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Is selected aquaporin expression in the chicken adrenal gland affected by disturbed sex steroid action?

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Original article

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In our earlier study, we demonstrated changes in the aquaporin 4 (AQP4) abundance in the reproductive system of hens following a fasting or tamoxifen (TMX; estrogen receptor modulator) treatment. In the present study, we examine the hypothesis that the expression of selected aquaporins (AQPs) changes in the avian adrenal gland under reduced circulating sex steroid hormone concentrations or a blockage of estrogen receptors. Accordingly, our aim was to examine the AQP1, AQP2, AQP4, AQP5 and AQP9 mRNA and/or protein expression and immunolocalisation in the adrenal glands of chickens after several days of fasting or TMX treatment. The hens were fasted or treated with TMX every day, until a pause in egg laying occurred in all the hens. Control hens were fed *ad libitum* or were treated with a vehicle, respectively. The hens were euthanised on Day 6 or 8 of the experiment, respectively, and the adrenal glands were collected. Subsequently, the gene and protein expressions of AQPs in the tissues were examined by quantitative real-time PCR and Western blotting, respectively. The fasting, which is accompanied by diminished plasma concentrations of sex steroids, caused a decrease in the relative abundance of AQP1, AQP2, and AQP9 mRNA transcripts, while the TMX treatment lowered the AQP2 and AQP4 mRNA transcript levels. However, the abundances of AQP proteins were not changed by the fasting and TMX treatment. Immunoreactivity for AQP1, AQP2, and AQP9 protein was localised to the adrenal gland chromaffin cells and adrenocortical cells; whereas for AQP4 it was localised exclusively to chromaffin cells. In summary, the selected AQPs seem to be important for the regulation of the water/glycerol transport required for the functioning of the hen's adrenal gland cells. An involvement of ovarian steroids, especially estradiol, in the regulation of the AQP expression is also suggested.

Key words: aquaporins, fasting, TMX, adrenals, hen.

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The adrenal glands play a key role in maintaining the physiological homeostasis of an organism by the secretion of important hormones and help an individual to survive under different environmental stressors. Stress disturbs the growth, development and reproduction processes, therefore affecting the well-being and production of domestic animals including egg-laying hens.

The avian adrenal glands consist of adrenocortical cells arranged in cords and adrenomedullary (chromaffin) cells, arranged in nests (islets) and surroun-

ded by a thin basal lamina. The chromaffin cell islets are located between the looped cords of the adrenocortical cells (Carsia 2022). The adrenocortical cells secrete aldosterone, angiotensin II and corticosterone (CORT) in response to the adrenocorticotropic hormone (ACTH). The chromaffin cells secrete epinephrine (E) and, in lower amounts, norepinephrine (NE) (Ohmori 1998; Unsicker 1973). CORT is the main corticosteroid released by the adrenal glands of birds (Carsia 2022; Deviche *et al*. 2014). In addition to CORT, the adrenal glands secret cortisol and are an important source of testosterone and estradiol (Tanabe *et al*. 1979).

Many factors have been shown to affect catecholamine and steroid secretion in the case of an acute stress response and a long stress response, respectively. Stimulation of the sympathetic-adrenomedullar axis leads to the secretion of E and NE (Ghosh *et al*. 2001; Konarska *et al*. 1989). A subsequent activation of the hypothalamic-pituitary-adrenocortical axis (HPA) results in corticosteroid secretion under pituitary ACTH stimulation released in response to the action of the hypothalamic corticotropin-releasing hormone (CRH) and arginine vasotocin (AVT) (Axelrod & Reisine 1984; Herman *et al*. 2016). AVT has a role in maintaining the water balance; thus, it may activate the HPA axis in cases of osmotic stress (Sharma *et al*. 2009). Corticosteroids are critical for maintaining the electrolyte and water balance (Carsia 2022). A high circulating CORT concentration may also suppress parental behavior and reduce an animal's reproductive success. On the other hand, ovarian/reproductive hormones, estradiol and testosterone, may affect the adrenal gland functions by involving the estrogen and androgen receptors present in this gland (Błachuta & Wrońska-Fortuna 2012; Kiezun *et al*. 2015). The functional properties of the adrenal glands and hormone release change, according to the physiological state of a bird.

