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Luciferase isozymes from the Brazilian *Aspisoma lineatum* (Lampyridae) firefly: origin of efficient pH-sensitive lantern luciferases from fat body pH-insensitive ancestors†

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Firefly luciferases usually emit green-yellow bioluminescence at physiological pH values. However, under acidic conditions, in the presence of heavy metals and, at high temperatures they emit red bioluminescence. To understand the structural origin of bioluminescence colors and pH-sensitivity, about 20 firefly luciferases have been cloned, sequenced and investigated. The proton and metal-binding site responsible for pH- and metal sensitivity in firefly luciferases was shown to involve the residues H310, E311 and E354 in firefly luciferases. However, it is still unclear how and why pH-sensitivity arose and evolved in firefly luciferases. Here, we cloned and characterized two novel luciferase cDNAs from the fat body and lanterns of the Brazilian firefly *Aspisoma lineatum*. The larval fat body isozyme (AL2) has 545 residues, and displays very slow luminescence kinetics and a pH-insensitive spectrum. The adult lantern isozyme (AL1) has 548 residues, displays flash-like kinetics and pH and metal sensitive bioluminescence spectra, and is at least 10 times catalytically more efficient than AL2. Thermostability and CD studies showed that AL2 is much more stable and rigid than the AL1 isozyme. Multialignment and modelling studies show that the E310Q substitution (E310 in AL2 and Q310 in AL1) may have been critical for the origin of pH-sensitivity in firefly luciferases. The results indicate that the lantern efficient flash-emitting pH-sensitive luciferases arose from less efficient glow-type pH-insensitive luciferases found in the fat body of ancestral larval fireflies by enzyme structure flexibilization and substitution at position 310.

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Introduction

Firefly luciferases catalyze the ATP-dependent oxidation of benzothiazole D-luciferin, eliciting the emission of different bioluminescence colors from green to yellow-orange depending on the species and life stages.[1,2] However, whereas at alkaline pH these luciferases elicit the emission of green-yellow light, at acidic pH, in the presence of heavy metals and at high temperatures they elicit red bioluminescence.[3,4]

To understand the structural origin of bioluminescence colors and pH-sensitivity, over 20 firefly luciferases have been cloned, sequenced, and investigated.[5–8] These luciferases were cloned from distinct species, tribes and subfamilies occurring around the world: the North-American *Photinus pyralis*[9] (Lampyrinae: Photinini), *Photuris pennsilvanica* (Photurinae),[10] and *Phausis reticulata* (Lampyrinae: Lampyrini);[11] the Eurasiatic species of *Luciola* spp.[12–14] (Luciolinae), *Lampyris* sp.[15] and *Pyrocoelia miyako*[16] (Lampyrinae: Lampyrini); and the South-American *Macrolampis* (Lampyrinae: Photinini), *Cratomorphus distinctus* (Lampyrinae: Cratomorphini)[17] and *Amydetes vivianii* (Amydetinae).[18] The three-dimensional structure was also determined for four beetle luciferases,[19–22] revealing important active site segments. Recently, the proton and metal-binding site responsible for pH and metal sensitivities has been identified in firefly luciferases, involving the conserved residues H(T) 310, E311 and E(N)354.[23]

Most firefly luciferases have been cloned from the adult stage lanterns and all of them are pH-sensitive. However, only a few luciferases have been cloned from the larval stage. In the larval stage of *P. pennsylvanica*, two isozymes were reported and cloned, one pH-sensitive and the other pH-insensitive.[10,24] Similarly, the larval and adult firefly luciferase isozymes of *Luciola cruciata*,[25]

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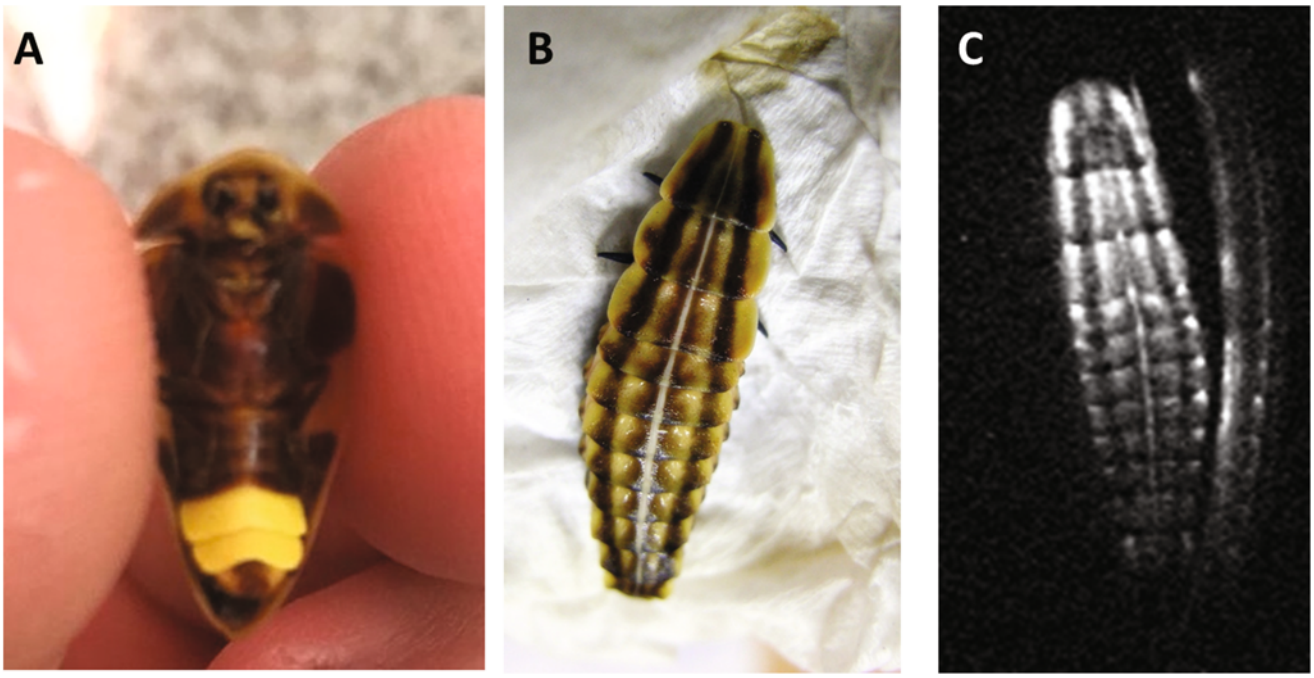


Fig. 1 Aspisoma lineatum firefly: (A) ventral side of the adult male showing the lantern; (B) dorsal side of the larva, and (C) CCD image of the weak continuous bioluminescence emitted by the fat body widespread in the larval body.

Luciola lateralis,[26] *Pyrocelia atrippenis*[27] and *Luciola parvula*[28] were also shown to be pH-insensitive and pH-sensitive respectively.

The firefly *Aspisoma lineatum* (Fig. 1) is common in the open fields of Southeastern Brazil. Its *in vivo* and *in vitro* bioluminescence spectra for the adult and larval stages[29,30] and its life cycle under laboratory conditions were reported.[31]

Previously, we have shown that the fat body of larval firefly *A. lineatum* is weakly bioluminescent, providing evidence that firefly lanterns are an extension of the fat body, and that photocytes arise from trophocytes.[30] We have also shown that there are two distinct isozymes occurring in the larvae, one which predominates in the lanterns and emits yellow-green light, and the other which predominates in the fat body and emits green light.[29,30] The transcriptional analysis of *A. lineatum* photogenic and non-photogenic tissues confirmed the presence of two distinct luciferase isozymes, which are homologous to *P. pennsilvanica* (PpL2) and *L. cruciat*a (LcL2) pH-sensitive and pH-insensitive luciferases.[32] Whereas the pH-sensing moiety and metal-binding sites have been already identified in firefly luciferases,[23] it is unclear how and why pH-sensitivity naturally evolved and was selected in firefly luciferases.

Therefore, considering that *A. lineatum* firefly has been recently used as a model for anatomical and biochemical studies, here we cloned the cDNA coding for the pH-sensitive and pH-insensitive isozymes which occur in the fat body and lanterns, and expressed, purified and characterized them. A comparison of these two luciferase isozymes leads us to identify the putative substitutions and structural features responsible for the origin of pH-sensitivity and evolution of firefly luciferases.

Materials and methods

Insects

Larval and adult *A. lineatum* fireflies were collected at the campus of Sorocaba of the Federal University of São Carlos (UFSCar), located in Sorocaba city, Brazil, from November to January.

