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**Fish Physiology and Biochemistry**

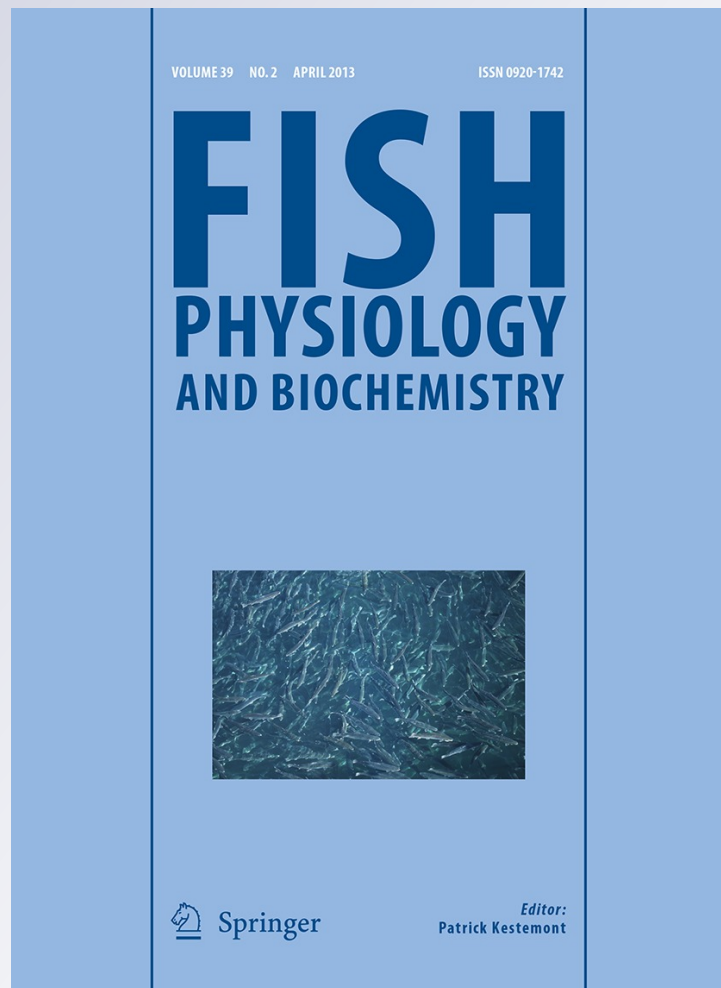
ISSN 0920-1742

Volume 39

Number 2

Fish Physiol Biochem (2013) 39:233-241

DOI 10.1007/s10695-012-9694-1



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# The sexually dimorphic adipose fin is an androgen target tissue in the brown trout (*Salmo trutta fario*)

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Received: 28 March 2012 / Accepted: 10 July 2012 / Published online: 25 July 2012  
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**Abstract** An investigation has been described on the relationship of body length, age and sex with adipose fin length and the number of androgen receptor (AR)-containing cells in the adipose fin as a secondary sexual characteristic for brown trout (*Salmo trutta fario*). Firstly, body and adipose fin lengths of 2- to 5-year-old brown trout were measured. Thereafter, these fish were killed by decapitation, then their sexes were determined, and adipose fins were excised. The cellular bases of AR binding activities in the adipose fins were analyzed with an antibody against human/rat AR peptide. Immunocytochemistry and western blotting techniques were performed with this antibody. Analysis of morphological measurements indicated that body length and age had a linear relationship with

adipose fin length. The coefficients of determination for the body length and age were 0.92 and 0.85 in the male fish and 0.76 and 0.73 in the female fish against the adipose fin length, respectively. At 2 years of age, cells in the adipose fin did not exhibit AR immunoreactivity. However, AR-immunopositive cells were abundant in the adipose fin of 3- to 5-year-old fish. Moreover, the number of AR-immunopositive cells was significantly ( $P < 0.05$ ) high in males and increased with age. These observations indicate that the adipose fin in the brown trout is a probable target for androgen action and that tissue function or development may to some extent be androgen dependent. In addition, it is likely that such an effect will be mediated by specific androgen receptors.

