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Eggshell structure in *Caiman latirostris* eggs improves embryo survival during nest inundation

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Egg inundation often results in poor hatching success in crocodylians. However, how tolerant eggs are to submergence, and/or how eggshell ultrastructure may affect embryo survival when inundated, are not well understood. In this study, our objective was to determine if embryo survival in *Caiman latirostris* is affected by eggshell surface roughness, when eggs are submerged under water. Tolerance to inundation was tested early (day 30) versus late (day 60) in development, using eight clutches (four per time treatments), subdivided into four groups: ($N = 9$ per clutch per treatment; $9 \times 4 = 36$ eggs per group). 'Rough' eggshell represented the natural, unmodified eggshell surface structure. 'Smooth' eggshell surface structure was created by mechanically sanding the natural rough surface to remove surface columnar elements and secondary layer features, e.g. irregularities that result in 'roughness'. When inundated by submerging eggs under water for 10 h at day 30, 'smooth' eggshell structure resulted in more than twice as many dead embryos (16 versus 6, smooth versus rough; $N = 36$), and fewer than half as many healthy embryos (6 versus 13, smooth versus rough, respectively; $N = 36$). By contrast, at day 60, inundation resulted in very low hatching success, regardless of eggshell surface structure. Only two hatchlings survived the inundation, notably in the untreated group with intact, rough eggshells. Inundation produced a high rate of malformations (58% at day 30), but did not affect hatchling size. Our results indicate that eggshell roughness enhances embryo survival when eggs are inundated early in development, but not late in development. Apparently, the natural surface 'roughness' entraps air bubbles at the eggshell surface during inundation, thereby facilitating gas exchange through the eggshell even when the egg is submerged under water.

1. Introduction

Embryonic mortality is high in different species of crocodylians, such as *Alligator mississippiensis* [1–3], *Crocodylus porosus* [4–6], *C. niloticus* [7], *Caiman crocodilus* [8], *Ca. yacare* [9], *C. johnstoni* [10] and *C. acutus* [11]. Nest depredation is a major cause of egg loss [12–15], but other factors such as weather, i.e. flooding and/or drought, are also important sources of egg mortality. Nest inundation is particularly significant in rainy years [3,12,16]. However, little is known about the tolerance of the embryos to flooding [6,17], or if this tolerance could be altered by eggshell structure. As development occurs, the eggshell gets thinner [18] and the number and size of pores increases [19,20]. These changes could be adaptations to avoid hypoxia, as hypoxia reduces hatching success [21] and/or has subsequent adverse effects on hatchlings

[22]. Furthermore, embryo tolerance to hypoxia changes during development [21].

Female broad-snouted caiman (*Ca. latirostris*) lay eggs from mid-December through January. After 70 days of incubation (depending on temperature [23]), hatching occurs from the end of February into mid-April [24]. Egg incubation occurs during the warm and rainy months [25,26], increasing the probabilities of embryonic death due to inundation. Female broad-snouted caiman use floating vegetation for nesting when available (they utilize the vegetation available to make the mount nest), because depredation in floating vegetation is lower [13,15], and also inundation is minimized if the nest floats on the water surface. During extreme rain events, the floating vegetation absorbs some water, and consequently, nests are sometimes inundated for short periods, typically for hours rather than for days.

Previous studies have related egg structure to nesting environment. Specifically, it has been suggested that ornamentation could have evolved to increase survival of dinosaur eggs incubated in high humidity nesting environments [27,28]. We hypothesized that the roughness (eggshell surface structure, microarchitecture of the outside of the eggshell) of caiman eggs has evolved to increase egg survival during these short-term inundations. Caiman eggs are characterized by having calcite micro-ornamentations with the form of columns or deposits that extend on different highs and are irregularly connected with adjacent columns [29]. On the eggshell surface, there are micro depressions and craters, producing concavities. These surface structures on the outer eggshell (craters and columns) form cavities. Typically, pores are centred in these concavities [29]. During inundation events, due to the superficial water tension, such cavities facilitate the formation of air bubbles that could improve embryo survival. Moreover, those structures could cushion air bubbles against higher hydrostatic pressure of water when eggs are submerged, compared with smooth surfaced eggs, devoid of any outer eggshell ultrastructure (M. S. Fernández 2014, personal communication).

