

Article

Isolation and Characterization of the *Arapaima gigas* Growth Hormone (ag-GH) cDNA and Three-Dimensional Modeling of This Hormone in Comparison with the Human Hormone (hGH)

Eliana Rosa Lima ¹, Renan Passos Freire ¹, Miriam Fussae Suzuki ¹, João Ezequiel Oliveira ¹, Vanessa Luna Yosidaki ¹, Cibele Nunes Peroni ¹, Thaís Sevilhano ¹, Moisés Zorzeto ², Lucas Simon Torati ³, Carlos Roberto Jorge Soares ¹, Igor Daniel de Miranda Lima ⁴, Thales Kronenberger ^{5,6,7,8}, Vinicius Gonçalves Maltarollo ⁴ and Paolo Bartolini ^{1,*}

- ¹ Instituto de Pesquisas Energéticas e Nucleares (IPEN-CNEN), Cidade Universitária, São Paulo 05508-000, SP, Brazil
- ² Piscicultura Raça, Canabrava do Norte 78658-000, MT, Brazil
- ³ EMBRAPA Pesca e Aquicultura, Loteamento Água Fria, Palmas 77008-900, TO, Brazil
- ⁴ Departamento de Produtos Farmacêuticos, Faculdade de Farmácia, Universidade Federal de Minas Gerais (UFMG), Av. Presidente Antônio Carlos, 6627, Belo Horizonte 31270-901, MG, Brazil
- ⁵ Institute of Pharmacy, Pharmaceutical and Medicinal Chemistry and Tübingen Center for Academic Drug Discovery, Eberhard Karls University Tübingen, Auf der Morgenstelle 8, 72076 Tübingen, Germany
- ⁶ Department of Oncology and Pneumology, Internal Medicine VIII, University Hospital Tübingen, Otfried-Müller-Straße 10, DE, 72076 Tübingen, Germany
- ⁷ Tübingen Center for Academic Drug Discovery & Development (TüCAD2), 72076 Tübingen, Germany
- ⁸ School of Pharmacy, Faculty of Health Sciences, University of Eastern Finland, 70211 Kuopio, Finland
- * Correspondence: pbartoli@ipen.br



Citation: Lima, E.R.; Freire, R.P.; Suzuki, M.F.; Oliveira, J.E.; Yosidaki, V.L.; Peroni, C.N.; Sevilhano, T.; Zorzeto, M.; Torati, L.S.; Soares, C.R.J.; et al. Isolation and Characterization of the *Arapaima gigas* Growth Hormone (ag-GH) cDNA and Three-Dimensional Modeling of This Hormone in Comparison with the Human Hormone (hGH). *Biomolecules* **2023**, *13*, 158. <https://doi.org/10.3390/biom13010158>

Academic Editor: Vladimir N. Uversky

Received: 7 November 2022

Revised: 15 December 2022

Accepted: 30 December 2022

Published: 12 January 2023

Abstract: In a previous work, the common gonadotrophic hormone α -subunit (ag-GTH α), the ag-FSH β - and ag-LH β -subunit cDNAs, were isolated and characterized by our research group from *A. gigas* pituitaries, while a preliminary synthesis of ag-FSH was also carried out in human embryonic kidney 293 (HEK293) cells. In the present work, the cDNA sequence encoding the ag-growth hormone (ag-GH) has also been isolated from the same giant Arapaimidae Amazonian fish. The ag-GH consists of 208 amino acids with a putative 23 amino acid signal peptide and a 185 amino acid mature peptide. The highest identity, based on the amino acid sequences, was found with the Elopiformes (82.0%), followed by Anguilliformes (79.7%) and Acipenseriformes (74.5%). The identity with the corresponding human GH (hGH) amino acid sequence is remarkable (44.8%), and the two disulfide bonds present in both sequences were perfectly conserved. Three-dimensional (3D) models of ag-GH, in comparison with hGH, were generated using the threading modeling method followed by molecular dynamics. Our simulations suggest that the two proteins have similar structural properties without major conformational changes under the simulated conditions, even though they are separated from each other by a >100 Myr evolutionary period (1 Myr = 1 million years). The sequence found will be used for the biotechnological synthesis of ag-GH while the ag-GH cDNA obtained will be utilized for preliminary Gene Therapy studies.

Keywords: *Arapaima gigas*; growth hormone; pirarucu; molecular modeling; in silico sequencing



Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Arapaima gigas (the Brazilian pirarucu) is a giant fish of the order of Osteoglossiformes, which is native to the Amazon River basin that can reach 3 m in length and weigh up to 250 kg. It is very important for human nutrition and extractivism in the region, but this species is in danger of disappearing because of the increasing human presence and exploitation by predatory fishing [1–3]. Commercial breeding is still incipient and established

reproductive centers face difficulties, especially due to the limited reproduction capacity of this species in captivity [4–10].

It is known that the two pituitary gonadotrophic hormones, the follicle-stimulating hormone (FSH) and the luteinizing hormone (LH), regulate reproductive processes such as gametogenesis and follicular growth in all vertebrates [11–14]. Given the extensive experience of our research group with human pituitary gonadotrophins, we have carried out the molecular cloning and characterization of *A. gigas* FSH and LH alpha- and beta-subunit cDNAs, together with a preliminary synthesis in HEK293 cells and a characterization of ag-FSH [15,16].

Growth hormones could be particularly important for *A. gigas* breeding since, in addition to the well-known effects on somatic growth and muscle mass increase, it also has immune and reproductive functions stimulating spermatogonial proliferation and accelerating the spermatogenic processes in fish [17,18]. In fact, it has been demonstrated that GH promotes testosterone and estradiol production in the catfish *Clarias batrachus*, exerting a reproductive role in this species [19]. For these reasons, we are currently collaborating with established *A. gigas* reproduction centers in order to carry out the molecular cloning and characterization of ag-GH cDNA.

A schematic image of *A. gigas* and the signaling pathway of hormones is presented in Figure 1.

For this purpose, we have employed a two-pronged approach: bioinformatics (“in silico”) to derive the ag-GH cDNA from the whole genomic sequence of pirarucu, available via the very comprehensive and useful work of Vialle et al. [20], together with the classical method based on pituitary extracted mRNA [16] to confirm the previously found sequence. Based on the bioinformatics approach, it is practically impossible to find specific 5’UTR or 3’UTR sequences. Since our vector constructions usually do not need the 5’UTR, we chose to work only with the 3’UTR, which is adequate for the detection of the poly(A) signal and poly(A) tail.

The cloning of the GH cDNA for different species of fish has been reported by some authors [21–24], while other authors also reported the related synthesis in different hosts [25–30].

According also to our phylogenetic studies [15,16], the fish GH sequences that are expected to have the highest percentages of identity with ag-GH are those related to *Anguilla anguilla* and *Acipenser baerii*.

Considering the importance that ag-GH has on somatic growth, muscular mass increase, and also on reproductive functions, our goal has been to obtain, for the first time, its cDNA sequence. This will be used for ag-GH biotechnological synthesis and preliminary gene therapy studies. As in our previous work on ag-FSH and ag-LH [16], we employed 3D modeling for ag-GH, permitting now a comparison of this structure with that of human GH, separated from ag-GH by an evolutionary period of more than 100 Myr [20,31,32].

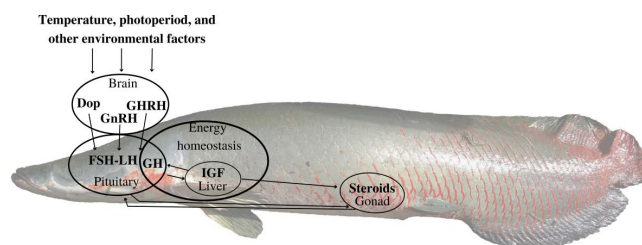


Figure 1. Schematic figure representing the main signaling pathways in the control of fish reproduction, adapted from Migaud et al. [33]. Environmental information (temperature, photoperiod, etc.), perceived in the brain (pineal, retina, and other receptors) initiates a reproductive cascade regulating the gonadotropin-releasing hormone (GnRH), which initiates the brain–pituitary–gonadal (BPG) cascade. While GnRH release stimulates FSH-LH release, dopamine (Dop) exerts an inhibitory effect. The growth hormone (GH) controlling the insulin-like growth factor (IGF) production in the liver is believed to be key a messenger in both energy homeostasis and gonadogenesis through the stimulation of gonadal sex steroids [19].

2. Materials and Methods

2.1. *A. gigas* Pituitary Gland Sources and the Detection of a First Partial Region of ag-GH

Pituitary glands were obtained from two fish farms. The first is located in São João da Boa Vista (São Paulo), and the processed material was used for studies regarding ag-LH and ag-FSH [16] and detection of a first partial region (~200 bp, “seq1”) of ag-GH, utilizing primer #1 and #2 (Table 1). Initially, the design of the two primers was based on conserved regions of the GH gene from three different fish species: *Anguilla anguilla*, *Cyprinus carpio*, and *Ictalurus punctatus*, available in GenBank under the accession numbers AY148493.1, M27000.1, and NM_001200245.1, respectively. For the selection of the species in this study, data previously obtained by our research group were used [16,17]. These GH sequences were aligned and the region that presented the closest profile for the three species was chosen as a safe region for the design of the primers used in this first step. The second pituitary collection was from Canabrava do Norte, Mato Grosso State, within the facilities of the Raça fish farm, collaborating with our project. The animals weighed 15–40 kg and the collected pituitary glands were 70–100 mg. These were stored in 1 mL of RNALater™ (ThermoFisher Scientific, Waltham, MA, USA), and transported to our laboratory in São Paulo in dry ice.

Table 1. Primers used in the cloning of *A. gigas* growth hormone.