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**Keywords** Adipose fin · Androgen receptor ·  
Secondary sexual characteristic · Brown trout

## Introduction

In mature fishes, internal differences between the sexes are generally obvious, but it is frequently difficult to distinguish the sexes externally. However, some fishes show sexual dimorphism (differences in the body shape or size) or dichromatism (color differences) when spawning (Moyle and Cech 2004). Salmonids are generally regarded as monomorphic

until they reach maturity, after which they develop sexually dimorphic characteristics. The best-known sexually dimorphic characteristics of many salmon and trout are dorsal hump size (Quinn et al. 2001), jaw length (Blair et al. 1993), skin thickness, tooth size (Johnson et al. 2006) and color (Craig and Foote 2001).

Morphological measurements have been taken in *Salmo trutta labrax* (Bardakçı et al. 1994) and *Salmo trutta macrostigma* (Çetinkaya 1999) in Turkey. Age, length and weight relationships, and condition factors for *Salmo trutta macrostigma* populations raised in natural settings have also been analyzed (Alp and Kara 2004; Kara et al. 2007), as have relationships between total length, standard length, fin length and weight (Arslan et al. 2004) and age, sex, growth and death rate (Arslan et al. 2007).

Androgens (testosterone and 11-ketotestosterone) stimulate spermatogenesis in the testes and play an important role in male sexual differentiation and the development of secondary (non-gonadal) sexual characteristics (Miura et al. 1991; Angus et al. 2001). They exert many of these effects by binding to and activating a specific androgen receptor (AR) in target cells. These ARs act as transcriptional factors by binding to hormone-regulatory elements of specific genes to trigger a cascade of transcriptional events. Although females produce primarily estrogenic steroid hormones, testosterone often can be detected in their plasma as an intermediate steroid. In males, the production of nuptial colors, breeding tubercles and other secondary sexual characteristics is stimulated by androgens (Borg 1994).

The adipose fin is a fleshy, dorsal appendage that is found in trout (Salmonidae), smelts (Osmeridae), lanternfish (Myctophidae) and various types of catfish and characins. It has been widely used by systematists in species descriptions (Helfman 1997) or to distinguish between otherwise similar species (Dahlberg and Phinney 1967; Martinenz 1984; Graca et al. 2008; Lundberg and Dabdul 2008). The location between the dorsal and caudal fins toward the caudal peduncle, the small size and the lack of stiffening rays make the function of this fin a mystery.

In the literature, previous studies on the adipose fin have addressed its influence on mate choice by females (Petersson et al. 1999), its hydrodynamic function in juvenile steelhead trout and catfish (Reimchen and Temple 2004; Temple and Reimchen

2008) and its sexual dimorphic characteristics, with males having a larger adipose fin in salmonids (Jones 1959; Beacham and Murray 1983; Naesje et al. 1988). Moreover, it has been suggested that the exaggerated size of male adipose fins in salmonids may serve to compensate for reduced swimming efficiency due to expression of the other secondary sexual characteristics such as dorsal humps and large jaws (Fleming and Gross 1994). The aim of this study was to assess whether the size of the adipose fin shows sex and/or age differences and to examine whether these differences are associated with differential expression of the androgen receptor.

## Materials and methods

### Animals

Five female and five male fish of each age group (2, 3, 4 and 5 years old), totally 40 brown trout, that had been reared at the research and extension center of the Department of Aquaculture at Atatürk University were used for the experiments. Fishes were acclimated for 1 month in 700-L circular fiberglass tanks with a constant flow of 1.5 L min<sup>-1</sup> of recirculated, aerated and dechlorinated tap water and separated by sex at 9 °C with light–dark cycles of 0618 hours. Dissolved oxygen, pH and temperature of the water used in the study were measured using a multiprobe (Model HQ40d, HACH Instruments). Total hardness and the amount of HCO<sub>3</sub><sup>-</sup> and Ca<sup>2+</sup> per liter were determined according to standard methods (APHA 1998). The fishes were fed with a daily ration at 1 % of their live body weight. Feeding times were centered in the middle of the light period. Fish treatment protocols were conducted according to Arena (2002).

