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RESEARCH

The immunolocalization of adiponectin and its receptors in the testis of the frog *Pelophylax bergeri*

Francesca Simoncelli[1] · Francesca Mercati[2] · Ines Di Rosa[1] · Elisa Palmioli[2,3] · Cecilia Dall'Aglio[2] · Daniele Marini[2,4] · Anna Fagotti[1]

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Abstract

In all vertebrates, reproductive strategies are achieved by modulation of the neuroendocrine system in a similar manner and with minor variations among the different classes. Most of the available information on amphibian testicular cycles derive from anurans, and among these, water frogs have been extensively studied in terms of reproductive mechanisms and sex steroid correlation. Adiponectin (AdipoQ) and its receptors—AdipoR1 and AdipoR2—are essential for most of the normal testicular and sperm functions. In this study, the identification of AdipoQ and its two receptors was carried out by immunohistochemistry in the testis of adult males of *Pelophylax bergeri*. The AdipoQ system was observed in the frog spermatogenic cysts, in both germinal and Sertoli cells, as well as in the rete testis. AdipoQ and AdipoR1 were localized in germ-line cells, from spermatogonia to round spermatids, while AdipoR2 was detected in the elongated spermatids, spermatozoa, and Sertoli cells. AdipoR1 was also observed in the intratesticular canals of the rete testis. This preliminary study shows the AdipoQ system's presence in the anurans' testis. The results obtained could be a starting point for future functional studies aimed at defining the physiological role of the AdipoQ system in frog testicular functions.

Keywords Adiponectin · AdipoR1 · AdipoR2 · Anuran spermatogenesis · Amphibians · Immunohistochemistry

Introduction

Adiponectin (AdipoQ) is a protein composed of 247 amino acids (Nguyen 2020), that was discovered in 1995 as an adipocyte-specific factor. In fact, like most adipokines, it is produced by white adipose tissue, as precisely demonstrated by the 3T3-L1 cell line (Fang and Judd 2018). AdipoQ is also called apM1 (adipose most abundant gene transcript I), Acrp30 (adipocyte complement-related protein), GBP28 (gelatin-binding protein 28), or AdipoQ (Caminos et al. 2008). AdipoQ is initially produced in monomeric form, and once circulating, undergoes a multimerization process, that

Francesca Simoncelli and Francesca Mercati have contributed equally to the work.

✉ Francesca Mercati
francesca.mercati@unipg.it

Francesca Simoncelli
francesca.simoncelli@unipg.it

Ines Di Rosa
ines.dirosa@unipg.it

Elisa Palmioli
elisa.palmioli@studenti.unipg.it

Cecilia Dall'Aglio
cecilia.dallaglio@unipg.it

Daniele Marini
daniele.marini3@studenti.unipg.it

Anna Fagotti
annalisa.fagotti@unipg.it

[1] Department of Chemistry, Biology and Biotechnology, University of Perugia, Via Elce di Sotto, 8, 06123 Perugia, Italy

[2] Department of Veterinary Medicine, University of Perugia, Via San Costanzo 4, 06126 Perugia, Italy

[3] Department of FISSUF, Ph.D. Course in "Ethics of Communication, Scientific Research and Technological Innovation" Medical-Health Curriculum, University of Perugia, Piazza G. Ermini, 1, 06123 Perugia, Italy

[4] Department of Organismal Biology, Evolutionary Biology Centre, Uppsala University, Norbyvägen 18A, 752 36 Uppsala, Sweden