All metabolic processes in tissues, including in adrenal gland cells, requires a proper balance of water and the transport of other molecules. The essential players in the maintaining of water availability and the cell volume are proteins known as aquaporins (AQPs). AQPs are a family of 13 membrane channel proteins (~ 25-35 kDa) that are permeable primarily to water and some other molecules, including glycerol and urea, across the cell membrane (Agre *et al*. 1993; Arrighi *et al*. 2016). AQPs are classified into three subgroups: water channels contain AQP1, AQP2, AQP4 and AQP5, among others; aquaglyceroporins include AQP3 and AQP9; and superaquaporins, i.e. AQP11 and AQP12. Many studies indicate that the functions of AQPs are also related to other functions, not only their fluid transport function (Li & Yang 2023; Verkman 2005). For example, they may be implicated in the swelling of tissues under stress, signal transduction and organelle physiology. In addition, AQP1, AQP3 and AQP5 are involved in the regulation of cell proliferation (Galán-Cobo *et al*. 2016). It is well established that AQPs participate in the regulation of processes in many organs including the avian reproductive system (Hrabia *et al*. 2020; Nowak *et al*. 2017; Skowronski *et al*. 2009; Socha *et al*. 2018; Tiwari *et al*. 2014; Wolak *et al*. 2024; Yang *et al*. 2016) and kidneys (Nishimura *et al*. 2007; Nishimura & Yang 2013; Sugiura *et al*. 2008; Yang *et al*. 2004; Yoshimura *et al*. 2011). By contrast, information about the involvement of AQPs in the regulation of the adrenal gland functions in birds is limited. So far, the presence of mRNA transcripts of AQP1 and AQP3 in the quail adrenal glands has been revealed (for a review, see Yang & Nishimura 2021). However, information about the physiological role of AQPs and the regulation of their gene and protein expression in the adrenal glands of birds is not known from the published data. Accordingly, the aim of this study was to investigate the mRNA transcript and/or protein abundance of selected water transporting AQP1, AQP2, AQP4 and AQP5, and of aquaglyceroporin AQP9, in the adrenal glands of chickens following fasting (reduced sex steroid levels in the blood plasma) or a tamoxifen (TMX, an estrogen receptor blocker) treatment. Additionally, the AQP1, AQP2, AQP4 and AQP9 proteins were localised in the adrenal gland cells.

Materials and Methods

Birds and experimental design

The animal experiments were performed according to a research protocol approved by the Local Animal Ethics Committee in Krakow, Poland (Approval No. 218/2015). Laying Hy-Line Brown hens, purchased from a local commercial farm, were caged individually under a photoperiod of 14 h light : 10 h dark, with free access to commercial feed and water.

In the first experiment, carried out as precisely described by Hrabia *et al*. (2020) and Wolak & Hrabia (2021), the hens (at the age of 32 weeks) were divided into two groups: those fed *ad libitum* (control; n=6); and those subjected to a pause in laying by complete food deprivation for 5 days (fasting; n=6).

The chickens were euthanised on Day 6 of the experiment. In the second experiment, performed as described by Wolak & Hrabia (2020) and Socha & Hrabia (2018), the control hens (n=8) were subcutaneously injected in the abdominal area (just below the breastbone) with a vehicle (ethanol), while the experimental hens (TMX; n=8) were treated with TMX (Sigma-Aldrich, Saint Louis, MO, USA) at a dose of 6 mg/0.3 ml ethanol/kg body weight. The chickens were treated daily until a pause in laying occurred in all the TMX-treated hens (the average day of the experiment: 5.8 ± 0.35) and were euthanised on Day 8 of the experiment. The control hens (laying) in both experiments were euthanised about 2 h after oviposition. The adrenal glands were rapidly harvested, frozen and stored at -80°C for a Western blot analysis, or were placed into RNAlater (Sigma-Aldrich) and stored at -20°C for a later quantitative real-time PCR (qRT-PCR). Additionally, the other tissue fragments (including the kidneys) of the control hens (n=3) were fixed in a 10% buffered formalin, dehydrated through graded ethanol solutions, cleared in xylene and embedded in paraffin wax. Microtome sections (6 µm thickness) were mounted onto microscope slides and were used for the immunohistochemical and immunofluorescent analysis.

RNA isolation and qRT-PCR analysis

The total RNA extraction, reverse transcription (RT) and qRT-PCR were performed as described previously (Wolak *et al*. 2024). Briefly, RNA was extracted from the collected tissues using TRI Reagent (Sigma-Aldrich). The total RNAs (1 µg) were reverse-transcribed with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's recommendations. The obtained cDNA was used in duplex qRT-PCR for AQP1, AQP2, AQP4, AQP5 or AQP9 and *18S* rRNA as a reference gene, in a 10 µl volume containing 5 µl of TaqMan Gene Expression Master Mix (Applied Biosystems), with 0.5 µl TaqMan Gene Expression Assays with a specific TaqMan MGB-probe and one pair of primers (*AQP1,* Assay ID: Gg03358590_g1, Genebank Accession No. NM_001039453.1, amplicon size 79 bp; *AQP2*, Assay ID: Gg07161814_m1, Genebank Accession No. AB358969.1, amplicon size 83 bp; *AQP4*, Assay ID: Gg03346640_m1, Genbank Accession No. NM_001004765.1, amplicon size: 87 bp; *AQP5*, Assay ID: Gg03323994_m1, Genebank Accession No. AJ829443.3, amplicon size 96 bp; *AQP9*, Assay ID: Gg03315007_m1, Genebank Accession No. AB359226.1, amplicon size 80 bp; Applied Biosystems), 0.5 µl of Eucaryotic *18S* rRNA Endogenous Control (pair of primers and TaqMan probe-labelled VIC/TAMRA, Cat. # 4310893E, amplicon size: 187 bp; Applied Biosystems), 3 µl of water and 1 µl of cDNA (10x diluted sample after the RT). The amplifications included an initial denaturation step at 50ºC for 2 min and 95ºC for 10 min, as well as 40 cycles at 95ºC for 15 s and at 60ºC for 1 min. Each sample was run in duplicate. Water (nuclease-free) as a negative control was used in all the reactions. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) was used to calculate the relative expression [RQ] levels of the AQP genes after normalisation to *18S* rRNA, and a calibration was done to the expression in the control chickens.

