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Immunohistochemical localization of sex hormone receptors in two *Raillietina* tapeworms

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ABSTRACT. Sex hormone receptors play critical roles in development and reproduction. However, it is not known whether they exist in *Raillietina* tapeworms, and if they do, whether they have a similar function to that in vertebrates. We examined the immunohistochemical distributions of androgen receptors (ARs), estrogen receptors (ERs), and progesterone receptors (PRs) in the tissues of two tapeworm species: *Raillietina echinobothrida* and *Raillietina tetragona*. Immunopositive ARs were found in the entire reproductive system of *R. echinobothrida*, including the testes, ovaries, and oocysts, and weakly immunopositive ERs and PRs were found in the testes, ovaries, and oocysts. Immunopositive ARs were also found throughout the entire reproductive system of *R. tetragona*, including the testes, ovaries, and oocysts, and weakly immunopositive ERs were in the testes and oocysts; the PRs were distributed in an immunonegative manner. The results show that androgens and their receptors play critical roles in reproductive system development in the two tapeworms. The immunoreactivity and

tissue localizations of the sex hormone receptors suggest that, in both species, they have similar functions as in vertebrates, and modulate reproduction.

Key words: *Raillietina echinobothrida*; *Raillietina tetragona*; Sex hormone receptor; Immunolocalization

INTRODUCTION

Sex hormone receptors play critical roles during both development and reproduction. Studies on sex hormone receptors have mainly focused on vertebrates (Fannon et al., 2001; González-Morán et al., 2008; Cleve et al., 2012; Wang et al., 2014; Fu et al., 2016); however, the number of studies that have investigated sex hormone receptors in invertebrates has increased in the last 20 years, before which it was believed that these receptors originated from deuterostome (Laudet et al., 1992). These receptor families are more ancient than previously thought (Thornton et al., 2003). Gagné and Blaise (2003) detected estrogen receptors (ERs) in the homogenate of freshwater *Elliptio complanata*, and ER-like immunoreactivity has been found in growing oocytes and auxiliary cells in close contact with the growing oocytes of the scallop *Patinopecten yessoensis* (Osada et al., 2003). Furthermore, Di Cosmo et al. (2002) identified a 17β-estradiol receptor in the reproductive system of female *Octopus vulgaris*, which had characteristics that resembled those of ERs in vertebrates (high affinity, specificity, and immunoreactivity). Moreover, a hormone-activated estrogen receptor has been found in the annelids *Platynereis dumerilii* and *Capitella capitata* (Keay and Thornton, 2009). Androgen receptors (ARs) and progesterone receptors (PRs) have also been identified in invertebrates (de Mendonça et al., 2000; Escobedo et al., 2010).

Tapeworms are platyhelminths, which played an important role in animal evolution (Králová-Hromadová et al., 2013; Yan et al., 2013). Regarding tapeworms, testosterone and 17β-estradiol have been found in *Taenia solium* and *T. crassiceps* cysticerci (Romano et al., 2003; Valdéz et al., 2006). In addition, androgen and progesterone have also been identified in the tissue homogenate of *R. tetragona* (Sun et al., 2010), and many key enzymes in the sex hormone biosynthesis pathway have been identified in tapeworms (Valdéz et al., 2006; Janer and Porte, 2007). Sex hormones perform their biological functions by binding to their receptors in vertebrates (Fannon et al., 2001). However, it is not known whether sex hormone receptors exist in *Raillietina* tapeworms. Therefore, we followed an immunohistochemical approach to detect ARs, ERs, and PRs in the tissues of two tapeworms, *R. echinobothrida* and *R. tetragona.*

MATERIAL AND METHODS

Tapeworms

Adult *R. echinobothrida* and *R. tetragona* tapeworms were obtained from the intestines of 106 chickens that were bought at Sanjiao Market, South China Agriculture University, China. After being washed and dissected, the intestines were doused with ddH_2O once the tapeworms were discovered. The tapeworms were removed after their scolexes had been anesthetized by ddH_2O and disjunct from intestine wall. After being rinsed several times with physiological saline, the tapeworms were fixed in 10% neutron-formalin for 8-24 h to maintain their natural state.