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is essential for the biological actions of the molecule. The monomeric form is modified into multiple isoforms: low-molecular weight (LMW) (trimer), middle-molecular weight (MMW) (hexamer), and high-molecular weight (HMW) (multimer); the latter is the most biologically active form (Caminos et al. 2008; Choi et al. 2020). To perform its functions, AdipoQ needs the bind with its two receptors, AdipoR1 and AdipoR2, first characterized by Yamauchi et al. (2003). The specific functions of AdipoQ depend on which receptor is expressed. AdipoR1 is more present in skeletal muscle with an affinity for the molecule's globular and trimeric forms, which causes an enhancement of fat β-oxidation and glucose uptake. AdipoR2 is expressed in the liver with an affinity for the hexameric and multimeric forms, leading to increased fat β-oxidation and decreased gluconeogenesis (Lago et al. 2007). Later, the receptors' expression has been also identified in the myocardium, macrophages, brain tissue, endothelial cells, lymphocytes, adipose tissue, and pancreatic β cells (Nguyen 2020). The AdipoQ presence has also been detected in several tissues including skeletal muscle, cardiomyocytes, osteoblasts, adrenal gland, placenta, and liver, and hence, the molecule could be considered a rather pleiotropic regulator of a large set of biological functions (Caminos et al. 2008). The AdipoQ system, given by the AdipoQ and its receptors, has been also detected in the testis of amniote models, showing its role in normal testicular and sperm functions, including the involvement in the regulation of spermatogenesis, gonadal steroid hormones, and sperm motility, as well as in the modulation of testicular oxidative stress (Caminos et al. 2008; Dutta et al. 2019). Furthermore, it has been proved that the AdipoQ system's deficiency can cause an impairment of testicular functions (Choubey et al. 2019).

Beyond laboratory animals, AdipoQ and its receptors have been described in several organs, including the testis, in a wide range of species, comprising mammals, birds, and teleost fish (Civitarese et al. 2004; Ocón-Grove et al. 2008; Kondo et al. 2011; Martínez-Barbitta et al. 2023). However, to the author's knowledge, no studies histologically describe the AdipoQ system in any organ or tissue in amphibians. These vertebrate anamniotes are characterized by a peculiar cystic spermatogenesis (Pudney 1995); hence, the study of the AdipoQ system in their testis could give new insights about the role of this molecule on testicular functions.

Accordingly, in this study, the presence and the localization of the AdipoQ system were evaluated for the first time on the testis of water frogs of peninsular Italy belonging to the B–H system (Berger 1988; Ludovisi et al. 2014).

Materials and methods

Study species

In peninsular Italy, water frogs constitute mixed populations consisting of the parental species *P. bergeri* and the hybrid *P.* kl. *hispanicus* (B–H system) (Berger 1988; Ludovisi et al. 2014). The latter is a kleptospecies derived from *P. bergeri* and *P. ridibundus* or *P.* kl. *esculentus* (Andreone et al. 2009). Certain authors classify *P. berger*i and *P.* kl. *hispanicus* as a subspecies of *P. lessonae* (Camerano, 1882) and *P.* kl. *esculentus*, respectively. Specifically, *P. lessonae lessonae* is recognized as a distinct subspecies found in northern Italy, while *P. lessonae bergeri* as a separate subspecies located in peninsular Italy (Crochet and Dubois 2004; Lanza et al. 2006; Canestrelli and Nascetti 2008; Sindaco and Razzetti 2021). For the sake of simplicity, we refer to the parental species as *P. bergeri* in this context. B–H system frogs breed in small water bodies located in two sub-basins of the Tiber River (Central Italy), one of which is the Lake Trasimeno basin (Ludovisi et al. 2014). These frogs are characterized by an annual testis cycle according to which spermatogenesis resurges gradually in the spring months, continues through the summer and autumn, and shows a brief winter stasis. Slight variations occur according to the different geographical areas where specimens are collected (Rastogi et al. 1976).

Sample collection

Adult males ($n = 10$) of *Pelophylax bergeri* were collected in the Lake Trasimeno basin (Central Italy) in September. The taxonomic determination of the captured frogs was carried out by Southern hybridizations of genomic DNA, performed by using the satellite DNA RrS1 as a molecular marker (Mosconi et al. 2005). To carry out the analysis, the animals were sacrificed with 1% buffered tricaine-methane sulfonate (MS-222, Sigma-Aldrich, MO, USA) and testes were removed and processed for histological examination.

Capture and handling of the specimens were conducted according to the permission granted by the Italian Ministry for Environment, Land and Sea Protection (0002093/PNM of 06.02.2015 and 0008363/PNM of 20.04.2018) and Italian Ministry of Health (08/2018-UT of 24/07/2018), respectively.