Protein extraction and Western blotting

A Western blot analysis for AQP1, AQP2, AQP4 and AQP9 was performed as described recently by Wolak *et al*. (2024). The protein concentrations in the tissue homogenates were estimated by the Bradford method with a Pierce Detergent Compatible Bradford Assay Reagent (Thermo Fisher Scientific, Rockford, IL, USA). Samples (50 µg of total protein) were mixed with a loading buffer and warmed at 99.9ºC for 7 min. After denaturation, the samples were loaded into 12% SDS-polyacrylamide gel, and the proteins were separated by electrophoresis under reducing conditions. The resolved proteins were transferred from the gel to a PVDF membrane using a semi-dry blotter (Thermo Scientific Pierce G2 Fast Blotter; Thermo Fisher Scientific) in FLASHBlot transfer buffer (Advansta, Menlo Park, CA, USA) for 7 min at a constant voltage of 25V. The membranes were then blocked for 60 min with 5% non-fat milk in Tris-buffered saline (TBS) with 0.1% v:v Tween-20 (TBST; pH 7.6). After washing, the membranes were incubated overnight at 4ºC with primary rabbit polyclonal antibodies for the AQPs (Table 1). The membranes were next washed and treated with a secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (60 min; Table 1). To examine them for loading controls, the membranes were stripped and reprobed with mouse monoclonal anti-β-actin HRP-conjugated IgG (Table 1). The sites of the antibody-antigen reaction were detected using enhanced chemiluminescence WesternBright™ ECL (Advansta) and were visualised using a ChemiDoc-It 410 Imaging system and VisionWorks Life Science software. The bands representing each sample were densitometrically quantified using the ImageJ v.1.8.0 program (developed at the National Institutes of Health; Bethesda, MD, USA). Relative abundances of AQP proteins were normalised to the β-actin in each corresponding data point.

Table 1

Primary and secondary antibodies used for Western blot (WB), immunohistochemistry (IHC) and immunofluorescence (IF)

Antibody	Serum	Host species	Vendor	Cat. no	WB dilution	IHC/IF dilution
Anti-AQP1	5% NGS	Rabbit	Abcam, Cambridge, UK	ab125041	1:500	1:100
Anti-AQP2	5% NGS	Rabbit	Bioss Inc., Woburn, MA, USA	bs-4611R	1:500	1:100
Anti-AQP4	5% NGS	Rabbit	Operon Biotechnologies, Tokyo, Japan	Custom-made	1:3000	1:250
Anti-AQP9	5% NGS	Rabbit	Invitrogen, Rockford, IL, USA	PA5-114872	1:1000	1:100
HRP anti-β-actin	–	Mouse	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA	Sc47778	1:500	–
HRP-anti rabbit IgG	–	Goat	Advansta, Menlo Park, CA, USA	170-6516	1:3000	–
Biotinilated anti rabbit IgG	–	Goat	Vector Laboratories, Burlingame, USA	BA-1000	–	1:300
DyLight 594 anti-rabbit IgG	–	Goat	Vector Laboratories, Burlingame, USA	Di-1594-1.5	–	1:150

Abbreviations: AQP – aquaporin; HRP – horseradish peroxidase; NGS – normal goat serum

Immunohistochemistry and immunofluorescence

The immunohistochemical localisation of AQP1, AQP2, AQP4 and AQP9 in the adrenal glands of the control hens was performed routinely, as previously described (Socha *et al*. 2018; Hrabia *et al*. 2020). Briefly, after the blocking of nonspecific binding sites with 5% normal goat serum in TBST, the sections were incubated overnight with primary antibodies against AQPs (the same as for the Western blot test; Table 1) diluted in TBST, washed in TBS and were incubated with secondary biotin-labelled goat anti-rabbit IgG (90 min, room temperature; Table 1), followed by an avidin-biotin-horseradish peroxidase complex (Vectastain ABC kit; 20 min; Vector Laboratories, Burlingame, USA). The colour reaction was developed by incubation with diaminobenzidine and an H_2O_2 solution. Additionally, sections were stained with hematoxylin QS (Vector Laboratories). In the immunofluorescent staining, after incubation with the fluorescent DyLight 594 secondary anti-rabbit antibody (Table 1), the sections were mounted with the VECTASHIELD Vibrance Antifade Mounting Medium with DAPI (Vector Laboratories). Negative controls were performed by a replacement of the primary antibody with normal rabbit serum or the TBST buffer. As a positive control, kidney tissue sections were used (the results are not shown). The tissue sections were examined under an Axio Scope. A1 light or a fluorescent microscope, and photographed using an Axiocam 503 colour camera and the ZEN 2.3 pro software (Carl Zeiss, Germany). The intensity of the immunoreactivity was estimated as strong, moderate, weak or very weak.

