

1 ***Differential disease susceptibility in experimentally reptarenavirus infected boa
2 constrictors and ball pythons***

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20 Running title: Reptarenaviruses cause IBD

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Abstract

Inclusion body disease (IBD) is an infectious disease originally described in captive snakes. It has traditionally been diagnosed by the presence of large eosinophilic cytoplasmic inclusions, and is associated with neurological, gastrointestinal and lymphoproliferative disorders. Previously, we identified and established a culture system for a novel lineage of arenaviruses isolated from boa constrictors diagnosed with IBD. Although ample circumstantial evidence suggested that these viruses, now known as reptarenaviruses, cause IBD, there has been no formal demonstration of disease causality since their discovery. We therefore conducted a long-term challenge experiment to test the hypothesis that reptarenaviruses cause IBD. We infected boa constrictors and ball pythons by cardiac injection of purified virus. We monitored progression of viral growth in tissues, blood, and environmental samples. Infection produced dramatically different disease outcomes in snakes of the two species. Ball pythons infected with Golden Gate virus (GoGV) and with another reptarenavirus displayed severe neurological signs within two months and viral replication was only detected in central nervous system tissues. In contrast, GoGV-infected boa constrictors remained free of clinical signs for two years despite high viral loads and the accumulation of large intracellular inclusions in multiple tissues including the brain. Inflammation was associated with infection in ball pythons but not in boa constrictors. Thus, reptarenavirus infection produces inclusions and inclusion body disease, although inclusions per se are neither necessarily associated with nor required for disease. Although the natural distribution of reptarenaviruses has yet to be described, the different outcome of infection may reflect differences in geographical origin.

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Importance

New DNA sequencing technologies have made it easier than ever to identify the sequences of microorganisms in diseased tissues, i.e. to identify organisms that *appear* to cause disease. But to be certain that a candidate pathogen *actually* causes disease, it is necessary to provide additional evidence of causality. We have done this to demonstrate that reptarenaviruses cause inclusion body disease (IBD), a serious transmissible disease of snakes. We infected boa constrictors and ball pythons with purified reptarenavirus. Ball pythons fell ill within two months of infection and displayed signs of neurological disease typical of IBD. In contrast, boa constrictors remained healthy over two years despite high levels of virus throughout their bodies. This difference matches previous reports that pythons are more susceptible to IBD than boas and could reflect the possibility that boas are natural hosts of these viruses in the wild.

57

58 **Introduction**

59 Inclusion body disease (IBD) has been a vexing problem in captive snake collections for
60 several decades (1). Classic clinical signs of IBD include neurological signs, regurgitation, and
61 secondary bacterial infections including stomatitis and pneumonia (2). More recently, several cases of
62 lymphoproliferative disorders have been associated with IBD in boa constrictors (2–5). Different
63 clinical outcomes have been described for boas and pythons, with pythons reportedly experiencing a
64 shorter, more severe, and more CNS-involved disease course (1, 2, 6, 7). Passage experiments
65 demonstrated IBD to be transmissible, but the etiological agent remained elusive until the recent
66 identification and isolation of arenaviruses from snakes diagnosed with IBD (1, 7–10).

67 Two major groups of arenaviruses (family *Arenaviridae*) have been identified: those that infect
68 mammals (genus *Mammarenavirus*) and those that infect snakes (genus *Reptarenavirus*) (11–13).
69 Arenaviruses share a number of common characteristics, including a bi-segmented ssRNA genome,
70 with two genes on each of the small (S) and large (L) genome segments in ambisense orientation (11,
71 12). One possibly distinguishing feature of reptarenaviruses is that simultaneous infection by multiple
72 viruses is common in captive snakes (14–16). Whether this is true in wild snakes is unclear, and in fact
73 there is no published information about the natural hosts of reptarenaviruses, although IBD has been
74 described in a number of captive snake of a number of species worldwide, and reptarenaviruses have
75 been identified in snakes on multiple continents (2, 6, 8–10, 14, 17–19).

76 There is strong indirect evidence that reptarenaviruses cause IBD. First, reptarenavirus RNA
77 detection and viral recovery is correlated with IBD diagnosis (8–10, 14). Second, cytoplasmic
78 inclusions, the historical diagnostic hallmark of IBD, contain reptarenavirus nucleoprotein (10, 14, 20,
79 21). Third, several independent metagenomic next-generation sequencing studies have not identified
80 other candidate etiologic agents (8–10, 14, 15). Nevertheless, apparently healthy snakes can be
81 infected with reptarenaviruses and even harbor inclusion bodies (22). In fact, 5 of the first 6 apparently
82 healthy boa constrictors that we obtained initially for this study proved to be already infected with
83 reptarenavirus. Clearly, infection does not always, or immediately, produce disease. Therefore, the
84 purpose of this study was to determine whether reptarenavirus infection *can* cause IBD, as formal
85 demonstration of disease causality, and as a step toward identification of viral and host determinants of
86 pathogenicity, and to study the outcome of reptarenavirus infection in snakes of multiple species.

87 We therefore experimentally infected boa constrictors (*Boa constrictor*) and ball pythons
88 (*Python regius*) with reptarenaviruses. We monitored infected snakes and uninfected controls. We
89 periodically collected blood samples and tissue biopsies to monitor virus replication, and collected
90 environmental samples to assess possible mechanisms of transmission. Infected boa constrictors
91 remained subclinical over two years despite high and disseminated viral loads and the accumulation of
92 inclusion bodies. In contrast, infected ball pythons exhibited severe neurological signs within two
93 months after infection with viral nucleic acid and protein detected only in the brain.

94

95 Results

96 To confirm absence of pre-existing virus infection in snakes to be used for experimental
97 infections, blood, lung, and liver biopsies were collected and examined histologically and tested for
98 reptarenavirus RNA by qRT-PCR and metagenomic NGS. Five of the first six boa constrictors we
99 obtained initially tested positive for viral RNA (designated boas A–F). Three additional boa
100 constrictors from a closed collection tested negative and were used for infection studies (boas G–I).
101 We infected snakes G and H with 4×10^5 fluorescent focus-forming units (ffu) of Golden Gate Virus
102 (GoGV), a prototypic reptarenavirus, by intracardiac injection (6). Virus had been purified from the
103 supernatant of infected JK boa constrictor cells (8). The third snake (boa I) was mock infected.
104 Following inoculation, snakes were monitored and blood samples and liver and lung biopsies were
105 periodically collected. Feces, urates, shed skin, and tank swabs were also collected to assess possible
106 routes of virus shedding (Fig. 1A).