Morphological staining and immunohistochemistry

After collection, samples were fixed in 4% paraformaldehyde solution in phosphate-buffered saline (0.1 M PBS, pH 7.4) at 4 °C. Subsequently, the fixed samples were dehydrated

in a series of ethanol solutions of increasing concentration and cleared in xylene to be included in paraffin wax. Five µm thick sections were cut with a rotatory microtome, mounted on poly-L-lysine-coated glass slides, and air-dried at 37 °C. Some sections of all specimens were stained with the hematoxylin–eosin solution to carry out a morphological evaluation.

The immunohistochemistry procedure on all samples was performed according to the following description (Dall'Aglio et al. 2021): the sections were subject both to dewaxing in xylene and to hydration through a series of ethanol concentrations until distilled water. Sections were microwaved for two 5-min cycles at 750 W in citrate buffer (pH 6.0) to expose the epitopes to the antibodies and immersed in a 3% hydrogen peroxide solution for 10 min to block the endogenous peroxidase activity. Incubation with 1:200 normal goat serum (S-1000-20, Vector Laboratories, Burlingame, CA, USA) was performed for 30 min to avoid non-specific bindings. Subsequently, different sections from the same sample were incubated overnight at room temperature (RT) with the following rabbit polyclonal primary antibodies: 1:100 anti-AdipoQ (MBS2028428, MyBioSource, CA, USA), 1:100 anti-AdipoR1 (LS-C151518, LifeSpan BioSciences, WA, USA), and 1:100 anti-AdipoR2 (ARP60819_P050, Aviva Systems Biology Corporation, CA, USA). The dilution used for each primary antibody was the best to obtain the intensity of the signal without background. On the second day, sections were incubated for 30 min with a 1:200 goat anti-rabbit biotin-conjugated (BA-1000, Vector Laboratories, Burlingame, CA, USA) secondary antibody. The avidin–biotin complex solution (PK-6100, Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA) was used to detect the immunological binding sites, which were revealed with DAB chromogen (SK-4100, DAB substrate kit, Vector Laboratories, Burlingame, CA, USA). Sections were also counterstained with hematoxylin.

Sections were washed with PBS between each incubation step, except after the addition of normal serum. Each step was performed at RT and slides were incubated in a humid chamber.

Negative control sections were incubated with normal rabbit IgG (I-1000-5, Novus Biological, Littleton, CO, USA) without applying the primary antibodies.

Results

The testis of *P. bergeri* we collected from the Lake Trasimeno basin in September still showed high spermatogenic activity, according to the frog seasonal testicular cycle (Fig. 1). The seminiferous lobules contained cysts of germinal cells, from spermatogonia to round spermatids, elongated spermatids sustained by Sertoli cells, and abundant spermatozoa detached from Sertoli cells. In some lobules residual released degenerating spermatozoa were observed in the lobule lumen.

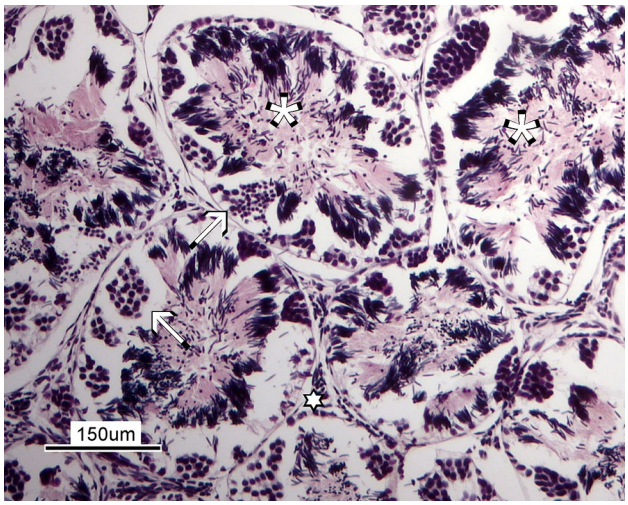

Fig. 1 HE stained testis of *P. bergeri*. In the seminiferous lobules cystic germ cells (arrow), spermatozoa (asterisk), and interstitial cells (star) are shown

In all samples examined, the immunohistochemical investigation highlighted the presence of the AdipoQ system at the level of the frog testis since the positivity was observed for the ligand AdipoQ and its two receptors AdipoR1 and AdipoR2.

