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Immunolocalization of activin and inhibin at different stages of follicular development in the lizard *Sceloporus torquatus*

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ABSTRACT

The activins and inhibins are glycoproteins with a role in the follicular development of vertebrates, that are found in follicular fluid and somatic follicular cells, with a different pattern among taxa. The principal function of activin (Act) is to modulate the follicle-stimulating hormone (FSH) synthesis and secretion, whereas inhibin (Inh) downregulates it. Both factors are modulators of intraovarian follicular recruitment, oocyte maturation, cell proliferation, and steroidogenic activity. Our aim was to characterize the immunolocalization of Act and Inh in the ovarian follicles during the reproductive cycle of the lizard *Sceloporus torquatus*. Act was detected in the granulosa cells and oocyte cortex in the different stages of follicular development. On the other hand, we identified Inh in the oocyte cortex and the cytoplasm of pyriform and small cells of previtellogenic follicles. Also, we found immunoreactivity in the oocyte cortex, theca, and small cells of vitellogenic and preovulatory follicles. Our data provide evidence that Act and Inh have changes related to the stage of follicular development. This dynamic appears to be conserved among vertebrates and is fundamental to ensure an adequate follicular development in this specie.

1. Introduction

To acquire the competence to interact with sperm and support embryo development, oocytes depend on proper interaction with somatic cells (granulosa and theca) and adjacent follicles [1,2]. Numerous studies in mammals have assessed the role of the members of the superfamily of transforming growth factor-β (TGF-β) in follicular recruitment, selection, dominance, and nuclear and cytoplasmic maturation [3–5].

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Activins and inhibins belong to this superfamily and are considered key regulators of follicular development [6–9]. Both are dimeric glycoproteins conformed by two disulfide-linked subunits: inhibins as heterodimers of α and β subunits (A or B), and activins formed by homodimers of β subunits, leading to the formation of diverse isoforms of activin A ($\beta_A\beta_A$), B ($\beta_B\beta_B$) and AB ($\beta_A\beta_B$) [10]. Each isoform has different dynamics in concentration and localization through the reproductive cycle [6,9,11–13]. Both cytokines have an antagonistic effect and have a pleiotropic function in the regulation of reproduction by influencing cell proliferation, differentiation, hormones secretion, and apoptosis [13]. Numerous studies have reported the presence of both factors in the somatic cells surrounding the oocyte [14–16].

In mammals, activins are involved in primordial follicles growth [11], oocyte maturation [17], granulosa proliferation [9,10], and modulation of follicular cell's steroidogenic activity [6,18]. On the other hand, Inh participates in downregulating FSH secretion and sex steroids synthesis [19]. It induces granulosa proliferation and decreases its apoptosis [20].

In non-mammal vertebrates, some studies begin to clarify the intraovarian role of these glycoproteins and the degree of similarity with mammals. In zebrafish, InhA allows an adequate folliculogenesis and ovulation rate [14]. Meanwhile activin induces the breakdown of germinal vesicle (GVBD) [21] and enhances steroidogenesis [22]. In *Rana pipiens,* Inh blocks GVBD and steroidogenesis [23]. In the laying hens, an increase in InhB prevalence is evident in the pre-hierarchical follicles and shifts to ActA dominance in preovulatory follicles [15]. Studies about intraovarian regulation of follicular development and particularly on Act and Inh in reptiles are scarce. To the best of our knowledge, there is only a group that reported the immunolocalization of Inh in the oocyte and pyriform cells cytoplasm of previtellogenic follicles of the lizard *Podarcis sicula* and suggested that it regulates follicular recruitment and hierarchy [24,25]. However, more research on lower vertebrates is required to provide a better framework for more detailed comparisons. *Sceloporus torquatus* is a viviparous lizard that inhabits the central region of Mexico [26]. Females present an autumn reproductive strategy: the vitellogenesis takes place in late summer, they ovulate in October–November, are pregnant during winter until the following spring and give birth in April–June [26–28]. According to histological criteria, Uribe et al. [29], classified this lizard's follicular development into nine stages. It is noteworthy that the follicular epithelium is multilayered and polymorphic and allows the characterization of the stage of development [29].