All the fishes were anesthetized using tricaine methanesulfonate (100 mg L<sup>-1</sup> MS-222 buffered with NaHCO<sub>3</sub>, pH 7.0). Total length and adipose fin length (cm) were measured using a digital caliper; body weights were measured using a microbalance to a precision of 0.01 g. Afterward, fish were killed by decapitation and surgically manipulated to see the gonads and determine their sex (Murray 2002). Gonads from fish were also removed and weighed to calculate gonadosomatic index (GSI) by the formula  $GSI = 100 G_W/W$ , where  $G_W$  is the gonad weight (g) and  $W$  is the total weight (g) (Wootton 1998).

The adipose fins were lastly excised and put in a 10 % formalin solution. Procedures for dehydrating, embedding and sectioning were carried out as described by Hisar et al. (2002).

### Immunocytochemistry (ICC)

An avidin–biotin–peroxidase technique with diaminobenzidine (DAB) as chromogen and biotinylated goat anti-rabbit immunoglobulin as secondary antibody was used. Immunostaining was performed using IgG antibody to androgen receptor (AR) (AR antibody; AR411, Dako; dilution rate: 1/50). Briefly, five-micron tissue sections attached onto the adhesive glass were deparaffinized and rehydrated. Sections were microwaved in target retrieval solution (pH: 9) for 20 min for antigen unmasking. After cooling and washing, the endogenous peroxidase activity was blocked by incubating 3 % H<sub>2</sub>O<sub>2</sub> for 15 min. The sections were then washed in a phosphate-buffered saline (PBS) solution (pH: 7.4) for 10 min. The sections were incubated with the primary antibodies for 60 min in room temperature. After rinsing the sections with PBS, the steps of biotinylated goat anti-polyvalent (Lab vision, Westinghouse, CA) secondary antibody, streptavidin peroxidase solution and DAB chromogen were performed. All sections were counterstained using Mayer's hematoxylin. Positively stained cell nuclei were counted.

All sections obtained from each block without sampling procedures were used for stereological analyses. The cells in the stained sections were counted using optic disector counting method of Howard and Reed (1998) at stereology workstation for stereological analyses. It is composed of a CCD digital camera (Optronics MicroFire), personal computer, computer-controlled motorized specimen stage (Bio-Precision MAC 5000 controller system) and a light microscope (Leica DM4000 B) with StereoInvestigator software (Microbrightfield, California, USA). Cells were counted using a 20× Leica Plan Apo objective. Each cell was counted according to the unbiased counting rules of optical disector.

### Protein extraction and western blotting

The frozen male and female brown trout fins were powdered in a mortar filled with liquid nitrogen, and the powder was suspended in lysis buffer containing

1 % Triton X-100, 50 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 10 mM  $\beta$ -glycerophosphate, 10 mM  $\beta$ -mercaptoethanol, 1 mM NaF, 1 mM PMSF and Protease Inhibitor Cocktail, EDTA-Free (Roche). The lysates were centrifuged at 13,000xg for 1 h, and then the precipitate was removed.