In this study, we investigated whether eggshell roughness affected hatching success when *C. latirostris* eggs were experimentally inundated for short time periods during early versus late development. Eggshell surface features were experimentally removed by sanding, creating a 'smooth' eggshell surface which tended to minimize entrapment of air bubbles at the outer eggshell surface during submergence under water. We hypothesized that embryonic survival and hatching success would be greater in eggs with surface 'roughness', and reduced for eggs 'smoothed' by mechanical sanding to remove surface irregularities.

2. Material and methods

Eight *Ca. latirostris* nests were collected from the wild within Santa Fe province, Argentina in December 2014, as part of the ranching programme 'Proyecto Yacaré'. At the time of harvest, egg viability was based on the presence of an opaque band [30]. Nests were located by active search by researchers within swamps on the floating vegetation and forest. Eggs were marked, removed from the nest and transported in containers (plastic tank of 20 l) to Proyecto Yacaré facilities (Laboratorio de Zoología Aplicada: Anexo Vertebrados-FHUC; UNL/MAS-PyMA). In the laboratory, one egg per clutch was opened to determine developmental stage; all eight nests were younger than stage 4 (4 days of incubation at 31°C; [30]). Clutch size ranged from 37 to 40 per nest. The eight nests were randomly separated into two groups of four clutches; each nest was split into four treatments (nine eggs per nest per treatment).

Eggshell roughness was modified when the eggs were about 5 days old, by a gentle sanding of the eggs until they felt smooth to touch. Sanding was done with a commercially available fine sand paper of aluminium oxide grade 150 (from a hardware shop) without rotating or changing the egg's orientation, in order to avoid mechanical trauma to developing embryos. Once sanded, eggs were brushed so no abraded material was able to fill pores (see figure 1*a*,*b*; SEM images of rough and smooth eggshell were taken at Laboratorio de Microscopía Electrónica (CICyTTP-CONICET). We weighed every egg before and after removing eggshell structures. Incubation of eggs was at 32 ± 1°C and high relative humidity (estimated over 90% RH, but not measured). During incubation, each egg was positioned with the bottom surface on vermiculite, and the top surface covered with nesting material. Each egg was submerged under tap water for a 10 h period (at room temperature, approx. 25°C). Four nests were inundated when they were 30 days old, and the other four nests inundated at 60 days of incubation. We weighed (nearest 0.1 g) every egg before the inundation, those inundated were weighed again after the treatment, and then completed their incubation in the same conditions as the treatments that were not inundated.

Prior to hatching, we isolated every egg in order to identify hatchlings. Once hatched, the snout-vent length (SVL) was measured (precision 1 mm) and weighed (0.1 g). We also recorded hatchlings with any abnormality or malformation, such as not absorbed vitelline sac, swollen jowls, spinal cord deformation and limbs absent/deformation (figure 2). Data on hatching success were analysed with Mix General Lineal Model, using the four treatments as fixed effects, nest or origin was used as a random effect and we used hatching success (hatch or not hatched, binomial mode) a dependent variable. We performed two analyses, one for the 30 days group, the other for the 60 days group. Data on body mass and hatchling size were analysed with General Lineal Model, using the four treatments as fixed effects, nest or origin was used as a random effect and we used SVL and mass as dependent variables. We performed two analyses, one for the 30 days group, other for the 60 days group. Using a Mixed General Lineal Model, we tested eggs inundated at 30 days using the four treatments as fix effects, nest or origin was used as a random effect and we used malformation (healthy or malformed, binomial mode) as the dependent variable.

3. Results

Egg sanding resulted in a weight reduction of 0.6 ± 0.2 g (range 0–1.6 g), and did not affect embryo development, because when not inundated, sanded (smooth eggs) and not sanded eggs (rough eggs) had similar hatching success (over 90%; $p = 0.3501$). Sanding eggs did not affect egg weight when inundated, sanded eggs increased 1.0 ± 0.7 g (range 0.2–4.2 g); control eggs increased 1.3 ± 0.7 g (range 0.3–3.5 g).