Chemicals and reagents

Mouse monoclonal antibody against ARs (110 kDa, clone No. AR441), rabbit monoclonal antibody against ERs (66 kDa, a synthetic peptide corresponding to residues near the N-terminus of the human estrogen receptor), and rabbit monoclonal antibody against PRs (clone No. Y85, a synthetic peptide corresponding to residues near the N-terminus of the progesterone receptor) were obtained from Epitomics®. None of the antibodies cross-reacted with the other two steroid hormone receptors. A SuperMark polymer-HRP kit (Ascend Biotechnology Co. Ltd., China) was used as a second antibody. The following buffers were used: 0.01 M citrate buffer solution, pH 6.0, and 0.01 M phosphate buffer solution, pH 7.4. The phosphate buffer solution was used as a negative control.

Paraffin section

The fixed samples were cut into small pieces (approximately 1-1.5 cm). The materials then underwent gradual alcoholic dehydration, paraffin imbedding, and serial sectioning (5-μm thickness). After adhesion, the sections were baked at 37°C overnight.

Immunohistochemistry

The immunolocalization procedure was similar to that used previously (Brattig et al., 2010; Kueakhai et al., 2011), with some modifications. Deparaffinated section slides were placed in 0.01 M citrate buffer solution, pH 6.0, and incubated for 10 min in a microwave. Slides were then incubated in 3% H_2O_2 for 10 min to block endogenous peroxidase. Antibodies against ARs, ERs, and PRs were incubated for 1 h at 25°C, or at 4°C overnight. Phosphate-buffered saline was used as a negative control. The paraffin sections were incubated with polymer-HRP biotin-free general-style second antibodies (SuperMark) for 20-30 min at 25°C. They were then reacted with Diaminobenzidine (DAB) for 1-2 min to colorate, dyed with haematoxylin for 1-2 min, and rinsed with water to remove the superfluous dye. The slides were subsequently dehydrated and mounted on neutral resin. A Leica DM2500 microscope was used to observe the slides and take photographs (Leica Microsystems, Wetzlar, Germany).

RESULTS

Three *R. echinobothrida* tapeworms and five *R. tetragona* tapeworms were found, with a total infection rate of 7.5%.

Distribution of sex hormone receptors in male reproductive organs

In *R. echinobothrida*, strongly immunopositive ARs were found in the testes of mature proglottids, and parenchymatous tissues and teguments were also stained with anti-AR antibodies (Figure 1A). Immunopositive ERs were in the testes; however, the PR reaction was weak (Figure 1C and E). In *R. tetragona*, ARs had a wide distribution; besides the testes, they were also present in the parenchymatous tissues and teguments (Figure 1B). The ERs exhibited a weak immunopositive reaction, and there were no PRs present (Figure 1D and F).