Aliquots containing 60  $\mu$ g of proteins were first denatured at 95 °C for 10 min and then used to perform SDS–PAGE. Proteins were immediately transferred to a PVDF membrane (Macherey–Nagel, GERMANY) by a Trans-Blot, SD Semi-Dry Transfer Cell system (BIORAD), at room temperature. After transfer, the membrane containing our samples was incubated in 3 % non-fat milk in TTBS (Tris Base 20 mM, NaCl 0.5 mM, Tween 20 0.005 %, pH 7.5) for 3 h, in order to block non-specific immunoabsorbent sites. The AR protein was detected by incubating the membrane overnight, at 4 °C, with a mouse monoclonal antibody anti-human AR (AR antibody; AR411, Dako; dilution rate: 1/250) and a mouse monoclonal antibody anti-human  $\alpha$ -tubulin (sc-5286, Santa Cruz; dilution rate: 1/1000) in TTBS with 1 % non-fat dry milk. After washing with TTBS, the membrane was stained for 2 h in 0.1 % Coomassie Brilliant Blue R-250 containing 50 % methanol, 10 % acetic acid and 40 % distilled water and then destained with many changes in the same solvent without dye.

### Statistical analysis

Student's *t*-test was used to compare means between sexes of each morphological feature. GSI was tested by one-way ANOVA and Duncan's multiple comparisons test. Multiple linear regression analysis of adipose fin length against body length and age was also carried out for brown trout. To examine the effects of age and sex and their interaction on AR-containing cells in the adipose fin, the numbers of AR-containing cells were subjected to Student's *t*-test. The results are expressed as mean  $\pm$  SD.

## Results

Some of the physical and chemical characteristics of the water used in the study were as follows: temperature,  $9 \pm 2$  °C; pH, 7.8; dissolved oxygen content, 8.9 mg L<sup>-1</sup>; HCO<sub>3</sub><sup>-</sup>, 2.11 mg L<sup>-1</sup>; Ca<sup>2+</sup>, 2.45

**Table 1** Some morphological characteristics of brown trout (*Salmo trutta fario*) in different ages

	Age	Female	Male	Significant of difference
Body length (cm)	2	23.8 ± 0.6	24.7 ± 0.7	0.22
	3	35.0 ± 1.7	34.7 ± 1.2	0.42
	4	41.8 ± 0.6	39.6 ± 2.9	0.32
	5	48.3 ± 3.2	49.4 ± 2.5	0.22
Body weight (g)	2	228 ± 8	227 ± 15	0.81
	3	637 ± 126	698 ± 119	0.09
	4	1175 ± 153	935 ± 252	0.39
	5	1885 ± 348	2021 ± 304	0.08
Adipose fin length (cm)	2	1.79 ± 0.08 <sup>a</sup>	2.28 ± 1.19 <sup>b</sup>	0.01
	3	2.21 ± 0.42 <sup>a</sup>	3.31 ± 0.29 <sup>b</sup>	0.05
	4	2.83 ± 0.11 <sup>a</sup>	3.52 ± 0.28 <sup>b</sup>	0.001
	5	2.86 ± 0.22 <sup>a</sup>	5.13 ± 0.52 <sup>b</sup>	0.000

Each value is the mean ± SD of 5 animals per groups. Means having different superscripts in the row differ significantly ( $P < 0.05$ )

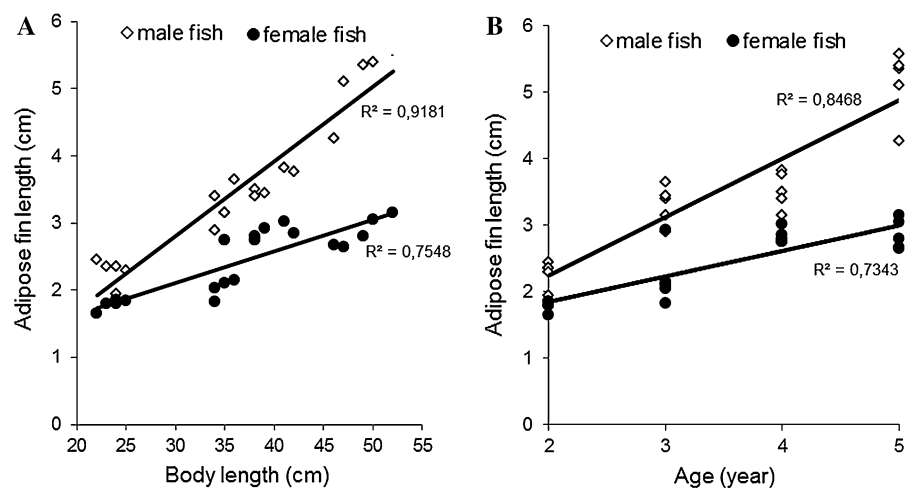
milliequivalent per liter; and total hardness, 102 mg as  $\text{CaCO}_3$ .