When submerged, eggs in which the outer eggshell surface was intact (natural), and unaltered by sanding, exhibited air bubbles (of different sizes) and they were distributed throughout the entire surface of eggshell, in contrast with those that were 'smoothed' by sanding off the outer irregularities (figure 1*c*,*d*). This observation supports the hypothesis that shell outer surface architecture facilitates trapping air bubbles during inundation. The entrapped air

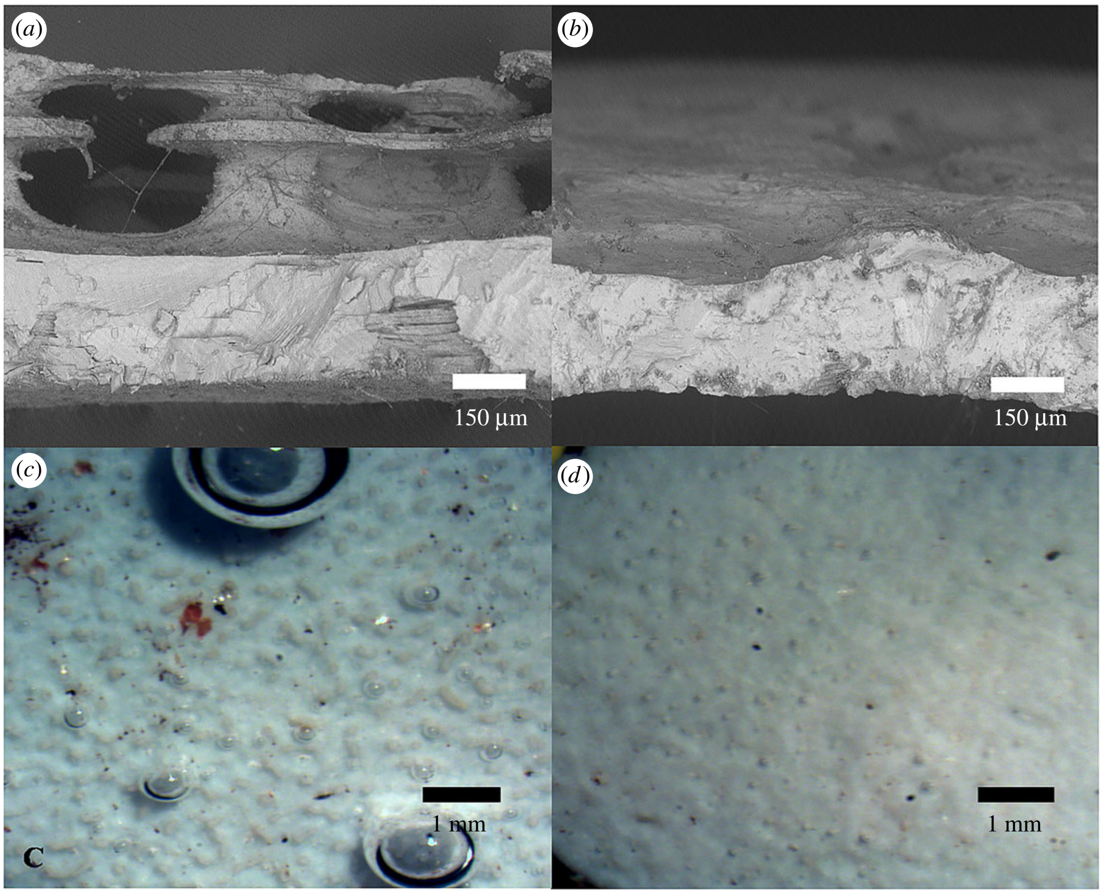

Figure 1. Enlarged cross-section (top, *a*,*b*) images of outer surface of a *Caiman latirostris* egg, 'rough' surface (*a*) showing interconnected columns and thin secondary shell layer atop columns, and 'smoothed' outer surface after sanding (*b*) to remove irregular features shown in upper left view. When submerged in water (bottom, *c*,*d*) large and small air bubbles are evident on the rough surface (*c*) but not on the 'smoothed' surface that was sanded (*d*). SEM images from CICyTTP-CONICET (Diamante, Entre Ríos, Argentina). (Online version in colour.)

