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Molecular Characteristics, Expression Patterns, and Response of Insulin-like Growth Factors Gene Induced by Sex Steroid Hormones in Blotched Snakehead (*Channa maculata*)

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Abstract: Insulin-like growth factors (IGFs) play central roles in the growth and development of vertebrates. Blotched snakehead (*Channa maculata*), an economically significant fish, exhibits obvious sexual dimorphism and achieves sexual maturity in one year. However, the role of IGFs in *C. maculata* remains unknown. Three *IGF* genes were identified in *C. maculata*, designated as *CmIGF1-1*, *CmIGF1-2*, and *CmIGF2*. The cDNA sequences of these genes are 1184, 655, and 695 bp, encoding putative proteins of 168, 131, and 215 amino acids, respectively, and all three proteins contain a conserved IGF domain. Quantitative real-time PCR (qPCR) revealed the predominant expression of *CmIGFs* in the liver of adult fish, with higher expression levels observed in males. Notably, *CmIGF1-1*, *CmIGF1-2*, and *CmIGF2* displayed analogous expression profiles in the liver across various developmental stages, peaking at 365 days after hatching (dah). Subsequently, 600 individuals at 75 dah, at an early developmental stage, were randomly divided equally into six groups and reared in aerated 2 m × 2 m × 2 m cement ponds at 26.0 ± 1.0 °C. Following a one-week acclimatization period, fish without observed abnormalities were intraperitoneally injected with either 17α-ethynylestradiol (EE_2) or 17α-methyltestosterone (MT) at a dose of 10 μg/g body weight. Three groups underwent short-term hormone treatment, and the remaining three groups underwent long-term hormone treatment, which included five injections at two-week intervals over ten weeks. The analysis of *CmIGFs* expression levels in the liver under different hormone treatments revealed that EE_2 suppressed the expression of *CmIGF1-1* and *CmIGF1-2* while promoting *CmIGF2* expression. In females, MT up-regulated the expression of *CmIGF1-1* and *CmIGF2* in a time-dependent manner, but consistently inhibited *CmIGF2* expression. In males, MT promoted the expression of *CmIGFs* in a time-dependent manner, reaching peak levels for *CmIGF1-1*, *CmIGF1-2*, and *CmIGF2* after 8, 10, and 2 weeks of injection, respectively. Additionally, *CmIGF1* and *CmIGF2* might exhibit a complementary relationship, with a compensatory increase in *CmIGF2* expression in response to low *CmIGF1* concentration. These findings highlight the potential key role of IGFs upon growth and their regulation by sex steroid hormones in *C. maculata*, providing a crucial foundation for future research aimed at elucidating the molecular mechanisms underlying the growth dimorphism between female and male blotched snakeheads.

Keywords: IGFs; gene cloning; growth dimorphism; expression analysis; hormone induction

Key Contribution: This study shows, for the first time, the presence of three insulin-like growth factor genes (*CmIGF1-1*, *CmIGF1-2*, and *CmIGF2*) in the economically significant blotched snakehead (*Channa maculata*), and elucidates their predominant expression in liver, with higher levels observed

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in males. Furthermore, it demonstrates the regulatory impact of sex steroid hormones (EE_2 and MT) on the expression of *CmIGFs*. These findings highlight the potential key role of IGFs in growth and their regulation by sex steroid hormones in blotched snakehead (*C. maculata*), providing a foundation for future research aimed at elucidating the molecular mechanisms underlying the growth dimorphism between female and male blotched snakeheads.

1. Introduction

The growth hormone/insulin-like growth factor (GH/IGF) axis plays a vital role in vertebrate growth, encompassing key molecules such as growth hormone (GH), growth hormone receptor (GHR), insulin-like growth factor (IGF), and their associated binding proteins and receptors, including IGF-binding proteins (IGFBPs) and IGF receptors (IGF1R and IGF2R) [1]. GH secretion is regulated by the anterior pituitary gland, which responds to various hormones released from the hypothalamus, notably growth hormone-releasing hormone (GHRH) and growth hormone-releasing peptide (GHRP). GH travels to the liver through the bloodstream, where it rapidly binds to GHR, initiating a cascade of cellular signaling events that culminate in the secretion of IGFs [2]. Subsequently, IGFs travel through body fluids to various tissues, promoting cell growth and differentiation. GH, GHR, and IGFs orchestrate biological growth and development through close coordination during the different development stages of an organism's lifespan [2,3].

IGFs have garnered considerable attention across various species because of their vital role in growth regulation. IGFs are highly conserved and primarily include two types, IGF-1 and IGF-2 [4]. In mammals, IGF-1 primarily governs postnatal growth and development, while IGF-2 plays a crucial role during embryonic development, influencing processes such as cell proliferation, differentiation, migration, and apoptosis [5]. The *IGF-1* gene in fish was first cloned from Coho salmon (*Oncorhynchus kisutch*) [6] and subsequently identified in other fish species, such as gopher rockfish (*Sebastes carnatus*) [7], Nile tilapia (*Oreochromis niloticus*) [8], zebrafish (*Danio rerio*) [9], tongue sole (*Cynoglossus semilaevis*) [10], yellow catfish (*Pelteobagrus fulvidraco*) [11], and spotted scat (*Scatophagus argus*) [12]. *IGF-1* exhibits widespread expression in various tissues, particularly expressed in the liver of gopher rockfish (*S. carnatus*) [7], Nile tilapia (*O. niloticus*) [8], yellow catfish (*P. fulvidraco*) [11], and spotted scat (*S. argus*) [12]. Additionally, in Nile tilapia (*O. niloticus*) and yellow catfish (*P. fulvidraco*), males exhibit higher *IGF-1* expression levels than females. On the other hand, *IGF-2* in fish was initially cloned from rainbow trout (*Oncorhynchus mykiss*) [13] and subsequently identified in other fish species, such as zebrafish (*D. rerio*) [9], medaka (*Oryzias latipes*) [14], and spotted scat (*S. argus*) [12]. Studies have reported the expression patterns of *IGF-2* during embryonic development in Nile tilapia (*O. niloticus*) [8], zebrafish (*D. rerio*) [9], medaka (*O. mykiss*) [14], and spotted scat (*S. argus*) [12]. In zebrafish (*D. rerio*), the expression of *IGF-2* initiates at the gastrula stage and exhibits a gradual increase throughout embryonic development [9], and the disruption of *IGF-2* results in embryonic lethality [15]. The expression profile of *IGF-2* mRNA closely mirrors that of *IGF-1*, expressed in diverse tissues, and particularly pronounced in the liver. Furthermore, *IGF-1* or *IGF-2* exhibit different subtypes in a few fish species, including *IGF-1a* and *IGF-1b* in giant grouper (*Epinephelus lanceolatus*) [16] and *IGF-2a* and *IGF-2b* in grass carp (*Ctenopharyngodon idellus*) [17].

The synthesis and secretion of sex steroid hormones by the gonads are pivotal in regulating the hypothalamus–pituitary–liver axis in vertebrates [18]. Cumulative evidence indicates that sex steroid hormones can impact the growth and development of vertebrates by modulating the synthesis and secretion of IGFs [18–20]. In rainbow trout (*O. mykiss*) [21], testosterone (T) induction has been shown to stimulate the mRNA expression of *IGF1* and *IGF2*, while treatment with 17α-estradiol (E_2) decreases *IGF1* expression. In Nile tilapia (*O. niloticus*), the long-term injection of E_2 in females

dramatically increases *IGF-1* expression levels in the liver [22], and MT strongly enhances *IGF-1* expression in males, albeit without significant effects on females [23]. These findings suggest that sex steroid hormones can regulate *IGFs* expression in fishes, with potential variation among species [24,25]. However, the underlying mechanisms remain unclear and warrant further investigation.

Blotched snakehead (*Channa maculata*), a member of the Channidae family, is widely farmed in China for its palatable taste, high protein content, and pharmacological properties [26]. Male snakeheads exhibit faster growth rates and bigger sizes than females; given the demand for large individuals in the market, it is imperative to understand the molecular mechanism of growth disparities between male and female individuals, which will provide a theoretical basis for selective breeding in production practices [27]. Previous studies have demonstrated the crucial role of the *GH* gene in the growth of blotched snakehead (*C. maculata*) [28]. This research aims to illustrate the function of the IGFs in the GH/IGF axis and explore their expression patterns during ontogeny and in adult tissues. Moreover, the effects of exogenous hormone induction via EE_2 and MT on the expression levels of *IGFs* in both male and female individuals were investigated. This work endeavors to provide insight into the basic function of IGFs and offers novel perspectives for exploring the molecular mechanisms underlying the growth disparities between male and female blotched snakehead.

2. Materials and Methods

2.1. Fish and Sampling

Blotched snakeheads in this study were reared in the fish laboratory of the Model Animal Research Center, Pearl River Fisheries Research Institute (Guangzhou City, Guandong Province, China). The fish were cultivated in an open system, with water quality parameters meeting aquaculture standards. Specifically, dissolved oxygen levels exceeded 6–8 mg/L, ammonia nitrogen remained below 0.5 mg/L, nitrite levels were less than 0.01 mg/L, and pH values were 7.0–7.5. Water exchange occurred every three days at a 20% exchange rate. To mimic natural environmental conditions, the lighting followed a 12 h day and night cycle. The fish were fed with a commercial diet provided by Nanhai Bairong Improved Aquatic Seed Co., Ltd. (FoShan City, Guangdong Province, China), comprising 45% protein, 5% fat, and 27% carbohydrates, ensuring the balanced nutritional intake that is suitable for snakehead growth requirements. The feeding ratio was adjusted according to the growth stage and size of the fish, following the manufacturer's recommendations. One-year-old male and female fish were randomly selected, respectively, and a small piece of fin tissue was sampled for the subsequent genomic DNA (gDNA) extraction and the cloning of gDNA sequences of *IGF* genes. Additionally, twelve tissue samples (gill (G), liver (L), spleen (S), intestines (I), middle kidney (MK), muscle (M), head kidney (HK), gonad [ovary (O)/testis (T)], heart (H), pituitary (P), hypothalamus (HY) and brain (B)) were collected from the blotched snakeheads (n = 3). Tissues were collected under sterile conditions, with each tissue individually removed using sterile instruments to prevent cross-contamination. The collected tissues were immediately snap-frozen in liquid nitrogen and stored at −80 °C to maintain RNA integrity for subsequent RNA extraction, cDNA cloning, and the assessment of *IGF* distribution patterns.