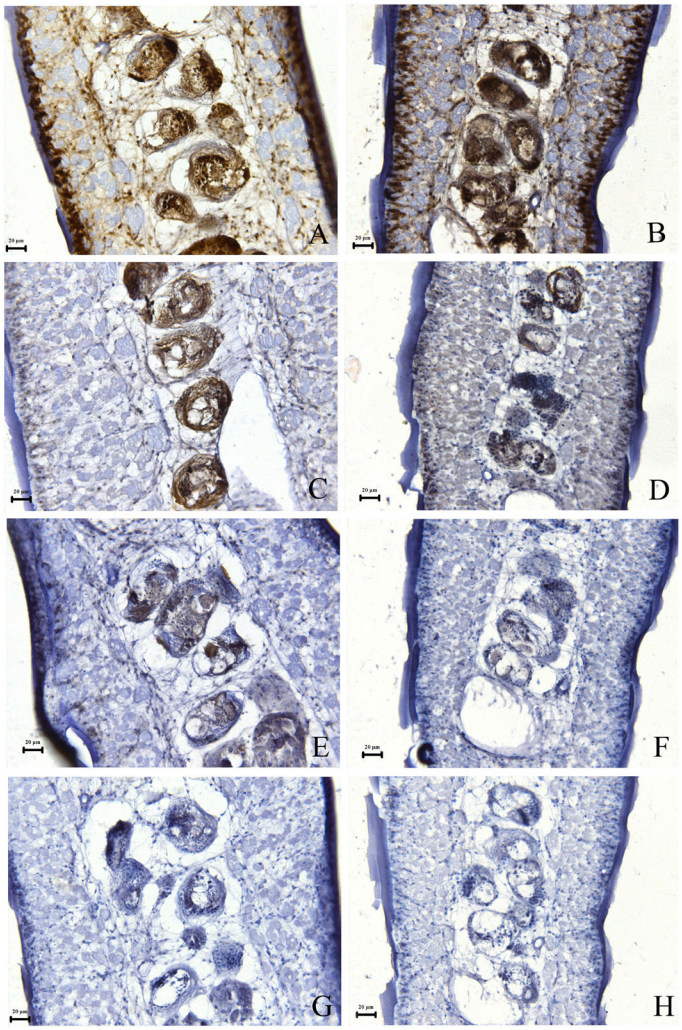

Figure 1. Distribution of sex hormone receptors in the testes of *Raillietina echinobothrida* and *Raillietina tetragona*. Immunopositive androgen receptors were found in the testes, parenchymatous tissues, and teguments of both *R. echinobothrida* (**A**) and *R. tetragona* (**B**); (**C**) immunopositive estrogen receptors (ERs) in the testes of *R. echinobothrida*; (**D**) immunopositive ERs in the testes and teguments of *R. tetragona*; (**E**) weakly immunopositive progesterone receptors (PRs) in the testes of *R. echinobothrida*; (**F**) immunonegative PRs in *R. tetragona*; (**G**) and (**H**) were controls for *R. echinobothrida* and *R. tetragona*, respectively. Scale bars (A-H) = 20 μm.

Distribution of sex hormone receptors in female reproductive organs

Immunopositive ARs were in the ovaries, vitellaria, and parenchymatous tissues of *R. echinobothrida* (Figure 2A), while the ERs and PRs only had weakly immunopositive reactions in the ovaries (Figure 2C and E). In *R. tetragona*, AR-immunopositive reactions were found in the ovaries (Figure 2B); neither ERs nor PRs were found (Figure 2D and F).

