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RESEARCH ARTICLE

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RNA/DNA ratio and expression of GHR1 and IGF-I hormones: A study on seasonal variation effect on growth characterization of male *Oreochromis niloticus*

ABSTRACT:

Nile tilapia (*Oreochromis niloticus*) is considered as one of the most economical and popular aquaculture species. One of the main concerns in tilapia aquaculture, as well as in the wild, is growth, because of its importance for the fish population, stock assessment and their production in aquaculture. Accordingly, this study was initiated to characterize growth on molecular basis. In the same respect, RNA/DNA ratio and GHR1/IGF-I as growth-related genes are known to be involved in weight gain alterations. To assess the correlation between growth, biochemical and hormonal conditions of fish during summer and winter. Adult male *O. niloticus* (100 – 250 gm) were collected from Bahr Shebeen Canal, for such investigation. The results revealed that the GHR1 and IGF-I mRNAs were directly proportional to age, length and weight. However, they were up regulated in summer than in winter. However, RNA/DNA ratio gave an observable positive correlation with age and length only with higher values in winter. In conclusion, this study supports the use of GHR1/IGF-I as reliable indices of growth and condition of *O. niloticus* in their natural habitat in addition to RNA/DNA ratio assessment.

KEY WORDS:

Oreochromis niloticus, GHR1, IGF-I, seasonal variation, RNA/DNA ratio.

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INTRODUCTION:

Oreochromis niloticus is known to adapt readily to a range of environmental factors such as salinity, low oxygen levels and can feed at different tropic levels when need arises (Canonico and Arthington, 2005). Male tilapias are mainly used in aquaculture, for growth problems (Trewavas, 1982). The growth of freshwater and marine fish species has been used to be assessed using RNA/DNA ratio (Khallaf *et al*., 1993; Caldarone, 2005; Mercaldo-Allen *et al*., 2006; Tanaka *et al*., 2008), mollusks (Fathallah *et al*., 2010), and crustaceans (Chícharo *et al*., 2007; Grimm *et al*., 2015). The temperature has a positive effect on growth rates, but it has a negative effect on RNA/DNA ratio (Ramirez *et al*., 2004). RNA/DNA ratio was affected by age and size as the ratio was increased continuously with size and the slight reductions occurred for larger individuals (Rooker and Holt, 1996; Iglesias *et al*., 2002). An increase of RNA/DNA with larval size has been also reported on herrings (Clemmesen, 1994). Growth hormones (GHs) were used also to examine the fish growth due to its important role in regulating different physiological processes including growth, metabolism, reproduction and osmoregulation (Reinecke *et al*., 2005; Amenyogbe *et al*., 2019). GHs stimulates liver production of the insulin-like growth factors (IGF1 and IGF2),

which mediate the anabolic actions of GHs (Wood *et al*., 2005; Reinecke, 2010). Seasonal variation has effects on body growth rates and circulating levels of growth hormones (Marchant and Peter, 1986). Several studies reported a close relationship between seasonal temperature and variations of GH or IGF-I levels (Marchant and Peter, 1986; Mingarro *et al*., 2002; Taylor *et al*., 2003). Some authors have demonstrated the involvement of the GH/IGF system in the growth-promoting effect of high temperatures, during the embryonic and the post larval period (Gabillard *et al*., 2003 & 2005). A positive relationship was found between IGF-I and growth rates (Beckman *et al*., 2004), and protein concentrations (Dyer *et al*., 2004). In accordance, this study was carried out to investigate the correlation of RNA/DNA ratio and GHR1/IGF-I to growth variation of *O. niloticus* in their natural habitat.

MATERIAL AND METHODS:

Samples collection:

Nile tilapia (Oreochromis niloticus) males of different body weights (100-250 gm) were collected randomly from Bahr Shebeen Canal during summer and winter months in 2016 and had been transferred to laboratory within one hour. Standard length to the nearest cm and total body weight to the nearest g and the fish scales to estimate the age were recorded for each fish. Moreover, parts of the liver and pectoral muscle tissues were kept frozen at - 80ºC for further analyses.