RNA extraction

The abdominal segments containing the adult lanterns were isolated and the tissue was homogenized in Trizol (1 mL for 50–100 mg of tissue). After centrifugation, the pellet was discarded and chloroform (200 μL per 1 mL of Trizol) was added to the sample. The aqueous phase containing the RNA was isolated and precipitation was performed by adding isopropanol (500 μL per 1 mL of Trizol) followed by washing with a 75% ethanol solution. Finally, total RNA was resuspended in RNAse-free water.

PCR cloning

To obtain the cDNA library, reverse transcription was performed using the "GoScript Reverse Transcriptase Kit (Promega)" according to the manufacturer's protocol. The sequences of *A. lineatum* luciferases were previously obtained from transcriptome analysis.[32] Therefore, specific primers containing restriction sites (BamHI and HindIII) at the extremities were designed. After PCR amplification of the target genes from the cDNA library, the inserts were digested with restriction enzymes, and purified using Wizard SV Gel and a PCR Clean-Up System (Promega, Madison) and then ligated into pColdII vector (Takara) using T4 ligase (Promega, Madison).

The following primers were designed: (AL1 forward) GGT CCA TGA GGA TCC ATG GAA TCG GAA GAT AAA AAT ATT GTT TGC GGT CC; (AL1 reverse) GTA TGG CTA TCG AAG CTT ACA ATT TGG ATT TCT TTG CCT TAA TTA AA; (AL2 forward) TCA CAG GAT GGA TCC ATG GAC GAT ACA AAT ATT TTA TAT GGG CCT AAA CC; and (AL2 reverse) GTA TCG TCG AAG CTT CTA AAG TTT AGA CTT AGG TTT TGA AAG TAT TT. After insertion of gene into the expression vector, the clones were confirmed by Sanger sequencing.

Luciferase expression and purification

Escherichia coli BL21 strain cells were transformed with the plasmid containing the cDNA of *A. lineatum* pH-sensitive (AL1) and pH-insensitive (AL2) luciferases. The cells were grown in LB medium/ampicillin liquid cultures at 37 °C until Abs_{600} = 0.4 and then induced with 0.5 mM IPTG at 16 °C overnight. The induced cells were harvested by centrifugation (at 2.000*g* for 15 minutes at 4 °C) and resuspended in 10 mL of cold extraction buffer (50 mM Tris-HCl, 1% Triton X-100, 10% glycerol, 300 mM NaCl, pH 8) supplemented with Anti-protease Cocktail (Roche). The lysis of cells was performed by sonication (Mixonix). After that, the homogenate was centrifuged and the enzymes were purified by nickel–agarose chromatography followed by overnight dialysis using 25 mM Tris-HCl buffer (pH 8) complemented with 10 mM NaCl, 0.1 mM EDTA, 10% glycerol, and 2 mM DTT. The quality of the purification process was analyzed by electrophoresis on 10% SDS-PAGE.

Luciferase activity measurements

The bioluminescence activities were measured in counts per second (cps) using an AB2200 luminometer (ATTO, Tokyo, Japan). The assays were performed by mixing 85 μL of buffer (0.1 M Tris-HCl, pH 8), 5 μL of 40 mM ATP/80 mM $MgSO_4$ solution, 5 μL of purified enzyme (~1 μg) and 5 μL of 10 mM D-luciferin (LH_2). Bioluminescence activity measurement using luciferyl–adenylate (LH_2–AMP) was performed by mixing 5 μL of LH_2–AMP to 90 μL of the same buffer and 5 μL of purified luciferase (~1 μg). All measurements were performed in triplicate and the average values were reported.

To analyze the effect of heavy metals on the luminescence activity, metal salts ($CdSO_4$, $HgCl_2$, $LiSO_4$, $CuSO_4$, $PbCl_2$, $ZnSO_4$ and $NiSO_4$) at 1 mM final concentration were added to the standard reaction mixture.

Kinetic characterization

The assays to determine K_M for luciferin were performed by mixing 5 μL of 80 mM ATP/40 mM $MgSO_4$ solution, 85 μL of 0.10 M Tris-HCl pH 8.0 buffer, 5 μL of purified luciferase (~1 μg) and luciferin at final concentrations between 0.01 and 1 mM. The assays to determine K_M for ATP were performed by mixing 5 μL of 80 mM $MgSO_4$ solution, 80 μL of 0.10 M Tris-HCl buffer (pH 8.0), 5 μL of purified luciferase (~1 μg), 5 μL of 10 mM luciferin and ATP at final concentrations in the range from 0.02 to 2 mM. The values were calculated using the Lineweaver–Burk plots. All measurements were performed in triplicate and the average values were reported.

Determination of k_{cat} and k_{ox}

The overall catalytic constant (k_{cat}) and the oxidative constant (k_{ox}) were determined from the overall specific bioluminescence activities with luciferin and ATP, or with luciferyl–adenylate, respectively. We calculated the ratio of luminescence activities in cps (counts per second), as a measure of V_{max}, and the number of luciferase molecules based on the specific bioluminescence activities measured for AL1 and AL2 luciferases using the equation $k_{cat} = V_{max}/[E]$.

All measurements were the result of three independent purifications and each luciferase assay was performed in triplicate. Because the absolute value of cps in photon per s could not be determined, the absolute values of k_{cat} and k_{ox} in s^{-1} could not be determined, and therefore the values were reported in cps (counts per second). Although these values are not absolute, they can be safely used as relative values of catalytic constants.

Bioluminescence spectra

Bioluminescence spectra were obtained using a Lumispectra spectroluminometer (ATTO, Tokyo, Japan). The reaction mixture was prepared using the same parameters as those for luciferase activity measurements. For heavy metal sensitivity analysis, 1 mM (final concentration) of metal salts ($CdSO_4$, $HgCl_2$ and $ZnSO_4$) were added to the standard reaction mixture. For pH sensitivity analysis, different buffers were used: 0.10 M sodium citrate (pH 5.0 and 5.5), 0.10 M sodium phosphate (pH 6.0, 6.5, 7.0 and 7.5) and 0.10 M Tris-HCl (pH 8.0).

Thermostability

The purified luciferases (pH-sensitive and pH-insensitive) were separated into aliquots with a standard concentration of ~0.2 mg mL^{-1} and incubated at 4 °C, 22 °C and 37 °C. The luminescence activity was measured at different times (0 h, 1 h, 3 h, 6 h, 12 h, 24 h, 48 h, 96 h and 120 h). For the pH-insensitive (AL2) luciferase, the thermostability was also analyzed at 50 °C (0–6 h) and 60 °C (0–15 min). The bioluminescence images were obtained using a *Canon Eos T7* photographic camera. The exposure time for photograph capture was 5 minutes.

CD spectra

Recombinant proteins were prepared at final concentrations of 3.5 μM (*Amydetes vivianii* and *A. lineatum* AL2) and 2.4 μM (*Macrolampis* sp2) in a solution consisting of 20 mM Na_2HPO_4, 120 mM NaCl, pH 7.5 and were analyzed at 20 °C. Circular Dichroism (CD) spectra were recorded on a Jasco J-815 spectral polarimeter (Japan Spectroscopic, Tokyo, Japan) equipped with a Peltier unit for temperature control. Far-UV CD spectra were obtained using 1 mm path length cuvettes. The spectra were presented as the average of ten scans recorded in the 190–260 nm wavelength range with 100 nm min^{-1} increment step, 10 s averaging time, 1 nm bandwidth and 0.5 s response time. The residual molar ellipticity was expressed in degree ×

cm^2 per decimole. The data were analyzed using the Dichroweb CD analysis program available at http://dichroweb.cryst.bbk.ac.uk[33] to obtain the estimates of the secondary structural composition of proteins. Moreover, the thermal transition was evaluated by CD at 222 nm wavelength in the 20–100 °C range. Proteins were dialyzed against 20 mM HEPES, pH 7.5, 150 mM NaCl, 10 percent glycerol and analyzed at concentrations of 3.5 μM (*Amydetes vivianii* and *A. lineatum* AL2) and 2.4 μM (*Macrolampis* sp2).

Bioinformatic analysis

Multialignment of luciferase primary structures was performed using the ClustalOmega program.[34] Sequence analysis (PI and MW) was performed using ProtParam.[35] The relative hydrophobicity or hydrophilicity of amino acid residues used the Creighton scale.[36]

Phylogenetic analysis

We performed the phylogenetic analysis of luciferase enzymes using MrBayes 3.2[37] and IQTree 1.5.6 software.[38] The amino acid sequences were aligned using MEGA 6.0 software.[39] The trees obtained using both these software were visualized using the software FigTree v.1.4.1.[40] The accession number of luciferases used in the analysis is provided in Table S1 (ESI†).