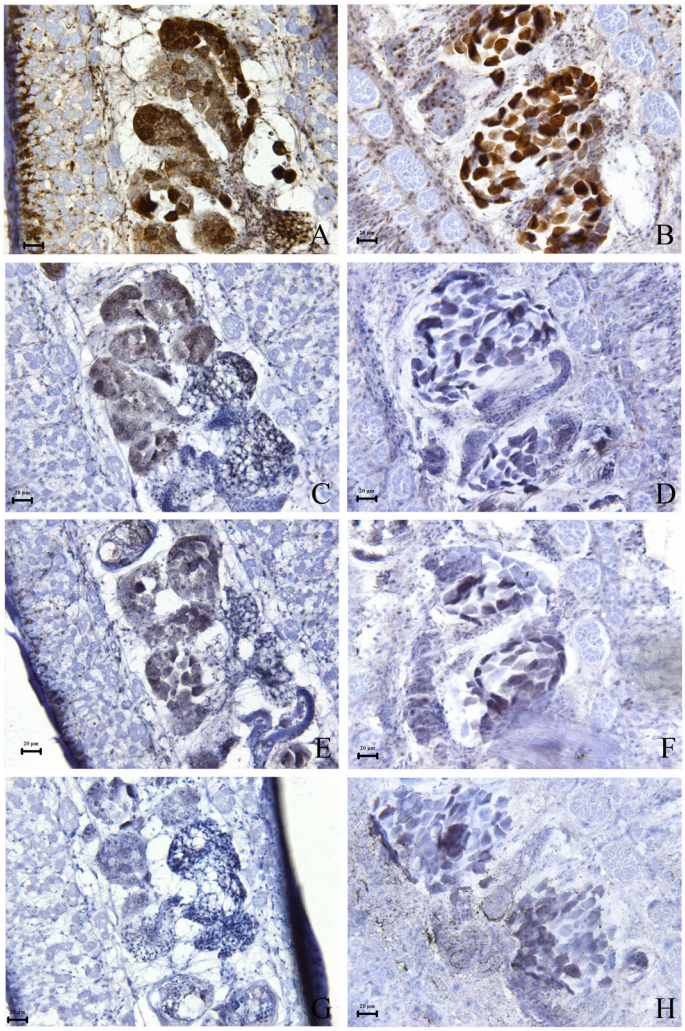

Figure 2. Distribution of sex hormone receptors in the ovaries of *Raillietina echinobothrida* and *Raillietina tetragona*. (**A**) Immunopositive androgen receptors (ARs) in the ovaries and teguments of *R. echinobothrida*; (**B**) immunopositive ARs in the ovaries of *R. tetragona*; (**C**) weakly immunopositive estrogen receptors (ERs) in the ovaries of *R. echinobothrida*; (**D**) no ERs were found in the ovaries of *R. tetragona*; (**E**) weakly immunopositive progesterone receptors (PRs) in the ovaries of *R. echinobothrida*; (**F**) immunonegative PRs in *R. tetragona*; (**G**) and (**H**) were controls for *R. echinobothrida* and *R. tetragona*, respectively. Scale bars (A-H) = 20 μm.

Distribution of sex hormone receptors in gravid proglottids

Immunopositive ARs were present in the eggs of *R. echinobothrida*, and egg capsules that were surrounded by parenchymatous tissue were also stained with anti-AR antibodies (Figure 3A). Both ERs and PRs were found in the eggs and egg capsules of *R. echinobothrida* (Figure 3C and E). In *R. tetragona* eggs, ARs had immunopositive reactions (Figure 3B), and ERs had weak immunopositive reactions (Figure 3D); however, there were no PRs (Figure 3F).

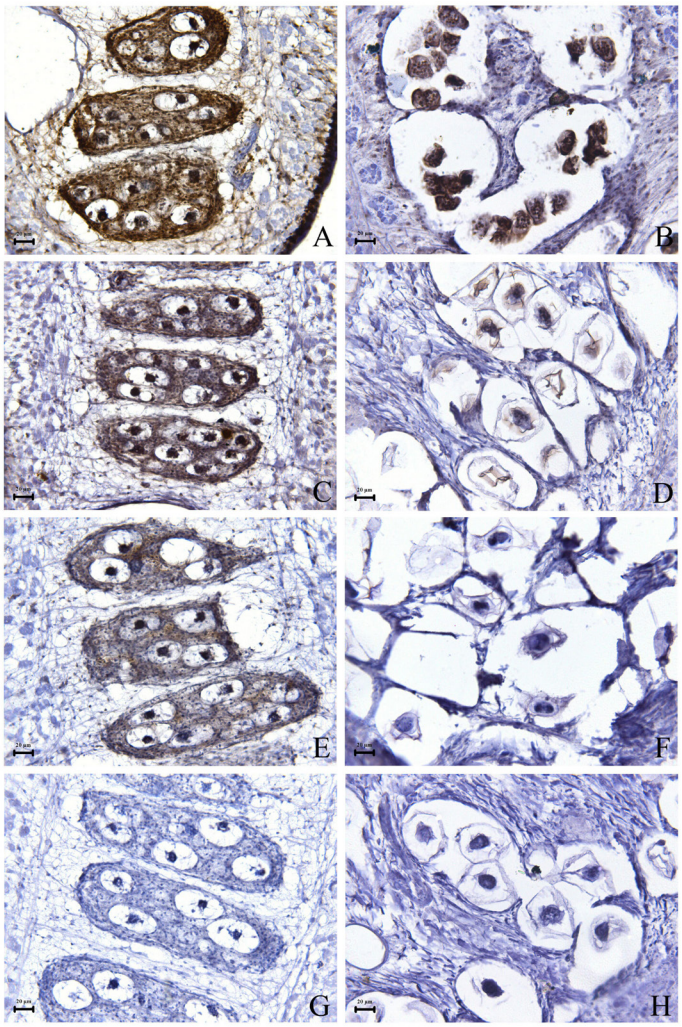

Figure 3. Distribution of sex hormone receptors in the gravid proglottids of *Raillietina echinobothrida* and *Raillietina tetragona*. (**A**) Immunopositive androgen receptors (ARs) were found in the eggs, egg capsules, and teguments of *R. echinobothrida*; (**B**) immunopositive ARs in the eggs of *R. tetragona*; (**C**) immunopositive estrogen receptors (ERs) in the eggs and egg capsules of *R. echinobothrida*; (**D**) weakly immunopositive ERs in the eggs of *R. tetragona*; (**E**) immunopositive progesterone receptors (PRs) in the eggs and egg capsules of *R. echinobothrida*; (**F**) immunonegative PRs in *R. tetragona*; (**G**) and (**H**) were controls for *R. echinobothrida* and *R. tetragona*, respectively. Scale bars (A-H) = 20 μm.