Electrophoretic pattern of nucleic acids (DNA, RNA) from tissue lysate:

Gels were prepared with 1.8% electrophoretic grade agarose (Sigma-Aldrich, Germany) and 0.2% polyvinyl pyrolidine (Sigma-Aldrich, Germany). The agarose and PVP were boiled with Tris borate EDTA buffer (1 × TBE buffer; 89 mMTris, 89 mm boric acid, 2 mm EDTA, pH8.8). Ethidium bromide (0.5 µg/ml) was added to the gel at 40ºC. Gels were poured and allowed to solidify at room temperature for 1h before the samples were loaded. Muscle tissues (20 mg) were collected from the area between the lateral line and dorsal fin were squeezed and lysed in 200 µl lysing buffer (50 mM NaCl, 1 mM Na_2EDTA, 0.5% SDS, pH 8.3) lysing buffer over night at 37ºC. For electrophoretic pattern of nucleic acids of tissue lysate, 20 µl of lysate muscle tissues was loaded in well. Electrophoresis with 1 × TBE running buffer at a constant voltage (50 V) for 30 min using small electrophoresis cell (Bio-Rad power pack 300, USA). DNA and RNA were visualized using a 312 nm UV light under a transilluminator. Gels were photographed using digital camera (Hassab El-Nabi *et al*., 2001).

Determination of RNA/DNA ratio:

RNA/DNA ratio varies among different tissues, and fish white muscles are considered the most sensitive for nucleic acid measurements (Foster *et al*., 1993). Hence, RNA/DNA ratio was estimated in muscle tissues of the adult fish samples through the optical density of DNA, RNA in tissue lysate. The intensity of DNA and RNA areas was measured by image J software, as a mean of optical density values.

Quantitative real-time polymerase chain reaction (qRT-PCR) for insulin growth factor-I (IGF-I) and growth hormone receptor-I (GHR1) mRNA:

In order to investigate the genetic effect on growth, the expression of IGF-I and GHR1 mRNA in liver samples was evaluated. The total RNA was purified from the liver samples using a RNeasy Plus Minikit (Qiagen, Valencia, CA). The Revert Aid™ H Minus Reverse Transcriptase (Fermentas, Thermo Fisher Scientific Inc., Canada) was used to prepare cDNA. Real-time PCR reactions were carried out using Power SYBR® Green (Life Technologies, CA) on an Applied Biosystems 7500 system under the following conditions: 95°C for 10 minutes (Holding stage) and 40 cycles of 95°C for 15 seconds (Denaturation stage) followed by 60°C for 1 minute (Annealing and extension stage). Relative values of gene expression were normalized to β-Actin as a house-keeping gene. Samples of cDNA were run in triplicate for real-time PCR analysis. The amount of change in gene expression was calculated from the obtained cycle threshold (CT) values provided from real-time PCR instrumentation. Primer sequences of the genes are provided in table 1.

Table 1. The sequences of primers used in qRT-PCR.

Gene	Gene Bank ID	Primer sequence 5′→ 3′
β-Actin	NM005159.4	F: GCCTCTCTGTCCACCTTCCA R: GGGCCGGACCCATCGTACT
IGF-I	Y10830.1	F: GTTTGTCTGTGGAGAGCGAGG R: GAAGCAGCACTCGTCCACG
GHR1	AY973232.1	F: CAGACTTCTACGCTCAGGTC R: CTGGATTCTGAGTTGCTGTC

F: Forward primer; R: Reverse primer

Qualitative assessment of total gene expresion by defferential display RT-PCR:

In order to assess the seasonal variation of total gene expression qualitatively, the prepared cDNAs were amplified by the PCR using three decamer arbitrary primers (Mahmoud *et al*., 2017). The primers sequences were illustrated in table 2. The DDRT-PCR products were separated on 2% agarose gel (Sigma-Aldrich, Germany) against DNA ladders (Thermo Scientific™ Gene Ruler™, US). The molecular weight of DDRT-PCR products was measured by imageJ software.