To analyze *IGF* expression levels in the liver, samples were collected from male and female blotched snakeheads (n = 5) at various developmental stages (45, 75, 105, 135, 165, 195, and 365 dah) after anesthetization. Genetic sex was identified by sex-specific molecular markers, as previously described [29]. All fish experiments were conducted following the regulations outlined in the National Institutes of Health guide for the care and use of laboratory animals (https://olaw.nih.gov/resources/publications/guide-care-2011.htm, accessed on 1 January 2024).

2.2. Hormone Treatment

Healthy individuals at 75 dah (n = 600; body length: 15.1 ± 0.4 cm; body weight: 67. 9 ± 4.3 g) were randomly divided into six groups, each comprising 100 individuals. These fish were reared in 2 m × 2 m × 2 m aerated cement ponds at 26.0 ± 1.0 °C. After a one-week acclimatization period, the fish with no abnormal clinical signs were subjected to further studies. Sex steroid hormones, EE_2 and MT (Aladdin, Shanghai, China) were dissolved in corn oil prior to intraperitoneal injection. Three groups underwent short-term hormone treatment: the first group received an intraperitoneal injection of MT (10 μg/g body weight); the second group was intraperitoneally injected with EE_2 (10 μg/g body weight); and the third group served as the control, receiving an intraperitoneal injection of corn oil [26]. Liver samples were collected from male and female individuals (n = 5) at 24, 48, 72, 96, 120, and 144 h post-injection. Genetic sex was determined using the same method as previously described [29]. The remaining three groups underwent a long-term hormone treatment, which consisted of five injections at two-week intervals over a ten-week period. At each injection, the fourth group received an intraperitoneal injection of MT (10 μg/g body weight), the fifth group received EE_2 (10 μg/g body weight) intraperitoneally, and the sixth group served as the control, receiving an intraperitoneal injection of corn oil. Liver samples were collected from male and female individuals (n = 5) at 2, 4, 6, 8, and 10 weeks post-injection, and genetic sex identification was performed as previously described [29]. Subsequently, all samples were immediately frozen in liquid nitrogen and stored at −80 °C before RNA extraction.

2.3. RNA Extraction and cDNA Synthesis

The total RNA was extracted from tissue samples utilizing TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The quality and quantity of RNA were assessed using a spectrophotometer (ThermoFisher, Waltham, MA, USA), and RNA integrity was detected by electrophoresis using a 1.0% agarose gel. Subsequently, total RNA extracted from tissue samples of different individuals under the same conditions was pooled in equal amounts for cDNA synthesis. The synthesis of the first-strand cDNA was performed utilizing the pooled RNA, a random hexamer primer (Takara, Osaka, Japan), and M-MLV Reverse Transcriptase (Promega, Fitchburg, WI, USA). To obtain both 5′- and 3′-RACE Ready cDNA, the SMART™ RACE cDNA Amplification Kit (Takara, Japan) was employed. Finally, cDNA template for qPCR was synthesized using the ReverTra Ace qPCR RT Kit (Toyobo, Tokyo, Japan) with the pooled RNA as the template.

2.4. Full-Length cDNA Cloning and Sequence Analysis for IGFs in Blotched Snakehead (C. maculata)

Three *IGF* genes were identified in the blotched snakehead genome (SRA Accession No. PRJNA730430) [30]. Primers were specifically designed to verify the predicted cDNA sequences. To obtain the 5′ and 3′ untranslated regions (UTRs), specific and adapter primers were designed using 5′-RACE- and 3′-RACE-ready cDNA as templates, with primers within the UTRs designed accordingly (Table 1). PCR amplification was performed by 2 × Super Pfx MasterMix (Cwbio, Taizhou, China). The amplified products of the expected size were purified using a Gel Extraction Kit (Omega, Norwalk, CT, USA). The purified products were cloned into a pMD-19T vector (Takara, Japan) and transformed into competent *Escherichia coli* DH5α cells (Takara, Japan). Positive colonies including the target fragments were sequenced by a commercial company (Tianyihuiyuan, Guangzhou City, Guangdong Province, China).

Sequence analysis of *Cm*IGFs was conducted using the Sequence Manipulation Suite (STS) (http://www.bio-soft.net/sms/, accessed on 10 January 2024). Domain features of the IGF proteins were predicted utilizing the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de, accessed on 15 January 2024).

Physicochemical properties of the IGF proteins were predicted by ExPASy-Protparam Tool (https://web.expasy.org/protparam/, accessed on 15 January 2024). IGF protein sequences from other species were retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/, accessed on 15 January 2024). Multiple amino acid sequence alignments were performed using ClustalX 2.1 (http://www.ebi.ac.uk/tools/clustalx2.1, accessed on 15 January 2024), and a phylogenetic tree was constructed using Mega 5.0 software (http://www.megasoftware.net/index.html, accessed on 15 January 2024) with the neighbor-joining method and bootstrap resampling (1000 replicates) [31].

Table 1. Primers used for *IGF* genes cloning and qPCR in blotched snakehead (*C. maculata*).

Primer Name	Sequence (5′~3′)	Application
IGF1-1-F1	CTCCTGTAGCCACACCCTC	Partial sequence obtaining
IGF1-1-R1	GAATGACTGTGTCCAGGTAAAG	
IGF1-2-F1	ATGGGCTGTATCTCCTGTAGTC	
IGF1-2-R1	AGTATTCTCGGCAAGTCGGT	
IGF2-F1	AGCAAAGATACGGACAGCAC	
IGF2-R1	GTTGACATAGTTATCCGTGGC	
IGF1-1-5′R-out	AAGCCTCTTTCTCCACACACAAACTGC	5′-RACE PCR amplification
IGF1-1-5′R-in	GCAGTGAGAGGGTGTGGCTACAGGAG	
IGF1-2-5′R-out	AGCGTGTGGGTTTACT	
IGF1-2-5′R-in	TGTCGACCAGCTCCACCC	
IGF1-1-3′F-out	CCTGCCAAGACTAACAAGCCAACTCG	3′-RACE PCR amplification
IGF1-1-3′F-in	GAGAACAACTAAGAGACCTTTACCTGGACACA	
IGF1-1-F2	CCTGTTCGCTAAATCTCACTTCTC	ORF identification
IGF1-1-R2	CATTTGTCCATTCGCTCCATC	
IGF1-2-F2	GGACTACAAGAGAGACGG	
IGF1-2-R2	TTTGTCCCTTCGCTCCAT	
IGF2-F2	AGCCAAATAACCCCCAACA	
IGF2-R2	AGCGGGCTCATTTGTGG	
IGF1-1-DL-F	CGCTCTTTCCTCTCAGTGGC	qPCR amplification
IGF1-1-DL-R	CCATAGCCTGTGGGTTTACTGA	
IGF1-2-DL-F	GTTTGTGTGTGGAGACAGAGGC	
IGF1-2-DL-R	GCACGCACAGAGTGAGTTGG	
IGF2-DL-F	GTCTTCGTCCAGTCGTTCGC	
IGF2-DL-R	TGTTGCCCCTGCTGGTTG	
β-actin-F	GCAAGCAGGAGTATGATGAG	
β-actin-R	TTGGGATTGTTTCAGTCAGT	
EF1α-F	GGGAGACCCACAATAACATCG	
EF1α-R	CCAGGCATACTTGAAGGAGC	

2.5. Cloning the Genomic Sequence of IGFs Genes in Blotched Snakehead (C. maculata)

The gDNA was extracted from fin tissues using the General AllGen Kit (CWBio, Taizhou, China) following the manufacturer's protocol. Specific primers (Table 2) were designed according to the predicted genome sequences of *IGFs* obtained from the blotched snakehead genome [30]. Subsequently, the genomic sequences of *CmIGFs* were amplified using these primers and the fin gDNA as the template. The PCR products were sequenced to obtain the genomic sequences of *CmIGFs*, which were then compared with the cDNA sequences to identify exons and introns based on the GT/AG principle.

Table 2. Primers used for genomic sequence amplification of blotched snakehead (*C. maculata*) *IGFs* gene.

Primer Name	Sequence (5′~3′)	Length (bp)
IGF1-1-gDNA-F1	TTTATGATTGGGTCACAGCA	1674
IGF1-1-gDNA-R1	GGACTCAGCAGGAATTACTCT	
IGF1-1-gDNA-F2	GTTACTTACTGGCAGGTTTT	1827
IGF1-1-gDNA-R2	TGTTTGGGTTCTACTCAATT	
IGF1-1-gDNA-F3	GGCGGCAAATTAGAGTTGTG	1848
IGF1-1-gDNA-R3	ATGGACGAACTGAGGTTACAAG	
IGF1-1-gDNA-F4	ACAAACGCTGTGAAGTGGTC	1764
IGF1-1-gDNA-R4	CAGGGAGCTACTTAATGCTTA	
IGF1-1-gDNA-F5	GGGTGATTTCACTGGGATGT	1962
IGF1-1-gDNA-R5	AACCTGTGGATTCTTGGAGC	
IGF1-1-gDNA-F6	TAGTCCCTGCCCAGCCGTAA	1997
IGF1-1-gDNA-R6	AGAAACAAAGCATAGGTGAA	
IGF1-1-gDNA-F7	TCGATTCCCCTGTCCCCTAA	2008
IGF1-1-gDNA-R7	GCCTGCGTTTCGACTTCACG	
IGF1-1-gDNA-F8	GATGTTTTAGGCAGCGTCTG	1588
IGF1-1-gDNA-R8	AACCGTGTTTTACTCTTTTAG	
IGF1-2-gDNA-F1	TGCCTTTGTAGTTTACCTTT	1829
IGF1-2-gDNA-R1	TAATTTGTCCCCTTTATTCG	
IGF1-2-gDNA-F2	ATGCTAGGACTGAAATGCTA	1727
IGF1-2-gDNA-R2	TAGATGATAAATAACGGGTA	
IGF1-2-gDNA-F3	GTCAGTGCTGTTCTTTCCAA	1155
IGF1-2-gDNA-R3	AAAAGGGGCTGTGCCTTGTT	
IGF1-2-gDNA-F4	AAGTGAAGCATTTCAAACTT	1546
IGF1-2-gDNA-R4	TAACTGGCAGAAGATGACTA	
IGF2-gDNA-F1	GGAGGAGCGATGGGTGGTGG	1677
IGF2-gDNA-R1	AGCGGCCCATTGTCAGTCCG	
IGF2-gDNA-F2	GTCTCAAGACTTCGTCCAGG	1623
IGF2-gDNA-R2	GTCTCAAGACTTCGTCCAGG	
IGF2-gDNA-F3	CCACTATGGGAAACAATGCC	1742
IGF2-gDNA-R3	AGGACTGCCACAGAAATCAC	

2.6. Quantitative Real-Time PCR (qPCR)

Gene-specific primers were designed for quantifying the expression of *CmIGFs* using qPCR. *β-actin* and *EF1α* genes were selected as reference genes according to our previous study [26,32]. qPCR was performed utilizing the StepOnePlus™ Real-Time PCR System (ABI, Los Angeles, CA, USA) by SYBR® Green Realtime PCR Master Mix (Toyobo, Osaka, Japan), and each sample was subjected to three replications. For normalization, the geometric mean of the Ct values of both *β-actin* and *EF1α* was calculated for each sample. The expression levels of *CmIGFs* in adult tissues and developmental stages were calculated by the $2^{-\Delta\Delta Ct}$ method [33]. The expression levels of *CmIGFs* in female gills were used as the baseline (1.0) for tissue expression pattern analysis, and the expression levels of *CmIGFs* in the liver of 45 dah males were used as the baseline (1.0) for the developmental expression pattern analysis. The expression levels of *CmIGFs* after hormone treatment were calculated using the $2^{\Delta Ct}$ method [26].