AdipoQ immunostaining was detected in germ-line cells, from spermatogonia to round spermatids, while elongated spermatids and detached sperm cells were negative to the ligand (Fig. 2).

The immunostaining for AdipoR1 was detected in germline cells from spermatogonia to round spermatids (Fig. 3). In addition, the epithelial cells of the intratesticular canals of the rete testis appeared positive (Fig. 3c). No signal was observed in interstitial tissue (Fig. 3a), in spermatozoa (Fig. 3a), in elongated spermatids (Fig. 3b), and Sertoli cells (Fig. 4).

The immunopositivity for AdipoR2 was detected in the abundant spermatozoa detached from Sertoli cells (Fig. 5a, b), as well as in elongated spermatids and Sertoli cells before the spermiation (Fig. 5c). The remaining germ cell stages, the residual degenerating released spermatozoa, the rete testis and the interstitial tissue were negative.

No staining was observed in the negative controls performed for all molecules analysed in the work (data not shown).

Discussion

In recent years, scientific interest has been focused on adipokines which are currently studied under many aspects of metabolism. Adipokines are ubiquitous molecules that

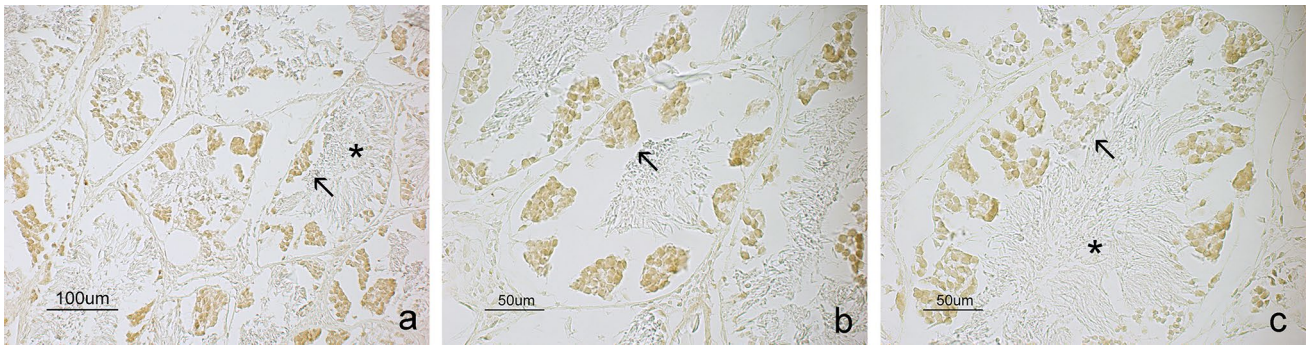

Fig. 2 AdipoQ immunostaining in *P. bergeri* testis. **a** The low magnification image shows numerous lobules where the germ cells appear positive to AdipoQ while sperm cells appear negative. **b**, **c** High magnification of a lobule. Arrows point out cysts containing AdipoQ-positive germ cells at different stages of spermatogenesis; asterisk points out detached AdipoQ-negative sperm cells together with fully released spermatozoa