Table 2. The sequences of primers used in DDRT-PCR.

Name	Primer sequence 5′ → 3′
OP-A03	AGTCAGCCAC
OP-B11	GTAGACCCGT
OP-C05	GATGACCGCC

RESULTS:

The correlation between RNA/DNA ratio and age, standard length and body weight:

In this study a marked proportional correlation was observed between RNA/DNA ratio in *O. niloticus* muscle tissues and both of age and standard length. This relation showed irregularities with weight (Fig. 1).

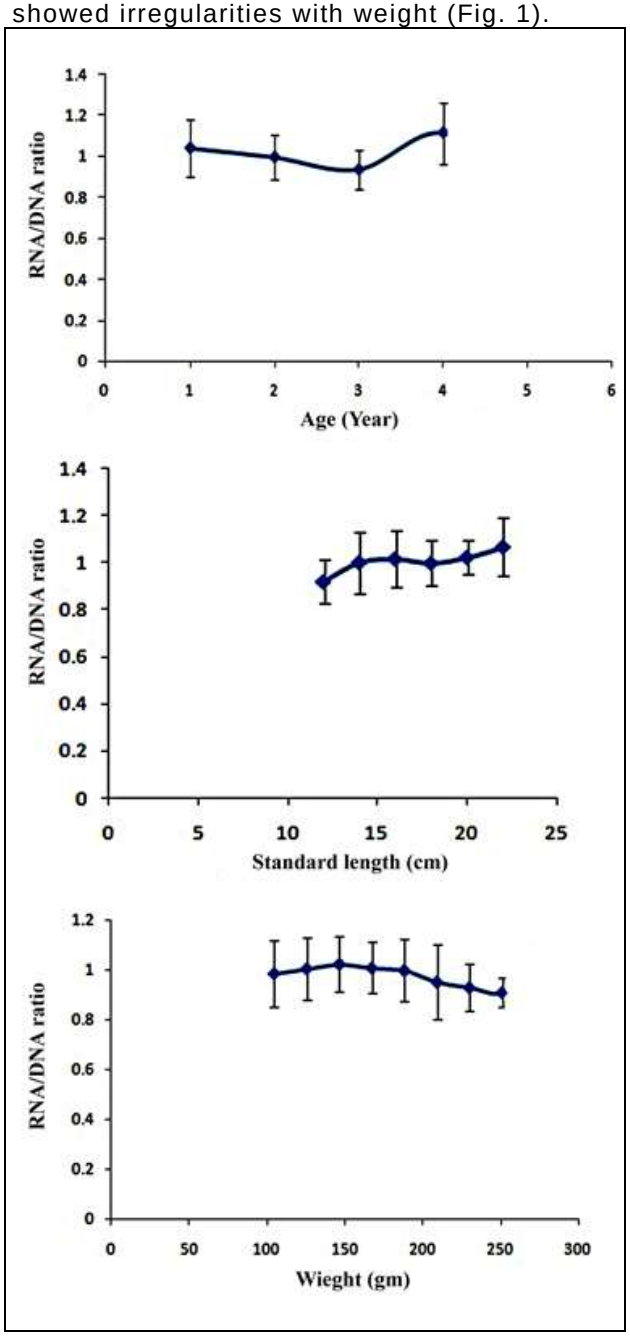


Fig. 1. The correlation between RNA/DNA ratio and age, standard length and weight in *O. niloticus* muscle tissues (n = 7). Data represents means; bars, standard deviation.