There is no sufficient data on the factors involved in the intraovarian control of follicular development in reptiles, which can shed light to the degree of conservation within the mechanisms involved in oocyte maturation among vertebrates. Our aim was to characterize the immunolocalization of Act and Inh in the ovarian follicles during the reproductive cycle of *Sceloporus torquatus*.

2. Material and methods

2.1. Animals

The population was located in the Sierra de Guadalupe State Park (19°37′ N, 99°12' W), State of Mexico. We collected by hand sexually mature females of *S. torquatus* (n = 15) (snout-vent length of 84.60 ± 7.04 mm and 16.06 ± 4.04 g of weight) during June to November under the scientific collecting license (SGPA/DGVS/00579/17 and SGPA/DGVS/02921/19) granted by the Secretaría del Medio Ambiente y Recursos Naturales. The females were transported to the laboratory of Reproductive Biology of the National Autonomous University of Mexico (UNAM, Faculty of Higher Studies Iztacala) in cloth bags and small cages. Lizards were maintained in enclosures (2.60 m × 5.0 m x 2.70 m) with natural photoperiod and temperature. The organisms were fed with crickets and mealworms with free access to food and water during the experimental period. Two months after surgery, we released females to their site of capture.

2.2. Sample collection and histology

All experimental procedures were conducted with the approval of the Bioethical Committee of the UNAM. We anesthetized the females (16 mg/kg sodium pentobarbital) to perform a paramedian celiotomy and obtain the ovarian follicles [30,31]. The tissues were processed for light microscopy as follows: began with fixation in Bouin's solution, dehydration through graded alcohols, clearing by cedar oil, and impregnation with paraffin wax. Tissues were serially sectioned and stained with Masson's trichome and Periodic Acid-Schiff (PAS) for histological studies. We classified the follicles according to the Uribe et al. [29], criteria that consider diameter, the morphology of the follicular epithelium, and yolk deposition.

2.3. Immunohistochemistry (IHC)

Sections 5 μm thick were cut from the paraffin-embedded follicles in each phase of the follicular development (five lizards per phase) and processed for IHC as described by Cruz-Cano et al., 2023 [32]. They were deparaffinized in xylol and rehydrated in descending grades of ethanol. The slides were heated in a water bath at 100 °C with 0.1 M citrate buffer (pH 6.0) for 20 min for antigen retrieval. The activity of endogenous peroxidase was quenched with an incubation in 0.3% H_2O_2 for 30 min and then rinsed in 0.1 M phosphate-buffered saline (PBS) (pH 7.4). To limit the background staining, we employed a preincubation for 30 min in a blocking solution containing one drop of horse serum and 5% bovine serum albumin in 2 ml of PBS. Slides were incubated with the polyclonal primary antibodies rabbit anti-activin A (Thermo Fisher PA5-100101) or rabbit anti-inhibin α (Thermo Fisher PA5-13681) diluted 1:75 in a humidified chamber at room temperature overnight. Tissues were washed with PBS, and incubated with biotinylated Pan-Specific Universal antibody (PK-8800, Vector Laboratories Inc., Burlingame, CA, USA) for 15 min. After a wash with PBS, the sections were incubated for 10 min with streptavidin/horseradish peroxidase complex. The antigens were localized using 0.03% diaminobenzidine