2.7. Statistical Analysis

The experimental data were displayed as mean (n = 3) ± standard error of the mean (S.E.M). Two-way ANOVA was conducted in SPSS (version 22.0; SPSS, Chicago, IL, USA).

When significant differences were observed among factors, one-way ANOVA was conducted, followed by Tukey's test. $p < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Characterization of CmIGFs

After PCR amplification and sequencing, a 1184-bp *CmIGF1-1* cDNA sequence (GenBank No. MW715800) was obtained. It included a 255-bp 5′-UTR, a 507-bp open reading frame (ORF) encoding 168 amino acids (aa), and a 452-bp 3′-UTR with RNA instability motifs (ATTTA), and a poly(A) tail. The analysis of the putative protein revealed the presence of a signal peptide (aa 1–43) and an IGF domain (aa 56–112) (Figure S1a). The genomic sequence of *CmIGF1-1*, obtained through amplification, sequencing, and alignment, spanned 13,504 bp. It consisted of five exons with lengths of 48, 187, 176, 36 and 60 bp, and four introns measuring 1301, 8800, 250 and 789 bp, respectively (Figure 1). It was in line with the GT/AG rule.

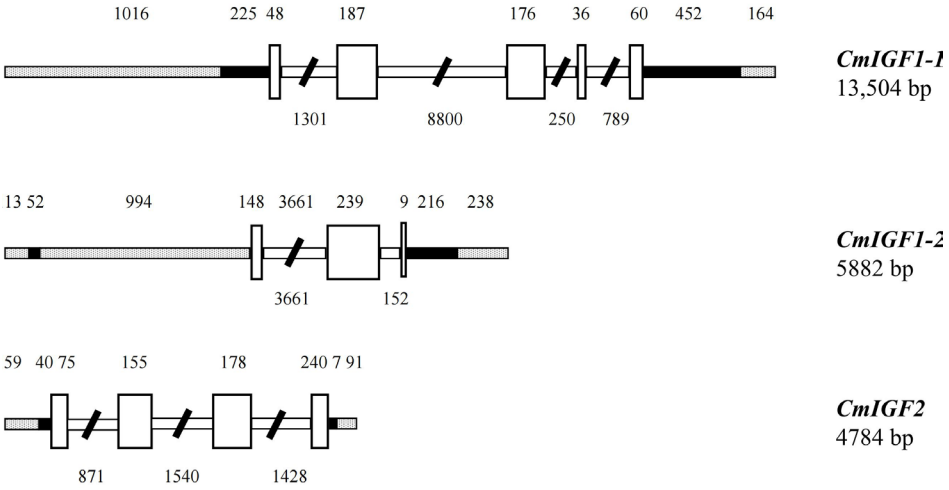

Figure 1. Genomic structure of *CmIGFs*. The shadow represents the flanking region, the black horizontal frame represents the untranslated region, the white vertical frame represents the exon, the white horizontal frame represents the intron, and the numbers above and below each schematic represent the length, respectively.

The homologous *CmIGF1-2* was also determined to be 675 bp in length (GenBank No. MW715801), comprising a 58-bp 5′-UTR, a 396-bp ORF encoding 131 aa, and a 211-bp 3′-UTR with a typical AATAA and a poly (A) tail. The bioinformatics analysis of the putative protein indicated the presence of a signal peptide (aa 1–26) and an IGF domain (aa 27–83) (Figure S1b). *Cm*IGF1-2 shared an amino acid sequence homology of 83.29% with *Cm*IGF1-1. After sequence identification, a genomic sequence of 5882 bp in length was obtained, comprising three exons (148, 239, and 9 bp) and two introns (3661 and 152 bp) following the GT/AG consensus rule (Figure 1).

The sequence of *CmIGF2* was determined to be 695 bp in length (GenBank No. MW715802) with an ORF of 648 bp encoding 215 aa, a 5′-UTR of 40 bp, and a 3′-UTR of 7 bp. The putative protein was determined to have an IGF domain (aa 53–110) and an IGF2_C domain (aa 147–202) (Figure S1c). When comparing the amino acid sequences of *Cm*IGF2 and *Cm*IGF1-1 with *Cm*IGF1-2, the similarities were 50.40% and 54.08%, respectively. The genomic sequence of *CmIGF2* was 4784 bp long, comprising four exons (75, 155, 178, and 240 bp) and three introns that were 871, 1540, and 1428 bp in size, respectively (Figure 1). All introns began with GT and ended with AG, which was consistent with the splicing rules between exons and introns in eukaryotes.

3.2. Multiple Alignments and Phylogenetic Analysis

*Cm*IGF1-1 exhibited the highest similarity to *Po*IGF1a (IGF1a in olive flounder (*Paralichthys olivaceus*)) (96.97%), followed by *Po*IGF1b (IGF1b in *P. olivaceus*) (91.82%) and *Sa*IGF1 (IGF1 in silthed sea bream (*Sparus aurata*)) (87.50%). Conversely, the lowest similarity was observed between *Cm*IGF1-1 and *Hs*IGF1b (IGF1b in human (*Homo sapiens*)) (50.05%) (Figure S2a). The protein sequence similarities between *Cm*IGF1-2 and other species varied, ranging from its highest homology with *Po*IGF1a (84.85%) to its lowest homology with *Hs*IGF1a (52.67%) (IGF1a in *H. sapiens*), as shown in Figure S2b. Further analysis indicated that the sequence similarity between *Cm*IGF1-1 and *Cm*IGF1-2 was only 73.28%. *Cm*IGF2 showed the highest identity with *Ca*IGF2 (IGF2 in *C. argus*) (98.68%) and *At*IGF2 (IGF2 in climbing bass (*Anabas testudineus*)) (90.73%), while it was the least similar to *Xl*IGF2a (IGF2a in African clawed frog (*Xenopus laevis*)) (44.98%) (Figure S2c). These results were corroborated by phylogenetic analysis (Figure 2). The phylogenetic tree suggested that these homologous proteins can be classified into two clades: IGF1 and IGF2. Each main clade consisted of four subclades, encompassing fishes, amphibians, birds, and mammals. Interestingly, the IGF1 and IGF2 proteins of fish appeared distinctly separated from those of other vertebrates. The phylogenetic tree reflected the consistent genetic relationships and evolution among species.

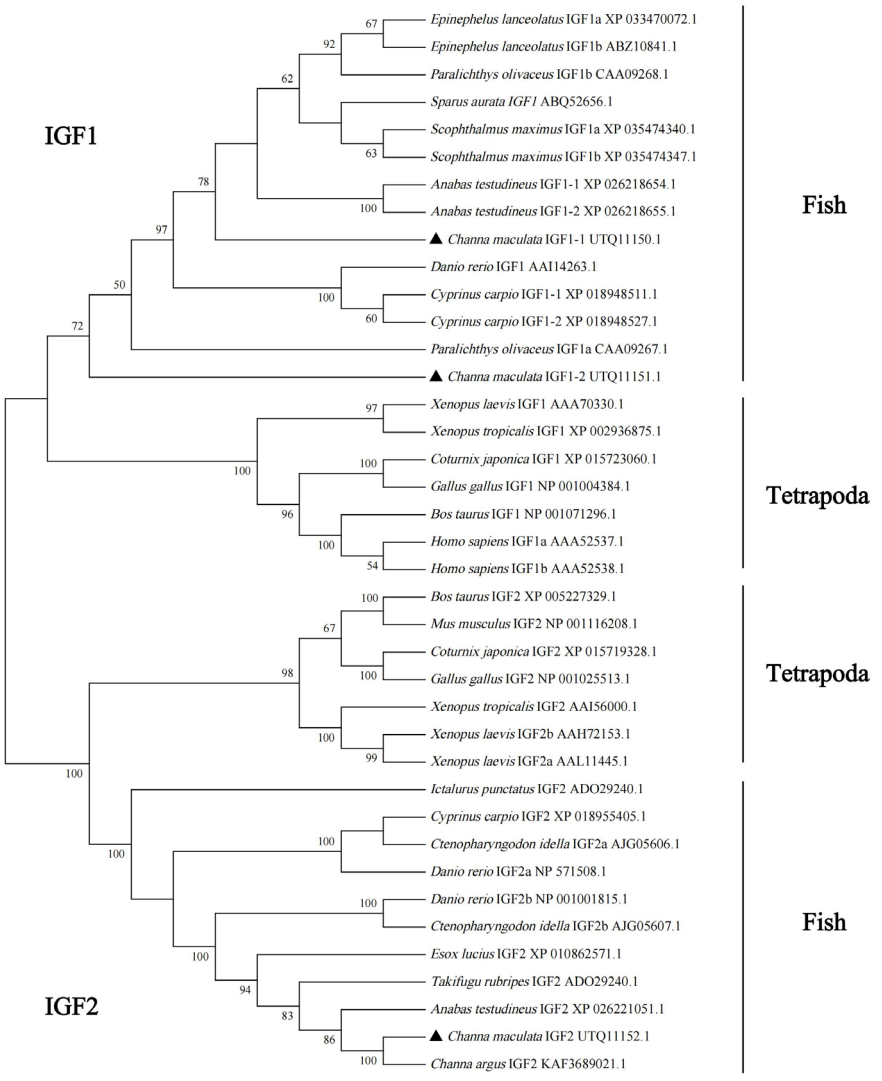

Figure 2. Phylogenetic relationship between the IGFs proteins in different species. A neighbor-joining phylogenetic tree was constructed using MEGA 5.0 software. The bootstrap values of the branches were obtained by testing the tree 1000 times and values were over 50% percent marked.

