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Bioassay on horseshoe crab embryo survivability in cultures contaminated by *Bacillus thuringiensis*

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Abstract. Horseshoe crab embryos are highly sensitive to temperature and microbial stress. In this bioassay, embryos were tested under ambient, constant temperature (32 °C), dark conditions and another set with *Bacillus thuringiensis*. Six developmental stages were recorded for ambient and constant temperature settings. Survivability (100%) was maximum only in ambient temperature, it declined for 32 ºC to 76 % and it was 70.8 % together with darkness. Constant 32 °C accelerated hatching to 37 days in comparison to 40–42 days for ambient group. Exposure to *B. thuringiensis* arrested the embryogenesis in first embryonic moult stage and survivability was only 32.8%. There was abnormal coloration from degraded eggshell and these embryos were reduced in size and weight. Later, the advanced embryo had impaired limb buds. Infection rates were lowest at ambient temperature with darkness (20%) and highest under 32 °C with darkness (67.2%). Basically, thermal manipulation was stressful to horseshoe crab embryos because the hatching success rate and immunity was lowered. The presence of *B. thuringiensis* affirmed that stress increased vulnerability to pathogens. Understanding the presence of combined stressors is critical for biodiversity conservation and aquaculture management of *C. rotundicauda*.

1 Introduction

The marine environment is no longer sustainable following the expansion of human activities like industrialisation, tourism, marine traffic, and global temperature change. Furthermore, climate warming has altered the dynamics of coastal areas worldwide, leading to an increasing prevalence of vector-borne incidences in tropical regions. Typically, microorganisms develop mutual or symbiotic relationships with the ecosystem, co-supporting a network comprising bacteria, viruses, fungi, and protists. This relationship can shift for metazoans, as viruses may function as both symbionts and pathogens [1].

For example, a crayfish (*Cherax quadricarinatus*) may carry Decapod penstylhamaparvovirus 1 and show no symptoms, but upon exposure, shrimp (*Penaeus*

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vannamei) could become infected with infectious hypodermal and haematopoietic necrosis. While a total of 102 agents comprising of 25 viruses, 33 bacteria, 23 protists, and 21 metazoans were responsible for notable diseases across plants, fish, corals, molluscs, crustaceans, echinoderms, turtles, and mammals, many of these diseases occur during early life stages or failed to gain widespread notice at the population level [2].

Assays were developed to understand the interactions of biological agents, from which a total of 182 half-effective concentrations involving *Penicillium* sp. and *Pseudialteromonas issachenkonii* were derived [3]. A separate assay revealed 12 bacterial extracts (*Bacillus* sp. and *Enterococcus* sp.) capable of 90 % to 100 % mosquito larvae mortality. Aside from product identification and industrial applications, bioassays are also used to evaluate growth performance, such as the exposure of *P. vannamei* post-larvae to *Vibro* sp. carrying VpPirAB genes. Although 100 % mortality was observed, histopathology revealed sloughing and detachment of the hepatopancreas, confirming the disease aetiology in contaminated hatcheries. A *Bacillus tequielensis* strain demonstrated 65 % efficacy for insecticidal activity against the mangrove pest insect (*Spodoptera frugiperda*), and these findings indicated its potential for endophytic remediation in mangrove plots. Importantly, however, not all Bacillus strains are beneficial for promoting the safe development and growth of a target species.

Meanwhile, horseshoe crabs are marine arthropods that endure a lengthy embryogenesis lasting 40 days, require 9–10 years for sexual maturity, and have a perceived lifespan of 15–18 years [4]. As an oviparous species, their embryos are left buried in the sand, enduring brief episodes of tidal exchange or otherwise experiencing periodic thermal shocks that are thought to expedite the embryonic moulting process. Essentially, with four embryonic moults, horseshoe crab embryos may pass through sensitive periods that can be identified using experimental assays. For instance, stock enhancement programmes encourage release strategies in optimal habitats, such as intertidal areas or near mangrove roots, specifically targeting low tide (when water temperatures are cooler) and preferably using individuals at the instar stages [5]. Feeding experiments indicated a blood meal containing 40 % iso-nitrogen and 14 MJ kg^{-1} as an optimal replacement for conventional feed containing 15 % to 25 % protein.

Early experiments outlined the development of horseshoe crabs, recording 16 moult cycles in 9 years for the male and 17 moult cycles over 10 years for the female of Limulus polyphemus, whereas *Tachypleus tridentatus* showed 15 and 16 moult cycles over 13 and 14 years, respectively, for the same sexual orders. While such experiments involved lengthy hatchery rearing, a simple tolerance experiment indicated optimum development below 60 ppt salinities and when water was maintained at 25–40 ºC [6]. Horseshoe crab embryos were also tested with cadmium, zinc, copper, mercury, and tributyltin, with results indicating 1% embryonic malformations, which included impaired walking legs. Generally, these embryos showed higher stress protein production and accumulated these hazards in their tissue; moreover, the adults could sequester toxic compounds to their eggs, indicating a risk to predators higher up in the food chain. The growth and performance of horseshoe crabs were also evaluated through assays that used raw seawater exchange to supply organic matter, supplemented by live *Artemia* nauplii. Simple experiments such as this revealed crucial support is needed to sustain sensitive early life stages up to 10 weeks, until hatchlings reach the 3rd instar stage. These assays also provided crucial information on developmental defects as well as growth performance. Fundamentally, horseshoe crabs have persisted for 450 million years with negligible evolution, which accounts for their ancient template that has allowed them to continue existing despite climatic events and coastal developments during the course of modernisation.

Given the limited experiments investigating post-fertilisation development and embryogenesis until hatching, much has been discovered concerning cellular changes and morphological variability. This includes the confirmed presence of the amoebocyte during stage 18 of embryonic development, which accounts for its increased tolerance against abrupt environmental changes, and the formation of the book gill, similar to the scorpion book lungs [7]. A subsequent experiment evaluated ex situ spawning ground sediment characteristics in relation to nesting preferences, egg release, and spawning frequencies. Spawning grounds with 0.25–1.00 mm sediments were most preferred by 45 female crabs, which released 27,266–30,859 eggs in 3 hours [18]. However, biotic assays on this arthropod remain limited. Although the arthropod can be fed with fish, squid, clams, shrimp, fish diets, and small crabs, it is susceptible to infection by algae, fungi, cyanobacteria, Gram-negative bacteria, and parasites, and currently has few known therapeutic options aside from removal, pharmacokinetics, oxytetracycline administration, and oral dosing.

