Zoomorphology (2017) 136:219–224
DOI 10.1007/s00435-017-0344-0

ORIGINAL PAPER

Developmental morphology of granular skin glands in pre-metamorphic egg-eating poison frogs

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Received: 8 November 2016 / Revised: 24 January 2017 / Accepted: 27 January 2017 / Published online: 20 February 2017

Abstract Parents in many taxa, including insects, molluscs, fish, snakes, and amphibians provision chemical defences, such as peptides, steroids, or alkaloids to their offspring to reduce the risk of predation. In most cases, those defences are transferred to offspring in the egg and gradually diminish throughout the larval period. Adult poison frogs sequester alkaloid-based defences from arthropod prey in granular skin glands. In at least one poison frog, *Oophaga pumilio*, mother frogs intermittently feed tadpoles until metamorphosis with nutritive eggs containing those alkaloid-based defences. However, alkaloids are not detected in tadpoles until they reach the middle stages of larval development. Here, we investigate the histology of a developmental series of *O. pumilio* tadpoles to determine whether their ontogenetic alkaloid profile coincides with granular gland development. Our findings suggest that alkaloid sequestration in tadpoles is delineated by the differentiation of rudimentary granular skin glands in epithelial tissue. The timing of differentiation of granular glands in this species coincides with other anurans. Thus, provisioning of chemical defences to offspring is likely constrained by developmental timing of derived structures that can effectively store those toxic or noxious compounds.

Keywords Alkaloid · Ontogeny · Poison gland · Provisioning · Tadpole

Introduction

Parents in many taxa reduce predation of young by provisioning offspring with chemical defences (Hutchinson et al. 2008; Stynoski et al. 2014a; Santos et al. 2016 and therein). While this behaviour and its associated chemical ecology is well studied in some invertebrates (e.g., Dussourd et al. 1988; Williams et al. 2011), less is known about vertebrates that endow eggs with chemical defences, such as some fish (Itoi et al. 2013), snakes (Hutchinson et al. 2008), salamanders (Hanifin et al. 2003), and frogs (reviewed in Gunzburger and Travis 2005). Even less is understood about more complex vertebrate systems in which chemical provisioning continues after hatching or birth, such as in the strawberry poison frog (*Oophaga pumilio*; Stynoski et al. 2014a). A complete understanding of the processes of sequestration, transport, and storage of diet-based chemicals in parents and offspring will require ecological, morphological, genomic, and functional perspectives. Such an understanding will play a key role in elucidating the evolution of chemical defence and the mechanisms behind the management of alkaloid compounds with important biological functions as well as real-world applications (e.g., medicines and agricultural toxins).

The unique skin of amphibians is filled with mucous, granular, and/or lipid glands (Toledo and Jared 1995; Angel et al. 2003; Delfino et al. 2010; Chammas et al. 2015). Granular (serous, poison) glands synthesise or sequester an incredible diversity of compounds that serve as defence against predators and pathogens (Saporito et al. 2012; Santos et al. 2016; Mina et al. 2015). Chemical defences are

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Electronic supplementary material The online version of this article (doi:10.1007/s00435-017-0344-0) contains supplementary material, which is available to authorized users.

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especially important to the survival of amphibians during the juvenile and adult phases and in some cases the egg phase (Gunzburger and Travis 2005; Saporito et al. 2012; Santos et al. 2016). However, tadpoles are thought to be less chemically defended, because they lack both yolk and granular glands in which to store toxic or noxious compounds (reviewed in Gunzburger and Travis 2005). Studies on the development of amphibian granular glands are limited (Angel et al. 2003; Gunzburger and Travis 2005; Delfino et al. 2010; Chammas et al. 2015). In anurans, ectoderm-derived precursor cells of glands begin to aggregate in pre-metamorphic stages (~30–38 of staging table by Gosner 1960), form an alveolus containing early granules in the initial metamorphic stages (~38–40), and become small adult-type glands with secretory product present during metamorphic climax (~41–46; see Fig. 1; Toledo and Jared 1995). Glands migrate from the epidermis to the dermis during the terrestrial juvenile phase (Toledo and Jared 1995).