Number	Direction	Name	Sequence
Primer 1	Sense	ag-GH1	5' TCA GCA AGA CCT CCC CA 3'
Primer 2	Antisense	ag-GH2	5' TTG AAG CTG CAT GTG GGA TCC TTG 3'
Primer 3	Sense	GSP1	5' GAG GCT TCT TAC TGG GCT CC 3'
Primer 4	Antisense	GSP6	5' AAG TGC AGT TGC TCT CCA CAA 3'
Primer 5	Antisense	AP	5' GGC CAC GCG TCG ACT AGT ACT TTT TTT TTT TTT TTT T 3'
Primer 6	Sense or Antisense	AUAP	5' GGC CAC GCG TCG ACT AGT AC 3'
Primer 7	Sense	GSP8	5' CTC TCA AGA CCT TCA GCG AGG 3'

2.2. In Silico Prediction Analysis

After Sanger sequencing of “seq1”, it was used to identify the position of ag-GH in the *A. gigas* genome [20] (NCBI access number GCA_900497675.1) using MegaBLAST [34]. After having found a unique location (scaffold 1040) and employing the gene prediction tool Gnomon pipeline [35], four exons were rearranged according to Splign and Chainer, allocating one single variant of 627 nt, which included the whole coding sequence of ag-GH. ClustalΩ pairwise alignment [36] using 44 fish GH coding sequences provided a similarity ranging between 35.8 and 92.8 %, confirming our data. The fish species and respective GenBank accession numbers are listed below (Table 2).

Table 2. Fish species and GenBank accession numbers of the sequences used in the amino acid percentage determinations and cDNA cloning of the fish growth hormone.

Organism	Nucleotide (mRNA)	Amino Acid	Superorder, Order, and Family
<i>Arapaima gigas</i>			Osteoglossomorpha, Osteoglossiformes, Arapaimidae
<i>Acipenser baerii</i>	JX947839.1	AFX71645.1	Chondrostei, Acipenseriformes, Acipenseridae
<i>Anguilla japonica</i>	M24066.1	AAA48535.1	Elopomorpha, Anguilliformes, Anguillidae
<i>Anguilla anguilla</i>	AY148493.1	AAN61122.1	Elopomorpha, Anguilliformes, Anguillidae
<i>Chanos chanos</i>	XM_030794045.1	XP_030649905.1	Ostariophysi, Cypriniformes, Cyprinidae
<i>Colossoma macropomum</i>	XM_036564141.1	XP_036420034.1	Ostariophysi, Characiformes, Serrasalminae
<i>Coregonus clupeaformis</i>	XM_041852232.2	XP_041708166.2	Protacanthopterygii, Salmoniformes, Salmonidae

Table 2. Cont.

Organism	Nucleotide (mRNA)	Amino Acid	Superorder, Order, and Family
<i>Electrophorus electricus</i>	XM_027026729.2	XP_026882530.1	Ostariophysi, Gymnotiformes, Gymnotidae
<i>Lates calcarifer</i>	X59378.1	CAA42022.1	Percoidei, Perciformes, Latidae
<i>Megalops cyprinoides</i>	XM_036552661.1	XP_036408554.1	Elopomorpha, Elopiformes, Megalopidae
<i>Myripristis murdjan</i>	XM_030057829.1	XP_029913689.1	Berycoidei, Beryciformes, Holocentridae
<i>Pangasianodon hypophthalmus</i>	JF303887.1	ADX99243.1	Ostariophysi, Siluriformes, Pangasiidae
<i>Paramormyrops kingsleyae</i>	XM_023835741.1	XP_023691509.1	Osteoglossomorpha, Osteoglossiformes, Mormyridae
<i>Scleropages formosus</i>	XM_018764341.2	XP_018619857.1	Osteoglossomorpha, Osteoglossiformes, Osteoglossidae
<i>Salmo trutta</i>	XM_029727357.1	XP_029583217.1	Protacanthopterygii, Salmoniformes, Salmonidae
<i>Salmo salar</i>	X14305.1	CAA32481.1	Protacanthopterygii, Salmoniformes, Salmonidae
<i>Salvelinus namaycush</i>	XM_038989124.1	XP_038845052.1	Protacanthopterygii, Salmoniformes, Salmonidae
<i>Toxotes jaculatrix</i>	XM_041062256.1	XP_040918190.1	Acanthopterygii, Perciformes, Toxotidae
<i>Salvelinus alpinus</i>	XM_024010963.1	XP_023866731.1	Protacanthopterygii, Salmoniformes, Salmonidae
<i>Monopterus albus</i>	AY265351.1	AAP42271.1	Acanthopterygii, Synbranchiformes, Synbranchidae
<i>Sebastes schlegelii</i>	U89917.1	AAB49492.1	Acanthopterygii, Scorpaeniformes, Sebastidae
<i>Solea senegalensis</i>	U01143.1	AAA60372.1	Acanthopterygii, Pleuronectiformes, Soleidae
<i>Paralichthys olivaceus</i>	D29737.1	BAA06159.1	Acanthopterygii, Pleuronectiformes, Paralichthyidae
<i>Thunnus thynnus</i>	X06735.1	CAA29914.1	Acanthopterygii, Perciformes, Scombridae
<i>Scomber japonicus</i>	GU138643.1	ACZ04987.1	Acanthopterygii, Perciformes, Scombridae
<i>Chrysophrys major</i>	AB904715.1	BAR88081.1	Acanthopterygii, Perciformes, Sparidae
<i>Epinephelus coioides</i>	AY513647.1	AAR97729.1	Acanthopterygii, Perciformes, Serranidae
<i>Morone saxatilis</i>	XM_035674082.1	XP_035529975.1	Acanthopterygii, Perciformes, Moronidae
<i>Dicentrarchus labrax</i>	GQ918491.1	ADB23477.1	Acanthopterygii, Perciformes, Moronidae
<i>Amphiprion melanopus</i>	HM135406.1	ADJ57589.1	Acanthopterygii, Perciformes, Pomacentridae
<i>Oreochromis niloticus</i>	KT387598.1	AMB21545.1	Acanthopterygii, Perciformes, Cichlidae
<i>Acipenser schrenckii</i>	KC460212.2	AGI96360.2	Chondrostei, Acipenseriformes, Acipenseridae
<i>Acipenser sinensis</i>	EU599640.2	ACC86123.1	Chondrostei, Acipenseriformes, Acipenseridae
<i>Ctenopharyngodon idella</i>	M27094.1	AAA58724.1	Ostariophysi, Cypriniformes, Cyprinidae
<i>Danio rerio</i>	AJ937858.1	CAI79040.1	Ostariophysi, Cypriniformes, Cyprinidae
<i>Hypophthalmichthys nobilis</i>	GQ497221.1	ADD62434.1	Ostariophysi, Cypriniformes, Cyprinidae
<i>Pimephales promelas</i>	AY643399.1	AAT91088.1	Ostariophysi, Cypriniformes, Cyprinidae
<i>Clarias gariepinus</i>	FJ823972.1	ACN97175.1	Ostariophysi, Siluriformes, Clariidae
<i>Ictalurus punctatus</i>	EU009499.1	ABS70461.1	Ostariophysi, Siluriformes, Ictaluridae
<i>Silurus meridionalis</i>	AF530481.2	AAP82934.1	Ostariophysi, Siluriformes, Siluridae
<i>Coregonus autumnalis</i>	X77245.1	CAA54461.1	Protacanthopterygii, Salmoniformes, Salmonidae
<i>Oncorhynchus mykiss</i>	NM_001124689.1	NP_001118161.1	Protacanthopterygii, Salmoniformes, Salmonidae
<i>Gadus morhua</i>	EU676171.1	ACD46080.1	Paracanthopterygii, Gadiformes, Gadidae
<i>Fundulus heteroclitus</i>	XM_012872083.3	XP_012727537.1	Acanthopterygii, Cyprinodontiformes, Fundulidae
<i>Trichogaster trichopterus</i>	AF157633.1	AAG60346.1	Acanthopterygii, Perciformes, Osphronemidae

2.3. Obtaining the Coding Region from the Pituitary Gland cDNA of *A. gigas*

After confirmation of the coding region in silico, new specific primers were designed using BLAST- NCBI, and a new PCR reaction was performed on pituitary extracted RNA, following the manufacturer's guidelines. The Platinum™ SuperFi™ II DNA Polymerase system (Invitrogen™, ThermoFisher Scientific, Waltham, MA, USA) was used. A primer sense (#3) and another antisense (#4) (Table 1) were used, obtaining, according to the Sanger

method, a fragment of approximately 600 bp that included the whole coding sequence of ag-GH. The latter perfectly matched the sequence previously obtained in silico.

2.4. Rapid Amplification of cDNA Ends (RACE) for Obtaining agGH 3'UTR

For rapid amplification of the 3' ends of the cDNA, the commercial 3' RACE System for rapid amplification of the cDNA ends kit (Invitrogen™, ThermoFisher Scientific, Waltham, MA, USA) was used. The primer for this reaction was also designed based on the sequence previously obtained in silico. From 5 µg of a “template strand” of total RNA extracted from the pituitary glands, a 3' RACE reaction was performed using 1 µL of PCR Anchor Primer (AP, #5, Table 1) (provided by the manufacturer) and 200U (1 µL) of the Go Script® Reverse Transcription System (Promega, Madison, WI, USA) to obtain single-stranded cDNA. PCR was performed on single-stranded cDNA using 1 µL of 10 µM specific primer sense (#7) together with the AUAP antisense primer (#6) and 10 µL of 10 µM Taq polymerase Platinum SuperMix High Fidelity® (ThermoFisher Scientific, Waltham, MA, USA). Cycling was performed as per the manufacturer's instructions. After completion of the reaction, the cDNA obtained was purified by agarose gel electrophoresis and sequenced.

2.5. Molecular Modelling

The initial three-dimensional (3D) models of *A. gigas* GH (ag-GH) were generated using the threading modeling method with a similar but updated protocol of previously reported work [16]. Five initial models were built using the SWISS-MODEL web server (<https://swissmodel.expasy.org/>, accessed on 26 June 2022) [37,38] based on the highest sequence identity with potential templates (PDB IDs: 3hhr [39], 1hwg [40], 1a22 [41], 1bp3 [42], and 1axi [43], with 49.72, 49.72, 49.16, 44.81, and 44.26% of identity, respectively). Subsequently, all five models were compared according to the global model quality estimate (GMQE) and QMEANDisCo global score [44], and the best-scored model was submitted to model refinement with DeepRefiner (<http://watson.cse.eng.auburn.edu/DeepRefiner/>, accessed on 6 August 2022) [45] and ModLoop (<https://modbase.compbio.ucsf.edu/modloop/>, accessed on 27 June 2022) [46] web servers. Additionally, a model obtained from AlphaFold [47,48] implemented on NMRbox [49] was also used to compare with other models. The refined models were also compared according to their validation metrics, such as the Q-MEAN scoring function [50] and Ramachandran plots [51] (Supplementary Figure S1), and the best-performing model according to the quality control was submitted to molecular dynamics (MD) simulations.