Homology modelling

Ab initio models of *Aspisoma lineatum* luciferase isozymes (AL1 and AL2) were generated using I-TASSER.[41] The effects of mutations on *Aspisoma lineatum* pH-insensitive luciferase were predicted by DynaMut[42] and Arpeggio[43] web servers, available at http://biosig.unimelb.edu.au/, implemented to analyze and visualize the impact of mutations. The ligand was docked to protein models using Blind Docking Server, BINDSURF,[44] available at http://bio-hpc.eu/software/blind-docking-server/. Furthermore, the molecular graphical visualization and analysis were performed using the PyMOL Molecular Graphics System, Version 2.3.3, Schrödinger, LLC.[45]

Results and discussion

cDNA and amino acid sequences

The ORF sequence of *A. lineatum* lantern luciferase has 1647 nucleotides and encodes 548 amino-acids. The calculated theoretical PI value of this polypeptide is 6.06 and its molecular weight is 60.45 kDa. The fat body luciferase sequence has 1648 nucleotides and encodes a shorter polypeptide with 545 amino acids. The molecular weight of this polypeptide is 60.49 kDa and its theoretical PI value is 6.89. Both amino acid sequences display a C′-terminal SKL peroxisomal targeting signal.[46] The respective primary structures share 57% identity and 66% similarity among themselves. The fat body isozyme AL2 is more similar to LcL2 from *Luciola cruciata*[25] (61% identity and 79% similarity) displaying a shorter amino-acid sequence than the lantern isozyme, which is pH-insensitive. These results indicate that these luciferases are paralogous enzymes that diverged after a duplication event considerable time ago.[27,47]

Molecular phylogeny

As expected, the phylogenetic analysis of the primary amino acid sequences of luciferases recovered the Lampyridae family as a monophyletic group (Fig. 2). The *Aspisoma lineatum* isoenzyme (AL1), which was cloned from the lanterns of an adult individual, was grouped as a sister group of the Photinini tribe (Macrolampis + Photinus)[48] within the Lampyrinae subfamily. Surprisingly, although *Aspisoma* genus was originally classified in the Cratomorphini tribe together with the *Cratomorphus* genus, the expected relationship was not found in the tree topology. In this topology, we observed four clades: I. Lampyrinae luciferases (*Aspisoma*, *Cratomorphus*, *Lucidina*, *Macrolampis*, *Photinus* and *Pyrocoelia*) + Amydetinae (*Amydetes*); II. Luciolinae (*Lampyroidea* and *Luciola*) + Photurinae luciferases (*Photuris*); III. Cyphonocerinae + Ototretinae (*Cyphonocerus*, *Drilaster* and *Stenocladius*) luciferases; and IV. Larval pH-insensitive luciferases cloned from distinct Lampyridae species (*Luciola cruciata*, *Luciola lateralis*, *Luciola parvula*, and *Photuris pennsylvanica*).

Therefore, based on the molecular and biochemical analyses performed here and in previous studies,[25,27–30] we distinguished two main groups within the firefly luciferase tree topology, a clade formed by the luciferases cloned from the lanterns (both adult and larval), which are pH-sensitive, and a second clade formed by the isozymes cloned from the fat-body, which are pH-insensitive. The second isoform AL2 seems to be basal to the lantern luciferase clade, indicating that this isozyme is more primitive.

Expression and purification

The recombinant luciferases were expressed in *E. coli* and purified by nickel–agarose chromatography. As expected, considering the molecular weight estimated by sequence analysis, both luciferases displayed similar MW values close to 60 kDa. However, the pH-insensitive enzyme had much higher expression and purification yields. The concentration of the purified pH-insensitive luciferase was ~0.7 mg mL^{-1} while that of the pH-sensitive luciferase was only ~0.03 mg mL^{-1} (Fig. 3). The difference in the expression efficiency of these luciferases in *E. coli* can be attributed to the different codon usage, which may affect expression inside bacteria cells.

Comparison of the bioluminescence activities and kinetic properties

The lantern and fat body isozymes displayed quite different kinetic and spectral properties. In the standard luminescence activity assay, the lantern luciferase displayed a flash-like luminescence kinetics, similar to that of other adult luciferases arising from firefly lanterns such as *Amydetes vivianii*,[18] *Macrolampis* sp2,[7] and *Photinus pyralis*[9] and others reported in the literature. On the other hand, the fat body isozyme displayed a much slower kinetics, with a half-rise time of 10 minutes and a half-life of several hours (Fig. 4).

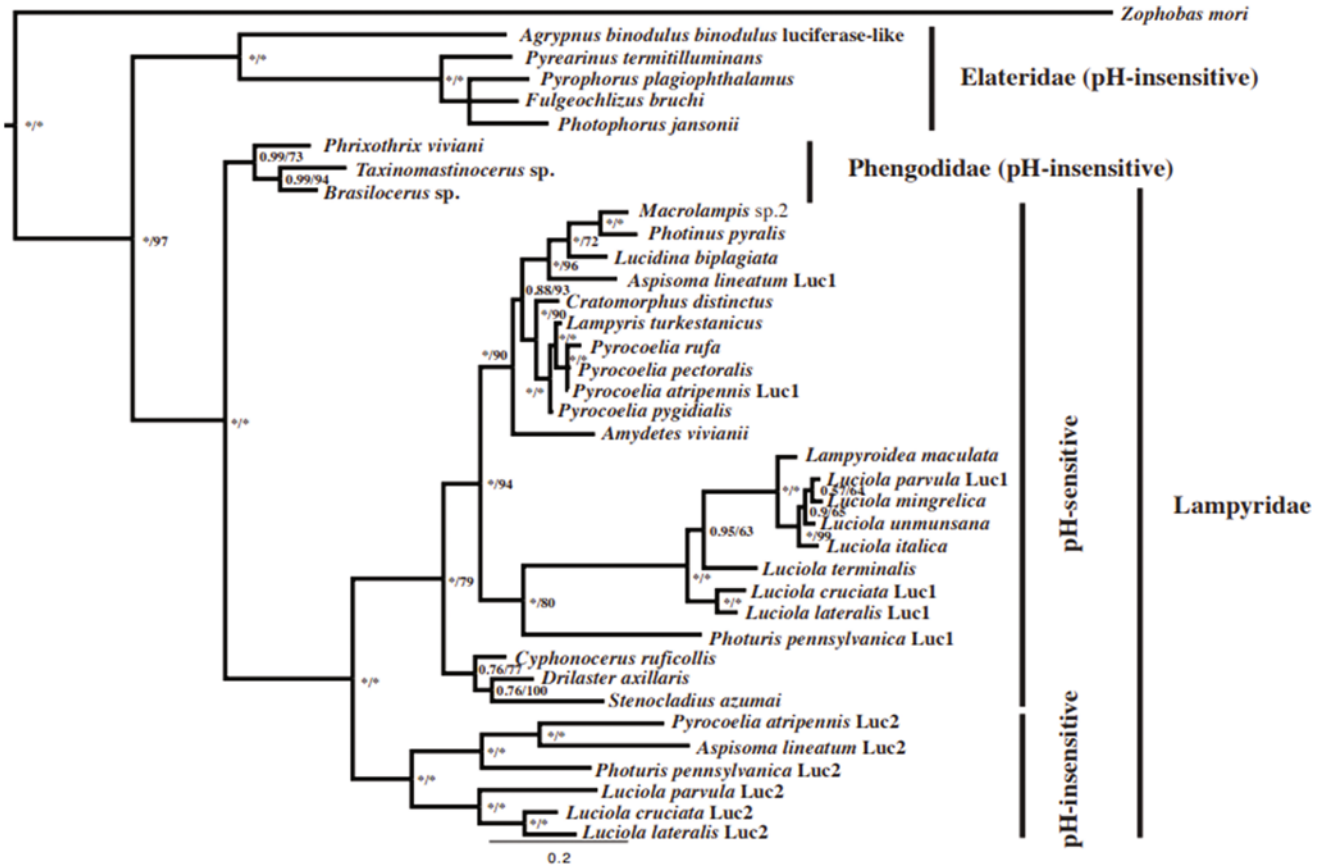


Fig. 2 Molecular phylogeny of firefly luciferases using Bayesian and maximum-likelihood approaches. The nodes well-supported with posterior probability of 1 and bootstrap of 100 were marked with *. GenBank accession number of luciferase primary sequence used: *P. termitilluminans* AF116843.1; *P. plagiophthalmus* AAQ11735.1; *P. jansonii* AB767301.1; *A. binodulus* luciferase-like enzyme BAF96580.1; *P. pyralis* AB644228.1; *L. biplagiata* AB535101; *C. distinctus* AY633557; *L. turkestanikus* AY742225; *P. atripennis* Luc1 LC215693.1; *P. rufa* AF328553.1; *P. pectoralis* EF155570.1; *P. pygidialis* EU826678; *L. mingrelica* S61961.1; *L. parvula* Luc1 L39929.1; *L. unmusana* AF420006.1; *L. itálica* DQ138966.1; *L. maculate* DQ137139.1; *L. terminalis* EU302126.1; *L. cruciata* Luc1 M26194.1; *L. lateralis* Luc1 X66919.1; *P. pennsylvanica* Luc1 D254416.1; *D. axillaris* AB604790.1; *S. azumai* AB644225.1; *C. ruficollis* AB5604789.1; *P. atripennis* Luc2 LC215694.1; *L. cruciata* Luc2 AB490793.1; *L. lateralis* Luc2 AB693934.1; *L. parvula* Luc2 AB812879.1; *P. pennsylvanica* Luc2 U31240.1; *P. vivianii* AF139644.1; *Brasilocerus* sp. FJ545728.1; *F. bruchi*, *Macrolampis* sp2, *A. vivianii*, *Taxinomastinocerus* sp and *Z. morio* – Not available.