Statistical analysis

The data was analysed using the nonparametric Mann-Whitney *U* test or the Student's t-test. Differences in the values were considered to be significant at $p < 0.05$. All the values were expressed as the mean ± SEM. The calculations were performed with SigmaPlot_V_13 (Systat Software Inc., USA).

Results

Expression of mRNA and protein for AQPs

The relative abundance of AQP1, AQP2 and AQP9 mRNA transcripts in the adrenal glands was lower by 79.3% ($p < 0.001$), 46.6% ($p < 0.05$) and 36%

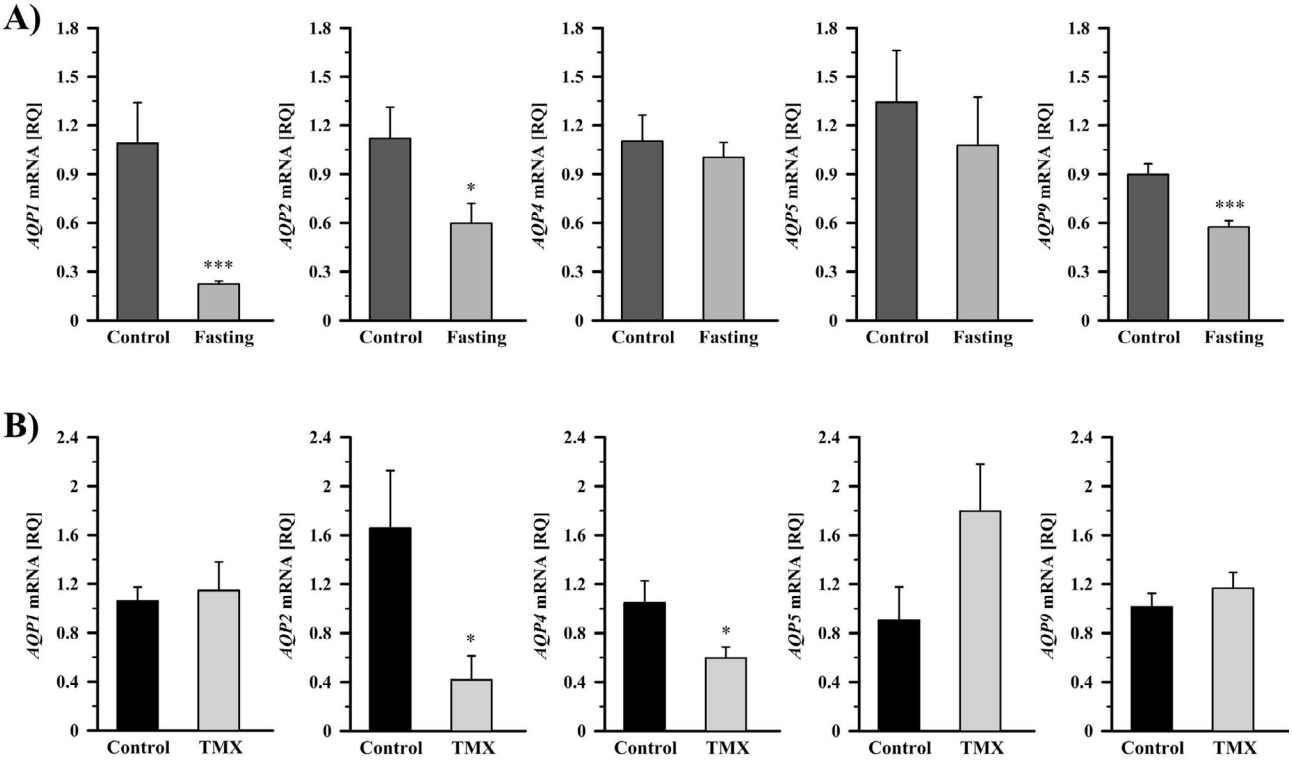

Fig. 1. Relative expression of AQP1, AQP2, AQP4, AQP5 and AQP9 mRNA in the chickens' adrenal glands following fasting (A) or a tamoxifen treatment (B). Each value represents the mean relative quality (RQ) ± SEM from six chickens (biological replicates) normalised to the expression of *18S* rRNA and standardised to the expression in the tissue of the control hens. Asterisks indicate significant difference between the control and treated groups (*$p < 0.05$, ***$p < 0.001$). Abbreviations: AQP – aquaporin; TMX – tamoxifen.