2.6. Molecular Dynamics

The selected ag-GH model was submitted to an MD simulation and compared to the human GH (hGH) model simulations (PDB ID: 1hwg, the template employed) as the control. The protein structures were prepared using Epik [52] by adjusting the ionization states of the amino acid residues and further subjecting them to restrained energy minimization (PrepWiz, Maestro v2019.1). The missing residues of hGH were inserted manually following the available amino acid sequence of the PDB file. The N- and C-termini were capped, and ions and waters of crystallization were removed. Disulfide bonds were set automatically. The final structure was minimized using PrepWizard and the OPLS4 force field [53], with movements constrained to a maximum variation of 0.3 Å.

MD simulations were carried out using Desmond [54] with the OPLS4 force field [53]. The simulated system encompassed the refined protein model, a predefined explicit solvent model for water (TIP3P [55]) and counterions (Na⁺ or Cl[−] adjusted to neutralize the overall system charge). The entire system was contained in a 13 Å³ cubic box with periodic boundary conditions. We used a time step of 1 fs and the short-range coulombic interactions were treated using a cut-off value of 9.0 Å, while the smooth particle mesh ewald method (PME) handled the long-range coulombic interactions [56]. Initially, the system was minimized using steepest descent followed by equilibration. All simulations were run in an NPT ensemble (T = 310 K, Nosé–Hoover method; $p = 1.01325$ bar, Martyna–Tobias–

Klein method) with default Desmond settings. Reversible reference system propagator algorithms (RESPA) with 2 fs, 2 fs, and 6 fs timesteps were used for bonded, near and far, respectively. A constant temperature of 310 K was maintained throughout the simulation using the Nosé–Hoover thermostat algorithm, together with the Martyna–Tobias–Klein Barostat algorithm to maintain 1 atm of pressure. After minimization and relaxation of the system, we proceeded with five independent production steps of 200 ns (total 1 μ s) for both systems, with randomly generated seeds. The concatenated trajectory of the five replicates for each system was analyzed according to RMSD and RMSF calculations for C-alpha atoms and secondary structure formation. In this sense, RMSD plots were generated using a single concatenated trajectory of 1 μ s represented by five replicas of 200 ns, while RMSF was represented by the average values calculated for each amino acid residue. Finally, clusterization of structures was performed to retrieve the most frequent conformation of both proteins.

2.7. Quantitative Similarity Comparison between the ag-GH Model and the Template (hGH)

Two distinct methods were employed to structurally compare the generated model and the selected template: ConSurf (https://consurf.tau.ac.il/consurf_index.php, accessed on 1 September 2022) [57–59] and 3D visualization of sequence similarity (SS3D) [60]. The ConSurf method calculates the evolutionary rate of each amino acid position of the protein to identify the most structurally and functionally important positions. In this context, the evolutionary rate is inversely proportional to the degree of conservation of a given site. The most populated cluster frames from the MD of the ag-GH and the hGH models were used as input.

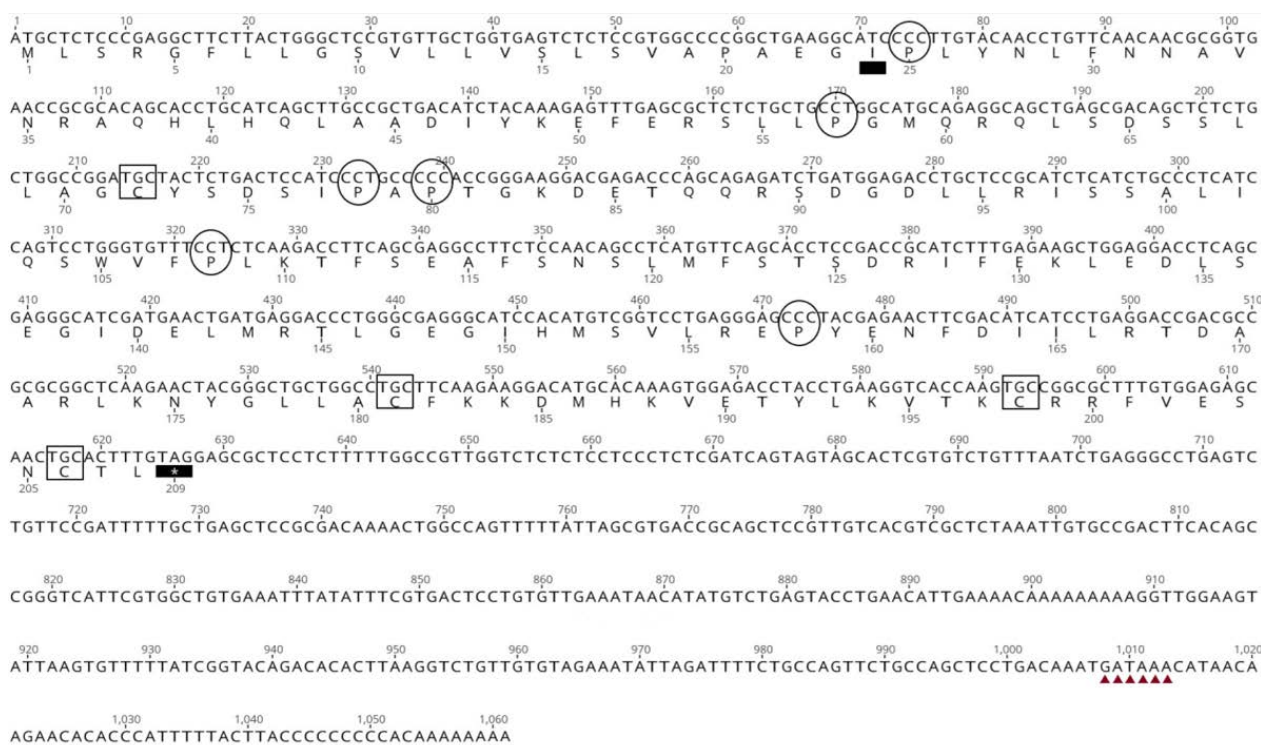
The SS3D method was carried out by comparing the ag-GH and hGH clusters from the MD results to the template crystallographic structure (PDB ID: 1hwg) to highlight any difference between these homologous proteins by combining sequence and structure information. At this step, a contact map of the α -carbons was generated with a cut-off distance of 0.6 nm. The SS3D value for each of the selected contacts was calculated employing the experimental structure of hGH (PDB ID: 1hwg) as the reference for comparing the structures. For this purpose, we selected residues whose α -carbon was within a radial distance of 1 nm from any of the residues in contact, considering only residues that persisted within this radius for more than 75% of the frames. Finally, the structures were mapped between 0 (dissimilar) and 1 (similar), according to the calculated SS3D value.

3. Results

3.1. cDNA Sequencing

The DNA sequences obtained in silico and via 3' RACE using RNA from two different pituitary glands were compared and aligned. The *A. gigas* GH cDNA sequence (Figure 2) was 1061 bp in total length, with an open reading frame of 627 bp, beginning with the first ATG codon at position 1 bp and ending with the stop codon at position 627, presenting a 434 bp 3'-UTR. A polyadenylation signal (GATAAA) was recognized 40 bp upstream from the poly(A⁺) tail. The coding region translates into a polypeptide of 208 amino acids, while the cleavage site for the putative signal peptide was between amino acids 23 and 24. The signal peptide prediction was based on Signal P 6.0 [61].

This corresponds to a mature peptide of 185 and a signal peptide of 23 amino acids. The mature peptide of ag-GH thus contains four conserved cysteines and six prolines (Pro², Pro³⁴, Pro⁵⁵, Pro⁵⁷, Pro⁸⁵, and Pro¹³⁵), three of which (underlined) are highly conserved (Figure 2). There is one potential glycosylation site at Asn¹⁸² that can be seen in all species, but one, in the alignment of the ag-GH mature peptide with the forty-one teleosts and three Acipenseriformes in Figure 3.



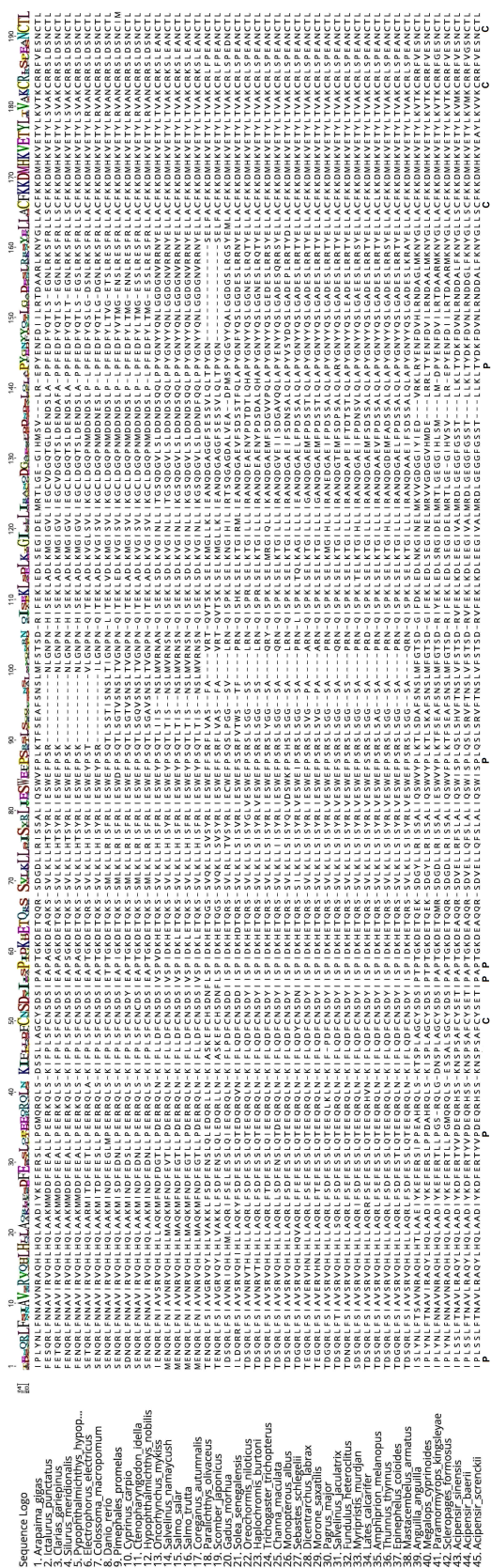


Figure 3. Alignment of the GH cDNA coding sequence from 45 fish species, including *Arapaima gigas*. The symbols indicate prolines (P) and cysteines (C).