The GenBank accession numbers of IGF proteins are given after the species names in the tree. The triangle represents IGF in blotched snakehead (*C. maculata*).

3.3. Tissue Distribution of CmIGFs

The expression levels of *CmIGFs* in male and female blotched snakeheads were assessed through qPCR. As depicted in Figure 3, similar expression patterns of *CmIGF1-1* (Figure 3a) and *CmIGF1-2* (Figure 3b) were observed, primarily expressed in liver (L), with low levels in spleen (S), and undetectable in other tissues. Interestingly, the expression levels of *CmIGF1-1* and *CmIGF1-2* in the liver (L) exhibited significant sexual dimorphism between males and females ($p < 0.01$). *CmIGF2* transcripts were strongly expressed in the liver (L), with higher expression in males compared to females ($p < 0.01$). Additionally, moderate expression levels were detected in spleen (S), intestines (I), and heart (H), with significant sex differences ($p < 0.01$), while low levels were observed in other tissues (Figure 3c).

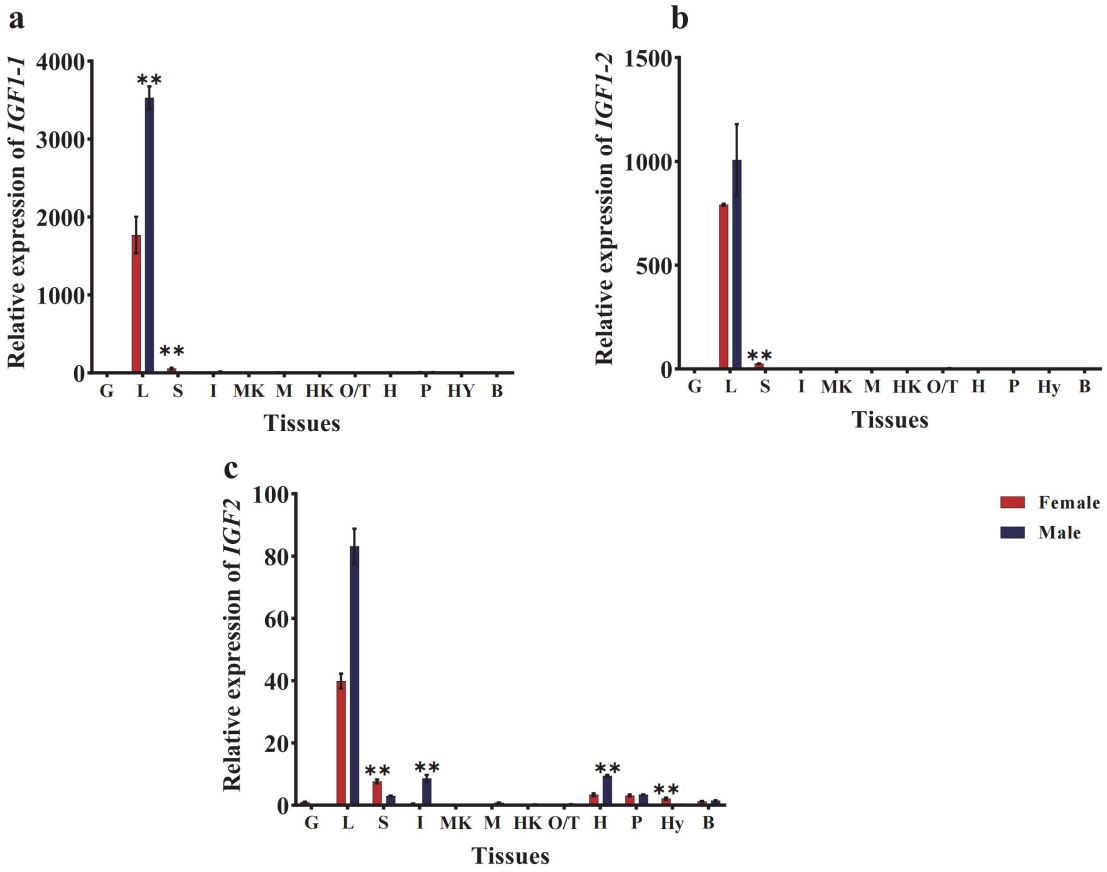

Figure 3. Gene expression patterns of *CmIGFs* in twelve tissues of males and females. (**a**) *CmIGF1-1*; (**b**) *CmIGF1-2*; (**c**) *CmIGF2*. The tissues are designated as follows: G (gills), L (liver), S (spleen), I (intestine), MK (middle kidney), M (muscle), HK (head kidney), O/T (ovary/testis), H (heart), P (pituitary), HY (hypothalamus), B (brain). *β-actin* and *EF1α* were the internal controls. Results represent the mean ± S.E.M ($n = 3$), and the relative expression levels in different tissues were the ratio of the expression in the female gills (G). Asterisks represent significant differences between males and females. ** $p < 0.01$.

3.4. CmIGFs Expression Patterns at Different Developmental Stages

In order to investigate the role of *CmIGFs* in growth, the expression levels of *CmIGFs* in liver (L) were analyzed across seven different developmental stages. Males reached body lengths of 9.6 ± 0.8 cm at 45 dah, 14.3 ± 0.8 cm at 75 dah, 21.3 ± 0.8 cm at 105 dah, 27.1 ± 1.2 cm at 135 dah, 29.1 ± 1.9 cm at 165 dah, 31.1 ± 1.3cm at 195 dah, and 44.7 ± 1.6 cm at

365 dah, and females reached body lengths of 9.7 ± 0.4 cm at 45 dah, 14.3 ± 0.7 cm at 75 dah, 20.5 ± 1.1 cm at 105 dah, 24.9 ± 1.5 cm at 135 dah, 28.0 ± 1.5 cm at 165 dah, 27.5 ± 2.8 at 195 dah, and 40.1 ± 1.3 cm at 365 dah. As shown in Figure 4, *CmIGFs* expression was higher in males than that in females across different developmental stages. In females, *CmIGF1-1* maintained low expression levels from 45 to 195 dah and peaked at 365 dah ($p < 0.01$). For male individuals, the transcription of *CmIGF1-1* started increasing from 75 to 105 dah and reached a tiny peak at 105 dah ($p < 0.01$). Subsequently, the expression levels decreased significantly from 105 dah to 195 dah before reaching the highest level at 365 dah ($p < 0.01$) (Figure 4a). The expression patterns of *CmIGF1-2* were similar to those of *CmIGF1-1* at developmental stages, peaking at 365 dah in both male and female individuals. From 45 to 195 dah, *CmIGF1-2* expression maintained a low level in females, while exhibiting a parabolic trend and peaking at 105 dah in males ($p < 0.01$) (Figure 4b). As for *CmIGF2*, the expression levels showed a considerable decline from the initial sample in both females and males, reaching the lowest levels at 135 dah. Then, they were gradually up-regulated and peaked at 365 dah ($p < 0.01$) (Figure 4c).

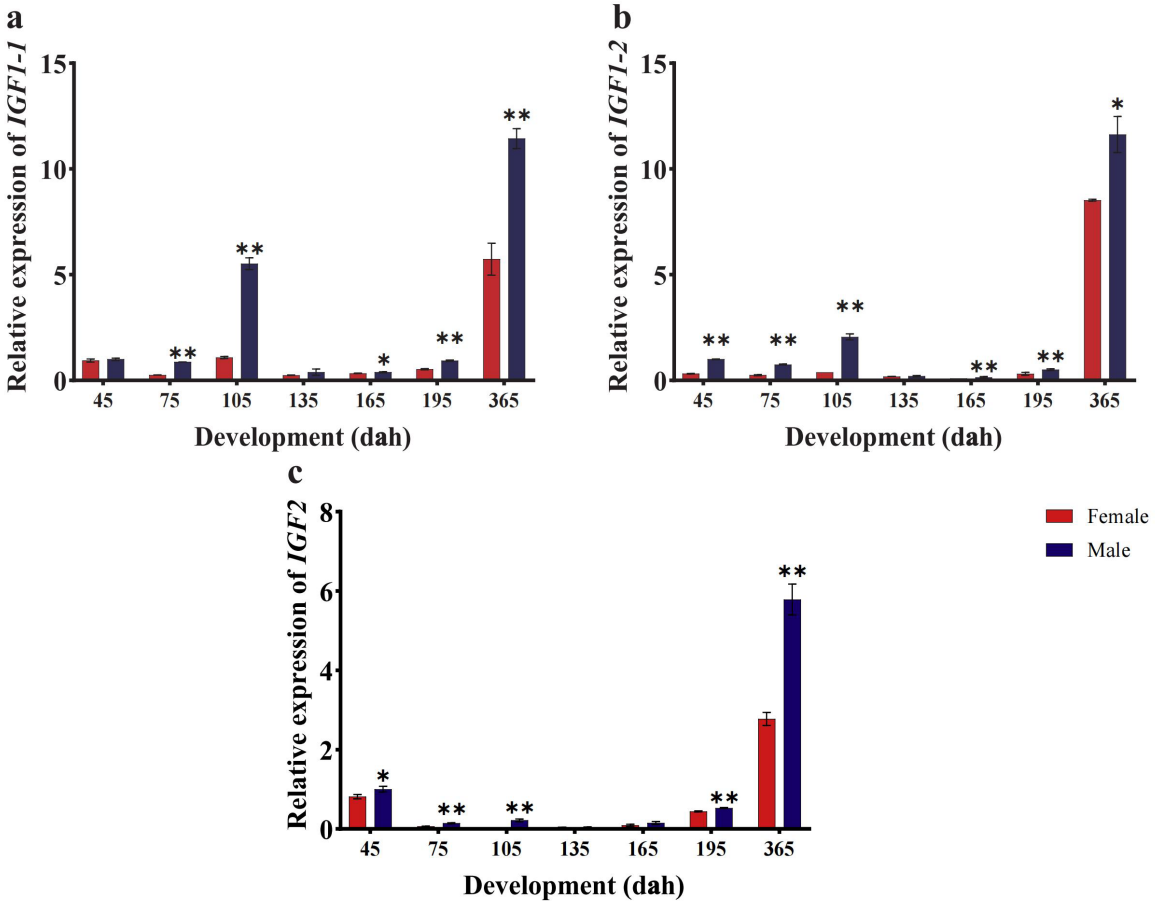

Figure 4. Gene expression profiles of *CmIGFs* in the liver of male and female individuals at different development stages. (**a**) *CmIGF1-1*; (**b**) *CmIGF1-2*; (**c**) *CmIGF2*. *β-actin* and *EF1α* were the internal controls. Results represent the mean ± S.E.M ($n = 3$), and *CmIGFs* transcription in the liver of 45 dah female served as the baseline (1.0). Asterisks represent significant differences between males and females. * $p < 0.05$, and ** $p < 0.01$.