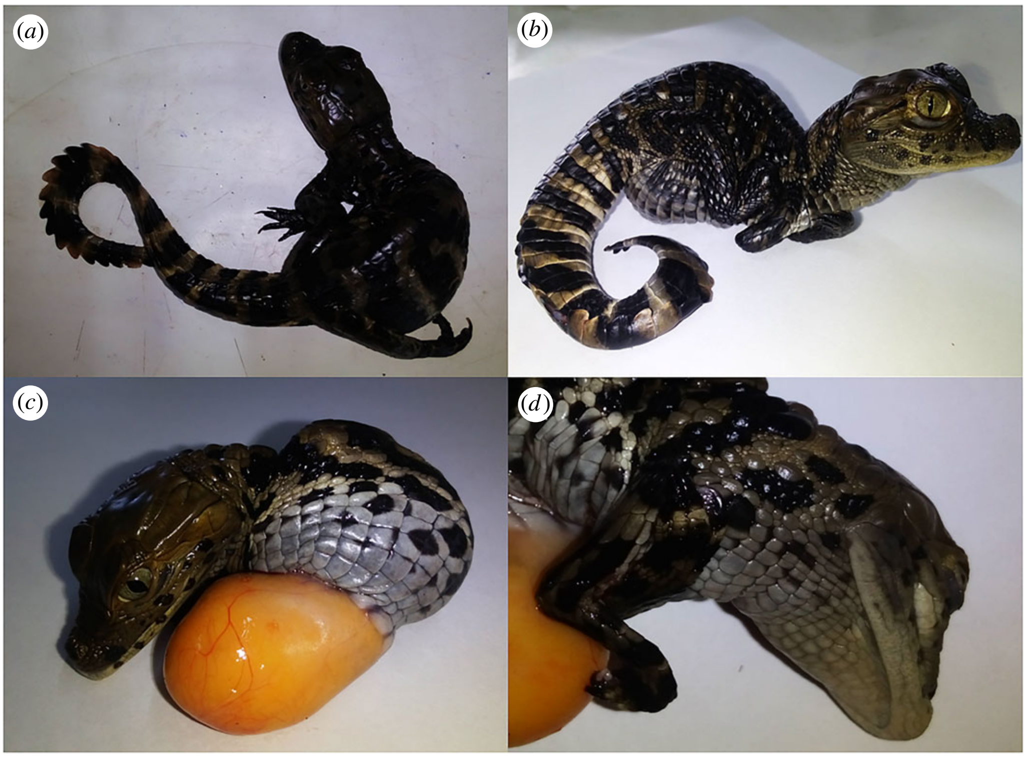

Figure 2. Malformations. Most common malformations were (*a*) spinal cord deformation, (*b*) limbs absent/deformation, (*c*) unabsorbed vitelline sac and (*d*) swollen jowls. (Online version in colour.)

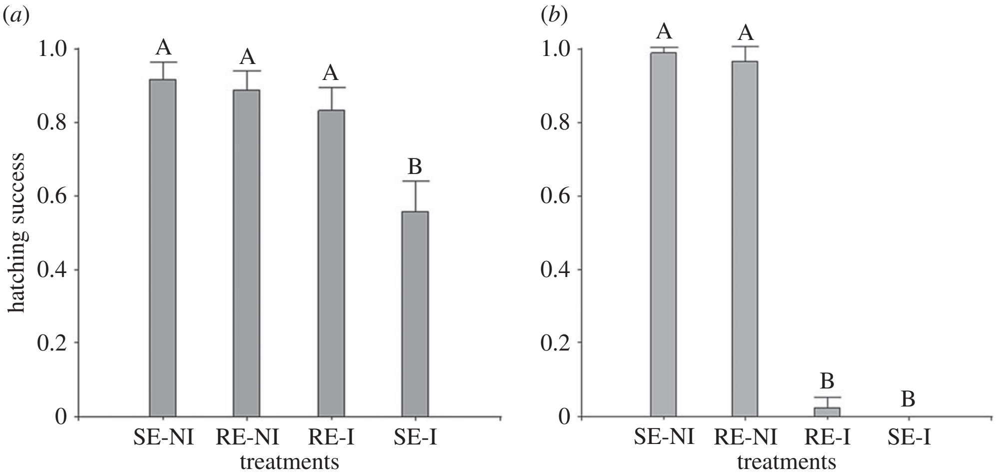

Figure 3. Hatching success of *Ca. latirostris* eggs for the four treatments (rough eggshell-non-inundated (RENI), rough eggshell-inundated (REI), smooth eggshell-non-inundated (SENI) and smooth eggshell-inundated (SEI)) when inundation occurs at 30 days (*a*), and at 60 days (*b*) of embryos' development. Differences are statistically at *p*-value lower than 0.05, and different letters indicate differences.

bubbles may act as oxygen reservoirs during submergence, resulting in an increase in egg survival during these short periods of flooding.