Table 3. Percentage identity of GH peptides between fish orders.

Superorder	Order	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. Osteoglossomorpha	Osteoglossiformes (3)	86.4	53.8	62.7	63.1	63.2	74.5	82.0	79.7	62.6	68.2	66.3	65.6	62.3	70.8
2. Acanthopterygii	Pleuronectiformes (2)	-	63.7	68.1	67.6	68.3	55.7	45.7	45.2	63.0	68.5	53.5	80.7	59.5	78.0
3. Acanthopterygii	Scorpaeniformes (1)		-	100	84.8	84.3	66.0	40.1	40.1	72.6	78.2	52.4	84.9	65.7	86.3
4. Acanthopterygii	Synbranchiformes (1)			-	100	86.3	67.2	40.1	40.1	72.1	77.7	52.9	84.9	66.7	86.5
5. Berycoidei	Beryciformes (1)				-	100	66.9	41.5	41.0	74.1	79.5	55.8	84.7	71.6	87.4
6. Chondrostei	Acipenseriformes (3)					-	97.2	81.5	79.4	62.2	66.6	69.0	64.8	65.7	68.4
7. Elopomorpha	Elopiformes (1)						-	100	86.6	69.5	78.6	49.1	75.5	38.4	82.8
8. Elopomorpha	Anguilliformes (1)							-	100	68.0	78.1	46.7	75.5	37.9	82.7
9. Ostaryophysi	Siluriformes (3)								-	95.2	82.7	88.8	71.0	71.9	78.8
10. Ostaryophysi	Cypriniformes (5)									-	92.9	89.2	68.5	77.6	78.3
11. Ostaryophysi	Characiformes (1)										-	100	78.3	49.0	87.2
12. Acanthopterygii	Perciformes (9)											-	84.8	62.3	70.8
13. Paracanthopterygii	Gadiformes (1)												-	100	85.6
14. Protacanthopterygii	Salmoniformes (6)													-	97.0

Table 4. Percentage identity of GH peptides of different species relative to *A. gigas*.

Common name	Scientific Name	Identity (%)	GenBank/NCBI
Pirarucu	<i>Arapaima gigas</i>	100.0	OP575308
Crocodile	<i>Crocodylus porosus</i>	61.5	AAB34999.1
Manatee	<i>Trichechus manatus</i>	60.9	XP_004374274.1
Australian parakeet	<i>Melopsittacus undulatus</i>	59.4	XP_005140349.2
Whale	<i>Balaenoptera physalus</i>	58.9	CAH42020.1
Dolphin	<i>Tursiops truncatus</i>	58.9	XP_033703592.1
Orca	<i>Orcinus orca</i>	58.9	XP_004275687.2
Mouse	<i>Mus musculus</i>	57.8	AAH61157.1
Ox	<i>Bos taurus</i>	56.3	AAX07134.1
Chimpanzee	<i>Pan troglodytes</i>	44.7	AAL72284.1
Human	<i>Homo sapiens</i>	44.7	KAI2584577.1

3.2. Three-Dimensional Model of agGH

The five initial models obtained from the SWISS-MODEL were very similar. The model generated using the structure of hGH determined by X-ray crystallography (PDB ID: 1hwg) as a template was chosen due to the slightly higher GMQE score. Two refined models were then obtained from the DeepRefiner and ModLoop servers. The model retrieved from the first method drastically improved the quality of the model concerning the Ramachandran plot (0% of outliers) and QMEAN Z-scores (Supplementary Figure S1). In addition, the refined model was compared with the template to validate several of the structural features before the MD simulations. Finally, the model obtained with AlphaFold improved the quality of C β atoms, all atoms, and solvation scores, but decreased the QMEAN Z-score and torsion score. Therefore, we followed the refined model from the SWISS-MODEL. The 3D structures of the selected model and the experimental template structure are very similar (Figure 4A). All secondary structures were conserved, and their superimposition shows low dissimilarity (RMSD 0.81 Å, where <1 is considered similar). Furthermore, the electrostatic surfaces of the two proteins were also similar with comparable charge distributions, in particular in the regions reported to bind the GH receptor (regions I, II, and III in Figure 4B). Therefore, the quality measurements and the structural comparison both suggested that the model selected was suitable for further analysis.

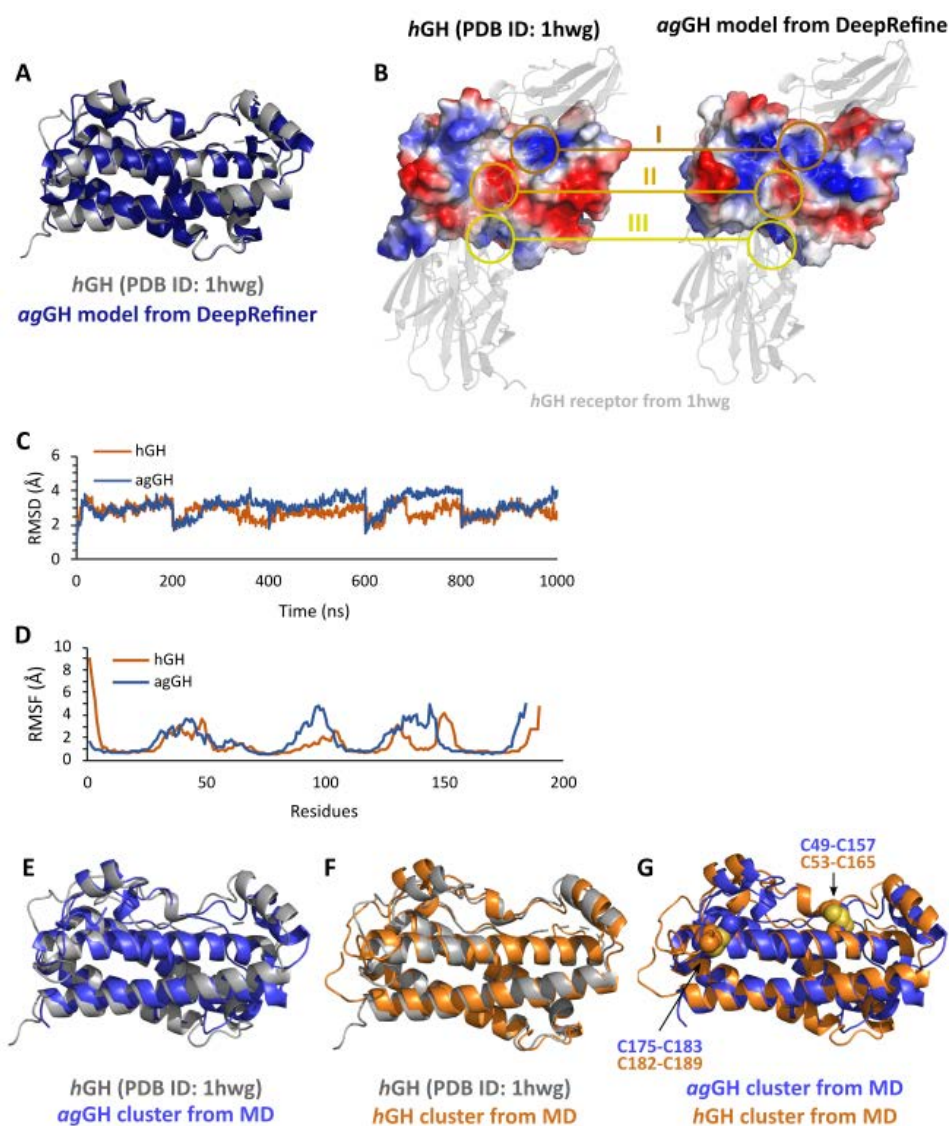


Figure 4. Superimposition of the template (PDB ID: 1hwg) and the refined structure (A). A comparison of the electrostatic potential surfaces (ranging from red, the electron-rich region, to blue, the electron-deficient region) of the template structure with that of the refined initial ag-GH model and with the PDB structure of the hGH receptor, shown as a transparent grey cartoon (B). RMSD (C) and RMSF (D) values were calculated for C-alpha atoms during the whole trajectories for both the ag-GH model (dark blue) and the hGH model (dark orange). The RMSD plot is presented as five subsequently concatenated 200 ns replicas resulting in a single plot of 1 μ s. Superimposition of the ag-GH model (blue) retrieved from the cluster analysis of the MD simulations with the experimental template structure (grey) (E). Superimposition of the hGH model (orange) retrieved from the cluster analysis of the MD simulations with the experimental template structure (grey) (F). Superimposition of the ag-GH model (blue) and the hGH model (orange) retrieved from the cluster analyses of the MD simulations. The two disulfide bonds are displayed in sphere representation (G).

The subsequent MD simulations of ag-GH and hGH displayed similar structural stability along the simulation timescale (Figure 4C), as well as the fluctuations of the alpha carbons of both proteins (Figure 4D). Taken together, these results suggest that the two proteins have similar structural properties, without major conformational changes under the simulated conditions. The most representative conformations of ag-GH and hGH maintained overall folding and secondary structures that were similar to those of the X-ray structure of hGH employed as the template (Figure 4E,F). Therefore, the proposed model

for ag-GH appears to be stable, indicative of the quality of the model generated by this procedure. Indeed, the MD simulations only promoted structure relaxation rather than causing major conformational changes between the refined model of ag-GH and the initial X-ray structure of hGH. Moreover, the two disulfide bonds of ag-GH and hGH present the same 3D position in the two proteins (Figure 4G).

