia (Jobling & Koskela, 1996; Mlglavs & Jobling, 1989; Tian & Qin, 2003; Wang et al., 2000). Thus, the CG response to fasting observed in *C. dimerus* seems to be caused by a metabolic change. Surprisingly, the CG response was already evident early during refeeding, where SGR(BW) was higher at day 3 of refeeding in refed fish, accompanied with a concomitant increment in FCE (Table 3). Moreover, at this point, the liver size was completely recovered as HSI and hepatocyte area were not different between control and refed fish (Table 4), and kept unchanged until third week (Table 4). It was previously demonstrated a reduction in liver HSI and hepatocyte area in *C. dimerus* after a four-week fasting period (Delgadin et al., 2015). Interestingly, differences in SGR and FCE were notably higher between refed and control fish at day 3 when compared to one-week refeeding (Table 3). On the other hand, SGR(SL) was not different between treatments by 3 days of refeeding, which points out that the first growth parameter to respond to fasting during refeeding is BW.

As the CG response was observed during the first week of refeeding, it was proposed that changes in GH/IGFs system should have occurred before to that period. To test that hypothesis, a three-day refeeding protocol was carried out. Surprisingly, no changes were observed between fed and refed fish on GHR1, GHR2, IGF-1 and IGF-2 both in liver and muscle. This might be unexpected, but gain relevance when considering the expression of these genes after fasting. A four-week fasting experiment was conducted previously in *C. dimerus* (Delgadin et al., 2015) and showed that GHR2 was upregulated in liver and muscle of fasted fish, while IGF-1 was downregulated only in muscle. By day 3 of refeeding, there were no differences in mRNA expression of those genes, suggesting that refeeding in fact restores its expression both in liver and muscle. On the other hand, liver and muscle GHR1 and hepatic IGF-1 were not altered after fasting (Delgadin et al., 2015) or refeeding. Gabillard et al. (2006) showed in *O. mykiss* a full restoration of the GH/IGFs system during refeeding, particularly muscle GHR1 and GHR2 that were increased in fasting were restored to control levels by 4 days of refeeding. Similar results were obtained in *O. mossambicus* (Fox et al., 2010), where hepatic IGF-1 expression reached control levels during refeeding.

Surprisingly, the expression of GHR1, GHR2 and IGF-2 was altered in muscle after 3 weeks of refeeding, where CG had already ceased. Indeed, GHR1 and IGF-2 were increased in three-week-refed fish, but not during the first 3 days of refeeding where CG took place. On the other hand, GHR2 is increased in four-week fasting fish (Delgadin et al., 2015), fall to control levels at day 3 of refeeding and is decreased in three-week-refed fish (Figure 4b). These expression profiles may suggest a long-lasting effect of refeeding on GH/IGFs system, with a first period of restoration of the gene expression matching the CG phase.

IGF-1 and IGF-2 behave differentially in fasting and refeeding. While muscle IGF-1 is downregulated in fasting (Delgadin et al., 2015), muscle IGF-2 is not affected. Moreover, after 3 weeks of refeeding, muscle IGF-2 is increased in refed fish, while muscle IGF-1 expression returns to control values. Surprisingly, liver IGF-1 and IGF-2 are not altered in these experiments, which point out that locally produced IGF-1 and IGF-2 may play different roles in somatic growth.

It is interesting to note that FCE and SGR are increased in three-day-refed fish and mirrored plasma and mRNA GH levels. On the third week of refeeding, both plasma and transcript GH levels did not differ between control and refed fish (Figure 2), following the FCE and SGR profile as well. Taking into account that GH is not affected by 4 weeks of fasting, these results suggest that refeeding induces an increase in GH in refed fish and that might be related to FCE and SGR variations. Additionally, it is interesting to note that circulating GH levels were not altered by fasting, a fact that does not follow the GH resistance model by which this hormone is increased after starvation. If the negative feedback of IGF-1 on pituitary GH secretion is present in *C. dimerus*, as in other teleosts (Duan, 1997; Wood et al., 2005), the lack of GH increments after fasting could be related to the absence of IGF-1 reduction in this situation (Delgadin et al., 2015). In agreement with this result, no changes in plasma GH levels were observed in *O. mossambicus* after fasting (Fox et al., 2010; Uchida et al., 2003).

In conclusion, an early CG phase is observed after fasting when feed is available to fasted fish. This response is not sufficient to completely restore body size of refed fish to control ones. During the CG phase, refed fish showed higher FCE and SGR along with a peak of GH mRNA expression and plasma levels, which suggests a strong association between them. Taken together, these results show that *C. dimerus* responds to refeeding by elevating GH during the anabolic phase.

ACKNOWLEDGEMENTS

We thank Mr Ignacio Nahuel for technical assistance. This work was supported by Universidad de Buenos Aires (grant number: 20020120100280 to PV), CONICET 2014-2016 (grant number 11220130100501CO to PV) and Agencia Nacional de Promoción Científica y Tecnológica (grant number: PID 020-2013 to SA).

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Delgadin TH, Simó I, Pérez Sirkin DI, Di Yorio MP, Arranz SE, Vissio PG. *Cichlasoma dimerus* responds to refeeding with a partial compensatory growth associated with an increment of the feed conversion efficiency and a rapid recovery of GH/IGFs axis. *Aquacult Nutr*. 2018;00:1–10. https://doi.org/10.1111/anu.12661