107 Similar to the boa constrictors, four ball pythons were obtained and confirmed negative for pre-
108 existing virus infection (pythons J–M). One ball python (L) was infected with 4×10^5 ffu of GoGV, and
109 a second (M) was co-infected with 2×10^5 ffu of GoGV and 2×10^5 ffu of a reptarenavirus isolated from
110 a boa constrictor that had exhibited stomatitis and anorexia and had been euthanized and diagnosed
111 post-mortem with IBD (snake #37 in (14)). Our rationale for co-infecting python M was twofold: to
112 assess the pathogenic potential of genetically diverse reptarenaviruses (the S segments of the two
113 viruses share ~74% pairwise nucleotide identity), and to conduct a preliminary investigation of
114 reptarenavirus multiple-infection, which is surprisingly common in captive snakes (14, 15). Feces,
115 urates, shed skin, and tank swabs were collected.

116 None of the boa constrictors developed clinical signs during the two-year experiment. All
117 snakes behaved normally and gained weight equivalently. The three boa constrictors were euthanized

118 at the end of the study period, 24 months post-inoculation. Complete post-mortem examinations were
119 performed and tissues were collected from all major organs for pathological examination and virus
120 detection.

121 In contrast, infected ball pythons exhibited severe clinical signs within ~2 months of infection.
122 Sixty-five days post-infection, python M developed an acute onset of neurological signs characteristic
123 of IBD, including lethargy, abnormal posture, and failure to recover from dorsal recumbency (**Fig. 1**
124 **inset**, and **Supplementary Video**). This snake was immediately euthanized. Three days later (68 days
125 post-infection) python L was observed to have focal dermatitis of unknown etiology on its right side.
126 Further evaluation revealed that the snake had paralysis of the caudal 80% of its body and did not
127 respond to hypodermic needle insertions in that area. It was unclear whether the dermatitis was related
128 to infection. The snake was immediately euthanized. The control ball pythons did not display any
129 clinical signs, and were euthanized at day 68 as well. Complete post-mortem examination of ball
130 pythons were performed, but no ante-mortem biopsies were collected because of rapid disease onset.

131 We used qRT-PCR to measure viral RNA levels in tissues. Despite the absence of clinical signs
132 in boa constrictors, high-level systemic virus replication was evident. Viral RNA was detectable in
133 blood samples throughout infection at concentrations that ranged from 10^3 to 10^{10} genome equivalents
134 per ml of blood (**Fig. 2A**). Viral RNA was detected in ante-mortem liver biopsies and in all post-
135 mortem tissues assayed: liver, lung, tonsil, spleen, kidney, colon, trachea, and brain (**Fig. 2B–C**).
136 Levels of viral RNA varied, but reached concentrations exceeding 100-fold the copy number of the
137 glyceraldehyde 3-phosphate dehydrogenase (GAPDH) control mRNA. Viral RNA was also detected in
138 feces, urates, and skin shed from boa constrictors collected throughout the two-year infection (**Fig. 3**).
139 Attempts to isolate virus from these ‘environmental’ samples were unsuccessful, perhaps because
140 samples may not have been processed or stored in a manner that preserved infectivity. These results
141 show that boa constrictors support high reptarenavirus loads in the absence of clinical signs and shed
142 detectable viral RNA in feces, urates, and skin.

143 In ball pythons, viral RNA was only detected in the central nervous system of both infected
144 snakes, but not in other tissues tested (blood, colon, liver, lung, and kidney; **Fig. 4A**). Segments of
145 genotype S2 and L2 were detected in the brain of snake L (**Fig. 4B**). Segments of genotype S6 and L3
146 were detected by qRT-PCR in the brain of snake M, which had been co-infected with GoGV (S2/L2)
147 and snake 37 virus (genotype S6/L3/L21) (**Fig. 4C**). We created shotgun NGS libraries from total
148 RNA extracted from the brains of the two infected ball pythons to confirm the absence of other
149 organisms that could be responsible for neurological signs and did not identify other candidate

150 pathogen sequences. Viral RNA was not detected in feces, urates, or shed-skin collected from ball
151 pythons.

152 We used fluorescence microscopy with an antibody raised against GoGV NP to visualize viral
153 protein in tissues. In necropsy tissues from infected boa constrictors, we observed large cytoplasmic
154 NP-positive inclusions in every tissue examined: heart, intestines, liver, kidney, and brain (**Figs. 5 & 6**).
155 Viral inclusions were also apparent in the liver biopsy samples from both boas taken at 16 weeks and
156 32 weeks post-infection, but were not evident in pre-infection tissues (**Fig. 5**).

157 In infected ball pythons, we did not detect NP-staining inclusions in any tissues except for brain
158 (Figs. 6 & 7). Anti-NP antibody staining was present in brain cells of ball python L, but in contrast to
159 the inclusions found in boa constrictor tissues including the brain, the staining appeared diffusely
160 cytoplasmic (compare **Figs. 6B** and **6C**). For the brain of python M, anti-NP staining was observed,
161 but the fixed slices from python M were not of sufficient quality for staining by DAPI, limiting our
162 ability to characterize infection in this specimen. Anti-NP staining was absent from all other ball
163 python necropsy tissues, including heart, kidney, intestines, and liver. (**Fig. 7**).

164 Gross and histopathological examinations were performed on euthanized snakes. In both
165 infected and control boa constrictors, gross lesions were mild or considered incidental. The most
166 notable histological change in boa constrictors was the presence of large eosinophilic inclusions in
167 tissues throughout the body, and in some tissues the majority of cells were affected. Most (boa H) to
168 virtually all (boa G) neurons in the brain and spinal cord had sharply demarcated inclusions (**Fig. 8**).
169 Inclusions were most dense in the retina, neurons, bile duct epithelium, ductuli efferenti, exocrine
170 pancreas, stomach, and kidney. Inclusion bodies were common in lymphocytes of all tissues in
171 infected boa H but not in boa G. Inclusions were also noted in peripheral ganglia, the optic nerve,
172 seminiferous tubules, oviductal glands, adrenal glands, hardarian glands, small intestine, respiratory
173 epithelium, pulmonary smooth muscle, cardiomyocytes, hepatocytes, and multiple vessels. Inclusions
174 were absent in the uninfected boa constrictor. Despite the abundant inclusions, little inflammation was
175 observed, and that which was observed was not considered related to infection.