Student's *t*-test revealed that there was a significant difference between sexes for only adipose fin length among the studied morphological characteristics, and adipose fins of male fish were larger on average than those of female fish at the same age (Table 1). Multiple linear regression analyses also indicated that body length and age had a linear relationship with adipose fin length ( $P < 0.05$ ). The coefficients of determination for the body length and age were 0.92 and 0.85 in the male fish and 0.76 and 0.73 in the female fish against the adipose fin length, respectively (Fig. 1). GSI values of the brown trout for both sexes in November 2011 are shown in Table 2. The mean GSI value varied from 9.8 to 10.6 in females and

2.3–2.5 in males. The GSI values were significantly lower in the 2-year-old female brown trout than in 3- or 4-year-olds.

Based on size, location and appearance, all AR-labeled cells are represented in Fig. 2 and Fig. 3. Anti-AR typically labeled cell nuclei. Some cell populations had reactions over both nucleus and cytoplasm, and fiber tracts were never labeled. Intracellular labeling patterns and size characteristics were specific for a given antibody in different adipose fin regions.

Labeling patterns were not obviously different for males and females. Moreover, no staining was observed in the adipose fin sections of 2-year-old brown trout. However, the intensity of AR-immunoreactive staining was significantly ( $P < 0.05$ , using

**Fig. 1** Relationship of adipose fin length with body length (a) and age (b) among mature male or female brown trout

**Table 2** GSI (%) values of female or male brown trout in different ages in November 2011

Age	GSI (%)	
	Female	Male
II	9.8 ± 0.3 <sup>a</sup>	2.3 ± 0.1
III	10.5 ± 0.2 <sup>b</sup>	2.5 ± 0.2
IV	10.6 ± 0.3 <sup>b</sup>	2.3 ± 0.1
V	10.1 ± 0.2 <sup>ab</sup>	2.3 ± 0.1

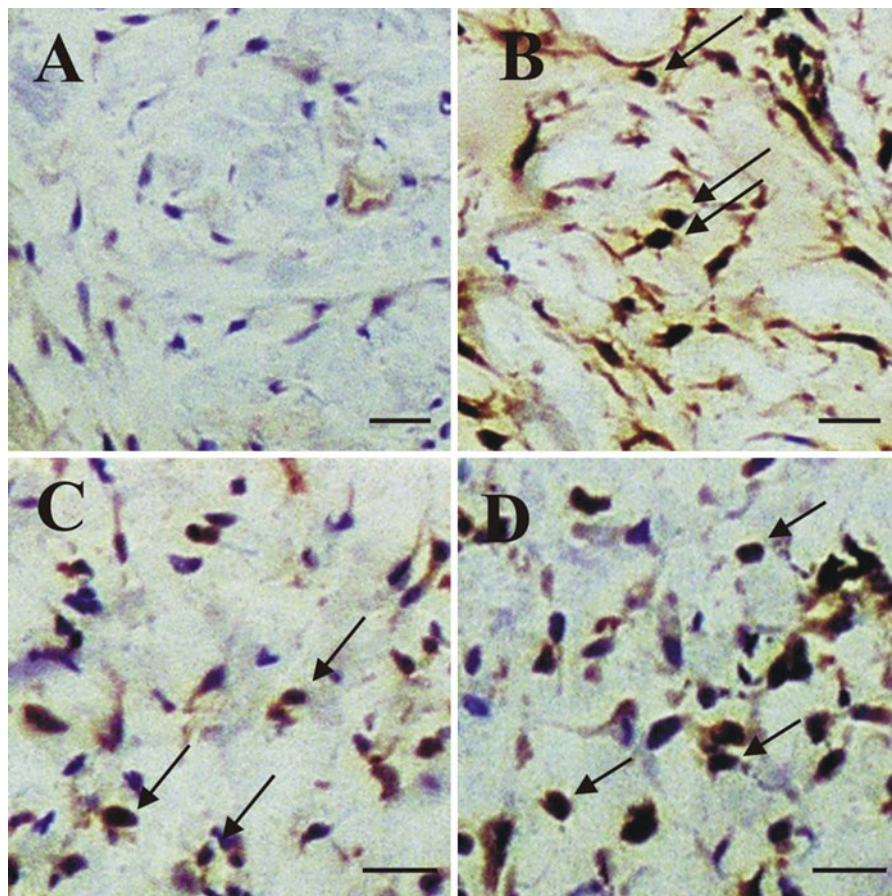
Each value is the mean ± SD of 5 animals per groups. Means having different superscripts in the column differ significantly ( $P < 0.05$ )