In *O. pumilio*, mother frogs visit larval offspring every 1–8 days across 6 or more weeks of development to provide unfertilised eggs as food, without which offspring starve (Brust 1993). Tadpoles of other species in this group, at least beyond the mid-larval stages, exhibit mouthparts that are specialized for an egg-based diet (Myers et al. 1984; Grant et al. 2006). Recent work demonstrated that mothers provision nutritive eggs with alkaloids derived from a diet of leaf-litter arthropods (Stynoski et al. 2014a), which effectively defend offspring from some predators (Stynoski et al. 2014b). Alkaloids were not detected in recently hatched tadpoles, but were detected in rising concentration and diversity after stage 34 (Stynoski et al. 2014a). It was suggested that this ontogenetic alkaloid profile may reflect temporal modulation of alkaloid provisioning by mother frogs or developmental shifts in tadpole sequestration ability in the skin or elsewhere (Stynoski et al. 2014a). In this study, we investigate the latter hypothesis.

Juvenile *O. pumilio* have small granular glands that increase dramatically in diameter at sexual maturity (Saporito et al. 2010). In related dendrobatid species in which alkaloid sequestration in detritus-eating tadpoles is not known (*Dendrobates auratus*, Angel et al. 2003; *Phyllobates bicolor*; Delfino et al. 2010), rudimentary glands are first seen in late pre-metamorphic stages. However, the larval morphology of granular glands has not been described in the obligatorily egg-feeding genus, *Oophaga*. Here, we describe the timing of granular gland morphogenesis in a developmental series of larval *O. pumilio* in an effort to determine whether the onset of alkaloid sequestration known in this species coincides with gland ontogeny. In addition, because skin alkaloids are diet-based, changes in tadpole oral structures across development could affect their ability to ingest and sequester alkaloids. Therefore, we also examine whether development of oral structures is concurrent with granular gland maturation and alkaloid sequestration.

Methods

Oophaga pumilio tadpoles were reared by captive-bred parents in tanks at Harvard University. Mother frogs were fed *Drosophila* flies and, therefore, were not provisioning alkaloids to tadpoles. Seven tadpoles (stages

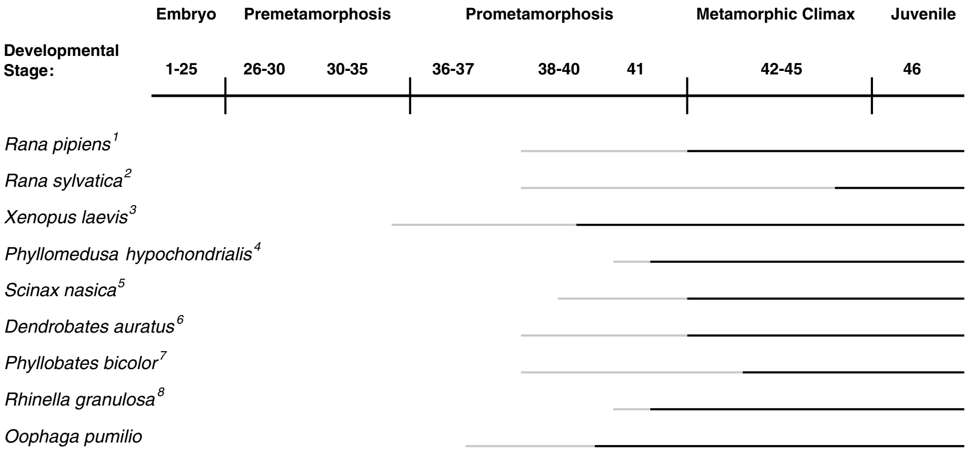

Fig. 1 Literature reports of timing of anuran granular skin gland development, indicating the first stage examined that contained structures. Early glands (*grey bars*) refer not only to a cluster of rudimentary gland cells, but to an alveolar configuration. Adult-like glands containing granules of secretory product (*black bars*) generally appear during or just prior to metamorphic climax (i.e., four legs present). Stages reported according to Gosner (1960), following conversion from Nieuwkoop and Faber (1956) or Taylor and Kollros (1946) staging tables when necessary using Table 2.1 in McDiarmid and Altig (1999). *1* Bovbjerg (1963), *2* Formanowicz and Brodie (1982) *3* Reilly et al. (1994), *4* Delfino et al. (1998), *5* Terreni et al. (2003), *6* Angel et al. (2003), *7* Delfino et al. (2010), *8* Chammas et al. (2015)