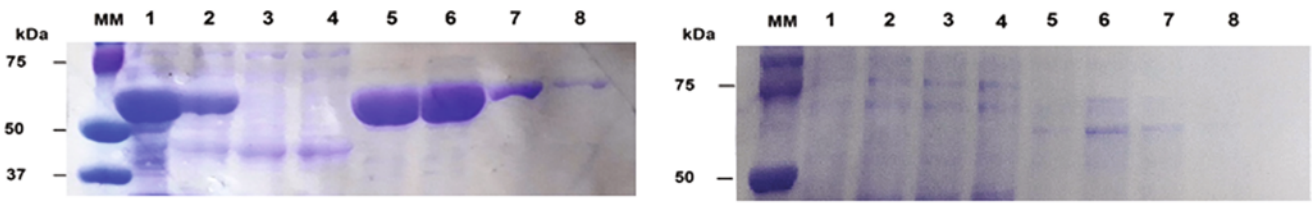


Fig. 3 SDS-PAGE analysis of *A. lineatum* fat body pH-insensitive (left) and lantern pH-sensitive (right) luciferase purification process. (**MM**) Molecular weight marker, (**1**) crude extract, (**2**) cell lysate, (**3**) first eluate containing the proteins that did not bind to the nickel–agarose resin, (**4**) washing, (**4–8**) eluted samples.

The differences in the luminescence reaction kinetics of AL1 and AL2 can be explained based on product inhibition, where the fast decay of flash-like kinetics is caused by inhibition by oxyluciferin and mainly dehydroluciferin and its adenylated form, dehydroluciferin–adenylate.[55,56]

The addition of coenzyme-A after the beginning of the reaction causes an increase in the luminescence intensity and a decrease of luminescence decay, resulting in a more sustained luminescence. The enhancing effect of CoA is most likely caused by the promotion of the alternative thioesterification reaction of CoA with luciferyl–adenylate, producing dehydroluciferyl–CoA, a much weaker inhibitor which is easily removed from the active site,[57–60] resulting in a more sustained luminescence intensity.

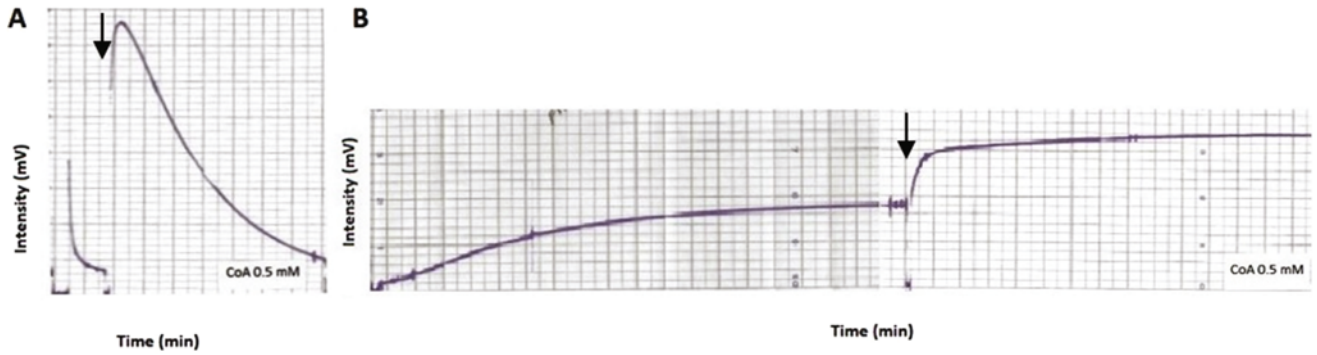


Fig. 4 Luminescence kinetics of *A. lineatum* firefly luciferase isozymes under standard reaction conditions without coenzyme-A and the same reaction after addition of coenzyme-A at a final concentration of 0.05 mM. The arrows indicate the moment when Co-A was added: (A) pH-sensitive adult lantern luciferase. (B) pH-insensitive fat body luciferase.

Bioluminescence activities and catalytic constants

Beetle luciferases are bifunctional enzymes that catalyze a two-step reaction: (1) adenylation of D-luciferin producing luciferyl–adenylate and (2) oxidation of luciferyl–adenylate (Scheme 1). Therefore, while the catalytic constant (k_{cat}) reflects the overall reaction, the measurements starting with presynthesized luciferyl–adenylate reflect only the oxidative reaction (k_{ox}).

The specific activity for AL1 was ~17 times higher than that for AL2. As expected, the catalytic constant (k_{cat}) for AL1 was higher than that for the fat body isozyme (Table 1). It is worth noting that the bioluminescence activity with luciferyl–adenylate and the catalytic oxidative constant (k_{ox}) (Fig. 5 and Table 1) for the pH-sensitive luciferase (AL1) were forty times higher than those for the pH-insensitive luciferase (AL2).

K_M for ATP and luciferin and catalytic efficiencies

The K_M values for luciferin and ATP were quite similar for both isozymes: for the pH-sensitive luciferase, the values were 4 μM and 7 μM for ATP and luciferin, respectively, and for the pH-insensitive isozyme (AL2), the values were 7 μM for ATP and 3 μM for luciferin (Table 1). Overall, the catalytic efficiency, which was much higher for the pH-sensitive isozyme AL1, indicates that the lantern isozyme AL1 is a considerably more efficient oxygenase and much brighter luciferase than the fat body isozyme AL2.

pH-Profile

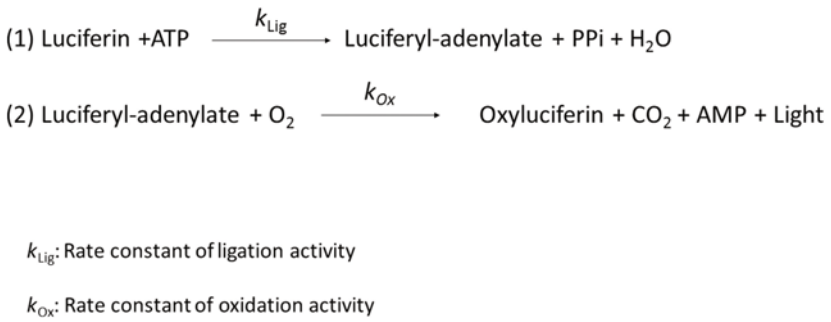
Although the pH-insensitive luciferase showed a slightly higher activity at higher pH values than the pH-sensitive isozyme, both enzymes showed a similar optimum pH value of about 8.0 (Fig. 6).

Bioluminescence spectra and pH-sensitivity

The adult lantern firefly luciferase AL1 emitted light in the yellow region with an emission peak at 573 nm at pH 8.0 and was pH-sensitive, shifting the spectrum to the red region with a peak at 605 nm at pH 6.0. On the other hand, the fat body isozyme AL2 emitted green-yellow light with a pH-insensitive spectrum peaking at 561 nm (Fig. 7). These values have a difference of approximately 15 nm from the previously reported *in vivo* and *in vitro* spectra of extracted native luciferase.[29,30] This difference is probably caused by the difference in calibration between equipment used.

The effect of heavy metals

Heavy metals such as copper, zinc, cadmium and mercury had a considerable inhibitory effect on the luciferase activity of both enzymes. Yet, this inhibitory effect is slightly more pronounced in the pH-insensitive luciferase (Fig. 8).



Scheme 1 Reactions catalyzed by beetle luciferases.