The correlation between RNA/DNA ratio and seasonal variation:

Total genomic DNA and total nucleic acids with/without RNase incubation of *Oreochromis niloticus* muscle tissues among the summer and winter samples were performed to evaluate the RNA and DNA contents (Fig. 2). The RNA/DNA ratios were calculated among the two seasons and the higher value of 1.04 ± 0.13 was recorded in winter while the value of 0.95 ± 0.09 was during summer with a decreased value of 8.5% (Fig. 3).

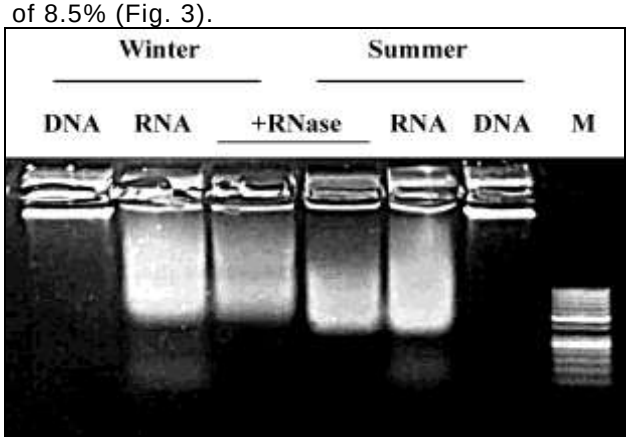


Fig. 2. Representative digital photograph of 1.8% TBE-agarose gel electrophoresis showing intact total genomic DNA and total nucleic acids with/without RNase incubation of *Oreochromis niloticus* muscle tissues among the summer and winter samples. M: marker (Thermo Scientific™ O'Gene Ruler™, US). Arrows point to the area of RNA without digestion with RNase.

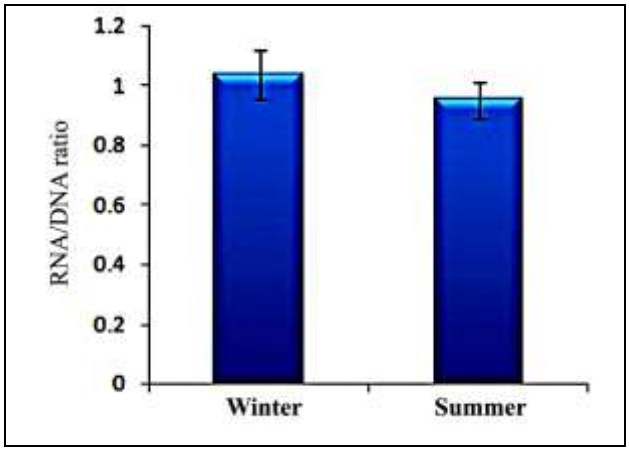


Fig. 3. Seasonal variations of RNA/DNA ratios of *O. niloticus* muscle tissues (n= 7). Data represents means; bars, standard deviation.

The correlation between seasonal variation and cDNA differential display:

As the evaluation of seasonal variation effect on total genes expression at the level of mRNA was the main objective of the current study, differential display method was used to characterize the genetic variation between summer and winter (Fig. 4). The results of differentially displayed cDNAs revealed that the total number of bands (transcripts) was 28 (molecular size, > 205~1010 bp). The eighty-three polymorphic bands (37.04%) were differentially displayed

with the used primers. However, OP-A03, OP-B11, and OP-C05 generated 42.86, 41.66, and 22.22% polymorphism (Table 3). Based on these results, the summer samples showed more activity of gene expression as observed as higher polymorphic banding.