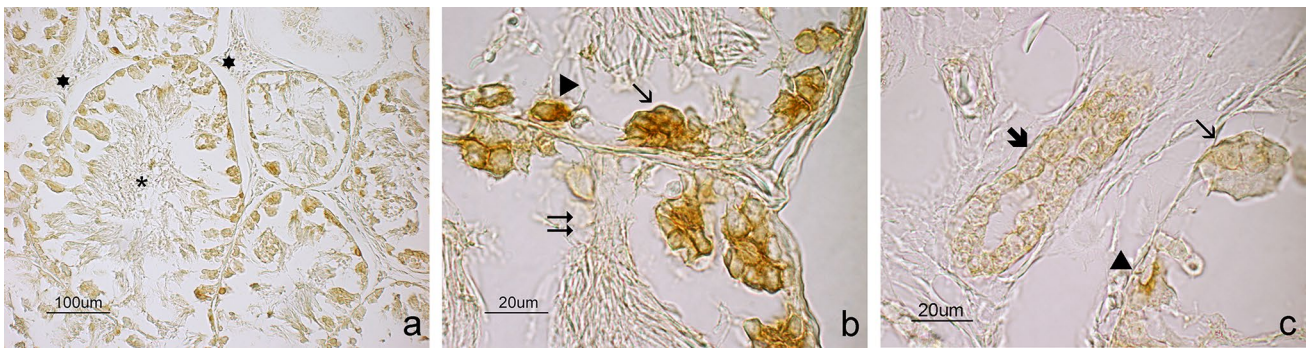

Fig. 3 AdipoR1 immunostaining in *P. bergeri* testis. **a** The low magnification image shows numerous lobules where cystic germ cells appear positive to AdipoR1 while spermatozoa (asterisk) appear negative. Star points out AdipoR1-negative interstitial tissue. **b** High magnification of AdipoR1-positive cystic germinal cells at different stages of differentiation, such as a primary spermatogonium (arrowhead) and secondary spermatogonia (arrow). Elongated spermatids sustained by Sertoli cells were not stained (double arrow). **c** High magnification of AdipoR1-positive intratesticular canal of the rete testis (thick arrow). Also, positive primary spermatogonium (arrowhead) and secondary spermatogonia (arrow) are shown

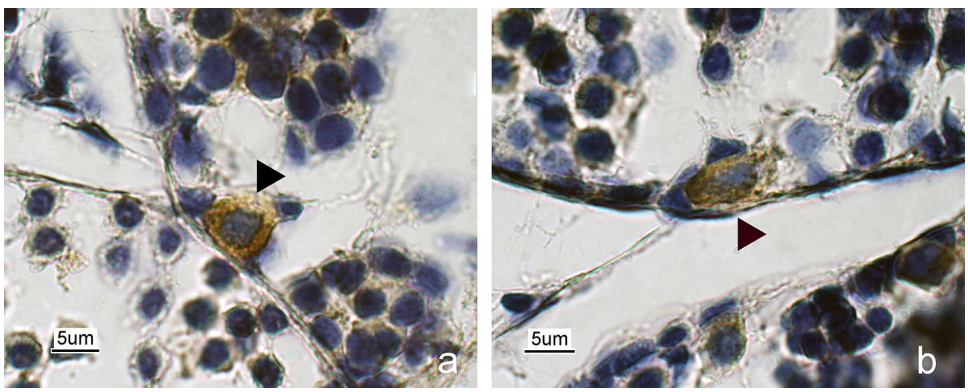

Fig. 4 AdipoR1 immunostaining in *P. bergeri* testis. The figure shows the morphological relationship between AdipoR1-positive primary spermatogonium (arrowhead) and AdipoR1-negative Sertoli cells, counterstained with hematoxylin. The primary spermatogonium appears as the biggest cell with a large nucleus while the Sertoli cells are placed around it and show a pyramidal nucleus

influence local tissue and whole organism physiology, such as AdipoQ (Trayhurn et al. 2006). AdipoQ system has been detected through the study of its gene transcripts and proteins in many vertebrates, including mammals, birds, and teleost fish (Tang et al. 2021). In amphibians, investigations concern only the mRNA expression during metamorphosis (i.e. Zhu et al. 2020). Particularly, in several amniotes, the AdipoQ system has been identified in the testis and its involvement in normal testicular and sperm functions has been demonstrated (Caminos et al.