Table 1 Comparative bioluminescence and kinetic properties of *A. lineatum* firefly luciferase isozymes with other beetle luciferases

Luciferase	λ_{max} [a] (nm) pH 8 [half-band]	λ_{max} [a] (nm) pH 6 [half-Band]	K_M (μM) ATP	K_M (μM) LH_2	Specific activity (10^9 cps mg^{-1}) [relative activity]	Oxidative activity (10^9 cps mg^{-1}) [relative activity]	k_{cat} [b] [standard error] (10^{-6} c s^{-1})	k_{ox} [b] [standard error] (10^{-6} c s^{-1})	k_{cat}/K_M [standard error] ATP	k_{cat}/K_M [standard error] (LH_2)	k_{ox}/K_M [standard error] ATP	k_{ox}/K_M [standard error] (LH_2)
Fireflies												
Amydetes vivianii[18]	547 [81]	583	9	9	890	730	109	81	12.1	12.1	9	9
Macrolampis sp2[18]	575 [86]	606 [77]	83	20	1198	1008	125	106	1.5	6.25	1.3	5.3
Photinus pyralis[18]	567 [81]	608 [80]	250	5	1037	910	116	102	0.46	23	0.4	20.5
Aspisoma lineatum Adult lantern	573 [88]	605 [94]	4	7	693	1766	69 [±7]	177 [±10]	11 [±3.2]	9.8 [±3.6]	44	25
Aspisoma lineatum Fat body	561 [88]	563 [88]	7	3	40	29	6 [±1.5]	4.7 [±1.75]	0.85 [±0.5]	2 [±0.9]	0.67	1.5
Click beetles												
Pyrearinus termitilluminans[18]	546 [87]	546	370	80	290	55	29	56	0.78	3.625	0.15	0.7
Railroadworms												
Phrixothrix hirtus[18]	626 [82]	626	230	7	70	65	8.3	7.8	0.04	1.2	0.034	1.11
Phrixotrix vivianii[18]	558 [89]	558	330	64	37.9	33	3.79	3.12	0.011	0.059	0.009	0.048

[a] The peak estimated error was ±2.5 nm. [b] The overall catalytic constant was calculated from the total light intensity in counts per second of the bioluminescence reaction starting with ATP and D-luciferin, whereas the catalytic constant of oxidation was calculated from the luminescence intensity using luciferyl–adenylate as the substrate.

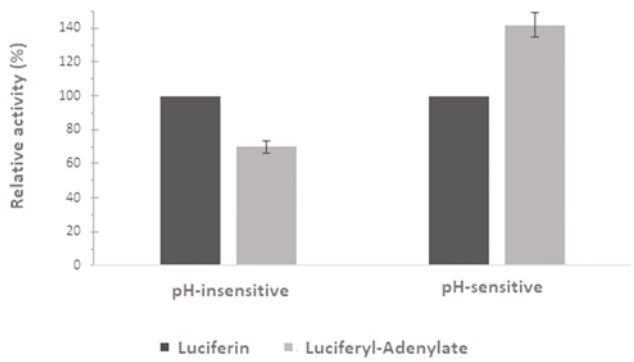


Fig. 5 Overall bioluminescence and oxidative activities of *A. lineatum* firefly fat body pH-insensitive (AL2) and lantern pH-sensitive (AL1) isozymes.

As expected, in the case of the adult lantern luciferase, the metals promoted the expected color change from green-yellow to red, similar to the effect of acidic pH values. It is worth noting that a smaller spectral shift was also observed for the pH-insensitive luciferase (Fig. 9), indicating that the enzyme, despite being pH-insensitive, still displays some degree of sensitivity to metals. Cadmium and mercury promoted the largest shifts and also the highest inhibition of the activity. For AL1, bathochromic shifts of 20 nm and 24 nm were observed for cadmium and mercury, respectively. For AL2, the shift was only 10 nm for both cadmium and mercury.

Thermostability

The thermostability of both enzymes were compared at 4 °C, 22 °C and 37 °C. The pH-insensitive isozyme was much more

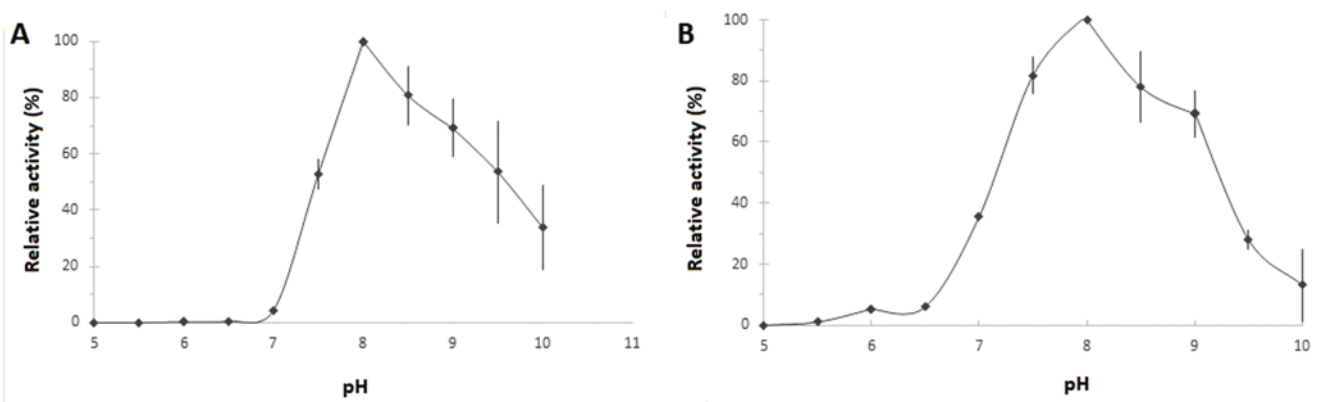


Fig. 6 Effect of pH on *A. lineatum* luciferase isozymes activities. (A) pH-Insensitive AL2 and (B) pH-sensitive AL1.

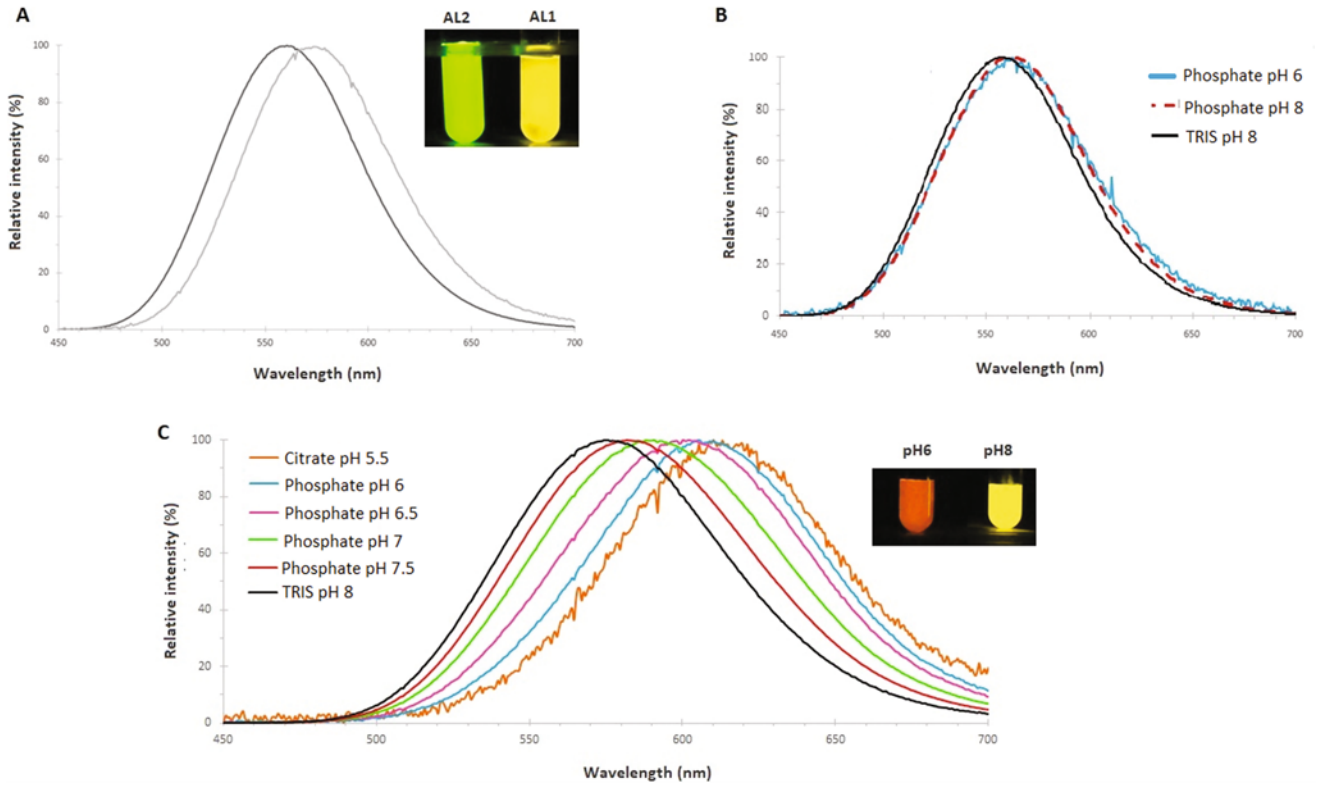


Fig. 7 (A) Bioluminescence spectra and color of *A. lineatum* larval fat body (the light grey line) and adult lantern (the dark grey line) luciferases; (B) the effect of pH on the bioluminescence spectra of the larval fat body luciferase AL2; (C) the effect of pH on the bioluminescence spectra of the adult lantern luciferase AL1.