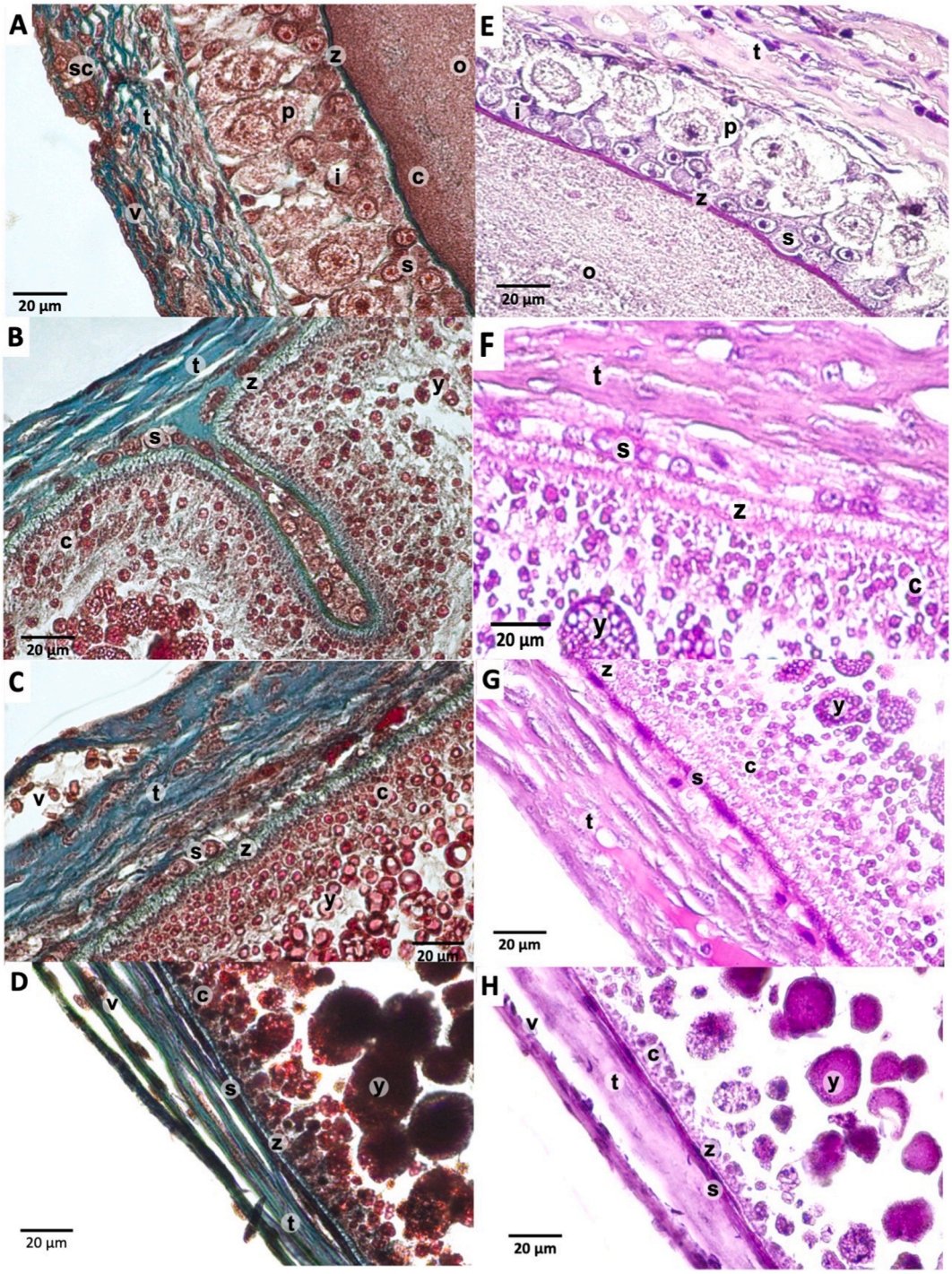

Fig. 1. Morphology and histochemistry of ovarian follicles in the lizard *S. torquatus*. Previtellogenesis: A) The ooplasm (o) is homogeneous with a well-defined cortex (c); there is a multilayered and polymorphic granulosa with small (s), intermediate (i) and pyriform (p) cells. The zona pellucida (z) and a fibrous theca (t) with blood vessels (v) and secretory cells (sc) are clearly distinguishable. E) The zona pellucida (z) and the granules in the cortex (c) are intense PAS-positive. Vitellogenesis (late): B) The oocyte contains yolk granules (y), and granulosa is comprised by a monolayer of small cells (s) that are abundant in follicular invaginations, the zona pellucida (z) is thicker C) the theca (t) has blood vessels (v) and the small cells (s) begin to decrease in size. F, G) Intense PAS-positive is observed in the small cells (s) and yolk granules (y). Preovulatory: D) The zona pellucida (z) decreases in thickness, and the small cells (s) are flattened in a single layer. The theca (t) decreases in height and blood vessels (v) (A-D Masson Trichrome) (E-H Periodic Acid Schiff). Magnification power: 40×.