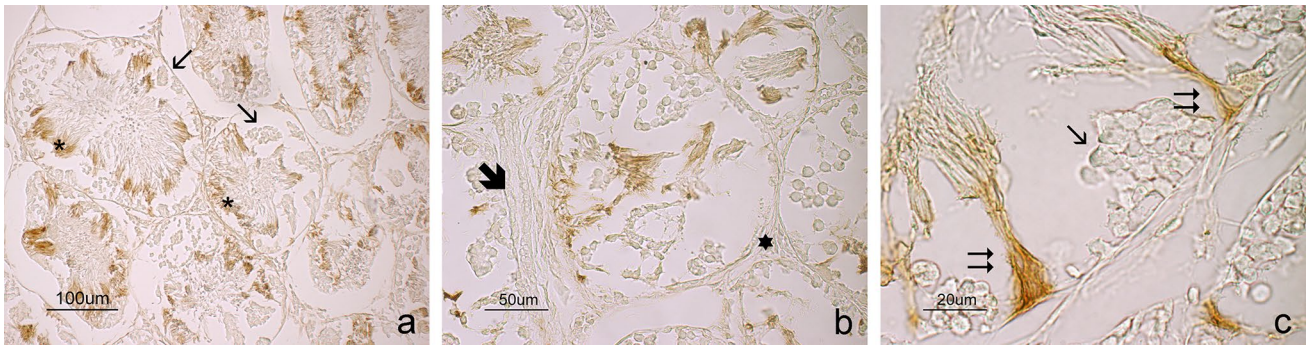

Fig. 5 AdipoR2 immunodetection in *P. bergeri* testis. **a** The low magnification image shows numerous lobules where spermatozoa detached from Sertoli cells (asterisk) are positive to AdipoR2 while spermatogenic cysts (arrow) appear negative. **b** Higher magnification of AdipoR2-negative intratesticular canal of the rete testis (thick arrow) and interstitial cells (star). **c** AdipoR2-immunopositivity localized in elongate spermatids sustained by AdipoR2-immunoreactive Sertoli cells with ovoid nucleus (double arrow), alternating with negative cystic cells (arrow)

2008; Ocón-Grove et al. 2008; Rahmanifar and Tabandeh 2012; Martínez-Barbitta et al. 2023). The current study is the first report that assesses the presence and the localization of AdipoQ and its receptors AdipoR1 and AdipoR2 in the testis of the water frog *Pelophylax bergeri*.

Although spermatogenesis is a conserved process in all vertebrates, there are differences in the mode of development and maturation of germ cells relative to the compartment in which these events take place. In amniotes (mammals, birds, and reptiles) spermatogenesis occurs in seminiferous tubules (acystic spermatogenesis); otherwise, in anamniotes (amphibians and fish) spermatogenesis takes place in cysts formed by Sertoli cells enclosing synchronously developing germ cells (cystic spermatogenesis) (Haczkiewicz et al. 2017; Roco et al. 2021; Svanholm et al. 2023). In addition, unlike the higher vertebrates, the germinal epithelium of the lower vertebrates has a transient nature, having to be replaced during successive breeding seasons, and requires the turnover of Sertoli cells (Pudney 1995; Roco et al. 2021).

Pelophylax bergeri examined in this study belongs, together with *Pelophylax* kl. *hispanicus*, to mixed populations of water frogs of peninsular Italy (B–H system) (Berger 1988) and are directly analogous to the central European *lessonae–esculentus* population system (L–E systems) (Uzzell and Berger 1975; Uzzell and Hotz 1979). Like these, water frogs of the B–H system have a potentially continuous type of spermatogenesis which implies that the process continues even during autumn (Lofts 1964; Rastogi et al. 1976, 1978; Chieffi et al. 1980). In addition, in *Pelophylax* water frogs, an annual cyclic pattern is also displayed by both interstitial cell and Sertoli cell activity (Lofts 1964; Rastogi 1976).

In the testes of *P. bergeri* sampled in September all the spermatogenic stages were present in the seminiferous lobules, and a considerable quantity of spermatozoa detached from Sertoli cells was observed; the interstitial tissue among lobules appeared poorly developed (Lofts 1964; Rastogi et al. 1976).