Lastly, we also analyzed the behavior of residues reported to bind to the GH receptor [40] in both models: Lys41, Gln46, Ser51, Glu56, Ser62, Lys64, Arg167, and Lys168 from hGH and Arg38, Asp42, Ala47, Asp52, Thr58, Arg60, Lys160, and Lys161 from agGH (Figure 5). Interestingly, the residues were conserved in terms of sequence alignment (Figure 5A) and according to the three-dimensional alignment as well (Figure 5B). In addition to the electrostatic potential surfaces that suggested a similar ability to bind to their receptor, the solvent-accessible surface area distribution of the above-mentioned residues was also very similar along the entire MD simulation (Figure 5C), suggesting the availability to interact with their receptor binding site instead of being buried.

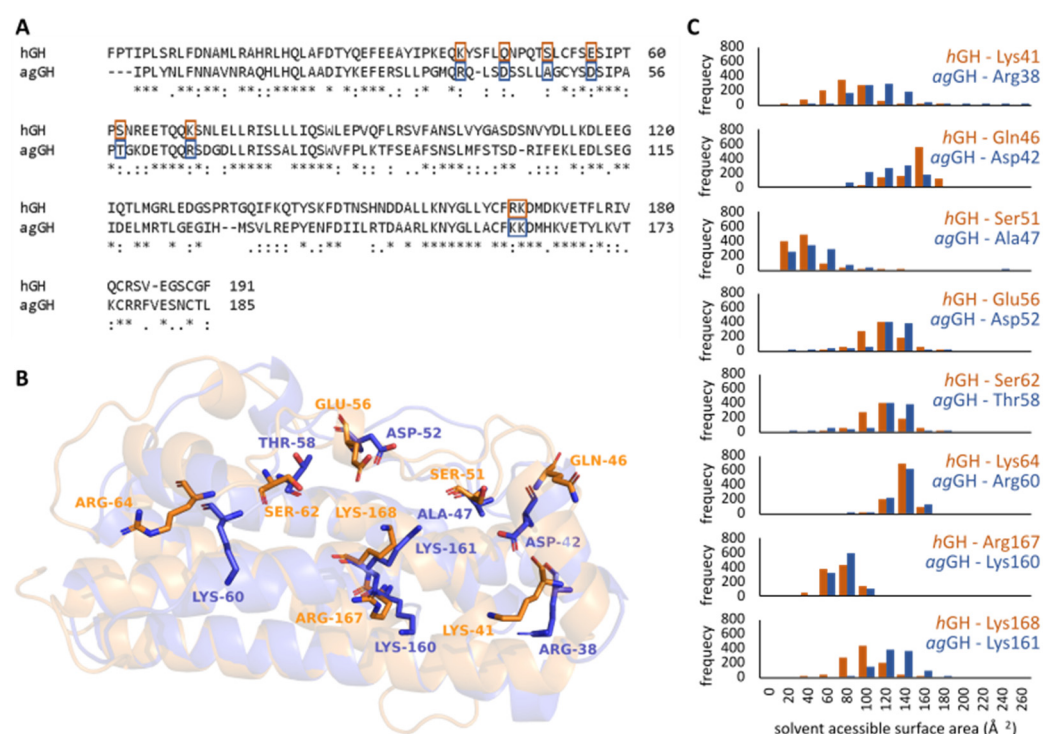


Figure 5. Alignment of hGH and agGH sequences using Clustal Omega [36], an * (asterisk) indicates positions which have a single, fully conserved amino acid residue, (colon): indicates residues with strongly similar properties, and (period) indicates residues with weakly similar properties (A). Alignment of three-dimensional structures highlighting the residues reported to be involved in binding at the receptor's binding site (B). Histograms of calculated solvent-accessible surface area distribution of each amino acid residue from both models during MD simulations (C).

3.3. Evolutionary Implications and Comparison of the Three-Dimensional Contacts of ag-GH and hGH Models

An independent comparison of the amino acid sequence conservation and the 3D similarity (Figure 6) confirmed the previous results. Thus, human (Figure 6A) and *A. gigas* (Figure 6B) GH models displayed similar amino acid conservation profiles in comparison with homologous proteins. Furthermore, the regions with the highest conservation scores were located at α -helices that interact with the GH receptor (Figure 4B). C-terminal residues could not be classified due to insufficient data.

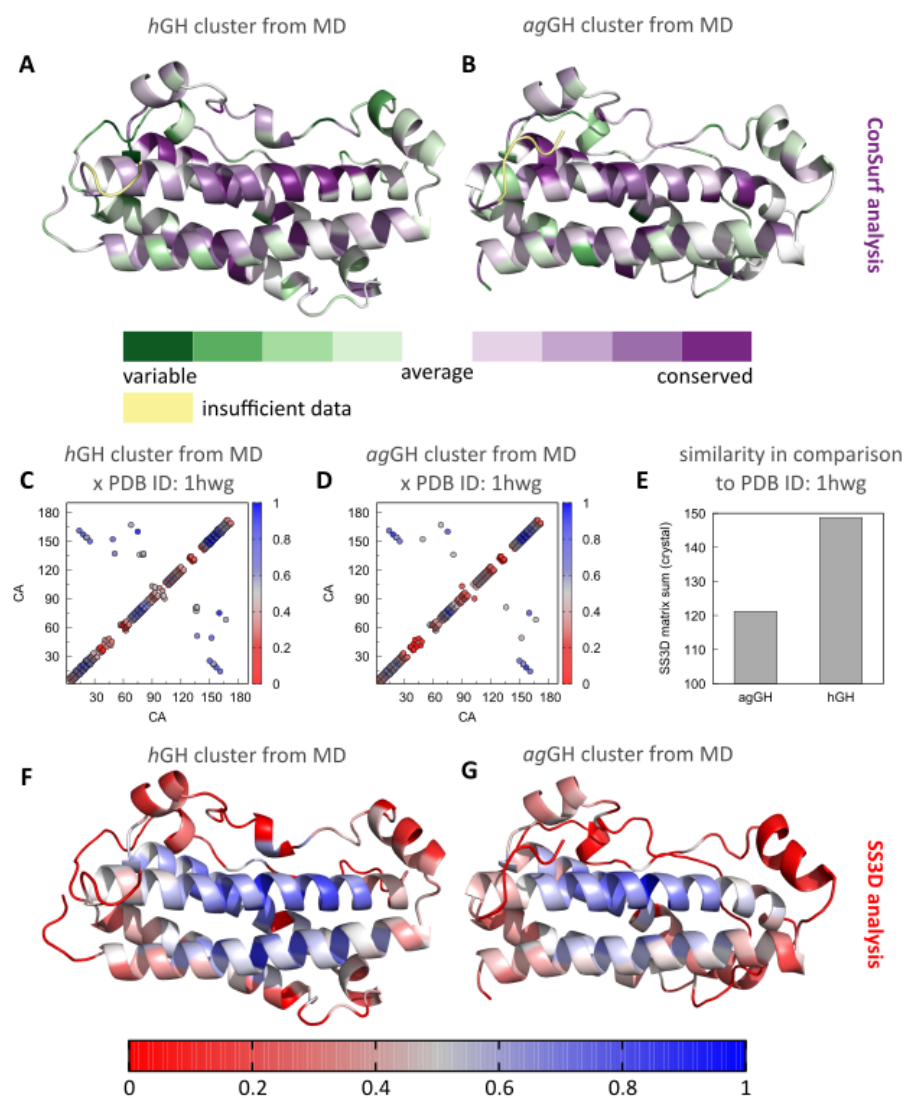


Figure 6. ConfSurf maps for the hGH model (A) and the ag-GH model (B) display the highly conserved regions of the proteins (purple) and the highly variable amino acid sites (green). Contact maps from the SS3D analysis for the hGH model (C) and the ag-GH model (D), as well as the overall SS3D similarity in comparison with the experimental template structure (PDB ID: 1hwg) (E), and the maps from the SS3D similarity analysis (F,G).

The same similarity profile was found in the SS3D studies. Both models were very similar to the experimental hGH structure of the template, in particular considering their α -helices (Figure 6F,G). In this regard, the contact matrices were very similar (Figure 6C,D) and the hGH model exhibited greater similarity to the hGH experimental structure (Figure 6E), as would be expected.

4. Discussion

For the first time, ag-GH cDNA has been sequenced and characterized, and also an *A. gigas* hormone was sequenced via in silico methods, and this sequence was compared with that obtained via the classical method. As far as we know, two homologous bioactive molecular structures, at an evolutionary distance of >100 million years, were never compared by three-dimensional modeling and molecular dynamics.

The characterization of ag-GH cDNA points to a protein with a putative signal peptide of 23 amino acid residues and a mature hormone of 185 amino acids. There are four cysteines (Cys⁴⁹, Cys¹⁵⁸, Cys¹⁷⁵, and Cys¹⁸³) that are perfectly conserved and located at

nearly the same position as those present in the GH of other vertebrates. One potential N-glycosylation site (Asn–Cys–Thr) is present at Asn182, separated from the stop codon only by a Leu. Although glycosylation is not usually found in GH molecules, it would be interesting to see whether ag-GH can be glycosylated in other eukaryotes. The same C-terminal (Asn–Cys–Thr–Leu) is very common in the GH cDNA of many species of fish, such as salmon [21], Japanese eel [26], pacu, giant catfish, channel catfish, bighead carp, silver carp [24,29] and prenanth's schizothoracin [30]. As a matter of fact, we can deduce from Figure 2 that only one species (*Pimephales promelas*) out of the forty-five considered does not have the same N-terminal ending.

The two disulfide bonds that can be formed by the four Cys are critical for the integrity and biological activity of the hormone. Consequently, the C-terminal region, where three out of four cysteines are located, should be particularly important for GH structure and function [62].

Six prolines, three of which are highly conserved, confirm that they can be essential for the protein structure since they tend to bend the local amino acid alignment and, therefore, fold the protein [16,63].

Table 3, comparing the pairwise identity of GH peptides between fish orders, shows that GH (38–89%) is less variable than FSH β (28–67%) and approximately as variable as LH β (47–89%) [16]. The greater ag-GH identity with Elopomorpha confirms our previous data obtained with ag-GTH α , ag-LH β , and ag-FSH β [15,16].