($p < 0.001$), respectively, in the fasted hens compared with the control hens. However, fasting did not affect the AQP4 and AQP5 mRNA transcript abundance (Fig. 1A). Treatment of the hens with TMX decreased ($p < 0.05$) the AQP2 and AQP4 mRNA transcript levels by 74.7% and by 43.1%, respectively, compared with the control hens (Fig. 1B). The expression of the AQP1, AQP5 and AQP9 mRNA transcript abundances did not change significantly ($p > 0.05$) in the adrenal glands after the TMX treatment.

The presence of AQP1, AQP2, AQP4 and AQP9 proteins in the chicken adrenal glands was found by Western blotting (Fig. 2). In the adrenal glands of both the control and the treated birds, bands of approximately 28-32 kDa (AQP1, AQP2, AQP4 and AQP9) were identified (Fig. 2A and B). An image analysis of all protein densities did not show significant differences in the AQP protein abundances in the adrenal glands of the fasted (Fig. 2A) or TMX-treated hens (Fig. 2B) compared with the control hens.

Immunohistochemical and immunofluorescent localisation of AQP proteins

As demonstrated by the immunohistochemistry and immunofluorescence, the moderate immunoreactivity for AQP1 and AQP2 was localised to the cell membrane and nuclear membrane of numerous cells in the adrenal gland chromaffin cell islets and in the nuclear membrane of a few adrenocortical cells (Fig. 3A, A', A" and B, B', B"). Moreover, a weak AQP2-positive immunoreaction was observed in the membrane of the adrenocortical cells (Fig. 3B, B', B"). Strong immunopositive staining for AQP4 was found in the membrane of the chromaffin cells (Fig. 3C, C', C"). An immunoreactivity for AQP9 was present in the chromaffin cell membranes, as well as in the membrane of the adrenocortical cells, on the side facing the adrenal sinusoids (Fig. 3D, D', D").