tetrahydrochloride (DAB; Vector Laboratories) in PBS and 0.1% H_2O_2. Two controls were performed to assess the specificity and effectiveness of the IHC: a negative by omitting the primary antibody and a positive with slides of mouse ovary.

2.4. IHC staining interpretation

We classified the intensities in each reproductive phase as: negative, weak, moderate, or intense for each slide in a double-blinded manner by three authors. Non concluding slides were omitted. We obtained the images with a reflex camera (Canon EOS Rebel T5i) mounted in an optic microscope (Nikon, Eclipse 100).

3. Results

3.1. Ovarian follicles histology

The females of *S. torquatus* exhibited a fall reproductive pattern, like the reported before [29]. As the reproductive cycle progressed, the ovarian follicles developed distinctive features. Previtellogenic follicles were comprised by an oocyte surrounded by a zona pellucida, polymorphic granulosa cells (small, intermediate, and pyriform) (Fig. 1A), and theca. The zona pellucida (z) of these follicles showed the presence of glycosaminoglycans, as seen in the positive Schiff reaction (Fig. 1E). With the onset of vitellogenesis, the height of the follicular epithelium decreased due to the pyriform cells' reduction and flattening, and small yolk platelets were evident in the oocyte cortex (Fig. 1B, C). In the theca, the height and the number of capillaries increased. Reduction of the small cells seemed to be associated with invaginations of the follicular layer (Fig. 1B). The PAS-positive reaction in these follicles was evident in the remaining small cells and the yolk (Fig. 1F, G). Granulosa was a monolayer of flattened-small cells and a vascularized theca in preovulatory follicles. Also, the zona pellucida appeared to decrease in thickness, and small cells were completely flattened (Fig. 1D). In this stage, the PAS-positive reaction was evident in the remnant small cells and the yolk (Fig. 1H).