When inundated, at day 30, eggs with a 'smooth' eggshell structure produced more than twice as many dead embryos (16 versus 6, smooth versus rough; $N = 36$ in each group), and less than half as many healthy embryos (6 versus 13, smooth versus rough respectively; $N = 36$ in each group). Inundated eggs with rough eggshells had a hatching success similar to that of not inundated eggs; smooth eggs that were inundated had lower hatching success than the other three treatments ($p = 0.0016$; table 1 and figure 3). On the other hand, at day 60 of embryo development, inundation decreased hatching success in both groups (with or without scraping eggshell surfaces) (the treatment smooth eggshell-inundated was excluded from our analyses because no caiman hatched from this group; $p = 0.0004$; table 1 and figure 3). Inundation (at day 30 or at day 60) or removal of eggshell roughness did not influence morphometric characteristics of hatchlings, all treatments presented similar SVL and mass (at day 30 SVL $p = 0.6612$, mass $p = 0.3011$; and at day 60 SVL $p = 0.0644$, mass $p = 0.6534$).

Inundation of eggs increases the percentage of malformations in hatchlings, independently if eggs are rough or smooth ($p = 0.0039$, table 1). At 60 days, two caiman hatched from rough eggshell inundated treatment; these two hatchlings died soon after hatching. Embryos from all of the smooth eggs that were inundated died prior to hatching (table 1). The most common malformations on inundated eggs were spinal cord deformation (11/31; figure 2*a*), limbs absent/deformation (8/31; figure 2*b*) and unabsorbed vitelline sac (6/31; figure 2*c*). The most common malformations on non-inundated eggs were unabsorbed vitelline sac (10/16), and swollen jowls (4/16; figure 2*d*).

Table 1. Number of healthy hatchlings and malformed hatchlings resulting from four experimental treatments, rough eggshells inundated, and smooth eggshells inundated; when inundation occurs at 30 day (italics) and at 60 days (bold) of embryos development. Experimental inundations of 10 h duration.

treatments	healthy	malformed
rough eggshell-non-inundated	*25*	*7*
rough eggshell-inundated	*13*	*17*
smooth eggshell-non-inundated	*25*	*8*
smooth eggshell-inundated	*6*	*14*
rough eggshell-non-inundated	**33**	**0**
rough eggshell-inundated	**2**	**0**
smooth eggshell-non-inundated	**34**	**1**
smooth eggshell-inundated	**0**	**0**

4. Discussion

Hatching success of eggs inundated at 30 days of incubation with rough eggshells was similar to 'non-inundated' treatments (with or without eggshell roughness). In the experimentally 'smoothed' eggs, in which the outer surface irregularities were removed by sanding, hatching success was reduced by 32% (compared with the mean of the other three treatments). This indicates that the unaltered natural, 'rough' eggshell structure is possibly related to a normal egg's ability to tolerate inundations or other suboptimal conditions such as if nesting material does not allow for proper gas exchange of the egg.

Sanding eggs did not affect egg weight when inundated, sanded eggs increased 1.0 ± 0.7 g (range 0.2–4.2 g); control eggs increased 1.3 ± 0.7 g (range 0.3–3.5 g). Because smooth eggs did not increase their weight when inundated compared with the rough eggs, this outer eggshell architecture seems to have little effect on water interchange, relative to submergence events. Larger embryos seem to be more susceptible to flooding, since at 60 days of incubation, hatching success when inundated was very low, less than 5% (figure 3*b*), irrespective of eggshell roughness. In this study, we recorded a hatching success higher than 90%, even in smooth eggs, when eggs were not subjected to experimental flooding. This result indicates that our procedure to remove eggshell roughness did not affect the normal development of the embryos.