Student's *t*-test) influenced by sex, as males had more AR+ cells than females (Fig. 4). Similar results were obtained using western blot assay. For instance, it could be seen that the protein expression of AR was

compared between 4-year-old male and female brown trout fins in Fig. 5.

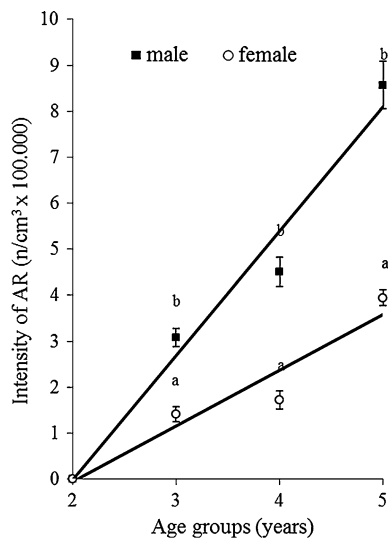
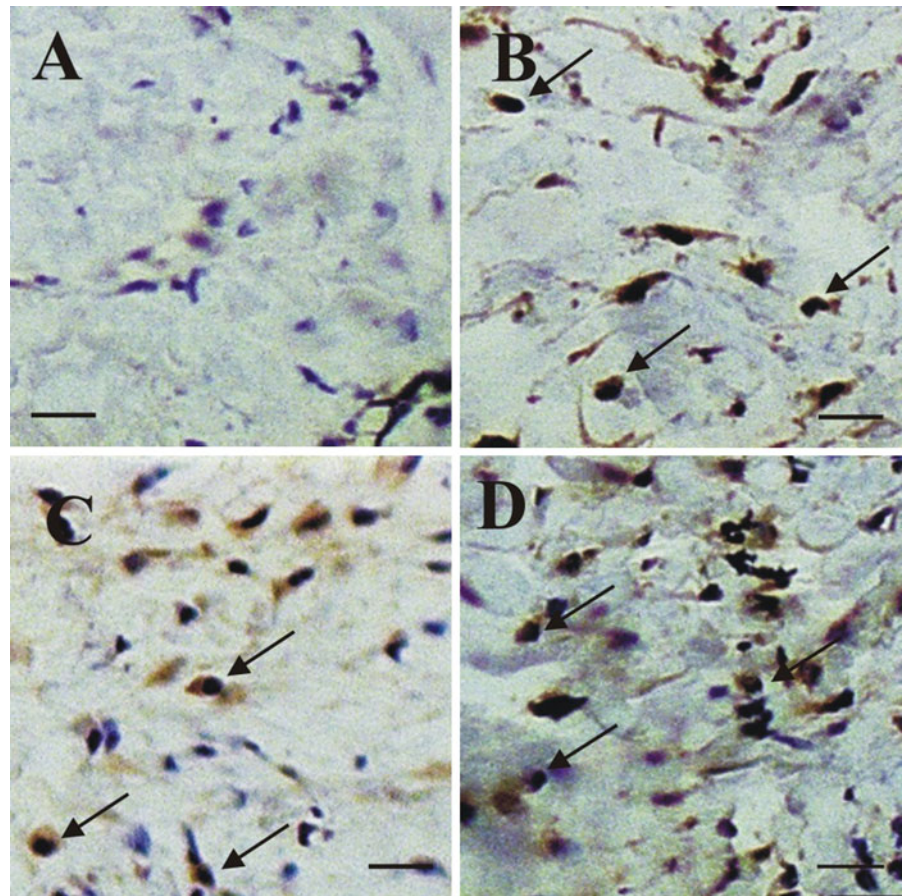
## Discussion