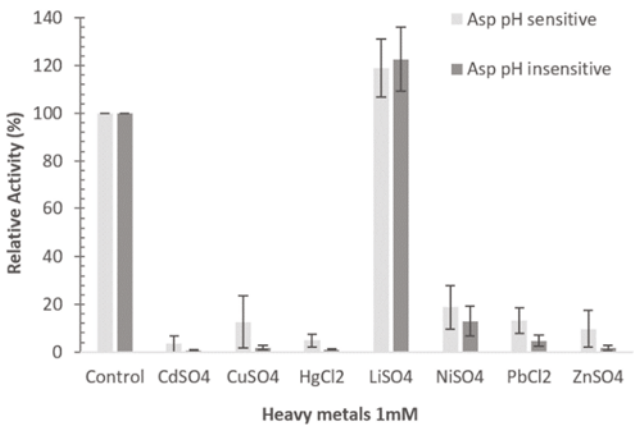


Fig. 8 Effect of heavy metals at a concentration of 1 mM on the *in vitro* luminescence activity. (Light grey) *A. lineatum* pH-sensitive luciferase AL1; (dark grey) pH-insensitive luciferase AL2.

stable than the pH-sensitive isozyme and showed a 48 h half-life of activity at 37 °C, whereas the pH-sensitive adult luciferase showed only a 1 h half-life of activity (Fig. 10A). Due to its higher thermostability at 37 °C, we also compared the stability of the pH-insensitive luciferase at higher temperatures. The half-lives at 50 °C and 60 °C were approximately 3 hours and 10 minutes, respectively (Fig. 10B and C). Among the recombinant luciferases previously tested in our laboratory, the pH-insensitive enzyme of *Pyrearinus termitilluminas* larval click beetle and the pH-sensitive enzyme of *Amydetes vivianii* firefly were the most thermostable, with half-lives of 11 hours[49] and 12 hours at 37 °C,[18] respectively. Therefore, these results indicate that the AL2 isozyme is likely the most stable wild-type luciferase ever reported. Such a higher stability is desirable for the application of luciferase as a bioanalytical reagent.

CD spectra indicate that the pH-insensitive AL2 luciferase is more rigid

The secondary structural composition (Table 2), calculated from the CD spectra of luciferases using the algorithm CDSSTR with the 7 dataset,[50] (Fig. 11A) showed that the pH-insensitive luciferase of *A. lineatum* AL2 and the less pH-sensitive luciferase of *A. vivianii* firefly show a lower α-helix content (21% and 33%, respectively) than the more pH-sensitive luciferase of *Macrolampis* sp2 firefly (34%). On the other hand, the β-sheet fraction (24%) was higher in the pH-insensitive *A. lineatum* AL2 luciferase.

The CD spectra and secondary structural content can be correlated with the melting temperature (T_m) and the correlation reflects the effect of temperature on the fraction of unfolded proteins (Fig. 11B). It is worth noting

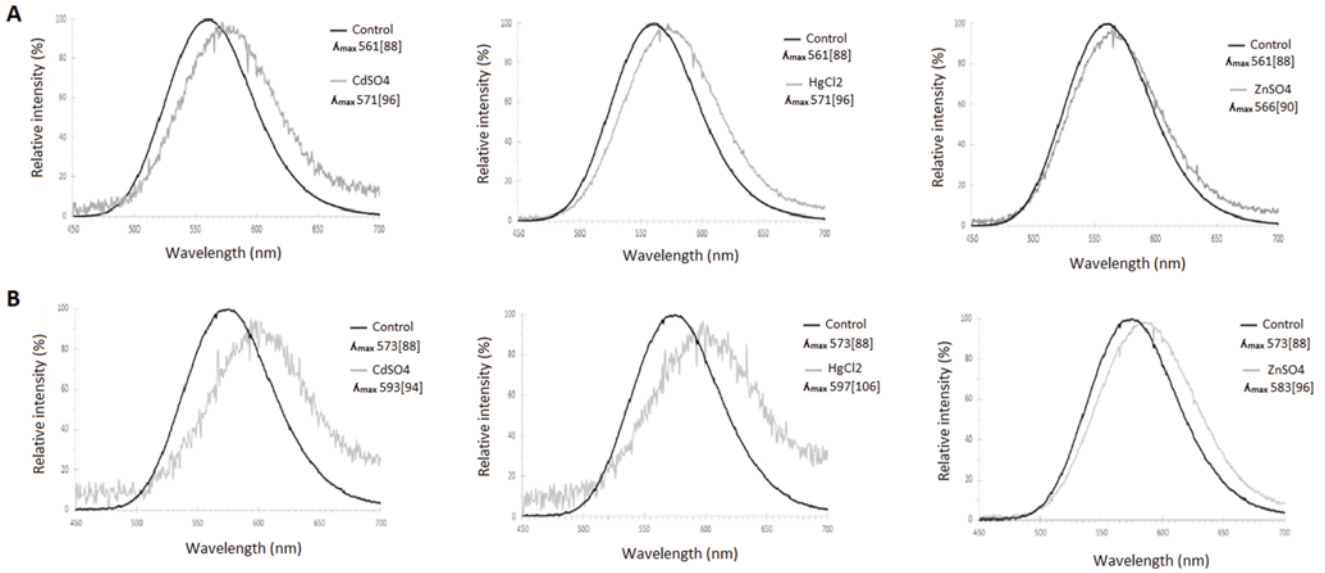


Fig. 9 Effect of different heavy metals at a concentration of 1 mM on the *in vitro* bioluminescence spectra of *A. lineatum* luciferases. λ_{max} [half-band] (nm). (A) Larval fat body pH-insensitive luciferase AL2 and (B) adult lantern pH-sensitive luciferase AL1.