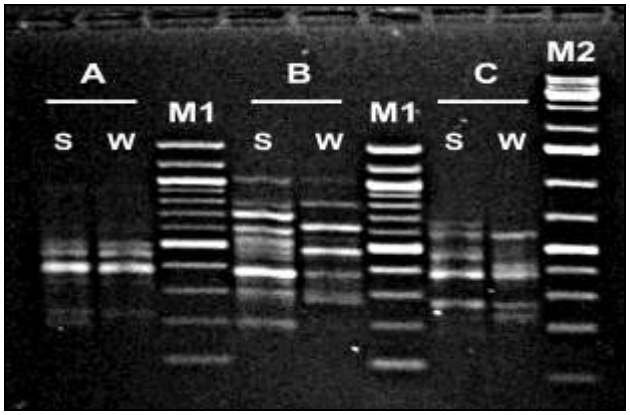


Fig. 4. Representative digital photograph of cDNAs differential display resolved by 2% agarose gel electrophoresis showing the correlation between seasonal variations and cDNAs differential display in *O. niloticus* livers (n = 7). Where S, summer; W, winter; A, OP-A03; B, OP-B11, and C, OP-C05 random decametric primers; M1, 50 bp and M2, 1 kb Plus DNA ladders (Thermo Scientific, O'Gene Ruler™, US).

Table 3. The analysis of generated differential display profile of *O. niloticus* livers among summer and winter samples.

Primer	Total No. of amplified bands	No. of polymorphic bands	Percentage of polymorphism
OP-A03	7	3	**42.86
OP-B11	12	***5	*41.66
OP-C05	9	2	22.22
Total	28	10	37.04

*Maximum % polymorphism, ** Minimum % polymorphism,*** Maximum no. of polymorphic bands.

The correlation between seasonal variation and GHR1 and IGF-I mRNA expression:

The GHR1 and IGF-I levels of gene transcription in livers of *O. niloticus* showed marked differences between the two seasons. The quantitative results of both GHR1 and IGF-I mRNA transcriptions were down regulated with percentage of 40 and 33%, respectively, in livers of winter group than that of the individuals in summer group (Fig. 5).

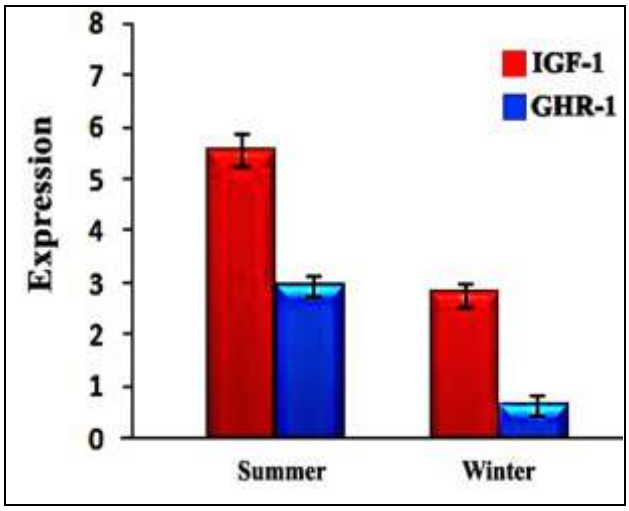
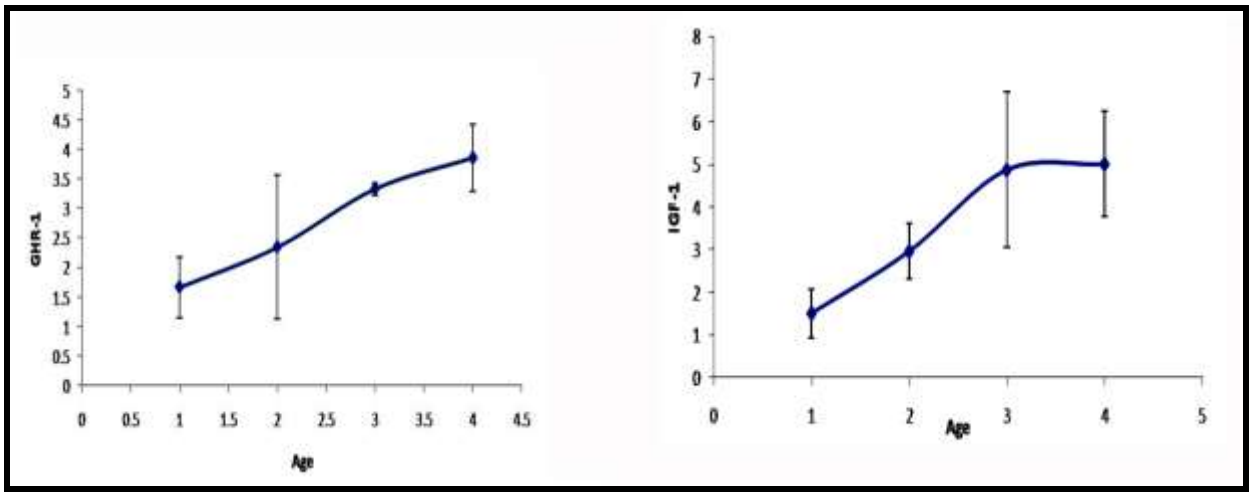