Brown trout is the only species among salmonid fishes in Turkish waters (Geldiay and Balık 1996). Age distributions of brown trout were generally 0–7 years in Turkish inland waters (Çetinkaya 1999). It was also reported that the age at first maturity of brown trout inhabiting Turkish waters was between 2 and 4 (Geldiay and Balık 1996). It was previously established that gonadal recrudescence and circulating androgens levels were maximum in November (Matty 1985). Similarly, in Turkey, the spawning time for brown trout was reported as November–December in some streams in the eastern Black Sea region (Tabak



**Fig. 2** Androgen receptor (AR)-labeled cells in the internal cell layer of the adipose fin of two (a)-, three (b)-, four (c)- and five (d)-year-old female brown trout. Scale bar, 15  $\mu$ m

**Fig. 3** Androgen receptor (AR)-labeled cells in the internal cell layer of the adipose fin of two (a)-, three (b)-, four (c)- and five (d)-year-old male brown trout. Scale bar, 15  $\mu$ m



**Fig. 4** Intensity of AR in adipose fin of male ( $n = 20$ ) and female ( $n = 20$ ) brown trout to the years of age [(differences within same age were indicated as lower-case letters ( $P < 0.05$ )]

et al. 2001) and November–January in the eastern Mediterranean region in Turkey (Alp et al. 2003). Age distributions of brown trout in the present study were 2–5 years, and all of them were matured. In addition, spawning time and GSI values demonstrated similarity with the other brown trout populations from Turkish fresh waters.

Development of practical and rapid methods for sexing live fish is important for determining production goals and estimating reproductive potential (Crim and Glebe 1990). In earlier researches on *Salmo trutta*, several morphometric parameters (head length, body weight, fork length, size of anal fin and abdomen) significantly differed between males and females (Grunchy and Vladykov 1968; ReyesGavilan et al. 1997; Baltacı et al. 2007; Demir et al. 2007). However, knowledge of these differences is not enough to manage trout broodstocks, since identifying sex by using many morphological measurements is time-consuming and



**Fig. 5** Western blotting of androgen receptor (AR) (96 kDa) (<http://www.uniprot.org>), comparing the protein expression of androgen receptor (AR) between 4-year-old male and female

brown trout fins.  $\alpha$ -tubulin (21 kDa) (<http://www.uniprot.org>) was used as the loading control

expensive. Therefore, specific morphometric values need to be developed to rapidly and accurately determine sex.