3.5. Effects of Short-Term Hormone Treatment on CmIGFs in Females

In female blotched snakehead, the expression of *CmIGF1-1* in the control group fluctuated from 24 h to 144 h and peaked at 48 h and 120 h ($F_{5,10} = 84.254$, $p < 0.01$), respectively. Upon the administration of EE_2, *CmIGF1-1* transcription was inhibited,

remaining lower than that in the control group ($F_{2,10}$ = 35.081, p < 0.01) until 144 h, when the expression levels of *CmIGF1-1* were comparable between the control and the EE_2 treatment group (Figure 5a). After MT treatment, the highest peak of *CmIGF1-1* expression occurred at 48 h, while it remained significantly lower than that in the control group ($F_{2,10}$ = 35.081, p < 0.01) (Figure 5a). Both EE_2 and MT administration down-regulated the expression of *CmIGF1-2* ($F_{2,10}$ = 48.841, p < 0.01), which was lower than that in the control group from 48 h to 120 h (Figure 5b). A marked increase was observed in *CmIGF1-2* expression at 144 h when it was significantly higher ($F_{5,10}$ = 60.233, p < 0.01) than that in the control group (Figure 5b). *CmIGF2* transcripts in EE_2-treated group were consistently lower ($F_{2,10}$ = 61.829, p < 0.01) than those in the control group until 120 h, when *CmIGF2* displayed equivalent levels in the control and the EE_2 treatment group. After treatment with MT, *CmIGF2* showed higher levels than those in the control group from 24 h to 48 h ($F_{2,10}$ = 61.829, p < 0.01), reaching the highest level at 120 h, after which it sharply dropped to the lowest level at 144 h ($F_{5,10}$ = 30.592, p < 0.01) (Figure 5c).

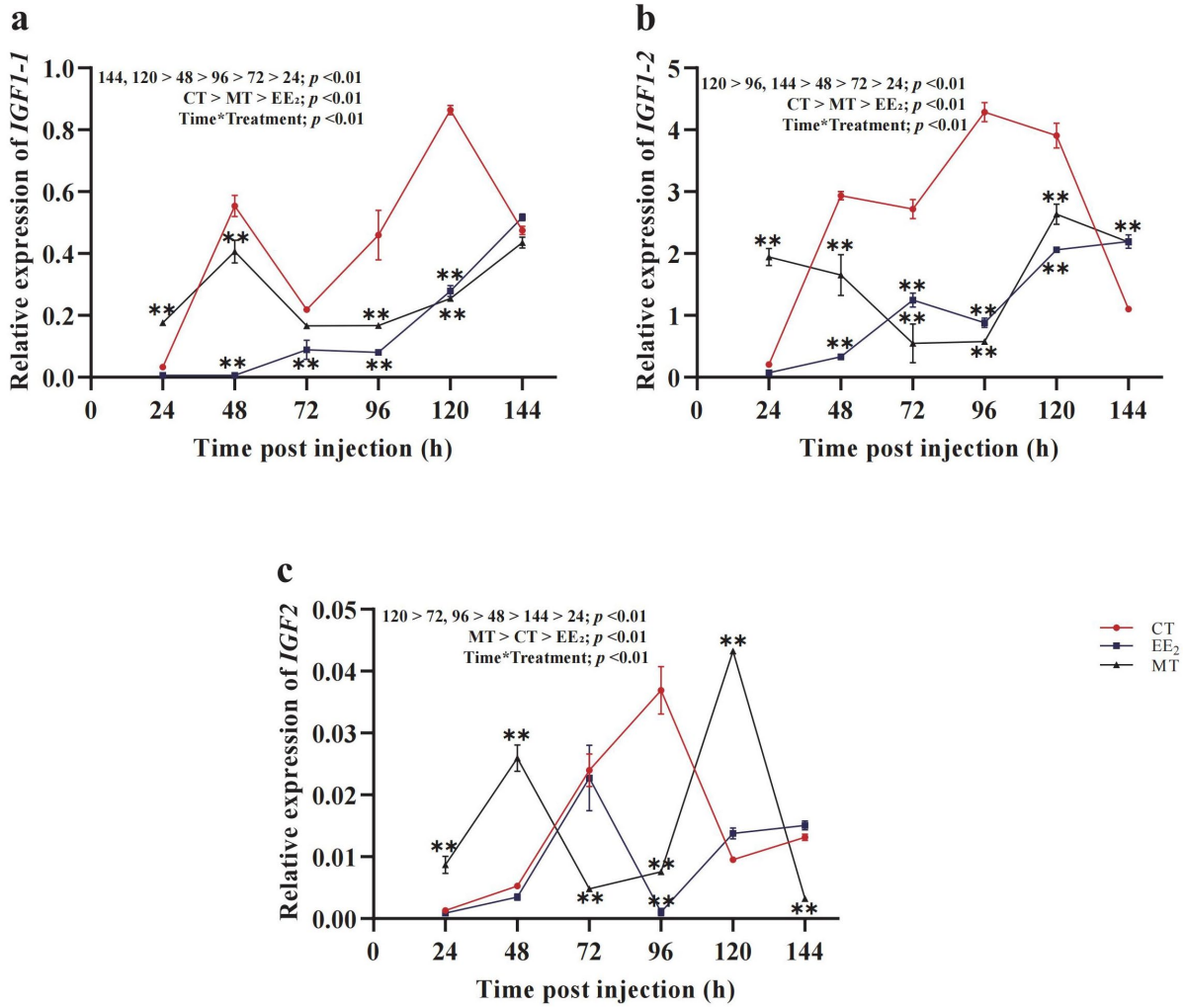

Figure 5. Effects of short-term hormone treatment on *CmIGFs* in female blotched snakehead. (**a**) *CmIGF1-1*; (**b**) *CmIGF1-2*; (**c**) *CmIGF2*. *β-actin* and *EF1α* were used as the internal controls for qPCR. Data are presented as mean ± S.E.M (n = 3). Asterisks represent significant differences between treatments and control at the corresponding time point for each dataset. ** p < 0.01.

3.6. Effects of Short-Term Hormone Treatment on CmIGFs in Males

In male individuals, *CmIGF1-1* transcripts showed a similar expression pattern, with the parabolic trends in both the control and EE_2-treated groups, gradually increasing and reaching their peak at 120 h, then sharply dropping to the initial level at 144 h ($F_{5,10}$ = 32.762,

$p < 0.01$) (Figure 6a). However, it showed fluctuating variation in the MT-treated group ($F_{2,10} = 54.339$, $p < 0.01$) (Figure 6a). In the control group, *CmIGF1-2* expression reached the highest value at 72 h and then gradually decreased and returned to the starting level at 144 h ($F_{5,10} = 54.207$, $p < 0.01$). *CmIGF1-2* expression displayed fluctuating changes in the EE_2-treated and MT-treated groups ($F_{2,10} = 33.880$, $p < 0.01$), with the expression level at 144 h being significantly higher than that in the control group (Figure 6b). *CmIGF2* expression exhibited a similar dynamic pattern of change in both the control and EE_2-treated groups, with *CmIGF2* being sharply down-regulated in the control group from 120 h, while showing a slight up-regulation ($F_{2,10} = 55.904$, $p < 0.01$) in the EE_2-treated group. After MT treatment, *CmIGF2* transcription gradually decreased and reached the lowest level at 96 h and then quickly rose to the highest level at 144 h, which was significantly higher ($F_{5,10}$ =92.615, $p < 0.01$) than that in the control group ($p < 0.01$) (Figure 6c).