Fig. 5. The correlation between seasonal variations with GHR1 and IGF- I mRNA expression in *O. niloticus* livers (n = 7). Data represents means; bars, standard deviation.

The correlation between GHR1 and IGF-I mRNA expression and age, standard length and weight:

A marked proportional correlation was observed between age, standard length and weight and the GHR1 and IGF-I levels of gene transcription in livers of *O. niloticus* in this study (Fig. 6).



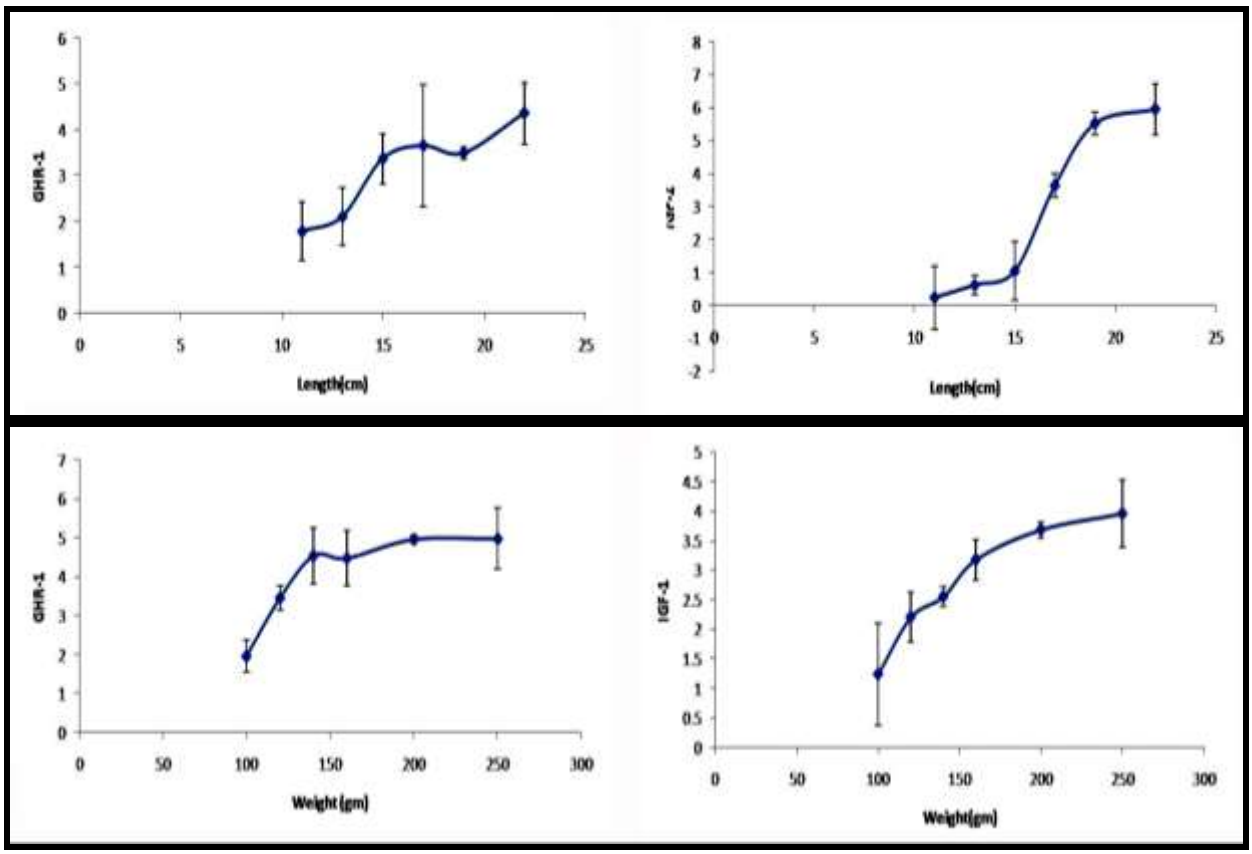


Fig. 6. The correlation of GHR1 and IGF-I mRNA expression and age, standard length and weight in *O. niloticus* livers (n = 7). Data represent means; bars, standard deviation.

DISCUSSION:

Tilapia is one of the most popular world's aquaculture species, representing high landings as wild or cultured fish in Egypt. However, since fish are poikilotherms, metabolic processes are influenced by ambient temperature. The impact of climate affects the aquaculture production. The synthesis of RNA and protein would be expected to accelerate as temperature increases with a corresponding rise in RNA/DNA ratio (Mathers *et al*., 1993; Canino, 1994). The present study revealed that the elevation in muscular RNA / DNA ratio occurred during winter. Spawning ceases during this season which seems to be a compensatory mechanism after saving the activities of reproduction for other vital process like growth (Khallaf *et al*., 1993). The decreased ratio during summer is recorded in this study (Bulow *et al*., 1978). This may be due to the high maintenance of required energy associated with high temperature and reduced food assimilation efficiency in addition to biochemical pathways shift during periods of higher temperatures and reduced levels of dissolved oxygen (Cech and Wohlschlag, 1975). According to NASA the last five years (2013 – 2018) are the warmest along recorded history of earth temperatures. Apparently, that interfered with the fish biology and growth. Such emphasis was indicated by Khallaf *et al.* (2016).