With regard to microbial infections, embryogenesis could be threatened by bacteria like *Shewanella putrefaciens*, *Bacillus cereus*, *Corynebacterium* sp., and *Enterococcus faecalis*, as well as fungi such as *Aspergillus* sp., *Aspergillus niger*, *Peniclillium* sp., *Gliocladium* sp., which cause the horseshoe crab eggshells to appear red, grey, or black. Eggs with abnormal colourations were also reported in another spawning ground, namely Johor Lama, where hatched larvae exhibited impaired or distorted body plans. The same outbreak occurred at Pantai Balok, the most studied spawning ground for horseshoe crabs in 2022–2024 surveys. Notably, this *Bacillus thuringiensis* possesses the cry1 toxin gene and is capable of digesting the proteins on the horseshoe crab egg envelope [8]. It is confirmed that *B. thuringiensis* is Gram-positive, typically discovered in soil, and may have a host-specific aetiology. A recent study indicated that this bacterium is likely to experience notable habitat gains globally by 2090 due to its use as a biopesticide. The entomopathogenic capability of *B. thuringiensis* and the presence of cry homologous toxins have made it an ideal insecticide. Crucially, however, experiments on growth performance under biotic influences have not yet been attempted. Therefore, this study aims to evaluate the embryogenesis of the horseshoe crab, *Carcinoscorpius rotundicauda*, in different culture assays alongside the administration of *B. thuringiensis* to examine host-specific developmental responses.

2 Methodology

2.1 Sampling

Fieldwork was conducted at Pantai Balok (N 03º 56.009′; E 103º 22.584′), a recognised spawning ground for horseshoe crabs on the east coast of Peninsular Malaysia. Sampling trips were scheduled in accordance with the monthly lunar cycle, when large numbers of horseshoe crabs migrate into shallow waters for amplexus during the high-tide breeding period. Potential nests were located by following the tracks left by adults on the mid- to high-beach face. A total of 15 nests were excavated at low tide between August 2022 and March 2024. Of the identified disturbances, nine nests actually contained eggs grouped in a single clutch, whilst 38 were determined to be false crawls (or abandoned nesting attempts). Freshly laid eggs were separated from the clutch using a sieve, counted, and subsequently transported to the laboratory for culture experiments.

2.2 Temperature assay

The collected eggs were subsequently raised at the hatchery. This process commenced with a two-day acclimatisation period before the initiation of the assay, which employed two distinct temperature regimes. The first regime used ambient conditions, where the temperature was permitted to fluctuate between daytime and night-time intervals (28–31 ºC). The second regime was maintained at a constant 32 ºC using heaters (Jager Aquarium Regler-Heizer N12350 220V-240V). Another set of eggs was maintained under the same regimes but were blacked-out to provide a consistently dark environment (Fig. 1a). For all experiments, each tank contained 250 eggs, and the water was retained at 30 ‰ salinity to accurately mimic the surface water of Pantai Balok. Finally, a portable aquarium air pump (AP-005 220-240V50/60H2) was used to oxygenate the water.

2.3 Bioassay

The soil bacteria, *Bacillus thuringiensis*, were initially cultured in nutrient broth for 24 hours. Following this, the culture underwent a ten-fold serial dilution, and the colony-forming units (CFU) were counted to quantify the inoculum. A dosage of 10^8 CFU in 1 ml of nutrient broth, consistent with the LC 50 recommendation, was subsequently introduced to the relevant conditions prepared in the temperature assay. Consequently, the experimental set-up comprised eight distinct conditions: ambient (A); ambient with bacterial exposure (A+B); ambient with permanent darkness (A+D); ambient with permanent darkness and bacterial exposure (A+D+B); constant 32 ºC (C); constant 32 ºC with bacterial exposure (C+B); constant 32 ºC with permanent darkness (C+D); and constant 32 ºC with permanent darkness and bacterial exposure (C+D+B). Conditions without bacterial addition served as control groups, whilst those with bacterial inoculation were designated as treatment groups (Fig. 1a). The embryos introduced into the assay were monitored over the maximum (more precise wording) incubation period for their development, which was 42 days post-fertilisation. We monitored the embryos daily until 100% hatching was achieved in the control groups. Recorded observations included developmental stages and duration, survivability, weight (using an analytical balance), length and width (measured with an electronic digital vernier calliper), and vulnerability, alongside any abnormal colour variation (often red colour) in the embryos.

2.4 Histology examination

Microscopic slides of all samples were prepared using the standard Haematoxylin and Eosin (H&E) protocol. Initially, samples were longitudinally sectioned (4 µm thickness) with a microtome, placed in labelled polypropylene cassettes, and fixed in 10% neutral buffered formalin (pH 7) for 24 hours. This was succeeded by a 12-hour tissue processing cycle that involved graded ethanol dehydration (70 %, 90 %, 95 %, and 100 %) and xylene clearing, carried out prior to hot paraffin wax infiltration (75 ºC). Subsequently, the specimens were embedded in paraffin blocks, trimmed, and sectioned into 4 µm ribbons. These ribbons were then expanded on a 40 ºC water bath, mounted onto Mayer's albumin-coated slides, fished out, and dried for 24 hours at 35 ºC. The H&E stain protocol was then modified to integrate additional steps for Sudan IV and Toluidine-O staining. This primary protocol modification included integrating three washing repeats between the alcohol and the haematoxylin staining steps. Following this, further modifications involved adjusting the soak timings to three times for Sudan IV and six times dipping for Toluidine-O to accurately distinguish proteins, haemocyanin, amoebocytes, and lipids. The final stages involved a ten-second xylene dip and air drying for three minutes. Mounting was achieved using DePeX (polystyrene in xylene), followed by the placement of a cover slip. Lastly, the specimens were inspected under an advanced compound microscope (Nikon eclipse 80i, USA) using 5X, 10X, and 20X magnifications (Fig. 1b & c).