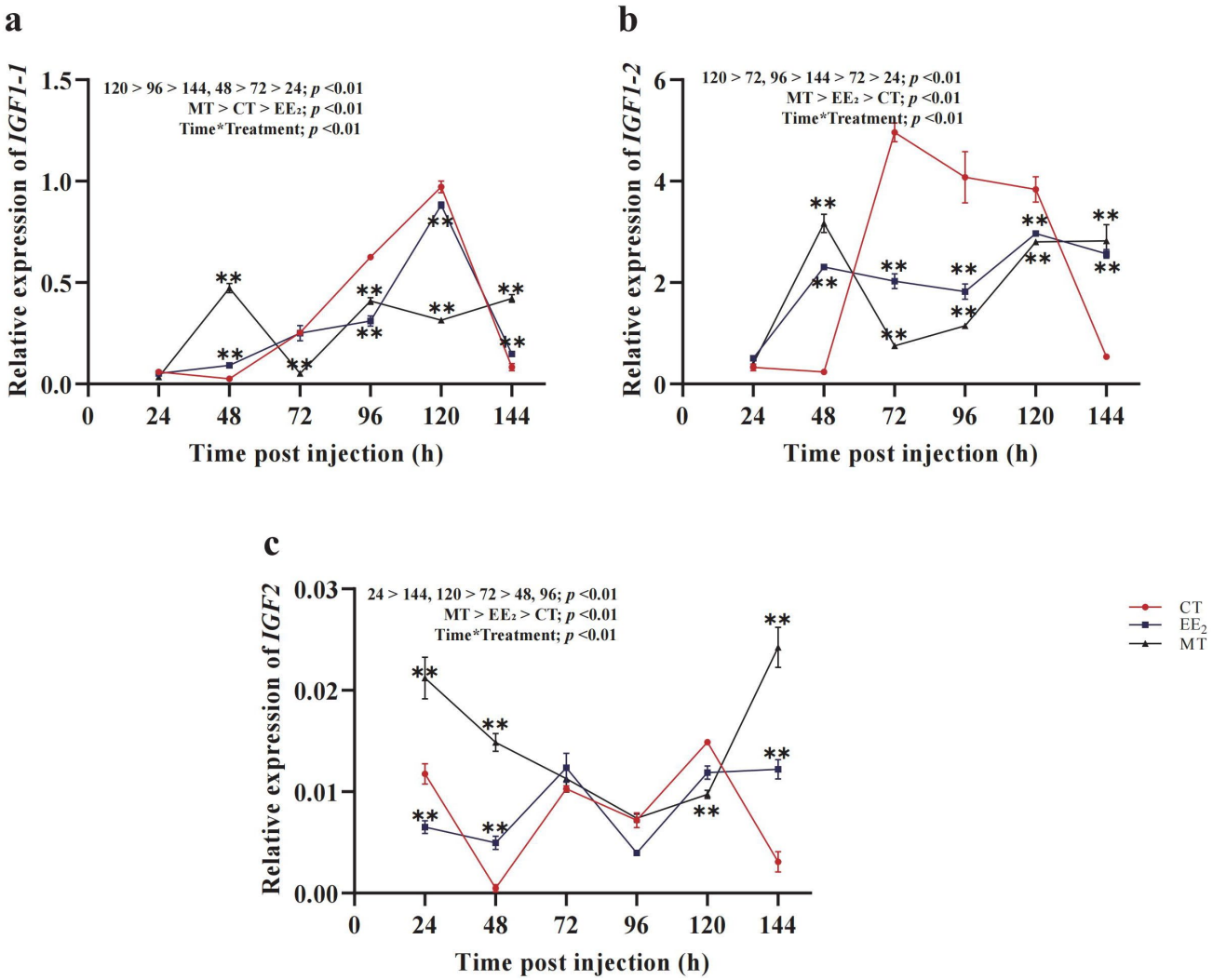

Figure 6. Effects of short-term hormone treatment on *CmIGFs* in male blotched snakehead. (**a**) *CmIGF1-1*; (**b**) *CmIGF1-2*; (**c**) *CmIGF2*. *β-actin* and *EF1α* were used as the internal controls for qPCR. Data are presented as mean ± S.E.M (n = 3). Asterisks represent significant differences between treatments and control at the corresponding time point for each dataset. ** $p < 0.01$.

3.7. Effects of Long-Term Hormone Treatment on CmIGFs in Females

In females, MT considerably boosted *CmIGF1-1* mRNA levels in comparison to the control group at 2 weeks ($F_{2,8} = 55.732$, $p < 0.01$), followed by a sudden drop to the lowest level at 4 weeks (Figure 7a). The highest level was detected at 8 weeks, which was not

significantly different from the control group (Figure 7a). Treatment with EE_2 resulted in consistently lower *CmIGF1-1* mRNA levels than controls throughout ten weeks, reaching the lowest level at 10 weeks and being significantly lower ($F_{4,8}$ = 62.65, $p < 0.01$) than that in the control group (Figure 7a). Hormone treatment (EE_2 and MT) led to the maintenance of low transcription levels of *CmIGF1-2*, which were significantly lower than that in the control group ($F_{2,8}$ = 69.249, $p < 0.01$) (Figure 7b). *CmIGF2* remained at a low expression level in the control group, while *CmIGF2* mRNA levels were considerably boosted ($F_{2,8}$ = 73.017, $p < 0.01$) after the administration of MT and EE_2, with the highest levels detected at 6 weeks and 8 weeks ($F_{4,8}$ = 74.372, $p < 0.01$), respectively, which were much higher than that in the control group (Figure 7c). Finally, the body weight and length of fish in the control and treated groups were measured at 10 weeks post-injection. Females treated with MT achieved an average body length of 21.4 ± 1.1 cm and a weight of 240.1 ± 9.2 g. Compared to the control group, which presented an average body length of 26.7 ± 1.8 cm and a weight of 331.5 ± 9.3 g, the growth rates were reduced by 19.8% in length and 27.4% in weight. Females treated with EE_2 reached an average body length of 19.2 ± 1.5 cm and a weight of 207.5 ± 8.7 g, indicating the reductions in growth rates of 28.0% in length and 37.3% in weight compared to the control group.

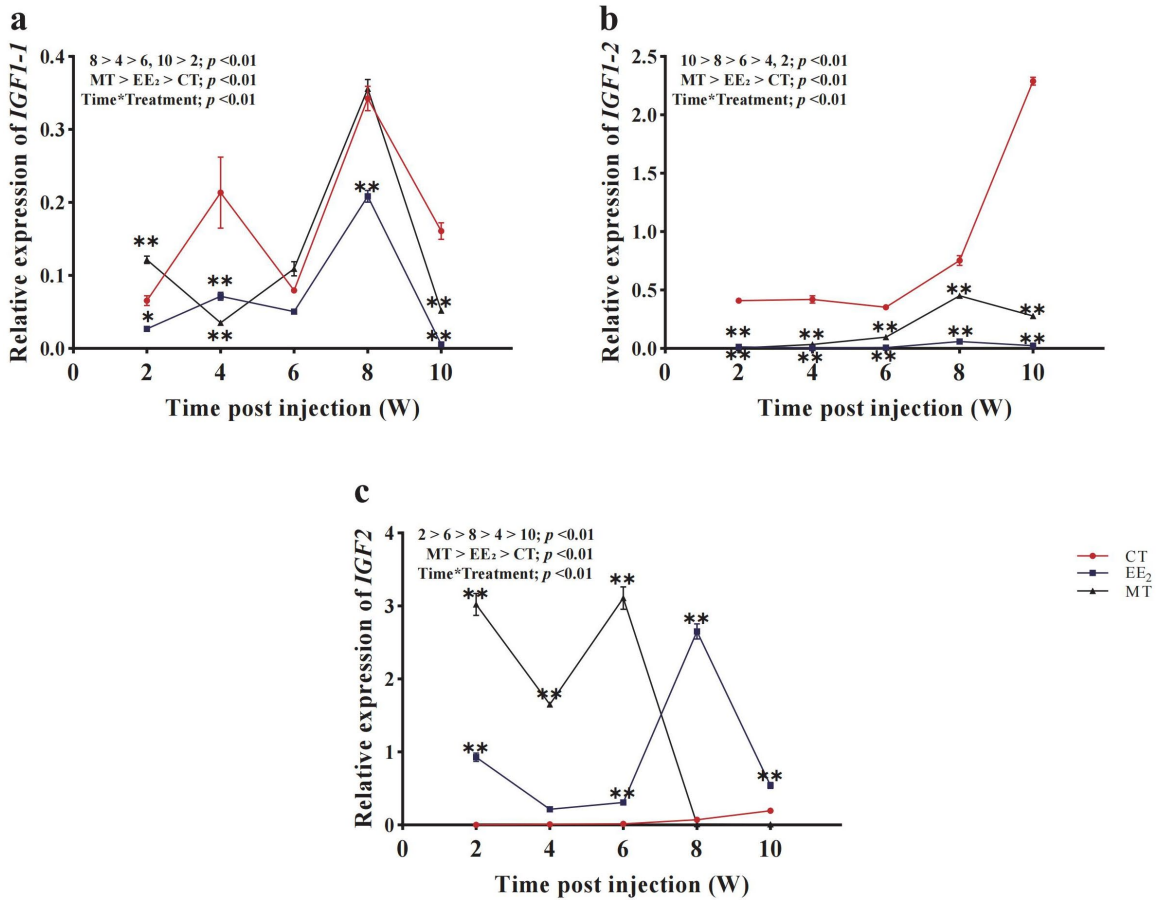

Figure 7. Effects of long-term hormone treatment on *CmIGFs* in female blotched snakehead. (**a**) *CmIGF1-1*; (**b**) *CmIGF1-2*; (**c**) *CmIGF2*. *β-actin* and *EF1α* were used as the internal controls for qPCR. Data are presented as mean ± S.E.M (n = 3). Asterisks represent significant differences between treatments and control at the corresponding time point for each dataset. * $p < 0.05$, and ** $p < 0.01$.

3.8. Effects of Long-Term Hormone Treatment on CmIGFs in Males

As shown in Figure 8a,b, the expression levels of *CmIGF1-1* ($F_{2,8}$ = 57.041, $p < 0.01$) and *CmIGF1-2* ($F_{2,8}$ = 62.436, $p < 0.01$) remained low in males after the administration of EE_2, being lower than those in the control group. Upon the administration of MT, the

transcription of *CmIGF1-1* ($F_{4,8}$ = 78.874, $p < 0.01$) and *CmIGF1-2* ($F_{4,8}$ = 43.802, $p < 0.01$) remained at low levels until 6 weeks, then increased sharply and reached their peaks at 8 and 10 weeks, respectively, which were significantly higher than those in the control group (Figure 8a,b). EE_2 and MT treatments up-regulated ($F_{2,8}$ = 69.567, $p < 0.01$) the expression levels of *CmIGF2* compared to the control group at 2 weeks (Figure 8c). After that, *CmIGF2* expression displayed a decreasing trend and achieved the lowest level at 10 weeks ($F_{4,8}$ = 58.721, $p < 0.01$) (Figure 8c). In contrast, *CmIGF2* transcripts dramatically increased from 4 weeks and reached the peak ($F_{2,8}$ = 69.567, $p < 0.01$) at 8 weeks with EE_2 administration and subsequently rapidly declined to the lowest level at 10 weeks ($F_{4,8}$ = 58.721, $p < 0.01$) (Figure 8c). At 10 weeks post-treatment, males treated with EE_2 achieved an average body length of 24.3 ± 1.6 cm and a body weight of 330.6 ± 7.4 g. Compared to the control group, which presented an average body length of 28.6 ± 1.3 cm and a body weight of 370.5 ± 8.8 g, the growth rates were reduced by 15.1% in length and 10.8% in weight. Males treated with MT reached an average body length of 33.7 ± 1.4 cm and a body weight of 406.2 ± 10.3 g, indicating the increased growth rates of 15.1% in length and 15.1% in weight compared to the control group.