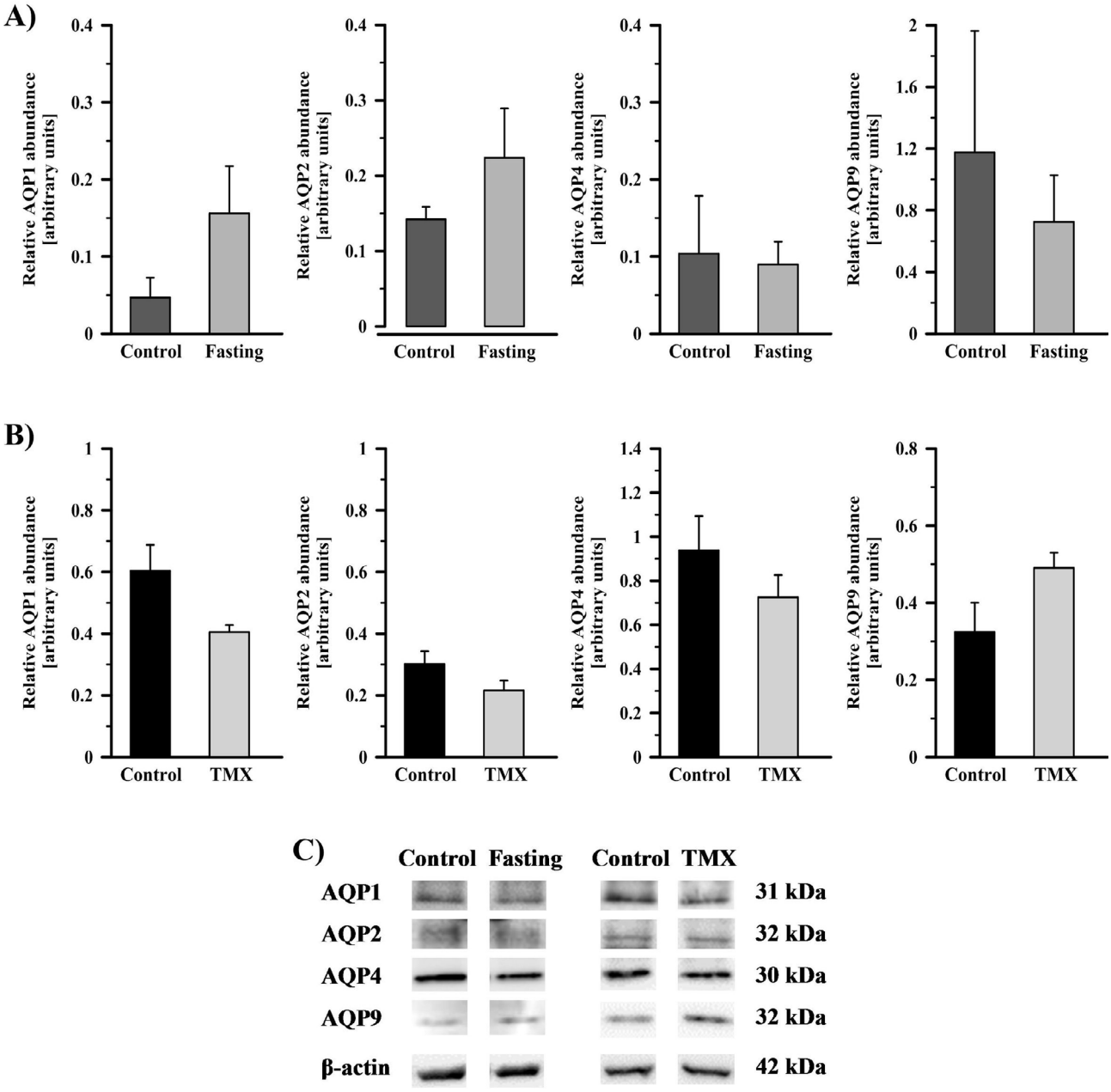

Fig. 2. Western blot analysis of AQP1, AQP2, AQP4 and AQP9 protein in the chickens' adrenal glands after fasting (A, C) or a tamoxifen treatment (B, C). The graphs show the relative abundance of AQPs in the adrenal glands. The densitometric analysis of the protein abundance was normalised against the corresponding β-actin. Data obtained from three to eight separate analyses is expressed as the mean ± SEM. N = six chickens (biological replicates). The images (C) are representative blots. Abbreviations: AQP – aquaporin; TMX – tamoxifen.

Discussion

The study revealed, for the first time, that the expression of selected AQPs at mRNA and/or protein levels in the adrenal glands of chickens following fasting for several days or after a TMX treatment. The present results extend the number of avian endocrine organs in which the presence of AQPs has been shown; that is, in the ovaries, testes and pancreas (Isokpehi *et al*. 2009; Nowak *et al*. 2017; Skowronski *et al*. 2009; Tiwari *et al*. 2014; Wolak *et al*. 2024). Our findings imply a possible involvement of AQP1, AQP2, AQP4, AQP5 and AQP9 in the regulation of the cell volume and the functioning of the chicken's adrenal glands during a deficiency in the ovarian steroid action. Following fasting, the concentration of sex steroid hormones in the blood plasma is reduced (Proszkowiec & Rząsa 2001; Socha *et al*. 2017), and