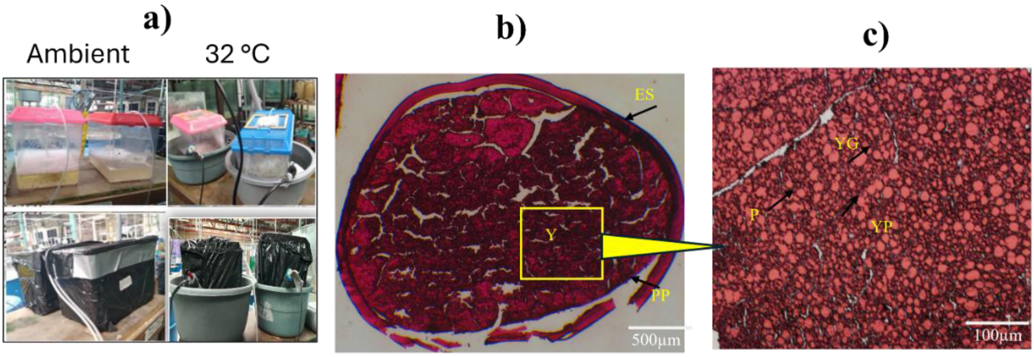

Fig. 1. A general view on the experimental setup and the organism tested. a) Bioassay setup for *Carcinoscorpius rotundicauda* embryos for all conditions including light, dark and with bacteria. b) longitudinal section of *C. rotundicauda* egg used for histological observations, c) higher magnification of egg yolk. ES= eggshell (outer chitinous layer), pp= periplasm (inner layer), Y= yolk, YG= yolk granules, YP= yolk platelets, P=pits.

2.5 Statistical analysis

Egg weight, volume, and surface area during embryogenesis were analysed using a two-way repeated measures ANOVA in GraphPad Prism software (version 5). A significance level of $P \leq 0.05$ was considered indicative of a substantial difference between means. It should be noted that the egg volume and egg surface area were calculated based on the embryo length and width measurements, employing specific equations as provided below. In addition, the developmental rate, survivability, and vulnerability were expressed as percentages and subsequently plotted using Microsoft Excel.

$$\text{Egg volume} = (0.6057 - 0.0018B)\,LB^2 \quad (1)$$

$$\text{Egg surface area} = (3.155 - 0.0136L + 0.0115B)\,LB \quad (2)$$

*L= length, B=width

3 Results

3.1 Bioassay findings

3.1.1 Life stages of embryogenesis

The developmental stages of *C. rotundicauda* eggs in control groups were divided into six categories represent in early developmental stage (ED), the first embryonic moult (M1; stage 18), the second embryonic moult (M2; stage 19), the third embryonic moult (M3; stage 20), the emergence of trilobite larvae (H; stage 21), and the first instar larvae (L) after 24 hours of hatching. During the ED stage (days 1–7, 1st week), the eggs appear olive green in colour, with cleavage nuclei emerging at the egg surface which contribute to the initiation of germ disk formation. Embryos of this stage were generally spherical and later transition into oval-like form as development progresses. In stage M1, after 7–14 days (by 2nd week), the first embryonic moult was characterized by cracking of the tough opaque chorion and the embryo appear enclosed by both outer opaque chorion and inner non-expanded extraembryonic shell. In 15–21 days or 3rd week, the embryos reached stage 19 or M2 where the second moult is characterised by chorion shedding that exposes the non-expanded extraembryonic shell (transparent membrane).

At this stage, a small undifferentiated embryo became clearly visible through the transparent membrane, with rudimentary limb buds that were initially rounded and later developed into pointed tips. This stage is regarded as the critical point for determining successful development, marking the transition from opaque to semi-translucent eggshell, swelling from perivitelline fluid and visibility of recognizable embryo. From days 22–28 (4th week or stage 20), the third embryonic moult (M3) occurs. The inner egg membrane was expanded tremendously where a moving embryo was visible. At this stage, the egg size increases by 120% due to cellular differentiation and uptake of water, constant larval movement was observed, and the prosoma and opisthosoma became distinct. By days 29–35 (5th week; stage 21), a well-developed embryo with distinguishable morphologically was ready to hatch as the trilobite larva. Finally, in days 36–42 (week 6), the trilobite larva moults into the first instar stage where the larvae resemble the adult form (Fig. 2a & Table 1). However, embryos exposed to *B. thuringiensis* failed to complete development under all tested conditions. Most of the embryos had their development arrested in the first embryonic moult, displaying abnormal coloration (often red) and gradually becoming smaller and more rounded in shape. Only a small proportion (~2%) advanced to the second embryonic moult (M2) and remained in this stage at the end of the experiment (day-35; Fig. 2b & Table 2).

3.1.2 Embryogenesis during the bioassay

In the ambient condition, early development (ED) was reached slightly earlier, on day 13. The first moult stage or M1 also lasted 13 days, beginning on day 8 and ending on day 20. Generally, M2 lasted 14 days, from day 14 to day 27, M3 lasted for 13 days, from day 21 to day 34, hatching (H) was shorter, lasting 13 days, from day 28 to day 40, and first instar (L) was reared for 6 days, beginning on day 35 and ending on day 40. Embryogenesis occurred until termination of experiment on day-40 (Fig. 3a). However, with the addition of *B. thuringiensis*, the ED stage prolonged, up to 30 days. Interestingly, embryos transitioned into the M1 stage within 11 days but unfortunately, they remained without change for 42 days. Only 2% transitioned to the M2 stage on day-35 and remained in this stage until day-42 (Fig. 3b). In dark conditions, the ED stage spanned 14 days although a majority of embryos transitioned into M1 by day-13. The M2 stage occurred from day-15 to day-28 while M3 stage occurred from day 22 to day-35 with both stages lasting 14 days apart. Hatching occurred by day-29 and lasted to day-42 and the hatched trilobite took 7 days from day day-36 to day-42 to completely transition into the first instar (Fig.3c). In dark condition, the embryos exposed to *B. thuringiensis* performed better where the ED stage lasted 28 days. The progression into stage M1 occurred by day-11 and for the remaining 97.2 %, they remained at this stage until day-42 PF. This ~2.8% took 31 days to transition into stage M2 (Fig.3d).