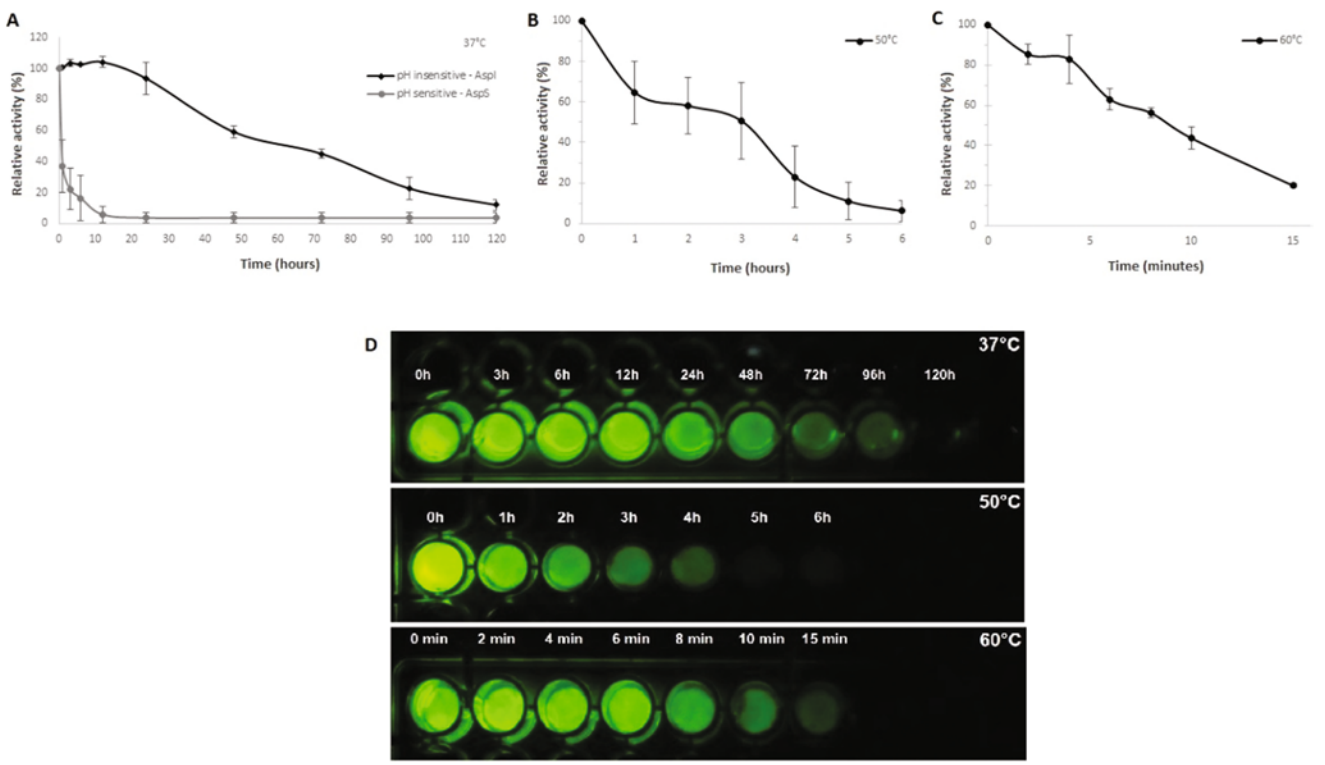


Fig. 10 (A) Comparison of the thermostability of *A. lineatum* luciferase isozymes at 37 °C. The light grey line represents the pH-sensitive enzyme AL1 and the black line represents the pH-insensitive enzyme AL2; (B) thermostability of the pH-insensitive isozyme at 50 °C; (C) thermostability of the pH-insensitive luciferase at 60 °C; (D) bioluminescence image of the pH-insensitive fat body *A. lineatum* luciferase at 37 °C, 50 °C and 60 °C.

that the *A. lineatum* pH-insensitive AL2 luciferase enzyme had a T_m value 20 °C higher than any other luciferases tested here.

Overall, these results clearly indicate that the pH-insensitive isozyme AL2 has a more rigid structure than pH-sensitive luciferases.

Table 2 Analyses of the structural dynamics of firefly luciferases using the CD data in Fig. 12A

	Fraction of each secondary structure						
Luciferase	Helix (%)	Strand (%)	Turn (%)	Unorderd (%)	NRMSD	Method	Basis sets
Aspisoma lineatum AL2	21	24	21	34	0.023	CDSSTR	7
Amydetes vivianii	33	19	22	26	0.020	CDSSTR	7
Macrolampis sp2	34	18	17	31	0.019	CDSSTR	7

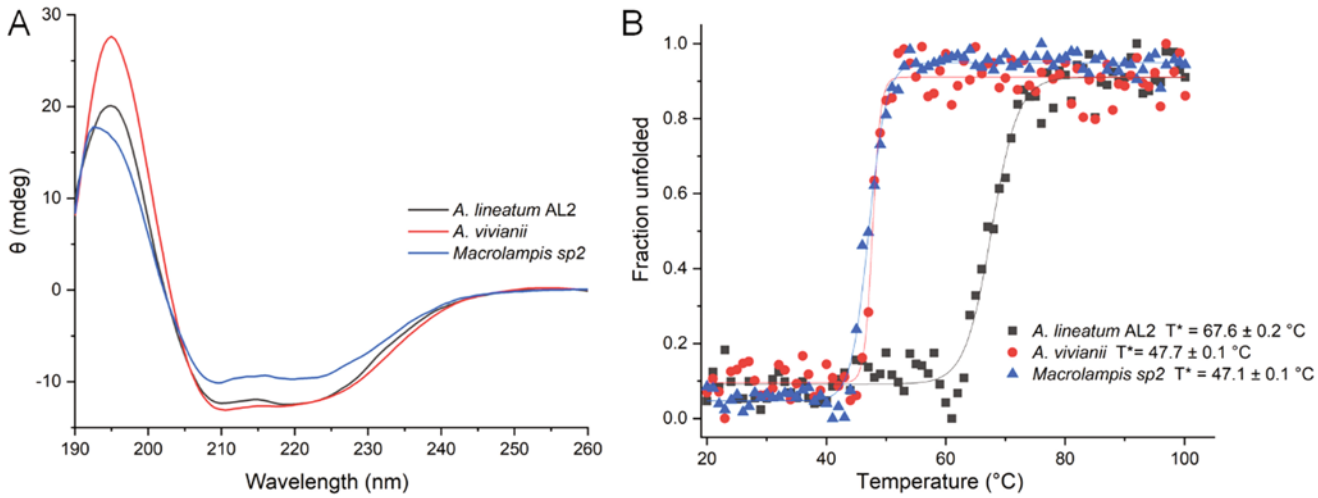


Fig. 11 CD spectra and the effect of temperature on protein unfolding: (A) secondary structural characterization of luciferase enzymes by CD spectroscopy. The far-UV spectrum was recorded in 20 mM Na_2HPO_4, 120 mM NaCl, pH 7.5 using an optical path length of 1 mm. (B) Thermal unfolding of luciferases was monitored by recording the change in the CD signal at 222 nm in response to heating. The temperature was increased from 20 to 100 °C at 1 °C per min. The fraction of unfolded protein and the T_m values (insert T^*) were calculated using OriginLab, Boltzmann function, and sigmoidal curve.

The critical pH-sensitive site 310 is substituted in AL1 and AL2

To identify the putative residues involved in pH-sensitivity determination, we performed a multiple sequence alignment of several pH-sensitive and pH-insensitive luciferases and the *A. lineatum* isoforms AL1 and AL2 (Fig. 12). Among the substitutions, the substitution of glutamine Q310 in the pH-sensitive isozyme AL1 by glutamate E310 in the pH-insensitive isozyme AL2 deserves special attention, because we have recently shown that this position is critical for metal binding and pH-sensitivity.[23]

It has been proposed that in some firefly luciferases under physiological conditions, H310 forms a salt bridge with E354, helping to keep a closed active site conformation favorable for green light emission. The disturbance of this salt bridge by external charges (protons and metals) partially opens and polarizes the active site, resulting in the red light emission.[23] Mutations in this position have also been shown to affect both pH and metal sensitivities.[23,54]

In most firefly luciferases, histidine is present at the above-mentioned position. However, *Luciola* spp luciferases have valine or threonine, whereas pH-insensitive railroad worm and click beetle luciferases have threonine, arginine or alanine. Threonine and glutamine hydroxyl and amide groups can still potentially coordinate metals such as zinc, and participate in hydrogen bonding networks with other pH-sensing residues, especially E354.

Modeling studies (Fig. 13) showed that the estimated distance between the side-chains of H310 and E354 (that interact with each other forming a salt bridge) in the pH-sensitive luciferase such as *A. vivianii* is 2.8 Å. For the pH-sensitive *A. lineatum* AL1, the predicted distance between the Q310 and E354 side chains is 4.1 Å. Although this value is considerably higher than that observed for *A. vivianii* luciferase (2.8 Å), it is close to that found for the pH-sensitive luciferase of *Macrolampis* sp2 that has the natural E354N substitution and a more red-shifted spectrum. However, for the pH-insensitive *A. lineatum* AL2, which has a glutamate at 310 position (E310), the predicted distance is 9.4 Å.

The presence of glutamate at position 310 in the pH-insensitive isozyme (AL2) is a much more drastic substitution due to the insertion of the negative charge at that position. Whereas the negative charge of the glutamate (E310) side-chain of the AL2 isozyme can still interact with divalent metals, explaining the reminiscent metal sensitivity, the presence of this negatively charged residue may interfere with the pH sensor due to

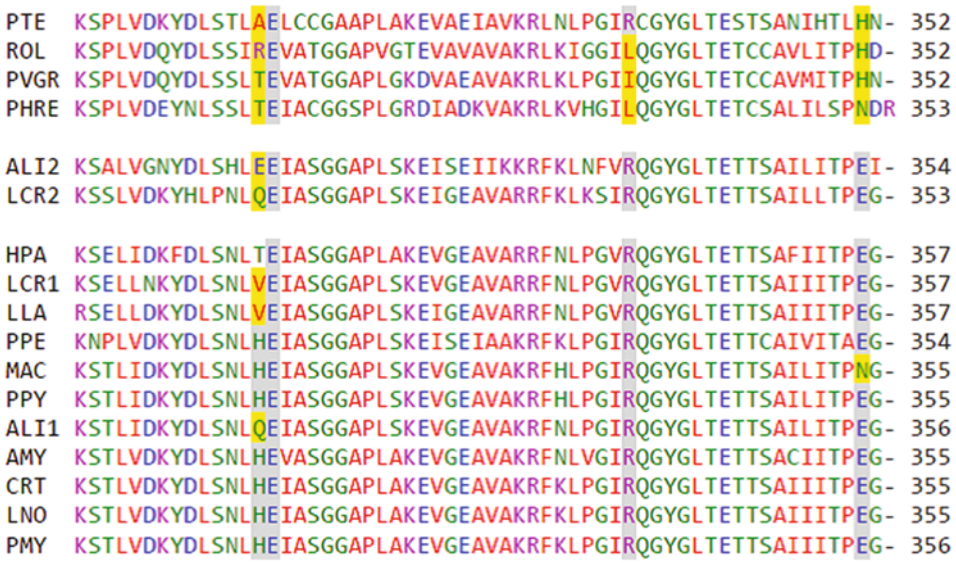


Fig. 12 Multiple sequence alignment of beetle luciferases. The pH sensor residues are highlighted in gray shadow and highlighted in yellow shadow the positions that contain amino acid substitutions. (PVGR) *Phirixothrix vivianii* green-emitting, (PHRE) *P. hirtus* red-emitting, (PTE) *P. termitilluminans*, (ALI2) *A. lineatum* AL2, (LCR2) *L. cruciata* Luc2, (HPA) *H. parvula*, (LCR1) *L. cruciata* Luc1, (PPY) *P. pyralis*, (MAC) *Macrolampis* sp2, (AMY) *Amydetes vivianii*, (CRT) *C. distinctus*, (ALI1) *A. lineatum* AL1, (LLA) *L. lateralis*, (PPE) *P. pennsylvanica*, and (PMY) *P. miyako*.