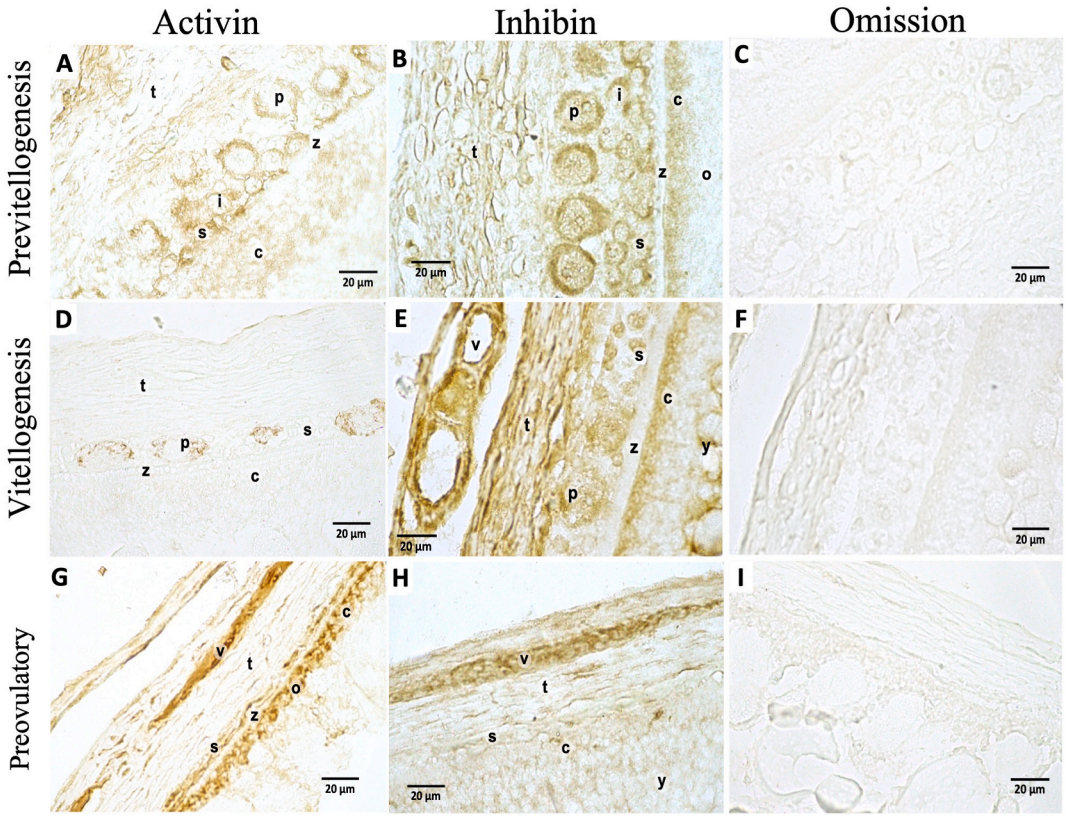

Fig. 2. Immunolocalization of activin (A, D, G) and inhibin (B, E, H) in the ovarian follicles. Previtellogenesis: A) Act immunostaining is moderate in small (s) and pyriform cells (p) weak staining is detected in the cortex (c); B) Intense Inh immunostaining in granulosa cells (s, i, p) cortex (c), and weak in theca (t). Vitellogenesis: D) Intense Act immunostaining in regressing pyriform (p) cells; E) Intense Inh immunostaining in granulosa cells (s, i, p) cortex (c) and theca (t). Preovulatory: G) Intense Act immunoreactivity in the flattened small cells (s), cortex (c) and blood vessels; H) Intense Inh immunoreactivity in the blood vessel (C, F, I omission controls). Magnification power: 40×.

3.2. Activin and inhibin immunolocalization in ovarian follicles

During the previtellogenic stage, the Act was moderately localized in the small and pyriform cells and was scarce in the oocyte cortex (Fig. 2A). With the follicular epithelium remodeling during the vitellogenic stage, we observed the immunoreactivity in regressing piriform cells (Fig. 2D), and weak signal in the cortex. Before the ovulation, an intense Act signaling was found in the blood vessels, the flattened small cells, and the cortex of the oocyte with a weak immunoreactivity at the cortex (Fig. 2G).

Previtellogenic follicles exhibited an intense Inh immunoreactivity in the granulosa cells (more intense in the pyriform cells) and oocyte cortex (Fig. 2B). In vitellogenic follicles, intense immunostaining spread out to the granulosa cells, theca, cortex, and yolk (Fig. 2E). In preovulatory follicles, intense immunoreactivity was localized in blood vessels and moderate in the yolk (Fig. 2H).

No immunostaining reaction was observed in the controls of each follicular development stage (Fig. 2C, F, I).

4. Discussion

This work contributes to the comprehensive and rational study of the ovarian activity and physiology in lizards, particularly *S. torquatus*. Our approach allowed us to study the presence of Act and Inh at different stages of follicular development without compromising the life and fertility of the females studied.

The morphology of the ovarian follicles in *S. torquatus* changed as the oocyte grew, as observed before for this lizard [29]. In the vitellogenic follicles, when the granulosa layers were reduced, we found invaginations of the follicular layer filled with small cells, as reported previously in *P. sicula* [33]. These invaginations may be related to granulosa cell reduction and flattening rather than proliferative properties [34–39]. Future studies should focus on the role of these invaginations in remodeling the follicular epithelium in lizards and determine the role of each cell type during oocyte maturation.