25, 25, 27, 32, 33, 40, and 41) were euthanised by application of topical 20% benzocaine and fixed in 4% paraformaldehyde in 1X phosphate buffered saline (PBS) overnight at 4 °C. The following day, tadpoles were cryoprotected in 30% sucrose in 1X PBS with 0.01% sodium azide and stored at 4 °C for 5 days until shipment to Colorado State University for sample processing.

We verified tadpole stage and photographed each tadpole and its mouthparts using an Olympus stereoscope, and then used the ImageJ 1.47t (NIH) software to measure total length (Table 1). We flash froze tadpoles in isopentane and sectioned in the transverse plane on a cryostat at 18 µm. We stained tissue with toluidine blue and counterstained with eosin. All tissue sections were examined with an Olympus microscope at 10-20X for the presence of developing granular or other glands.

Results

Tadpoles at stages 25–27 did not show evidence of glands, nor any skin structures resembling rudimentary granular glands or mesenchymal cell clusters, as in other studies (Toledo and Jared 1995; Angel et al. 2003; Delfino et al. 2010; Chammas et al. 2015; Fig. 2). At stages 32–33, tadpole epidermis contained some clustered mesenchymal cells, but no mature structures. At stages 40–41, the epidermis contained numerous small granular glands with alveoli that were just beginning to migrate to the dermis. The alveoli contained translucent granules as in Angel et al. (2003) (Fig. 3).

Early (stage 25–27) tadpoles had a keratinised upper jaw sheath, but no keratodont rows (Fig. 4). Later tadpoles (stage 32–41) possessed one posterior keratodont row, the oral disc papillae were more elaborate and thick, and the upper jaw sheath was larger and more serrated.

Discussion

We provide histological evidence that the developmental morphology of the granular skin glands in *Oophaga pumilio* coincides with the species' ontogenetic alkaloid profile (Stynoski et al. 2014a). Here, we show that granular skin glands first develop between tadpole stages 32 and 40 in *Oophaga pumilio*. Past work detected maternally derived alkaloids in tadpoles after stage 34 and in increasing amounts up to stage 45, but not yet at stage 25 (Stynoski et al. 2014a). Therefore, it is likely that pre-metamorphic morphogenesis of skin structures—rather than modulation of alkaloid provisioning to nutritive eggs by mother frogs—delimits the onset of alkaloid sequestration, although the alkaloid content of trophic eggs across tadpole development remains to be determined.

Tadpoles are thought to be most vulnerable during metamorphic climax (stages 42–45) due to higher caloric content, reduced swimming ability with four bulky limbs, and energetic allocation to morphological transformation (Wassersug and Sperry 1977; Arnold and Wassersug 1978). Acquiring defensive chemicals prior to metamorphosis, whether via biosynthesis as in some other amphibians or through sequestration of egg-derived alkaloids as in *O. pumilio* (reviewed in Saporito et al. 2012; Santos et al. 2016), should benefit metamorphosing tadpoles (Stynoski et al. 2014b). An additional row of keratodonts and a stronger and sharper upper jaw sheath appear concurrently with early granular glands (as seen in other maturing dendrobatid tadpoles; Myers et al. 1984; Grant et al. 2006). Although these tadpoles eat eggs throughout the larval period (Brust 1993; Stynoski 2009), these oral structures may aid late stage tadpoles in better tearing and anchoring on to them (de Sousa et al. 2014), perhaps increasing consumption of alkaloid-containing material once they can sequester those compounds.