The relationship between embryo development and the effect of inundation (larger, older embryos being more susceptible) could be related to the higher oxygen demand of a developing crocodylian embryo [21]; this has been also observed in other species [31–33]. Oxygen demand starts to increase exponentially at about 60% of the incubation period (day 43 approx.), and the peak demand for oxygen occurs very late in development, i.e. at 90% of incubation (in our case, estimated to be day 64 approx.; extrapolated from [34]). However, there are certain limits to embryonic tolerance to flooding, prolonged inundation will probably kill all embryos, irrespective of its age/stage [1,6]. In the light of results presented here, a similar experimental protocol which tested embryonic tolerance midway through development (at approx. 45 days for this species), rather than early (30 days) when oxygen demand is very low, or later in development (60 days) when oxygen demand is very high might be more instructive. It is likely that the shell architecture entrapping air bubbles at the outer shell surface may be of maximum benefit to embryos experiencing inundation during the period of exponential increases in oxygen consumption during this middle period, rather than during the initial stage because at initial period oxygen demand is very low, or later when oxygen demand is maximal, and demand is so high that bubbles are unable to provide oxygen to embryos needs.

Our study demonstrated that in *Ca. latirostris*, the natural, irregular outer shell architecture, characterized in this study as a 'rough' eggshell surface, provides the embryo within the egg with a higher tolerance to inundation, when compared with the lower tolerance shown in eggs in which the outer shell architecture has been experimentally removed by 'sanding' off the irregularities consisting of columns and/or a secondary shell layer. When submerged, embryonic death could result from water entry into the egg or from lack of oxygen, i.e. hypoxia. Our results support the idea that hatching success is related to gas exchange via the eggshell surface layer. 'Rough' eggshells produced more and larger air bubbles than 'smooth' eggshells when submerged, thus providing an oxygen reservoir during the brief time (hours rather than days) when the nest is flooded (figure 1*c*,*d*). These bubbles could also act as 'bubble gills' facilitating oxygen diffusion, similar to 'bubble gills' of diving water insects and spiders. According to Fernández *et al.* [29], roughness originates from the discontinuous superposition of calcareous layers, consisting of adjacent columns of different heights on the underlying shell surface. These columns are interconnected, and overlaid in places with a secondary thin shell outer layer. Additional structural and functional details of the shell structure of *C. latirostris* are outlined in [18].

We note that as embryonic development proceeds, the thickness of the calcareous layer of the eggs decreases (up to 20%), mostly after the middle third of incubation (day 50 approx.); similar observations have been made in other species such as the American alligator [20,35,36]. Also in both species, there was an increase in size (from 0.05 to 0.1 mm) and number of pores [18,20,37]. These changes in the eggshell could facilitate gas interchange between the embryo and the atmosphere [20,37,38,39]. Even though the pores increase in number and size, water uptake does not appear to be a major factor. In our experimental design, mass increase after inundation was similar in 30 day versus 60 day embryos.

Hatchling size and body mass were not affected by the experimental submergence in our treatments, but inundation affected both hatching success and the occurrence of malformations. Of these, the most common ones were spinal cord deformation, limbs absent/deformation or unabsorbed vitelline sac. These deformities probably reduce hatchling survival in nature, because there are difficulties emerging from the nest, obtaining food and/or avoiding predators. Thus, inundation acts in multiple ways to increase embryo mortality directly, and to reduce neonate survival through increases in hatchling malformation. We conclude that eggshell roughness mitigates the embryo loss and/or damage caused by early inundation, but inundation late in development tends to be fatal without regard to egg outer shell texture.

Ethics. All the embryos were treated following the Reference ethical framework for biomedical research: ethical principles for research with laboratory, farm and wild animals (CONICET 2005). Embryos used in this work are part of the harvest of a sustainable management programme that uses wild Caiman populations, approved by Provincia de Santa Fe (Law 11820), registered in Dirección de Fauna Silvestre de la Nación (following resolutions No 283/00 y 03/04), fulfils CITES normative and follow recommendations of the Crocodile Specialist Group (CSG/SSC/IUCN). These caiman populations are not endangered. The research project (#2523384) was approved by Universidad de Tamaulipas, México and CONACYT (Consejo Nacional de Ciencia y Tecnología), México.

Data accessibility. Data and code used to generate the results are available in the electronic supplementary material.

Authors' contributions. J.W.L., C.I.P. and M.S.S. conceived the study. C.I.P. and M.S.S. designed the study and analysed the data. A.L., C.C.-L., C.I.P., J.W.L., M.S.S. and P.M.L.L. have interpreted results, drafted the manuscript, and they critically revised the manuscript. C.C.-L. and P.M.L.L. participated in performing most of the experiment. All authors gave their final approval for publication.

Competing interests. The authors declare no competing interests.

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