Follicular development is regulated by multiple factors [2,40] in which Act and Inh have a critical role in oocyte maturation [6,9,11,13]. However, the information available on the molecules that play a role in reptiles is scarce and limited to model species in other vertebrates. Our study corroborates the presence of these proteins in the ovarian follicles of *S. torquatus*. Also, the localization of each factor is related to the stage of follicular development where they participate in female reproductive physiology at different levels [10]. The participation of the granulosa layer in follicular development is corroborated by the presence of Act, Inh, and other intraovarian factors such as gonadotropin releasing-hormone (GnRH), gonadotropin release inhibitory hormone (GnIH), bradykinin [41], sex steroid receptors [32,42,43], SCF, C-kit [33], and its receptors in lizards.

In the previtellogenesis, the polymorphic granulosa is a trademark of Squamata follicular epithelium [24,34,44]. Of the three cellular types recognized, the pyriform cells have a critical role in supporting the oocyte maturation [45] and providing small molecules to the germ cell via intercellular bridges [35,36,46,47]. Act presence in this stage, suggests that it may participate by inducing oocyte competence and maturation while modifying the activity of follicular cells and their response to FSH [3,15,21,48,49]. Also, Act could facilitate the proliferation of follicular epithelium as reported in mammals [50]. We found Act immunoreactivity at the stage of vitellogenesis when pyriform cells regressed. We suggest this factor might be acting synergistically with other factors such as GnRH and their receptors via DNAse activation to induce their apoptosis [39,51] and the transition to the vitellogenic state [14]. In the preovulatory phase, the small cells, cortex, and blood vessels showed marks that may reflect their role in steroidogenesis [10,52]. The registered peak of estradiol concentrations with the onset of ovulation in this species confirms this fact [28,32]. In the hen, the increase of activin is related to the selection of preovulatory follicles to ovulate [53], and in lizards, it may have a similar function by increasing receptor appearance [54]. To the best of our knowledge, this work is the first evidence of the presence of Act in lizards. However, the steroidogenic activity of each cell type has not been addressed yet.

In the case of Inh, this factor also is also secreted by the follicular cells and has multiple roles in the reproductive physiology of females [55]. In mammals, the main recognized effect is to diminish the FSH concentration and limit the Act effects in the hypophysis [56] hence controlling the number of follicles recruited and ovulated [9]. A similar phenomenon is observed in the lizard *P. sicula,* where this protein controls the number of oocytes recruited from germinal beds [24,44]. Furthermore, the amount of Inh in this phase may be limiting the growth of newly recruited and subordinate follicles [3,11,57,58] keeping the gonads in a seasonal refractory state [59]. Inh blocks GVBD and modifies steroidogenic activity like that reported in fishes and frogs [23,60]. We consider that the events of follicular dominance can be studied in the squamate model considering the benefits of a seasonal proliferation of the oogonia in the germinal bed and the polymorphic granulosa. It can shed light on the roles according to the granulosa cell type. Finally, this protein diminution may be associated with steroidogenic activity in the preovulatory follicles [49].

A modification in the expression and concentrations of Act and Inh suggests that the events regulated by these glycoproteins are conserved among taxa and indispensable for an adequate follicular development [22]. Our results indicate Act activity is more intense in oocyte and granulosa cells, then it changed to the cortex of the oocyte with the vitellogenesis. The similar behavior in both molecules could reflect their interaction as a regulatory system of follicular development in *S. torquatus* during the annual reproductive cycle in a natural population. For future research we suggest the use of more specific methods of protein detection with a quantifiable nature to understand the regulatory mechanisms of follicular development in reptiles.

5. Conclusions

Our observations suggest that the Act and Inh system can modulate the ovarian activity of this lizard as in other vertebrates. The immunoreactivity was more evident in the granulosa cells, particularly in the pyriform cells and the cortex. Moreover, the temporal localization of these proteins is related to the stage of follicular development and might be related to oocyte maturation. However, an

experimental approach with a more specific and quantifiable detection system is needed to elucidate the role of each factor in the different cell types and phases.

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Author contribution statement

Norma Berenice Cruz-Cano: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Uriel Ángel Sánchez-Rivera: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Carmen Álvarez-Rodríguez; Romeo Eduardo Loya-Zurita; Yabín Josué Castro-Camacho: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Martín Martínez-Torres: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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