It is noteworthy that ag-GH exhibits a 44.7% sequence identity with the corresponding human hormone (hGH), and a 57.8% identity with a mouse GH. These values suggest the possibility of bioactivity determination via the classical *in vivo* bioassay based on dwarf “little” mice (lit/lit) [64], a hypothesis that still needs to be verified experimentally using purified ag-GH against the WHO's hGH International Standard. If this can be confirmed, it would be useful to set up an ag-GH standard for inter-laboratory comparisons and future production and purification. Even though immunological activity does not reflect *in vivo* biological activity, we found that a Western blot, based on anti-hGH antiserum, worked perfectly using ag-GH as an antigen (data not presented).

The theoretical protocol consisted of homology modeling followed by molecular dynamics simulations using multiple replicas with long timescale (200 ns or more) simulations to ensure that the final results were representative in terms of folding and structural motions [65–67]. Furthermore, in the simulations, both systems (ag-GH and hGH as control) presented similar conformational movements, as indicated by the RMSD and RMSF values (Figure 4C,D). The observed minor differences in the RMSD were not unexpected since different models of the same protein can display RMSD differences during long simulations [68].

The analysis of protein structures in terms of the most populated cluster (Figure 4E,F) indicated that ag-GH and hGH have very similar foldings, even after structural relaxation during the molecular dynamics simulation. In addition, the similarity of the more important structural features of the protein, such as the receptor binding regions and the disulfide bonds (Figure 4B,G, respectively), suggests that the functionality of the two proteins is conserved [69–71].

The function, interaction, and folding of proteins depend on a select group of specific amino acids and these tend to be conserved throughout evolution or replaced by residues with similar biophysical characteristics that still preserve their biological role [72]. Therefore, in order to confirm the consistency of the structures generated, the trajectories obtained from the MDs were analyzed by two different approaches.

The first analysis performed was to assess the evolutionary similarity through phylogenetic relationships between homologous sequences. Indeed, both hGH and ag-GH were found to have a very similar conservation profile when compared to homologous proteins, with conservation regions that coincided with each other (Figure 6A,B). Although there was only approximately a 50% sequence similarity between hGH and ag-GH, the most conserved region coincides exactly with the site responsible for the interaction with the GH

receptor. As previously shown by other works, this result is evidence that proteins share their functional roles [58,73].

To visualize details regarding the three-dimensional similarity and differences of the structures, the second approach analyzed a combination of sequence and structural similarity. Thus, ag-GH and hGH showed an SS3D value similar to that of the crystallographic structure of hGH (Figure 6E), which indicates that the two protein models have comparable residue identities around their α -carbon contacts. The SS3D tool indicates important sites involved in carrying out the function of homologous proteins, with these regions showing a high degree of spatial similarity [60]. Again, the region of greatest similarity corresponded to the site of interaction with the GH receptor, further strengthening the evidence that the proteins share a common function (Figure 6F,G).

Taken together, the results (predicted fold, electrostatic potential surface, evolutionary similarity, and 3D similarity) reinforce our hypothesis that the similarity of the proteins is related not only to their 3D structures, but also to their functionality as well.

In addition to the novelty of having cloned and sequenced ag-GH for the first time, there are practical aspects related to our ongoing collaboration with the two pirarucu fishing stations. Since fish GH is known for its effects on somatic growth and muscle mass increase and its action on reproduction, we are developing a preliminary gene therapy approach. A practical limitation is that repeated injections highly stress and decrease the appetite of these fish, so usual modes of GH administration are not really recommended. We will, therefore, study the effects of i.m. administrations of ag-GH cDNA, injecting only once a month or even less often, on ag-GH endogenous levels and mass increase, as our research group studied in dwarf “little” mice [74–76], and little/scid mice [77,78].

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/biom13010158/s1>, Figure S1. Comparison of Ramachandran plots and QMEAN scores of the SWISS-MODEL’s selected model, the two refined ones, and the model obtained from AlphaFold.

Author Contributions: Conceptualization, E.R.L. and P.B.; methodology, R.P.F. and M.F.S.; validation, J.E.O., V.L.Y., T.S. and C.N.P.; formal analysis, I.D.d.M.L., T.K. and V.G.M.; investigation, L.S.T. and M.Z.; resources, P.B. and C.R.J.S.; data curation, E.R.L. and R.P.F.; writing—original draft preparation, P.B., C.N.P. and V.G.M.; writing—review and editing, E.R.L. and P.B.; supervision, P.B.; project administration, P.B.; funding acquisition, P.B. and C.R.J.S. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the São Paulo State Research Foundation-FAPESP, São Paulo, Brazil, research project No. 2017/26369-1, No. 2020/10435-8, and No. 2020/16549-5; by CNPq, fellowship No. 169008/2018-5, No 2020/10435-8, No 305839/2021-7, and No. 305839/2021-7; and by CAPES, fellowship No. 88887.506371/2020-00 and No. 88887.624106/2021-00.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: The authors thank the CSC-IT Center for Science, Finland, for the very generous computational resources, the Baden-Württemberg Ministry of Science, as part of the Excellence Strategy of the German Federal and State Governments, Germany, the means of the program TüCAD2, and the “Pró-reitoria de Pesquisa” of the UFMG and FAPEMIG.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Borella, M.I.; Venturieri, R.; Mancera, J.M. Immunocytochemical identification of adenohypophyseal cells in the pirarucu (*Arapaima gigas*), an Amazonian basal teleost. *Fish. Physiol. Biochem.* **2009**, *35*, 3–16. [CrossRef]
2. Cavole, L.M.; Arantes, C.C.; Castello, L. How illegal are tropical small-scale fisheries? An estimate for Arapaima in the Amazon. *Fish. Res.* **2015**, *168*, 1–5. [CrossRef]
3. Watson, L.C.; Stewart, D.J.; Kretzer, A.M. Genetic diversity and population structure of the threatened Giant Arapaima in southwestern Guyana: Implications for their conservation. *Copeia* **2016**, *104*, 864–872. [CrossRef] [PubMed]

4. Garcia, A.; Tello, S.; Duponchelle, F. Patterns of commercial fish landings in the Loreto region (Peruvian Amazon) between 1984 and 2006. *Fish. Physiol. Biochem.* **2009**, *35*, 53–67. [\[CrossRef\]](#) [\[PubMed\]](#)
5. Chu-Koo, F.; Dugue, R.; Aguilar, M.A.; Daza, A.C.; Bocanegra, F.A.; Veintemilla, C.C.; Duponchelle, F.; Renno, J.F.; Tello, S.; Nunes, J. Gender determination in the Paiche or Pirarucu using plasma vitellogenin. 17 beta-estradiol and 11-ketotestosterone levels. *Fish. Physiol. Biochem.* **2009**, *35*, 125–136. [\[CrossRef\]](#) [\[PubMed\]](#)
6. Mylonas, C.C.; Fostier, A.; Zanuy, S. Broodstock management and hormonal manipulations of fish reproduction. *Gen. Comp. Endocrinol.* **2010**, *165*, 516–534. [\[CrossRef\]](#) [\[PubMed\]](#)
7. Castello, L.; Stewart, D.J.; Arantes, C.C. Modeling population dynamics and conservation of arapaima in the Amazon. *Rev. Fish. Biol. Fish.* **2011**, *21*, 623–640. [\[CrossRef\]](#)
8. Torati, L.S.; Varges, A.P.S.; Galvão, J.A.S.; Mesquita, P.E.C.; Migaud, H. Endoscopy application in broodstock management of *Arapaima gigas* (Schinz, 1822). *J. Appl. Ichthyol.* **2016**, *32*, 353–355. [\[CrossRef\]](#)
9. Torati, L.S.; Migaud, H.; Doherty, M.K.; Siwy, J.; Mullen, W.; Mesquita, P.E.C.; Albalat, A. Comparative proteome and peptidome analysis of the cephalic fluid secreted by *Arapaima gigas* (Teleostei: Osteoglossidae) during and outside parental care. *PLoS ONE* **2017**, *12*, e0186692. [\[CrossRef\]](#)
10. Torati, L.S.; Lima, A.F.; Kirschnik, L.N.G.; Migaud, H. Endoscopy and cannulation as non-invasive tools to identify sex and monitor reproductive development in *Arapaima gigas*. *Copeia* **2019**, *107*, 287–296. [\[CrossRef\]](#)
11. Huang, H.; Zhang, Y.; Huang, W.; Li, S.; Zhu, P.; Liu, Y.; Yin, S.; Lin, X.; Lin, H. Molecular characterization of marbled eel (*Anguilla marmorata*) gonadotropin subunits and their mRNA expression profiles during artificially induced gonadal development. *Gen. Comp. Endocrinol.* **2009**, *162*, 192–202. [\[CrossRef\]](#) [\[PubMed\]](#)
12. Levavi-Sivan, B.; Bogerd, J.; Mañanós, E.L.; Gómez, A.; Lareyre, J.J. Perspectives on fish gonadotropins and their receptors. *Gen. Comp. Endocrinol.* **2010**, *165*, 412–437. [\[CrossRef\]](#) [\[PubMed\]](#)
13. Schulz, R.W.; de França, L.R.; Larteyre, J.J.; Le Gac, F.; Chiarini-Garcia, H.; Nobrega, R.H.; Miura, T. Spermatogenesis in fish. *Gen. Comp. Endocrinol.* **2010**, *165*, 390–411. [\[CrossRef\]](#)
14. Senthilkumaran, B. Recent advances in meiotic maturation and ovulation: Comparing mammals and pisces. *Front. Biosci.* **2011**, *16*, 1898–1914. [\[CrossRef\]](#)
15. Faria, M.T.; Carvalho, R.F.; Sevilhano, T.C.A.; Oliveira, N.A.J.; Silva, C.F.P.; Oliveira, J.E.; Soares, C.R.J.; Garcez, R.; Santo, P.R.; Bartolini, P. Isolation of the pituitary gonadotrophic α -subunit hormone of the giant Amazonian fish: Pirarucu (*Arapaima gigas*). *Fish. Phys. Biochem.* **2013**, *39*, 683–693. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Sevilhano, T.; Carvalho, R.F.; Oliveira, N.A.; Oliveira, J.E.; Maltarollo, V.G.; Trossini, G.; Garcez, R.; Bartolini, P. Molecular cloning and characterization of pirarucu (*Arapaima gigas*) follicle-stimulating hormone and luteinizing hormone β -subunit cDNAs. *PLoS ONE* **2017**, *12*, e0183545. [\[CrossRef\]](#)
17. Canosa, L.F.; Chang, J.P.; Peter, R.E. Neuroendocrine control of growth hormone in fish. *Gen. Comp. Endocrinol.* **2007**, *151*, 1–26. [\[CrossRef\]](#)
18. Franz, A.C.; Oliver, F.; Kollner, B.; Shved, N.; Link, K.; Casanova, A.; Wenger, M.; D’Cotta, H.; Baroiller, J.F.; Ullrich, O.; et al. Endocrine and local IGF-I in the bony fish immune system. *Biology* **2016**, *5*, 9. [\[CrossRef\]](#)
19. Gopal, R.N.; Kumar, P.; Lal, B. Temperature dependent action of growth hormone on somatic growth and testicular activities of the catfish, *Clarias batrachus*. *Gen. Comp. Endocrinol.* **2014**, *195*, 125–131. [\[CrossRef\]](#)
20. Vialle, R.A.; de Souza, J.E.S.; Lopes, K.D.; Teixeira, D.G.; Sobrinho, P.D.A.; Ribeiro-dos-Santos, A.M.; Furtado, C.; Sakamoto, T.; Silva, F.A.O.; de Oliveira, E.H.C.; et al. Whole genome sequencing of the pirarucu (*Arapaima gigas*) supports independent emergence of major teleost clades. *Genome Biol. Evol.* **2018**, *10*, 2366–2379. [\[CrossRef\]](#)
21. González-Villaseñor, L.I.; Zhang, P.; Chen, T.T.; Powers, D.A. Molecular cloning and sequencing of coho salmon growth hormone cDNA. *Gene* **1988**, *65*, 239–246. [\[CrossRef\]](#) [\[PubMed\]](#)
22. Chang, Y.S.; Liu, C.S.; Huang, F.L.; Lo, T.B. The primary structures of growth hormones of three cyprinid species: Bighead carp, silver carp and grass carp. *Gen. Comp. Endocrinol.* **1992**, *87*, 385–393. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Hong, Y.; Scharlt, M. Sequence of the growth hormone (GH) gene from the silver carp (*Hypophthalmichthys molitrix*) and evolution of GH genes in vertebrates. *Biochim. Biophys. Acta* **1993**, *1174*, 285–288. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Pinheiro, J.S.; Wolff, J.L.C.; Araujo, R.D.; Hilsdorf, A.W.S. Molecular cloning and sequence analysis of growth hormone cDNA of Neotropical freshwater fish Pacu (*Piaractus mesopotamicus*). *Genet. Mol. Biol.* **2008**, *31*, 381–384. [\[CrossRef\]](#)
25. Sekine, S.; Mizukami, T.; Nishi, T.; Kuwana, Y.; Saito, A.; Sato, M.; Itoh, S.; Kawauchi, H. Cloning and expression of cDNA for salmon growth hormone in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **1985**, *82*, 4306–4310. [\[CrossRef\]](#)
26. Saito, A.; Sekine, S.; Komatsu, Y.; Sato, M.; Hirano, T.; Itoh, S. Molecular cloning of eel growth hormone and its expression in *Escherichia coli*. *Gene* **1988**, *73*, 545–551.
27. Tsai, H.J.; Chen, H.M.; Lo, C.F. Secretory synthesis of active recombinant fish growth hormone by insect cells using a baculovirus vector. *Can. J. Fish. Aquat. Sci.* **1994**, *51*, 1–7. [\[CrossRef\]](#)
28. Tsai, H.J.; Hsieh, M.H.; Kuo, J.C. *Escherichia coli*-produced fish growth hormone as a feed additive to enhance the growth of juvenile black seabream (*Acanthopagrus schlegelii*). *J. Appl. Ichthyol.* **1997**, *13*, 79–82. [\[CrossRef\]](#)
29. Sciara, A.A.; Rubiolo, J.A.; Somoza, G.M.; Arranz, S.E. Molecular cloning, expression and immunological characterization of pejerrey (*Odontesthes bonariensis*) growth hormone. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **2006**, *142*, 284–292. [\[CrossRef\]](#)