176 Pathological examinations of ball pythons revealed a markedly different picture than in boas,
177 characterized by central nervous system inflammation and a general lack of obvious inclusions. No
178 gross lesions were detected in python M. Regionally extensive dermatitis was found in python L, the
179 cause and significance of which was unknown. The most significant histopathologic findings were
180 inflammatory changes in the brain, spinal cord, and ganglia of both infected ball pythons (**Fig. 9**).

181 Infected pythons had mild to moderate lymphocytic encephalitis, lymphocytic ganglioneuritis, and
182 lymphocytic, histiocytic, and granulocytic meningoencephalitis. Neuronal necrosis and neuronophagia
183 were also present (**Fig. 9**). At the site of the dermatitis observed on infected python L, multiple
184 variably sized foci of necrosis with heterophilic infiltrates were observed. Other histologic changes
185 included moderate lymphocytolysis in multiple lymphoid organs and minimal lymphocytic biliary
186 dochitis (python M). In infected ball pythons, the presence of inclusion bodies was equivocal, with
187 possible viral inclusions observed in neurons and rare bile ducts of infected python M (**Fig. 10**). In
188 both pythons, multiple types of epithelial cells had eosinophilic granular material within the cytoplasm.
189 Although these “granules” were suggestive of inclusions, the material was generally more lightly
190 stained and indistinct when compared to typical inclusions of IBD. In control snakes (J and K), no
191 significant gross or microscopic lesions were observed.

192

193 Discussion

194 Reptarenaviruses were first identified in cases of IBD, and substantial but indirect evidence
195 suggested that infection causes disease (8–10, 14, 15). While infection of both boa constrictors and ball
196 pythons resulted in the presence of detectable viral replication, we noted a stark contrast between the
197 outcome in the two types of snakes. During two years of infection, boa constrictors maintained high
198 levels of viremia (10^3 – 10^{10} viral copies per mL of blood) and accumulated widespread
199 intracytoplasmic inclusions. Despite the high viral load and numerous inclusion bodies, boas did not
200 display overt clinical signs by the time they were euthanized, and there was a notable absence of
201 inflammation. In contrast, infection of ball pythons produced dramatic clinical signs over the course of
202 only ~60 days. In pythons, inclusions were extremely rare, virus was only detected in the CNS, and
203 pronounced inflammation was observed. These findings are by and large concordant with those of two
204 IBD transmission experiments in Burmese pythons and boa constrictors that were conducted prior to
205 the identification of reptarenaviruses (1, 7). Additional studies will be required to untangle the factors
206 underlying this species-specific clinical outcome. It is also likely that not all snakes (even of the same
207 species) respond identically to infection, and additional studies using larger numbers of infected snakes
208 could reveal variability in clinical outcomes that our study, with its relatively small numbers, missed.

209 It is not clear whether the infected boa constrictors would have eventually progressed to disease,
210 and if so, over what time period. There are many examples of viruses that only produce disease after a
211 long chronic period. For instance, HIV-1 infection typically only progresses to AIDS after years of
212 mainly subclinical infection. It is possible that a longer chronic phase, secondary infection, stress, or

213 other triggers are necessary for IBD progression in boa constrictors and other less susceptible snakes.
214 Nevertheless, reptarenavirus infection in ball pythons produced neurological signs typical of IBD, and
215 these viruses remain the leading candidate etiologic agent for IBD in all snakes.

216 One possible explanation for the chronic subclinical infection in boa constrictors is that they are
217 a reservoir host for reptarenaviruses in the wild (23). Boa constrictors (family *Boidae*) are native to the
218 Americas and ball pythons (family *Pythonidae*) are found in Africa. It is possible that reptarenaviruses
219 have co-evolved with and adapted to their natural reptile hosts in the Americas, as is the case for the
220 New World lineage of mammal-infecting arenaviruses (12, 24, 25). Additional sampling of wild
221 snakes will address this possibility.

222 It is possible that reptarenavirus genotype influences clinical outcome. Indeed, a large number
223 of genetically diverse reptarenaviruses have been described, and it is possible that some
224 reptarenaviruses would produce different disease outcomes than those observed here. For instance a
225 reptarenavirus not studied here might cause disease in boa constrictors but not ball pythons. It would
226 therefore be imprudent to extrapolate from these results to all reptarenaviruses. Nevertheless, prior
227 studies have observed a strong connection between snake species and IBD clinical course, whereas no
228 connection between reptarenavirus genotype and clinical outcome has been noted to date (1, 2, 7, 9, 14,
229 15). And, in our experiment, ball pythons infected with different reptarenavirus genotypes exhibited
230 similar clinical signs: python L with GoGV and python M with GoGV and 'snake 37 virus' both
231 displayed severe neurological signs. We identified a subset of the inoculated genome segment
232 genotypes in python M's brain (S6/L3), indicating that genotype combinations S2/L2 and S6/L3
233 produced similar disease, and that experimental infection could be used to further investigate intra-host,
234 inter-reptarenavirus genotype dynamics.

235 One of our motivations for co-infecting python M with GoGV and 'snake 37' virus was to
236 begin to investigate the phenomenon of multiple unbalanced reptarenavirus infections (14, 15). This
237 phenomenon is surprisingly common in captive snakes and is characterized by intrahost virus
238 populations composed of multiple distinct viral genotypes, and by an imbalance between the numbers
239 of S and L segment genotypes in a single infection. For instance, the 'snake 37' virus inoculum was
240 composed of 3 genetically distinct reptarenavirus segments: S6, L3, and L21 (GoGV is simply S2 and
241 L2). This virus was isolated from an infected boa constrictor, and the 3 segments replicate as an
242 ensemble in culture (14). In our survey of reptarenavirus diversity, S6 was by far the most prevalent S
243 segment genotype, both at a population level and in individual snakes, suggesting it may be
244 outcompeting S segments of other genotypes (14). That S6 was the only S genotype detected in the

245 brain of co-infected ball python M supports this suggestion, but larger studies will be necessary for a
246 more conclusive investigation of this intriguing phenomenon.