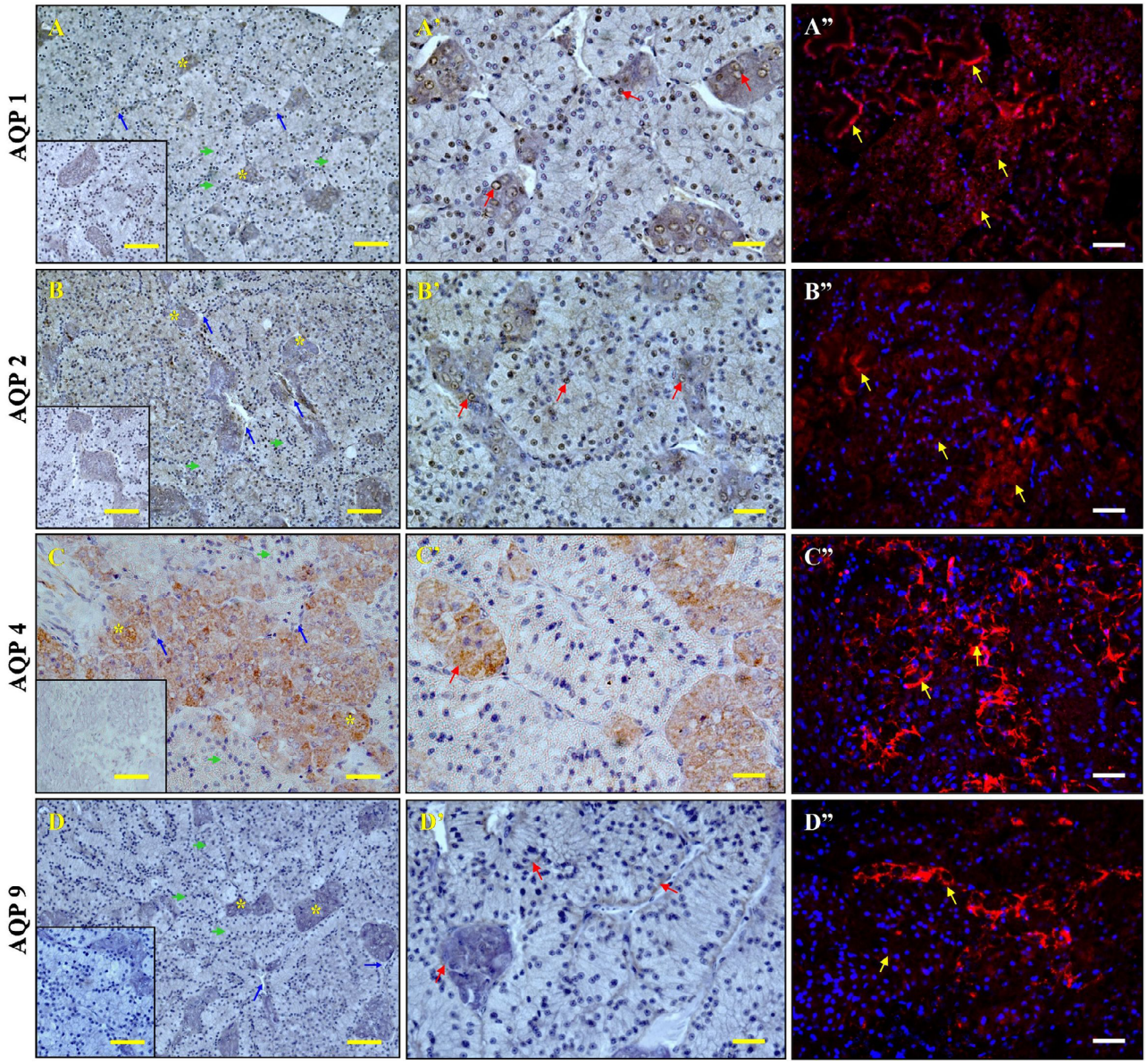

Fig. 3. Representative micrographs of immunohistochemical and immunofluorescent localisation of AQP1, AQP2, AQP4, and AQP9 proteins in the chickens' adrenal glands. Staining was performed with DAB and counterstaining with hematoxylin (A ,A', B, B', C, C', D, D'). Red arrows indicate positive immunoreactivity. Green arrowheads indicate looped cords of the adrenocortical cells. The yellow asterisk indicates a chromaffin cell islet. Blue arrows indicate sinusoids. The inserts in A, B, C and D show a negative control. A positive (red fluorescence) reaction for AQP proteins is indicated by yellow arrows (A", B", C", D"). The cell nuclei were counterstained with DAPI (blue fluorescence). The scale bars represent 50 µm (A, B, C, D) and 20 µm (A', A", B', B", C', C", D', D"). Abbreviation: AQP – aquaporin.

following TMX treatment the of estradiol is disrupted due to an estrogen receptor blockage. Moreover, a TMX treatment inhibits the ovarian progesterone synthesis (Rzasa *et al*. 2009).

In this study, we found an adrenal gland cell-dependent localisation of AQPs. Whereas AQP1, AQP2 and AQP9 were present in the membrane of medullar and adrenocortical cells, AQP4 was found exclusively in the chromaffin cells. Such a differential localisation of the examined AQPs is likely related to the different functions of particular AQPs. The presence of AQPs in both types of adrenal gland cells may be attributable to the regulation of water and/or glycerol homeostasis, as well as to the ion balance

associated with these cell activities, just like in the kidneys of mammals and birds. As an example, the AQP2, which is specifically localised in cells of the collecting ducts of kidney (Fushimi *et al*. 1993; Yang *et al*. 2004) has been proposed to mediate water influx; while AQP3 and AQP4 mediate the efflux of water from these cells (Castle 2005). In turn, AQP9, with an amino acid sequence similar in chickens and mammals, is permeable to glycerol and urea (Sagiura *et al*. 2008). Thus, these AQPs may play a pivotal role in water and glycerol transport, including in the chickens' adrenal cells. The abundant localisation of AQP4 in chromaffin cells indicates that chromaffin cells are the main cells in the chickens' adrenal glands where AQP4 may exert a physiological role. These results seem unsurprising, since the adrenal chromaffin cells and the postganglionic sympathetic neurons originate from a common cell population (Shtukmaster *et al*. 2013). AQP4 is the main AQP in the brains of different organisms including chickens (Isokpehi *et al*. 2009; Yoshimura *et al*. 2011) where it regulates, among other things, the neuronal activity by buffering ion exchanges, releasing/receiving transmitters or through a repolarisation of the water flux (Yoshimura *et al*. 2011). The adrenal chromaffin cells release catecholamines; thus, AQP4 may be involved in the regulation of the synthesis and/or release of these molecules under stressful conditions.