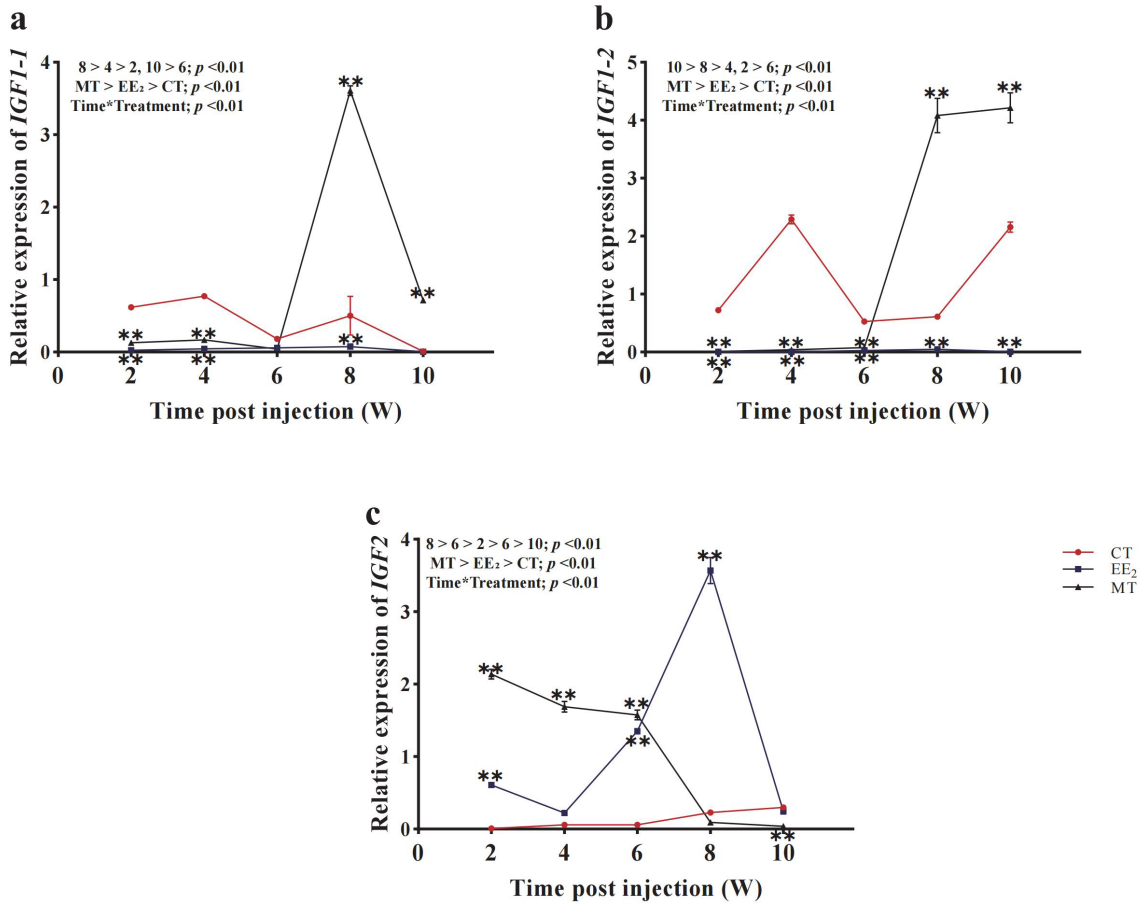

Figure 8. Effects of long-term hormone treatment on *CmIGFs* in male blotched snakehead. (**a**) *CmIGF1-1*; (**b**) *CmIGF1-2*; (**c**) *CmIGF2*. *β-actin* and *EF1α* were used as the internal controls for qPCR. Data are presented as mean ± S.E.M (n = 3). Asterisks represent significant differences between treatments and control at the corresponding time point for each dataset. ** $p < 0.01$.

4. Discussion

Growth, one of the most crucial economic traits in farmed fishes, plays a vital role in the advancement of aquaculture. In the present study, *IGFs*, which are known to be involved in growth regulation, were characterized in blotched snakehead (*C. maculata*). Consistent with findings in zebrafish (*D. rerio*) [9] and giant grouper (*E. lanceolatus*) [16], two copies of *IGF1* were identified in blotched snakehead, designated as *CmIGF1-1* and *CmIGF1-2*, located on chromosomes 15 and 10, respectively [30]. The *IGF1* gene in many fish species, including giant grouper (*E. lanceolatus*) [16], ussuri catfish (*Pseudobagrus ussuriensis*) [34], and zebrafish (*D. rerio*) [9], typically consists of five exons and four introns, with intron 2 being the longest. In our study, *CmIGF1-1* also consisted of five exons and four introns, with intron 2 being the longest at 8800 bp. In contrast, *CmIGF1-2* manifested a more concise structure, with solely three exons and two introns. The structural differences between the two gene products may potentially stem from divergent splicing methodologies [35]. The *IGF2* gene differs from *IGF1* in exon–intron composition in ussuri catfish (*P. ussuriensis*) [34] and yellow catfish (*P. fulvidraco*) [11], typically consisting of four exons and three introns. In this study, *CmIGF2* comprised four exons and three introns. The alignment of amino acid sequences revealed conserved IGF domains in both IGF1-1 and IGF1-2 across species. Furthermore, IGF2 exhibited conserved IGF and IGF2_C domains, which play a vital role in protein folding, maintaining proper secondary spatial structure, and facilitating normal physiological functions [36]. Evolutionary analysis revealed that IGF1 and IGF2 clustered together among all fish species, diverging from those of tetrapods, which encompass mammals, birds, and reptiles. Our findings are consistent with both systematic evolutionary analysis and traditional taxonomy.

Two distinct variant forms have been identified in *CmIGF1*, whereas *CmIGF2* was expressed as a single form, consistent with observations in giant grouper (*E. lanceolatus*) [16] and olive flounder (*P. olivaceus*) [37]. In contrast, in zebrafish (*D. rerio*) [9], both *IGF1* and *IGF2* present two variants, a disparity that may stem from gene duplication. This suggests that the retention of gene duplication among species may be selective, reflecting the diversity of evolutionary histories and genomic adaptabilities [38]. *CmIGF1-1* and *CmIGF1-2* exhibited notable expression levels in liver, consistent with the previous findings in spotted scat (*S. argus*) [25], giant grouper (*E. lanceolatus*) [16], and Japanese sea bass (*Lateolabrax japonicus*) [39], indicating the significant role of liver cells in IGF1 secretion. *CmIGF2* was broadly distributed across tissues, although it showed predominant expression in the liver. A high expression of *IGF2* has been observed in the gonads of Chinese sturgeon (*Acipenser dabryanus*) [40] and the gills of redbanded seabream (*Pagrus Auriga*) [24]. The observed variation in the predominant tissue for *IGF2* mRNA expression among different fish species may be attributed to interspecific differences. Sex-biased differences in the expression of *CmIGFs* were observed, with higher levels in male livers than in females, consistent with reports in yellow catfish (*P. fulvidraco*) [11] where *IGFs* expression levels were higher in the livers of males with faster growth rates than female individuals. Sexual dimorphism in growth has been observed in European eels (*Anguilla anguilla*) [41] and tongue sole (*C. semilaevis*) [42], with females demonstrating faster growth rates than males. In the late juvenile stage of European eels (*A. anguilla*) [41] and the larval phase of tongue sole (*C. semilaevis*) [10], the expression levels of *IGF1* and *IGF2* are significantly higher in females compared to males. In addition to *IGF1* and *IGF2*, a gonad-specific IGF family gene (*IGF3*) has been isolated in the gonads of turbot (*Scophthalmus maximus*) [4], zebrafish (*D. rerio*) [9], orange-spotted grouper (*Epinephelus coioides*) [43], and common carp (*Cyprinus carpio*) [44], uniquely expressed in ovaries and testes. *IGF3* commences at the early stage of sex determination and differentiation and remains high in expression throughout gonadal development, offering insights into fish gonadal development, particularly in economically valuable species. However, the expression profiles of *CmIGF1-1*, *CmIGF1-2*, and *CmIGF2* across various tissues did not reveal any gonad-specific expression patterns like *IGF3*. Additionally, the comparison of

the amino acid sequences of IGF3 from zebrafish (*D. rerio*) retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov/, accessed on 20 January 2024) with those of *Cm*IGF1-1, *Cm*IGF1-2, and *Cm*IGF2 showed low similarity levels, at only 25.6%, 22.3%, and 43.1%, respectively. Furthermore, the *IGF3* gene was not identified in the genome of blotched snakehead (*C. maculata*) (SRA Accession No. PRJNA730430) [30], suggesting the potential absence of IGF3 in blotched snakehead (*C. maculata*), warranting further investigation.

CmIGFs were detected in all tissues sampled from 45 to 365 dah, with global expression levels higher in males than in females. The expression of *CmIGF1-1* and *CmIGF1-2* gradually increased with growth and culminated in a minor peak at 105 dah, similar to observations in yellow catfish (*P. fulvidraco*) [11] and spotted scat (*S. argus*) [25]. Subsequently, a gradual decrease in the expression of *CmIGF1-1* and *CmIGF1-2* was observed from 135 dah, potentially due to a substantial portion of energy from feed being used for gonadal development [28,45]. *CmIGF1-1* and *CmIGF1-2* revealed the highest expression levels at 365 dah, corresponding to sexual maturity in blotched snakehead (*C. maculata*), with energy primarily directed toward growth. Furthermore, *CmIGF1-1* and *CmIGF1-2* exhibited elevated expression levels in male snakehead compared to females, possibly due to deferred sexual maturity in males and preferential energy allocation toward growth [46]. Similar expression patterns of *IGF1* have observed in Nile tilapia (*O. niloticus*) at different developmental stages [47], showing temporal and spatial variations with the gradual elevation of hepatic expression levels during the initial growth stages, followed by a minor peak, rapid decline, and resurgence to peak levels in the late sampling period. *CmIGF2* expression was high at 45 dah and decreased to a minimum at 135 dah and then gradually increased to peak at 365 dah, suggesting a crucial role of *IGF2* in early growth and maturation in blotched snakehead (*C. maculata*). Similar results have been reported in aucha perch (*Siniperca chuatsi*) [48] and dark sleeper (*Odontobutis potamophila*) [49]. However, *IGF2* exhibits different expression profiles in mammals, where it is highly expressed during early embryonic development and subsequently diminishes [5], suggesting a broader role of IGF2 in fishes beyond embryonic growth and development. Our results suggest that differences in growth between male and female individuals may be connected with sexually dimorphic expression levels of *IGFs*, warranting further investigation.