247 Despite the name IBD, the connection between inclusions and disease is clearly not
248 straightforward. It is now well established that reptarenavirus infection produces the inclusions
249 associated with IBD (8, 10, 22). However, inclusions do not necessarily indicate disease and disease
250 does not require inclusions. Inclusions can be found in apparently healthy snakes, and in infected ball
251 pythons, viral nucleoprotein was cytoplasmic but not in inclusion bodies. We speculate that inclusion
252 bodies may accumulate slowly, and given the rapid disease onset in ball pythons, inclusions may not
253 have had enough time to form. Indeed, the granular appearance of cytoplasmic anti-NP staining in
254 python tissues is reminiscent of the staining pattern observed in boa JK cells shortly after infection (8).
255 Thus, reptarenavirus infection produces inclusions and inclusion body disease, but inclusions per se are
256 not pathognomonic for IBD, despite assertions to that effect (16).

257 This study has implications for control of IBD in captive snake populations. Our data suggest
258 that large quantities of virus may be shed in feces, urates, and skin. Thus, infected boas could be
259 actively transmitting virus during a chronic and subclinical period, confounding disease control and
260 quarantine measures. It would be prudent to separate boa constrictors and pythons until the boa
261 constrictors have been confirmed by molecular methods to be free of reptarenaviruses, which have
262 now been unambiguously linked to disease in ball pythons.

263

264 Materials and Methods**265 Ethics Statement**

266 This study including protocols for the care, handling, and infection of animals was approved by
267 the University of California, Davis Institutional Animal Care and Use Committee (IACUC protocol
268 #17450).

269 Preparation of Virus Stocks

270 Virus stocks for inoculation were prepared by infecting JK boa constrictor cells with low-
271 passage stocks of Golden Gate virus (GoGV (8)) or “snake 37 virus”, the virus population isolated
272 from snake #37 (14). Ten-cm dishes of infected JK cells were cultured as described (8). Supernatant
273 was collected at 4, 7, 10, and 13 days post infection and stored at -80°C. Viral RNA was purified from
274 supernatant using the Zymo Research viral RNA Kit and screened for viral RNA levels by qRT-PCR
275 as described below. Supernatants with the highest viral RNA levels were pooled and clarified by
276 centrifugation at 930g for 5' at room temperature. Clarified supernatants were filtered through a 0.22
277 µm filter, and underlaid with a 30% sucrose cushion in a centrifuge bottle (Beckman Coulter
278 #355618). Viruses were concentrated by ultracentrifugation at 140,000g in a Thermo Fisher F50L-
279 8x39 rotor for 2 hours at 4°C. Supernatant was decanted and the pellet re-suspended in 1 to 2 ml PBS.
280 Aliquots were stored at -80°C and titrated using a fluorescent focus assay as described previously (26).

281 Snake Husbandry and Monitoring

282 Three adult boa constrictors (*Boa constrictor*; one male control, one male infected, one female
283 infected) and four adult ball pythons (*Python regius*; two female control, one female infected, one male
284 infected) were used for this study. Control and infected snakes were housed in separate buildings, were
285 handled independently, and each animal had its own tank and supplies. Snakes were allowed to
286 acclimate to their housing following procurement for three weeks prior to start of the study. Whole
287 blood was collected for overall health assessment and for arenavirus RNA qRT-PCR prior to inclusion
288 in the study. During the acclimation and study periods, the snakes were monitored twice daily for
289 overall health. Animals that exhibited any abnormal neurological (star gazing, head tilt, tongue
290 flicking), gastrointestinal (regurgitation, diarrhea, constipation), or respiratory clinical signs, or that
291 repeatedly declined food, or that exhibited steady body weight loss, were to be euthanized.

292 Liver and Lung Biopsies

293 After the acclimation period, liver and lung biopsy samples were collected under isoflurane
294 anesthesia. Snakes were again anesthetized and surgical lung and liver biopsies were collected at 4
295 and 8 months post-inoculation. Biopsies were examined histopathologically and for reptarenavirus
296 RNA by qRT-PCR and metagenomic NGS.

297 **Snake Innoculation and Blood Sample Collection**

298 Several weeks after the initial biopsies were collected, mock or experimental infections were
299 administered by intracardiac injection in 200 µl PBS under general anesthesia (anesthetic protocol
300 identical to that described for biopsies). We chose this route of infection because the natural routes of
301 reptarenavirus transmission in the wild remain unknown and because prior studies have shown that
302 reptarenaviruses replicate in blood cells (20). Thereafter, every 14 days for the 1st 3 months, 0.3-0.5 ml
303 whole blood samples were collected via cardiotocesis with manual restraint, using a 25 g needle on a
304 1 or 3 cc syringe. A minimum of 3 blood smears were made and the remaining blood was collected in
305 lithium-heparin tubes and stored at -80°C until testing. At 2 months, 3 months, and 18 months, an
306 additional 0.25 ml whole blood sample was collected into an K₂ EDTA tube for a complete blood
307 count (and biochemistry panel at 18 months). After 3 months, blood was collected monthly for 9
308 months, then every 3 months during the second year of the study.

309 **Euthanasia and Post-mortem Examination**

310 Snakes were euthanized using 100 mg/kg pentobarbital administered intracardiac while under
311 isoflurane anesthesia after either exhibition of clinical signs, or at the end of the study. A full post-
312 mortem examination was performed.

313 Sections of brain, spinal cord, trachea, lung, liver, kidney, spleen, pancreas, adrenal glands,
314 gonads, heart, tonsil and complete gastrointestinal tract were collected and placed in 10% buffered
315 formalin, fixed and processed as 5 µm sections and stained with H&E. A second identical set of
316 tissues were flash frozen immediately in liquid nitrogen and stored at -80 °C.