For the constant 32 °C exposure, stage ED lasted for 11 days despite the first transition into stage M1 by day 7. This stage lasted 7 days with the last embryo transition on day 18. Meanwhile, M2 spanned 13 days, from day 12 to day 24, M3 lasted 13 days, from day 18 to day 30, H lasted 18 days, from day 25 to day 42, L was observed for 7 days, between days 31 and 37 (Fig. 2d; Fig. 4a). However, with the addition of *B. thuringiensis*, ED lasted for 34 days. The first transition into stage M1 occurred on day 9 and the embryos remained in this stage for 34 days even until the experiment ended by day-42 (Fig. 4b). For the batch in constant 32 °C and darkness, the ED stage lasted for 11 days while the transition to stage M1 begun on day-7 and continued to day-18. About 13 days was needed for embryos to reach stage M2 which begun on day-12 to day day-24. The first transition to stage M3 occurred on day-18 and lasted to day-30. Egg hatching begun on day 25 and it continued daily until day-42. The first instar was observed by day-31 and all embryos were completely in instar stage by day-37 (Fig. 4c). However, when *B. thuringiensis* was added to this experimental design, the ED stage lasted for 34 days. The first transition to stage M1 occurred on day-34 and the embryos remained in this stage until day-42 (Fig. 4d).

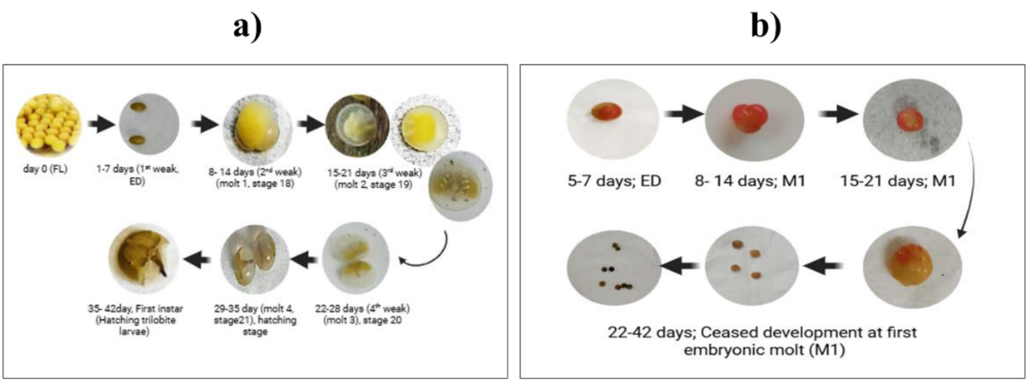

Fig. 2. Visual inspections on the embryos of *Carcinoscorpius rotundicauda*. a) The appearance of developing *C. rotundicauda* embryos, b) The appearance of *C. rotundicauda* embryos after infection. FL= freshly laid eggs, ED= early developmental stage, M1= first embryonic moult, M2= second embryonic moult, M3= third embryonic moult, H= hatching of trilobite larvae, L= first instar stage).

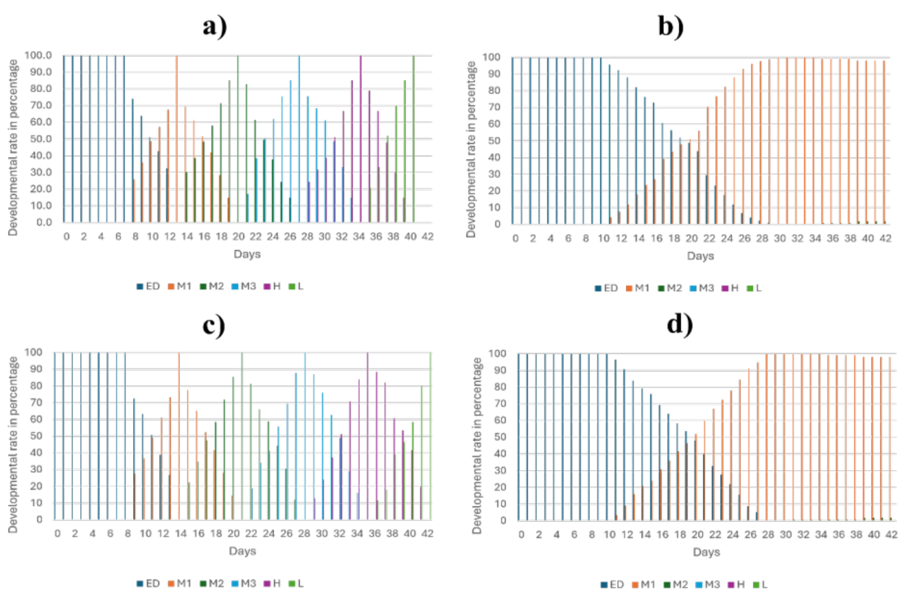

Fig. 3. Development of *Carcinoscorpius rotundicauda* embryos characterised by different embryonic moult stages. a) ambient condition, b) ambient condition with bacteria, c) ambient temperature with darkness, d) and ambient temperature with darkness and bacteria.