In this study, the RNA / DNA ratio has been increased as the fish get older, from age I to age IV. This is similar to the finding of Buckley and Bulow (1987) and Mourya *et al.* (2007) who reported that the ratio is higher in older fish compared to younger ones. RNA/DNA ratio has also been shown to vary with age (Clemmesen, 1987).

On the other hand, Haines (1973) found that age of fish was found to have an important effect on RNA–DNA ratio, with the ratio being higher in younger fish where the RNA / DNA ratio were higher in I -year-old fish than in II -year-old fish.

The increased ratio was also correlated with length in this study (Clemmesen, 1994; Rooker and Holt, 1996; Iglesias *et al*., 2002).

In this study, the RNA/DNA ratio was curvilinearly correlated with temperature. However, the results showed that the linear effect of temperature did not explain the variability in the RNA/DNA ratio. The RNA/DNA ratio was found to nonlinearly increase with elevated temperature (16 – 36°C) (Wang *et al*., 2017). Furthermore, the positive effect of temperature on growth rates and the negative effect on RNA/DNA ratio of sardine larvae (Ramirez *et al*., 2004) support the effect of seasonal variation in the finding of this study especially in the results of DDRT-PCR which support the enhanced total gene expression in summer than winter. The higher temperature in the last five years (as stated by NASA), certainly has an

adverse effect in interfering with the metabolism and growth of the fish, since it might exceed the optimum temperature range required for proper growth.

In teleost fishes, the liver is a major target organ for the endocrine action of pituitary growth hormone (GH) (Wood *et al*., 2005; Reinecke, 2010). GH stimulates liver production of the insulin-like growth factors (IGF1 and IGF2), which mediate the anabolic actions of GH. Several studies have reported a close relationship between seasonal temperature and variations of GH or IGF-I levels (Mingarro *et al*., 2002; Taylor *et al*., 2003).