317 **Immunofluorescence Staining and Imaging**

318 Paraffin mounted slides were deparaffinized with the following series of three minute washes:
319 mixed xylenes (x2), 50% mixed xylenes to 50% ethanol, 100% ethanol (x2), 95% ethanol, 70%
320 ethanol, 50% ethanol, and deionized water (x2). Antigen retrieval followed with a 30-minute
321 incubation at 99 °C in EDTA buffer (1mM EDTA with 0.05% Tween-20). Slides were then rinsed
322 three times with deionized water, and washed in 50 mM Tris, pH 7.6; 150 mM NaCl (TBS) containing
323 0.025% Tween-20 for 5 minutes (x2). Permeabilization was done in PBS with 0.1% Triton-X for 5

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324 minutes and followed with 5 minute washes in TBS with 0.05% Tween-20 (TBS-T) (x4). After
325 washing, the slides were blocked in blocking buffer (5% Donkey Serum, 1% BSA in TBS) for 20
326 minutes and incubated overnight at 4 °C in anti-nucleoprotein primary antibody (8) at a 1:1000 dilution
327 in TBS with 1% BSA, followed by washing in TBS-T for 5 minutes (x4). Donkey anti-rabbit Alexa
328 Fluor 488 (Thermo Fisher A-21206) secondary antibody was then applied at a 1:400 solution in TBS
329 with 1% BSA for 30 minutes at room temperature in the dark. Finally slides were washed in TBS-T
330 (x4) and mounted using Prolong Antifade Mounting with DAPI (Thermo Fisher Scientific P36931).
331 Imaging was preformed on a Zeiss Axio Scan using a 20x lens or on a Nikon Ti microscope with a
332 Andor Zyla 4.2 sCMOS spinning disk camera with a 100x lens. Image processing was done using the
333 Zeiss software, Zen Microscopy and ImageJ (27).

334 **RNA Extraction**

335 RNA was extracted from solid tissue samples, feces, urate, and shed skin samples as previously
336 described (14). Purified and DNase treated RNA samples were resuspended in 50µl of RNase/DNase
337 free water and quantified fluorometrically. To extract RNA from blood, 250µl of whole blood was
338 added to a 2 ml tube containing 1 ball bearing and 1 ml of Trizol reagent (Invitrogen) and
339 homogenized using the TissueLyzer (Quiagen) for 2-3 min at 30 Hz. Homogenized samples were
340 mixed with 200µl of chloroform, incubated at room temperature for 2 minutes, and centrifuged for 10
341 min at 12,000 x g at 4°C. The aqueous phase was mixed with 450µl cold isopropanol and incubated at
342 4°C for 1 hour. Samples were centrifuged for 10 min at 12,000g at 4°C, and the supernatant decanted.
343 Precipitated RNA was washed with 1 ml of 75% ethanol and incubated for 10 min at 4°C. RNA was
344 pelleted by centrifugation for 10 min at 12,000g at 4°C. Ethanol was removed and the pellet allowed to
345 air dry before resuspending in 80µl of RNase/DNase free water. Samples were DNased using 20 units
346 of DNaseI (NEB) and incubated at 37°C for 30 min. To DNase treated samples, 100µl of
347 phenol/chloroform/isoamyl alcohol mixture (125:24:1 pH 4.3) was added and incubated at room
348 temperature for 15 min, then centrifuged for 3 min at 12,000g at 4°C. The aqueous phase was
349 transferred to a new 1.5 ml tube and RNA was precipitated using the GlycoBlue coprecipitant protocol
350 (Ambion) with a prolonged incubation step of 30 min. Samples were DNase treated twice, followed by
351 phenol/chloroform extraction and glycoblue coprecipitation.

352 **Illumina Sequencing and Data Analysis**

353 Sequencing libraries were prepared from RNA and analyzed as previously described (14).
354 Sequencing data have been deposited in the SRA (bioproject accession PRJNA383000).

355 **qRT-PCR**

356 RNA (500 ng) was added to 1 μ l of 250 μ M random hexamer oligonucleotide, and incubated at
357 65°C for 5 minute. Master mix was added to final concentrations of 1X reaction buffer, 5mM
358 dithiothreitol, 1.25mM (each) deoxynucleoside triphosphates (dNTPs), and 0.5 μ l of Superscript III
359 reverse transcriptase (Life Tech). Reactions were incubated for 5 minutes at 25 °C, then for 5 minutes
360 at 42°C and then for 15 minutes at 70°C. cDNA was diluted to 100 μ l (1:10) in water. Each qPCR
361 reaction contained 5 μ l diluted cDNA, 1X Hot FirePol Mix Plus (Solis Biodyne), and 0.5 μ M of each
362 primer. qPCRs were run on a Roche LightCycler 480 instrument with thermocycling conditions: 15
363 min at 95°C, 40 cycles of 10 sec at 95°C, 12 sec at 60°C and 12 sec at 72°C. Viral RNA levels were
364 calculated using linearized plasmid standard curves. Primer sequences for qPCR were: S6: MDS-558
365 TTGATCTTCAGTCAGGACTTTACG & MDS-559 RACCTTGGTTCCACTGCTG; L3: MDS-530
366 ATGAGTGAGYCGACCTCCATAG & MDS-531 CRAGTGCCAATGATGTAAGAGAA; L21:
367 MDS-538 CCTCCATTGGCCTAACAACT & MDS-539 CAAGAGCAAGAGAGGTCAGAGAG;
368 S2: MDS-554 CGGTGAATCCTAGTGAGGAG & MDS-555 CTACCTTGGACCCACTGGAA; L2:
369 MDS-532 CGRCTCCACCGCCATT & MDS-533 GAGTGCTAGTGARGAAAGAGATCC; L13:
370 MDS-785 TGTCACAATGATGACCCTCAA & MDS-786 GGGCCAGTGATGAGAGAGAC;
371 GAPDH: MDS-921 AATATCTGCCCATCAGCTG & MDS-923 GTTTCCAAGAGCGTGATCC.
372 In some instances, Sanger sequencing was used to verify qRT-PCR products.

373

374 **Figure Legends**

375

Figure 1. Timeline of experimental reptarenavirus infection of boa constrictors and ball pythons.