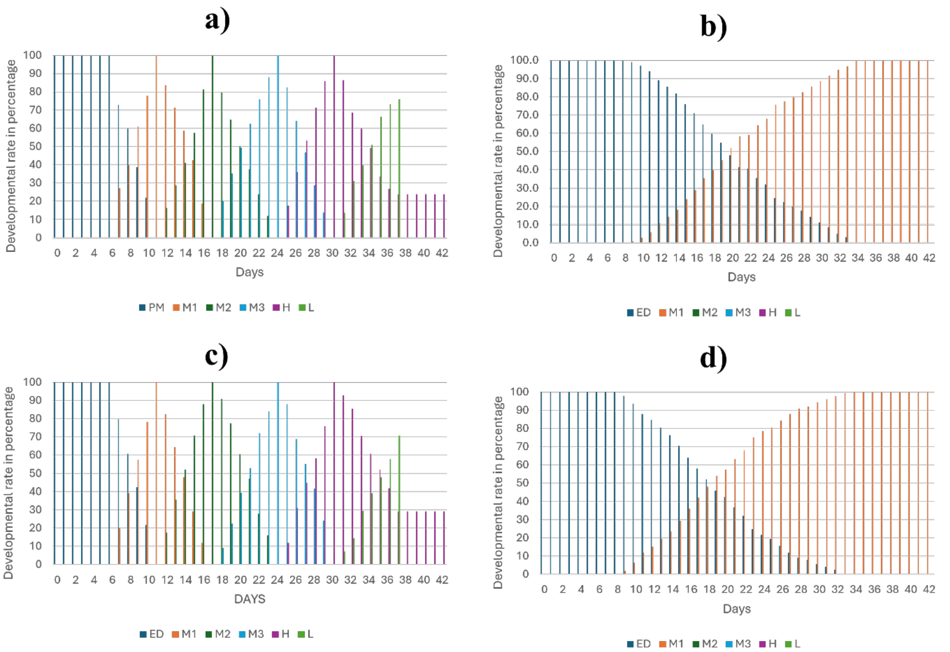

Fig. 4. Development of *Carcinoscorpius rotundicauda* embryos characterised by different embryonic moult stages. a) constant temperature (32 °C), b) constant temperature (32 °C) with bacteria, c) constant temperature (32 °C) with darkness, and d) constant temperature (32 °C) with darkness and bacteria.

3.1.3 Survival of developing embryo in bioassay

Survivability test was conducted to estimate persistence of embryos to continue developing after infection by *B. thuringiensis*. In control groups (ambient temperature, 32ºC and darkness with both temperature settings) *C. rotundicauda* embryos maintained 100% survival for 42 days because trilobite larvae hatched out and moulted to the first instar. However, in constant 32 °C, the survivability was 76% because not all embryos transitioned to first. Comparatively at 32 °C and darkness, the survivability reduced to 70.8% due to prolonged hatching; after day-35 (Figure 4.a). For all temperature assays, including darkness, the addition of bacteria reduced the survivability to 70.8% (fluctuating temperature, a), 80% (a+darkness), 40.8% (c or 32ºC), and 32.8% (c+darkness; Fig. 5a).

3.1.4 Vulnerability of developing embryo in bioassay

A vulnerability test was conducted to identify the most critical embryonic stages of *C. rotundicauda* for infection during the moult cycle. The assessment was based on the appearance of abnormal coloration on the eggshell, typically red, which was considered indicative of bacterial infection. In all control groups, the vulnerability score remained 0 across all embryonic moults throughout the incubation period (Table 1). In contrast, eggs exposed to *B. thuringiensis* in the treated groups exhibited varying degrees of vulnerability. The highest scores were recorded in regimes with 32 °C (59.2%), together with darkness (67.2%) and followed by ambient temperature (29 %) and together with darkness (29.2%). The vulnerability score indicated the first 14 days of the experiment as most sensitive and despite some embryos developed to stage M2, the inability to surpass this stage by 42 is an indication of ongoing vulnerability that threatened their development (Table 2).

3.1.5 Weight of developing embryo in bioassay

Embryo weight was monitored throughout the incubation period (7, 14, 21-, 28-, 35-, and 42-days) as an indicator of successful *C. rotundicauda* embryogenesis. For embryos maintained in ambient conditions and ambient temperature with darkness, these embryos showed a steady increase in weight up to day-28, reaching peak values of 133.99 mg and 148.32 mg, respectively. Thereafter, a sharp decline was observed at day-35 and day-42, with weights decreasing to 72.05 mg and 65.01 mg. In conditions like ambient and together with darkness, the embryo final weights were 78.42 mg and 72.62 mg in respective order. In 32 °C, maximum weight gain was on day-28 at 81.48 mg and with darkness it was 89.17 mg but by day-35 and day 42, the weight of embryos in the same order became 61.33 mg and 56.24 mg, and 59.83 mg and 53.97 mg, respectively (Fig. 4b). However, the presence of bacteria in this experiment led to uneven distribution of weight across all development stages. For instance, weigh gain was witness by day-14 in the order ambient (a; 41.35 mg), A +darkness (43.53 mg), 32 ºC (37.64 mg) and with darkness (36.36 mg). For the same order, the final weights reduced to 28.55 mg, 28.12 mg, 22.63 mg, and 21.72 mg. Embryo weight differed significantly across all days between control and treatment groups ($P< 0.001$) (Fig. 5b).

3.1.6 Egg volume of developing embryo in bioassay

Egg volume was assessed as an additional indicator of successful growth in *C. rotundicauda* embryos, with measurements taken at regular incubation intervals (7, 14, 21-, 28-, 35-, and 42-days PF) based on embryo length and width. In the control groups maintained under both ambient and 32 °C conditions, embryos exhibited a steady increase in egg volume, reaching their maximum by day 42 (403.39 mm^3, 424.65 mm^3, 255.99 mm^3, and 200.60 mm^3) in the order ambient condition, ambient temperature with darkness, constant 32 °C, and constant 32 °C with darkness (Fig. 4c).

In contrast, embryos exposed to bacterial treatment showed only a slight increase in egg volume up to day 14 PF, with consistently lower values than those in the non-bacterial groups. Notably, embryos reared under constant temperature with bacterial exposure displayed slightly lower volumes than those at ambient conditions with bacterial exposure. Their dimensions were 32.61 mm^3, 34.48 mm^3, 22.26 mm^3, and 16.66 mm^3 in the order ambient condition with bacteria, ambient temperature with darkness and bacteria, constant 32 °C with bacteria, and constant 32 °C with darkness and bacteria. Thereafter, egg volume gradually declined toward day 42. Differences were significant on most days between control and treatment groups ($P < 0.001$), with exceptions on day 14 for C vs. C+B and C+D vs. C+D+B (no significance), day 7 (weak significance, $P < 0.05$ for C vs. C+B and C+D vs. C+D+B) as shown in Fig. 5c.