Past studies have shown that alkaloid concentration and diversity are correlated with the diameter of granular skin glands in juvenile and adult *O. pumilio* (Saporito et al. 2010). It is not known whether the quantity of

Table 1 Lengths and staging characteristics of tadpoles examined in developmental series of *Oophaga pumilio*

Gosner stage	Stage characteristics	Total length (mm)
25	No gills or limb buds	10.0
25	No gills or limb buds	10.4
27	Hind limb buds >½ and <1 times as long as wide	14.9
32	Hind limb buds "mitten" shaped	18.1
33	Hind limb buds with 3 early digits	17.9
40	Subarticular tubercles and cloacal tail piece, no forelimbs	21.4
41	Forelimbs visible under skin	21.9

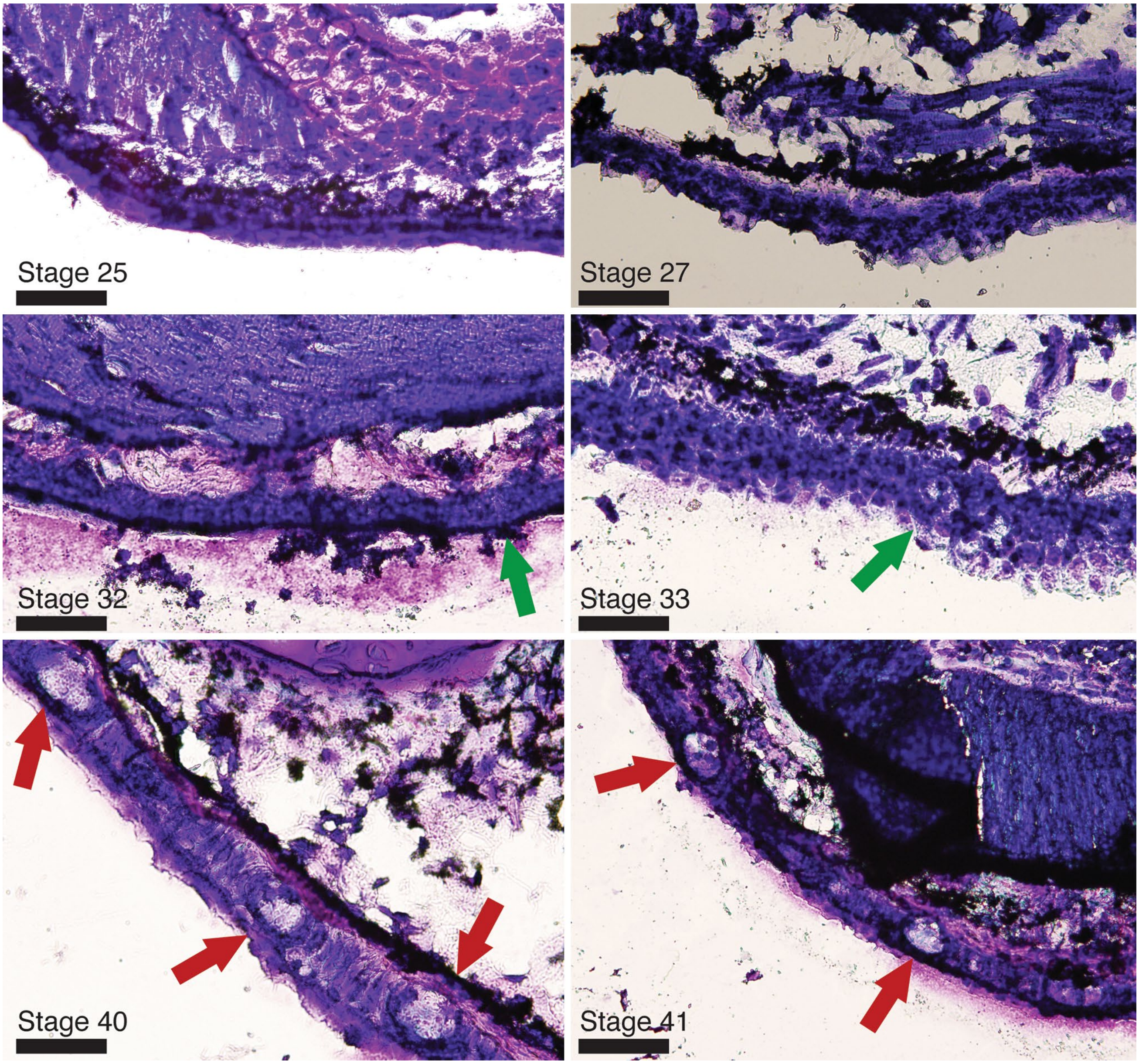

Fig. 2 Microphotographic images (×20) of granular skin gland development in *Oophaga pumilio*, which starts with clusters of epithelial cells in stage 32 and alveoli forming in stage 33 (*green arrows*). By stages 40–41, glands containing granules (*red arrows*) are visible. Gland morphogenesis complements this species' ontogenetic alkaloid profile in which alkaloids were detected in tadpoles stage 34 and above, but not in stage 25. *Scale bars* measure 50 μm

alkaloid that is consumed or provisioned can influence the timing of differentiation, growth, or ultimate size of granular glands or vice versa (Saporito et al. 2010; Stynoski et al. 2014a). In the current study, granular glands differentiated and were forming granules (presumably alkaloid-free) in the absence of alkaloid consumption. Angel et al. (2003) also found that captive-reared tadpoles of confamiliar *Dendrobates auratus* were producing "secretory aggregate" in their glands as early as stage 38. The chemical identity of such granules as well as those seen in stage 40 and 41 *O. pumilio* tadpoles in the current study are not likely to be a toxic or unpalatable chemical, as captive-reared dendrobatids at any stage are well known to be neither toxic nor noxious (Saporito