376 Time of pre- and post-infection biopsies (bx) and blood samples tested are indicated. Inset images of
377 representative infected boa constrictor and ball python at the end of their respective study periods.
378

379

Figure 2. Boa constrictors had persistently high viral loads in all tissues. Viral RNA levels were

380 quantified by qRT-PCR **(A)** Blood viral RNA levels. S2/L2: viral genome segment genotypes. **(B)**
381 Tissue viral RNA levels for snake G and **(C)** snake H. w.p.i: weeks post infection. Samples from the
382 uninfected boa constrictor were negative.
383

384

Figure 3. Viral RNA is detectable in feces, urates, and shed skin from infected boa constrictors.

385 Viral RNA was detected by qRT-PCR. Viral RNA was not detected in any feces, urate, or skin samples
386 collected from ball pythons. *The positive result for this fecal sample from the control snake may have
387 resulted from sample mislabeling; no other sample from this control animal ever tested positive.
388

389

Figure 4. Viral RNA was detectable in infected ball python brains. Viral and cellular RNA levels

390 were quantified by qRT-PCR. **(A)** Reptarenavirus RNA was detected in brain but not other tissues.
391 Viral RNA levels were normalized to levels of GAPDH mRNA in ball python L **(B)** and M **(C)** brains.
392 Samples from uninfected snakes were negative. Controls: virus 37: virus 37 inoculum; GoGV: GoGV
393 inoculum; J brain: uninfected python J brain. nd: not detected.
394

395

Figure 5. Reptarenavirus nucleoprotein-positive inclusions were detected in livers of infected boa

396 **constrictors throughout infection.** **(A)** Biopsy and necropsy liver sections from infected and
397 uninfected boa constrictors were stained with anti-NP antibody (green) and DAPI (blue). **(B)** Necropsy
398 heart, kidney, and intestine sections were stained as in (A). Scale bars = 10 μ M.
399

400

Figure 6. Reptarenavirus nucleoprotein detected in the brain of infected snakes. Brain sections

401 were stained with anti-NP antibody (green) and with DAPI (blue). Sections from uninfected ball
402 pythons J **(A)**, infected ball python L **(B)**, and infected boa constrictor H **(C)**. Top, lower left, and
403 lower right panels display increasingly zoomed images of the same sections. Scale bars = 2000, 200,
404

405 and 20 μM . Contrast speckled cytoplasmic staining in infected ball python cells (B) with inclusions in
406 infected boa constrictor cells (C).

407

408 **Figure 7. Reptarenavirus nucleoprotein was not detected in non-CNS tissues of infected ball**
409 **pythons.** Necropsy tissues from infected ball python L were stained with anti-NP antibody (green)
410 and DAPI (blue). These images are representative of negative staining of all non-CNS tissues from all
411 ball pythons. Scale bars = 50 μM .

412

413 **Figure 8. Inclusions were evident in infected boa constrictor brains.** Images of hematoxylin and
414 eosin (H&E) stained brain sections from indicated boa constrictors. Infected boa constrictors (G and
415 H) had numerous, brightly eosinophilic, cytoplasmic viral-inclusion bodies (arrow heads) within
416 neuronal cell bodies and glial cells of the brain. Similar inclusions were found within cells of nearly
417 every organ examined. No inflammation was associated with the inclusions. The uninfected boa
418 constrictor (boa I) did not have inclusions. The inset shows a magnified view of the boxed regions.
419 Scale bars = 50 μm .

420

421 **Figure 9. Inflammation in infected ball python central nervous system tissues.** Arenavirus
422 infected pythons (L and M) had moderate lymphocytic, histiocytic, and granulocytic inflammation
423 (asterisk) within the brain, spinal cord, and ganglia. Necrotic neurons were occasionally seen (arrows).
424 No inflammation was detected in the uninfected python (J). HE. Scale bars = 50 μm . Central canal (C).

425

426 **Figure 10. Bile duct inclusions in ball python M.** Small eosinophilic cytoplasmic inclusions (arrows)
427 were seen in rare bile duct epithelial cells of the infected ball python M. H&E stained tissue section;
428 scale bar = 50 μm .