3.1.7 Egg surface area of developing embryo in bioassay

Egg surface area was calculated from egg length and width measurements as an additional indicator of successful growth in *C. rotundicauda* embryos. In the control group, egg surface area was decreased between day-7 and day-14 PF, followed by a gradual decline as development continued on day 15 onward to days 35–42 days. Among the control conditions, the steepest decline occurred at ambient temperature with permanent dark, followed by ambient condition, then 32 °C, and lastly 32 °C with permanent dark. In contrast, eggs exposed to bacterial treatment showed an early decline in surface area between day 7 and day 14 after which seems stabilized across all conditions. Most days showed significant differences between the control and treatment groups (P < 0.001), except on day 14 for C+D vs. C+D+B, where no significant difference was detected, and on day 7, where only weak significance was observed (P < 0.05 for both C vs. C+B and C+D vs. C+D+B), as illustrated in Fig. 5d.

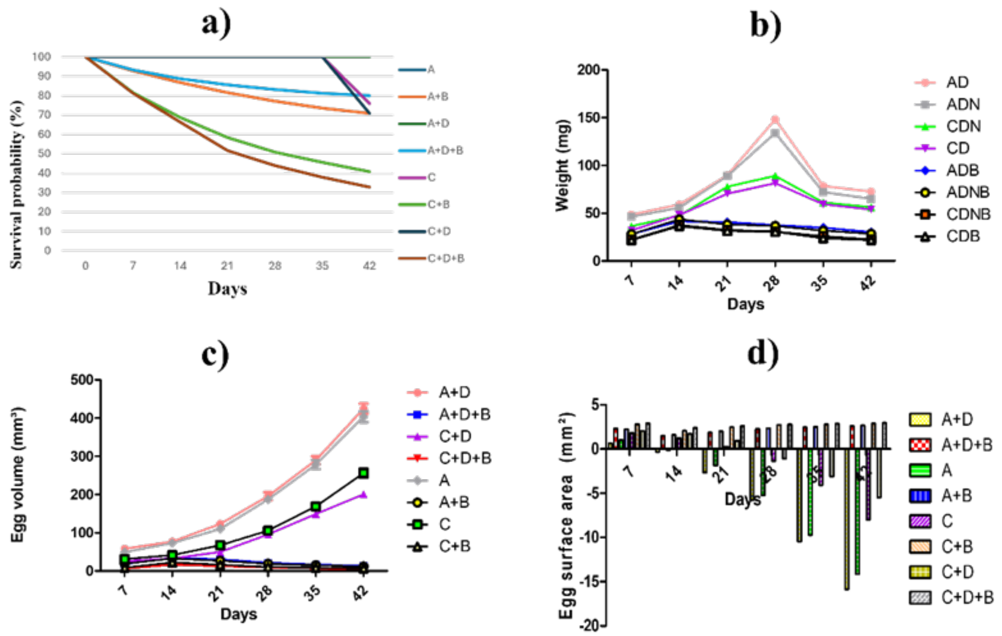

Fig. 5. Associations to *Carcinoscorpius rotundicauda* embryogenesis during the assays. a) Survival of *C. rotundicauda* embryos where n=250 eggs for each group. b) weight of *C. rotundicauda* developing embryo in bioassay, c) egg volume of *C. rotundicauda* developing embryo in bioassay, and d) egg surface area of *C. rotundicauda* developing embryo in bioassay. * A+D= Ambient temperature with permanent dark, C+D= Constant temperature 32 °C with permanent dark, A+D+B= Ambient temperature with permanent dark and bacterial exposure, C+D+B= Constant temperature 32 °C with permanent dark and bacterial exposure, A= Ambient temperature with day-night interval, C= Constant temperature 32 °C with day-night interval, A+B= Ambient temperature with day-night interval and bacterial exposure, and C+B= Constant temperature 32c° with day-night interval and bacterial exposure. * In eggs exposed to *B. thuringiensis*, embryos ceased at M1 persisted during the same period in which control group embryos advanced toward hatching.

Table 1. Developmental stages of *Carcinoscorpius rotundicauda* embryos, egg morphology and the vulnerability score. G= green, M= moult, RO= round, O= oval, T= trilobite, OP= opaque, TR= transparent, + indicates presence of feature, and - indicates absence of feature.

Condition		A+D						A						C+D						C					
Stage		ED	M1	M2	M3	H	L	ED	M1	M2	M3	H	L	ED	M1	M2	M3	H	L	ED	M1	M2	M3	H	L
General features	Colour	G	M	M	M	M	M	G	M	M	M	M	M	G	M	M	M	M	M	G	M	M	M	M	M
	Shape	Ro	O	O	O	O	T	Ro	O	O	O	O	T	Ro	O	O	O	O	T	Ro	O	O	O	O	T

Condition		A+D						A						C+D						C					
Stage		ED	M1	M2	M3	H	L	ED	M1	M2	M3	H	L	ED	M1	M2	M3	H	L	ED	M1	M2	M3	H	L
	Eggshell appearance	OP	OP	TR	TR	TR	-	OP	OP	TR	TR	TR	-	OP	OP	TR	TR	TR	-	OP	OP	TR	TR	TR	-
Specific features	Limb bud formation	-	-	+	+	+	+	-	-	+	+	+	+	-	-	+	+	+	+	-	-	+	+	+	+
	Surface cracking	-	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-
	Extra-embryonic shell expansion	-	-	+	+	+	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	+	+	+	-
	Movement	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	+	+	+
	Segmentation	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	+	+	+
Vulnerability (%)		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 2. Development stages of *Carcinoscorpius rotundicauda* embryos, egg morphology and the vulnerability score after the addition of bacteria. R= red, B= black, RO= round, O= oval, OP= opaque, + indicates presence of external feature, and - indicates absence of external feature. *Embryos that ceased at M1 were retained until embryos in control group advanced toward hatching.