Sex steroid hormones are assimilated by fish and influence the expression of genes associated with growth, thereby regulating protein synthesis, cellular proliferation, and overall growth [50,51]. Previous studies have suggested interactions between sex steroid hormones and components of the GH/IGF axis [11,18,21,25,27]. However, these interactions are varied, and the reason for the variety among these studies remains unclear. In this study, both *CmIGF1-1* and *CmIGF1-2* were down-regulated in response to EE_2 treatment in both sexes, consistent with findings in rainbow trout (*O. mykiss*) where E_2 treatment reduces the hepatic expression levels of *IGF1* [21]. Additionally, EE_2 inhibits *GH* expression in the pituitary of blotched snakehead (*C. maculata*) [27], indicating that EE_2 down-regulates hepatic *CmIGF1* transcription levels in both sexes, probably by diminishing the expression of *CmGH*. After EE_2 stimulation, the expression of *CmIGF2* was up-regulated in both sexes, which differs slightly from the results in Nile tilapia (*O. niloticus*), where E2 stimulation increased *IGF2* expression in females but had no significant effect on *IGF2* expression in males [18]. Conversely, in spotted scat (*S. argus*), E2 treatment increased the transcription of both *IGF1* and *IGF2* in males, while in females, *IGF1* was up-regulated and *IGF2* was down-regulated [25]. These findings indicate that the transcriptional levels of *IGFs* are influenced by sex steroid hormones and exhibit variation among different fishes, potentially attributable to differences in experimental methods (such as administration method, concentration, and exposure duration) and developmental stages.

The ability of androgens to regulate the GH/IGF axis is supported by several observations of this study. In females, *CmIGF1-1* expression initially increased after MT

treatment for up to 2 weeks, but eventually, its expression was inhibited, while the expression of *CmIGF1-2* remained inhibited. Additionally, *CmIGF2* expression was significantly up-regulated in females before 6 weeks, but eventually, its expression was inhibited. Comparable results were observed in other fish species. In female yellow catfish (*P. fulvidraco*), *IGF-1* and *IGF-2* were up-regulated in the liver after 2–3 weeks of MT treatment and then declined, significantly falling to a lower level than the control group by 4 weeks [11]. Similarly, in female Nile tilapia (*O. niloticus*), following T treatment, *IGF1* expression initially increased, then significantly dropped below the control group by 3 weeks [18]. Such fluctuations may be due to the resistance of endogenous estrogen to exogenous androgen, thereby regulating *IGFs* expression [35,52]. *CmIGF1-1* and *CmIGF1-2* expression levels were significantly up-regulated in males treated with MT after 6 weeks. It is possible that the anabolic effect of MT leads to an increase in food intake in males, thus enhancing energy allocation for growth in males [53]. Similar findings have been observed in rainbow trout (*O. mykiss*), where T directly increases steady-state *IGF-1* and *IGF-2* expression levels [21]. Likewise, *IGF1* and *IGF2* are provoked by T treatment in male Nile tilapia (*O. niloticus*), with expression increasing 7–21 days after injection [18]. However, the responses of *CmIGF1-1* and *CmIGF1-2* to MT stimulation differed after 8 weeks, suggesting that MT regulates the expression of GH/IGF axis genes in a time-dependent manner. Additionally, MT treatment significantly up-regulated *CmIGF2* expression compared to the control group before 6 weeks, and subsequently, it gradually decreased to below the control level. We suggest that exogenous sex steroid hormones stimulate IGFs via IGF-1R in blotched snakehead (*C. maculata*), resulting in a compensatory increase in *CmIGF2* expression in response to low *CmIGF1* concentration, as IGF-1R has higher binding affinity for IGF1 than for IGF2 [54]. The specific pathways through which MT regulates the expression of *CmIGFs*, thereby affecting the growth of blotched snakehead, are currently unclear. Perhaps in the future, we can conduct a deeper investigation by using liquid chromatography–mass spectrometry (LC–MS) to detect changes in sex steroid hormones in both males and females during hormone treatment processes.

Sexual dimorphism in growth is governed by a complex interplay of factors, including the differential expression of GH/IGF axis genes, the influence of exogenous sex steroid hormones, and various other elements such as environmental conditions, the abundance of IGF receptors, interactions among growth axis genes, and the modulation of endogenous hormones. Future research projects will focus on delving deeper into the mechanisms underlying growth dimorphism between male and female snakehead.

5. Conclusions

The full-length sequences of *CmIGFs* were cloned, and their sequence characteristics and gene expression patterns were thoroughly scrutinized. The investigation unveiled that *CmIGFs* closely resembled those documented in bony fishes, exhibiting the typical IGF domain of the IGF family. Gene expression analyses showed the predominant expression of *CmIGFs* in the male liver, signifying that the liver serves as the primary site for the synthesis and secretion of IGFs. Furthermore, the study showed that exogenous sex steroid hormones, EE_2 and MT, exerted significant modulatory effects on the expression of *CmIGFs* genes. Specifically, EE_2 was observed to suppress the expression of *CmIGF1-1* and *CmIGF1-2* while promoting the expression of *CmIGF2*. In females, MT up-regulated the expression of *CmIGF1-1* and *CmIGF2* in a time-dependent manner, while consistently inhibiting the expression of *CmIGF1-2*. Interestingly, MT was found to promote the expression of *CmIGFs* in males in a time-dependent manner, and *CmIGF1* and *CmIGF2* may exhibit a complementary relationship, with a compensatory increase in *CmIGF2* expression in response to low *CmIGF1* concentration. These findings lay the groundwork for future investigations aimed at unraveling the molecular mechanism underlying the growth dimorphism between female and male blotched snakeheads.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes9040120/s1. Figure S1: Nucleotide and putative amino sequences of *Cm*IGF1-1 (a), *Cm*IGF1-2 (b), and *Cm*IGF2 (c). The sequence numbers of nucleotide (lower row) and putative amino acid (upper row) are shown on the left. The translation initiation codons and stop codons are in bold. The motif-associated mRNA instability (ATTTA) is shown as a double underscore. The poly-adenylation signal sequence (AATAA) is shown as a wavy line. The signal peptide is shown as an underscore. The IGF domain is marked with a green background. The IGF2_C domain is marked with a yellow background. The box represents the IGF-1R recognition sequence. Figure S2: Multiple protein alignments of *Cm*IGF1-1 (a), *Cm*IGF1-2 (b), and *Cm*IGF2 (c) in various species. The amino acid sequences of IGF1s from typical organisms were aligned using the Clustal X 2.1 program. The black shade represents 100% identity, dark gray represents 80% identity, and the IGF domain is marked by a green box. *At*IGF1-1 stands for IGF1-1 protein in *A. testudineus* (Protein ID. XP_026218654.1), *At*IGF1-2 stands for IGF1-2 protein in *A. testudineus* (Protein ID. XP_026218655.1), *At*IGF2 stands for IGF2 protein in *A. testudineus* (Protein ID. XP_026221051.1), *Bt*IGF1 stands for IGF1 protein in *B. taurus* (Protein ID. NP_001071296.1), *Bt*IGF2 stands for IGF2 protein in *B. taurus* (Protein ID. XP_005227329.1), *Ca*IGF1a stands for IGF1a protein in *C. argus* (Protein ID. KAF3696413.1), *Ca*IGF2 stands for IGF2 protein in *C. argus* (Protein ID. KAF3689021.1), *Cc*IGF1-1 stands for IGF1-1 protein in *C. carpio* (Protein ID. XP_018948511.1), *Cc*IGF1-2 stands for IGF1-2 protein in *C. carpio* (Protein ID. XP_018948527.1), *Cc*IGF2 stands for IGF2 protein in *C. carpio* (Protein ID. XP_018955405.1), *Cm*IGF1-1 stands for IGF1-1 protein in *C. maculata* (Protein ID. UTQ11150.1), *Cm*IGF1-2 stands for IGF1-2 protein in *C. maculata* (Protein ID. UTQ11151.1), *Cm*IGF2 stands for IGF2 protein in *C. maculata* (Protein ID. UTQ11152.1), *Ci*GF2a stands for IGF2a protein in *C. idella* (Protein ID. AJG05606.1), *Ci*GF2b stands for IGF2b protein in *C. idella* (Protein ID. AJG05607.1), *Cj*IGF2 stands for IGF2 protein in *C. japonica* (Protein ID. XP_015719328.1), *Dr*IGF1 stands for IGF1 protein in *D. rerio* (Protein ID. AAI14263.1), *Dr*IGF2a stands for IGF2a protein in *D. rerio* (Protein ID. NP_571508.1), *Dr*IGF2b stands for IGF2b protein in D. rerio (Protein ID. NP_001001815.1.), *El*IGF1a stands for IGF1a protein in *E. lanceolatus* (Protein ID. ABZ10840.1), *El*IGF1b stands for IGF1b protein in *E. lanceolatus* (Protein ID. ABZ10841.1), *Gg*IGF1 stands for IGF1 protein in *G. gallus* (Protein ID. NP_001004384.1), *Gg*IGF2 stands for IGF2 protein in *G. gallus* (Protein ID. NP_001025513.1), *Hs*IGF1a stands for IGF1a protein in *H. sapiens* (Protein ID. AAA52538.1), *Hs*IGF1b stands for IGF1a protein in *H. sapiens* (Protein ID. AAA52537.1), *Ip*IGF2 stands for IGF2 protein in *I. punctatus* (Protein ID. ADO29240.1), *Mm*IGF2 stands for IGF2 protein in *M. musculus* (Protein ID. NP_001116208.1), *Po*IGF1a stands for IGF1a protein in *P. olivaceus* (Protein ID. CAA09267.1), *Po*IGF1b stands for IGF1b protein in *P. olivaceus* (Protein ID. CAA09268.1), *Sa*IGF1 stands for IGF1 protein in *S. aurata* (Protein ID. ABQ52656.1), *Sm*IGF1a stands for IGF1a protein in *S. maximus* (Protein ID. XP 035474340.1), *Sm*IGF1b stands for IGF1b protein in *S. maximus* (Protein ID. XP 035474347.1), *Tr*IGF2 stands for IGF2 protein in *T. rubripes* (Protein ID. ADO29240.1), *Xl*IGF1 stands for IGF1 protein in *X. laevis* (Protein ID. AAA70330.1), *Xl*IGF2a stands for IGF2a protein in *X. laevis* (Protein ID. AAL11445.1), *Xl*IGF2b stands for IGF2b protein in *X. laevis* (Protein ID. AAH72153.1), *Xt*IGF1 stands for IGF1 protein in *X. tropicalis* (Protein ID. XP_002936875.1), and *Xt*IGF2 stands for IGF2 protein in *X. tropicalis* (Protein ID. AAI56000.1).

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