429

430

431

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436

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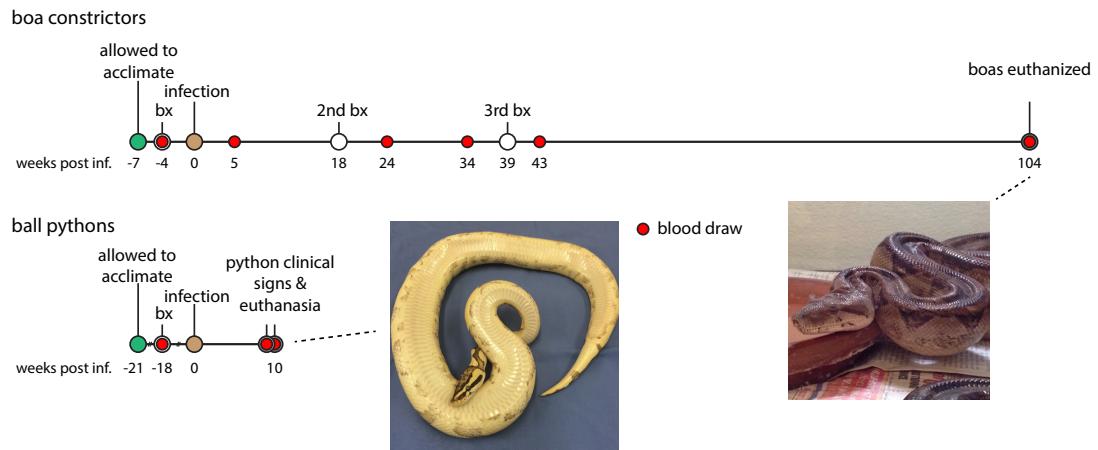
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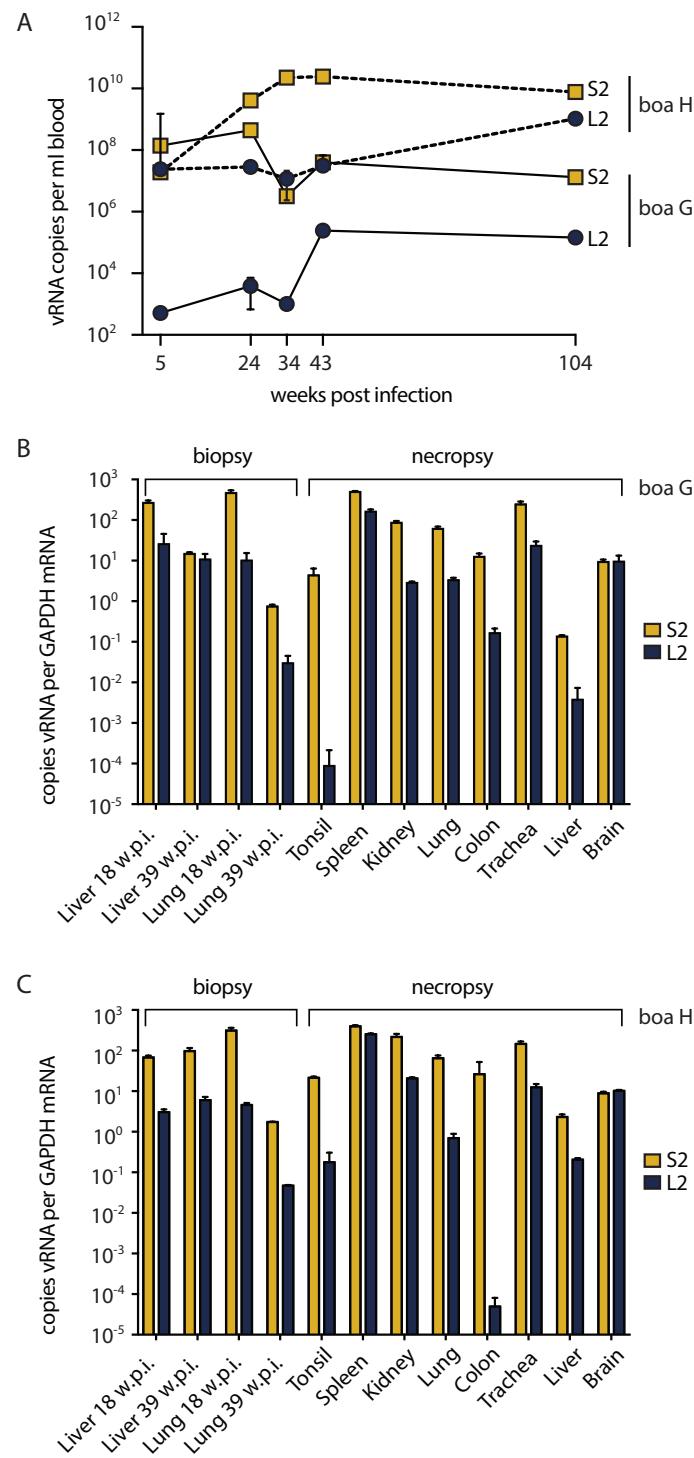
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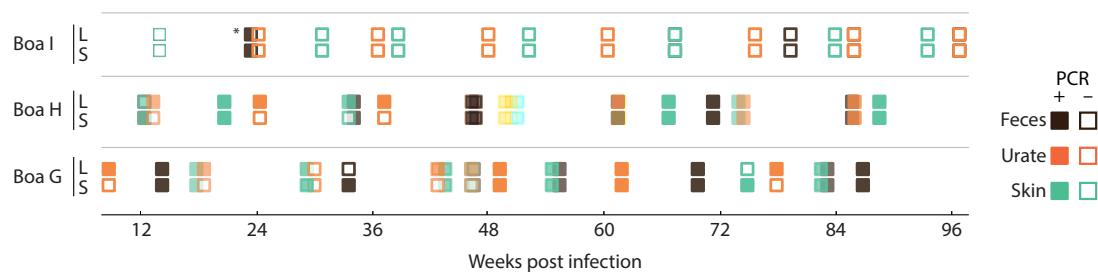