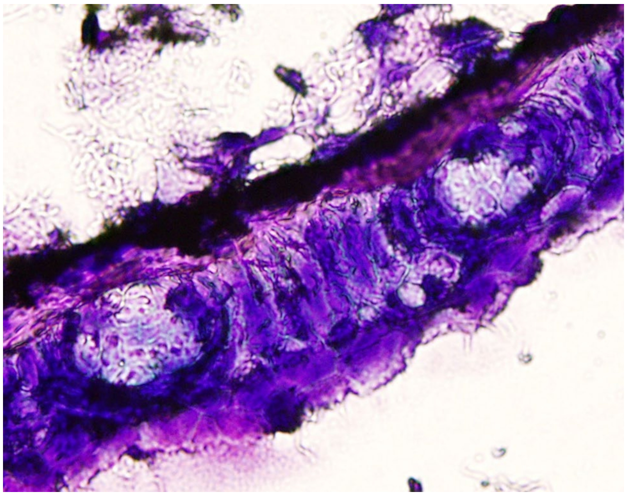

Fig. 3 Microphotographic image of granular skin glands in stage 40 *Oophaga pumilio* tadpole showing the presence of translucent granules

et al. 2012; Santos et al. 2016 and therein); perhaps, these granules help to maintain exogenous alkaloids when available. Adults of a dendrobatid frog that does not sequester alkaloids, *Mannophryne* [=*Colostethus*] *trinitatis*, also exhibit granules in granular glands (Neuwirth et al. 1979; Grant et al. 2006; Santos et al. 2016). Future work using labeled alkaloids or other chemical imaging methods would confirm the ability of *O. pumilio* tadpoles to store alkaloids in early glands and/or in other tissues.

The differentiation of granular glands in the late premetamorphic stages coincides temporally across anurans, including *O. pumilio*, with no obvious trends of heterochrony. In amphibian taxa basal to dendrobatids, such as the bufonid toads, larvae generate granular glands and synthesise chemical defences concurrently in pre-metamorphic development (Chammas et al. 2015). In later life stages, those granular glands are mostly used for storage of chemicals with minimal biosynthesis (Angel et al. 2003). The ability seen in poison frogs to sequester and store lipid-soluble diet-based alkaloids has evolved independently in at least five amphibian families (Saporito et al. 2012; Santos et al. 2016), perhaps from co-opted glands that historically were biosynthesising other chemical defences, such as peptides, steroids, and biogenic amines, which in turn are thought to have evolved from regulatory mucous glands (Neuwirth et al. 1979; Daly et al. 1987; Toledo and Jared 1995). Ontogenetic functional and transcriptomic analyses that explain uptake and storage in a comparative context would illuminate the repeated evolution of alkaloid sequestration in tadpole and frog skin.