In all the adult males of *P. bergeri* the immunolocalization of AdipoQ and its receptors in the testis has provided results that are correlated with the characteristic reproductive pattern of the frogs. The immunoreactivity of both AdipoQ and AdipoR1 on the cystic germ cells, from spermatogonia to round spermatids, let us hypothesize a potential involvement in the development of the germ cells inside the cysts. In addition, the presence of AdipoR1 in frog intratesticular canals of the rete testis suggests that the receptor could be involved in sperm transport. AdipoR2 immunostaining was observed in both Sertoli cells with ovoid nucleus and elongated spermatids sustained by them, as well as in spermatozoa detached from Sertoli cells but not fully released in the lobule lumen. Based on such results, it might be assumed that AdipoR2 is involved in frog cystic cell maturation toward the new generation of spermatozoa. The lack of staining of the frog interstitial cells is probably circumstantial, due to the fact that they had not fully resumed their secretory activity yet, despite in *Pelophylax* water frogs it starts at the time of our animal sampling (Lofts 1964; Rastogi et al. 1978; Izzo et al. 2006). Therefore, it will be necessary to analyze water frog testis with interstitial cells showing a higher secretory activity.

Although preliminary, the results obtained in this study are a starting point for future functional studies, also taking into account other periods of the seasonal spermatogenic cycle, aimed to define the physiological role of AdipoQ system in frog testicular functions, as well as reported in amniotes (Caminos et al. 2008; Ocón-Grove et al. 2008). The annual spermatogenic cycle of *Pelophylax* water frogs is deeply conditioned by environmental and endogenous endocrine cues, along the hypothalamus–pituitary–gonad axis (Rastogi 1976; Minucci et al. 1992; Di Fiore et al. 2020). The endocrine route is integrated within the testis

by internal modulators that regulate the germ cell progression and functions (Pierantoni et al. 2002; Meccariello et al. 2020). Recently, the modulation of the testicular cell-to-cell interactions by the Cx43 protein and kisspeptin system has been demonstrated to be involved in the anuran spermatogenesis (Izzo et al. 2006; Chianese et al. 2017). Similarly, the AdipoQ system could act as a modulator in the anuran reproductive processes acting through a paracrine and/or autocrine mechanism (Martin 2014). In *P. bergeri*, the presence of AdipoQ and receptor 1 in the cystic germ cells, from spermatogonia to round spermatids, suggests an autocrine mechanism of the molecule towards them while the presence of only receptor 2 in the Sertoli cells, elongated spermatids sustained by them, and detached spermatozoa suggests a paracrine action on these other cells. In addition to the autocrine and paracrine mechanisms, it is necessary to consider the possibility of an endocrine mechanism. AdipoQ in mammals is mainly secreted by white adipose tissue and is present in the circulation at high concentrations among other adipokines (Arita et al. 1999). As far as the amphibians are concerned, an important nutritional reserve for the gonads is the closely associated fat bodies that, in addition, seem to be included in the hypothalamus–hypophysial–gonadal axis, taking part in the reproductive processes (Chieffi et al. 1980). Considering the presence of AdipoR1 and AdipoR2 in the *P. bergeri* cysts, it cannot be excluded that an endocrine mechanism interacts with the local autocrine/paracrine one in the spermatogenesis and gonadal functions. Accordingly, the molecule may be produced by the fat bodies of the frog and intervene in gonadal development and functions. However, there are currently no studies attesting to the production of adipokines by fat bodies.

Adipokines in amphibians remain unexplored despite numerous studies that have demonstrated their importance and multiple actions in regulating several metabolic mechanisms in other vertebrate classes. Accordingly, due to the absence of information on the AdipoQ system in the testis of anurans, this study may open a new research topic facing with the role of the molecule in their reproductive biology.

Author contributions Francesca Simoncelli: conceptualization, investigation, resources, visualization, writing—original draft; and Writing—review and editing. Francesca Mercati: conceptualization, investigation, resources, validation, visualization, writing—original draft; and writing—review and editing. Ines Di Rosa: validation, visualization, writing—original draft; and writing—review and editing. Elisa Palmioli: investigation, validation, visualization, writing—original draft; and writing—review and editing. Cecilia Dall'Aglio: resources, writing—review and editing. Daniele Marini: investigation, writing—review and editing. Anna Fagotti: conceptualization, resources, supervision, visualization, writing—original draft; and writing—review and editing.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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