30. Li, C.; Chen, X.; Zhang, Y.; Ye, H.; Liu, T. Molecular and expression characterization of growth hormone/prolactin family genes in the Prenant's schizothoracin. *Mol. Biol. Rep.* **2011**, *38*, 4595–4602. [\[CrossRef\]](#)
31. Lundberg, J.G.; Chernoff, B. A Miocene Fossil of the Amazonian Fish *Arapaima* (Teleostei, Arapaimidae) from the Magdalena River Region of Colombia-Biogeographic and Evolutionary Implications. *Biotropica* **1992**, *24*, 2–14. [\[CrossRef\]](#)
32. Rainer, F.; Pauly, D. (Eds.) Arapaimidae. *FishBase*. August 2013. Available online: <http://www.fishbase.org/Summary/FamilySummary.php?Family=Arapaimidae> (accessed on 1 June 2022).
33. Migaud, H.; Davie, A.; Taylor, J.F. Current knowledge on the photoneuroendocrine regulation of reproduction in temperature fish species. *J. Fish Biol.* **2010**, *76*, 27–68. [\[CrossRef\]](#) [\[PubMed\]](#)
34. Altschul, S.F.; Madden, T.L.; Schaffer, A.A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D.J. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* **1997**, *25*, 3389–3402. [\[CrossRef\]](#) [\[PubMed\]](#)
35. Souvorov, Y.; Kapustin, B.; Kiryitin, V.; Chetvernin, T.; Tatusova, T.; Lipman, D. *Gnomon—NCBI eukaryotic gene prediction tool*. National Center for Biotechnology Information; NCBI: Bethesda, MD, USA, 25 February 2010.
36. Sievers, F.; Wilm, A.; Dineen, D.; Gibson, T.; Karplus, K.; Li, W.; Lopez, R.; McWilliam, H.; Remmert, M.; Soding, J.; et al. Fast, scalable generation of highquality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol.* **2011**, *7*, 539. [\[CrossRef\]](#)
37. Waterhouse, A.; Bertoni, M.; Bienert, S.; Studer, G.; Tauriello, G.; Gumienny, R.; Heer, F.T.; de Beer, T.A.P.; Rempfer, C.; Bordoli, L.; et al. SWISS-MODEL: Homology modelling of protein structures and complexes. *Nucleic Acids Res.* **2008**, *46*, W296–W303 (2018). [\[CrossRef\]](#)
38. Biener, S.; Waterhouse, A.; de Beer, T.A.P.; Tauriello, G.; Studer, G.; Bordoli, L.; Schwede, T. The SWISS-MODEL Repository—New features and functionality. *Nucleic Acids Res.* **2017**, *45*, D313–D319. [\[CrossRef\]](#)
39. De Vos, A.M.; Ultsch, M.; Kossiakoff, A.A. Human growth hormone and extracellular domain of its receptor—Crystal-structure of the complex. *Science* **1992**, *255*, 306–312. [\[CrossRef\]](#)
40. Sundstrom, M.; Lundqvist, T.; Rodin, J.; Giebel, L.B.; Milligan, D.; Norstedt, G. Crystal structure of an antagonist mutant of human growth hormone, G120R, in complex with its receptor at 2.9 angstrom resolution. *J. Biol. Chem.* **1996**, *271*, 32197–32203. [\[CrossRef\]](#)
41. Clackson, T.; Ultsch, M.H.; Wells, J.A.; de Vos, A.M. Structural and functional analysis of the 1:1 growth hormone: Receptor complex reveals the molecular basis for receptor affinity. *J. Mol. Biol.* **1998**, *277*, 1111–1128. [\[CrossRef\]](#)
42. Somers, W.; Ultsch, M.; De Vos, A.M.; Kossiakoff, A.A. The X-ray structure of a growth hormone-prolactin receptor complex. *Nature* **1994**, *372*, 478–484. [\[CrossRef\]](#)
43. Atwell, S.; Ultsch, M.; De Vos, A.M.; Wells, J.A. Structural plasticity in a remodeled protein-protein interface. *Science* **1997**, *278*, 1125–1128. [\[CrossRef\]](#) [\[PubMed\]](#)
44. Studer, G.; Rempfer, C.; Waterhouse, A.M.; Gumienny, R.; Haas, J.; Schwede, T. QMEANDisCo—Distance constraints applied on model quality estimation. *Bioinformatics* **2020**, *36*, 1765–1771. [\[CrossRef\]](#) [\[PubMed\]](#)
45. Shuvo, M.H.; Gulfam, M.; Bhattacharya, D. DeepRefiner: High-accuracy protein structure refinement by deep network calibration. *Nucleic Acids Res.* **2021**, *49*, W147–W152. [\[CrossRef\]](#) [\[PubMed\]](#)
46. Fiser, A.; Sali, A. ModLoop: Automated modeling of loops in protein structures. *Bioinformatics* **2003**, *19*, 2500–2501. [\[CrossRef\]](#)
47. Jumper, J.; Evans, R.; Pritzel, A.; Green, T.; Figurnov, M.; Ronneberger, O.; Tunyasuvunakool, K.; Bates, R.; Zidek, A.; Potapenko, A.; et al. Highly accurate protein structure prediction with AlphaFold. *Nature* **2021**, *596*, 583–589. [\[CrossRef\]](#)
48. Varadi, M.; Anyango, S.; Deshpande, M.; Sreenath, N.; Natassia, C.; Yordanova, G.; Yuan, D.; Stroe, O.; Wood, G.; Laydon, A.; et al. AlphaFold Protein Database: Massively expanding the structural coverage of protein-sequence space with high-accuracy models. *Nucleic Acids Res.* **2021**, *50*, D439–D444. [\[CrossRef\]](#)
49. Maciejewski, M.W.; Schuyler, A.D.; Gryk, M.R.; Moraru, I.I.; Romero, P.R.; Ulrich, E.L.; Eghbalian, H.R.; Livny, M.; Delaglio, F.; Hoch, J.C. NMRbox: A resource for biomolecular NMR computation. *Biophys. J.* **2017**, *112*, 1529–1534. [\[CrossRef\]](#)
50. Benkert, P.; Biasini, M.; Schwede, T. Toward the estimation of the absolute quality of individual protein structure models. *Bioinformatics* **2011**, *27*, 343–350. [\[CrossRef\]](#)
51. Hooft, R.W.W.; Sander, C.; Vriend, G. Objectively judging the quality of a protein structure from a Ramachandran plot. *Comput. Appl. Biosci.* **1997**, *13*, 425–430. [\[CrossRef\]](#)
52. Shelley, J.C.; Cholleti, A.; Frye, L.L.; Greenwood, J.R.; Timlin, M.R.; Uchimaya, M. Epik: A software program for pK(a) prediction and protonation state generation for drug-like molecules. *J. Comp. Aided Mol. Des.* **2007**, *21*, 681–691. [\[CrossRef\]](#)
53. Lu, C.; Wu, C.J.; Ghoreishi, D.; Chen, W.; Wang, L.L.; Damm, W.; Ross, G.A.; Dahlgren, M.K.; Russell, E.; Von Barger, C.D.; et al. OPLS4: Improving force field accuracy on challenging regimes of chemical space. *J. Chem. Theory Comput.* **2021**, *17*, 4291–4300. [\[CrossRef\]](#) [\[PubMed\]](#)
54. Bowers, K.J.; Chow, E.; Xu, H.; Dror, R.O.; Eastwood, M.P.; Gregersen, B.A.; Klepeis, J.L.; Kolossvary, I.; Moraes, M.A.; Sacerdoti, F.D.; et al. Scalable algorithms for molecular dynamics simulations on commodity clusters. In Proceedings of the 2006 ACM/IEEE Conference on Supercomputing, Tampa, FL, USA, 11–17 November 2006; ACM: New York, NY, USA, 2006. [\[CrossRef\]](#)
55. Jorgensen, W.L.; Chandrasekhar, J.; Madura, J.D.; Impey, R.W.; Klein, M.L. Comparison of simple potential functions for simulating liquid water. *J. Chem. Phys.* **1983**, *79*, 926–935. [\[CrossRef\]](#)
56. Darden, T.; York, D.; Pedersen, L. Particle mesh Ewald—An n.log(n) method for Ewald sums in large systems. *J. Chem. Phys.* **1993**, *98*, 10089–10092. [\[CrossRef\]](#)