Condition		A+D+B						A+B						C+D+B						C+B					
Stage		ED	M1	M2	M3	H	L	ED	M1	M2	M3	H	L	ED	M1	M2	M3	H	L	ED	M1	M2	M3	H	L
General features	Colour	R	R	R	R	R	R-B	R	R	R	R	R	R-B	R	R	R	R	R	R-B	R	R	R	R	R	R-B
	Shape	RO	O	O	O	RO	RO	RO	O	O	O	RO	RO	RO	O	O	O	RO	RO	RO	O	O	O	RO	RO
	Eggshell appearance	OP	OP	OP	OP	OP	OP	OP	OP	OP	OP	OP	OP	OP	OP	OP	OP	OP	OP	OP	OP	OP	OP	OP	OP
Specific features	Limb bud formation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Surface cracking	-	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-
	Extra-embryonic shell expansion	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Movement	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Segmentation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Vulnerability (%)		6.8	4.4	3.2	2.4	2	1.2	7.2	6	5.2	4.4	3.6	2.8	18.4	12.8	10.4	7.6	5.2	4.8	18.8	14.8	14.8	7.6	6	5.2

3.2 Histological observations of developing embryo in bioassay

After 7 days into the experiment, there was variability in periplasm thickness and yolk distribution, with pit formations (hemispherical depressions) scattered across the embryonic surface (Fig. 6a). These structures result from interactions between the periplasm and the underlying yolk layer following the cortical reaction of fertilization. At this stage, multiple nuclei were visible. It was caused by successive superficial cleavages within cytoplasmic masses that later developed into blastomeres. These blastomeres, concentrated at the embryonic periphery, contribute to blastula formation and marked the onset of germ disks. The yolk became organised into discrete masses.

After 14 days, in stage M1, the germ disk expands and develops irregular furrows extending across the entire surface (Fig. 6b). These structures initiate segmentation which then corresponds to the later formation of limb buds and future body segments. At this stage, the opaque chorion cracks to expose the non-expanded extra-embryonic shell, within which fibrous bundles are visible. By day 21, embryonic development advances into organogenesis. This is evident with the production of blood cells (amoebocytes) associated with isolated yolk masses positioned at the embryonic periphery or within connective tissue. In later stages, these cells become confined to the haemocoel cavities or the heart. Additional developmental features include the differentiation of the opisthosoma and prosomatic appendages, such as limb buds. The opisthosomal appendages, including the first and second branchial appendages, begin separating into distinct gill lamellae, while other gills remain clustered in the gill book. Furthermore, endoskeletal elements (cartilaginous structures) emerge as solid outgrowths from the ventral wall of the branchial appendages (Fig.6c&d).

In contrast, infected eggs (Fig. 6a–f) initially appeared to follow a similar developmental pattern to normal eggs during the early stage and M1, with yolk organized into distinct central and peripheral masses. However, these eggs simultaneously exhibited clear degradation of the chitinous eggshell, leading to eroding characteristics and pigmented lesions on the shell (exoskeleton). These lesions often referred to as blebs, box burns, or black spots seemed to originate from the embryo's surface and are likely the result of embryonic autolysis. Comparatively, embryos infected by bacteria were indicated with arrested development on day-14 or stage M1 and failed to progress to M2. This was a condition marked by agenesis when compared with normal embryos at the same stage (Fig. 6g).

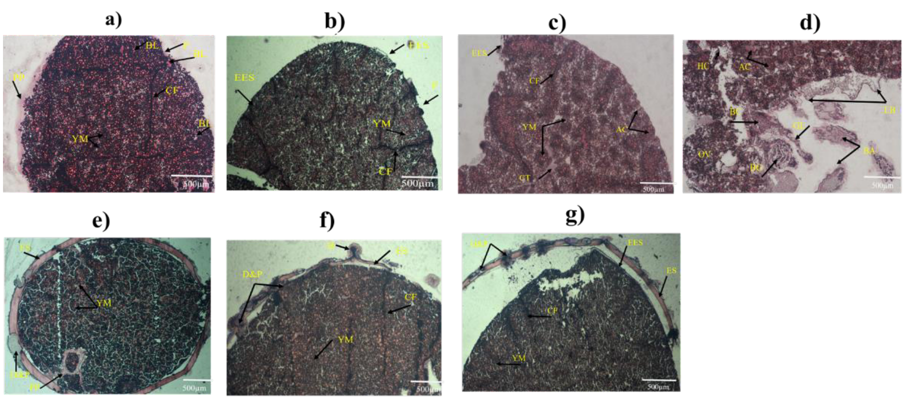

Fig. 6. Longitudinal sectioning of *Carcinoscorpius rotundicauda* embryos after experimentation. a) day 7 (early develop stage), b) day 14 (1st embryonic moult), c) day 21 (2nd embryonic moult dorsal view), d) day 21 (2nd embryonic moult, ventral view), e) after bacterial infection on day 7 (early develop stage), f) day 14 (1st embryonic moult) and g) day 21 PF (ceased develop at 1st embryonic moult or M1). PP= periplasm, P= pits, BL= blastomeres cells, YM= yolk mass, F= furrows, CF= chitinous fibrous bundles, EES= non- extraembryonic shell, CT= connective tissue, GL= gill lamella, BG =book gill, GL= gill lamella, LB= limb bud, BA= branchial appendages, C= cartilage, OV= opisthosomal vent-rum , AC=

amoebocytes, HC= hemocoel cavity, D&P= degradation and pigmentation of eggshell, B= Blebs formation.

Discussion

Carcinoscorpius rotundicauda embryos reared under ambient and ambient-dark conditions successfully completed development, progressing through four embryonic moults and hatching as trilobite larvae with ≈100% success over 40–42 days. This outcome is consistent with other reports of 41–42 days for embryogenesis and near-complete hatching under ambient conditions [9]. Early development was characterised by key milestones like shallow periplasm, uneven yolk, and pit formation marked the cortical reaction and blastoderm initiation within 7 days, followed by germ disk formations in the early blastula stages [10]. By Day 14, the first embryonic moult (M1) was evident through furrows and chorion cracking, correlating with embryo expansion after germ disk completion, yolk mobilisation, and the onset of differentiation for later limb bud formation and body segmentation [11]. By Day 21, organogenesis had produced limb buds, gills, an endoskeleton, and immune cells, consistent with circulatory and immune maturation [7]. In contrast, elevated temperature (32 °C) accelerated development, shortening the time-to-hatching by five days to ~37 days. However, not all embryos adapted well, as hatching success was reduced to 76 %, or 70.8 % in addition with constant darkness. Sustained warming can narrow the thermal safety margin, increase late-stage mortality, and ultimately restrict embryonic development, as observed in *Limulus polyphemus* and other horseshoe crabs with a safety margin generally ≥35 °C [6].