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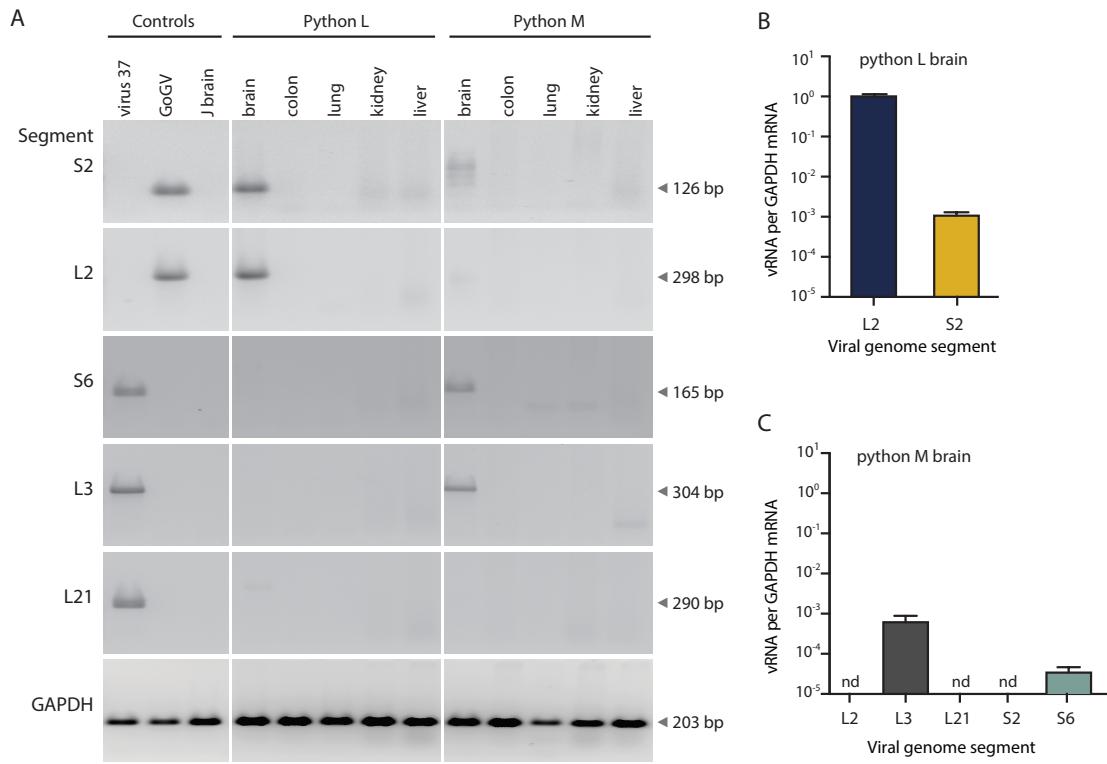
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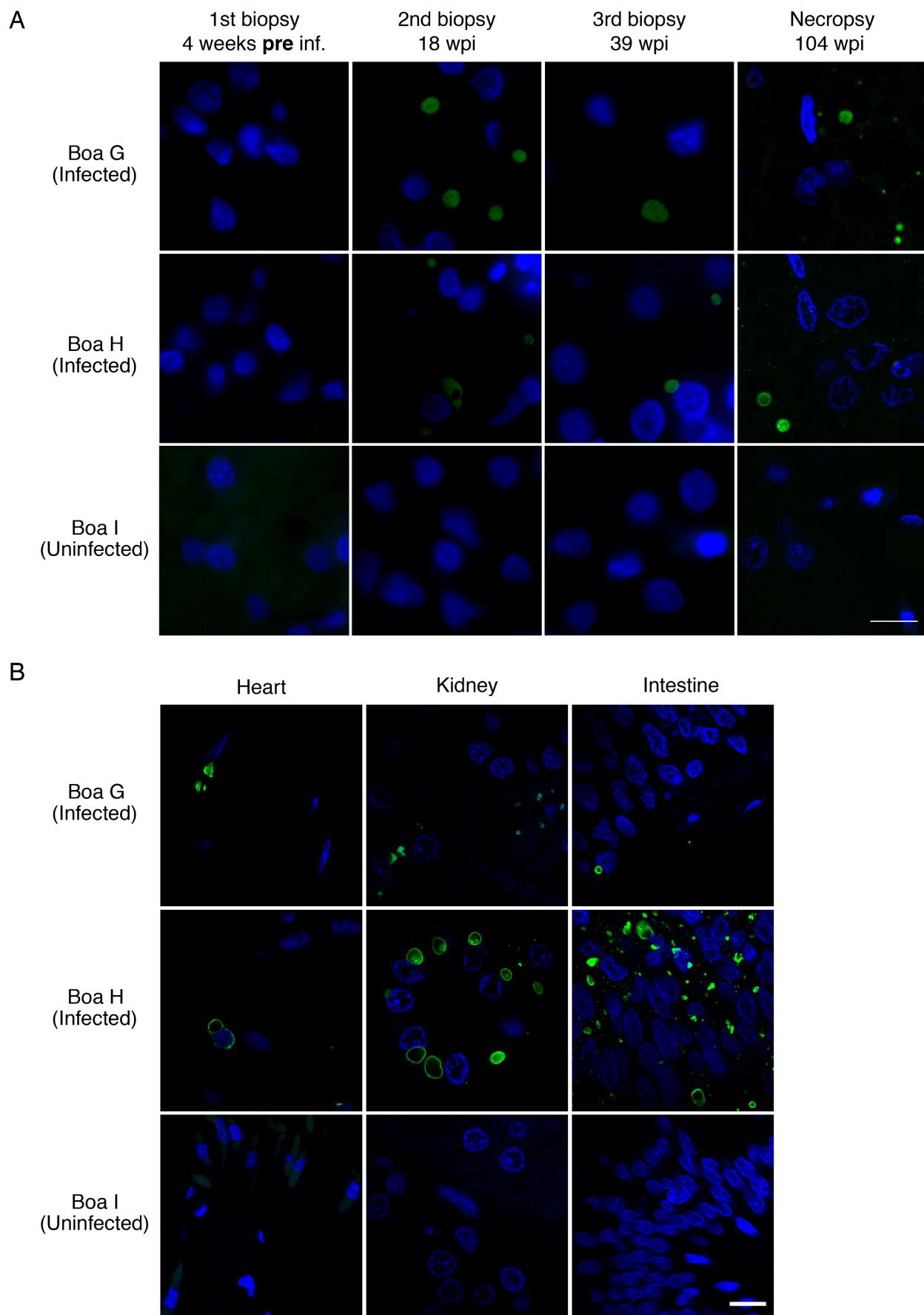
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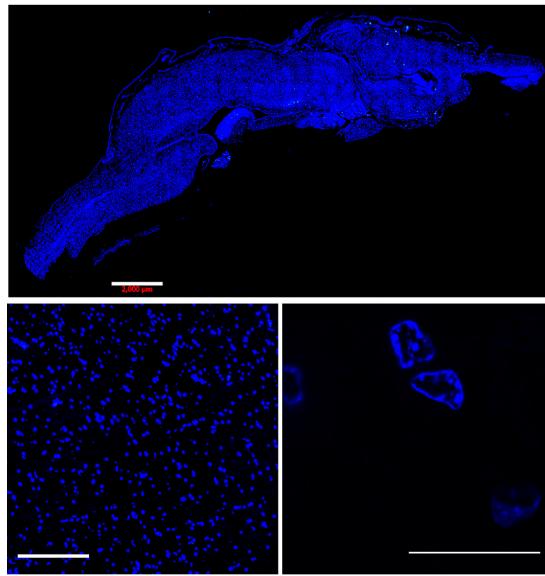




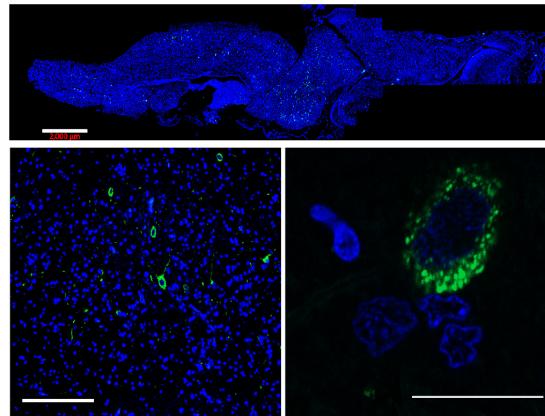




A Ball python J - uninfected



B Ball python L - infected



C Boa constrictor H - infected