electrostatic repulsion with E354, as suggested by modeling studies. Therefore, the E310Q substitution may have been one of the critical substitutions responsible for the origin of pH-sensitivity in firefly lanterns luciferases.

The origin of efficient pH-sensitive luciferases in fireflies

The origin and evolution of firefly luciferases and of the bioluminescence system in fireflies remain challenging mysteries in modern biology.

Previously, we have shown that *A. lineatum* larvae display a weak continuous bioluminescence throughout the body, besides the lanterns that display controllable and much more intense flash-like bioluminescence. The diffuse glow has been reported in other larval fireflies too, but it was not so evident as in *A. lineatum* larvae, because other firefly larvae usually display darker pigmentation throughout the body. We have shown that the diffuse bioluminescence is produced from a green emitting luciferase isozyme more abundant in the fat body,[29] specifically from the trophocytes, the putative ancestral cells that may have originated the lantern photocytes.[29,30]

To investigate the origin of pH-sensitive luciferases and the ontology of the light organs, we used the information of former transcriptional analysis of *A. lineatum* firefly adult and larval lanterns and fat body[32] to clone the cDNAs of the pH-sensitive luciferase isozyme from the lanterns (AL1) and the pH-insensitive isozyme from the fat body (AL2). According to the transcriptional analysis, the luciferase AL1 is found predominantly in both adult and larval lanterns, whereas the pH-insensitive isozyme is predominantly found in the larval fat body and eggs.[25,32]

The AL2 isozyme displays a glow type luminescence kinetics, and is catalytically less efficient than the adult lantern isozyme AL1, which is pH-sensitive and displays a flash-like kinetics. These results and phylogenetic analysis confirmed that the pH-insensitive isoform AL2 is more primitive than AL1.

It is worth noting that the kinetic and spectral properties of AL2 resemble those of other luciferases found in the lateral lanterns of railroadworms (*Phengodidae*),[51] starworms (*Ragophtalmidae*)[52] and larval click beetles (*Elateridae*),[53] which are also pH-insensitive and glow-type, providing compelling evidence that the pH-insensitive luciferases were the ancestral luciferases which occurred in the lateral lanterns of more ancestral bioluminescent Elateroidea larvae.[27,47]

The predominant occurrence of a primitive isozyme AL2 in the fat body reinforces our previous hypothesis that the weakly glowing fat body was the ancestral tissue that originated the photogenic tissue of lanterns.[30] Such weaker and continuous bioluminescence may have arisen as a consequence of some accidental metabolic conditions in the trophocytes, such as fatty acid or pigment metabolism, acquiring a new adaptive biological advantage, with later specialization in the photogenic tissue of small lateral lantern spots, which are common in nowadays railroadworm and click beetle larvae, and finally giving rise to larger firefly larval lanterns.[47]

On the other hand, the luciferases of the group AL1, which are predominantly found in nowadays firefly lanterns, may have evolved later from a flexibilization of the protein scaffold and substitutions like E310Q at the bottom of the luciferin binding site, originating luciferases which are catalytically more efficient but also more sensitive to factors such as pH and temperature, and displaying flash-like kinetics. All these properties seem to be better suited for emission of brighter and physiologically controllable flashes, as observed in live

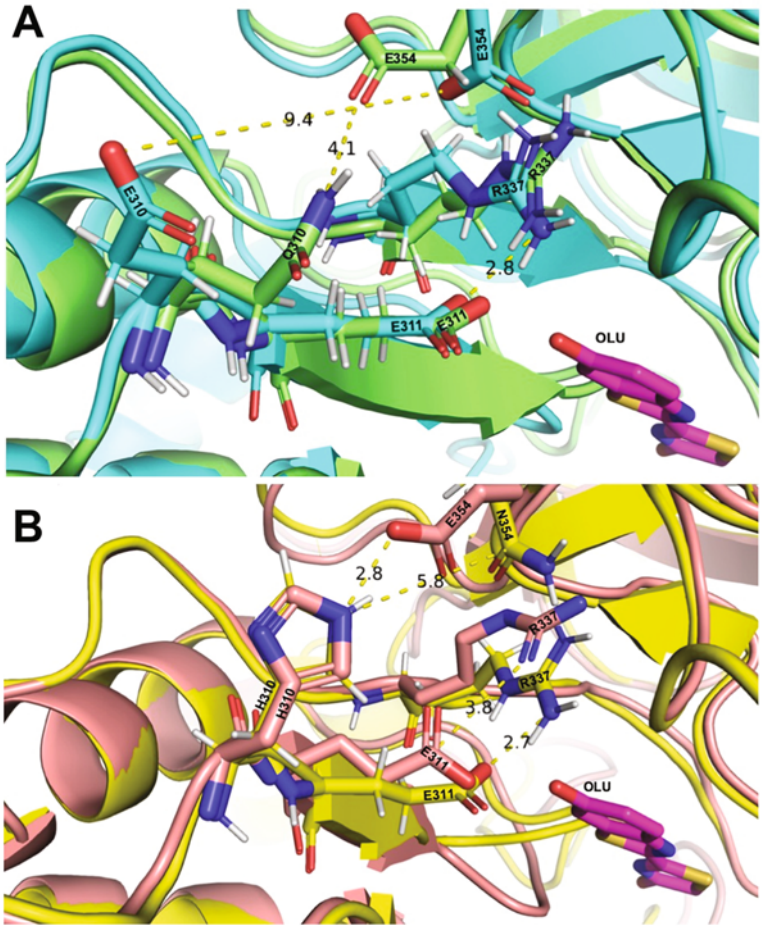


Fig. 13 Modeling of the active site of *A. lineatum* luciferases showing the residues of the pH sensor of firefly luciferases. (A) Overlapping of the AL1 models (green) that have the H310Q substitution and the AL2 (blue) that have the H310E substitution. (B) Models of *A. viviani* (rosé) and *Macrolampis* sp2 (yellow) luciferases for comparative purposes. The prediction of the 3D structure obtained by I-Tasser and OLU position was suggested by docking simulations using BINDSURF.

fireflies. Somehow, the pH-sensitivity of lantern luciferase could be linked to a higher catalytic efficiency and brightness, and to the flash-like kinetics. It is possible that pH may play a physiological role in flash control, considering that during intense bioluminescence, pH may become more acidic as a consequence of ATP hydrolysis and CO_2 production[1,2] in the lantern's photocytes. Further studies are required to better understand the physiological and biological functions of pH-sensitivity in firefly luciferases.

Concluding remarks

We cloned two new luciferases from the fat body (AL2) and lanterns (AL1) of the Brazilian *Aspisoma lineatum* firefly. We showed that the lantern luciferase AL1 displays yellow emission, is pH-sensitive and displays a flash-type kinetics, whereas the fat body isozyme AL2 emits in the green region, is pH-insensitive, displays a glow-type kinetics and is catalytically much less efficient than the lantern luciferase. The pH-insensitive isozyme AL2 is much more thermally stable and rigid than the lantern isozyme AL1. The E310Q substitution is apparently one of the substitutions that may have been responsible for the origin of pH-sensitive phenotype in lantern firefly luciferases. These results indicate that the lantern luciferases arose from the fat body ancestral luciferases through flexibilization of the protein scaffold and mutations at the bottom of the luciferin binding site, leading to more efficient flash emitting and pH-sensitive luciferases. The much higher stability, pH-insensitivity and glow type-kinetics make the AL2 isozyme very interesting as a reporter gene for bioimaging purposes.

Conflicts of interest

There are no conflicts to declare.

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