57. Ashkenazy, H.; Abadi, S.; Martz, E.; Chay, O.; Mayrose, I.; Pupko, T.; Ben-Tal, N. ConSurf 2016: An improved methodology to estimate and visualize evolutionary conservation in macromolecules. *Nucleic Acids Res.* **2016**, *44*, W344–W350. [[CrossRef](#)]
58. Celniker, G.; Nimrod, G.; Ashkenazy, H.; Glaser, F.; Martz, E.; Mayrose, I.; Pupko, T.; Ben-Tal, N. ConSurf: Using evolutionary data to raise testable hypotheses about protein function. *Isr. J. Chem.* **2013**, *53*, 199–206. [[CrossRef](#)]
59. Ashkenazy, H.; Erez, E.; Martz, E.; Pupko, T.; Ben-Tal, N. ConSurf 2010: Calculating evolutionary conservation in sequence and structure of proteins and nucleic acids. *Nucleic Acids Res.* **2010**, *38*, W529–W533. [[CrossRef](#)]
60. Lima, I.; Cino, E.A. Sequence similarity in 3D for comparison of protein families. *J. Mol. Graph. Model.* **2021**, *106*, 107906. [[CrossRef](#)] [[PubMed](#)]
61. Teufel, F.; Armenteros, J.J.A.; Johansen, A.R.; Gislason, M.H.; Pihl, S.I.; Tsirigos, K.D.; Winther, O.; Brunak, S.; von Heijne, G.; Nielsen, H. SignalP 6.0 predicts all five types of signal peptides using protein language models. *Nat. Biotechnol.* **2022**, *40*, 1023–1025. [[CrossRef](#)] [[PubMed](#)]
62. Venugopal, T.; Anathy, V.; Pandian, T.J.; Gong, G.Z.; Mathavan, S. Molecular cloning of growth hormone-encoding cDNA of an Indian major carp, *Labeo rohita*, and its expression in *Escherichia coli* and zebrafish. *Gen. Comp. Endocrinol.* **2002**, *125*, 236–247. [[CrossRef](#)]
63. Morgan, A.A.; Rubenstein, E. Proline: The distribution, frequency, positioning, and common functional roles of proline and polyproline sequences in the human proteome. *PLoS ONE* **2013**, *8*, e53785. [[CrossRef](#)]
64. Bellini, M.H.; Bartolini, P. In vivo bioassay for the potency determination of human growth hormone in dwarf “little” mice. *Endocrinology* **1993**, *132*, 2051–2055. [[CrossRef](#)] [[PubMed](#)]
65. Maltarollo, V.G.; Shevchenko, E.; de Miranda Lima, I.D.; Cino, E.A.; Ferreira, G.M.; Poso, A.; Kronenberger, T. Do go chasing waterfalls: Enoyl reductase (FabI) in complex with 6inhibitors stabilize tetrameric structure and opens water channels. *J. Comp. Inf. Model.* **2022**, *62*, 5746–5761. [[CrossRef](#)] [[PubMed](#)]
66. Kronenberger, T.; Serafim, M.S.M.; Tonduru, A.K.; Maltarollo, V.G.; Poso, A. Ligand accessibility insights to the dengue virus NS3-NS2B protease assessed by long-timescale molecular dynamics simulations. *ChemMedChem* **2021**, *16*, 2524–2534. [[CrossRef](#)] [[PubMed](#)]
67. Ferreira, G.M.; Pillaiyar, T.; Hirata, M.H.; Poso, A.; Kronenberger, T. Inhibitor induced conformational changes in SARS-CoV-2 papain-like protease. *Sci. Rep.* **2022**, *12*, 11585. [[CrossRef](#)]
68. Fan, H.; Mark, A.E. Refinement of homology-based protein structures by molecular dynamics simulation techniques. *Protein Sci.* **2022**, *13*, 211–220. [[CrossRef](#)] [[PubMed](#)]
69. Kayano, A.M.; Simoes-Silva, R.; Medeiros, P.S.M.; Maltarollo, V.G.; Honorio, K.M.; Oliveira, E.; Albericio, F.; da Silva, S.L.; Aguiar, A.C.C.; Krettli, A.U.; et al. BpMP-1, a new metalloproteinase isolated from *Bothrops brazili* snake venom with *in vitro* antiparasitic properties. *Toxicon* **2015**, *106*, 30–41. [[CrossRef](#)]
70. Zaqueo, K.D.; Kayano, A.M.; Simoes-Silva, R.; Moreira-Dill, L.S.; Fernandes, C.F.C.; Fuly, A.L.; Maltarollo, V.G.; Honorio, K.M.; da Silva, S.L.; Acosta, G.; et al. Isolation and biochemical characterization of a new thrombin-like serine protease from *Bothrops pirajai* snake venom. *Biomed. Res. Int.* **2014**, 595186. [[CrossRef](#)]
71. Martins, W.; Baldasso, P.A.; Honorio, K.M.; Maltarollo, V.G.; Ribeiro, R.I.M.A.; Carvalho, B.M.A.; Soares, A.M.; Calderon, L.A.; Stabeli, R.G.; Caballol, M.A.O.; et al. A novel phospholipase A(2) (D49) from the venom of the *Crotalus oreganus abyssus* (North American Grand Canyon rattlesnake). *Biomed. Res. Int.* **2014**, 654170. [[CrossRef](#)]
72. Cooper, M.; Brown, C.D. Qualifying the relationship between sequence conservation and molecular function. *Genome Res.* **2008**, *18*, 201–205. [[CrossRef](#)]
73. Naidu, C.K.; Suneetha, Y.; Reddy, P.S. Computational analysis of molt-inhibiting hormone from selected crustaceans. *Comp. Biochem. Physiol. Part D Genom. Proteom.* **2013**, *8*, 292–299. [[CrossRef](#)]
74. Cecchi, C.R.; Higuti, E.; Oliveira, N.A.J.; Lima, E.R.; Jakobsen, M.; Dagnaes-Hansen, F.; Gissel, H.; Aagaard, L.; Jensen, T.G.; Jorge, A.A.L.; et al. A novel homologous model for gene therapy of dwarfism by non-viral transfer of the mouse growth hormone into immunocompetent dwarf mice. *Curr. Gene Ther.* **2014**, *14*, 44–51. [[CrossRef](#)] [[PubMed](#)]
75. Cecchi, C.R.; Higuti, E.; Lima, E.R.; Vieira, D.P.; Squair, P.L.; Peroni, C.N.; Bartolini, P. Efficient non-invasive plasmid-DNA administration into tibialis cranialis muscle of “little” mice. *Curr. Gene Ther.* **2017**, *17*, 230–235. [[CrossRef](#)] [[PubMed](#)]
76. Lima, E.R.; Cecchi, C.R.; Higuti, E.; de Jesus, G.P.P.; Gomes, A.M.; Zacarias, E.A.; Bartolini, P.; Peroni, C.N. Optimization of mouse growth hormone plasmid DNA electrotransfer into tibialis cranialis muscle of “little” mice. *Molecules* **2020**, *25*, 5034. [[CrossRef](#)] [[PubMed](#)]
77. Higuti, E.; Cecchi, C.R.; Oliveira, N.A.J.; Vieira, D.P.; Jensen, T.G.; Jorge, A.A.L.; Bartolini, P.; Peroni, C.N. Growth responses following a single intra-muscular hGH plasmid administration compared to daily injections of hGH in dwarf mice. *Curr. Gene Ther.* **2012**, *12*, 437–443. [[CrossRef](#)] [[PubMed](#)]
78. Higuti, E.; Cecchi, C.R.; Oliveira, N.A.J.; Lima, E.R.; Vieira, D.P.; Vieira, D.P.; Jensen, T.G.; Jorge, A.A.L.; Bartolini, P.; Peroni, C.N.; et al. Partial correction of the dwarf phenotype by non-viral transfer of the growth hormone gene in mice: Treatment age is critical. *Growth Horm. IGF Res.* **2016**, *26*, 1–7. [[CrossRef](#)] [[PubMed](#)]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.