The present study showed that the levels of IGF-I expression positively correlated to the GHR expression (Kajimura *et al*. 2004; Tymchuk *et al*., 2009). Furthermore, rise in temperature has its effect on GHR and IGF-I. They have the highest value during summer in agreement with the records of Marchant and Peter (1986). In addition, Gabillard *et al*. (2006) reported on rainbow trout that rising in temperature increased the GHR gene expression only in the liver, which probably participates in the increase of circulating IGF-I and could play a key role in the growth-promoting effect of temperature. In addition, Mingarro *et al*. (2002) built close positive correlation between IGF-I and thermal-unit growth coefficient (TGC) in gilthead sea bream.

The present study shows direct relationship between IGF-I and age, standard length and weight in parallel to the hypothesis of "dual effector theory of action" by Green *et al*. (1985) since GH increases growth directly by promoting the differentiation of precursor cells, which then become responsive to IGF-I. Moreover, the IGF-I in plasma and liver was strongly correlated with size and condition factor for both salmonid species (Tymchuk *et al*., 2009). A stronger relationship between IGF-I and growth rate compared with IGF-I and size was also found (Niu *et al*., 1993; Moriyama, 1995; Beckman *et al*., 2004) and IGF-I treatment increases growth in Chinook salmon (McCormick *et al.,* 1992; Beckman *et al.,* 1998), tilapia (Chen *et al*., 2000). The same results were found in different species (Dyer *et al.,* 2004).

In this study, evidences suggest that measuring IGF-I and GHR1 gene expression may provide a useful tool for monitoring fish growth rate independently to RNA/DNA ratio.

Conflict of interest:

The authors declare that there is no conflict of interest with respect to the research, authorship, and/or publication of this article.

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رنا/دنا والتعبير الهرمونى جى اتش آر و آى جى اف: دراسة على تأثير التغير الفصلي على خصائص النمو لذكور اوريوكروميس *نيلوتيكس*

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تعتبر سمكة البلطي النيلي (*أوريوكروميس نيلوتكس*) أحد أكثر الأحياء المائية أهمية اقتصادية والأكثر استخداما في المزارع السمكية. حيث يعتبر النمو من أهم الشواغل لتربية اسماك البلطي وكذلك في البرية. بدأت هذه الدراسة باستخدام عوامل بيو جزيئية لوصف النمو، ومن المعروف أن نسبة الأحماض النووية الريبوزية/ الحمض النووي الديوكسي ريبوزي والجينات المرتبطة بالنمو كمستقبل هرمون النمو-1 وعامل النمو شبيه الانسولين -1 تشترك في تغيرات زيادة الوزن. ولتقييم العلاقة بين النمو والظروف الكيمائية الحيوية والهرمونية للأسماك خلال فصول الصيف والشتاء. تم جمع الأسماك من الذكور (100-250 جم) من قناة بحر شبين حيث اظهرت النتائج أن كلا من مستقبل هرمون النمو-1 وعامل النمو شبيه الانسولين -1 يتناسب طرديا مع العمر والطول والوزن وكانت أعلى قيمة لهم في فصل الصيف مقارنة بالشتاء. أما بالنسبة لقياس نسبة الاحماض النووية الريبوزية /الحمض النووي الديوكسي ريبوزي كانت النتائج طردية مع العمر والطول فقط وكانت أعلى قيمة في فصل الشتاء مقارنة بالصيف. تدعم هذه الدراسة استخدام مستقبل هرمون النمو-1 وعامل النمو شبيه الانسولين -1 كمؤشر لنمو أسماك البلطي في بيئتها الطبيعية وكذلك نسبة الأحماض النووية الريبوزية /الحمض النووي الديوكسي ريبوزي.