In this study, it was determined that adipose fin length exhibited a linear relationship with body length and age. However, male adipose fins grew much more than those of female fishes at the same age. Similar to our results, Topal et al. (2010) showed that the most significant morphologic feature of brown trout in determining sex was adipose fin length by using discriminant and chi-square automatic interaction detector analysis methods. It was also determined that the ratio of adipose fin length to fork length was the best predictor of sex in chinook salmon (Merz and Merz 2004). Westley et al. (2008) reported population differences in the size of sockeye salmon (*Oncorhynchus nerka*) adipose fin; however, the phenotypic and genetic connection between the adipose fin and the other sexually dimorphic traits such as body depth, body length and jaw length was unclear. Taking all of this information into account, it is reasonable to hypothesize that probability of a non-reproducing trout being male or female could be predicted from the adipose fin length. However, further study would be necessary to confirm this hypothesis.

Salmon and trout species show spectacular reproductive behavior, which includes intra- and intersexual interactions. In some studies, it has been suggested that salmonid females choose their mates on the size of the male's adipose fin (Järvi 1990; Petersson and Järvi 1997). Petersson et al. (1999) also reported that while no significant differences in the plasma levels of testosterone (T) and 11-Ketotestosterone (11-KT) between the chosen males and those not chosen was observed, the dominant (spawning) males had significantly higher plasma levels of T and 11-KT both before and after the experiment.

In many instances, 11-KT has been identified experimentally as the most potent androgen in teleost

fish. However, T may also mediate androgen action; levels of T can exceed those of 11-KT, and T is known to be capable of binding to a putative fish androgen receptor (AR) with high affinity. This interpretation is supported by the results of a study in which it was demonstrated that 11-KT was not bound by plasma, skin cytosol and skin nuclear extract, whereas T was bound with high affinity to these fractions in mature male and immature male and female brown trout during a single spawning cycle (Pottinger 1988).

Androgenic hormones are essential for the morphological specification of fish, but little is known about the developmental mechanisms of secondary sexual characteristics. Androgen hormone actions require direct binding to ARs. As in previous reports of studies on goldfish (Gelinias and Callard 1993, 1997), it can be concluded from our experiment that brown trout AR is sufficiently homologous to its mammalian counterparts to cross-react specifically with antibodies raised to human or rat antigens.

Adipose fin has no rays, but the connective tissue in dermis is thickly developed. The dermis is composed of two layers, the upper *stratum spongiosum*, which is a loose network of collagen and reticulin fibers, contiguous with the epidermal basement membrane and containing the pigment cells, mast cells and cells of scale beds and also the scale, and *stratum compactum*, the collagenous dense matrix (Pottinger 1987).

The data obtained during the course of the present study indicate that brown trout fibroblast cells in both male and female dermis contain two sites for androgens. These are located in the cytosolic fraction and nuclear fraction of the cells. Binding of androgens in the nuclear fraction and cytosolic fraction shows characteristics consistent with the presence of steroid receptor, and there are common regulatory mechanisms for AR that is activated by binding of androgens in the cytoplasm and then translocating into the nucleus. In previous studies, ARs have been identified

in several species of fish like the three-spined stickleback (Olsson et al. 2005), fathead minnow (Wilson et al. 2004), kelp bass and Atlantic croaker (Sperry and Thomas 1999). Moreover, in skin and the electric organ, the presence of androgen receptors has been linked to sexually dimorphic patterns of development, while androgen binding in the brain is suggestive of a behavioral role (Bass et al. 1986; Pottinger 1987; Pasmanik and Callard 1988).

To address the role of AR, we determined not only the intracellular labeling patterns of AR but also the number of AR-immunolabeled cells in both male and female adipose fin tissues of brown trout. No staining was observed in the adipose fin sections of 2-year-old brown trout. However, the number of AR-immunolabeled cells in male fish was significantly higher than in females. This finding was also confirmed by western blot assay, which shows that the protein expression of androgen receptor is higher in males than females (Fig. 5). These data suggest that the amount of AR in adipose fin tissue is involved in a response of the tissue to sex-specific effects, rather than an overall change in reproductive status of the brown trout.

In conclusion, growth and AR immunoreactivity characteristics in trout adipose fins have offered an entry point for research on sexual differentiation and androgen-signaling pathways during the expression of secondary sexual characteristics.

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