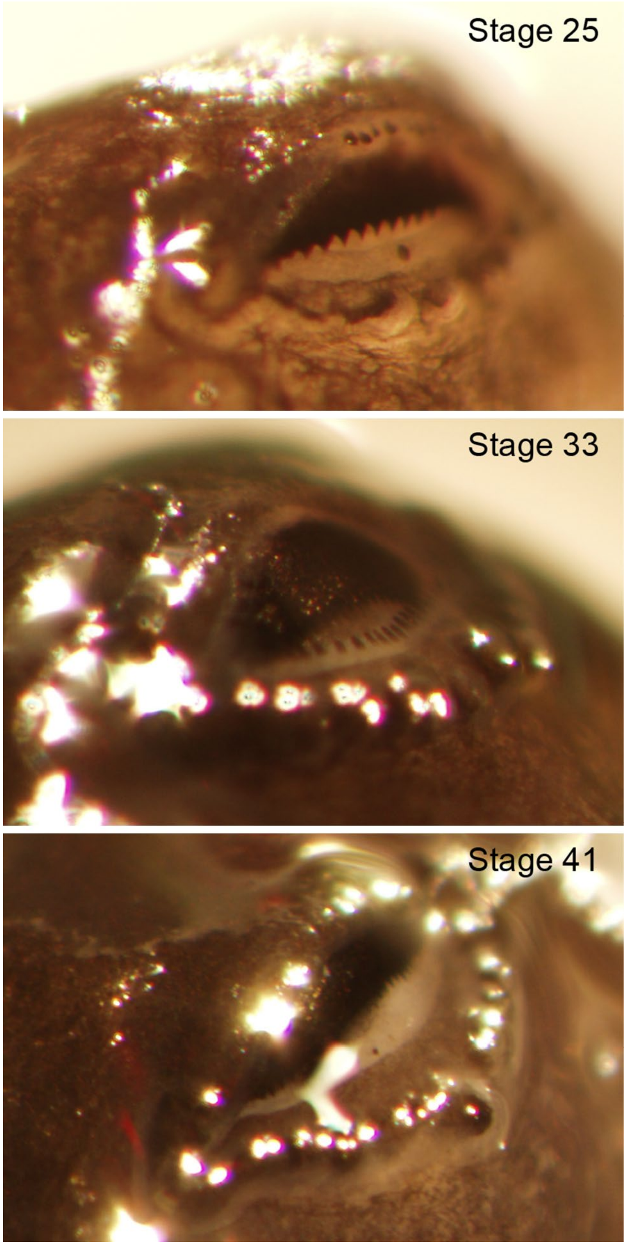

Fig. 4 Stereophotographic images (×3) of the mouthparts of *Oophaga pumilio* tadpoles at stages 25, 33, and 41. Development includes elaboration of oral disc and its papillae, expansion and further serration of the upper jaw sheath, and the addition of a posterior larval keratodont row

Acknowledgements The authors thank Kim Hoke for equipment use and Alexandre-Benoit Roland for animal care. JLS is supported by an American Association for University Women American Postdoctoral Fellowship, and LAO is supported by a Bauer Fellowship from Harvard University, the L'Oreal Women in Science Fellowship, the William F. Milton fund from Harvard Medical School, a Konishi Research Grant from the International Society for Neuroethology, and the National Science Foundation (IOS-1528866).

Author contribution JLS processed samples and wrote the manuscript. LAO acquired samples and edited the manuscript. Both designed the study.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the Institutional Animal Care and Use Committee of Harvard University (Protocol 12-10-01). This article does not contain any studies with human participants performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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