Exposure to *B. thuringiensis* produced a strikingly different outcome, marked by signs of eggshell blebs, erosion, pigmentation anomalies, and autolysis. A vast majority of embryos (98%) failed to progress beyond the M1 stage, suggesting that the bacterium disrupted the perivitelline environment, inducing developmental arrest and autolysis [6]. These pathological effects likely stemmed from *B. thuringiensis* toxins and chitinolytic enzymes that damaged epithelia, altered osmotic balance, and facilitated pathogen invasion [12]. Similar toxin-induced eggshell damage, such as emulsification and electron-dense blebs, has been reported in *Trichostrongylus colubriformis* following *B. thuringiensis* exposure. Since chitinases degrade cuticle and peritrophic membranes, this enzyme acts as a virulence factor, compromising the host defences and promoting successful bacterial establishment in the egg [12].

These 98 % of infected embryos also exhibited red colouration and shrinkage. Developmental bottlenecks are well-documented in horseshoe crabs, where the M1 stage was characterised by high metabolic demands, rupturing of the opaque chorion, and yolk reorganisation. These were observations associated with thinning of the chorion envelope which inevitably increased vulnerability [7, 13]. This vulnerability at the M1 stage may be linked to a weak immune defence, as amoebocytes are largely 'absent'. Robustness increases in progressive stages like M2 (second embryonic moult onward) due to tissue specialisation, as seen in *L. polyphemus* and *T. tridentatus* embryos during M2 (stages 15–21 of the Sekiguchi scale) [13]. The threat is further reduced with the presence of immature amoebocytes during the M3 stage. Therefore, it is not surprising that bacterial (e.g., *Bacillus* spp.) or fungal infections, alongside blebs, erosion, and arrested development, are frequently discovered in horseshoe crab nests [8].

Progressive mortality was clearly observed with *B. thuringiensis* exposure, and was exacerbated when combined with other stressors. Mortality was high at the constant temperature (32 °C; 59.2 %) and worsened under darkness (67.2 %). While temperature and light effects on metabolism and membrane properties may have acted as minor co-factors influencing the rate of embryogenesis in *C. rotundicauda*, it was similar to how light provides cues and temperature triggered the moulting process in *L. polyphemus* and *T. gigas* larvae.

In addition, the exposure to *B. thuringiensis* caused an early and persistent reduction in surface area. This pattern signifies developmental arrest before M2, which prevents organogenesis and cuticle remodelling, leaving embryos pale, undersized, and static. The synergistic effects of constant darkness may be related to the absence of photoperiodic cues that perhaps trigger embryonic moults. Previous studies noted that while *L. polyphemus* embryos showed accelerated development above 30 °C, they also exhibited increased heat shock proteins when salinity was altered. Other research linked temperatures above 32 °C, combined with factors like pH and salinity to inducing stress in embryos.

Stress often triggers organism susceptibility to pathogens. While *C. rotundicauda* embryos (control) showed steady weight and volume gains during the first 28 days, ambient temperature embryos achieved larger dimensions than those at 32 °C. This increase in weight and dimension correlated with a reduction in egg surface area during Days 7–14, confirming 'normal' morphogenesis because the rate of tissue accretion was proportional to yolk depletion. This process alters the egg geometry through compaction and fluid balance, as described for the M2 stage in *L. polyphemus* and *Tachypleus* spp. [14]. Weight gain and size increase align with late embryonic morphogenesis, where yolk depletion supports somatic growth during organogenesis; tissue and organs are relatively denser than yolk material [7, 11]. Embryos typically weigh less between Days 35–42 as the extra-embryonic membrane thins and releases perivitelline fluid to facilitate hatching [7]. Comparatively, only the 2 % of *B. thuringiensis*-exposed embryos successfully moulted to M2, while the remaining 98 % were arrested at M1. All infected embryos gained weight only until ~Day 14 before experiencing a decline due to shrinkage. Under stress, these embryos likely accelerated their metabolism through yolk utilisation, resulting in poor energy balance. Developmental arrest at M1 then prevented the mass and volume gains required for organogenesis, as energy was redirected toward managing stress and immunity. Poor energy reserves and the presence of pathogen toxins caused tissue damage and catabolism, evidenced by egg size shrinking and weight reduction. Natural selection, by forcing yolk depletion at a rate unequal to tissue synthesis, likely limited the development of the 98 % of embryos, mirroring observations during thermal-accelerated development in *L. polyphemus* [11, 15]. The ability of *B. thuringiensis* to digest embryonic envelope proteins (classes H, B1, B2, and 'rest') likely facilitated bacterial invasion, compromising osmotic balance and catalysing yolk reserves through toxin activity. This aligns with red-egg pigmentation, microbial infection, and arrested moulting seen in *T. gigas* embryos. Overall, temperature acted as an abiotic modifier by increasing metabolic demands and reducing early immune capacity, while darkness potentially disrupted the photoperiodic cues regulating moult timing. The prolonged development observed was caused by these co-factors acting together to increase vulnerability.

Conclusion

Under normal conditions, embryos of *Carcinoscorpius rotundicauda* progressed successfully through six defined developmental stages (early developmental stage, M1 to M3 moults, hatching, and the first instar larva), completing embryogenesis within 42 days. This was confirmed by high survival rates (>70%–100%) and "normal" development, evidenced by steady increases in egg volume and weight, decreasing surface area, and characteristic histological transitions like chorion shedding and organogenesis. In contrast, exposure to *Bacillus thuringiensis* severely impaired embryogenesis, causing developmental arrest at the first embryonic moult (M1). This was indicated by abnormal colouration, diminished weight and volume, increased surface area, reduced survivability (as low as 32.8% under 32 °C and darkness), and histological evidence of eggshell degradation and cellular autolysis. Only ~2%

of exposed embryos progressed beyond M1, highlighting the first 14 days as the most critical period of infection. Overall, *B. thuringiensis* disrupts normal morphogenesis and compromises embryo viability across all temperature and light regimes, suggesting that warmer climates may favour pathogens over host survivability. Further research is necessary to investigate the mechanistic pathways of *B. thuringiensis* in inducing this developmental arrest, focusing on immune responses and eggshell integrity, to inform mitigation measures for oviparous arthropods in changing environments.

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