The adrenal gland plays a key role in maintaining the physiological balance of birds and helping them to survive during environmental changes (Fadl *et al*. 2024). Thus, we hypothesised that the stress associated with the food unavailability would affect the AQP abundance in the adrenal glands, as a stress-responding organ. Food is critical for proper growth, sexual maturation and for egg-laying in hens (Chin *et al*. 2013). An insufficiency of food acts as a stressor and directly stimulates the HPA axis, with an increase in glucocorticoid synthesis (Fokidis *et al*. 2013; Lynn *et al*. 2010). In this study, a food restriction for five days caused a decrease in the AQP1, AQP2 and AQP9 mRNA transcript abundances compared to the control, but did not affect the AQP4 and AQP5 mRNA transcript levels in the adrenal glands. The protein abundance of the examined AQPs was not affected by fasting. This stable expression of the AQP4 mRNA in adrenals was unexpected. Our previous study revealed a reduction in the AQP4 gene and protein expression in the oviducts of chickens following fasting (Socha *et al*. 2018). However, this discrepancy may be attributable to different organs and the specificity of their response to fasting, as well as to the duration of the fasting. For example, AQP4 mRNA expression in the kidneys of chickens was not influenced by water-deprivation for 1 day or by salt-loading (Sugiura *et al*. 2008), while it decreased in the kidneys after two days of dehydration treatment (Saito *et al.* 2005). At the same time the AQP4 mRNA expression increased in the hypothalamus, and no significant differences were observed in the proventriculus and breast muscle (Saito *et al.* 2005). The results presented in the current study differ from those recently reported by Greene *et al*. (2024), in which the mRNA expression of several AQPs, including AQP2 and AQP9, was upregulated by heat stress in the hypothalamus of broilers and in their ancestor jungle fowl, concomitantly with a decrease in the AVP mRNA abundance, the AQP2-regulating factor. That differential expression of AQPs is probably associated with different tissues and the stress-induced factors, as well as by the different roles of particular AQPs in the adrenal glands of birds. In another study, the expression of numerous AQPs in the kidneys and/or whole blood of chickens was affected by stressors such as water deprivation (Orlowski *et al*. 2017). Furthermore, water deprivation increased the AQP2 and AQP3 mRNA abundances in the kidneys of chicks (Sugiura *et al*. 2008). The results obtained in this study provide additional data about the expression of several AQPs in under stressful conditions other organs, i.e. the adrenal gland, and suggest that water channels may have an important role in water/ion/glycerol homeostasis, as well as in the adaptation to different stressors in hens kept in farms and those living in natural areas.

In female birds, fasting also reduces the circulating ovarian sex steroid concentrations and reduces egg production (Hocking *et al*. 2002; Proszkowiec & Rzasa 2001; Socha *et al*. 2017). On the other hand, the adrenal glands of birds are sex steroid hormone-responsive organs (Błachuta & Wrońska-Fortuna 2012; Kiezun *et al*. 2015). Moreover, it has been strongly suggested in chickens, that estradiol by its receptors is an important mediator between the HPA and hypothalamo-pituitary-gonadal axis during the recovery after short-term fasting-induced stress in chickens (Błachuta & Wrońska-Fortuna 2012). It is becoming increasingly clear that ovarian sex steroids orchestrate the AQP expression (Grzesiak *et al*. 2016; He *et al*. 2006; Kordowitzki *et al*. 2020; Skowronski *et al*. 2011; Skowronska *et al*. 2015). Thus, further in the study, we examined whether the blockage of estrogen receptors with TMX treatment, and in consequence the limitation in estrogen action, the regression of the reproductive system, and a pause in egg-laying, would affect the AQP abun-

dances in the adrenal glands. We found that there was a reduction in AQP2 and AQP4 mRNA transcript levels in the hen adrenal glands after TMX treatment, without significant changes in different AQP protein abundances. Thus, a vriation in various AQP mRNA transcript abundances might be attributed to different regulatory mechanisms in the hen adrenal glands and the different physiological roles of each examined AQP that might play a role in this organ. We suggest that there are two possibilities for the decrease in AQP2 and AQP4 mRNA expression in the adrenal glands following the TMX treatment. First, there may be a decrease in the estradiol action and plasma progesterone concentrations (Rząsa *et al*. 2009), which under normal conditions may stimulate AQP synthesis (Jablonski *et al*. 2003; Lindsay & Murphy 2006; Yang *et al*. 2016). Second, changes in there may be a biological activity of the adrenal gland cells as a result of a steroid influence deficiency. However, such assumptions need to be confirm in birds in future studies, but in women, an elevated psychological stress is a predictor of reduced estrogen concentrations (Roney & Simmons 2015). Furthermore, pharmacological blockage of androgen or estrogen signaling leads to permanent modifications of the adrenal glands in the rat adulthood of both sexes (Lagunas *et al*. 2022). In respect to AQP4, the results obtained are in line with the previous observations of a noticeable decline in AQP4 transcript abundance in the oviducts of hens treated with TMX (Socha *et al*. 2018). The present results further indicate the possibility that sex steroid hormones, especially estradiol, are implicated in the mechanisms driving the expression of water channel genes, including in the adrenal glands of hens.

Conclusions

In conclusion, our results show, for the first time, that selected AQP mRNA and protein expression depends on the cell type and the physiological state of the chicken's adrenal glands. By the regulation of water/ions/glycerol transport, AQPs may contribute to the control of this organ's activity and adaptation to different stressors. Since the adrenal gland responds to different types of stress by the release of glucocorticoid and catecholamines to alleviate the adverse effects, the participation of AQPs may be crucial in the release of these molecules. Moreover, the results provide a new insight into the understanding of the hormonal control of AQP expression. A relationship between the action of steroid hormones, especially estradiol, and AQP gene expression is suggested.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Research concept and design A.H.: Collection and/or assembly of data: A.H., D.W., K.F., M.G., N.S., A.S.; Data analysis and interpretation: A.H., D.W., K.F.; Writing the article: A.H.; Critical revision of the article: M.G., N.S., A.S.; Final approval of article: A.H., D.W., K.F., M.G., N.S., A.S.

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