DISCUSSION

Sex hormones and their receptors widely exist in vertebrates, and play an important role in the processes of development and reproduction. Sex hormones corresponding to those in vertebrates have been reported in several invertebrates, particularly in arthropods and

mollusks (Janer and Porte, 2007; Morris and Spradling, 2012; Zhang et al., 2012). Regarding tapeworms, there is evidences that testosterone and estrogen are present in the cysticerci of *T. solium* and *T. crassiceps* (Romano et al., 2003; Valdéz et al., 2006). The cysticerci can synthesis androgens using progesterone as a precursor (Jiménez et al., 2006). Sun et al. (2010) found testosterone and progesterone in the tissues of *R. tetragona*. In the present study, we found ARs, ERs, and PRs in the tissues (although not in the same tissue) of *R. echinobothrida* and *R. tetragona*. However, whether sex hormone receptors behave in the same way as in vertebrates during tapeworm development and reproduction requires further investigation.

Escobedo et al. (2004) found that 17β-estradiol and progesterone could stimulate the reproduction and infectivity of *T. crassiceps* cysticerci. However, testosterone and dihydrotestosterone inhibit their reproduction and reduce their motility and infectivity. 17β-estradiol and progesterone levels increase, whereas testosterone and dihydrotestosterone levels decrease (Escobedo et al., 2004). Schistosomes express a homologous androgen receptor that affects androgen binding (de Mendonça et al., 2000). In the present study, ARs were found in the male and female reproductive systems and gravid proglottids of both tapeworm species, which suggests that androgens and their receptors function throughout the entire developmental stages of the reproductive systems of both tapeworms, including the initial stage, spermary development, ovary development, and fertilization.

Regarding ERs, both α and β ER isoforms are expressed in *T. crassiceps* (Escobedo et al., 2004). We found immunopositive ERs in the testes and eggs of *R. echinobothrida*; however, there were only weakly immunopositive ERs in the ovaries. In the tissues of *R. tetragona*, weakly immunopositive ERs were found in the testes and eggs. Our results are very different from those in vertebrates, in which ERs are abundant in female reproductive organs. Reproductive function mediated by ERs requires a combination of estrogen and its corresponding receptor. However, in the two *Raillietina* tapeworms there were only weakly immunopositive ERs, suggesting that ERs are not important for tapeworms, at least in the development of the female reproduction system.

Strongly immunopositive PRs were found in the eggs and egg capsules of *R. echinobothrida*; however, only weakly immunopositive PRs were present in the testes and ovaries. No PRs were found in the tissues of *R. tetragona*. It is possible that the antibody against human PR that was used in this study may have had different reaction specifications when recognizing the PRs of the two tapeworms. Escobedo et al. (2004) designed primers for PR-A and PR-B based on the most conserved regions of sequenced PR genes in most of the species in the NIH Gene Data Bank to clone the PRs from *T. crassiceps*. Their results showed that there was no PR expression (neither isoform A nor B) (Escobedo et al., 2004). However, in another study, a protein (*T. solium* PR, TsPR) was found that could recognize progesterone and mediate its effects. TsPR only matches to the PR-B isoform (Escobedo et al., 2010). Whether PRs exist in tapeworms requires further investigation, which should involve more species of tapeworm.

In conclusion, we investigated the immunoreactivity and immunolocalization of ARs, ERs, and PRs in *R. echinobothrida* and *R. tetragona*. ARs were expressed throughout the entire development of the reproductive system in both tapeworms, including the male and female reproductive organs and gravid proglottids; however, the expression patterns of ERs and PRs varied greatly between the two species, depending on the tissue type. To the best of our knowledge, this is the first study conducted on the immunolocalization of sex hormone receptors in *Raillietina* tapeworms. Confirmation of the existence of the sex hormone

receptors, together with their localizations, would further our understanding of the effects of sex hormone receptors on the development, reproduction, and evolution of platyhelminths. The localization patterns of the different sex hormone receptors and their effects on development and reproduction in tapeworms require further investigation.

Conflicts of interest

The authors declare no conflict of interest.

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